Neurology Asia 2020; 25(3) : 279 – 284

Japanese encephalitis virus: Biological clones from a clinical isolate quasispecies show differing neurovirulence *in vitro* and in a mouse model

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Abstract

The Japanese encephalitis virus (JEV), a leading cause of encephalitis, exists as quasispecies in clinical isolates. Using a limiting dilution method combined with immunohistochemistry to detect viral antigens, 10 biological clones were isolated and purified from a clinical JEV isolate (CNS138/9) derived from an autopsy brain. These biological clones were tested for neurovirulence in SK-N-MC and NIE-115 neuronal cells, and a 2-week-old, footpad-infected, JE mouse model. Nine clones were found to be neurovirulent; one clone neuroattenuated. Although further studies are needed to determine genotypic differences, if any, in these clones, the limiting dilution purification and neurovirulence testing methods described herein should be useful for phenotypic studies of quasispecies of neurotropic viruses in general, and JEV and other flaviviruses in particular.

Keywords: Japanese encephalitis virus, quasispecies, biological clones, limiting dilution

INTRODUCTION

Japanese encephalitis virus (JEV) is an enveloped, single-stranded positive-sense RNA arbovirus belonging to the family *Flaviviridae* and genus *Flavivirus*. The approximately 11 kb genome comprises capsid, pre-membrane and envelope (E) genes that encode for 3 corresponding structural proteins, respectively, and 7 non-structural genes (NS1, NS2A and B, NS3, NS4A and B, and NS5). The open reading frame is flanked by 5' and 3' untranslated regions.[1-3]

JEV is one of the leading causes of mosquito-borne viral encephalitides with an annual global estimate of 68,000 Japanese encephalitis (JE) cases, and 13,600 to 20,400 deaths in affected areas.[4] In endemic areas, JE largely involves children, but in non-endemic areas, all age groups are at risk of infection.[1] With a 1:25 to 1:1000 symptomatic to asymptomatic ratio[1,5], JE fatality rate ranges from 25% to 50%, and more than 50% of the survivors suffer permanent neurological sequelae.[6]

Similar to other flaviviruses like dengue virus (DENV) and West Nile virus (WNV), JEV replicates in the absence of proof-reading and repair of newly synthesized viral RNAs. Hence, routinely isolated viruses exist as a quasispecies which is a mixture of viral strains or biological clones with closely-similar genomes.[7,8] These biological clones may result in phenotypic differences including alterations in cell tropism, virulence, host range, and resistance to antiviral agents and host immune responses.[7,9] Isolation of biological clones from a flavivirus quasispecies population is possible, as had been demonstrated in WNV.[10-12] Since JEV is very difficult to isolate because of low and transient viraemia in humans, relatively little work has been done on its quasispecies.

One method that is commonly used for virus purification is the viral plaque assay. It uses serial dilutions of viruses to infect susceptible cell monolayers, and the application of a semi-solid nutrient medium overlay to prevent virus from spreading to nearby uninfected cells, resulting in a distinct plaque that can be easily visualized after staining. The method is useful for viruses that are able to produce good visible cytopathic effects (CPE).[13] Unfortunately, JEV infection does not produce good CPE and plaque formation, so the plaque assay is unsuitable for its purification.

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Date of submission: 18 February 2020, Date of acceptance: 28 March 2020

In this paper, we describe a limiting dilution method (slightly modified from the conventional plaque assay) to successfully obtain 10 biological clones from a rare JEV clinical isolate (CNS138/9) from the brain of an autopsy case of JE. Moreover, these clones were investigated for relative neurovirulence using neuronal cell cultures and a novel, footpad-infected mouse model of JE.[14]

METHODS

Cell lines

Human neuroepithelioma cells (SK-N-MC) (ATCC-HTB-10, USA) and mouse neuroblastoma cells (NIE-115) (ATCC-CRL-2263, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, USA), supplemented with 10% fetal bovine serum (FBS) (Hyclone, Fisher Scientific, USA) and 50 μg/mL gentamycin. *Aedes albopictus* cells (C6/36) (ATCC-CRL-1660, USA) were cultured in Roswell Park Memorial Institute medium (RPMI-160) (Sigma-Aldrich, USA), supplemented with 10% FBS and 50 μg/mL gentamycin.

