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BRIEF REPORT

Multiple amino acid variations in the nonstructural proteins of swine Japanese encephalitis virus alter its virulence in mice

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Abstract In a previous study, we performed serial brain-to-brain passages of swine Japanese encephalitis virus in mice and sequenced the complete genomes of the F5 and F20 passaged mouse-adapted variants. In the current study, we analyzed the differences between their genome sequences and found 12 amino acid substitutions in the nonstructural proteins. We also assessed the growth characteristics of these two variants in mammalian cells *in vitro* and *in vivo*. Our investigations revealed that the F20 variant had enhanced growth characteristics and modified virulence compared with the F5 variant. We therefore conclude that multiple amino acid substitutions in the nonstructural proteins of swine Japanese encephalitis virus alter its virulence in mice.

Keywords Japanese encephalitis virus · Swine · Mouse · Adaptation · Pathogenicity

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Japanese encephalitis virus (JEV) is an important mosquito-borne member of the genus *Flavivirus*, family *Flaviviridae*. JEV can infect humans and a variety of animals and is a major cause of viral encephalitis in eastern and southern Asia [2, 4, 6, 7, 9, 10, 13, 14, 16]. Pigs can harbor JEV and act as an amplifying host; therefore, human infections are closely associated with the prevalence of JEV in pigs. The disease caused by JEV in swine is characterized by abortion, stillbirth and weak offspring in pregnant sows, orchitis in boars, and neurological symptoms in piglets. Serological surveys on swine farms in eastern and southern Asia have indicated that JEV infection is widespread in pigs and causes great economic losses [4, 8, 13].

In 1988, we isolated and identified a strain of swine JEV, designated strain HW, from aborted fetal piglet brain tissue on a swine farm in Wuhan, China. In a previous study, we acquired different passages of mouse-adapted JEV variants by serial brain-to-brain passage in mice and determined the complete genome sequences of the F5 (designated HW, GenBank accession number: AY849939) and F20 (designated HWe, GenBank accession number: EF107523) passage variants. In this paper, we analyzed differences between the two genome sequences of these mouse-adapted variants and assessed their growth characteristics in mammalian cells *in vitro* and *in vivo*.

The JEV genome is a single-stranded, positive-sense RNA molecule, approximately 11 kb in length. It contains one long open reading frame (ORF) encoding, from 5′ to 3′, three structural proteins, namely, the capsid (C), membrane (M) and envelope (E) proteins, and the nonstructural proteins (NS1 to NS5) [11, 15]. The ORF is flanked by 5′ and 3′ non-coding regions (NCRs) approximately 95 and 585 nucleotides long, respectively. Sequence analysis showed that the genomes of strains HW and HWe were 10,977 and 10,976 nucleotides in length, respectively, because strain

HWe harbored a one-nucleotide deletion in the 3′ NCR (data not shown). Genome sequence comparisons revealed 33 nucleotide changes between the HW and HWe genomes, 29 of which were located in the coding region and four of which were located in the NCR. Of the 33 nucleotide changes, 12 (36%) resulted in amino acid substitutions within the viral proteins (Table 1). These were distributed across the nonstructural proteins, and included three codon changes in NS1 and NS2, one in NS3, one in NS4 and four codon changes in NS5 (Table 1).

We then investigated whether these 12 amino acid substitutions affect viral growth characteristics in mammalian cells *in vitro* and *in vivo*. First, viral propagation in primary hamster kidney cells was investigated by analyzing multiple replication cycles. Primary hamster kidney cells were inoculated with viruses of strain HW or HWe at a multiplicity of infection (MOI) of 0.01. After incubation at 37°C for 60 min, the virus suspension was removed and minimum essential medium (MEM) was added. Culture medium was collected every 12 h for 96 h after infection. The virus titer of each sample, expressed as the 50% tissue culture infectious dose ($TCID_{50}$), was calculated by the Reed–Müench method. Figure 1 shows the growth profiles of the HW and HWe viruses in primary hamster kidney cells. A significant difference was observed between the growth curves of the HW and HWe viruses. Although both HW and HWe viruses could be detected by 12 h post-infection and the viral titer peaked about 72 h post-infection, growth of the HWe virus was markedly accelerated compared with the growth of HW virus.

