Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad

Masaharu Takahashi,1 Tsutomu Nishizawa,1 Akira Yoshikawa,2 Shin Sato,3 Norio Isoda,3 Kenichi Ido,3 Kentaro Sugano3 and Hiroaki Okamoto1

1Immunology Division and Division of Molecular Virology, Jichi Medical School, Tochigi-Ken 329-0498, Japan
2Japanese Red Cross Saitama Blood Center, Saitama-Ken 338-0001, Japan
3Department of Gastroenterology, Jichi Medical School, Tochigi-Ken 329-0498, Japan

Two distinct hepatitis E virus (HEV) isolates, designated HE-JI3 and HE-JI4, were identified in a single patient with acute hepatitis in Japan, who had not travelled abroad. The HEV load of HE-JI3 at admission was $10^2$ copies/ml, but that of HE-JI4 was tenfold higher at $10^3$ copies/ml. The viraemia of HE-JI4 persisted for up to 16 days from admission, whereas HE-JI3 disappeared at 9 days after admission. The entire nucleotide sequence of the HE-JI4 isolate and partial nucleotide sequences of open reading frames (ORFs) 1 and 2 of the HE-JI3 isolate were determined. The full-length nucleotide sequence of HE-JI4 consisted of 7171 nucleotides excluding the poly(A) tail and contained ORF1 encoding 1684 amino acids, ORF2 encoding 671 amino acids and ORF3 encoding 114 amino acids. Sequence and phylogenetic analyses of the HEV genomes indicated that HE-JI4 was most closely related to an HEV isolate (T1) of genotype IV with the same strategy for translation of ORF2 and ORF3, but which differed from it by 16.5% over the entire genome. The HE-JI3 isolate showed the highest nucleotide identity (88.6–95.1%) to the genotype III HEVs, having higher identity to human and swine HEV isolates from the United States (US1, US2 and swUS1) than to those reported thus far from Japan (JRA1 and swJ570). The two co-infecting strains of HE-JI3 and HE-JI4 identified from the single patient shared only 80.1% nucleotide identity. These results indicate that multiple genotypes of HEV co-circulate in Japan, and that genotype IV comprises a remarkably heterogeneous group of HEVs.

Introduction

Hepatitis E virus (HEV) is a small, non-enveloped virus that has a single-stranded, positive-sense RNA genome of approximately 7.2 kb and contains a short 5' untranslated region (UTR), three open reading frames (ORFs) and a short 3' UTR terminated by a poly(A) tract (Reyes et al., 1990; Tam et al., 1991). HEV is the causative agent of acute hepatitis E, which is considered to be endemic in many developing countries in Asia and Africa and in Mexico where the disease usually occurs as epidemics (Purcell & Emerson, 2001). Sporadic cases of HEV infection have also been reported in non-endemic, industrialized countries, where the occurrence is usually associated with travel to endemic countries (Dawson et al., 1992; Herrera et al., 1993). Recently, however, several HEV isolates have been obtained from patients with acute hepatitis who live in industrialized countries and who have no history of travel to an area of endemicity (Erker et al., 1999; Kwo et al., 1997; Schlauder et al., 1999, 2000; Worm et al., 2000).

The full genomic sequence of HEV was first published in 1991 for a strain from Burma (Tam et al., 1991), which had greater than 93% nucleotide identity across the genome to additional isolates obtained from other Asian countries including China, India, Nepal and Pakistan (Aye et al., 1993; Panda et al., 2000; Gouvea et al., 1998; Tsarev et al.,...
et al., 2000). In contrast, the Mexican isolate that was implicated in an outbreak that occurred in Mexico in 1986 is distinct from the Burmese variants and constitutes a second genotype (Huang et al., 1992). Recently, a third group of HEVs has been identified in patients with acute hepatitis in the USA, Argentina, Austria, Italy, Greece, Spain and the UK (Schlauder et al., 1999, 2000; Zanetti et al., 1999; Wormald et al., 2000; Pina et al., 2000; Wang et al., 2001a). Extensive diversity has also been reported among isolates from sporadic cases of HEV infection in China and Taiwan that are distinct from the original Chinese isolates and they constitute a fourth group (Wang et al., 1999, 2000; Hsieh et al., 1999). Accordingly, HEV sequences have tentatively been classified into four major genetic groups (genotypes I–IV) (Schlauder & Mushahwar, 2001).

In Japan, hepatitis E is rarely reported and most, if not all, cases of hepatitis E observed thus far have been regarded as ‘imported’ hepatitis. Recently, an HEV strain (JRA1) of genotype III was isolated from a Japanese patient with acute hepatitis of unknown aetiology who had never been aboard (Takahashi et al., 2001). However, the extent of the genetic diversity of HEV in Japan remains to be elucidated. In the present study, we identified two distinct genotypes (III and IV) of HEV from a single Japanese patient with acute hepatitis who had not been abroad and characterized the HEV isolates molecularly and phylogenetically to understand better the genomic diversity and molecular epidemiology of HEV in non- endemic countries.

Methods

Subject. In March 2000, a 45-year-old Japanese male with acute hepatitis who had never been outside Japan presented to our hospital and was followed for 193 days (see Table 2). At admission, the patient had an elevated alanine aminotransferase (ALT) level of 2345 IU/l, an elevated aspartate aminotransferase (AST) level of 2055 IU/l and bilirubinemia with a bilirubin level of 101 mg/dl. Abnormal bilirubin levels persisted until 33 days after admission. He was negative for serum markers of hepatitis A, B and C viruses (HAV, HBV and HCV), but positive for IgM antibodies to HEV (anti-HEV) by an enzyme immunoassay using a recombinant virus-like particle (Li et al., 1997) as an antigen probe. The IgM antibody response was strongly positive at admission and then decreased rapidly, but was detectable at a low titre for 109 days following admission. He continued to be positive for anti-HEV IgG until the end of the observation period and there was no discernible decrease in the IgG antibody titre. The clinical presentation and liver histology of this patient have been described (Sato et al., 2002). Periodic serum samples obtained during the acute and convalescent phases from the patient had been collected and stored at –80 °C for subsequent testing.

