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Tissue tropism and molecular characterization of a Japanese encephalitis virus strain isolated from pigs in southwest China

Lei Yuan[a,b,1], Rui Wu[a,b,1], Hanyang Liu[a,b], Xintian Wen[a,b], Xiaobo Huang[a,b], Yiping Wen[a,b], Xiaoping Ma[a,b], Qigui Yan[a,b], Yong Huang[a,b], Qin Zhao[c], Sanjie Cao[a,b,*]

[a] Research Center of Swine Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China
[b] Laboratory of Zoonosis, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China
[c] College of Food Science, Sichuan Agricultural University, Ya'an 625014, China

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ABSTRACT

Since September 2012, an epidemic has been spreading among swine in a pig farm located in Sichuan province, southwest China, which has resulted in abortion, stillbirth, and fetal mummification. The brains of stillborn pigs were collected and a previously unknown Japanese encephalitis virus (JEV), namely SCYA201201, was isolated. According to the results of agarose gel diffusion precipitation, indirect immunofluorescence analysis, neutralization testing, reverse transcription PCR (RT-PCR) amplification, and physical and chemical testing, the virus was conformed to have the characteristics of JEV. The virus titer in BHK-21 cells was $10^{8.47}$ PFU/ml and the median lethal dose (LD50) to 3-week-old and 7-day-old mice was 1.99 log10 and 1.02 log10 PFU/LD50, respectively. The results of tissue tropism for mice showed that the viral load in the brain was significantly higher than other organs, indicating that the isolate was strongly neurotropic. Additionally, the complete genome sequence of the isolate was determined and compared with other JEV strains. Phylogenetic analysis showed that the isolate belongs to genotype I and may be an imported virus. The isolate had 88.4% nucleotide identity with the Chinese vaccine strain SA14-14-2. However, there were 69 amino acid substitutions compared with the strain SA14-14-2. Some substitutions indicated that SCYA201201 was highly neurovirulent and infective, in accordance with the results of animal testing.

1. Introduction

Japanese encephalitis is a zoonotic, mosquito-borne, viral disease caused by Japanese encephalitis virus (JEV), which belongs to the family Flaviviridae, genus Flavivirus (Van den Hurk et al., 2009). At present, JEV infection remains the main cause of viral encephalitis in Asia (Erlanger et al., 2009). According to a estimate in 2011, 67,900 human JEV infections occur annually in endemic countries, with an incidence of 1.8 cases per 100,000 persons. Approximately 33,900 (50%) of these cases occur in China and approximately 51,000 (75%) occur in children aged 0–14 years (incidence, 5.4 cases per 100,000 children). JEV can cause human viral encephalitis, which is characterized by fever, chills, headache, spasm, and mental disturbances. The mortality rate can reach as high as 30% and approximately 30% to 50% of survivors will have permanent neurological sequelae (Campbell et al., 2011; Ghosh and Basu, 2009). Other than vaccination and mosquito eradication, there are no specific drugs or therapies for treatment of JEV infection (Huang et al., 2011). In recent years, JEV geographical endemic areas have expanded to Pakistan, Western Indonesia, Papua New Guinea, and northern Australia (Mackenzie and Williams, 2009).

After mosquito inoculation, JEV replicates in skin Langerhans dendritic cells, which transport the virus to the lymph nodes. Following induction of primary viremia, the virus spreads to other peripheral organs, where virus replication further enhances viremia and promotes entry into the central nervous system through the blood circulation (Li et al., 2012).

The JEV genome contains a 5' untranslated regions (UTR), followed by 10,296-nucleotide coding region and a 3' UTR. The only open reading frame (ORF) is translated into a large polyprotein that is cleaved into three structural and seven non-structural proteins by viral and cellular proteases (Nam et al., 2002). The JEVs are generally classified into five genotypes based on phylogenetic analysis of the nucleotide sequences of the C/PrM and E genes (Solomon et al., 2003).

* Corresponding author.
E-mail address: sdrcenter@sina.com (S. Cao).
[1] These authors contributed equally to this study.

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Table 1
Primers for RT-PCR amplification.

Primer	Genomic position	Sequence (5′–3′)	Amplification size (bp)
P1	240-265	GCTCTTATCACGTTCTTCAAGTTTAC	754
P2	969-993	TTCCCAGACAATTAAAACTGTAAGC	
JEV-1f	1-28	AGAAGTTTATCTGTGTGAACTTCTTGGC	1765
JEV-1r	1738-1765	GGAGGCCTCCCTCCTGTGACCCAAGAGC	
JEV-2f	1676-1699	GGAGAAACAGAGAACTCCTCATGG	1330
JEV-2r	2981-3005	GTGTTCTCCTCTCTAATTTTCAGCC	
JEV-3f	2884-2907	GATGGACCCGAGACAAAGGAATGC	1613
JEV-3r	4472-4496	GGAACACCGGGATCATCAATCAAGT	
JEV-4f	4404-4427	GATGGAGGCTGCAATCACAGGAAG	1625
JEV-4r	6005-6028	GGTATTCATCTCCAACTTGGTT	
JEV-5f	5938-5960	CTTGGAAACCCATCCCCCATAAC	1503
JEV-5r	7416-7440	CACTTGGCCGACTTTCTTTTGCATC	
JEV-6f	7334-7357	CAGCGGCTGGCATAATGAAGAATG	1542
JEV-6r	8852-8875	CTTCCTTAGTGCACAAGCGGGGTC	
JEV-7f	8797-8822	GAAGTGCTCAACGAGACCACCAACTG	1442
JEV-7r	10215-10238	CTGCCACACCAGATGTCCTCACGC	
JEV-8f	10126-10150	GTCTGGAACAGGGTATGGATTGAAG	840
JEV-8r	10939-10965	AGATCCTGTGTTCTTCCTCACCACCAG	

