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Small plaque size variant of chikungunya primary isolate showed reduced virulence in mice

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Abstract

Background: Plaque size is a common feature of viral characterization. Small plaque size is used as a marker of attenuation for live-attenuated vaccine development.

Objective: To investigate whether the naturally occurring plaque size variation reflects virulence of the variants of chikungunya virus (CHIKV).

Methods: We selected and purified a variant with small plaque size from the primary isolate. The viral variant was tested for the plaque morphology, *in vitro* growth kinetics and mouse neurovirulence in comparison with the parental wild type.

Results: The small plaque size variant showed stable homogenous small plaques after 4 plaque purifications. The small plaque virus grew slower and to the lower titer when compared with wild type virus. After 21 days of infection, mice that received small plaque virus showed 98% survival rate while 74% of mice survived after infected with wild type virus.

Conclusion: The small plaque size variant of CHIKV can be obtained by plaque purification and the virus displays decreased virulence.

Keywords: Chikungunya virus, arbovirus, plaque purification, small plaque variant, mouse virulence.

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Introduction

Chikungunya virus (CHIKV) belongs to the genus *Alphavirus* in the family *Togaviridae*. It is a re-emerging arbovirus that causes an acute febrile illness with skin rashes, myalgia and/or arthralgia. While the fever and rash are self-limited and resolve within a few days, the arthalgia can be prolonged from months to years. Hence, the name chikungunya, which originated from Makonde language meaning that which bend, reflects the arthritic symptoms of the disease.[1,2] CHIKV was isolated for the first time in Africa in 1955 and has become epidemic periodically since then. The virus can be divided into 3 distinct groups by phylogenetic analysis: the West African, the Asian and the East/Central/South African (ECSA) genotypes according to their original geographical distribution. The ECSA virus with an alanine to valine mutation at the amino acid position 226 of the E2 envelope gene has caused multiple massive outbreaks in various regions starting in the La Reunion Islands in 2005. Afterward, the virus spread to Asia causing over a million cases in the following years.[3-5] The Asian genotype started invading the Americas in 2013 causing massive and ongoing outbreaks in various countries in Central, South America and the Caribbean. The ECSA virus is now the dominant virus all over the Africa and Asia and the Asian genotype is the dominant virus in the Americas.[4,6-9] Although a number of CHIKV vaccine candidates are being developed, no effective vaccine is currently available for clinical use.

Plaque assay is a classical method for viral quantification and characterization. Plaques are translucent areas on monolayer cell culture covered with semisolid or solid media after viral infection. They are caused by localized cell lysis. Their size and shape depend on viral replication rate, capacity for spreading

to neighboring cells and cell killing. Small plaque size indicates lower rates of viral replication, spread and cell killing, and therefore suggests lower virulence.[10] Viruses with smaller plaque sizes usually have lower fitness, and are selected against in natural conditions. Therefore, they are usually not maintained in large quantity in natural viral quasispecies. Viruses with small plaques sizes can be generated and selected by serial passages in cell lines from non-natural host species. Adaptation to a new host species can cause some variants to be de-optimized in the natural host species and have small plaque phenotype.

Primary isolates of CHIKV containing variants with different plaque sizes were previously reported.[11,12] We also observed viral variants with different plaque morphology including small plaque sizes in CHIKV primary isolate. It is curious how small plaque size variants with supposedly lower fitness were maintained in natural viral quasispecies. Plausible explanations include that the plaque size may not represent *in vivo* growth conditions, and that cooperation among variants with different plaque sizes may be required for optimal *in vivo* replication and transmission fitness. If the plaque size did not represent *in vivo* growth condition, and small plaque size variants had similar fitness as larger plaque size variants, they would be similarly virulent in an animal model. Although mouse is not a natural host for CHIKV, suckling mice have been used as an animal model for CHIKV and other arboviruses, especially in testing for attenuation phenotype of live-attenuated vaccine candidates.[13,14] In order to explore whether the small plaque CHIKV variant has reduced virulence *in vivo*, we purified a CHIKV small plaque size variant from a primary isolate and tested its pathogenicity in suckling mice.