Detection of HEV RNA in serum. Total RNA was extracted from 100 μl of serum with TRIZOL LS Reagent (Invitrogen), reverse-transcribed with SuperScript II RNase H– reverse transcriptase (Gibco BRL) and an antisense primer (HE008) (Table 1) specific for the HEV ORF2 sequence, and then subjected to nested PCR in the presence of TaKaRa Ex Taq (TaKaRa Shuzo). A part of the ORF1–ORF2 overlapping sequence was amplified using the primer pairs HE015 and HE008 for the first round and HE016 and HE009 for the second round. The primers used were derived from well-conserved regions among 19 known human and two known swine HEV sequences [B1 and B2 in Burma, C1, C2, C3, C4, C5 and C6 in China, I1, I2 and I3 in India, Ne1 in Nepal and P1 and P2 in Pakistan of genotype I (the abbreviations used for the HEV isolates are in accordance with the recent report by Schlauder & Mushahwar, 2001); MEX–14 in Mexico of genotype II; US1 and US2 in the USA and JRA1 in Japan of genotype III; and T1 in China of genotype IV, as well as swine HEV isolates of genotype III (swUS1 in the USA and swJ570 in Japan) (see Table 3 for the DDBJ/EMBL/GenBank accession no. of each isolate)]. PCR amplification was carried out for 35 cycles in the first round [94 °C for 30 s (additional 2 min in the first cycle), 55 °C for 30 s, 72 °C for 90 s (additional 7 min in the last cycle)] and for 25 cycles in the second round under the same conditions except that extension was carried out for 60 s. The size of the amplification product of the first-round PCR was 763 bp and that of the second-round PCR was 704 bp. The amplification products were electrophoresed on a 1.5% (w/v) NuSieve 3:1 agarose gel (FMIC BioProducts), stained with ethidium bromide and photographed under UV light.

Type-specific quantification of HEV RNA. Based on the nucleotide sequence of the amplification products of HE016 and HE009, two distinct sets of primers were designed to detect HEV RNA in a type-dependent manner. For detection of HE-JI3-type HEV, nested RT–PCR was performed with primers HE113 and HE114 in the first round and HE115 and HE116 in the second round (Table 1), which generated amplification products of 405 bp and 168 bp, respectively. Similarly, for detection of HE-JI4-type HEV, nested RT–PCR was carried out using primers HE123 and HE118 in the first round and HE124 and HE120 in the second round, which generated amplification products of 405 bp and 168 bp, respectively. The highest dilution of extracted RNA (10−6) found positive was estimated and was converted to the titre/ml of serum.

Amplification of the full-length HE-JI4 genome. Total RNAs extracted from the serum sample (500 μl) obtained at admission were subjected to RT–PCR for amplification of a central 7 kb sequence of the HEV genome (HE-JI4), using primers whose sequences were derived from well-conserved regions of the reported human and swine HEV sequences, as well as from sequences obtained during the amplification procedure. The central portion of the HE-JI4 genome was divided into seven overlapping sections: nt 69–1270 (1202 nt), nt 1078–3073 (1996 nt), nt 3048–3830 (783 nt), nt 3824–5256 (1433 nt), nt 5008–5929 (922 nt), nt 5907–6527 (421 nt) and nt 6316–7073 (758 nt) (primer sequences excluded), and their sequences were determined separately.

The extreme 5′ end sequence of HE-JI4 (nt 1–88) 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 was treated with calf intestinal phosphatase followed by tobacco acid pyrophosphatase, and was then ligated to an RNA adapter supplied in the kit. This was used as a template to synthesize cDNA with an HEV-specific antisense primer, HE035, and SuperScript II RNSase H– reverse transcriptase. The cDNA was then amplified by nested PCR with TaKaRa Ex Taq 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 7060–7171 excluding the poly(A) tail] was attempted by the RACE method with a 20-mer primer (#167: 5′ CGTCAGCAGATCATGTTAGC 3′) representing a part of a 41-mer oligonucleotide containing (T)13 (SSP-T: 5′ AAAGATCCCTCGACATCGATAATACG(T)13 3′) and an HEV-speci-
Table 1. Positions and nucleotide sequences of oligonucleotide primers

