INTRODUCTION

Hepatitis E virus (HEV) was discovered in 1983 by immune electron microscopy (Balayan et al., 1983) and was first cloned in 1990 (Reyes et al., 1990). It is the sole member of the genus Hepevirus in the family Hepeviridae (Emerson et al., 2004). Transmission of HEV occurs primarily by the faecal–oral route via contaminated water supplies in developing countries where sanitation is suboptimal (Purcell & Emerson, 2001; Smith, 2001). Accumulating lines of evidence have indicated that hepatitis E is a zoonosis (Harrison, 1999; Meng, 2003; Meng et al., 1997a, 1998, 2002; Okamoto et al., 2001; Nishizawa et al., 2003, 2005; Tei et al., 2003; Yazaki et al., 2003; Sonoda et al., 2004). In addition, recent studies have indicated that zoonotic food-borne transmission of HEV from domestic pigs, wild boar or wild deer to humans may occur as autochthonous infection in Japan (Li et al., 2005; Matsuda et al., 2003; Tamada et al., 2004; Tei et al., 2003; Yazaki et al., 2003).

The HEV virion is 27–34 nm in diameter and does not possess an envelope. The genome of HEV is a single stranded, positive-sense RNA of approximately 7.2 kb (Tam et al., 1991). Based on the genomic variability noted among HEV isolates, HEV sequences have been classified into four genotypes: genotype 1 consists of epidemic strains in developing countries in Asia and Africa; genotype 2 has been described in Mexico and Africa; genotype 3 is widely distributed in the world and has been isolated from sporadic cases of acute hepatitis E and/or domestic pigs in the USA, European countries and Japan; and genotype 4 contains strains from humans and/or domestic pigs in Asian countries including China, Taiwan and Japan (Schlauer & Mushahwar, 2001; Lu et al., 2006; Inoue et al., 2006).

Propagation and production of HEV in vitro have been attempted in various continuous cell lines (Huang et al., 1992, 1995, 1999; Kazachkov et al., 1992; Li et al., 1996; Meng et al., 1996, 1997a; Wei et al., 2000) and in primary hepatocytes from non-human primates (Arankalle et al., 1988; Kane et al., 1984; Tan et al., 1996a, b, 1997; Tsarev et al., 1994). However, the lack of an efficient cell-culture system for HEV has greatly hampered detailed analysis of the virus replication cycle in infected cells to resolve many important questions. Recently, Emerson et al. (2005, 2006) reported a quantitative cell-culture assay for neutralization tests and thermal stability tests by detecting infected culture cells by immunofluorescence microscopy. However, high-titre HEV was not detected in the culture supernatant. Using
a faecal suspension with high HEV load (2.0 \times 10^7 \text{ copies} \text{ ml}^{-1}) as an inoculum, obtained from a Japanese patient who contracted domestic infection of genotype 3 HEV, we developed an efficient cell-culture system for HEV in a hepatocarcinoma cell line (PLC/PRF/5), which yielded the highest HEV load of up to 10^9 \text{ copies} \text{ ml}^{-1} in the culture supernatant and succeeded in propagating five generations of serial passages of culture supernatant. In addition, as preliminary applications of this culture system for HEV, we examined the thermal stability of HEV and the neutralizing activity of serum samples containing IgG-class and/or IgM-class HEV antibodies obtained from patients with clinical or subclinical HEV infection.

**METHODS**

**Virus.** We used an HEV strain (JE03-1760F) in faecal specimens obtained at the acute phase from a 67-year-old Japanese patient with chronic renal failure who contracted domestic infection of genotype 3 HEV in 2003, just before starting maintenance haemodialysis. The faecal suspension in Tris/HCl buffer (10 mM, pH 7.5) was centrifuged at 6200 \text{ g} at 4 °C for 10 min and the resulting clear supernatant was aliquoted as virus stocks and stored at −80 °C. The HEV RNA titre of the virus stock was estimated to be 2.0 \times 10^7 \text{ copies} \text{ ml}^{-1} by the method described below. Prior to inoculation of the faecal suspension, the virus stock was subjected to purification by passage through microfilters with a pore size of 0.45 and 0.22 \mu m (Millers-GV; Millipore).

