REVIEW

Culture systems for hepatitis E virus

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Abstract The lack of an efficient cell culture system for hepatitis E virus (HEV) has greatly hampered detailed analyses of this virus. The first efficient cell culture systems for HEV that were developed were capable of secreting infectious HEV progenies in high titers into culture media, using PLC/PRF/5 cells derived from human hepatocellular carcinoma and A549 cells derived from human lung cancer as host cells. The success achieved with the original genotype 3 JE03-1760F strain has now been extended to various HEV strains in fecal and serum samples obtained from hepatitis E patients and to HEV strains in fecal and serum samples and liver tissues obtained from pigs and wild boar across species barriers. In addition, infectious HEV cDNA clones of the wild-type JE03-1760F strain and its variants have been engineered. Cell culture-generated HEV particles and those in circulating blood were found to be associated with lipids and open reading frame 3 (ORF3) protein, thereby likely contributing to the assembly and release of HEV from infected cells both in vivo and in vitro. The ORF3 protein interacts with the tumor susceptibility gene 101, a critical cellular protein required for the budding of enveloped viruses, through the Pro, Ser, Ala, and Pro (PSAP) motif in infected cells; ORF3 is co-localized with multivesicular bodies (MVBs) in the cytoplasm of infected cells, thus suggesting that HEV requires the MVB pathway for the egress of virus particles. This article reviews the development of efficient cell culture systems for a wide variety of infectious HEV strains obtained from humans, pigs, and wild boar, and also provides details of a new model for virion egress.

Abbreviations

CPE	Cytopathic effect
DN	Dominant negative
dpi	Days post-inoculation
ESCRT	Endosomal complex required for transport
HCV	Hepatitis C virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
kb	Kilobases
mAb	Monoclonal antibody
mutLSAL	A PASP mutant with two amino acid substitutions (Pro to Leu) in the PSAP motif of the ORF3 protein
MVB	Multivesicular body
ORFs	Open reading frames
RT-PCR	Reverse transcription-polymerase chain reaction
siRNA	Small interfering RNA
TGN	Trans-Golgi network
Tsg101	Tumor susceptibility gene 101
UTR	Untranslated region
Vps4	Vacuolar protein sorting-associated protein 4

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Introduction

Hepatitis E virus (HEV) is the causative agent of acute or fulminant hepatitis, which occurs in many parts of the world, principally as a water-borne infection in developing

countries in Asia, Africa, and Latin America where sanitation conditions are suboptimal, and zoonotically in many industrialized countries including the United States, European countries, and Japan [1–6]. Hepatitis E has long been described as a self-limiting hepatitis that never progresses to chronicity [3]. However, since the identification of a case of chronic hepatitis E in Europe, chronicity has been documented in immunocompromised solid-organ transplant recipients and HIV-infected individuals [7–9].

HEV is classified in the genus *Hepevirus* within the family *Hepeviridae* [10]. The virion measures 27–34 nm in diameter and is believed to be non-enveloped, although HEV particles in circulating blood and culture supernatants are associated with lipids, as described in detail below. The HEV genome consists of a single-stranded, positive-sense RNA measuring approximately 7.2 kilobases (kb) in length, which is capped and polyadenylated [11, 12]. It contains a short 5′ untranslated region (UTR), three open reading frames (ORFs: ORF1, ORF2, and ORF3), and a 3′UTR. ORF1 encodes non-structural proteins including methyltransferase, papain-like cysteine protease, helicase, and RNA-dependent RNA polymerase [13, 14]. ORF2 and ORF3 overlap, and the ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA measuring 2.2-kb in length [15]. The ORF2 protein is the viral capsid protein, which works for particle assembly, binding to host cells, and eliciting neutralizing antibodies; the crystal structure of a truncated recombinant ORF2 protein has been elucidated [16, 17], but the structure of the whole capsid protein has not yet been resolved [18]. The ORF3 protein, which is a small phosphoprotein made of 113 or 114 amino acids (aa), is necessary for virion release [19, 20], as is described in detail below.

