Pathological findings in a mouse model of Japanese encephalitis infected via the footpad

[1]Tzeh Long Fu *BSc*, [2]Kien Chai Ong *PhD*, [1*]Kum Thong Wong *MBBS FRCPath*

[1]*Department of Pathology and* [2]*Department of Biomedical Science, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia*

Abstract

We have developed and characterised a mouse model of Japanese encephalitis virus (JEV) infection via footpad inoculation in order to better mimic viral transmission by mosquito bites. Two-week-old and 5-week-old mice consistently developed signs of infection such as ruffled fur, weight loss, hunchback posture, tremors, mask-like facies and occasionally, hindlimb paralysis at 4 days post infection (dpi) and 11-13 dpi, respectively. Most of the animals died within 24 to 48 hours following the onset of signs of infection, with mortalities of 100% and 33.3% in 2-week-old and 5-week-old mice, respectively. Mild meningitis and variable parenchymal inflammation with formation of microglial nodules, focal necrosis and neuronophagia, and perivascular cuffing by inflammatory cells were observed in the caudate nucleus, putamen, thalamus, cerebral cortex, brainstem, and spinal cord. Viral antigens/RNA were demonstrated by immunohistochemisty and *in situ* hybridization, respectively, in most of these areas as well as in the hippocampus and cerebellum, albeit more focally. The pathological findings in this mouse model were generally similar to human Japanese encephalitis (JE) and other established JE models but perhaps, compared to other JEV mouse models, it demonstrates lethal encephalitic infection more consistently. We believe that our mouse model should be useful to study the pathogenesis of JE, and for testing anti-viral drugs and vaccines

INTRODUCTION

Japanese encephalitis virus (family: *Flaviviridae*; genus: *Flavivirus*) is an enveloped, single-stranded, positive-strand RNA virus of approximately 11kb. It was first isolated from the brain of a fatal case of encephalitis in 1934 in Japan.[1] Japanese encephalitis virus (JEV) and other encephalitis-associated flaviviruses, including West Nile virus (WNV) and Murray Valley encephalitis virus (MVEV), are antigenically closely-related, and comprises the JE serogroup of flaviviruses.[2] In terms of incidence, morbidity and mortality, JE is the most important viral encephalitis in Asia, with a higher incidence reported in children. Yearly, there are about 68,000 cases and 10,000-15,000 deaths.[2] JEV is transmitted by mosquitoes, primarily *Culex tritaeniorhynchus*, in a natural life cycle involving wild birds and pigs. After being bitten and infected by mosquitoes, humans are essentially dead-end hosts because significant viremia that can pass on the virus to other mosquitoes does not occur.

Most human JEV infections are asymptomatic[3] although mild symptoms that includes lethargy, fever, headache, anorexia, nausea, abdominal pain, vomiting and diarrhoea may occur.[4,5] Encephalitis is manifested clinically as altered consciousness, photophobia, nuchal rigidity, masked facies, muscle rigidity, cranial nerves palsies, abnormal movements and seizures.[6-8] Sensory impairment has been reported but appears to be rare.[9] Brain imaging studies have demonstrated prominent abnormalities in the thalamus[10,11], and also in the basal ganglia, midbrain, pons, cerebellum and cerebral cortex. Significant sequela in survivors in the form of severe cognitive and language impairment (20%) and persistent motor deficits (30%) may occur.[12] Pathological changes in the human central nervous system (CNS) consist of meningitis and parenchymal inflammation characterised by microglial nodules, neuronophagia, and perivascular cuffing by inflammatory cells.[13-15] JEV antigens and/or RNA were localised mainly in neurons, suggesting that neuronal viral cytolysis is important for pathogenesis.[13,15,16]

Experimental JEV infections have been investigated in the mouse, rat, hamster, monkey, rabbit and guinea pig models using different routes of infection, including intraperitoneal, intranasal,

Address Correspondence to: Prof. Wong KT, Department of Pathology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. Email: wongkt@ummc.edu.my

intracerebral, intradermal, subcutaneous and intravenous routes. Generally, neuronal infection in the CNS has been confirmed in some of these models.[17] Theoretically, footpad infection could more closely mimic the conditions of human infection by mosquito bites. Surprisingly, as far as we know, there is only one previous report of a mouse model infected by footpad inoculation in which 8- to 12-week-old mice showed varying degrees of neuroinvasion with 60% of mortality.[18] In this study, we describe 2-week-old and 5-week-old Japanese encephalitis (JE) mouse models that could be infected via footpad inoculation that mimics the natural route of infection. The pathological findings in this model were characterised to gain further insights into the infectious disease pathology, and to extend our knowledge on JEV neuroinvasion and pathogenesis.

