Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food

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Among ten patients who contracted sporadic acute or fulminant hepatitis E between 2001 and 2002 in Hokkaido, Japan, nine (90 %) had a history of consuming grilled or undercooked pig liver 2–8 weeks before the disease onset. We tested packages of raw pig liver sold in grocery stores as food in Hokkaido for the presence of hepatitis E virus (HEV) RNA by RT-PCR. Pig liver specimens from seven (1?9 %) of 363 packages had detectable HEV RNA. Partial sequence analyses revealed that the seven swine HEV isolates belonged to genotype III or IV. One swine HEV isolate (swJL145) from a packaged pig liver had 100 % identity with the HE-JA18 isolate recovered from an 86-year-old patient in Hokkaido. Two swine HEV isolates (swJL234 and swJL325) had 98?5–100 % identity with the HE-JA4 isolate obtained from a 44-year-old patient in Hokkaido. These results indicate that inadequately cooked pig liver may transmit HEV to humans.

Hepatitis E virus (HEV) is an unclassified virus that is the causative agent of hepatitis E in many developing countries in Asia, Africa and Latin America where hepatitis E is an important public health concern (Purcell & Emerson, 2001). HEV is transmitted primarily by the faecal–oral route and waterborne epidemics are characteristic of hepatitis E. Recent studies have documented that sporadic acute hepatitis E also occurs among individuals in industrialized countries with no history of travel to areas endemic for HEV (Harrison, 1999; Purcell & Emerson, 2001; Smith, 2001). The genome of HEV is a single-stranded, positive-sense RNA of approximately 7·2 kb in length and contains a short 5′ untranslated region (UTR), three open reading frames (ORF1, ORF2 and ORF3) and a short 3′ UTR terminated by a poly(A) tract (Reyes et al., 1990; Tam et al., 1991). HEV sequences have tentatively been classified into four major genotypes (I–IV) (Schlauder & Mushahwar, 2001). The majority of HEV infections are caused by genotype I in several developing countries in Asia and Africa and by genotype II in Mexico and Nigeria, while only isolated cases of infection with HEV of genotype III or IV have been described in the USA, European countries, Argentina, China and Taiwan (Hsieh et al., 1999; Pina et al., 2000; Schlauder et al., 1998, 1999, 2000; Wang et al., 1999, 2000, 2001; Worm et al., 2000; Zanetti et al., 1999). In Japan, multiple HEV strains of genotype I, III or IV have been isolated from Japanese patients with sporadic acute or fulminant hepatitis E (Takahashi et al., 2001, 2002a, b; Mizuo et al., 2002; Suzuki et al., 2002).

Risk factors for HEV infection among patients with sporadic cases of hepatitis E in industrialized countries have not been defined. Recently, we found that nine of ten patients who contracted sporadic acute or fulminant hepatitis E in 2001 and 2002 in Hokkaido, where hepatitis E is most prevalent in Japan, had ingested grilled or undercooked pig liver 2 weeks to 2 months before the disease onset. Therefore, in the present study, we tested packages of raw pig liver sold in grocery stores in Hokkaido as food for the presence of HEV RNA, and determined and analysed the partial nucleotide sequences of HEV isolates obtained from these pig livers and those recovered from patients with sporadic acute or fulminant hepatitis E to determine whether hepatitis E in Japan is likely to be food-borne.
A total of 38 patients with a clinical diagnosis of sporadic acute or fulminant hepatitis were seen at two city hospitals in Sapporo and Kitami on Hokkaido Island, Japan, between January 2001 and December 2002. Hepatitis A was diagnosed in three patients, type B acute hepatitis in five patients and type C acute hepatitis in only one patient. Among the remaining 29 patients with acute or fulminant hepatitis of non-A, non-B, non-C aetiology, ten patients (34%) had anti-HEV IgG and IgM antibodies detectable by in-house ELISA (Mizuo et al., 2002) and were diagnosed with acute or fulminant hepatitis E (see Table 1). Serum samples were obtained from the ten patients at admission and stored at −20 °C or below until testing. Informed consent was obtained from each patient. A total of 363 packages of raw pig liver, obtained from domestic pigs raised in Hokkaido and sold as food, was purchased from 25 grocery stores in Sapporo and Kitami on Hokkaido Island, Japan, between 19 December 2002 and 26 February 2003. The pig liver in each package had been processed as slices or a block of 103–819 g (mean 207.4 g). Several pieces of tissue specimens (100–200 mg) were obtained from each package and stored at −80 °C until testing.