Materials and methods

Cells and viruses

C6/36 cell from *Aedes albopictus* larvae was maintained at 28°C in minimum essential medium (MEM; Gibco, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone, UK) and 100 units/ml of penicillin and 100 µg/ml of streptomycin. Vero cell, an African green monkey kidney epithelial cell line, was maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% FBS and 100 unit of penicillin/streptomycin per ml.

CHIKVs were isolated in C6/36 cells from the sera of patients in Phang-nga, Thailand in 2009. CHIKV isolate 021, 025 and 039 were propagated in C6/36 cells. The culture media of the infected cells were harvested at 2 or 3 days after infection and stored at -80°C until use. CHIKV isolate 025, designated as WT-CHK025, was used for small plaque purification.

Plaque assay

Viruses were 10-fold serially diluted in BA-1 medium (1xM-199E, 1 M Tris-HCl pH 7.6, 2% (w/v) BSA, 100 units of penicillin/streptomycin per ml, 0.075% (w/v) $NaHCO_3$) and inoculated onto monolayer of Vero cells in 6-well plate. The cells were incubated for 2 hr at 37°C and then overlaid with nutrient agarose (0.8%agarose (SeaKem LE, USA) containing Earle's balanced salts supplemented with 0.5% (w/v) yeast extract, 2.5% lactalbumin hydrolysate, 3% FBS) and incubated at 37°C, 5% CO_2 for 5 days. The cell layer was stained with the second overlay containing 1% neutral red and incubated as mentioned above for 16-18 hr for plaque observation. For plaque size measurement, the cells were fixed with 8% formaldehyde in PBS at room temperature for 2 hours, the solid media was removed and the cells were stained with crystal violet. Thirty plaques of each virus were measured for the diameter in millimeter.

Selection and purification of small plaque virus

For small plaque purification, Vero cells were infected with WT-CHK025 passage 4 for plaque assay as described above. After 6 days of infection and additional 16-18 hr of incubation with the second overlay containing 1% neutral red, individual small plaques from the terminal dilution were picked using sterile pipette tips, suspended in serum-free medium and inoculated directly onto fresh C6/36 cells. The virus was continually propagated in C6/36 cells for 3 more passages and subsequently subjected to another plaque purification. The process was repeated until homogenous small plaque morphology was observed (**Figure 2**). This virus was designated as CHK-S.

Viral growth kinetics

Monolayers of C6/36 cells were infected with WT-CHK025 or CHK-S at MOI of 0.01. The viruses were adsorbed at room temperature for 2 hours. Then the cells were washed with PBS for 3 times and maintained in fresh complete media at 28°C. Supernatants of infected cells were collected at 0, 6, 12, 24, 48 and 72 hours post-infection (hpi). The titers of the infectious viruses were determined by standard plaque assay using Vero cells.

Viral pathogenicity in suckling mice

Pregnant ICR mice were purchased from National laboratory animal center and housed in the Institute of Molecular Biosciences animal facility. Groups of 50 and 55 suckling mice from 10 females were intracranially injected (i.c.) with 10^3pfu of WT-CHK025 or CHK-S, respectively. The mice were observed daily for any sign of sickness for 21 days. Moribund mice were euthanized. Kaplan-Meier survival plot of each group was made using Prism software (GraphPad software). The animal experiment was performed under Institutional Animal Care and Use Committee-approved protocols (#MB-ACUC 2016/006).

Results

Selection of small plaque variant of CHIKV

CHIKVs were isolated from the sera of patients and subjected to standard plaque assay. All three CHIKV primary isolates, 021, 025 and 039, showed plaque size heterogeneity with the diameter ranges of 0.5-2.8, 0.5-5 and 0.5-3 mm., respectively (**Figure 1**). The isolate 025, designated as WT-CHK025, was used as a parental virus for selection of small plaque variant. After single plaque purification and 3 passages of amplification in C6/36 cells, passage 7 of the virus still showed heterogeneity in plaque morphology (**Figure 2**). Therefore, the virus was continually subjected to 3 additional plaque

purifications until uniform small plaque size variant was obtained and designated as CHK-S. The distribution of the plaque size heterogeneity was quite stable when propagated in C6/36 cells as shown for virus passage 14 and 15 (**Figure 2**). In contrast, we previously observed that the small plaque variant gradually decreased when the virus was propagated in Vero cells.[15]