METHODS

All animal experiments and associated protocols described in this manuscript were approved by the Animal Care and Use Committee, University of Malaya, and carried out in accordance with institutional guidelines (Ethics No.: 2013-05-07/PATHO/R/WKT).

Virus stock and titration

Mosquito *Stegomyia albopicta* C6/36 cells grown in RPMI-1640 (Sigma, USA) medium, supplemented with 10% fetal bovine serum (FBS) (FLOWLAB, Australia), were used for viral stock preparation and titration. The JEV (prototype Nakayama strain) stock used throughout the study were harvested from C6/36 cells after propagation for 7 days. All infected cells were maintained in RPMI-1640 maintenance medium supplemented with 5% FBS. Stock virus quantitation was done using a limiting dilution assay[19] combined with immunohistochemistry (IHC) to detect viral antigens in infected cell monolayers instead of the usual detection of viral cytopathic effects (CPE) to ensure greater reliability and accuracy since CPE may be difficult to determine. As described previously[20], sets of four wells of C6/36 cells were inoculated with tenfold serial dilutions of the virus (10^{-1}–10^{-8}) and 4 uninfected wells served as negative controls. The plate was then incubated for 7 days prior to IHC to detect viral antigens. At 7 days post infection (dpi), following washing and formalin fixation, the cells were routinely stain based on standard IHC method[13] except antigen retrieval step. All wells were then examined for

the presence of viral antigens by visual viewing under inverted microscope (Figure 1A) and the number of infected wells at each dilutions was recorded. The titre, 50% cell culture infectious dose ($CCID_{50}$), was determined using a modified Karber method.[21]

Animal Experiments

All experiments using live virus were conducted in a biosafety level 2 laboratory. The general susceptibility of two groups of Institute of Cancer Research (ICR) mice (n=6 per group) of different ages (2 and 5-week-old) was investigated. Each mouse was inoculated via the left hindlimb footpad virus (10^6 $CCID_{50}$/ml) in a volume of 20μl using a 0.3-ml Becton Disckinson Micro-Fine insulin syringe, 31 gauge needle (Becton and Dickinson, USA) The mice were then observed twice daily for signs of infection. Mice were sacrificed as soon as they showed severe signs of infection, whereas mice with no signs of infection were sacrificed at 21 dpi. Tissues were harvested from all animals for pathological analysis.

Histopathology

The whole animals were fixed in 10% neutral buffered formalin and cut transversely to obtain 8-10 tissue blocks each so that most tissues could be examined. Tissue blocks were first decalcified in 5% formic acid overnight and then routinely processed.[22] Four μm-thick sections from each block were stained with haematoxylin and eosin for light microscopy, IHC and *in situ* hybridisation (ISH) to detect viral antigens and RNA, respectively.

Immunohistochemistry (IHC)

IHC was performed as previously described. Briefly, dewaxed and rehydrated tissue sections were antigen-retrieved by boiling in Tris-EDTA buffer (pH 9.0, 0.05% Tween20) for 30 minutes. After overnight incubation with primary rabbit polyclonal antibody to JEV envelope protein (Dilution, 1:2500, GTX125867, GeneTex Inc., Taiwan), secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (DAKO, Denmark) for 30min followed by 3-3' diaminobenzidine tetrahydrochloride (DAB) (DAKO, Denmark), the sections were counterstained with Harris haematoxylin. Formalin-fixed parrafin embedded (FFPE) blocks of JEV-infected mouse brains were used as positive controls. Mock-infected mouse brains and mouse

brains infected with WN, tick born encephalitis (TBE) and dengue (DEN) viruses (courtesy of Noriyo Nagata, National Institute of Infectious Diseases (NIID), Japan) in the form of FFPE blocks were used as negative controls. Duplicate negative control IHC assays were also done by omitting the primary antibody.

In situ hybridisation *(ISH)*

As IHC to detect viral antigens was only flavivirus-specific, ISH was performed as previously described to localise viral RNA to confirm JEV infection.[13] Dewaxed and rehydrated tissue sections were treated with 0.2N hydrochloric acid and proteinase-K and hybridised overnight with JEV-specific DNA probes[13] in a standard hybridisation solution at 42°C. This is followed by sequential washing in saline-sodium citrate and incubation with 0.5% blocking reagent, alkaline phosphatase-conjugated, anti-digoxigenin antibody and nitroblue tetrazolium/5-bromo-4-chloro-indol-3-indolyl phosphate (Roche diagnostics, Germany) steps. The tissues were counterstained with Mayer haematoxylin. The positive and negative tissue controls were the same as the IHC assay. A "minus probe" ISH assay was included as a negative control.