**Cell-culture.** A total of 21 established cell lines including the PLC/PRF/5 (CRL-8024), A549 (CCL-185), HepG2 (HB-8065), HuH7 (RCB1366), IEC-6 (CRL-1592), NUGC-4 (ICRBO834), MDCK (CCL-34), MDBK (CCL-22), P19 (CCL-1825), LLC-MK2 (CCL-7), B3H1 (CRL-1443), C2C12 (CRL-1772), HEK293 (CRL-1573), L929 (RCB1422), HT-1080 (ICRBR9131), SK-N-MC (HTB-10), GOTO (ICRBO612), C6 (CCL-107), CV1 (CRL-10478), HeLa (CCL-13) and MCF (ICRBO314) cells, which were available from the ATCC or RIKEN Cell Bank (RIKEN BioResource Center), were used in the present study. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10\% (v/v) heat-inactivated fetal calf serum (FCS; PAA Lab GmbH), 100 \text{ U} penicillin G ml^{-1}, 100 \text{ \mu g} streptomycin ml^{-1} and 2.5 \text{ \mu g} amphotericin B ml^{-1}, at 37 °C in a humidified 5\% \text{ CO}_2 atmosphere. For virus infection, confluent cells were trypsinized and diluted 1:4 in medium and 2.0 ml was added to wells (diameter of 3.5 cm) of a six-well microplate (IWAKI) 1 or 2 days before virus infection.

**Virus inoculation and passage.** Monolayers of cultured cells in a six-well microplate were washed three times with 1 ml PBS(-), containing 0.2\% (w/v) BSA (Sigma-Aldrich), and 0.2 ml of the filtered virus stock that had been diluted with PBS(-) was inoculated on the cells. In serial passages, 0.2 ml culture supernatant that had been filtered through a microfilter with a 0.22 \mu m pore size was inoculated on a monolayer of PLC/PRF/5 cells. One hour after inoculation at room temperature, the solution was removed and 2 ml maintenance medium was added. The maintenance medium used for virus culturing consisted of 50\% DMEM and 50\% medium 199 (Invitrogen) containing 2\% (v/v) heat-inactivated FCS and 30 mM MgCl_2 at final concentration; other supplements were the same as in the growth medium. Culturing was performed at 35.5 or 37.0 °C in a humidified 5\% \text{ CO}_2 atmosphere. On the day after inoculation, the cells were washed five times with 1 ml PBS(-) and 2 ml maintenance medium was added. Then, starting on day 2 after inoculation and then every other day, one-half (1 ml) of the culture medium was replaced with fresh maintenance medium and the medium collected was stored at −80 °C until virus titrations were performed. In this study, duplicate, triplicate or quadruplicate sets of inoculum were inoculated in parallel on cells cultivated in a six-well plate. The HEV load was determined for all or selected series of culture supernatants from the inoculated wells and representative data were adopted.

**Detection and quantification of HEV RNA.** For detection of HEV RNA in serum samples, faecal supernatants and culture media, nested RT-PCR with primers targeting the ORF2 region of HEV RNA was performed as described previously (Mizuo et al., 2002). The size of the amplification product of the first-round PCR was 506 bp and that of the second-round PCR was 457 bp. The nested RT-PCR assay that we used has the capability of amplifying all four known genotypes of HEV strains reported thus far (Mizuo et al., 2002; Takahashi et al., 2003; Yazaki et al., 2003). Quantification of HEV RNA was performed by real-time detection RT-PCR according to the method described previously (Iothikumar et al., 2006), with a slight modification. In brief, total RNA was extracted from 10–100 \mu l serum sample, faecal supernatant or culture medium with TRIzol-LS reagent (Invitrogen) and subjected to real-time RT-PCR with a QuantiTect Probe RT-PCR kit (Qiagen) using sense primer (5'-GGGTTTTCTGGGTGCA-3'), antisense primer (5'-AGGGTTGTTGGATGAA-3'), and a probe consisting of an oligonucleotide with a 5'-reporter dye (FAM) and a 3'-quencher dye (TAMRA) (5'-FAM-TGATTCTAGCCCTTCG-TAMRA-3') on an ABI Prism 7700 Sequence Detector (Applied Biosystems). Thermal cycler conditions were 50 °C for 30 min at stage 1; 95 °C for 15 min at stage 2; and 50 cycles of 94 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s at stage 3. The reproducibility of the quantitative assay was assessed by testing each sample in duplicate and the mean value was adopted.