The discoveries of animal strains of HEV from domestic pigs, wild boar, deer, mongoose, rabbits, rats, bats, chickens, and fish (trout) have significantly broadened the host range and genomic diversity of HEV [5, 21–29], and the existence of at least four putative genera in the family *Hepeviridae*, one comprising human HEV genotypes and closely related animal viruses, and the other three including viruses from rodent (rat), chiropteran (bat), and avian (chicken) hosts, has been suggested [28]. The trout hepevirus might correspond to a separate taxonomic unit of higher rank, e.g., a subfamily [30]. Hepatitis E is now a recognized zoonotic disease, and several species of animals, such as swine, wild boar, and deer, are considered to serve as reservoirs of HEV strains that infect humans [31–36]. The ingestion of uncooked or undercooked meat and the viscera of farmed pigs and wild boar may be the major route of HEV infection in industrialized countries, including Japan [5, 6, 37]. Four major genotypes of HEV that infect humans have been identified [38, 39]. Genotypes 1 and 2 have so far been isolated only from humans and are

mainly seen in developing countries. Genotypes 3 and 4 are zoonotic, and have been identified in many sporadic cases affecting middle-aged and elderly men in industrialized countries [4, 39–41]. Although two HEV strains (JBOAR135-Shiz09 and wbJOY_06) of new unrecognized genotypes, provisionally designated genotypes 5 and 6, respectively, that cluster closely with human HEV strains but are markedly divergent from rat, bat, and avian HEV strains, have been detected from wild boar in Japan, it remains unknown whether these boar strains can infect humans across species [42, 43].

HEV has been regarded as a virus that is impossible to culture in vitro, given the tremendous difficulties in developing a cell culture system. There are no small animal models of infection, and macaques are inaccessible or unaffordable for most researchers in the field. The 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 the prevention of HEV infection. This article reviews the breakthrough in the establishment of cell culture systems for various HEV strains from humans, pigs, and wild boar, and provides details of a new model for virion egress.

Establishment of cell culture systems for HEV strains of genotypes 3 and 4 found in feces and serial passages of their progenies in cultured cells

HEV usually replicates to low titers in vivo, and growing it in cell culture has proven to be exceedingly difficult. However, a breakthrough has been achieved recently using a fecal specimen from a sporadic case of acute hepatitis E in Japan that contained a very high load of HEV (JE03-1760F strain of genotype 3: 2.0×10^7 copies/ml), which thus enabled the establishment of a robust culture system for HEV. The fecal suspension was used as an inoculum, and the replication capacity of HEV was then evaluated in 21 established cell lines derived from humans, monkeys, cows, dogs, rats, and mice, including three human hepatocellular carcinoma cell lines (HepG2, Huh7, and PLC/PRF/5 cells). The JE03-1760F strain was found to replicate efficiently in two cell lines; PLC/PRF/5 (Alexander) and A549 from human lung cancer [44]. Of note, although A549 cells are not hepatocytes, HEV was shown to be distributed not only in the liver, but also in the lung, kidney, spleen, and even colon in infected pigs [45]. The presence of HEV in bile, lymph nodes, and tonsils in infected pigs has also been reported [46, 47].

Upon inoculation of the JE03-1760F strain, progeny viruses were excreted into the culture media in titers reflective of the HEV inoculum. HEV grew efficiently following the seeding of 1.0×10^5 copies (genome copies per cells: approximately 0.1) in the wells of a six-well microplate containing monolayers of PLC/PRF/5 or A549 cells, and the viral load in the culture medium reached

10^8 or 10^7 copies/ml, respectively, on day 50. The first successful propagation of the JE03-1760F strain in PLC/PRF/5 and A549 cells may have been ascribable to the markedly high HEV titer in the inoculum. RNA viruses generally exist as quasispecies [48]. A sample with a high titer has an increased probability of containing variant(s) with advantageous mutations needed to permit the infection of a cultured cell. Notably, the JE03-1760F strain harbored the 29 point mutations with 6 non-synonymous mutations found in the virus in feces, which were not possessed by any of the reported genotype 3 HEV strains [49].