RESULTS

The 2-week-old mice (n=6) started to show signs of infection at 4 dpi with death occurring between 5-6 dpi. Mortality was 100%. Mice from the 5-week-old group showed signs of infection between 11-13 dpi and a mortality rate of 33.3%. Signs of infection including ruffled fur, weight loss, hunchback posture, tremors, mask-like facies, and occasional hindlimb paralysis (Figure 1B) that did not differ significantly between groups. Infected mice were smaller and showed weight loss of 10-30% compared to mock-infected mice.

No macroscopic abnormalities in the footpads, oral cavities and internal organs were observed in infected mouse groups. Microscopic examination of the CNS showed mild to moderate meningitis and variable parenchymal inflammatory cell infiltration with formation of occasional microglial nodules (Figure 1C), focal necrosis, neuronophagia, and perivascular cuffing by inflammatory cells (Figure 1D), mainly in neuronal areas, such as cerebral cortex, thalamus, cerebellum, brainstem and spinal cord. Moderate neuronal loss and degenerated/necrotic neurons (Figure 1C) observed in cerebrum of the frontal lobe, were less prominent in other lobes. There were no viral inclusions or significant changes in the CNS white matter and non-CNS organs. No significant pathological changes were observed in 5-week-old mice that survived.

Viral antigens/RNA were detected in numerous neuronal cell bodies and processes in most of the neuronal areas, including caudate nucleus, putamen, cerebral cortex, brainstem and thalamus (Figure 1E-F). The hippocampus and cerebellum only rarely demonstrated neuronal viral antigens/RNA, and in the cerebellum, positive neurons were detectable mainly in the molecular layer and Purkinje cells (Figure 1G). In the spinal cord, antigens/RNA were localised predominantly in the gray matter, involving both anterior and posterior horn cells severely. (Figure 1H). No significant pathological changes or viral antigens/RNA were detected in mock-infected mice and infected mice showing no signs of infection. The JEV, MVEV and WNV-infected controls were all IHC positive, whereas normal mouse, TBE and DEN virus-infected brain tissues were negative with no significant background stanining. Since there was cross reactivity of the primary antibody to other flaviviral encephalitides, a specific ISH assay was used to confirm JEV infection and positive staining was obtained only in JEV controls.

DISCUSSION

We succesfully developed 2-week-old and 5-week-old mouse models of JE infected via footpad inoculation. Footpad inoculation is a combination of subcutaneous and intradermal routes[23] and hence is a preferable route of infection in a JE model[18], as well as in other mosquito-transmitted flaviviral encephalitides such as WN encephalitis and MVE.[24,25] The latter animal models showed viral neurotropism and occasional involvement of non-CNS organs and peripheral nervous system.[24,25] In our model, the highest density of viral antigens/RNA were localised primarily to neuronal bodies and processes in most of the neuronal areas including caudate nucleus, putamen, cerebral cortex, brainstem, thalamus and spinal cord. These results are in general agreement with human autopsy findings, where the preferential localization of JEV antigens were in the thalamus and brainstem.[15,16] Other parts of the brain including hippocampus, cerebellum and temporal cortex were also found to be infected significantly in human cases.[16] Our mouse model demonstrated only mild and focal viral antigen/
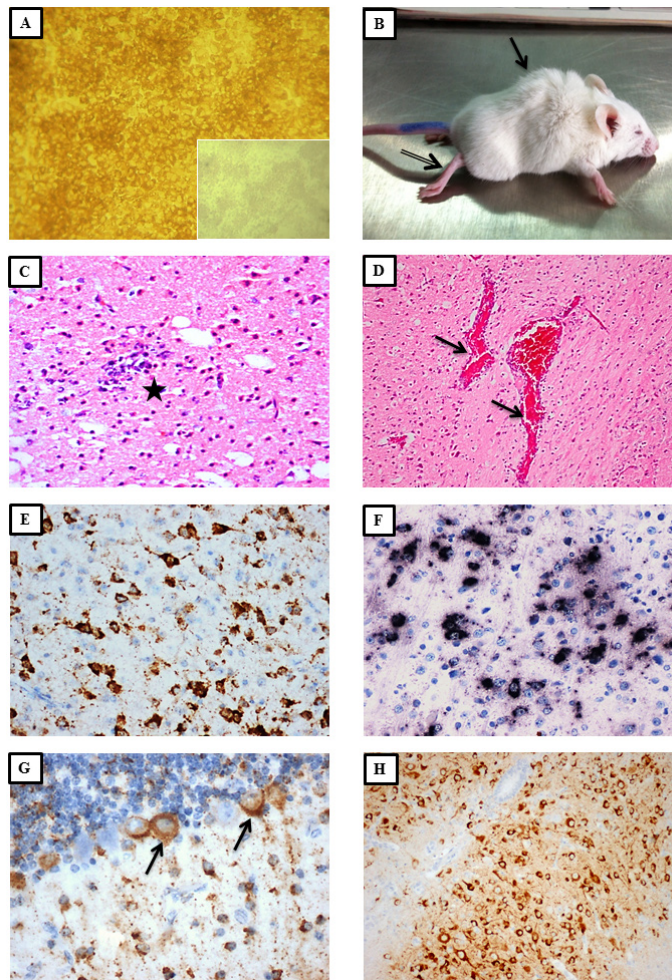
Figure 1. Viral titration, signs of infection and pathology of Japanese encephalitis virus in footpad mouse model. (A) JEV-infected C6/36 cells shows widespread of positive antigens, no antigen detected and no background staining in control (inset). (B) JEV-infected mouse shows a hunchback posture (black arrow), fur ruffling and hind-limb paralysis (double-line arrow). (C) Inflammatory cells and perivascular cuffing (D) in the cerebral cortex. Viral antigens/RNA in thalamus (E-F) and viral antigens in cerebellar Purkinjee cells (G) with minimal surrounding inflammation. (H) Viral antigens in gray matter of spinal cord. H&E stains (C-D), Immunoperoxidase with hematoxylin counterstain (A, E, G, H) and in situ hybridization with Mayer hematoxylin counterstain (F). Magnification: X10 objective (D, H); X20 objective (C, E, F); X40 objective (G).

