Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99 % identity over the entire genome

Tsutomu Nishizawa,1 Masaharu Takahashi,1 Hitoshi Mizuo,2 Haruko Miyajima,3 Yuhko Gotanda3 and Hiroaki Okamoto1

1Immunology Division and Division of Molecular Virology, Jichi Medical School, Tochigi-Ken 329-0498, Japan
2Department of Internal Medicine, Kin-ikyo Chuo Hospital, Hokkaido 007-0870, Japan
3Japanese Red Cross Saitama Blood Center, Saitama-Ken 338-0001, Japan

The full-length genomic sequences were determined of Japanese swine and human hepatitis E virus (HEV) isolates (swJ13-1 and HE-JA1, respectively) with 100 % identity in the partial sequence of open reading frame (ORF) 2 (ORF2, 412 nt). swJ13-1 was isolated from a 4-month-old farm pig born in Hokkaido, Japan, in 2002 and HE-JA1 was recovered from a 55-year-old patient who lived in Hokkaido and who had contracted sporadic acute hepatitis E in 1997. Both isolates consisted of 7240 nt, excluding the poly(A) tail, and contained three ORFs (ORFs 1–3) that encoded proteins of 1707, 674 and 114 aa. The overall nucleotide sequence identity between them was 99-0 % and the deduced amino acid sequence identities of ORFs 1–3 were 99-8, 100 and 100 %, respectively. The high degree of genomic similarity observed between swine and human HEV isolates in a restricted area of Japan further supports the finding that sporadic hepatitis E in Japan is a zoonosis.

Hepatitis E virus (HEV), the causative agent of hepatitis E, is a single-stranded, positive-sense, naked RNA virus (Purcell & Emerson, 2001a). Hepatitis E is an important public concern in many developing countries in Asia, Africa and Latin America, wherein both epidemic and sporadic forms exist (Purcell & Emerson, 2001a). Recent studies have documented that HEV-associated hepatitis also occurs among individuals in industrialized countries with no history of travel to areas endemic for HEV (Harrison, 1999; Purcell & Emerson, 2001a; Schlauder & Mushahwar, 2001; Smith, 2001) and that HEV is a potential zoonotic virus, as suggested by the close genetic relationship between human and swine viruses (Erker et al., 1999; Hsieh et al., 1999; Meng et al., 1997; Pina et al., 2000; Wu et al., 2002) and experimental cross-species infection of swine HEV to a chimpanzee and rhesus monkeys (Meng et al., 1998). The genome of HEV is approximately 7·2 kb long and contains a short 5’ untranslated region (UTR), three open reading frames (ORFs 1–3) and a short 3’ UTR terminated by a poly(A) tract (Reyes et al., 1990; Tam et al., 1991). Based on sequence analyses, the HEV isolates identified worldwide have been classified tentatively into four major genotypes (Schlauder & Mushahwar, 2001). These include genotypes I (HEV isolates from several countries in Asia and Africa), II (HEV isolates from Mexico and Nigeria), III (HEV isolates from the United States, European countries and Argentina) and IV (HEV isolates from China and Taiwan) (Hsieh et al., 1999; Pina et al., 2000; Schlauder et al., 1998, 1999, 2000; Wang et al., 1999, 2000, 2001a; Worm et al., 2000; Zanetti et al., 1999). In Japan, multiple HEV strains of genotype III or IV have been isolated from patients with sporadic acute or fulminant hepatitis of non-A, non-B, non-C aetiology who had not travelled abroad (Aikawa et al., 2002; Mizuo et al., 2002; Suzuki et al., 2002; Takahashi et al., 2001, 2002a, b) and from farm pigs (Okamoto et al., 2001; Takahashi et al., 2003). In our previous study, we identified a pair of Japanese swine and human HEV isolates (swJ13-1 and HE-JA1, respectively) of genotype IV with 100 % identity in the 412 nt sequence of ORF2 (Takahashi et al., 2003). The genomic characteristics of swine and human HEV isolates are not understood fully and the complete nucleotide sequence of a genotype IV swine HEV has not been determined thus far. Therefore, in the present study, we determined and analysed the full-length genomic sequences of the swJ13-1 and HE-JA1 strains to clarify the genomic characteristics of a genotype IV swine HEV and the relatedness of the swine and human HEV isolates over the entire genome.