Nucleotide positions refer to the 7171 nucleotide sequence of the HE-JI4 isolate obtained in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Polarity</th>
<th>Nucleotide</th>
<th>Specificity</th>
<th>Nucleotide sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE008</td>
<td>Antisense</td>
<td>5276–5301</td>
<td>Universal‡</td>
<td>5’ GGGTTGTGGTGATATAGGGGA 3’</td>
</tr>
<tr>
<td>HE009</td>
<td>Antisense</td>
<td>5257–5278</td>
<td>Universal</td>
<td>5’ GGAGTGGCGAAGGGCTGAGAATC 3’</td>
</tr>
<tr>
<td>HE015</td>
<td>Sense</td>
<td>4535–4557</td>
<td>Universal</td>
<td>5’ TGGAAAGACAYCTCYGTTGAGCC 3’</td>
</tr>
<tr>
<td>HE016</td>
<td>Sense</td>
<td>4571–4593</td>
<td>Universal</td>
<td>5’ TAGAAYACYGCTGGAAYATGGC 3’</td>
</tr>
<tr>
<td>HE033</td>
<td>Antisense</td>
<td>5257–5278</td>
<td>Universal</td>
<td>5’ GCTGCTAGAGCTGCTGCTGCG 3’</td>
</tr>
<tr>
<td>HE034</td>
<td>Antisense</td>
<td>5257–5278</td>
<td>HE-JI4</td>
<td>5’ CAAGGCCAGTTGCGGCGCTGC 3’</td>
</tr>
<tr>
<td>HE035</td>
<td>Sense</td>
<td>4535–4557</td>
<td>Universal</td>
<td>5’ CAGTCAGAGGCTGCTGAGCTG 3’</td>
</tr>
<tr>
<td>HE038</td>
<td>Sense</td>
<td>4571–4593</td>
<td>HE-JI4</td>
<td>5’ CAGTCAGAGGCTGCTGAGCTG 3’</td>
</tr>
<tr>
<td>HE040</td>
<td>Antisense</td>
<td>6367–6389</td>
<td>Universal</td>
<td>5’ TGGCGGRMCTATNGCTCCTGACACATC 3’</td>
</tr>
<tr>
<td>HE044</td>
<td>Sense</td>
<td>5884–5906</td>
<td>Universal</td>
<td>5’ TGGCGGRMCTATNGCTCCTGACACATC 3’</td>
</tr>
<tr>
<td>HE090</td>
<td>Sense</td>
<td>1–125</td>
<td>Universal</td>
<td>5’ TGGCGGRMCTATNGCTCCTGACACATC 3’</td>
</tr>
<tr>
<td>HE092</td>
<td>Sense</td>
<td>13–36</td>
<td>Universal</td>
<td>5’ TGGCGGRMCTATNGCTCCTGACACATC 3’</td>
</tr>
<tr>
<td>HE094</td>
<td>Antisense</td>
<td>5257–5278</td>
<td>Universal</td>
<td>5’ TGGCGGRMCTATNGCTCCTGACACATC 3’</td>
</tr>
<tr>
<td>HE111</td>
<td>Sense</td>
<td>5894–5915</td>
<td>Genotype III</td>
<td>5’ GYTCGGGTTGACCCAGGGCGGT 3’</td>
</tr>
<tr>
<td>HE113</td>
<td>Sense</td>
<td>4723–4744</td>
<td>Genotype III</td>
<td>5’ CAAATTGAAGGTTGATTACCGC 3’</td>
</tr>
<tr>
<td>HE114</td>
<td>Antisense</td>
<td>5115–5130</td>
<td>Genotype III</td>
<td>5’ GCAGACCACATGGTGGTCGAYGCC 3’</td>
</tr>
<tr>
<td>HE115</td>
<td>Sense</td>
<td>4791–4810</td>
<td>Genotype III</td>
<td>5’ GCAGACCACATGGTGGTCGAYGCC 3’</td>
</tr>
<tr>
<td>HE116</td>
<td>Antisense</td>
<td>4939–4958</td>
<td>Genotype III</td>
<td>5’ GCAGACCACATGGTGGTCGAYGCC 3’</td>
</tr>
<tr>
<td>HE118</td>
<td>Antisense</td>
<td>5107–5126</td>
<td>Genotype IV</td>
<td>5’ GCAGACCACATGGTGGTCGAYGCC 3’</td>
</tr>
<tr>
<td>HE120</td>
<td>Antisense</td>
<td>4939–4958</td>
<td>Genotype III</td>
<td>5’ GCAGACCACATGGTGGTCGAYGCC 3’</td>
</tr>
<tr>
<td>HE121</td>
<td>Antisense</td>
<td>484–506</td>
<td>Genotype III</td>
<td>5’ GCAGACCACATGGTGGTCGAYGCC 3’</td>
</tr>
<tr>
<td>HE123</td>
<td>Sense</td>
<td>4723–4743</td>
<td>Genotype IV</td>
<td>5’ GCAGACCACATGGTGGTCGAYGCC 3’</td>
</tr>
<tr>
<td>HE124</td>
<td>Sense</td>
<td>4791–4810</td>
<td>Genotype IV</td>
<td>5’ GCAGACCACATGGTGGTCGAYGCC 3’</td>
</tr>
</tbody>
</table>

* R = A or G; Y = T or C; H = A, T or C; K = G or T; N = G, A, T or C; M = A or C; and B = G, T or C.
† Universal primer sequence was derived from the well-conserved region of known HEV genomes of four distinct genotypes (I–IV).

Table 2. Laboratory parameters and type-specific quantification of HEV RNA in serum samples obtained periodically from the patient with HEV-associated hepatitis

<table>
<thead>
<tr>
<th>Days after admission</th>
<th>Total bilirubin (mg/dl)*</th>
<th>ALT (IU/l)*</th>
<th>AST (IU/l)*</th>
<th>HE-JI3 type (genotype III)</th>
<th>HE-JI4 type (genotype IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.1</td>
<td>2345</td>
<td>2055</td>
<td>1 × 10²</td>
<td>1 × 10³</td>
</tr>
<tr>
<td>5</td>
<td>11.0</td>
<td>1469</td>
<td>444</td>
<td>4 × 10¹</td>
<td>1 × 10³</td>
</tr>
<tr>
<td>6</td>
<td>10.4</td>
<td>1021</td>
<td>210</td>
<td>4 × 10¹</td>
<td>1 × 10³</td>
</tr>
<tr>
<td>7</td>
<td>7.9</td>
<td>702</td>
<td>142</td>
<td>1 × 10²</td>
<td>1 × 10³</td>
</tr>
<tr>
<td>9</td>
<td>8.5</td>
<td>416</td>
<td>89</td>
<td>—</td>
<td>4 × 10¹</td>
</tr>
<tr>
<td>12</td>
<td>7.1</td>
<td>182</td>
<td>71</td>
<td>—</td>
<td>4 × 10¹</td>
</tr>
<tr>
<td>14</td>
<td>6.0</td>
<td>126</td>
<td>57</td>
<td>—</td>
<td>4 × 10¹</td>
</tr>
<tr>
<td>16</td>
<td>4.9</td>
<td>99</td>
<td>57</td>
<td>—</td>
<td>1 × 10³</td>
</tr>
<tr>
<td>22</td>
<td>2.5</td>
<td>49</td>
<td>34</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>33</td>
<td>1.2</td>
<td>19</td>
<td>19</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>61</td>
<td>0.9</td>
<td>18</td>
<td>18</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>109</td>
<td>0.6</td>
<td>18</td>
<td>18</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>193</td>
<td>0.5</td>
<td>16</td>
<td>15</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