The initial success was followed by the establishment of a culture system with the use of another fecal suspension, from a patient with fulminant hepatitis E, containing the HE-JF5/15F strain of genotype 4 at a high titer (1.3×10^7 copies/ml) [50]. The HE-JF5/15F strain was propagated and passaged more efficiently than the JE03-1760F strain [51]. Epidemiological surveys suggest that HEV of genotype 4 is significantly more frequently associated with a severe form of hepatitis than HEV of genotype 3 [52–54]. The high replicative activity of HEV genotype 4, reproduced in the culture system for the HE-JF5/15F strain of this genotype, is expected to be useful for elucidating the viral factors associated with the development of fulminant hepatitis E in infected patients.

Furthermore, HEV progenies of the genotype 3 JE03-1760F strain in the culture supernatant grew efficiently through many generations of passages in PLC/PRF/5 and A549 cells (53 generations of passages as of June 2012), with the highest HEV RNA titer in the culture media being 10^8–10^9 copies/ml (unpublished observations). In addition, HEV progenies of the genotype 4 HE-JF5/15F strain in the culture supernatant replicated efficiently through many generations of passages in PLC/PRF/5 and A549 cells (33 generations of passages as of June 2012), with the highest HEV loads in the culture supernatants being 10^9–10^{10} copies/ml (unpublished observations). Shukla et al. [55] described the adaptation of the Kernow C1 strain, a genotype 3 HEV strain purified from the feces of a chronically infected patient, to growth in human hepatoma cells (HepG2/C3A) selected for a virus recombinant that contained an insertion of 174 ribonucleotides (58 amino acids) of a human ribosomal protein gene. However, no such insertion or deletion of nucleotides was observed in the genomes of the cell-culture adapted JE03-1760F and HE-JF5/15F strains during the long-term passages. No cytopathic effect (CPE) was observed in the PLC/PRF/5 and A549 cells during these serial passages of the JE03-1760F and HE-JF5/15F strains, despite differences in the durations of the cell culture and the changing profile of the HEV load in each passage.

Although HepG2 and Huh7 cells are not permissive for the wild-type JE03-1760F and HE-JF5/15F strains of feces

origin, recent studies have demonstrated that both these cell lines are capable of supporting the successful propagation and passages of cell-culture adapted JE03-1760F and HE-JF5/15F strains (unpublished observations).

Propagation of HEV strains found in circulating blood in cultured cells

HEV is an emerging infectious threat to blood safety. There have been a number of publications delineating this threat by providing evidence of the transmissibility of this virus through transfusions. Transfusion-transmitted HEV infection has been reported not only in developing countries (genotype 1) [56], but also in industrialized countries, including Japan and the United Kingdom (genotypes 3 and 4) [57–60], thus suggesting that HEV in circulating blood can also grow in cultured cells. Therefore, this study examined whether HEV strains in serum samples could replicate in PLC/PRF/5 and A549 cells and release infectious progeny viruses into the culture media; this was investigated in relation to the HEV load, genotype, and co-existence of HEV antibodies [61]. The study revealed that various HEV strains of genotype 1, 3, or 4 in the serum samples obtained from patients with autochthonous or imported hepatitis E could infect and replicate efficiently in PLC/PRF/5 and A549 cells. Notably, HEV strains in all serum samples tested, with or without concurrent HEV antibodies, were successfully propagated in cultured cells when inoculated at an HEV load of $\geq 10^5$ copies per well in a six-well microplate (Fig. 1). Progeny viruses of serum-derived HEV strains in the culture supernatant were successfully passaged in PLC/PRF/5 and A549 cells, thus indicating that the HEV progenies of serum origin released from cultured cells were infectious, similar to those of feces origin [61].

