Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004

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To characterize Japanese encephalitis virus (JEV) strains recently prevalent in Japan, JEV surveillance was performed in pigs from 2002 to 2004. Eleven new JEV isolates were obtained and compared with previous isolates from Japan and other Asian countries. All of the isolates were classified into genotype 1 by nucleotide sequence analysis of the E gene. Two new isolates with different levels of neurovirulence and neuroinvasiveness, but with only one nucleotide difference in the E gene, Sw/Mie/34/2004 and Sw/Mie/40/2004, were isolated at the same farm on the same day. Sw/Mie/40/2004 displayed higher neurovirulence and neuroinvasiveness in mice than the other four new isolates. Another new isolate, Sw/Hiroshima/25/2002, was neutralized by antiserum to Beijing-1 at a level similar to the homologous Beijing-1 strain, whilst seven other new isolates were neutralized at 10-fold-lower titres. However, there were no amino acid differences in the E protein among these eight isolates. The present study indicated that the 11 new JEV isolates were genetically similar, but biologically and serologically heterogeneous.

The GenBank/EMBL/DDBJ accession numbers for the E gene and 3′ NTR sequences of the 14 JEV isolates determined in this study (→ indicates ‘data not available’) are: Sw/Hiroshima/46/1998 (AB174837, →), Sw/Hiroshima/38/2000 (AB174838, →), Sw/Kagawa/24/2002 (AB112708, →), Sw/Kagawa/27/2002 (AB112707, AB231626), Sw/Hiroshima/25/2004 (AB231465, AB231621), Sw/Shizuoka/33/2002 (AB112703, AB231620), Sw/Shizuoka/39/2002 (AB112704, →), Sw/Chiba/68/2002 (AB112705, AB231622), Sw/Mie/41/2002 (AB112709, AB231623), Sw/Okinawa/285/2003 (AB238693, AB238694), Sw/Kagawa/35/2004 (AB231464, AB231627), Sw/Mie/34/2004 (AB231462, AB231624), Sw/Mie/40/2004 (AB231463, AB231625), JaTAn 1/94 (AB237171, AB237172).
**Japanese encephalitis virus** (JEV) is an arthropod-borne flavivirus. Approximately 50,000 cases of Japanese encephalitis (JE) with 10,000 deaths, mostly among children, are reported annually, mainly in China, South-East Asian countries and India (Tsai, 2000). The high fatality rate, frequent neuropsychiatric sequela in survivors and serious clinical symptoms make JE a significant public-health problem in many Asian countries (WHO, 1998). The WHO has therefore targeted JE as having high priority for second-generation vaccine development, and encouraged strengthening of JEV surveillance in Asian countries (Tsai, 2000). In recent decades, JE patients have been reported in previously non-endemic areas, including Badu Island in the Torres Strait, Australia, in 1995 (Hanna et al., 1996) and Papua New Guinea in 1997 (Spicer et al., 1999).

In Japan, more than 100 cases of JE were reported annually in the 1960s. The number of cases has decreased dramatically and fewer than 10 cases have been reported annually since the 1990s, due mainly to vaccination and to changes in rice-farming and pig-farming procedures. However, a high percentage of naive pigs seroconvert to JEV every year in most regions of Japan, from Okinawa to Honshu Island (Infectious Diseases Surveillance Center, 2004). Thus, JEV is still circulating in Japan and people are exposed to JEV.

Kuwayama et al. (2005) reported that JEV RNA was detected in cerebrospinal fluid samples from four of 57 aseptic meningitis cases from 1999 to 2002 in Hiroshima prefecture, Japan.

The genomic RNA of JEV is positive-sense, single-stranded and capped at the 5' end. It is approximately 11 kb in length and contains one long open reading frame. The gene order is 5'-C-preM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. The 5' and 3' non-translated regions (NTRs) are approximately 100 and 600 nt, respectively (Hashimoto et al., 1988; Sumiyoshi et al., 1987). The flavivirus E protein mediates a number of important biological functions (Rey et al., 1995), such as induction of neutralizing antibodies, protective immunity (Ali & Igarashi, 1999; Magada & Takegami, 1999), virulence (Zhao et al., 2003) and cellular tropism (McMinn, 1997; Paranjpe & Banerjee, 1996). To date, five genotypes have been reported based on the nucleotide sequence of the E gene (Hasegawa et al., 1994; Pyke et al., 2001; Solomon et al., 2003; Williams et al., 2000).