**Western blot analysis.** For detection of HEV capsid proteins, 10 \mu l culture medium or diluted faecal supernatant as a control was mixed with an equal volume of 2× gel-loading buffer with SDS and β-mercaptoethanol and a total of 15 \mu l solution was subjected to SDS-PAGE in a 5–15\% gradient gel (Bio-Rad), followed by transfer onto a nitrocellulose filter membrane (Hybond-EC; Amersham Biosciences). The membrane was immersed in Tris-buffered saline (TBS) [10 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.1\% (v/v) Tween-20] containing 5\% ECL blocking agent (Amersham Biosciences) and, after washing with TBS, incubated at room temperature for 1 h with 10 \mu g anti-HEV ORF2 mouse monoclonal antibody ml^{-1} (IgG1 subclass: Hyb-H6210) that had been raised against the recombinant HEV ORF2 protein expressed in the pupae of silkworm (Mizuo et al., 2002), as the primary antibody. After washing, the membrane was incubated with ECL anti-mouse IgG, horseradish peroxidase-linked species-specific whole antibody from sheep (1:2500; Amersham Biosciences) and examined using a chemical luminescence system (ECL Western blotting detection reagents and analysis system; Amersham Biosciences). ECL DualVue Western Blotting Markers (Amersham Biosciences) were used as protein markers.

**Study of the thermal stability of HEV.** One hundred microlitres of virus stock was dispensed to each of five MicroAmp reaction tubes (0.5 ml; Applied Biosystems). The tubes were incubated at 56 °C for 30 min, 70 °C for 10 min, 95 °C for 1 min or 95 °C for 10 min in a GeneAmp PCR System 2400 (Perkin Elmer). As control, a tube containing 100 \mu l virus stock was kept at room temperature (25 °C) for 30 min. The heat-treated virus and the control were diluted with PBS(-) to 6.0 \times 10^7 copies in 0.2 ml solution and inoculated on a monolayer of PLC/PRF/5 cells in a six-well microplate. The protocol after infection and maintenance of cultured cells were as described in the virus inoculation and passage section.

**Neutralization test.** Serum samples (nos 1–3) that were positive for IgG, IgM and IgA classes of anti-HEV antibodies by in-house
ELISA (Takahashi et al., 2005) were obtained from three patients with sporadic acute hepatitis E during the convalescent phase (Table 1). Three other serum samples (nos 4–6) containing only the IgG-class of anti-HEV antibody were obtained from patients with HEV infection 3.0, 8.7 or 24.0 years after disease onset (Kuno et al., 2003; Tokita et al., 2003; Mitsui et al., 2004). One additional serum sample (no. 7) was obtained from an individual with detectable anti-HEV IgG. The relative titres of anti-HEV IgG, IgM or IgA anti-bodies were determined by end-point ELISA according to the methods described previously (Tokita et al., 2003; Takahashi et al., 2005); i.e. the serum dilution that gave the absorbance (measured at the wavelength of 450 nm) of each cut-off point was estimated by testing multiple dilutions of the serum. All serum samples used were negative for HEV RNA.

The serum samples were purified by passage through a microfilter with 0.45 μm pore size and then a microfilter with 0.22 μm pore size, prior to use in the neutralization test. A solution of 0.2 ml containing the same amount of virus stock (6.0 × 10^4 copies) and each of various serum samples diluted with PBS(-) at 1:5, 1:50, 1:500 or 1:5000, was incubated at room temperature for 1 h and then inoculated on monolayers of PLC/PRF/5 cells in a six-well microplate. After 1 h, the supernatant was removed and 2 ml maintenance medium was added. The protocol after infection and maintenance of cell-culture was as described above.

