**TITLE:**

**A Murine Model of Dengue Virus-induced Acute Viral Encephalitis-like Disease**

**AUTHORS & AFFILIATIONS:**

Ting-Jing Shen1,2, Ming-Kai Jhan1,2, Jo-Chi Kao1,2, Min-Ru Ho1,2, Tsung-Ting Tsai1,2, Po-Chun Tseng1,2, Yung-Ting Wang1,2, Chiou-Feng Lin1,2,3

1Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei 110, Taiwan

2Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei 110, Taiwan

3Center of Infectious Disease and Signaling Research, National Cheng Kung University, Tainan 701, Taiwan

**Corresponding Author:**

Chiou-Feng Lin: cflin2014@tmu.edu.tw

**Email Addresses of Co-authors:**

Ting-Jing Shen: [bibobibo410@hotmail.com](mailto:bibobibo410@hotmail.com)

Ming-Kai Jhan: [williamjhan2730@gmail.com](mailto:williamjhan2730@gmail.com)

Jo-Chi Kao: [b614101036@tmu.edu.tw](mailto:b614101036@tmu.edu.tw)

Min-Ru Ho: [f250761@yahoo.com.tw](mailto:f250761@yahoo.com.tw)

Tsung-Ting Tsai: [minna1129@gmail.com](mailto:minna1129@gmail.com)

Po-Chun Tseng: [iluc0720@hotmail.com](mailto:iluc0720@hotmail.com)

Yung-Ting Wang: [olivia760717@gmail.com](mailto:olivia760717@gmail.com)

Chiou-Feng Lin: [cflin2014@tmu.edu.tw](mailto:cflin2014@tmu.edu.tw)

**KEYWORDS:**

Murine, dengue virus, encephalitis, neurotoxicity, intracerebral injection, intraperitoneal injection, suckling mice

**Abbreviations:**

BBB blood-brain barrier

CNS central nervous system

D2R dopamine D2 receptor

DENV dengue virus

ICR Institute of Cancer Research

NS nonstructural protein

PFU plaque forming units

RNA ribonucleic acid

SD standard deviation

TNF-α tumor necrosis factor α

WHO World Health Organization

**SUMMARY:**

Here, we prepare a protocol for creating an immunocompetent ICR murine model of CNS infection to display the development of neuropathy. Monitoring acute viral encephalitic disorders by the identical disease scores could be performed for showing DENV-induced neuropathy in vivo.

**ABSTRACT:**

Dengue virus (DENV), an arthropod-borne virus transmitted by mosquitoes, may cause the severe disease known as dengue hemorrhagic fever, which is characterized by lethal complications due to plasma leakage, ascites, pleural effusion, respiratory distress, severe bleeding, and organ impairment. A few cases of DENV infection present with neurological manifestations; however, studies have not explored DENV-induced neuropathogenesis further. In this study, we present a protocol to use an immunocompetent outbred Institute of Cancer Research (ICR) mouse for investigating the induction of central nervous system (CNS) infection with DENV followed by the progression of acute viral encephalitis-like disease.

**INTRODUCTION:**

Dengue virus (DENV), an arthropod-borne virus of the *Flaviviridae* family, contains a positive-sense RNA genome that encodes three viral structural proteins (capsid, premembrane, and envelope) and seven viral nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The four serotypes of DENV (DENV1-4), which infect approximately 390 million people annually, cause a global burden even though governments have directed substantial efforts toward mosquito vector and disease control 1. Currently, protective vaccines and therapeutic antiviral drugs are under development and require further long-term validation 2. In clinical practice, although dengue patient with central nervous system (CNS) infection is rare, it is needed for further exploring the diversity of dengue disease development 3. Further investigation and validation are needed; notably, the World Health Organization (WHO) has included the involvement of CNS impairment, such as cognitive impairment, convulsions, encephalopathy, and encephalitis, in the classification of severe dengue 3,4. Constructing animal models of DENV infection is indispensable for exploring the neuropathogenesis of DENV infection.

For generating CNS infection by DENV, several studies have executed different routes of DENV infection, including (1) intracerebral inoculation of C57BL/6 mice who received 4×103 plaque forming units (PFU) of non-adapted DENV3 5,6, (2) intraperitoneal inoculation of BALB/c mice who received 7×104 PFU of in vitro neuroadapted DENV4 7, (3) intracerebral inoculation of Swiss mice who received 1×105 PFU of in vivo neuroadapted DENV1 8, and (4) intracerebral and intraperitoneal coinoculation of ICR suckling mice who received 1×106 PFU of non-adapted DENV2 9. According to the findings 5-9, DENV infection in mice result in viral replication in the brain, leading to acute viral encephalitis-like syndromes, behavioral changes accompanied by limb paralysis and postural instability, CNS neurotoxicity and inflammation, general and localized plasma leakage through the blood-brain barrier (BBB), and lethality. All of results from these studies 5-9 have shown the ability of DENV to infect the CNS and the induction of acute viral encephalitis-like disease following infection.