* Normal ranges: total bilirubin, 0.2–1.0 mg/dl; ALT, 4–30 IU/l; AST, 11–30 IU/l.
fic sense primer (HE038) on cDNAs that had been reverse-transcribed from poly(A)+ RNAs with the 41-mer oligonucleotide with (T)\textsubscript{n} according to the method described previously (Okamoto \textit{et al}., 2000).

\[ \text{Amplification of partial sequences of the HE-JI3 genome}\]

A part of the ORF1 sequence of the HE-JI3 genome was amplified by nested RT–PCR using the primer pairs HE090 and HE094 in the first round and HE092 and HE121 (genotype III-specific) in the second round (Table 1), as described above. The size of the amplification product from the first-round PCR was 567 bp and from the second-round PCR was 494 bp. Similarly, a part of the ORF2 sequence of the HE-JI3 genome was amplified by nested RT–PCR with primer pairs HE044 and HE040 in the first round and HE111 and HE122 (both genotype III-specific) in the second round; they generated amplification products of 506 bp and 481 bp, respectively.

\[ \text{Cloning and sequence analysis of PCR products}\]

The amplification products were sequenced on both strands either directly or after cloning into pT7Blue\textsuperscript{T} vector (Novagen), using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). Sequence analysis was performed using Genetyx-Mac version 10.1.6 (Software Development and open version 1.1.1 from the DNA Data Bank of Japan (DDBJ); National Institute of Genetics, Mishima, Japan) (Ina, 1994). Sequences were aligned by \textsc{clustal w} (version 1.8) (Thompson \textit{et al}., 1994). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987), based on the partial nucleotide sequences of the ORF1 and ORF2 regions, or the entire nucleotide sequence of the HEV genome. Bootstrap values were determined on 1000 resamplings of the data sets (Felsenstein, 1985). The final tree was obtained using the \textsc{treeview} program (version 1.6.6) (Page, 1996).

\[ \text{Results}\]

\[ \text{Identification of two HEV variants from a single patient}\]

Thirteen serial serum samples obtained from a patient with acute hepatitis of unknown aetiology were tested for the presence of HEV RNA by nested RT–PCR. Eight serum samples obtained up to 16 days after the day of admission were positive for HEV RNA (Table 2). The amplicons obtained from the serum at admission were subjected to molecular cloning and sequence analysis. Among the six clones obtained, five clones were > 99% similar to each other, but the remaining one clone differed from them by 20–7% in the 659 nt sequence, suggesting the coexistence of two distinct genotypes of HEV in the patient. The minor clone had the highest similarity of 95–3% with the US1 isolate of genotype III (Schluder \textit{et al}., 1998), whereas the remaining five major clones were closest to the T1 isolate of genotype IV (Wang \textit{et al}., 2000), with an identity of 87–5%.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Genotype/isolate & \multicolumn{2}{|c|}{HE-JI4 (genotype IV)} & \multicolumn{2}{|c|}{HE-JI3 (genotype III)} \\
\cline{2-5}
\multicolumn{1}{|c|}{(accession no.)} & Full-length & ORF1 & ORF2 & ORF3 & Part of ORF1 & Part of ORF2 \\
\hline
\multicolumn{1}{|c|}{Genotype I} & \multicolumn{4}{|c|}{HE-JI4} & \multicolumn{2}{|c|}{HE-JI3} \\
\multicolumn{1}{|c|}{14 isolates\textsuperscript{†}} & \multicolumn{4}{|c|}{(7171 nt)} & \multicolumn{2}{|c|}{(659 nt)} \\
\hline
\multicolumn{1}{|c|}{} & (798–820) & (906–921) & (737–764) & (956–971) & & \\
\hline
\multicolumn{1}{|c|}{Genotype II} & \multicolumn{2}{|c|}{MEX-14 (M74506)} & \multicolumn{2}{|c|}{MEX-14 (M74506)} & \multicolumn{2}{|c|}{MEX-14 (M74506)} \\
\multicolumn{1}{|c|}{} & 733 & 707 & 790 & 836 & 771 & 764 \\
\multicolumn{1}{|c|}{} & (806) & (897) & (746) & & & \\
\hline
\multicolumn{1}{|c|}{Genotype III} & \multicolumn{2}{|c|}{US1 (AF060668)} & \multicolumn{2}{|c|}{US1 (AF060668)} & \multicolumn{2}{|c|}{US1 (AF060668)} \\
\multicolumn{1}{|c|}{} & 733 & 713 & 802 & 864 & 954 & 943 \\
\multicolumn{1}{|c|}{} & (840) & (924) & (839) & & (100) & (100) \\
\multicolumn{1}{|c|}{} & & & & & & \\
\multicolumn{1}{|c|}{Genotype IV} & \multicolumn{2}{|c|}{T1 (AJ727108)} & \multicolumn{2}{|c|}{T1 (AJ727108)} & \multicolumn{2}{|c|}{T1 (AJ727108)} \\
\multicolumn{1}{|c|}{} & 835 & 820 & 876 & 953 & 805 & 814 \\
\multicolumn{1}{|c|}{} & (933) & (972) & (921) & & (971) & (971) \\
\multicolumn{1}{|c|}{HE-JI4 (AB080575)} & \multicolumn{2}{|c|}{—} & \multicolumn{2}{|c|}{—} & \multicolumn{2}{|c|}{—} \\
\multicolumn{1}{|c|}{} & — & — & — & & — & — \\
\multicolumn{1}{|c|}{HE-JI4 (AB080575)} & \multicolumn{2}{|c|}{—} & \multicolumn{2}{|c|}{—} & \multicolumn{2}{|c|}{—} \\
\multicolumn{1}{|c|}{} & — & — & — & & — & — \\
\hline
\end{tabular}
\caption{Comparison of the two distinct HEV isolates obtained in the present study (HE-JI4 and HE-JI3) with 21 human and swine HEV isolates whose entire or nearly entire genomic sequence is known.}
\end{table}