**RESULTS**

**Inoculation of HEV on various cultured cells**

Faecal supernatant containing an HEV strain (JE03-1760F) was inoculated on each of the 21 selected cell lines and cultured at 35.5°C or 37.0°C. HEV could propagate in only PLC/PRF/5 and A549 cells among the 21 cell lines (Fig. 1). HEV RNA was first detected in the culture medium on day 12 or 14 after inoculation and continued to be detectable up to the end of the observation period of 38 days in the culture media of both PLC/PRF/5 and A549 cells. When HEV was inoculated on PLC/PRF/5 cells and maintained at 35.5°C, the HEV viral load was the highest throughout the observation period. Based on these results, culture of HEV was carried out using the PLC/PRF/5 cell line at a temperature of 35.5°C thereafter.

**Serial passages of HEV in PLC/PRF/5 cells**

As the first step in serial passages of HEV (passage 0), a faecal supernatant containing an HEV strain (JE03-1760F) was inoculated on PLC/PRF/5 cells (Table 2). HEV RNA became detectable in the culture supernatant on day 10 after inoculation at 1.1 × 10^4 copies ml^-1 and its load increased to 6.4 × 10^5 copies ml^-1 on day 28 (Fig. 2). The first passage (passage 1) on a fresh monolayer of PLC/PRF/5 cells was carried out using 28 days culture medium that was filtered through a 0.22 μm microfilter. Contrary to our expectation, HEV RNA was not detectable in the harvested culture medium 2 weeks later, but was first detected on day 36 after inoculation and reached a maximum load of 8.6 × 10^6 copies ml^-1 on day 56. For the second passage, the 56 days culture medium of passage 1 was inoculated on a fresh monolayer of PLC/PRF/5 cells. HEV RNA was first detected in the culture

![Fig. 1. Quantification of HEV RNA in culture supernatants of PLC/PRF/5 or A549 cells maintained at 35.5°C or 37.0°C that were inoculated with a faecal supernatant containing an HEV (JE03-1760F) strain and cultured for the indicated number of days.](http://vir.sgmjournals.org)
medium on day 16 and increased to $5.8 \times 10^6$ copies ml$^{-1}$ on day 88. In the third, fourth and fifth passages, progeny were first detectable in the culture medium on day 12 or 14 after inoculation and grew in a similar manner as those in passage 0 and passage 2. The HEV RNA titre in the culture medium increased to $2.4 \times 10^6$ copies ml$^{-1}$ on day 58 of the fifth passage. During these serial passages, no cytopathic effect (CPE) was observed in the PLC/PRF/5 cells, which continued to produce HEV progeny for at least 88 days as observed in passage 2.

**Inoculation of HEV at different viral loads**

The HEV RNA titre in the inoculum after filtration that was used for passage 1 was, at $3.6 \times 10^4$ copies per well, the lowest of the five passages (Table 2), suggesting that the day of the initial appearance of HEV in the culture medium may be affected by the HEV RNA titre in the inoculum. To elucidate the reason why HEV appeared 2 weeks later in the culture medium of passage 1 than in those of the other four passages, different amounts of HEV ($2.4 \times 10^4$, $3.2 \times 10^4$, $6.4 \times 10^4$, $1.6 \times 10^5$ or $8.6 \times 10^5$ copies per well) were inoculated on fresh monolayers of PLC/PRF/5 cells and the HEV viral load was measured in the culture media until day 60 after inoculation (Fig. 3). When HEV was inoculated at $2.4 \times 10^4$ or $3.2 \times 10^4$ copies per well, HEV RNA was first detected in the collected culture media on day 22 or 24 after inoculation. On the contrary, when HEV of $6.4 \times 10^4$ or more copies per well was inoculated, HEV initially appeared in the culture medium on day 12 or 14 after inoculation. The greater the amount of HEV inoculated, the more rapidly HEV in the culture medium increased. On day 60 after inoculation of HEV of $8.6 \times 10^5$ copies per well, the amount of HEV RNA in the culture medium was the highest at $8.6 \times 10^7$ copies ml$^{-1}$. Based on these results, the viral load of HEV to be inoculated on PLC/PRF/5 cells for efficient propagation of HEV was considered to be $6.0 \times 10^4$ or higher copies per well in our culture system.