For full-length sequencing, total RNA was extracted from 300 µl swine serum (swJ13-1) or 400 µl human serum (HE-JA1) using TRizol-LS reagent (Invitrogen). The RNA preparation obtained was reverse-transcribed and subjected to...
nested PCR using the primers listed in Table 1. The central 7 kb sequence of each of the swJ13-1 and HE-JA1 genomes was divided into six overlapping sections and amplified as follows: nt 37–1270 (1234 nt) (primer sequences excluded) was amplified with primers HE090 and HE012 in the first round and HE092 and HE014 in the second round (HE090-HE012 and HE092-HE014); nt 1150–3142 (1993 nt) with primers HE011-HE136 and HE134-HE027; nt 3117–3899 (783 nt) with primers HE047-HE048 and HE049-HE050; nt 3889–5325 (1437 nt) with primers HE030-HE008 and HE031-HE009; nt 5251–6007 (757 nt) with primers HE067-HE036 and HE059-HE006; and nt 5985–7142 (1158 nt) with primers HE044-HE166 (5' - AAGGATCCGTCGACATC - 3'), representing a part of primer SSP-T (described below), and HE112-HE064. The extreme 5'-end sequence (nt 1–67) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM-RACE) with the First Choice RLM-RACE kit (Ambion), as described previously (Okamoto et al., 2001). Briefly, the extracted RNA that was treated with calf intestinal phosphatase and then with tobacco acid pyrophosphatase was ligated to an RNA adapter supplied in the kit and this was used as a template to synthesize cDNA with an HEV-specific antisense primer, HE035, and SuperScript II RNase H' reverse transcriptase (Invitrogen). The cDNA was then amplified by nested PCR with Takara Ex Taq (TaKaRa Shuzo) and the following primers: two RNA adaptor primers supplied in the kit were used as forward primers and HE034 and HE033 were used as reverse primers in the first and second round, respectively. Amplification of the extreme 3'-end sequence [nt 7117–7240 excluding the poly(A) tail] was attempted by the RACE method with a 20-mer primer (#167, 5'-CCGACATCGATAATACG-3') representing a part of a 41-mer oligonucleotide containing (T)15 [SSP-T, 5'-AAGGATCCGTCGACATCGATAATACG(T)15-3'] and an HEV-specific sense primer (HE024) on cDNAs that had been reverse-transcribed from poly(A)+ RNAs with the 41-mer oligonucleotide with (T)15, according to the method described previously (Okamoto et al., 2000). To avoid contamination during PCR procedures, the guidelines of Kwok & Higuchi (1989) were strictly observed. Amplification products were sequenced on both strands either directly or after cloning into pT7BlueT-Vector (Novagen) and sequence analysis was performed as