The highest sequence identity in each region of the genome is indicated in bold type. The percentage amino acid identity is shown in parentheses.

* An overlapping sequence encoding parts of ORF1 (161 amino acids), ORF2 (46 amino acids) and ORF3 (58 amino acids) is indicated.

† The accession numbers of the 14 isolates are AF051830, AF076239, AF185822, D10330, D11092, D11093, L08816, L25547, L25595, M73218, M80581, M94177, X98292 and X99441.
Mixed HEV infection in Japan

Fig. 1. (a) Comparison of the HEV sequences containing potential initiation codons for ORF2 and ORF3. All HEV isolates whose entire or nearly entire genomic sequence is known including the HE-JI4 isolate obtained in the present study were compared. Nucleotide positions are in accordance with the entire 7171 nt sequence of the HE-JI4 isolate. Potential initiation codons of ORF2 are indicated by shaded boxes and those of ORF3 are shown by open boxes. The inserted U residue that is found exclusively in the T1 and HE-JI4 isolates of genotype IV is marked with a closed triangle. The termination codon (UGA) for ORF1 is overlined. See Table 3 for the accession no. of each isolate. (b) Comparison of the N-terminal sequence of HEV ORF2 proteins. The N-terminal sequence of the ORF2 protein in representative HEV isolates of genotypes I–III and two genotype IV isolates (T1 and HE-JI4) are aligned for comparison. The potential N-terminal amino acid residue (M) of each ORF2 protein is boxed. Dashes indicate amino acids that are identical to the top sequence and slashes denote deletion of amino acids. (c) Comparison of the N-terminal sequence of HEV ORF3 proteins. The potential N-terminal amino acid residue (M) of each ORF3 protein is boxed.

admission, the HEV load of the HE-JI3 type was \(10^2\) copies/ml, while that of the HE-JI4 type was tenfold higher at \(10^3\) copies/ml. The HE-JI4-type HEV remained detectable in the serum for 16 days after admission, while the viraemia of HE-JI3 persisted for 7 days after admission.

Characterization of the entire HE-JI4 genome

To determine the full-genome nucleotide sequence of the predominant isolate (HE-JI4) from this patient, nine overlapping cDNA sequences covering the entire genome were amplified by conventional PCR or RACE and molecularly cloned. Each cDNA sequence was completely identical to the overlapping sequence(s) of neighbouring regions and had the higher identity with the respective partial sequence of a reported genotype IV isolate (T1) than those of genotype III isolates (US1, US2, swUS1, JRA1 and swJ570) (4.6–9.3% higher in the nine sequences compared), suggesting that these nine cDNA sequences were derived from the HE-JI4 type and not from the HE-JI3 type.

The HE-JI4 isolate had a genomic length of 7171 nt, excluding the poly(A) tract at the 3’ terminus, and possessed three major ORFs similar to reported human and swine HEV isolates. ORF1, ORF2 and ORF3 had a coding capacity of 1684 amino acids (nt 26–5077), 671 amino acids (nt 5086–7098) and 114 amino acids (nt 5105–5446), respectively. Of interest is the fact that, as in the T1 isolate (Wang et al., 2000), the HE-JI4 isolate had the insertion of a single nucleotide (U) at position 5090, which affected both ORF2 and ORF3 (Fig. 1a). Assuming that translation starts from the first AUG codon in the ORF, the ORF2 of HE-JI4 starts nine nucleotides downstream of ORF1, whereas the ORF2 in all reported isolates of genotypes I–III begins 41 nucleotides downstream of ORF1. The first initiation codon of ORF3 in HE-JI4 is 28 nucleotides downstream of ORF1, contrasting with the ORF3 in reported isolates of genotypes I–III, which overlaps ORF1 by one nucleotide. Consequently, HE-JI4 has an additional 11 amino acids in the N terminus of the ORF2 protein and is comparable to the T1 isolate, which has an additional 14 codons in ORF2 (Fig. 1b). The predicted size of the ORF3 of HE-JI4 is identical
Fig. 2. Phylogenetic tree constructed by the neighbour-joining method of the entire nucleotide sequence of 22 HEV isolates. Since the amount of sequence that has been identified for different isolates is different, the overlapping 7137 nt sequence of 21 reported human and swine HEV isolates and that of the HE-JI4 isolate obtained in the present study, which is indicated in bold type, are compared (see Table 3 for the names of the isolates and relevant accession nos). The bootstrap values were ≥ 90% for all nodes in 1000 resamplings of the data; the values for the major nodes are indicated as percentage numbers.