Biological clone isolation and purification

A clinical isolate (CNS138/9) from the brain of a deceased JE patient in Sarawak, Malaysia, previously isolated and maintained in C6/36 cells[15], was purified by a limiting dilution method. The C6/36 cells seeded in 96-well plates ($1.5x10^4$ cells/well), were infected with 10-fold serial dilutions of virus stock in quadruplicate sets (12 dilutions: 10^{-1} to 10^{-12}). At 7 days post-infection (dpi), supernatant was collected from each well. The remaining cells attached to the wells were stained by immunohistochemistry (IHC) to detect the presence of viral antigens as proof of infection. This was necessary because infected C6/36 cells do not show obvious CPE. Supernatants from wells that were IHC-positive and were infected with the lowest concentrations of virus, were used for one more round of limiting dilution purification as before to obtain the final purified biological clones.

For IHC, cells were methanol-fixed, followed by application of anti-JEV primary antibody (Jath-160; courtesy of Dr. Takasaki, National Institute of Infectious Diseases, Japan) at 1:5000 dilution and incubated overnight at 4°C. This was followed by 30 mins ENVISION HRP conjugated goat anti-mouse secondary antibody (Dako, Denmark) incubation at 1:1 dilution at RT. Chromogen development was done using 3,3'-diaminobenzidine at 1:50 dilution (Dako, Denmark).

Virus titration by $CCID_{50}$ assay

To obtain adequate virus stocks, all purified biological clones obtained were propagated once in C6/36 cells and virus-titrated using a previously described $CCID_{50}$ assay[16], with slight modifications, In brief, C6/36 cells seeded in 96-well plates ($1.5x10^4$ cells/well) were infected with 10-fold serial dilutions (12 dilutions: 10^{-1} to 10^{-12}) of JEV in quadruplicates, and after 7 dpi observed for the presence of viral antigens by IHC as before. Viral titres were calculated using Karber's method.[17]

Biological clone infection in neuronal SK-N-MC and NIE-115 cells

JEV infectivity was initially evaluated in SK-N-MC cells at multiplicity of infection (MOI) of 5, 10, 15 and 20. Since at MOI of 10, the majority of the cells were infected (> 75% viral antigen-positive cells), and no significant changes were observed in control cells exposed to equal amounts of UV-inactivated viruses, an MOI of 10 was used for *in vitro* neurovirulence testing.

SK-N-MC cells seeded into 24-well plates ($8x10^4$ cells/well) overnight were infected with all the 10 purified biological clones at an MOI of 10. Unbounded viruses were removed by washing with phosphate buffered saline (PBS, pH7.4) after an hour pre-absorption. Uninfected controls and infected cells were monitored up to 120 hours post-infection (hpi). Based on cell shrinkage, rounding up and detachment as evidence of progressive CPE and neurovirulence, 9 biological clones (JEV-V1 to JEV-V9) were classified as neurovirulent while 1 clone (JEV-V10) was found to be neuroattenuated because the latter showed no or minimal CPE.

To reconfirm neurovirulence, JEV-V1 was selected at random as a representative neurovirulent clone for comparative studies with JEV-V10 using 2 cell cultures, SK-N-MC and NIE-115. For each cell type, cells seeded into 12-well plates (10^5 cells/well) for a day, were infected in triplicates with JEV-V1 and JEV-V10 at MOI of 10, respectively. At 120 hpi, supernatants were collected from a total of 3 independent sets of experiments for virus titration. Moreover, as further proof of infection, cells were methanol-fixed for IHC detection of viral antigens as before, even though, unlike C6/36 cells, CPE was distinct enough in these 2 cell lines to indicate infection.

Biological clone infection in a JE mouse model

To compare *in vivo* neurovirulence of the 10 biological clones, groups of 2-week-old ICR mice (Ethic Number: 2016-170819/PATHO/PS/WKT, 2017-200106/PATHO/R/WKT) were each infected on the right hind leg footpad with 20 μL of 10^6 $CCID_{50}$/ml of JEV-V1 (n=4), JEV-V10 (n=4), and JEV-V2 to JEV-V9 (n=3 per clone), respectively. This mouse model was recently described by our group as an excellent model for JE because it closely recapitulated human CNS pathology, and was infected via the skin, thus mimicking a mosquito bite.[14] The uninfected control group (n=4) was inoculated with 20 μL PBS.