Table 1 Nucleotide and amino acid substitutions in Japanese encephalitis virus strain HWe

Genome segment	Substituted nucleotides	Substituted amino acids
5'-NCR	32 (C to G)	–
Membrane	626 (C to G)	–
	929 (A to G)	–
NS1	2710 (C to T)	Phe – Leu (78)
	2996 (G to A)	–
	3515 (T to C)	–
	3603 (T to C)	Phe – Leu (376)
	3669 (C to T)	Leu – Phe (398)
NS2	3805 (T to C)	Val – Ala (31)
	3812 (C to A)	Asn – Lys (33)
	4528 (G to T)	Ser – Ile (272)
NS3	4661 (C to T)	–
	4694 (G to A)	–
	5309 (C to A)	–
	5626 (C to T)	Thr – Ile (340)
NS4	6844 (C to T)	Ser – Leu (127)
	7151 (T to C)	–
	7238 (G to A)	–
	7334 (G to A)	–
	7583 (A to G)	–
	7614 (C to T)	–
	7622 (A to T)	–
NS5	7945(G to A)	Cys – Tyr (90)
	8585 (T to C)	–
	9156 (C to T)	–
	9389 (T to C)	–
	9766 (T to A)	Leu – Gln (697)
	9975 (C to T)	Arg – Trp (767)
	10008 (T to C)	Cys – Arg (778)
	10428 (T to C)	–
3'-NCR	10609 (A to G)	–
	10701 (G to –)	–
	10972 (A to G)	–

Amino acid positions are as per the sequence of JEV HW polyprotein

Next, we evaluated the ability of the HW and HWe viruses to replicate in a mammalian host (Table 2). Following anesthesia, four-week-old BALB/c mice were inoculated intracerebrally with a 20-μl virus suspension (10^2 $TCID_{50}$) of HW or HWe viruses. Brain tissue specimens were then collected from the mice on days 2–5 post-infection, and the virus was titrated in primary hamster kidney cells. The viral titer in the brain tissue specimens, expressed as the $TCID_{50}$, was calculated by the Reed-Müench method. Although the HW and HWe viruses both replicated well in mice at days 2–5 post-infection, the HWe virus replicated at a faster rate, and the viral titers were significantly higher in the HWe-infected group than in the HW-infected group.

To evaluate the morbidity and mortality associated with the HW and HWe viruses in mice, we infected BALB/c mice aged 4 weeks intracerebrally with the viruses at 10^2

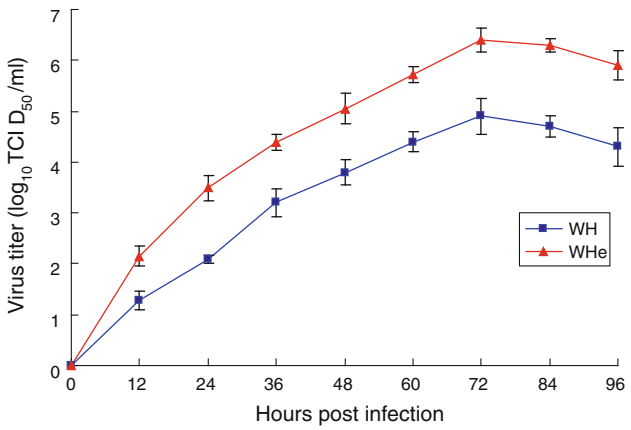

Fig. 1 Growth curves of HWe virus on primary hamster kidney cells. Primary hamster kidney cells were infected at an MOI of 0.01 with HWe (▲) or HW (■) viruses. At the indicated time points, infectious particles present in the supernatants were titrated using $TCID_{50}$ assays. Values are means ± SD

Table 2 Viral replication of HW and HWe viruses on different days in mice

Virus	Virus titer (log_{10} $TCID_{50}$/g, mean ± SD)			
	2 days	3 days	4 days	5 days
HW	2.13 ± 0.45	3.25 ± 0.20	4.34 ± 0.19	5.02 ± 0.34
HWe	2.75 ± 0.22[a]	4.16 ± 0.18[a]	5.48 ± 0.35[a]	6.27 ± 0.21[a]

BALB/c mice aged 4 weeks were inoculated intracerebrally with HW or HWe viruses at 10^2 $TCID_{50}$. On the indicated days after infection, viruses in mouse brains were titrated by $TCID_{50}$ assay on primary hamster kidney cells. The results are shown as means ± SD