Fig. 3. Phylogenetic tree constructed by the neighbour-joining method of the partial nucleotide sequence of (a) the ORF1 region (287 nt) of 33 human and swine HEV isolates and (b) the ORF2 region (301 nt) of 63 human and swine HEV isolates. In (a), in addition to the 22 HEV isolates indicated in Fig. 2, ten reported isolates of genotype III and HE-JI3 obtained in the present study, for which the 287–402 nt ORF1 sequence has been determined, were included for comparison. The accession nos of the ten reported isolates are: AF110387–AF110389, AF195064, AF195065, AF215661, AF264009, AF264010, AF279122 and AJ315768. Similarly, in (b), 40 reported isolates of genotype III or IV, for which the 301–1497 nt ORF2 sequence has been determined, were added for comparison. They are deposited under accession nos AB073910, AB073911, AF103940, AF117275–AF117281, AF134916, AF134917, AF151962, AF151963, AF195061–AF195063, AF296162–AF296167, AF302068, A344171, A344172, A344177, A344179–A344181, A344183–A344186, A344188 and A344191–A344194. Bootstrap values of more than 70% are indicated for the major nodes.

Comparison of HE-JI3 and HE-JI4 genomes with known complete genomes

Comparison of the HE-JI4 genome against reported HEV genomes of genotypes I–IV whose entire or nearly entire nucleotide sequence is known revealed that it is closest to an HEV strain (T1) of genotype IV, with identities of 83.5, 82.0, 87.6 and 95.3% in the nucleotide sequences of the full genome, ORF1, ORF2 and ORF3, respectively (Table 3). The HE-JI4 isolate was only 73.3–74.9% similar to the human and swine HEV isolates of genotypes I–III. The phylogenetic tree to that of the T1 isolate, but it is nine codons shorter than that of other reported isolates of genotypes I–III (Fig. 1c). There is another in-frame AUG codon in the ORF2 and ORF3 of HE-JI4 downstream; if these in-frame AUG codons were used as the start codons, ORF2 and ORF3 would encode 660 and 112 amino acids, respectively.

The 5’ UTR of HE-JI4 comprised 25 nucleotides, with a sequence starting with GCAGACCAC, and was identical to that of the T1 strain of genotype IV. In contrast, the 3’ UTR sequence of the HE-JI4 genome consisted of 70 nucleotides and had an appreciable diversity, differing by 18.8–30.6% compared with the 18 HEV isolates of genotypes I–IV whose extreme 3’ end sequence is known.
constructed based on the full genomic sequence confirmed that HE-JI4 belongs to genotype IV and is nearest to T1 (Fig. 2).

The nucleotide sequences of three non-overlapping regions (447 nt, 659 nt and 436 nt) of the HE-JI3 genome were determined and compared with the respective regions in reported HEV genomes. HE-JI3 was closest to genotype III HEVs, with the highest similarity of 94.3–95.4% at the nucleotide level to the US1 isolate rather than to an HEV isolate of Japanese origin (JRA1; 86.0–91.4% identity). When compared with the respective regions in the co-infecting HE-JI4 isolate, HE-JI3 was only 79.3–81.4% identical.

Comparison with partial ORF1 or ORF2 sequences of reported human and swine HEV isolates

The following partial ORF1 sequences of HEVs of genotype III have been reported: seven human HEV isolates from European countries (Austria, Italy, Greece, Spain and the UK) (Schlauder et al., 1999; Wormald et al., 2000; Pina et al., 2000; Wang et al., 2001a), two human HEV isolates from Argentina (Schlauder et al., 2000) and one swine HEV isolate from New Zealand (Garkavenko et al., 2001). Phylogenetic analysis of the common 287 nucleotides within the 5’-terminal region of ORF1 indicated that the HE-JI3 isolate was nearest to US1 among all genotype III isolates whose entire or partial nucleotide sequence is known (Fig. 3a). Partial ORF2 sequences of HEVs of genotype IV are available for 21 human HEV isolates in China (Wang et al., 2001b) and eight human and three swine HEV isolates in Taiwan (Wu et al., 2000). When the common 301 nt sequence of HE-JI4 was compared with that of the human and swine strains and the T1 isolate, the HE-JI4 isolate shared nucleotide identities of only 83.0–89.0%. The phylogenetic tree constructed based on the partial ORF2 sequence of 301 nt confirmed that HE-JI4 belonged to genotype IV and that it was clearly separate from known genotype IV isolates from China and Taiwan (Fig. 3b). Recently, Arankalle et al. (2002) reported 12 swine isolates of HEV of genotype IV in India, which shared only 81.3–84.3% identity with HE-JI4 in the partial ORF2 sequence of 241–263 nucleotides, suggesting that HE-JI4 belongs to a new subgroup of genotype IV that is separate from other subgroups to which the Chinese, Taiwanese and Indian isolates are classifiable.

Discussion

In the present study, two distinct HEV sequences were recovered from a single patient with acute hepatitis in Japan who had never travelled abroad, despite the rarity of clinical HEV infection in this non-endemic country. One (HE-JI4) of the two HEV variants was segregated to genotype IV and the other (HE-JI3) to genotype III. Takahashi et al. (2001) first reported an HEV strain of genotype III (JRA1) from a Japanese patient with acute hepatitis who had no history of travelling abroad. That the JRA1 strain is indigenous to Japan is supported by the identification of a swine HEV isolate (swJ570) from a farm pig in Japan (Okamoto et al., 2001), which had the highest similarity to the JRA1 isolate among the HEV isolates thus far identified. These four human and swine isolates of Japanese origin (JRA1, HE-JI3, HE-JI4 and swJ570) shared only 74.5–89.4% nucleotide identity, indicating that heterogeneous HEV strains circulate in Japan.