In order to define the characteristics of JEV isolates recently prevalent in Japan, we performed JEV surveillance in pigs in 10 prefectures from 2002 to 2004. In total, 11 JEV isolates were obtained from swine serum samples during this period. The nucleotide sequences of the E gene and 3' NTR were determined for these 11 new JEV isolates. They were then compared with a selection of JEV strains representing each genotype.

Swine blood samples were collected at farms in 10 prefectures in Japan and serum was separated. The serum samples were diluted and inoculated onto confluent Vero cell monolayers. Following adsorption for 1 h, fresh medium was added and the cells were incubated at 35 °C in 5% CO2. The cells were checked daily for cytopathic effect (CPE). When 50% CPE appeared, culture supernatant fluids were harvested and cellular debris was removed. Culture supernatants were examined for JEV by RT-PCR and/or real-time RT-PCR assays with primers JEE562s (5'-CTGGAGYTGTGARCCAAGGA-3') and JEEn623c (5'-GAHCCCCACGGTCATGA-3') and probe JEEn585p (5'-FAM-ACTRAACACTGAAGCGT-MGB-3') as described previously (Ito et al., 2004). JEV isolates were kept at −80 °C until use. JEV isolates were propagated similarly in Vero cells. Viral RNA was extracted from culture supernatant fluid by using a High Pure Viral RNA kit (Roche Diagnostics) and used for the synthesis of viral cDNA by using SuperScript III reverse transcriptase (Invitrogen).

The E gene and 3' NTR of the JEV genome were amplified by the use of a thermostable blend Taq DNA polymerase (TOYOBO), with primers JE955f (5'-TGYTTGTCCGCCTCCGGCTTA-3') and JE2536r (5'-AAGATGCCACTTCCACA-YCT-3') for amplification of the E gene and JE10141f (5'-TGGATTGAAGAAAATGAATGGATG-3') and JE10965r (5'-AGATGCTGTGTTCTTCTC-3') for amplification of the 3' NTR. PCR products were sequenced by using the ABI PRISM BigDye Terminator version 3.1 system and analysed by using an ABI PRISM 3100-Avant Genetic Analyzer (both from Applied Biosystems). Nucleotide sequences of the E gene were used to infer amino acid sequences by using GENETYX gene-analysis software (Genetyx Corporation). Multiple sequence alignments and phylogenetic analysis were done by using the CLUSTAL_X program (Thompson et al., 1994). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Nucleotide sequences of the 3' NTR of 15 JEV strains were also aligned by using GENETYX software.

The JEV strains used in this study are shown in Fig. 1(a). The names of 13 JEV strains analysed in this study were assigned according to the new nomenclature, which we proposed at the meeting of the WHO Steering Committee for the Development of JE and Other Flavivirus Vaccines in 2002. The nomenclature includes JEV(species)/host/place of isolation/isolate number/year of isolation, in order to clarify the origin of the isolate by name. The other 23 JEV strains analysed in this study were recorded as the original name published, with the name according to our nomenclature in parentheses (Fig. 1a).

In order to understand the evolutionary patterns of JEV, including the 11 new JEV isolates, we analysed a total of 36 JEV strains and Murray Valley encephalitis (MVE) virus (Fig. 1a) and constructed a phylogenetic tree on the basis of nucleotide sequences of the E protein, which plays essential biological roles in JEV infection (Takegami, 2003). All of the 11 new JEV isolates from swine were included in genotype 1 (G1) (Fig. 1a) and it was confirmed that the major genotype of JEV isolates changed from G3 to G1 in Japan in the early 1990s, as reported previously (Ma et al., 2003; Nga et al., 2004; Yoshida et al., 2005).
that the same genotype shift occurred in Korea (Nam et al., 1996; Yang et al., 2004) and in northern Vietnam (Nga et al., 2004). Twenty-four of the 28 G1 JEV strains isolated after 1992 appeared to form a new subcluster, which is enclosed by a dashed box in the phylogenetic tree (Fig. 1a). Furthermore, an amino acid change, threonine to methionine, was found at position E-129 in all of the 11 new isolates and in 13 old isolates that clustered in the new subcluster of G1, whereas three of the four old isolates, B2239, M859 and Th2372, grouped into the old subcluster of G1 and retained threonine at this position (data not shown). In addition, JEV strains belonging to G2, G3 and G4 also possessed threonine at E-129. One G1 JEV isolate, K91P55, possessed isoleucine at E-129, as does the Muar strain, belonging to G5 (Ali & Igarashi, 1997). These results suggested that the amino acid replacement at E-129 was a unique feature of the new JEV G1 isolates. There were no amino acid differences in the E protein among nine of the 11 new isolates, except for two isolates, Sw/Chiba/88/2002 and Sw/Okinawa/285/2003. The unique amino acids were valine for Sw/Chiba/88/2002 at position E-33 and alanine for Sw/Okinawa/285/2003 at position E-423, whereas the other new isolates contained isoleucine and threonine at the respective positions.