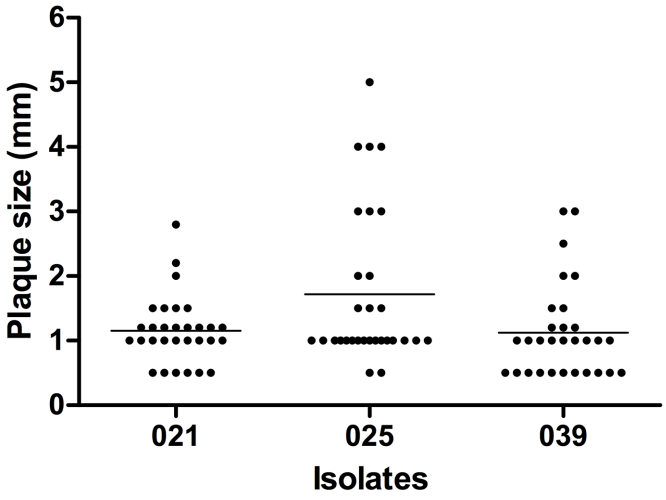

Figure 1. Plaque sizes of CHIKV isolates. CHIKV primary isolate number 021, 025 and 039 were subjected to plaque assay in Vero cells and incubated for 6 days. Thirty plaques of each virus were measured for diameters in millimeters. Each dot represents one plaque; horizontal lines are averages.

In vitro replication of wild type and small plaque variant of CHIKV

To test whether CHK-S has any growth defect, replication kinetics of the small plaque variant was studied in C6/36 cells in comparison to the wild type CHIKV. The titers of both viruses could be detected at 12 hpi in which the titer of CHK-S was approximately 100 times lower than that of WT-CHK025. Although the titer differences between those 2 viruses could be observed at every time points throughout the experiment, the gap became closer as the infection proceed. CHK-S yielded a maximum titer at 10^9 pfu/ml at 72 hpi, which was a log lower than that of WT-CHK025. This result indicates that small plaque variant can grow efficiently but not as good as its parental wild type CHIKV.

Virulence of wild type and small plaque variant of CHIKV

Persistence of small plaque variants in natural CHIKV quasispecies suggested that either the small plaque variant had normal replication kinetics and fitness *in vivo* or they were required to cooperate with the large plaque variant to obtain optimal replication and/or transmission fitness *in vivo*. If the small plaque variant had normal replication kinetics and fitness *in vivo* and only showed retarded replication *in vitro*, it should have similar virulence *in vivo* as compared to the wild type virus. To test the hypothesis, WT-CHK025 and CHK-S were intracranially injected into suckling mice at a dosage of 10^3 pfu/mouse, which is the dose that used to test CHIKV virulence for vaccine candidates. The mice were kept for observation for 3 weeks and the numbers of survived mouse were used to plot