[a] Significantly different from HW groups ($P < 0.05$) by Student's t test

$TCID_{50}$. Body weight and survival of the mice were recorded daily for 14 days post-infection (Fig. 2). All mice infected with HW and HWe viruses exhibited clinical signs including decreased activity, huddling, hunched posture, ruffled fur and viral encephalitis, but mice infected with HWe virus exhibited earlier and more severe symptoms compared with those infected with HW virus. Mice in the HWe group lost weight faster (Fig. 2A), and all of the mice in this group died within seven days of infection (Fig. 2B). Mice infected with the HW virus lost weight at a slower rate, and the time of death was delayed in these mice (Fig. 2A and B). Taken together, these results indicated that the HWe virus had higher virulence in mice than the HW virus.

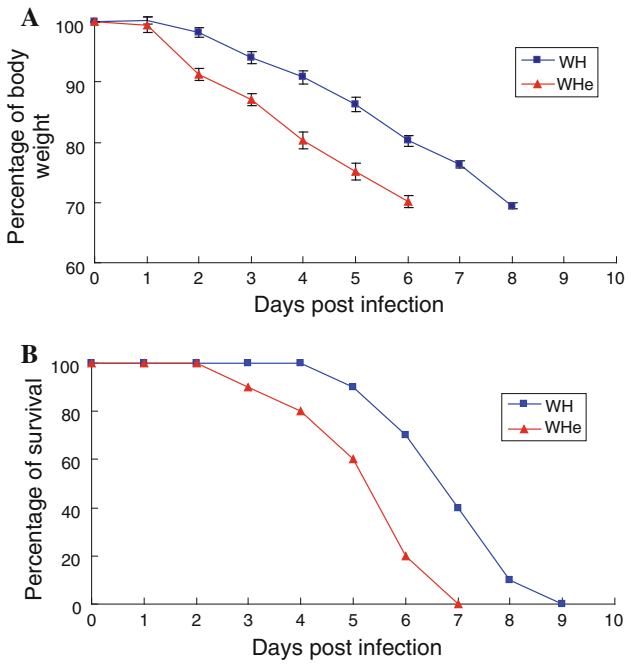

Fig. 2 Pathogenicity of HWe virus in mice. BALB/c mice aged 4 weeks were inoculated intracerebrally, after anesthesia, with HWe (▲) or HW (■) viruses at 10^2 $TCID_{50}$. Body weight (**A**) and survival (**B**) of mice were recorded daily for 14 days after infection

The nonstructural proteins of JEV play a role in viral replication, assembly, release and virulence. Venkatramana *et al.* reported that virus-specific cytolytic antibodies to NS1 protein effectively reduce the viral output from infected cells [3]. Lin *et al.* suggested that the NS5 protein is an interferon (IFN) antagonist that may play a role in blocking IFN-stimulated signaling via the activation of protein tyrosine phosphatases (PTPs) during JEV infection [5]. Other reports have identified the NS3 and NS5 proteins as major components of the viral RNA replicase complex associated with the 3′ NCR of genomic RNA in the initiation of viral replication [1, 12, 17]. In this study, we showed that 12 amino acid variations in the NS protein-encoding region of the JEV genome significantly altered viral growth characteristics and virulence. Further studies are now required to determine which of these amino acid mutations, or combinations of mutations, are responsible for altering viral characteristics and virulence.

In conclusion, we report that increased virulence of JEV in mice is associated with accelerated growth in mammalian cells and extended tissue tropism *in vivo*. We also report that multiple amino acid substitutions determine the virulence of JEV in mice. It is yet to be determined which of these substitutions, or combinations of substitutions, affect virulence of the virus in mice, and reverse genetics is currently being employed to address this issue.