Total RNA was extracted from 100 μl human serum using TRIZOL LS reagent (Invitrogen) and dissolved in 10 μl nuclease-free distilled water. For the pig livers, a piece (100 mg) was homogenized and total RNA was extracted using TRIZOL reagent and dissolved in 100 μl nuclease-free distilled water. The RNA preparation thus obtained (10 μl) was subjected to nested RT-PCR with ORF2 primers as described previously (Mizuo et al., 2002). RNA that had been extracted from each pig liver specimen contaminated with HEV was serially diluted 10-fold in distilled water containing 20 μg glycogen ml⁻¹ (Roche Diagnostics) and tested for HEV RNA by nested RT-PCR as described above. The titre of HEV RNA was expressed as the highest dilution (10⁻³⁶) testing positive. To confirm the presence of HEV RNA, a part of the ORF1 region was amplified by nested RT-PCR with ORF1 primers as described previously (Mizuo et al., 2002). To avoid contamination during PCR procedures, the guidelines of Kwok & Higuchi (1989) were strictly observed. The amplification products were sequenced directly on both strands and sequence analysis was performed as 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).

Of the ten patients studied (Table 1), eight developed acute hepatitis E and had peak total bilirubin levels of 5.6–26.0 mg dl⁻¹, peak alanine aminotransferase levels of 1276–4100 IU l⁻¹ and peak aspartate aminotransferase levels of 1193–4650 IU l⁻¹. The abnormal liver function test values normalized rapidly within 1 month in seven patients, but persisted for longer than 3–5 months in the remaining patient (Patient 9); who was 86 years of age and had severe prolonged jaundice. Two patients (Patients 6 and 8) contracted fulminant hepatitis E; Patient 6 died of hepatic failure 56 days after the disease onset. In Patient 8, the episode of encephalopathy resolved 2 weeks after admission, but severe jaundice and coagulopathy persisted for longer than 2.5 months and the patient died of hepatic failure 105 days after the onset of hepatitis E. The sera from all ten patients at admission were positive for HEV RNA. The HEV isolates obtained from two patients were of genotype III and those from the remaining eight patients were of genotype IV.

Table 1. Profiles of the 10 patients who contracted sporadic acute or fulminant hepatitis E

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Date of disease onset</th>
<th>Name of HEV isolate</th>
<th>Yes or no</th>
<th>Place</th>
<th>Frequency (last day of consumption)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>AH</td>
<td>22 May 2001</td>
<td>HE-JA12</td>
<td>Yes</td>
<td>At home</td>
<td>Two to three times a year (1 or 2 months ago)</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>AH</td>
<td>30 May 2001</td>
<td>HE-JA13</td>
<td>Yes</td>
<td>At home</td>
<td>Twice a month (2 weeks ago)</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>AH</td>
<td>13 November 2001</td>
<td>HE-JA14</td>
<td>Yes</td>
<td>At home</td>
<td>Two to three times (1 or 2 months ago)</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>AH</td>
<td>14 July 2002</td>
<td>HE-JA15</td>
<td>Yes</td>
<td>At home</td>
<td>Two to three times (1 or 2 months ago)</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>AH</td>
<td>16 August 2002</td>
<td>HE-JA16</td>
<td>Yes</td>
<td>At home</td>
<td>Once a month (1 month ago)</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>FH</td>
<td>28 September 2002</td>
<td>HE-JF4</td>
<td>Unknown</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
<td>AH</td>
<td>8 November 2002</td>
<td>HE-JA17</td>
<td>Yes</td>
<td>At home</td>
<td>Only once (41 days ago)</td>
</tr>
<tr>
<td>8</td>
<td>58</td>
<td>FH</td>
<td>23 November 2002</td>
<td>HE-JF5</td>
<td>Yes</td>
<td>At home</td>
<td>One to two times a year (1 month ago)</td>
</tr>
<tr>
<td>9</td>
<td>86</td>
<td>AH</td>
<td>30 November 2002</td>
<td>HE-JA18</td>
<td>Yes</td>
<td>At home</td>
<td>Seven consecutive days (19 days ago)</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>AH</td>
<td>19 December 2002</td>
<td>HE-JA19</td>
<td>Yes</td>
<td>At</td>
<td>Once a month (1 month ago)</td>
</tr>
</tbody>
</table>