Based on our current findings 9-15, we have created a murine model of DENV infection as an *in vivo* platform to examine the therapeutic efficiency of targeted agents/factors against viral replication as well as neurotoxicity. Here, we report the protocol we utilized to create an immunocompetent outbred ICR mouse to study CNS infection and to monitor the development of neuropathies with different severities caused by DENV. The results show the significant progression of encephalitis-like disease in DENV-infected mice in a time-dependent manner.

**PROTOCOL:**

Experimental protocols of animal study according to guidelines established by the Ministry of Science and Technology, Taiwan were approved by the Institutional Animal Care and User Committee of National Defense Medical Center (IACUC number: 16-261).

1. **Infection Procedure**
   1. Prepare non-adapted DENV2 (strain PL046) stocks 9, originally obtained from the Centers for Disease Control in Taiwan, ranging from 2.5×107 to 1×109 PFU/ml.
   2. Dilute the virus stock to 1×106 PFU with Roswell Park Memorial Institute 1640 medium (RPMI) to a total volume of 40 μL.
   3. Fill one 0.3-mL syringe fitted with a 30-gauge needle with 10 μL (2.5×105 PFU) of diluted virus, and fill another 0.3-mL syringe fitted with a 30-gauge needle with 30 μL (7.5×105 PFU) of diluted virus.
   4. Seven-day-old ICR suckling mouse was held and conducted the following steps:

1.4.1. For intracerebral injection, mice was held in a prone position by pressing the auricle between the index finger and thumb, and intracerebrally injected 10 μL diluted virus into the lambda area, the point at the intersection of the sagittal and lambdoid suture 16.

1.4.2. After intracerebral injection, mice was held in a supine position by using the index finger and thumb and intraperitoneally injected 30 μL diluted virus into the murine abdominal gently.

NOTE: To avoid cannibalism, 75% alcohol is used to make temporarily loss on mother’s olfactory sensation. Additionally, covering the litters with their mother’s stools and urines is suggested.

* 1. Lay the suckling mice back in their cages and wait for 5 min to check their safety poststimulation with the avidity of mice, including walking and mother milk sucking. Basically, mice represent normal activity poststimulation without harmful effects following the technical challenge.
  2. Animals are maintained in the Institutional Animal Care and User Committee of National Defense Medical Center, Taiwan. We next evaluate the daily progress of the mice in terms of body weight (by using Microbalance), acute viral encephalitis-like disease (by disease scoring as described below), and survival rate accordingly 9-15.

1. **Disease Scoring**
2. Monitor the grade of acute viral encephalitis-like illnesses as Score 0 for healthy mice; Score 1 for minor illness including weight loss, reduced mobility, and a hunchback body orientation; 2 for limbic seizure; Score 3 for limbic weakness, including moving with difficulty and anterior limb or posterior limb weakness; Score 4 for paralysis; and Score 5 for death.

NOTE: Once the score reaches 3, the disease symptoms are considered to be evolving rapidly.

1. Plot disease scoring, according to 2.2., for each day as a curve-based figure by using the mean ± SD of the daily test scores in each group.

**REPRESENTATIVE RESULTS:**

Severe dengue-associated neurological complications have been reported in patients for dengue pathogenesis 4. Although the cases are rare in clinic, creating an immunocompetent murine model of DENV infection can be used not only for studying immunopathogenesis but also for exploring CNS infection, neuroinflammation, neurotoxicity, and acute viral encephalitis-like disease. In this study, according to our current model, as summarized in the Protocol-Infectious Procedure section 9-15, seven-day-old ICR suckling mice were inoculated concurrently with DENV2 through intracerebral microinjection (2.5×105 PFU) and intraperitoneal injection (7.5×105 PFU) (**Figure 1**). Following infection, we performed numerous analyses to show the presence of dsRNA replication, viral protein expression, and virus release in DENV-infected brain 9-15. These experiments demonstrated that we established a successful *in vivo* model of DENV infection, replication, and release.

To evaluate the induction of acute viral encephalitis-like illnesses, as shown in the Protocol-Disease Scoring section 9-15, we monitored the changes in body weight in DENV-infected mice. Compared with the body weight changes in the mock group (mice were inoculated with RPMI medium only), **Figure 2** shows an increase in mock group but a significant (*P* < 0.01) decrease in body weight in infected mice. According to the symptoms of encephalitis-like illnesses, including hunchback posture, limbic seizure, limbic weakness, paralysis, and death, as existed in DENV-infected mice, **Figure 3** shows a significant (*P* < 0.05) increase in clinical score in the DENV-infected mice. The survival assay in **Figure 4** shows a time-dependent reduction in the survival rate (*P* < 0.001) of the DENV-infected mice. The data indicate the establishment of an infectious model of DENV in the CNS, which shows the progression of acute viral encephalitis-like illnesses.