**Western blot analysis with an ORF2 protein-specific mouse monoclonal antibody**

Western blots of the day 0 and day 12 culture media did not show any bands, but those of the day 18, 36, 50 and 68 culture media with HEV viral loads of $3.8 \times 10^4$ to $1.5 \times 10^8$ copies ml$^{-1}$ showed two bands of 65 kDa (major) and 74 kDa (minor) (lanes 3–6 in Fig. 4), the difference being most likely due to the absence/presence of glycosylation (Li et al., 1997). Their migration on SDS-PAGE was indistinguishable from that of faecal supernatant used as an inoculum in the present study (lanes 7 and 8 in Fig. 4).
Thermal stability of HEV

The same amounts of HEV inoculum were incubated at 95°C for 10 min, 95°C for 1 min, 70°C for 10 min, 56°C for 30 min or room temperature (25°C) for 30 min. They were diluted to $6.0 \times 10^7$ copies per well and inoculated on PLC/PRF/5 cells. When the HEV inoculum was incubated at 95°C for 10 min, 95°C for 1 min or 70°C for 10 min prior to inoculation on PLC/PRF/5 cells, HEV RNA was not detectable in the culture medium throughout the observation period of 50 days after inoculation (Table 3). However, when the HEV inoculum was incubated at 56°C for 30 min or room temperature (25°C) for 30 min prior to inoculation, HEV RNA was first detected in the culture medium on day 20 or day 16, respectively.

Neutralization test

Three serum samples (nos 1–3) (Table 1) containing IgG, IgM and IgA classes of anti-HEV antibodies obtained from patients who had contracted infection of HEV of genotype 3, 1 or 4, respectively, were tested for their ability to neutralize an HEV strain of genotype 3 (JE03-1760F) in the present culture system. Each serum sample, diluted at 1 : 2.5 with PBS(-), was mixed with an equal volume of the diluted virus stock containing $6.0 \times 10^8$ copies of HEV, kept at room temperature for 60 min and inoculated on monolayers of PLC/PRF/5 cells in a six-well microplate. In each well containing the serum sample of no. 1, 2 or 3, HEV RNA was not detectable throughout the observation period of up to 50 days after inoculation. However, in wells with the control serum (anti-HEV-negative), HEV RNA was first detectable on day 14 post-inoculation and continued to be positive ($> 10^6$ copies ml$^{-1}$) up to the end of the observation period.

One hundred microlitres each of serum samples containing IgG-class anti-HEV only (nos 4–7 in Table 1) diluted at 1 : 2.5, 1 : 25, 1 : 250 or 1 : 2500, was mixed with an equal volume of the diluted virus stock ($6.0 \times 10^8$ copies) and kept at room temperature for 60 min. When the serum samples obtained from patients (nos 4–6) 3.0 to 24.0 years after the onset of HEV infection were used, HEV RNA was not detectable in the culture supernatant up to 50 days after inoculation at the final dilutions of 1 : 5 and 1 : 50, but did become detectable in the culture supernatant on day 10 or 12 after inoculation at the final dilutions of 1 : 500 and 1 : 5000 (Table 4). In the serum sample (no. 7) with only anti-HEV IgG antibody that was obtained from an individual whose period of HEV infection is unknown, the culture supernatants were negative for HEV RNA throughout the observation period of 50 days when mixed at the final dilution of 1 : 5, but had detectable HEV RNA on day 12 and thereafter when mixed at the final dilutions of 1 : 50 and 1 : 500.