Sw/Mie/34/2004 and Sw/Mie/40/2004 were isolated from two serum samples collected at the same pig farm in Mie prefecture on the same day, 4 August 2004. These two isolates were located in the same branch of the phylogenetic tree, with one nucleotide difference in the E gene, as indicated by filled arrows (Fig. 1a). However, these two isolates demonstrated different levels of neurovirulence and neuroinvasiveness. Two other pairs of JEV isolates, Sw/Kagawa/24/2002 and Sw/Kagawa/27/2002, and Sw/Shizuoka/33/2002 and Sw/Shizuoka/39/2002, were isolated from the same farms in Kagawa and Shizuoka prefectures on the same day. They were located in the same branches of the phylogenetic tree. They were genetically identical, with 100% nucleotide similarity in the E gene, as indicated by empty arrows in Fig. 1(a). Chen et al. (1990) reported that JEV isolates from the same geographical region at close time points were very similar, but that genetic variation occurred among JEV isolates from diverse regions or those isolated at different time points in the same region. Interestingly, we obtained two new JEV strains from swine serum samples collected at the same farm on the same day, but they demonstrated different levels of virulence, suggesting that they are different strains.

The nucleotide sequences of the 3’ NTRs of 10 JEV isolates were determined and compared with those of five strains belonging to G1–G4 available in GenBank (Fig. 1a). These 10 isolates included nine new isolates and one previous isolate, JaTan1/94. The JEV isolates analysed in this study are printed in bold type in Fig. 1(b). The nucleotide alignment revealed two nucleotide regions: the variable region, which consists of approximately 60 nt, immediately downstream of the translation stop codon, and the conserved region, which consists of about 340 nt, at the extreme 3’ end (Gritsun et al., 1997). The polymorphic patterns of nucleotide deletions in the 3’ NTR variable region are demonstrated in Fig. 1(b). For convenience, codes a–e were assigned to the deletions, with the number of deleted nucleotides in parentheses. Deletions unique to each of the four genotypes were defined in the alignment, being different in position and length. The 10 JEV isolates and Ishikawa strain showed five deletions, with a code of a(2)-b(13)-c(1)-d(1)-e(2). This was a characteristic deletion pattern among the G1 JEV strains. Additionally, Sw/Kagawa/35/2004 showed a novel deletion in the variable region, which extended from nt 1 to 10 in code c. To confirm the result, the 3’ NTR sequences were also determined by direct RNA extraction from the two swine serum samples from which Sw/Kagawa/35/2004 and Sw/Mie/34/2004 were isolated. The sequences were the same as those determined for the isolates. However, the biological function(s) of this additional deletion in this strain remains unknown.

Eight new JEV isolates were analysed serologically by neutralizing assays with a hyperimmune mouse serum to strain Beijing-1 (Fig. 2). Neutralization activity was assessed by PRNT50 in Vero cells, as used for the governmental
potency test of the JE vaccine at NIID, Tokyo, Japan. The titre, expressed as the logarithm (log 10) of the reciprocal serum dilution, was calculated based on plaque numbers compared with those in the control wells, containing only a virus–diluent mixture. The PRNT50 titre was calculated by the method of Reed & Muench (1938). Hyperimmune mouse serum, collected from DDY mice immunized with a current vaccine strain, Beijing-1, was used as a control antiserum. Mice were immunized by intraperitoneal injections with the strain at 2 week intervals (0.5 ml per mouse per dose). In total, four injections were given: the inactivated antigen for the first injection, and live JEV for the second, third and fourth injections. Two weeks after the last injection, mice were bled and serum was separated and used as antibody. Beijing-1 and Nakayama-NIH, both of which belong to G3, were also included as reference strains. Anti-Beijing-1 serum neutralized the homologous Beijing-1 strain at a titre of 3.6 log10. The neutralizing-antibody titres to seven new isolates and Nakayama-NIH strain were about 10-fold lower (approx. 0.8–1. log10) than that to the Beijing-1 strain. It was of particular interest that the Sw/Hiroshima/25/2002 strain, which belonged to G1, was neutralized with a titre of 3.4 log10, as high as that for Beijing-1. The results were consistent in three independent experiments. However, there were no amino acid differences in the E protein between Sw/Hiroshima/25/2002 and the seven other isolates, as mentioned above. These results suggested that the epitopes responsible for neutralization variation may be located in part in the M protein, or that the difference in neutralizing titre is due to other reasons.