All animals were monitored twice daily up to 21 days post-infection (dpi). Animals that developed severe signs of infection or were moribund, were euthanized while animals that remained healthy were euthanized at the end of the observation period. Kaplan-Meier survival curves were plotted for each group of animals.

RESULTS

In the 1st round of limiting dilution purification, 4 JEV biological clones were isolated from the dilution of 10^{-6}. Then these 4 clones were subjected to 2nd round of limiting dilution purification which yield a total of 9 biological clones from the dilution of 10^{-5} and 1 biological clone from the dilution of 10^{-4}, respectively. Initial results that showed that SK-N-MC infection with 9 out of the 10 biological clones were more neurovirulent (JEV-V1 to JEV-V9) than JEV-V10, was confirmed by the representative JEV-V1, which showed progressive CPE (cell shrinkage, rounding up and detachment) (Figure 1A, 1B), while the JEV-V10 infection showed minimal or no CPE. Furthermore, the majority of JEV-V1-infected cells that remained attached were viral antigen-positive but only focal viral antigens in JEV-V10 infected cells at 120 hpi (Figure 1C, 1D). JEV-V1 titres which ranged from mean log 3.1 to 4.9 (Figure 2) were also significantly ($P \leq 0.05$) higher than JEV-V10 titres of mean log 0.4 to 3.5 $CCID_{50}$/ml.

All animals infected with JEV-V1 to JEV-V9 showed signs of infection resulting in severe infection/moribund stage/mortality (Figure 3) from 6-9 dpi. Signs of severe infection included ruffled fur, hunched back posture, paralysis and seizures. On the other hand, only 50% of JEV-V10 infected animals were severely infected, but later from 9-11 dpi (Figure 3). All the mock-infected animals control remained healthy.

DISCUSSION

Like all RNA viruses, flaviviruses including the JEV, can exist as quasispecies which comprise a complex mixture of virions with closely-related but non-identical genomes that undergo continuous mutations due to competitive selection and cooperation between the biological clones.[7,8] This genomic diversity is well known to confer different phenotypic characteristics such as alterations in host range/cell tropism, virulence, and resistance to antiviral agents and immune responses.[7,9] For example, deep sequencing of the DENV from patients' sera during the 2001-2002 Cuban epidemic showed genetic diversities that could have contributed to disease severity.[18] In a WNV study, 40 biological clones showing *in vitro* phenotypic diversities in C6/36 cells have been isolated from the parental WNV-CP40 strain, with 1 of the clones showing distinctive higher relative fitness.[10,11] Unfortunately, we do not have information regarding its neurovirulence.

In this study, we have purified 10 JEV biological clones from a rare clinical isolate from the brain of a fatal JE case using the limiting dilution method combined with IHC. The principle behind this method is that with increasing dilutions, individual biological clones become separated from each other, and thus after a second round of limiting dilution purification, a homogenous biological clone was obtained. IHC to detect the presence of viral antigens as proof of infection was done as CPE was not evident in C6/36 cells, in contrast to the more distinct CPE in SK-N-MC and NIE-115 cells. Differences in host cell responses to infection may be contributory factors to the differences observed in these cell lines.[19-21]

Nine of the 10 clones were shown to be neurovirulent by *in-vitro* cell cultures in 2 commonly used neuronal cell lines, and confirmed by our recently-described mouse model.[14] This model mimicked a mosquito bite in human infections by viral inoculation into the mouse footpad, and appears to model JE very well. However, one of the clones, JEV-V10, showed neuroattenuation *in vivo* as only half the animals developed severe infection and at a later time point too. Further work is needed to investigate if this was due to genomic mutations, which would in turn, help us identify the genomic determinants of JEV neurovirulence. It is conceivable that the relative abundance of neurovirulent clones in a brain-derived clinical isolate could suggest that JEV may have been preselected to be more neuroinvasive. However, we recognise that the

JEV quasispecies in this study had been laboratory-passaged about 4 times, may have undergone some mutations for this reason. Nonetheless, since it is extremely difficult to culture JEV from human blood because viraemia is often low and transient, investigations into a rare brain isolate obtained at autopsy offers opportunities to further understand neurovirulent/neuroinvasive biological clones.