The source of the two HEV variants in this Japanese patient is unclear. His wife and daughter who live with the patient were negative for anti-HEV, when tested 40 days after disease onset in the patient. Furthermore, the patient and his family members tested negative for antibodies to HAV, which is another water-borne hepatitis virus, and none of them had any history of travelling outside Japan. Therefore, the possibility of intrafamilial HEV transmission can be excluded. Since the patient had no history of blood transfusion, tattooing, or drug abuse by injection, transmission via a parenteral route can also be excluded. As the chef of a sushi shop, he used to eat raw fish and other uncooked seafood, which were caught in Japan or imported from many countries of the world, including China, Taiwan and the USA, where HEV of genotype III or IV circulates. In China, an HEV strain of genotype I is also prevalent and was the source of an epidemic HEV infection (Aye et al., 1993; Bi et al., 1993; Yin et al., 1993). However, genotype I HEV was not detected in our patient. As indicated in Fig. 3(b), the HE-JI4 isolate obtained from our patient in the present study belonged to genotype IV, but was located on a new branch and was considered to be in a separate subgroup from those of all known genotype IV isolates from China and Taiwan. HE-JI4 shared less than 85% identity with the recently reported swine isolates from India (Arankalle et al., 2002), indicating that HE-JI4 can be segregated to a separate subgroup from the Chinese, Taiwanese and Indian strains. Taken altogether, we would like to speculate that HE-JI4 is indigenous to Japan and that it was not imported from China, Taiwan or other countries through an unknown route.

In the USA, only genotype III HEVs from humans and pigs have been reported (Meng et al., 1997; Schlauder et al., 1998). Of interest is the fact that the HE-JI3 isolate that was obtained along with the HE-JI4 isolate from our patient in the current study was nearer to the US human and swine strains, with the highest nucleotide identity of 95% with the US1 isolate, than to a human isolate (JRA1) or swine isolate (swJ570) that are believed to be indigenous to Japan (Takahashi et al., 2001; Okamoto et al., 2001). This result indicates that HE-JI3 may be a US strain. However, the finding of mixed HEV infection of genotypes III and IV at admission in our patient seems to indicate that the infectious material was initially contaminated with both genotypes of HEV strains. Hence, it is tempting to speculate that the HE-JI3 strain was ‘imported’ from the USA in the past and has then evolved and circulated in Japan, and that both the HE-JI4 of genotype IV and HE-JI3 of genotype III are now indigenous to Japan. To support this issue, large-scale studies on patients with acute hepatitis of unknown aetiology are needed in Japan.
Although our patient did not report contact with animals such as pigs or rats, evidence is accumulating that hepatitis E is zoonotic in non-endemic countries (Harrison, 1999). In the USA and Taiwan, where hepatitis E is not endemic in humans, zoonotic spread of HEV is suspected, as the swine and human HEV isolates in each country belong to the same genotype and are closely related to each other (Meng et al., 1997; Hsieh et al., 1999) and cross-species infection has been documented (Meng et al., 1998; Erker et al., 1999). In contrast, in India, where hepatitis E is endemic in humans, the human HEV isolates belong to genotype I, whereas the swine isolates belong to genotype IV (Arankalle et al., 2002). Potential zoonotic infection of HEV is also supported by several recent reports that veterinarians working with swine are at higher risk of HEV infection than are normal blood donors in the USA and other countries (Meng et al., 2002), and that anti-HEV antibodies are highly prevalent in commercial swine populations in Canada, Australia and New Zealand (Yoo et al., 2001; Chandler et al., 1999; Garkavenko et al., 2001). An HEV-like agent has been recovered from chickens with 'big liver and spleen disease' in Australia or those with 'hepatitis-splenomegaly syndrome' in the USA, but avian HEV is genetically related to, but clearly distinct from, known human and swine strains of HEV, displaying less than 60% nucleotide sequence identity (Payne et al., 1999; Haqshenas et al., 2001).

In Japan, only three strains of swine HEV of genotype III (swJ570, swJ681 and swJ791) have been isolated from 60- to 90-day-old farm pigs (Okamoto et al., 2001). Swine HEV strains of the HE-JI3 type of genotype III and the HE-JI4 type of genotype IV have not been recovered from pigs in Japan thus far. Therefore, further epidemiological evidence is required to prove zoonosis of HEV from swine to humans, or vice versa, in Japan by means of isolation of completely identical strains from both humans and pigs.

The overall nucleotide sequence divergence among HEV isolates whose entire or nearly entire sequence is available was less than 27% and the amino acid sequence divergence of ORF1 was less than 20%. In particular, much of the variation in the nucleotide sequence of the HEV genome occurs in the third base of triplet codons, leading to a silent mutation. Similar to genotype III (Schlauder & Mushahwar, 2001), genotype IV seems to represent a remarkably heterogeneous group of HEV isolates, with the percentage nucleotide divergence between the T1 and HE-JI4 isolates being 16.5% over the entire genome. If nucleotide divergence of 15% is considered as the criterion for subgroup classification, T1 and HE-JI4 may have the status of distinct subgroups. Such genotypic classification of HEV awaits additional studies and requires a decision by the International Committee on Taxonomy of Viruses (ICTV).

In conclusion, two distinct HEV strains of genotypes III and IV that may be indigenous to Japan were identified from a single patient with acute hepatitis in Japan, where clinical HEV infection is rare, but where polyphyletic HEV strains of genotypes III and IV co-circulate. Whether the difference in HEV genotype and co-infection of different genotypes affect the pathogenesis and outcome of HEV infection deserves further analysis.

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.