*All ten patients were male.
†AH, acute hepatitis; FH, fulminant hepatitis.
‡Patients 2, 8 and 10 also ingested undercooked pig intestine/colon twice a month (2 weeks ago), once a month (1 month ago) and once a month (1 month ago), respectively.
§Frequency or number of times of consumption of pig liver (last day of consumption of pig liver in relation to the day of onset of hepatitis E).
All ten patients had no history of travel outside Japan or contact with travellers who had been abroad or foreigners, no contact with pigs and no history of blood transfusion in the preceding 6 months. Of particular interest, Patient 9 reported consumption of cooked pig liver 19–25 days before the onset of hepatitis E. According to his detailed statement at admission, he lived alone and, for the first time, had purchased processed raw pig liver from a grocery store located near his residence and then fried slices of pig liver at home and ingested it for 7 consecutive days. He became aware of jaundice and felt general malaise 19 days after the last day of consumption of the pig liver. His report prompted us to interview the remaining nine patients to ask whether they had consumed pig liver before the onset of illness. Surprisingly, eight of the nine patients recalled eating grilled pig liver 2 weeks to 2 months before the onset of hepatitis E (Table 1). For Patient 6, who died due to hepatic failure, in reply to our interview, his wife told us that they had never eaten pig liver at home, but she could not deny that he could have consumed undercooked pig liver while drinking alcohol at Japanese-style bars; he had had a history of alcohol intake for 30 years. When we interviewed 22 randomly selected patients with acute hepatitis of non-E aetiology who were seen at our hospitals between January 2001 and December 2002, they all denied consumption of pig livers before the disease onset, indicating that consumption of pig livers is significantly associated with the occurrence of hepatitis E among patients with acute or fulminant hepatitis (0/22 versus 9/10, P < 0·0001; Fisher’s exact test).

To investigate whether raw pig liver as food is contaminated with HEV, a total of 363 packages of raw pig liver, purchased from grocery stores in Hokkaido where the patients lived, were tested for HEV RNA. Pig liver specimens from seven patients (swJ15-5), AB094276 (swJ18-2), AB094263 (swJ15-8), AB094255 (swJ13-2) and AB094209 (swJ2-3).  

*DDBJ/EMBL/GenBank accession numbers of the known isolates are AB082562 (HE-JA6), AB082565 (HE-JA9), AB082560 (HE-JA4), AB094260 (swJ15-5), AB094276 (swJ18-2), AB094263 (swJ15-8), AB094255 (swJ13-2) and AB094209 (swJ2-3).

†Obtained from Patient 9 in the present study.

Table 2. Viral load and genotype of HEV in the pig livers and nucleotide sequence identities of swine HEV isolates obtained in the present study

<table>
<thead>
<tr>
<th>Pig liver no.</th>
<th>Store</th>
<th>Date of sale</th>
<th>Package (weight)</th>
<th>HEV RNA (copies g⁻¹)</th>
<th>HEV genotype</th>
<th>Name of HEV isolate</th>
<th>HEV isolate with the highest nucleotide identity among the known isolates (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
<td>A</td>
<td>12 January 2003</td>
<td>A block (313 g)</td>
<td>10⁵</td>
<td>III</td>
<td>swJL82</td>
<td>HE-JA6 (94-4) swJ15-5 (94-4)</td>
</tr>
<tr>
<td>97</td>
<td>B</td>
<td>12 January 2003</td>
<td>Sliced (103 g)</td>
<td>10⁶</td>
<td>III</td>
<td>swJL97</td>
<td>HE-JA9 (92-9) swJ18-2 (92-7)</td>
</tr>
<tr>
<td>98</td>
<td>B</td>
<td>12 January 2003</td>
<td>Sliced (103 g)</td>
<td>10⁷</td>
<td>III</td>
<td>swJL98</td>
<td>HE-JA9 (92-9) swJ18-2 (92-7)</td>
</tr>
<tr>
<td>131</td>
<td>C</td>
<td>1 February 2003</td>
<td>Sliced (288 g)</td>
<td>10²</td>
<td>III</td>
<td>swJL131</td>
<td>HE-JA6 (91-0) swJ15-8 (92-2)</td>
</tr>
<tr>
<td>145</td>
<td>D</td>
<td>1 February 2003</td>
<td>Sliced (146 g)</td>
<td>10⁴</td>
<td>IV</td>
<td>swJL145</td>
<td>HE-JA18* (100) swJ13-2 (90-8)</td>
</tr>
<tr>
<td>234</td>
<td>E</td>
<td>14 February 2003</td>
<td>Sliced (107 g)</td>
<td>10⁵</td>
<td>III</td>
<td>swJL234</td>
<td>HE-JA4 (98.5–100) swJ2-3 (93.4–95.9)</td>
</tr>
<tr>
<td>325</td>
<td>F</td>
<td>15 February 2003</td>
<td>A block (320 g)</td>
<td>10⁴</td>
<td>III</td>
<td>swJL325</td>
<td>HE-JA4 (98.5–100) swJ2-3 (93.4–95.9)</td>
</tr>
</tbody>
</table>