**FIGURE LEGENDS:**

**Figure 1. Experimental design for dengue virus (DENV) infection in suckling mice.** Seven-day-old ICR suckling mice were inoculated with DENV2 PL046 by concurrent intracranial and intraperitoneal injections. We measured changes in body weight, disease score, and mortality.

**Figure 2. The body weight in suckling mice during dengue virus (DENV) infection.** In DENV2-infected ICR suckling mice (*n*=12), changes of body weight per day were measured. Following a Wilcoxon signed rank test, values are presented as the mean ± SD. \*\* *p <* 0.01.

**Figure 3. The encephalitic disease score in suckling mice during dengue virus (DENV) infection.** Following DENV2 infection in ICR suckling mice (*n*=12), time-kinetic changes in clinical scores with hunchback posture (score 1), limbic seizure (score 2), limbic weakness (score 3), paralysis (score 4), and death (score 5), were measured. Following a Wilcoxon signed rank test, values are presented as the mean ± SD. \* *p <* 0.05.

**Figure 4. The survival rate in suckling mice during dengue virus (DENV) infection.** Time-kinetic changes in survival rates were measured in DENV2-infected ICR suckling mice (*n*=12). Following a log-rank test, values are presented as the mean ± SD. \*\*\* *p <* 0.001.

**DISCUSSION:**

DENV infection has been detected in the CNS of severe dengue patients 3,17, suggesting the possibility of the manifestation of acute viral encephalitis occurred during dengue pathogenesis. Here, we report an *in vivo* murine model of DENV infection for studying the involvement of CNS dysfunction in severe dengue, particularly with a focus on DENV-induced acute viral encephalitis-like illnesses. As compared with the previous models, particularly for one-route infection (intravenous only, intraperitoneal only, or intracerebral only), an extremely high titer (1 x 108 PFU) of DENV is utilized in immunocompetent mice. However, in immunocompromised mice, a relative low but variant titer (1 x 105 to 1 x 108 PFU) of DENV can be performed 18-20. In this study, we used two-route infection to induce concurrent CNS and systemic infection with low titer (IC 2.5 x 105 PFU and IP 7.5 x 105 PFU) of DENV in immunocompetent ICR suckling mice. The progression of encephalitis-like disease is completely reproducible 9-15. The possible limitation of this study is therefore considered by using a concurrent intracranial and intraperitoneal injection. Although it is artificial, however, consistent with previous works by others 5-8 and ourselves 9-15, DENV is actively replicated in the experimental murine brain 9-15, and the possible effects of viral factors and host responses on neuroinflammation as well as neurotoxicity are therefore of interest.

For neuropathogenesis, the stimulation of neuronal cell apoptosis is triggered by DENV infection as well as CNS inflammation, such as microglial cell activation followed by pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) production 8,12,15. Direct neurotoxicity caused by DENV infection and indirect neuroinflammation initiated by host immune responses are speculated to be involved in dengue encephalitis. Acute viral encephalitis is caused by virus-induced CNS inflammation of the brain3,21. Common clinical symptoms are high fever, headache, stiff neck and back, vomiting, and confusion, while severe cases also develop seizures, paralysis, and coma 21. By monitoring the changes in scores with hunchback posture, limbic seizure, limbic weakness, paralysis, and death, the development of these illnesses shows the progression of acute viral encephalitis-like disease in DENV-infected mice 9-15. Generally, as shown in the Protocol-Disease Scoring section, those DENV-infected mice with a score of 3 rapidly progressed to paralysis and death. The pathogenic mechanisms causing these illnesses require further investigation; however, it is speculated that neuronal dysfunction followed by neuropathy induction is involved 21. Targeting viral replication as well as TNF-α-mediated CNS inflammation 10-14, at least in part, confers therapeutic effects against the progression of dengue-associated acute viral encephalitis-like disease. The protocol of this study could confer significant application for creating an encephalitis-like pathological model of DENV infection.

Some studies have described the induction of intracerebral hemorrhage induced by DENV infection in the brain 22,23. We previously showed the induction of BBB disruption in DENV-infected mice 9. Although DENV is not recognized as a neurotropic virus, DENV can produce effective infection in neuronal cells *in vivo* and *in vitro* 8,11. Dopamine D2 receptor (D2R) is speculated to be a neuronal receptor for DENV infection, and pharmacologically targeting D2R effectively reduces DENV infection in neuronal cells *in vivo* and *in vitro* as well as DENV-induced encephalitic illnesses 11,24. Possible therapeutic strategies against acute viral encephalitis are important to develop so that severe dengue cases with neurological complications can be treated. For the future application, our protocol of disease model using DENV infection in the brain may become an *in vivo* platform to screen not only the viral and host factors associated with neuropathogenesis but also possible antiviral and anti-encephalitic drugs. Regarding the incidence of variant neurological disorders caused by DENV infection 25, by our established protocol, the difference between the variable types and/or strains of DENV for causing neurological dysfunction is needed to validate.

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**DISCLOSURES:**

The authors have nothing to disclose.

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