DISCUSSION

Establishment of a practical cell-culture system that facilitates the propagation of HEV in vitro is critical for virological characterization as well as for studies on prevention of HEV infection. Several in vitro culture systems for HEV replication, such as the human embryo lung diploid cell strain (2BS), A549, PLC/PRF/5, HepG2 and primary hepatocytes from non-human primates (chimpanzees, cynomolgus macaques, tamarins and African green monkeys), have been reported (Kane et al., 1984; Arankalle et al., 1988; Huang et al., 1992, 1995, 1999; Kazachkov et al., 1992; Tsarev et al., 1994; Li et al., 1996; Meng et al., 1996, 1997a; Tam et al., 1996a, b, 1997; Wei et al., 2000). However, none of these culture systems can provide high-titre HEV in the
culture medium; therefore, they cannot be used for the biophysical and virological studies of HEV. In the present study, using a faecal suspension (the JE03-1760F strain/genotype 3) with high HEV load (2.0 \( \times 10^7 \) copies ml\(^{-1} \)) as an inoculum, we tested 21 cell lines including A549, HepG2 and PLC/PRF/5 cells that had been reported to support in vitro replication of HEV (Huang et al., 1995, 1999; Li et al., 1996; Meng et al., 1996, 1997a; Wei et al., 2000), for the possible development of an efficient culture system for HEV. Of note, a high load of HEV was yielded from the culture supernatant of cultivated A549 and PLC/PRF/5 cells on day 12 to day 14 post-inoculation and thereafter, but HEV RNA was not detectable in the culture supernatant from the remaining 19 cell lines up to the end of the observation period of 30 days. Upon comparison of the load of HEV in culture supernatant obtained from A549 and PLC/PRF/5 cells that had been cultured at two distinct temperatures (35.5 or 37.0 °C), the highest yield of HEV was obtained in the culture supernatant of PLC/PRF/5 cells maintained at 35.5 °C throughout the observation period. Consequently, in the current study, we selected PLC/PRF/5 and 35.5 °C as the suitable cell line and culturing temperature, respectively.

Although the PLC/PRF/5 cell line has been used for the same purpose as the present study since the late 1980s (Pillot et al., 1996, 1998, 1999), the use of serum samples containing IgG-class antibodies to HEV from pedigreed and non-pedigreed patients who contracted clinical or subclinical HEV infection 3 to 24 years ago to confirm the neutralization of HEV in culture supernatants was also examined. The neutralization experiments were performed using serum samples diluted at 1 : 2.5, 1 : 25, 1 : 250 or 1 : 2500, and control serum diluted at 1 : 2.5, mixed with an equal volume of the diluted inoculum (6.0 \( \times 10^4 \) copies), kept at room temperature for 60 min, incubated on monolayers of PLC/PRF/5 cells and cultured for up to 50 days after inoculation. HEV RNA titre in the culture supernatant obtained on the indicated day after inoculation was measured.

Table 3. Effect of exposure to different temperatures on the infectivity of an HEV strain (JE03-1760F)

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>HEV RNA titre (copies ml(^{-1} )) in culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 °C, 30 min*</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>6.8 ( \times 10^2 )</td>
</tr>
<tr>
<td>18</td>
<td>1.2 ( \times 10^5 )</td>
</tr>
<tr>
<td>20</td>
<td>6.6 ( \times 10^5 )</td>
</tr>
<tr>
<td>30</td>
<td>4.7 ( \times 10^4 )</td>
</tr>
<tr>
<td>40</td>
<td>7.1 ( \times 10^5 )</td>
</tr>
<tr>
<td>50</td>
<td>1.2 ( \times 10^6 )</td>
</tr>
</tbody>
</table>

*HEV inoculum was incubated at the indicated temperature for 1–30 min, diluted to 6.0 \( \times 10^4 \) copies in 0.2 ml and inoculated on PLC/PRF/5 cells.

Table 4. Neutralization of an HEV strain (JE03-1760F) with serum samples containing IgG-class antibodies to HEV from pedigreed patients who contracted clinical or subclinical HEV infection 3 to 24 years ago or from a non-pedigreed individual

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>HEV RNA titre (copies ml(^{-1} )) in culture supernatant*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control serum</td>
</tr>
<tr>
<td></td>
<td>No. 4†</td>
</tr>
<tr>
<td>1:50</td>
<td>1:500</td>
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<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>3.2 ( \times 10^2 )</td>
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<tr>
<td>12</td>
<td>4.8 ( \times 10^4 )</td>
</tr>
<tr>
<td>14</td>
<td>7.0 ( \times 10^5 )</td>
</tr>
<tr>
<td>18</td>
<td>5.0 ( \times 10^6 )</td>
</tr>
<tr>
<td>20</td>
<td>6.8 ( \times 10^7 )</td>
</tr>
<tr>
<td>30</td>
<td>2.2 ( \times 10^4 )</td>
</tr>
<tr>
<td>40</td>
<td>4.0 ( \times 10^5 )</td>
</tr>
<tr>
<td>50</td>
<td>9.0 ( \times 10^5 )</td>
</tr>
</tbody>
</table>