### Table 1. Positions and nucleotide sequences of oligonucleotide primers

Nucleotide positions are in accordance with the 7240 nt sequence of the swJ13-1 and HE-JA1 isolates obtained in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Polarity</th>
<th>Nucleotide position</th>
<th>Nucleotide sequence (5'→3')*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE033</td>
<td>Antisense</td>
<td>68–88</td>
<td>TAGAGCTGCTGCTGCTGATAGCC</td>
</tr>
<tr>
<td>HE034</td>
<td>Antisense</td>
<td>74–93</td>
<td>GCTGCTAGAGCTGCTGCTGCTC</td>
</tr>
<tr>
<td>HE035</td>
<td>Antisense</td>
<td>89–109</td>
<td>CAGGCGGAGGATTTGCGCTGCC</td>
</tr>
<tr>
<td>HE090</td>
<td>Sense</td>
<td>1–25</td>
<td>GAGAGGACACRTATATGTGTGCGAYGCC</td>
</tr>
<tr>
<td>HE012</td>
<td>Antisense</td>
<td>1283–1306</td>
<td>CTGRCGGGCRCGGGATRTARTTACGC</td>
</tr>
<tr>
<td>HE092</td>
<td>Sense</td>
<td>13–36</td>
<td>TGGTGTGCGAYGCGCATTGAGGCCCA</td>
</tr>
<tr>
<td>HE014</td>
<td>Antisense</td>
<td>1271–1296</td>
<td>GGGATRTARTACGCCAGACACTTC</td>
</tr>
<tr>
<td>HE011</td>
<td>Sense</td>
<td>1046–1068</td>
<td>ATGACGTACTYCGYGGYATTAG</td>
</tr>
<tr>
<td>HE015</td>
<td>Sense</td>
<td>3155–3178</td>
<td>ATGGTGTGAGGCGACGGGCGCTC</td>
</tr>
<tr>
<td>HE013</td>
<td>Sense</td>
<td>1126–1149</td>
<td>TGCAATTTGACTGCTGTATACCTGC</td>
</tr>
<tr>
<td>HE027</td>
<td>Antisense</td>
<td>3143–3165</td>
<td>AGGHRGGRCCTCATTCAATSACAC</td>
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<tr>
<td>HE047</td>
<td>Sense</td>
<td>3059–3079</td>
<td>CGTGTAGTTGGTAACCTGCTG</td>
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<tr>
<td>HE048</td>
<td>Antisense</td>
<td>3941–3961</td>
<td>TGGCGCCATACAGCACAGTGTAC</td>
</tr>
<tr>
<td>HE049</td>
<td>Sense</td>
<td>3096–3116</td>
<td>CGTCTTATAACACACACACCG</td>
</tr>
<tr>
<td>HE050</td>
<td>Antisense</td>
<td>3900–3920</td>
<td>TAGCGACACTATACAGCTGAG</td>
</tr>
<tr>
<td>HE051</td>
<td>Sense</td>
<td>3860–3882</td>
<td>GAGAGCTGACAGGCGCTBCCTCCTA</td>
</tr>
<tr>
<td>HE008</td>
<td>Antisense</td>
<td>5345–5370</td>
<td>GGGGTTGTGTTGATGATATAGGGGA</td>
</tr>
<tr>
<td>HE031</td>
<td>Sense</td>
<td>3860–3888</td>
<td>GARCGGCGCTBCTCTAYMTGCC</td>
</tr>
<tr>
<td>HE009</td>
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<td>5326–5347</td>
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<td>HE067</td>
<td>Sense</td>
<td>4990–5011</td>
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<td>HE036</td>
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<td>6337–6359</td>
<td>GCCACGAAATACTCTGCTGCG</td>
</tr>
<tr>
<td>HE006</td>
<td>Sense</td>
<td>5230–5250</td>
<td>CCTATGTYGCGGGCGGACCACCC</td>
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<tr>
<td>HE059</td>
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<td>6008–6028</td>
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<td>HE044</td>
<td>Sense</td>
<td>5953–5975</td>
<td>CAAGGHTGGCGYTCGTTGAGAC</td>
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<tr>
<td>HE112</td>
<td>Sense</td>
<td>5963–5984</td>
<td>GYTCGKTGGAGACCTGCTGTTG</td>
</tr>
<tr>
<td>HE064</td>
<td>Antisense</td>
<td>7143–7163</td>
<td>TCCCGGGTTTTACCCACTCTTC</td>
</tr>
<tr>
<td>HE024</td>
<td>Sense</td>
<td>7093–7116</td>
<td>CAGGCGTGYGCTTTTYCAGTCTACT</td>
</tr>
</tbody>
</table>

*R = A or G; Y = C or T; H = A, T or C; S = G or C; M = A or C; K = G or T.
described previously (Okamoto et al., 2001). A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) and the final tree was obtained using the TreeView program, version 1.6.6 (Page, 1996).

The swJ13-1 and HE-JA1 isolates each had a genomic length of 7240 nt, excluding the poly(A) tract at the 3′ terminus, and possessed three major ORFs similar to reported human and swine HEV isolates (Meng et al., 1997; Tam et al., 1991; Wang et al., 2000). In each isolate, ORFs 1–3 had a coding capacity of 1707 (nt 26–5146), 674 (nt 5146–7167) and 114 aa (nt 5174–5515), respectively, with the same strategy for translation of ORFs 2 and 3 as the reported genotype IV human HEV isolates whose entire nucleotide sequence is known (T1 and HE-JI4) (Wang et al., 2000; Takahashi et al., 2002b). The 5′ UTR of swJ13-1 and HE-JA1 each comprised 25 nt with the same sequence as that in the T1 and HE-JI4 strains. The 3′ UTR sequence of the swJ13-1 and HE-JA1 genomes each consisted of 70 nt [excluding the poly(A) tail]. The swJ13-1 and HE-JA1 isolates showed an overall nucleotide identity of 99.0-7% (7168/7240). The nucleotide sequences of the 5′ UTR of the two isolates were 100% identical to each other. The nucleotide sequence of the 3′ UTR of the two isolates differed by 1 nt within the 70 nt sequence, although this region showed appreciable diversity among the current two isolates and the four HEV isolates of genotype IV whose extreme 3′ end sequence is known, differing by up to 21-4% identity. The HE-JI1 isolate had the nucleotide G at nt 7177, while the swJ13-1 isolate had a T at this position, similar to JAK-Sai and T1 of human origin and swJ570 of swine origin (Fig. 1a). The swJ13-1 and HE-JA1 isolates were 98.9-7% (5063/5121), 99-4 (2009/2022) and 99.7-7% (341/342) similar to each other in the nucleotide sequence of ORFs 1–3, respectively. In other words, the two isolates differed as follows: by 58 nt in ORF1 (9 nt in the first codon position, 4 nt in the second codon position and 45 nt in the third codon position); by 13 nt in ORF2 (4 nt in the first codon position and 9 nt in the third codon position); and by only 1 nt in ORF3 (in the third codon position). At the amino acid sequence level, the two isolates were 99.8-7% (1703/1707), 100 and 100% identical to each other in ORFs 1–3, respectively. Two of the four amino acid differences were recognized within the hypervariable region of ORF1 and one each of the remaining two amino acid differences was seen just upstream and downstream, respectively, of this particular region. Of note, amino acid residues at positions 516 (Asn), 726 (Phe), 758 (Leu) and 938 (Thr) in ORF1 of the swJ13-1 isolate were unique to this isolate and they were not found in human HEV isolates of genotype IV or in other swine HEV isolates (Fig. 1b).