Five new G1 isolates and the Beijing-1 and Nakayama-NIH JEV strains were examined for neurovirulence and neuroinvasiveness (Table 1). Groups of 3-week-old female DDY mice (n=6) were inoculated intracerebrally with 30 µl, or intraperitoneally with 100 µl, ten-fold serially diluted virus solution. Mice were observed for 2–3 weeks after inoculation. Neurovirulence and neuroinvasiveness were assessed by 50 % lethal dose (LD50). LD50 was determined for each virus by the method of Reed & Muench (1938). DDY mice were purchased from Japan SLC Inc. All mice were maintained in a pathogen-free environment. All experiments were conducted in accordance with the Fundamental Rules for Animal Experiments (NIID, Tokyo, Japan). Beijing-1, a current vaccine strain, displayed high neurovirulence and neuroinvasiveness, with 0.98 and 2.54 log10 p.f.u. per LD50, respectively, whilst Nakayama-NIH, a former vaccine strain, displayed low neurovirulence, with 2.96 log10 p.f.u. per LD50, and did not kill mice when inoculated by the intraperitoneal route (i.e. >106 p.f.u. per LD50). Three

**Table 1.** Comparative analysis of neurovirulence and neuroinvasiveness of five new swine isolates and two vaccine (reference) strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Passage history</th>
<th>Neurovirulence*  (log10 p.f.u. per LD50)</th>
<th>Neuroinvasiveness† (log10 p.f.u. per LD50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beijing-1</td>
<td>smb37‡</td>
<td>0.98</td>
<td>2.54</td>
</tr>
<tr>
<td>Nakayama-NIH</td>
<td>amb29smb4§</td>
<td>2.96</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td><strong>2002 swine isolates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sw/Hiroshima/25/2002</td>
<td>Vero 5‖</td>
<td>2.48</td>
<td>5.13</td>
</tr>
<tr>
<td>Sw/Mie/41/2002</td>
<td>Vero 2</td>
<td>3.60</td>
<td>5.63</td>
</tr>
<tr>
<td><strong>2004 swine isolates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sw/Mie/34/2004</td>
<td>Vero 2</td>
<td>2.12</td>
<td>3.72</td>
</tr>
<tr>
<td>Sw/Mie/40/2004</td>
<td>Vero 2</td>
<td>0.75</td>
<td>2.67</td>
</tr>
<tr>
<td>Sw/Kagawa/35/2004</td>
<td>Vero 2</td>
<td>2.88</td>
<td>5.12</td>
</tr>
</tbody>
</table>

*Examined by intracerebral inoculation
†Examined by intraperitoneal inoculation.
‡Viruses were obtained after 37 passages through suckling mouse brain (smb).
§Viruses were obtained after 29 passages through adult mouse brain (amb) and four passages through smb.
‖Viruses were obtained after five passages on Vero cells.
new G1 isolates, Sw/Hiroshima/25/2002, Sw/Mie/41/2002 and Sw/Kagawa/35/2004, showed low levels of neurovirulence and neuroinvasiveness, with 2.48–3.60 and 5.12–5.63 log10 p.f.u. per LD50, respectively. On the other hand, Sw/Mie/40/2004 displayed high levels of neurovirulence and neuroinvasiveness, with 0.75 and 2.67 log10 p.f.u. per LD50, respectively, and Sw/Mie/34/2004 showed intermediate levels. These results indicate that Sw/Mie/40/2004 is a JEV isolate with high neurovirulence and neuroinvasiveness, like Beijing-1, and that there are significant differences in neurovirulence and neuroinvasiveness among JEV isolates belonging to G1. Nevertheless, there were no amino acid differences in the E protein between Sw/Mie/40/2004 and four other new G1 isolates. These results suggest that some other gene(s) contributed in part to the levels of neurovirulence and neuroinvasiveness, along with the E protein.

There has been widespread opinion that the dramatic decrease in the annual number of JE cases since the early 1990s is due to the attenuation of JEV in addition to other factors. However, the present study revealed that there is a recent G1 JEV isolate with high neurovirulence and neuroinvasiveness in mice (Table 1) and that there is also a wide range of diversity among JEV strains isolated in Japan from 2002 to 2004. It is therefore important to continue to isolate JEV strains from pigs, mosquitoes and humans and to compare multiple features of these isolates. Further studies will be needed to understand completely the genetic and biological characteristics of JEV strains circulating in Japan today.

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