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References

1. Chen CJ, Kuo MD, Chien LJ, Hsu SL, Wang YM, Lin JH (1997) RNA-protein interactions: involvement of NS3, NS5, and 3′ noncoding regions of Japanese encephalitis virus genomic RNA. J Virol 71:3466–3473
2. Huang JH, Lin TH, Teng HJ, Su CL, Tsai KH, Lu LC, Lin C, Yang CF, Chang SF, Liao TL, Yu SK, Cheng CH, Chang MC, Hu HC, Shu PY (2010) Molecular epidemiology of Japanese encephalitis virus, Taiwan. Emerg Infect Dis 16:876–878
3. Krishna VD, Rangappa M, Satchidanandam V (2009) Virus-specific cytolytic antibodies to nonstructural protein 1 of Japanese encephalitis virus effect reduction of virus output from infected cells. J Virol 83:4766–4777
4. Lim SI, Kweon CH, Tark DS, Kim SH, Yang DK (2007) Sero-survey on Aino, Akabane, Chuzan, bovine ephemeral fever and Japanese encephalitis virus of cattle and swine in Korea. J Vet Sci 8:45–49
5. Lin RJ, Chang BL, Yu HP, Liao CL, Lin YL (2006) Blocking of interferon-induced Jak-Stat signaling by Japanese encephalitis virus NS5 through a protein tyrosine phosphatase-mediated mechanism. J Virol 80:5908–5918
6. Nerome R, Tajima S, Takasaki T, Yoshida T, Kotaki A, Lim CK, Ito M, Sugiyama A, Yamauchi A, Yano T, Kameyama T, Morishita I, Kuwayama M, Ogawa T, Sahara K, Ikegaya A, Kanda M, Hosoya Y, Itokazu K, Onishi H, Chiya S, Yoshida Y,

Tabei Y, Katsuki K, Tabata K, Harada S, Kurane I (2007) Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. J Gen Virol 88:2762–2768
7. Nidaira M, Taira K, Onodera I, Morikawa T, Itokazu K, Kudaka J, Ohno A (2007) Detection of Japanese encephalitis virus antibody in a pig on Yonaguni Island, where all pigs were slaughtered in 1997. Jpn J Infect Dis 60:70–71
8. Nidaira M, Taira K, Okano S, Shinzato T, Morikawa T, Tokumine M, Asato Y, Tada Y, Miyagi K, Matsuda S, Itokazu K, Kudaka J, Nakamura M, Tamanaha K (2009) Survey of Japanese encephalitis virus in pigs on Miyako, Ishigaki, Kume, and Yonaguni Islands in Okinawa, Japan. Jpn J Infect Dis 62:220–224
9. Nitatpattana N, Dubot-Peres A, Gouilh MA, Souris M, Barbazan P, Yoksan S, de Lamballerie X, Gonzalez JP (2008) Change in Japanese encephalitis virus distribution, Thailand. Emerg Infect Dis 14:1762–1765
10. Ohno Y, Sato H, Suzuki K, Yokoyama M, Uni S, Shibasaki T, Sashika M, Inokuma H, Kai K, Maeda K (2009) Detection of antibodies against Japanese encephalitis virus in raccoons, raccoon dogs and wild boars in Japan. J Vet Med Sci 71:1035–1039
11. Sumiyoshi H, Mori C, Fuke I, Morita K, Kuhara S, Kondou J, Kikuchi Y, Nagamatu H, Igarashi A (1987) Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. Virology 161:497–510
12. Ta M, Vrati S (2000) Mov34 protein from mouse brain interacts with the 3′ noncoding region of Japanese encephalitis virus. J Virol 74:5108–5115
13. Yamanaka A, Mulyatno KC, Susilowati H, Hendrianto E, Utsumi T, Amin M, Lusida MI, Soegijanto S, Konishi E (2007) Prevalence of antibodies to Japanese encephalitis virus among pigs in Bali and East Java, Indonesia, 2008. Jpn J Infect Dis 63:58–60
14. Yamanaka T, Tsujimura K, Kondo T, Yasuda W, Okada A, Noda K, Okumura T, Matsumura T (2006) Isolation and genetic analysis of Japanese encephalitis virus from a diseased horse in Japan. J Vet Med Sci 68:293–295
15. Yang DK, Kim BH, Kweon CH, Kwon JH, Lim SI, Han HR (2004) Molecular characterization of full-length genome of Japanese encephalitis virus (KV1899) isolated from pigs in Korea. J Vet Sci 5:197–205
16. Yoshida Y, Tabei Y, Hasegawa M, Nagashima M, Morozumi S (2005) Genotypic analysis of Japanese encephalitis virus strains isolated from swine in Tokyo, Japan. Jpn J Infect Dis 58:259–261
17. Yun SI, Choi YJ, Song BH, Lee YM (2009) 3' cis-acting elements that contribute to the competence and efficiency of Japanese encephalitis virus genome replication: functional importance of sequence duplications, deletions, and substitutions. J Virol 83:7909–7930