Comparison of the swJ13-1 and HE-JA1 genomes against 28 reported HEV genomes of genotypes I–IV, whose entire or nearly entire nucleotide sequences are known, revealed that they were closest to HE-JI4, a genotype IV human HEV isolate of Japanese origin (Takahashi et al., 2002b), with identities of 97-7–97-5%, but were only 84.0-7% similar to the prototype genotype IV HEV isolate (T1) of Chinese origin (Wang et al., 2000) in the nucleotide sequence of the full genome. In contrast, the swJ13-1 and HE-JA1 isolates were only 73-5-75-6% similar to the human and swine HEV isolates of genotypes I, II and III. The phylogenetic tree constructed based on the full genomic sequence confirmed that swJ13-1 and HE-JA1 belong to genotype IV and are most closely related to HE-JI4 (Fig. 2). When compared with swine HEV strains of genotype IV whose partial nucleotide sequence has been reported, the swJ13-1 isolate shared nucleotide identities of 97-8–99-8% with eight other isolates of Japanese origin in the nucleotide sequence of 412 nt (accession nos AB094219–AB094225 and AB094235). In contrast, swJ13-1 shared only 84-0–86-7% identities with five HEV isolates obtained from pigs in China in the nucleotide sequence of 300 nt (accession nos AJ428852–AJ428856), only 82-9–85-9% identities with four swine HEV isolates in Taiwan in the nucleotide sequence of 185–346 nt (accession nos AF077004, AF117280, AF117281 and AF302068) and merely 78-8–82-5% identities with 12 swine HEV isolates in Western India in the nucleotide sequence of 244–263 nt (accession nos AF324501–AF324504, AF405765–AF405767 and AF407560–AF407564). In addition, swJ13-1 was only 85-4–86-6% similar to six human HEV isolates of Vietnamese origin in the nucleotide sequence of 329 nt (accession nos AB075965–AB075970). These results indicate that Japanese swine HEV isolates of genotype IV are clearly separate from other subgroups of genotype IV to which the Chinese, Indian, Taiwanese and Vietnamese isolates are classifiable (Arankalle et al., 2002; Hijiikata et al., 2002; Hsieh et al., 1999; Wang et al., 2002; Wu et al., 2002). When compared with reported HEV strains of genotypes I–IV in the amino acid sequences of the gene products of ORFs 2 and 3 that are known to have several immunoreactive domains (Khudyakov et al., 1994; Yarbough et al., 1991), the swJ13-1 and HE-JA1 isolates shared 96-9–99-6% identities with other isolates of the same genotype but 89-5–93-9% identities with swine and human HEV isolates of genotypes I–IV, to the overall amino acid sequence of ORF2, and shared only 73-7–78-2, 76-3, 81-2–86-6 and 91-2–98-2% identities with HEV isolates of genotypes I–IV, respectively, over the entire amino acid sequence of ORF3. These results indicate that the sensitivities of immunoassays for antibodies to HEV may be increased by including antigens derived from different genotypes, as reported by Wang et al. (2001b), although it has been recently reported that the N-terminally truncated ORF2 antigens from the Meng strain of swine HEV (genotype III) and the Sar-55 strain of human HEV (genotype I), differing by approximately 5% at the amino acid level, are interchangeable with respect to their ability to detect antibodies to HEV (Engle et al., 2002).