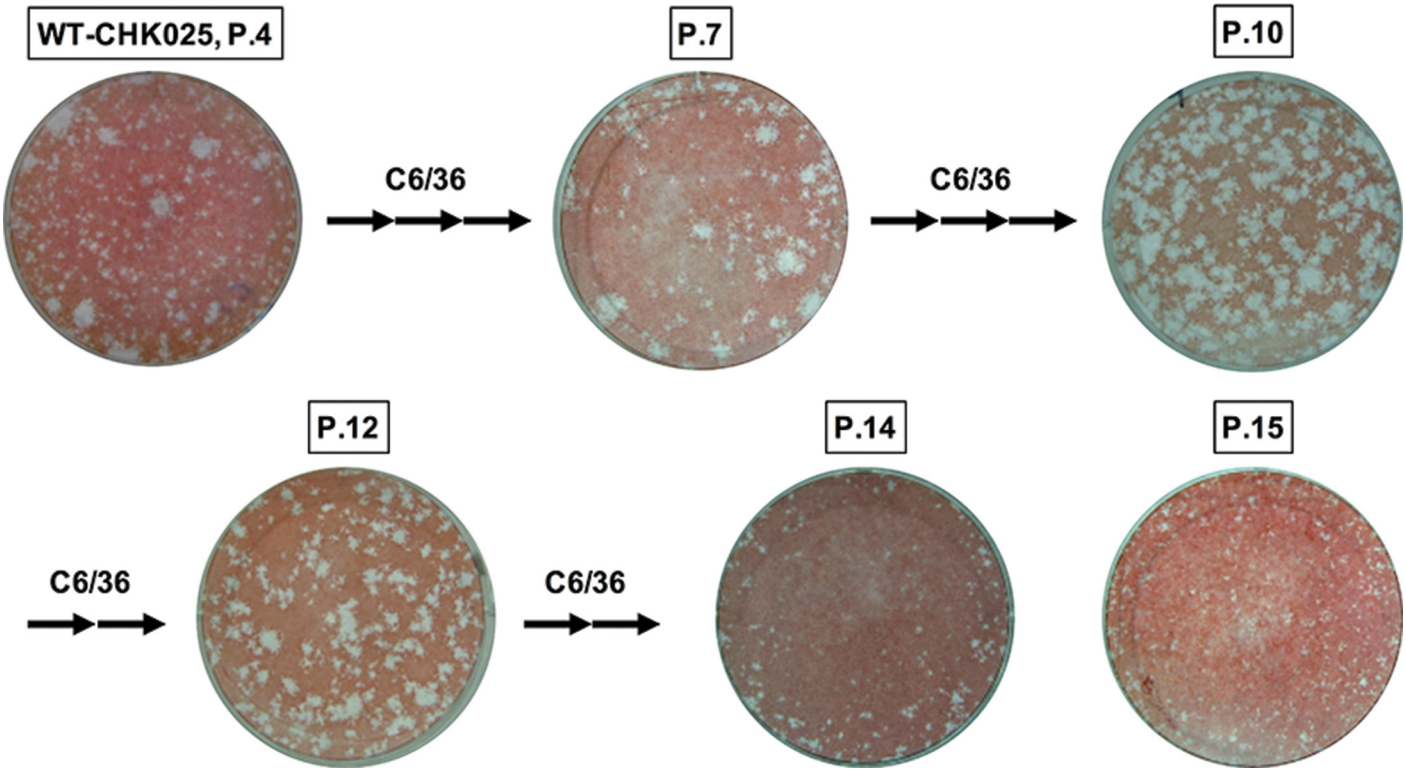

Figure 2. Plaque morphologies of CHIKV during the small plaque selection process. Small plaque variant was selected from WT-CHK025 passage 4 and continuously propagated using C6/36 cells. Plaque purification and propagation were repeated until passage 15, which showed a uniform stable small plaque morphology, designated as CHK-S. Standard plaque assays were undertaken using Vero cells with 6 days incubation. Each circle represents one well of a 6-well plate.

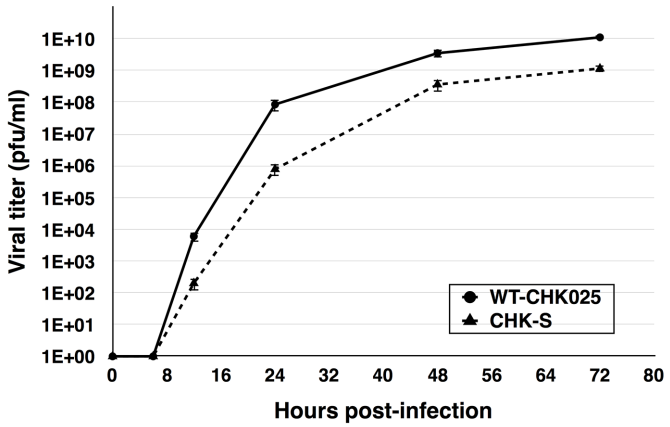

Figure 3. Growth kinetics of WT-CHK025 and CHK-S. C6/36 ells were infected with the WT-CHK025 (circle) or CHK-S (triangle) at MOI of 0.01. The supernatants were collected at 0, 6, 12, 24, 48 and 72 hours post-infection and subjected to plaque assay in Vero cells in duplicate. Data are mean (±SD) values of duplicate samples in one of 2 independent experiments with comparable results.