RNA found in the hippocampus and cerebellum. No viral antigen/RNA was detected in glial cells and endothelial cells, despite evidence that these cells may support viral replication *in vitro*[26] and in some fatal cases.[16] The relatively widespread distribution of viral antigens/RNA in the model suggests a haematogenous route of infection similar to human JE.[15,16] It is likely that no detectable replicating virus in non-CNS organs were observed in our model because the tissues were harvested at late time points. Further studies at earlier time points may needed to investigate this. Compared to most of the other established JEV models, in terms of CNS and non-CNS infection, our results are generally similar.

Our data showed that 2-week-old mice succumbed to JEV infection with 100% mortality whereas in older 5-week-old mice, the mortality was lower at 33.3%. This is consistent with the observations following subcutaneous infection in another study in which older animals (38 to 284-day-old mice) are more resistant to infection.[27] In another study of 8 to 12-week-old C57BL/6 mice infected via the footpad with the Nakayama strain, using a wide range viral titers (10 to 10^5 plaque-forming unit)[18] for infection, a

mortality rate of approximately 60% was reported suggesting that the severity of JEV pathogenesis may be influenced by other factors including viral titer, mouse strain and virus strain.[17] Further investigations are needed to study the various factors that could impact on JEV infection in our mouse models.

In summary, 2-week-old and 5-week-old mouse models appear to recapitulate the major findings in human JE, including neurological signs and infection of various brain regions. The main advantage of our mouse model is that the CNS tissues could be consistently infected after footpad inoculation, whereas other reported mouse models mainly use intracerebral inoculation routes which are rather artificial. Our mouse model showed lethal encephalitic infection mimicking the progression of the disease from peripheral tissues to the CNS. Moreover, with better pathological characterisation of the mouse model for JEV, we would be in a better position to study viral pathogenesis, host factors and immune response, since autopsies of patients with JEV encephalitis are very hard to come by. All these unique features qualify this mouse model to be used to evaluate JEV anti-viral drugs and vaccines.