Characterization of HEV particles in culture supernatant, circulating blood, and feces

An examination of the physicochemical properties of HEV particles in the culture supernatant revealed the HEV particles in cell culture to have a buoyant density of 1.15–1.16 g/ml in sucrose gradients [62], which was identical to the finding in serum samples, irrespective of the presence or absence of circulating anti-HEV antibodies, but markedly lower than that in feces, which peaked at 1.27–1.28 g/ml [61] (Table 1). The great majority (more than 90 %) of HEV particles in the circulation were free of immunoglobulins even in the presence of IgM anti-HEV antibodies [61]. Similar to cell culture-generated HEV particles, HEV particles in serum were non-neutralizable

Fig. 1 Quantification of hepatitis E virus (HEV) RNA in culture supernatants of PLC/PRF/5 cells after inoculation of serum samples at a viral load on the order of 10^6 (*upper panel*) or 10^5 (*lower panel*) copies per well, containing HEV of the indicated genotype. The *asterisks in parentheses* indicate serum samples with anti-HEV antibodies. See reference [61] for further details
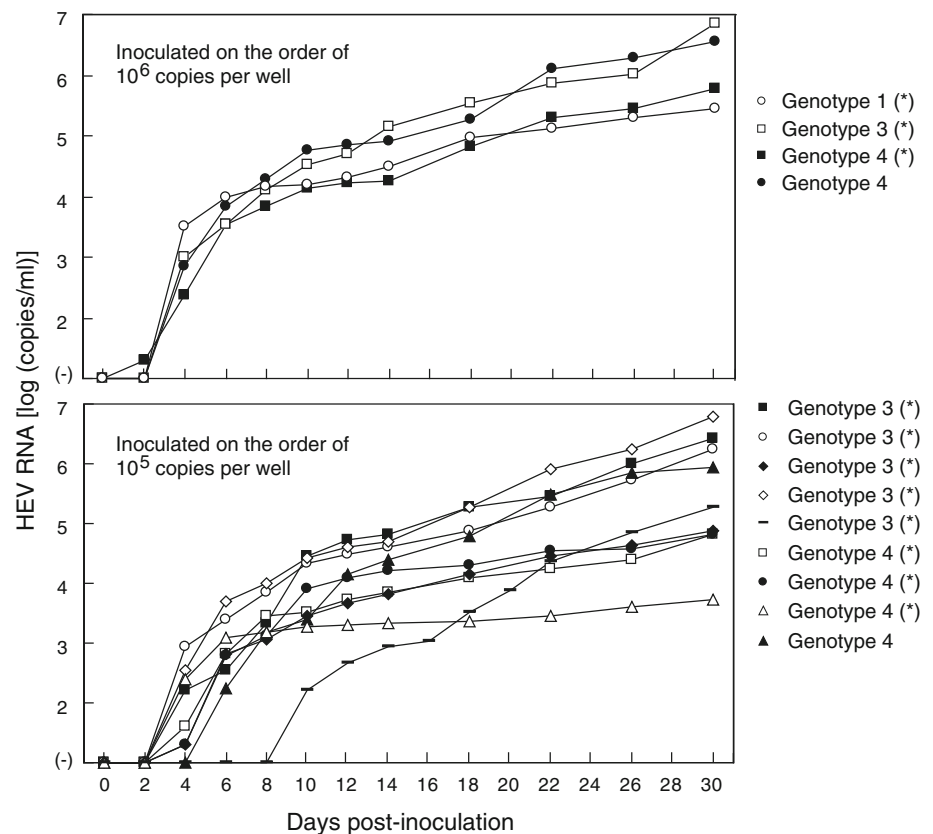
by immune sera and anti-ORF2 monoclonal antibodies (mAbs) that could definitely neutralize the infection of HEV in feces in the cell culture system [44, 62], and few or no virus particles in either the serum or cell culture were captured by anti-ORF2 mAb and anti-ORF3 mAb. Interestingly, however, after treatment with a detergent such as deoxycholic acid, Tween 20, or NP-40, the binding efficiency of HEV particles in serum and culture supernatant to both anti-ORF2 and anti-ORF3 mAbs markedly increased [61], thus suggesting that the HEV virion in both the serum and culture medium possesses the ORF3 protein on its surface, in association with lipids [19].