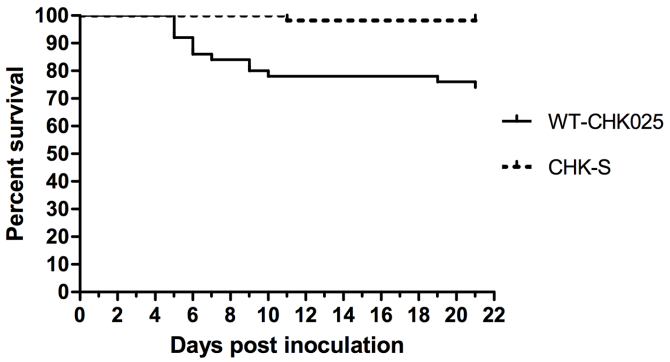

Figure 4. Neurovirulence of WT-CHK025 and CHK-S. Groups of suckling mice were intracranially inoculated with 103 pfu of WT-CHK025 (solid line) or CHK-S (dashed line). Mice were daily observed for any sign of sickness and numbers of survival mice were recorded for 21 days. The Kaplan-Meier plot shows the percentage of surviving mice on each day.

Kaplan-Meier survival curves (**Figure 4**). All but one mouse that received CHK-S survived at the end of the 3-week period. In contrast, mice infected with WT-CHK025 succumbed to the infection and started dying on day 5 post-infection with the overall mortality rate at 26%.

Discussion

Because of their extremely high mutation rate, RNA viruses usually exist as population of related variants known as quasispecies.[16] Viral variants in quasispecies are different from one another by only a few mutations. They compete in environment with selective pressures resulting in equilibrium with distribution of variants according to their fitness. Variants with reduced fitness can therefore exist only as a minor fraction in quasispecies. Persistence of CHIKV variant with small plaque size and reduced replication kinetics as a major subpopulation in CHIKV primary isolate and during multiple passaging in C6/36 cells is therefore intriguing. Our data showing reduced virulence in suckling mice indicate that the small plaque variant had also reduced *in vivo* fitness. This together with the persistence of small plaque variant in C6/36 cells and not in Vero cells suggest that replication cycle in mosquito vector may play an important role in maintaining the small plaque variant in natural infection.[15] While large plaque virus may replicate better and have higher fitness in mammalian hosts, small plaque virus may provide some selective advantage when the virus is transmitted to mosquito vector.[12] Alternating replication in mammalian host and mosquito vector provides unique selective environment and constraint for the viral quasispecies. Previously published data on another arbovirus, West Nile virus, showed that viruses from mosquitoes are more diverse than viruses from birds.[17] This suggested that the vector part of the transmission cycle provided higher positive selective pressure than the cycle in vertebrate host.

The persistence of CHK-S after multiple passages in C6/36 despite its lower replication kinetics when compared to WT-CHK025 is also intriguing. This suggests that the small plaque variant may be able to outcompete the large plaque variant when infecting the same cell by an unknown mechanism. Alternatively, small and large size variants may cooperate in a way that provides selective advantage for maintaining small plaque variant. Cooperation of variants within quasispecies has been described in poliovirus and enterovirus 71 pathogenesis.[18,19]

Several possible mechanisms could contribute to the small plaque phenotype of the virus. Previous study on West Nile virus showed amino acid substitutions in NS4B protein, a small poorly defined protein, associated with small plaque phenotype and the resulting virus induced less innate and adaptive immune responses in mice when compare to wild type virus.[20,21] Comparative sequence alignment of large and small plaque variants of CHIKV primary isolate from the Comoros Island showed 2 amino acid differences in the nsP2 protease and nsP3 protein. However, those particular amino acid residues can be observed in other isolates as well.[12] Therefore it is not clear whether these specific sequences are required for small plaque phenotype. To get an answer for our small plaque size variant, the whole genome sequencing, sequences comparison between the variants and confirmation by reverse genetics shall be performed in the future.

Our data provide some insight into the heterogeneous plaque size of CHIKV primary isolates. In addition, attenuation phenotype shown in the small plaque size variant suggests that selection and purification of small plaque virus from CHIKV primary isolates may provide a good live-attenuated vaccine candidate.

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Contribution

RP designed the study and prepared the manuscript; TJ performed the experiment; SY and SU provided materials and reagents.

Conflict of interest

All of the authors declare no conflict of interest.