In 1949, JEV was first isolated in China and, since then, many JEV are isolated from humans, mosquitoes, and pigs and have been found in different geographical areas. Most JEV isolates reported in China before 2001 belonged to genotype III. More recently, JEV genotype I has been frequently isolated in northern and western China (Gao et al., 2013). Pigs, as the reservoir hosts of JEV, play a critical role during the transmission of the virus between mosquitoes and humans. In China, JEV has become a major pathogen of breeding disorders of pigs, and leads to severe economic losses to the swine industry. Meanwhile, the rapid development of the swine industry in China has increased the risk of JEV outbreaks. However, JEV strains originating from pigs are very limited. In this study, one JEV strain was isolated from the brain tissues of aborted piglets collected from a pig farm located in Ya'an city, Sichuan province, China, in 2012. The virulence and tissue tropism of the isolate were assessed using a mouse model, the complete genome was sequenced, and the phylogenetic relationships and amino acid mutations with other JEV strains from around the world were analyzed.

2. Materials and methods

2.1. Ethics statement

All procedures performed in the present study involving animals were approved by the Institutional Animal Care and Use Committee of Sichuan Agriculture University (Approval Number BK2014-047), Sichuan, China and followed the guidelines of the National Institutes of Health.

2.2. Sample collection

From June to October 2012, an epidemiological survey of JEV in swine in Sichuan province, China, was commenced. A midget pig farm that included a total of 128 sows with no JEV vaccine immunization located in the suburbs of Ya'an city in western Sichuan province was found to be infected with JEV in September 2012. Fetal abortion, stillbirth, and mummification occurred in 37 sows and most of the remaining sows were infertile. A total of eight brain samples of stillborn piglets and 30 serum samples from sows and breeding pigs were collected. The results showed that the all of brain samples were infected with JEV and 73% (22/30) of the serum samples were positive for JEV. All of the brain samples were detected for other viruses which can lead sow breeding obstacle, such as classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PPRSV), pseudorabies virus (PRV) and porcine parvovirus (PPV), and all the results were negative.

2.3. Viral culture

Baby hamster kidney (BHK-21) cell were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) calf serum, 100 U penicillin/ml and 100 mg streptomycin/ml at 37 °C in 5% CO2. Female Kunming mice were purchased from Chengdu Institute of Biological Products (Chengdu, China), were maintained in animal holding laboratory under controlled condition (temperature of 25 ± 10 °C, humidity of 40 ± 10%) with free access to standard mouse diet and water. Live JEV vaccine for swine was purchased from Wuhan Keqian Animal Biological Products Company (Wuhan, China) and maintained at a temperature of −70 °C.

The brain tissues of aborted and stillborn pigs were homogenized in phosphate-buffered saline (PBS) to produce a 50% (w/v) suspension. The supernatants were centrifuged at 12,000 × *g* for 20 min at 4 °C and strained through a filter with 0.22-μm pores. Five-day-old suckling mice were intracerebrally injected with 25 μl of the filtrate and symptoms of infection were observed every 12 h. On post-injection day 5, brain tissues from the infected mice were collected and homogenized. Then, the filtrate of the brain tissue suspension was harvested for the next round of inoculation. After three rounds in suckling mice, the filtrates of brain tissue suspensions were inoculated into baby hamster kidney cells(BHK-21) and incubated at 37 °C for 1.5 h for virus adsorption. The filtrate was removed, mixed with DMEM supplemented with 2% (v/v) calf serum, and incubated at 37 °C under an atmosphere of 5% CO2 for 120 h. Then, the cultures were harvested for the next round of inoculation until a stable cytopathic effect (CPE) was observed and the viruses were isolated using a conventional plaque purification technique.

2.4. Virus identification

The isolate were confirmed as JEV by agarose gel diffusion precipitation (AGID) analysis, indirect immunofluorescence analysis (IFA), and neutralization testing using a polyclonal antibody developed in rabbits immunized with an attenuated JEV vaccine. RT-PCR was performed with a C/PrM gene-specific primer pair targeting nucleotides 240–993. In addition, the cultures of isolated virus were subjected to RT-PCR or PCR for detection of some common causative agents, such as CSFV, PPRSV, PRV, PPV and *Streptococcus*