The detergent-treated HEV virions in the serum and culture medium were partially neutralized by anti-HEV antibodies, which may be ascribable to the incomplete exposure of ORF2 and ORF3 proteins after treatment with detergent only. The cell culture-produced and serum HEV particles treated with both detergent and protease, with a buoyant density of 1.27-1.28 g/ml in sucrose (Table 1), can be neutralized by an anti-HEV immune serum and anti-ORF2 mAb, thus indicating that virions treated with lipid solvent and proteases, possessing the same characteristics as virions from feces, are neutralizable by anti-HEV immune sera. The ORF3 protein is required for virion release from cultured cells. Taken together, these findings indicate that it is very likely that HEV particles are released

from both infected cultured cells (in vitro) and infected hepatocytes (in vivo) as lipid-associated virions, accompanied by ORF3 protein, and that the ORF3 protein and lipids are dissociated from the virion after shedding in the bile duct, which contain detergent (deoxycholic acid), and then in the duodenum, which contain protease (trypsin) secreted from the pancreas (Fig. 2).

HEV attaches to the host cell via a specific high-affinity receptor and enters the cytoplasm by clathrin-mediated endocytosis [63]. However, virtually nothing is known about the mechanism by which HEV enters susceptible cells. It is interesting that non-neutralizable HEV particles in serum samples and culture supernatant, which are associated with lipids and band at 1.15-1.16 g/ml in sucrose gradients, can bind to cultured cells, although inefficiently, and can be propagated in cultured cells [19]. Furthermore, a lipid solvent did not abolish or increase the infectivity of the HEV progenies obtained from infected cells, and both the cell culture-generated HEV particles and those that were treated with detergent and/or protease were propagated in the cultured cells with nearly identical efficiency [61], regardless of the presence or absence of the ORF3 protein and lipids on the surface. These findings, taken together, suggest that it is unlikely that only a fraction of HEV particles in the serum samples and culture media, partially unaccompanied by lipids, can bind to the

Table 1 Changes in the buoyant density and antigenicity in hepatitis E virus (HEV) particles of distinct origin after treatment with deoxycholic acid and/or trypsin

HEV particles	Treatment		
	None	Deoxycholic acid[a]	Deoxycholic acid + trypsin[a]
Culture supernatant			
Density in sucrose (g/ml)	1.15–1.16	1.21–1.24	1.27–1.28
Detectability by			
Anti-ORF2 mAb	No	Yes	Yes
Anti-ORF3 mAb	No	Yes	No
Neutralizability by			
Immune sera	No	Yes	Yes
Anti-ORF2 mAb	No	Yes	Yes
Serum			
Density in sucrose (g/ml)	1.15–1.16	1.21–1.24	1.27–1.28
Detectable by			
Anti-ORF2 mAb	No	Yes	Yes
Anti-ORF3 mAb	No	Yes	No
Neutralizability by			
Immune sera	No	Yes	Yes
Anti-ORF2 mAb	No	Yes	Yes
Feces			
Density in sucrose (g/ml)	1.27–1.28	1.27–1.28	1.27–1.28
Detectability by			
Anti-ORF2 mAb	Yes	Yes	Yes
Anti-ORF3 mAb	No	No	No
Neutralizability by			
Immune sera	Yes	Yes	Yes
Anti-ORF2 mAb	Yes	Yes	Yes

ORF2 open reading frame 2, *mAb* monoclonal antibody

[a] Treated with 0.1 % deoxycholic acid and/or 0.1 % trypsin

cell surface receptors and enter susceptible cells. In order to provide a plausible explanation(s) for this intriguing observed phenomenon, future studies must therefore elucidate whether and how non-neutralizable HEV particles in the serum and culture supernatant can enter susceptible cells.