References

1. Cavrini F, Gaibani P, Pierro AM, Rossini G, Landini MP, Sambri V. Chikungunya: an emerging and spreading arthropod-borne viral disease. J Infect Dev Ctries. 2009;3(10):744-52.
2. Thiboutot MM, Kannan S, Kawalekar OU, Shedlock DJ, Khan AS, Sarangan G, et al. Chikungunya: a potentially emerging epidemic? PLoS Negl Trop Dis. 2010;4(4):e623.
3. Josseran L, Paquet C, Zehgnoun A, Caillere N, Le Tertre A, Solet JL, et al. Chikungunya disease outbreak, Reunion Island. Emerg Infect Dis. 2006;12(12):1994-5.
4. Pulmanausahakul R, Roytrakul S, Auewarakul P, Smith DR. Chikungunya in Southeast Asia: understanding the emergence and finding solutions. Int J Infect Dis. 2011;15(10):e671-6.
5. Sreekumar E, Issac A, Nair S, Hariharan R, Janki MB, Arathy DS, et al. Genetic characterization of 2006-2008 isolates of Chikungunya virus from Kerala, South India, by whole genome sequence analysis. Virus Genes. 2010;40(1):14-27.
6. Sudeep AB, Parashar D. Chikungunya: an overview. J Biosci. 2008;33(4): 443-9.
7. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 2007;3(12):e201.
8. Ahmed S, Francis L, Ricketts RP, Christian T, Polson-Edwards K, Olowokure B. Chikungunya virus outbreak, Dominica, 2014. Emerg Infect Dis. 2015;21(5):909-11.
9. Sam IC, Kummerer BM, Chan YF, Roques P, Drosten C, AbuBakar S. Updates on chikungunya epidemiology, clinical disease, and diagnostics. Vector Borne Zoonotic Dis. 2015;15(4):223-30.
10. Levitt NH, Ramsburg HH, Hasty SE, Repik PM, Cole FE, Jr., Lupton HW. Development of an attenuated strain of chikungunya virus for use in vaccine production. Vaccine. 1986;4(3):157-62.
11. Lim CK, Nishibori T, Watanabe K, Ito M, Kotaki A, Tanaka K, et al. Chikungunya virus isolated from a returnee to Japan from Sri Lanka: isolation of two sub-strains with different characteristics. Am J Trop Med Hyg. 2009;81(5):865-8.
12. Wasonga C, Inoue S, Rumberia C, Michuki G, Kimotho J, Ongus JR, et al. Genetic divergence of Chikungunya virus plaque variants from the Comoros Island (2005). Virus Genes. 2015;51(3):323-8.
13. Bhamarapravati N, Yoksan S. Live attenuated tetravalent dengue vaccine. Vaccine. 2000;18 Suppl 2:44-7.
14. Haese NN, Broeckel RM, Hawman DW, Heise MT, Morrison TE, Streblow DN. Animal Models of Chikungunya Virus Infection and Disease. J Infect Dis. 2016;214(suppl 5):S482-S7.
15. Onnome S, Ubol S, Pulmanausahakul R, editors. Effects of Vero and C6/36 cells upon CHIKV phenotypes. Proceedings of the 2nd ASEAN plus three graduate research congress; 2014 February 5-7; Bangkok, Thailand.[place unknown]:[publisher unknown]:2014.
16. Lauring AS, Andino R. Quasispecies theory and the behavior of RNA viruses. PLoS Pathog. 2010;6(7):e1001005.
17. Fitzpatrick KA, Deardorff ER, Pesko K, Brackney DE, Zhang B, Bedrick E, et al. Population variation of West Nile virus confers a host-specific fitness benefit in mosquitoes. Virology. 2010;404(1):89-95.
18. Meng T, Kwang J. Attenuation of human enterovirus 71 high-replication -fidelity variants in AG129 mice. J Virol. 2014;88(10):5803-15.
19. Pfeiffer JK, Kirkegaard K. Increased fidelity reduces poliovirus fitness and virulence under selective pressure in mice. PLoS Pathog. 2005;1(2):e11.
20. Welte T, Xie G, Wicker JA, Whiteman MC, Li L, Rachamallu A, et al. Immune responses to an attenuated West Nile virus NS4B-P38G mutant strain. Vaccine. 2011;29(29-30):4853-61.
21. Wicker JA, Whiteman MC, Beasley DW, Davis CT, McGee CE, Lee JC, et al. Mutational analysis of the West Nile virus NS4B protein. Virology. 2012;426(1):22-33.