*Each serum sample diluted at 1 : 2.5, 1 : 25, 1 : 250 or 1 : 2500 or control serum diluted at 1 : 2.5, was mixed with an equal volume of the diluted inoculum (6.0 \( \times 10^4 \) copies), kept at room temperature for 60 min, inoculated on monolayers of PLC/PRF/5 cells and cultured for up to 50 days after inoculation. HEV RNA titre in the culture supernatant obtained on the indicated day after inoculation was measured.

†HEV RNA was negative in the culture supernatants obtained on day 0 to day 50 post-inoculation when mixed with an equal amount of 1 : 2.5 dilution of serum sample.

‡NT, Not tested.
1987; Meng et al., 1996, 1997a), the precise reason why the high HEV load of up to $10^8$ copies ml$^{-1}$ was detected in the culture supernatant of cultivated PLC/PRF/5 cells in the current study is unknown. However, we speculate that the availability of a faecal suspension with high HEV load ($2.0 \times 10^7$ copies ml$^{-1}$) was vital for establishment of an efficient culture system for HEV in the present study. In support of our speculation, when we used other faecal suspensions with low HEV load, HEV could not be propagated on PLC/PRF/5 cells in repeated experiments (data not shown). Since the genotype 3 HEV strain used in this study could present higher infective capabilities than other HEV strains, the possibility of differences in the multiplication efficiencies of diverse HEV strains in cell-culture should also be considered.

In the present study, five generations of serial passages (passages 1–5) of culture supernatant were successfully carried out (Fig. 2). Prior to inoculation on PLC/PRF/5 cells, each inoculum (culture supernatant) for passage 1, 2, 3, 4 or 5 was purified by passage through a microfilter of 0.22 μm pore size. It is reasonable to consider that the filtered solution of inoculum does not contain cells or cell debris derived from long-term cultivation of PLC/PRF/5 cells and it is likely that serial passage was achieved by infection of virions produced by the infected cells to fresh cells in the next generation. These results suggest that the infected cells in the serial passages continued to replicate infectious HEV virions. Of note, progeny were detected approximately 2 weeks after inoculation and the HEV load increased to $10^6$–$10^7$ copies ml$^{-1}$ within 1 or 2 months in passages 2–5. However, in passage 1, HEV RNA was first detected in the culture supernatant on day 36 after inoculation. In order to clarify the reason for the delayed appearance of HEV in passage 1, the HEV RNA titre of the inoculum for each passage after treatment with the microfilter was determined. To our surprise, the HEV RNA titre of the inoculum after filtration was lower than that before filtration, probably due to non-specific absorption of HEV to the filter membrane. The real HEV RNA titre in the inoculum used for passage 1 was, at $3.6 \times 10^4$ copies per well, the lowest of the five passages, suggesting that the day of the initial appearance of HEV in the culture medium may depend on the HEV RNA titre of the inoculum. To confirm this notion, diluted faecal supernatants with various HEV loads were inoculated on PLC/PRF/5 cells. In the present system, the day on which HEV RNA became detectable in the culture supernatant was dose-dependent: i.e. it became detectable on day 12 or day 14 post-inoculation when a faecal suspension containing $6.4 \times 10^4$ or more copies per well was inoculated, and on day 24 or day 26 when a faecal suspension containing $2.4 \times 10^4$ or $3.2 \times 10^3$ copies per well was inoculated. In addition, the greater the amount of HEV inoculated, the more rapidly the HEV load in the culture supernatant increased, reaching higher levels. Therefore, in our cell-culture system using faecal supernatant containing the JE03–1760F strain as the inoculum, inoculation of $6.0 \times 10^4$ copies of HEV per well is recommended for efficient replication of HEV. In some reports (Huang et al., 1992, 1999; Li et al., 1996), the occurrence of HEV propagation was proven by observation of CPE. However, in our experiments, CPE was not observed in any of the cultured cells supporting replication of HEV. In our culture system, HEV RNA continued to be detected up to 4 months post-inoculation (data not shown), as long as HEV-infected PLC/PRF/5 cells were alive and continued to produce HEV progeny.