In conclusion, the limiting dilution purification and neurovirulence testing methods described herein should be useful for investigations into phenotypes of JEV biological clones. These

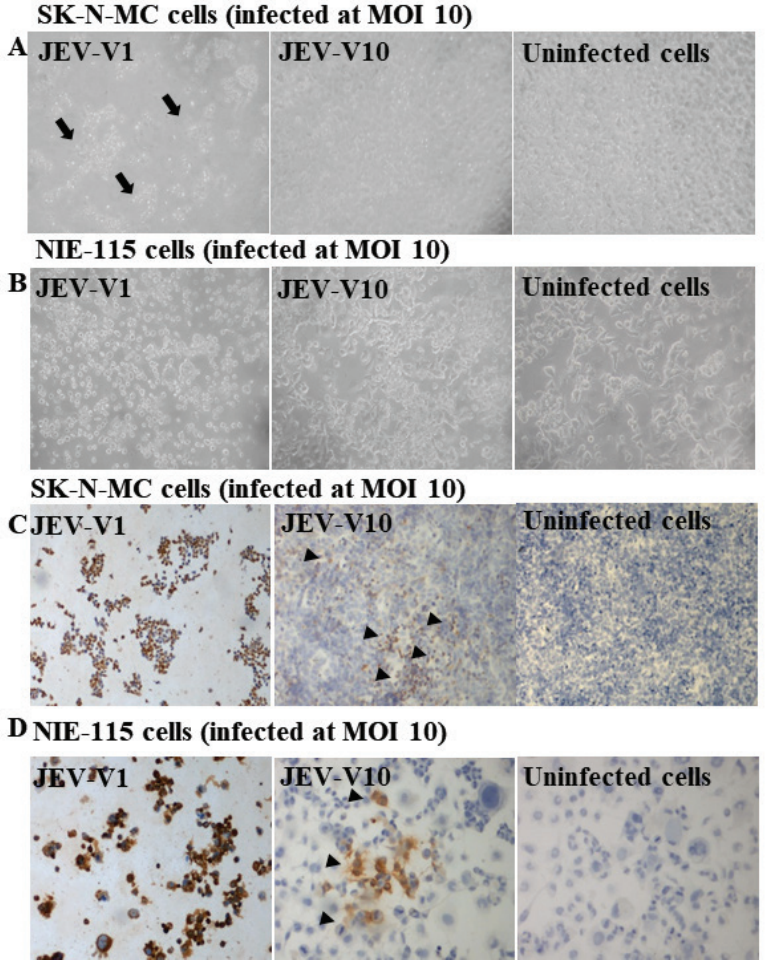

Figure 1. Cell morphology and immunohistochemistry-detected viral antigens after 120 hours post-infection by JEV-V1 and JEV-V10 biological clones.
(A) In SK-N-MC cells infected at MOI of 10, the cytopathic effect (CPE), which consisted of cell clumping, shrinkage and detachment (arrows), was noted in JEV-V1-infected cells but CPE was minimal or absent in JEV-V10-infected and uninfected cells.
(B) In NIE-115 cells infected at MOI of 10, CPE consisting mainly of cell rounding was observed in the majority of JEV-V1-infected cells, but CPE was minimal or absent in JEV-V10 infected and uninfected cells
(Note: Generally, NIE-115 cells do not achieve 100% confluency as observed in SK-N-MC cells).
(C) In SK-N-MC cells infected at MOI of 10 with JEV-V1, viral antigens (brown) were detected in the majority of remaining undetached cells. Only focal viral antigens were detected in JEV-V10-infected cells (brown, arrow head), viral antigens were absent from uninfected cells.
(D) In NIE-115 cells, infected at MOI of 10 with JEV-V1, the majority of remaining undetached cells were viral antigen-positive (brown). Focal viral antigens were detected in JEV-V10-infected cells (brown, arrow head). Viral antigens were absent from uninfected cells.
(A-D, original magnification: 10x objective).