DISCLOSURE

REFERENCES

1. Monath TP. Japanese encephalitis--a plague of the Orient. *N Engl J Med* 1988; 319:641-3.
2. Campbell GL, Hills SL, Fischer M*, et al.* Estimated global incidence of Japanese encephalitis: a systematic review. *Bull World Health Organ* 2011; 89:766-74, 74A-74E.
3. Dutta K, Rangarajan PN, Vrati S, Basu A. Japanese encephalitis: pathogenesis, prophylactics and therapeutics. *CURRENT SCIENCE* 2010; 98:22.
4. Tsai T, Vaughn D, Solomon T. Flaviviruses (Yellow fever, Dengue, Dengue hemorrhagic fever, Japanese encephalitis, West Nile encephalitis, St Louis encephalitis, tick-borne encephalitis). *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases. th ed, Elsevier, Philadelphia* 2005;1927-50.
5. Kabilan L, Rajendran R, Arunachalam N*, et al.* Japanese encephalitis in India: an overview. *Indian J Pediatr* 2004; 71:609-15.
6. Chaudhuri N, Shaw BP, Mondal KC, Maity CR. Epidemiology of Japanese encephalitis. *Indian Pediatr* 1992; 29:861-5.
7. Burke D, Monath T. Flaviviruses. *Fields virology* 2001;1:1043-125.
8. Solomon T, Vaughn DW. Pathogenesis and clinical features of Japanese encephalitis and West Nile virus infections. *Curr Top Microbiol Immunol* 2002; 267:171-94.
9. Misra UK, Kalita J, Jain SK, Mathur A. Radiological and neurophysiological changes in Japanese encephalitis. *J Neurol Neurosurg Psychiatry* 1994; 57:1484-7.
10. Kalita J, Misra UK. Comparison of CT scan and MRI findings in the diagnosis of Japanese encephalitis. *J Neurol Sci* 2000; 174:3-8.
11. Verma R. MRI features of Japanese encephalitis. *BMJ Case Rep* 2012; 2012.
12. Mackenzie JS, Gubler DJ, Petersen LR. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat Med* 2004; 10:S98-109.
13. Wong KT, Ng KY, Ong KC*, et al.* Enterovirus 71 encephalomyelitis and Japanese encephalitis can be distinguished by topographic distribution of inflammation and specific intraneuronal detection of viral antigen and RNA. *Neuropathol Appl Neurobiol* 2012; 38:443-53.
14. Fujii Y, Kitaura K, Nakamichi K, Takasaki T, Suzuki R, Kurane I. Accumulation of T-cells with selected T-cell receptors in the brains of Japanese encephalitis virus-infected mice. *Jpn J Infect Dis* 2008; 61:40-8.
15. Johnson RT, Burke DS, Elwell M*, et al.* Japanese encephalitis: immunocytochemical studies of viral antigen and inflammatory cells in fatal cases. *Ann Neurol* 1985; 18:567-73.
16. Desai A, Shankar SK, Ravi V, Chandramuki A, Gourie-Devi M. Japanese encephalitis virus antigen in the human brain and its topographic distribution. *Acta Neuropathol* 1995; 89:368-73.
17. Kimura T, Sasaki M, Okumura M, Kim E, Sawa H. Flavivirus encephalitis: pathological aspects of mouse and other animal models. *Vet Pathol* 2010; 47:806-18.
18. Larena M, Regner M, Lee E, Lobigs M. Pivotal role of antibody and subsidiary contribution of CD8+ T cells to recovery from infection in a murine model of Japanese encephalitis. *J Virol* 2011; 85:5446-55.
19. Fischinger PJ, Dunlop NM, Blevins CS. Identification of virus found in mouse lymphomas induced by HIX murine oncornavirus. *J Virol* 1978; 26:532-5.
20. Schoepp RJ, Beaty BJ. Titration of dengue viruses by immunofluorescence in microtiter plates. *J Clin Microbiol* 1984; 20:1017-9.
21. Kärber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Archiv f. experiment. Pathol. u. Pharmakol* 1931; 162:480-83.
22. Ong KC, Badmanathan M, Devi S, Leong KL, Cardosa MJ, Wong KT. Pathologic characterization of a murine model of human enterovirus 71 encephalomyelitis. *J Neuropathol Exp Neurol* 2008; 67:532-42.
23. Kamala T. Hock immunization: a humane alternative to mouse footpad injections. *J Immunol Methods* 2007;328:204-14.

24. Hunsperger EA, Roehrig JT. Temporal analyses of the neuropathogenesis of a West Nile virus infection in mice. *J Neurovirol* 2006; 12:129-39.

25. McMinn PC, Dalgarno L, Weir RC. A comparison of the spread of Murray Valley encephalitis viruses of high or low neuroinvasiveness in the tissues of Swiss mice after peripheral inoculation. *Virology* 1996; 220:414-23.

26. Chen CJ, Ou YC, Lin SY*, et al.* Glial activation involvement in neuronal death by Japanese encephalitis virus infection. *J Gen Virol* 2010; 91:1028-37.

27. Miura T. A new method for a potency test of Japanese encephalitis vaccine. Direct challenge method on suckling mice by subcutaneous inoculation. *Bull World Health Organ* 1970; 43:553-7.