Recent studies have indicated that zoonotic food-borne transmission of HEV from domestic pigs, wild boar or wild deer to humans may occur as domestic infection in Japan, where some people ingest uncooked or undercooked meat or viscera (such as raw liver and colon/intestines) (Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Tamada et al., 2004; Li et al., 2005). Pig liver specimens from seven (1.9 %) of 363 packages sold in local grocery stores in Hokkaido had detectable HEV RNA. Of interest, one swine HEV isolate (swJL145) obtained from a packaged pig liver was 100 % identical to the virus recovered from an 86-year-old patient who had contracted sporadic hepatitis E after ingestion of undercooked pig liver, suggesting that consumption of undercooked pig liver/intestine is a potential risk factor for HEV infection (Yazaki et al., 2003). Since little was known about the thermal stability of HEV, the newly developed culture system for HEV was used to examine the thermal stability of HEV. In the current study, HEV in the faecal suspension was inactivated by incubation at 70 °C for 10 min or at 95 °C for 1 min; however, upon incubation at 56 °C for 30 min, the HEV was still infectious, corroborating the previous report by Emerson et al. (2005). In the present study, the faecal suspension was heat-treated without the addition of proteins as stabilizing factors. Therefore, the temperature that would be required to inactivate virus embedded in an uncooked or undercooked meat or viscera from infected pigs, wild boars or deer is expected to be higher than that estimated in the present study.

In vitro neutralization tests for HEV were reported by two groups of investigators in 1997, but these tests have shortcomings. Tam et al. (1997) used primary hepatocytes isolated from cynomolgus monkeys for inoculation of HEV, but primary hepatocytes from this monkey are not available to most laboratories and the cells need to be cultured under fastidious conditions. Meng et al. (1997a) used PLC/PRF/5 cells for propagating HEV, as in our current study, but they detected only those antibodies that block binding of virus to cells; antibodies that neutralize at a step after binding would not be seen as neutralizing in their test. In our cell-culture system, only replicating viruses are detected, thus ensuring that a biologically relevant receptor is used and that neutralization post-attachment can be detected, similar to a recently reported neutralization assay for HEV that identifies the virus-infected cells (HepG2/C3A) by immuno-fluorescence microscopy (Emerson et al., 2006). A previous study indicated that all convalescent serum samples from rhesus monkeys that had been experimentally infected with
HEV genotype 1, 2, 3 or 4 neutralized the genotype 1 virus (Emerson et al., 2006). In the present study, serum samples with IgG, IgM and IgA classes of anti-HEV antibodies that were obtained from patients infected with HEV of genotype 1, 2 or 3 during the convalescent phase neutralized a genotype 3 virus, confirming that HEV antibodies are broadly cross-reactive. In addition, serum samples obtained from patients with IgG anti-HEV antibodies 8.7 or 24.0 years after the onset of HEV infection, that were detectable by ELISA using recombinant ORF2 protein expressed in the pupae of silkworm (Mizu et al., 2002; Takahashi et al., 2005), also prevented propagation of HEV in PLC/PRF/5 cells, suggesting the presence of long-lasting HEV antibodies with neutralizing activity in individuals with past HEV infection.

In conclusion, using a faecal suspension with a high HEV load of $2.0 \times 10^7$ copies ml$^{-1}$, we developed an efficient cell-culture system for HEV in PLC/PRF/5 cells, with an HEV RNA titre of up to $10^8$ copies ml$^{-1}$ in the culture supernatant. HEV progeny released in the culture supernatant were passaged five times serially in PLC/PRF/5 cells. The cell-culture system developed for HEV would be useful for resolving many important questions regarding the biophysical and virological characteristics of HEV and for facilitating vaccine research.

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