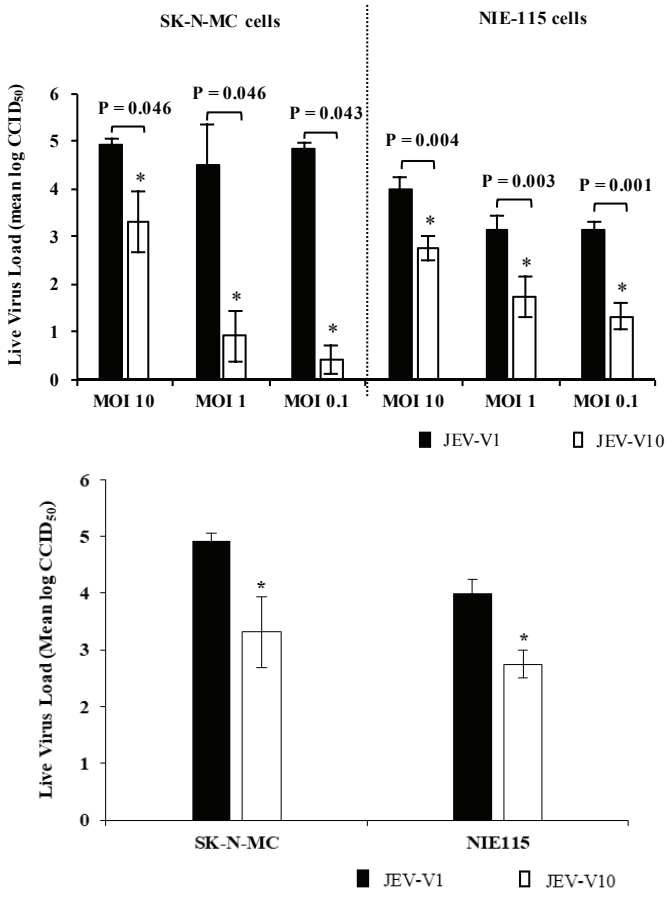

Figure 2. Infection of SK-N-MC and NIE-115 cells with JEV-V1 and JEV-V10.
(A) At MOI of 10, JEV-V1 clearly showed significant ($P \leq 0.05$*) increased growth compared to JEV-V10 at 120 hours post-infection (hpi).

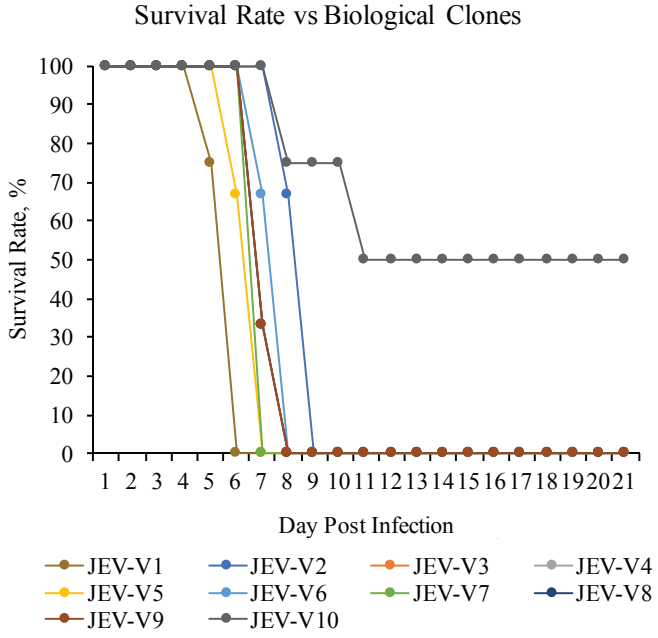

Figure 3. Survival graphs of mice footpad-infected with the 10 biological clones. All mice infected with 20 μL of 10^6 JEV-V1 to JEV-V9 succumbed to the infection from 6 to 9 days while the survival rates were 50% for JEV-V10 up to 21 days observation.

methods can be easily applied to quasispecies of other flaviviruses and other neurotropic viruses.

DISCLOSURE

Financial support: This work was supported by the HIR grant, University Malaya [Project number: UM.C/625/1/HIR/MOHE/MED/06] and Postgraduate Research Fund, University Malaya [Project number: PG285-2016A].

Conflict of interest: None

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