* DDBJ/EMBL/GenBank accession numbers of the known isolates are AB082562 (HE-JA6), AB082565 (HE-JA9), AB082560 (HE-JA4), AB094260 (swJ15-5), AB094276 (swJ18-2), AB094263 (swJ15-8), AB094255 (swJ13-2) and AB094209 (swJ2-3).
† Obtained from Patient 9 in the present study.
human HEV isolates (HE-JA6 or HE-JA9), with nucleotide sequence identities of 91.0–94.4%. A phylogenetic tree was constructed based on the common 412 nucleotide sequence of human HEV isolates of Japanese or non-Japanese origin, including those obtained in the present study and the seven swine HEV isolates obtained in the present study (Fig. 1). As illustrated in Fig. 1, swJL145 was most closely related to HE-JA18 and segregated into a cluster including HE-JA3 (Mizuo et al., 2002) and JKK-Sap (Takahashi et al., 2002a) as well as HE-JA12, HE-JA13, HE-JA14, HE-JA17, HE-JA18, HE-JA19, HE-JF4 and HE-JF5, which were isolated from Patients 1–3 and 6–10 in the present study. Interestingly,
all ten human HEV isolates in this cluster were obtained from patients living in Hokkaido, indicating that the ‘swJL145-like’ strain is predominant and circulating in Hokkaido. Similarly, the swJL234 and swJL325 isolates were closest to HE-JA4 and grouped into a cluster including HE-JA4 (Mizuō et al., 2002), JKN-Sap and JMY-Haw (Takahashi et al., 2002a) as well as HE-JA15 and HE-JA16, which were recovered from Patients 4 and 5, respectively, in the present study. Of note, all five of these human HEV isolates were obtained from patients who lived in Hokkaido, indicating that the ‘swJL234- or swJL325-like’ strain is also predominant and co-circulating with the ‘swJL145-like’ strain in Hokkaido. When compared with swine HEV isolates of Japanese and non-Japanese origin whose entire or partial nucleotide sequence is known, the seven swine HEV isolates obtained from raw pig liver in the present study were close in nucleotide sequence is known, the seven swine HEV isolates of Hokkaido. When compared with swine HEV isolates of non-Japanese origin whose entire or partial nucleotide sequence is known, the seven swine HEV isolates (accession nos AB089824) determined for these HEV isolates (accession nos AB108652–AB108666). swJL145 was 100 % similar to HE-JA18, and the swJL234 and swJL325 isolates had identical sequences to each other, with three common ambiguity codes of Y, both of which were 99–100 % identical to HE-JA4.

Although risk factors for acquiring sporadic acute or fulminant hepatitis E have not been recognized for patients in industrialized countries, it is probable that HEV transmission occurs via zoonosis and/or foods in industrialized countries where sanitation systems are well established. Zoonotic spread of HEV has been suggested as human and swine HEV strains are closely related genetically (Erker et al., 1999; Hsieh et al., 1999; Huang et al., 2002; Meng, 2000; Meng et al., 1997, 1998; Okamoto et al., 2001; Pina et al., 2000; Wang et al., 2002; Wu et al., 2002), and experimental cross-species infections of swine HEV to non-human primates and that of human HEV to swine have been demonstrated (Erker et al., 1999; Halbur et al., 2001; Meng et al., 1998). In our previous study, we found a high prevalence of swine anti-HEV IgG among Japanese pigs of 2–6 months of age (58 % or 1448/2500) (Takahashi et al., 2003) and identified a pair of Japanese swine and human HEV isolates (swJ13-1 and HE-JA1, respectively) of genotype IV with 99 % identity over the entire genome (Nishizawa et al., 2003). However, no direct evidence of HEV infection from swine to humans in clinical cases of hepatitis E has been provided thus far, and none of our patients with hepatitis E in the current study had contact with pigs or other animals such as rats, mice, dogs, cows, sheep or goats that could also potentially serve as reservoirs (Favorov et al., 1998, 2000; Kabrane-Lazizi et al., 1999; Tien et al., 1997).