References


E virus.
and experimental evidence for cross-species infection by swine hepatitis
40
Clinical Microbiology
normal blood donors in the United States and other countries.
antibodies to hepatitis E virus in veterinarians working with swine and in
71
Journal of
Research Communications
289
Biochemical and Biophysical
(1999).
Sato, S., Ido, K., Isoda, N., Hirasawa, T., Sato, A., Iino, S., Hozumi, M.,
A case of domestically infected acute hepatitis E. *Acta Hepatologica

Schlauder, G. G., Dawson, G. J., Erker, J. C., Kwo, P. Y., Knigge, M. F.,
virus isolated from a patient with acute hepatitis reported in the United
Europe: evidence for additional genotypes of HEV. *Journal of Medical
Schlauder, G. G., Frider, B., Sookoian, S., Castano, G. C. & Mushahwar,
Takahashi, K., Iwata, K., Watanabe, N., Hatahara, T., Ohta, Y., Baba,
virus strain that may be indigenous to Japan. *Virology* 287, 9–12.
Tam, A. W., Smith, M. M., Guerra, M. E., Huang, C., Bradley, D. W., Fry,
and sequence of the full-length viral genome. *Virology* 185, 120–130.
Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W:
improving the sensitivity of progressive multiple sequence alignment
through sequence weighting, position-specific gap penalties and weight
Tsarev, S. A., Emerson, S. U., Reyes, G. R., Tsareva, T. S., Legters,
a prototype strain of hepatitis E virus. *Proceedings of the National
Academy of Sciences* 89, 559–563.
Caudill, J. D., Snellings, N. J., Begot, L., Innis, B. L. & Longer, C. F.
Wang, Y., Ling, R., Erker, J. C., Zhang, H., Li, H., Desai, S., Mushahwar,
in Chinese patients with acute hepatitis. *Journal of General Virology* 80,
169–177.
Wang, Y., Zhang, H., Ling, R., Li, H. & Harrison, T. J. (2000). The
complete sequence of hepatitis E virus genotype 4 reveals an alternative
strategy for translation of open reading frames 2 and 3. *Journal of General
Virology* 81, 1675–1686.
Wang, Y., Levine, D. F., Bendall, R. P., Teo, C. G. & Harrison, T. J.
Wang, Y., Zhang, H., Li, Z., Gu, W., Lan, H., Hao, W., Ling, R., Li, H.
& Harrison, T. J. (2001b). Detection of sporadic cases of hepatitis E virus
(HEV) infection in China using immunomaps based on recombinant
open reading frame 2 and 3 polypeptides from HEV genotype 4. *Journal
of Clinical Microbiology* 39, 4370–4379.
Worm, H. C., Schlauder, G. G., Wurzer, H. & Mushahwar, I. K.
(2000). Identification of a novel variant of hepatitis E virus in Austria:
genetic and sequence of the full-length viral genome.
Schlauder, G. G., Carpenter, H. A., Murphy, P. J., Rosenblatt, J. E., Dawson,
a new isolate acquired in the United States. *Mayo Clinic Proceedings* 72,
1133–1136.
Li, T., Yamakawa, Y., Suzuki, K., Tatsumi, M., Razak, M. A. A.,
Uchida, T., Takeda, N. & Miyamura, T. (1997). Expression and self-
assembly of empty virus-like particles of hepatitis E virus. *Journal of
Virology* 71, 7207–7213.
Meng, X.-J., Purcell, R. H., Haubur, P. G., Lehman, J. R., Webb, D. M.,
novel virus in swine is closely related to the human hepatitis E virus.
*Proceedings of the National Academy of Sciences* 94, 9860–9865.
Meng, X.-J., Haubur, P. G., Shapiro, M. S., Govindarajan, S., Bruna,
J. D., Mushahwar, I. K., Purcell, R. H. & Emerson, S. U. (1998). Genetic
and experimental evidence for cross-species infection by swine hepatitis
Meng, X. J., Wiseman, B., Elvinger, F., Guenette, D. K., Toth, T. E.,
Engle, R. E., Emerson, S. U. & Purcell, R. H. (2002). Prevalence of
antibodies to hepatitis E virus in veterinarians working with swine and in
normal blood donors in the United States and other countries. *Journal of
Clinical Microbiology* 40, 117–122.
Okamoto, H., Nishizawa, T., Tawara, A., Takahashi, M., Kishimoto, J.,
marrow cells from an infected individual. *Biochemical and Biophysical
Research Communications* 279, 700–707.
Okamoto, H., Takahashi, M., Nishizawa, T., Fukai, K., Muramatsu, U.
& Yoshikawa, A. (2001). Analysis of the complete genome of indigenous
swine hepatitis E virus isolated in Japan. *Biochemical and Biophysical
Research Communications* 289, 929–936.
trees on personal computers. *Computer Applications in the Biosciences*
12, 357–358.
Panda, S. K., Ansari, I. H., Durgapal, H., Agrawal, S. & Jameel, S.
(2000). The in vitro-synthesized RNA from a cDNA clone of hepatitis
(1999). Sequence data suggests big liver and spleen disease virus (BLSV)
is genetically related to hepatitis E virus. *Virology* 169, 125.
identified in serum from humans with acute hepatitis and in sewage
Virology*, 4th edn, pp. 3051–3061. Edited by D. M. Knipe, P. M. Howley,
Philadelphia: Lippincott Williams and Wilkins.
Reyes, G. R., Purdy, M. A., Kim, J. P., Luk, K. C., Young, L. M., Fry,
K. E. & Bradley, D. W. (1990). Isolation of cDNA from the virus respon-
sible for enterically transmitted non-A, non-B hepatitis. *Science* 247,
1330–1339.
method for reconstructing phylogenetic trees. *Molecular Biology and
Sato, S., Ido, K., Isoda, N., Hirasawa, T., Sato, A., Iino, S., Hozumi, M.,
A case of domestically infected acute hepatitis E. *Acta Hepatologica


Received 6 March 2002; Accepted 25 March 2002