Cross-species infection of HEV has been documented (Erker et al., 1999; Meng et al., 1998) and evidence that hepatitis E is a zoonosis is accumulating (Balayan, 1997; Harrison, 1999; Meng, 2000; Meng et al., 1999, 2002; Smith, 2001; Huang et al., 2002). A prototype swine HEV strain of genotype III isolated in the United States (the Meng strain) showed...
nucleotide sequence identity of 91.8% to a human HEV strain of US origin (US2) over the entire genome (7242 nt) (Erker et al., 1999; Haqshenas & Meng, 2001; Meng et al., 1997). Similarly, Taiwanese swine and human isolates of genotype IV HEV (swT74 and T821, respectively) were closely related with 97.3% identity in the 185 nt sequence within ORF2 (Hsieh et al., 1999). Wu et al. (2002) also showed that HEV strains present in Taiwanese farm pigs were genetically closely related to human HEV found in hepatitis patients in Taiwan, with a pair of swine and human HEV isolates (TW11SW and TW8E-2, respectively) having the highest identity of 99.0% in the 304 nt ORF2 sequence. Pina et al. (2000) reported the genetic identification of novel genotype III HEV strains from the sera of hepatitis patients (VH1 and VH2) and from sewage samples of animal origin from a slaughterhouse (E11) in Spain. The E11 strain was
most closely related to the Spanish VH1 and VH2 strains of human HEV, with 92–1–94·0 % identities over 304 nt of the ORF2 fragment. Recently, Wang et al. (2002) reported that Chinese swine HEV strains most closely resembled viruses isolated from Chinese patients with sporadic acute hepatitis, focusing on a pair of swine and human HEV strains in Beijing, China (G8 and T1, respectively), with 99·3 % identity over 300 nt of the ORF2 region. These reports suggest that in particular geographical regions, the HEV strains isolated from pigs and humans are genetically closely related. In the present study, we determined the full-length nucleotide sequences of the swJ13-1 and HE-JA1 isolates of HEV genotype IV and found that they had the same genomic length of 7240 nt, excluding the poly(A) tract at the 3′ terminus, and 99·0 % identity over the entire genome. On the amino acid sequence level, swJ13-1 and HE-JA1 shared 99·8, 100 and 100 % identities in ORFs 1–3, respectively, indicating that swJ13-1 and HE-JA1 express a capsid protein with an identical amino acid sequence and that they are antigenically indistinguishable from each other. Taken together with the reported evidence of the close relationship between HEV strains detected in pigs and those from patients with sporadic acute hepatitis E, our present data emphasize further that HEV is a potential zoonotic virus.

The Japanese swine HEV isolate, swJ13-1, was isolated in August 2002 from a serum sample obtained from a 4-month-old pig raised in a swine farm near Sapporo in Hokkaido. On the other hand, the Japanese human HEV isolate, HE-JA1, was recovered from a 55-year-old man who lived in Sapporo City in Hokkaido and who had contracted sporadic acute hepatitis E in December 1997. The infected patient in the current study had no history of travel to areas endemic for HEV, no known contact with persons who had travelled to endemic areas and no contact with farm pigs. There are accumulating lines of evidence that a number of animal species other than pigs, such as rats, mice, dogs, cows, sheep and goats, may act as natural reservoirs of HEV (Favorov et al., 1998, 2000; Kabrane-Lazizi et al., 1999; Purcell & Emerson, 2001b; Tien et al., 1997). Therefore, it is tempting to speculate that cross-species transmission occurs from pigs to other animals and that these animals act as vehicles to transfer HEV from pigs to humans, since HEV viraemia was detected at a high frequency of 15 % (113/750) among 3-month-old pigs and antibodies to HEV were highly prevalent among 4- to 6-month-old pigs in Japan (89 % or 1110/1250) in our previous study (Takahashi et al., 2003). Recently, He et al. (2002) reported that rodents were infected with a genotype I HEV with 95–96 % identities in the 405 nt ORF2 sequence with human HEV strains circulating in Nepal. Therefore, such studies should be expanded to the domestic and wild animals present in industrialized countries that have a higher chance of contact with humans to elucidate the infectious source(s) and route(s) for acquiring sporadic hepatitis E in low endemic countries, including Japan.

In conclusion, the finding that a pair of swine and human HEV strains of genotype IV with 99 % identity over the entire genome and with 99·8, 100 and 100 % identities in the amino acid sequences of ORFs 1–3, respectively, was present in a restricted area in Hokkaido where clinical HEV infection occurs most frequently in Japan (Mizuo et al., 2002), further supports the notion that HEV is a potential zoonotic virus. However, no direct evidence of HEV infection from swine to humans has been obtained. Further clinical and ecological studies on HEV infection in humans and candidate animals to clarify how domestic HEV strains...
circulating in animal species are transmitted to humans are warranted.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan. We are grateful to Professor M. Mayumi for advice and encouragement during this study.

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