As described in a recent review article by Smith (2001), it is likely that foods can act as vehicles for transmission of HEV. The occurrence of acute hepatitis E in an individual in eastern Sicily after consumption of shellfish obtained from a beach was reported by Carapace et al. (1997). Similarly, Mecnik et al. (2001) reported an Israeli who acquired acute HEV infection possibly as a result of eating raw shellfish. Hartmann et al. (1998) attributed acute hepatitis E in a Turkish international living in Germany to ethnic foods brought as a gift by a Turkish visitor. However, no case of hepatitis E has been unequivocally shown to be due to food consumption. In the present study, nine of ten patients who developed sporadic acute or fulminant hepatitis E had consumed raw liver 2 weeks to 2 months before the onset of the illness. Since the pig livers they ingested were not available for testing, we purchased 363 packets of raw pig livers as food from grocery stores near the residences of our subjects in Hokkaido and showed that a certain proportion of packaged pig livers (1·9 % or 7/363) was contaminated with HEV. The incubation period for hepatitis E is considered to be 2–9 weeks and, therefore, we speculate that inadequately cooked pig liver contaminated with HEV was the source of the patients’ infection. Patient 10 in the present study actually reported ingestion of undercooked pig liver. Our speculation is supported by the evidence that one genotype IV HEV isolate (swJL145) obtained from a package of pig liver had a high nucleotide sequence identity, 97·8–100 %, with ten HEV isolates recovered from patients living in Hokkaido, including those from eight patients in the present study (Fig. 1), and that two genotype III HEV isolates (swJL234 and swJL325) from two distinct packages of pig liver had high nucleotide sequence identity, 96·6–100 %, with five human HEV isolates obtained from patients who lived in Hokkaido, including those from two patients in the present study (Fig. 1). With regard to the four patients with hepatitis E living in Hokkaido in our previous study (Mizuō et al., 2002), in reply to our interview, the ‘HE-JA2’ and ‘HE-JA4’ patients told us that they ingested undercooked pig liver several times or once a year, respectively; the last consumption date was 1 or 2 months before the onset of hepatitis E. However, the ‘HE-JA1’ patient denied consumption of pig liver, but said that he preferred to consume inadequately grilled pig intestine or colon once a month. Patients 2, 8 and 10 in the present study also reported ingestion of undercooked pig intestine/colon together with pig liver. HEV is shed into faeces for transmission by the faecal–oral route. Furthermore, it has been shown that both swine HEV and human HEV replicate in the colon and intestines (Williams et al., 2001). Therefore, undercooked pig intestine or colon contaminated with HEV may be another source of transmission of HEV. To support our speculation further, the following questions have to be answered. (i) Why were family member(s) of Patients 1–5, 7 and 8 who ingested pig liver with the patient at home not infected with HEV during the same period? (ii) Why did patients who had consumed pig liver in the past (Patients 1, 2, 5, 8 and 10) acquire HEV infection on this occasion? (iii) Do packaged pig livers sold in grocery stores as food contain infectious virus or just viral RNA? The extremely low chance
of acquiring HEV infection by ingestion of pig liver may be explained by the observed low frequency and by the low viral load in HEV-contaminated pig livers. Interestingly, a recent experimental transmission study of swine HEV to susceptible pigs showed that the risk of contracting swine HEV infection through consuming uncooked, virus-contaminated pork is extremely small (Kasorndorkbua et al., 2002). The extent of grilling may affect the risk of acquiring HEV infection via consumption of pig liver. In some patients’ families, the patient’s spouse ingested only well-cooked pig liver. Detection of HEV RNA by RT-PCR in raw pig liver in grocery stores does not necessarily mean that the contaminated swine HEV in the packaged pig livers is infectious. Therefore, further studies are needed to determine whether the packaged pig livers contain infectious virus.

In conclusion, we would like to speculate that sporadic acute or fulminant hepatitis E in Hokkaido, where clinical HEV infection is most prevalent in Japan, is transmitted by ingestion of inadequately cooked pig liver contaminated with HEV, based on our patients’ dietary habits and the evidence that raw pig livers that are available in grocery stores located near our patients’ residences were contaminated with HEV having a high nucleotide sequence identity to human HEV isolates recovered from patients living in Hokkaido. Therefore, although our present results may raise further public health concerns for HEV zoonosis, extended studies must be performed to determine to what extent pig livers in grocery stores are contaminated with HEV, not only in Hokkaido but also in other areas in Japan. Since we unequivocally found the presence of HEV among pig livers for sale as food in the present study, pig livers should be cooked well before ingestion to prevent the occurrence of possible food-borne hepatitis E in Japan.

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