Propagation of HEV strains in cell culture across species barrier

Shukla et al. [55] recently showed that genotype 3 viruses infected swine cells (LLC-PK1) more efficiently than human cells (HepG2/C3A). A549 cells can also support the replication of swine HEV of genotype 4 in a fecal specimen [64]. However, it remains unclear whether swine HEV strains of genotypes 3 and 4 recovered from domestic pigs and wild boar can grow as efficiently as human HEV strains in human cultured cells, irrespective of the source of the inoculum virus. Therefore, to investigate this problem, liver homogenate, serum and fecal specimens obtained from HEV-infected domestic pigs and wild boar were employed as inocula, and various swine and boar HEV strains were cultivated in A549 and PLC/PRF/5 cells and their replication efficiency was evaluated in relation to the viral load and origin of the inoculum [65].

Inoculation of three HEV RNA-positive liver homogenate samples with a higher HEV load (4.0×10^4–6.6×10^5 copies per well) obtained from raw pig liver, sold as food that had been purchased from grocery stores and kept frozen at -80 °C [6], released HEV progeny viruses into the culture medium. Figure 3a shows that HEV RNA was first detected in the culture medium of A549 cells on the 2nd to 6th day post-inoculation (dpi), with a viral load of 40–220 copies/ml, and continued to increase thereafter, with the highest titer of 1.1–7.3×10^7 copies/ml on 30 dpi. HEV progenies were also released into the culture medium upon the inoculation of fecal and serum samples [65].

Efficient viral multiplication was observed upon the inoculation of four liver homogenate samples containing boar HEV at an HEV load of 9.8×10^5–6.6×10^6 copies per well [65]. HEV RNA was initially detected on day 2 and reached the highest titer of 9.8×10^7–3.5×10^8 copies/ml on day 50 (Fig. 3b). Overall, HEV progenies were released into the culture medium when swine and boar
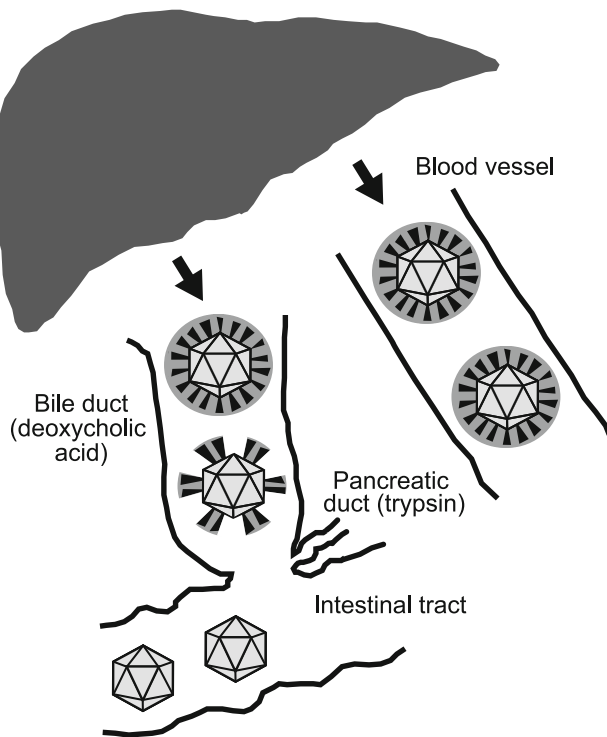
Fig. 2 Schematic diagram of the "enveloped" and "non-enveloped" HEV particles in infected hosts. HEV particles excreted from the liver into the circulation are likely to be present as "enveloped" virus with cellular membranes and open reading frame 3 (ORF3) proteins on their surfaces, while the ORF3 protein and cellular membranes are dissociated from the virion after shedding in the bile duct, which contains detergent (deoxycholic acid) and then in the duodenum, which contains protease (trypsin), both secreted from the pancreas

HEV strains were inoculated at an HEV load of $\geq 2.0 \times 10^4$ copies per well, irrespective of the inoculum source, although the HEV RNA titers in the culture medium of 30 dpi differed markedly, ranging from 1.0×10^2 to 1.1×10^7 copies/ml, by inoculum, when inoculated at viral loads ranging from 2.0×10^4 to 1.1×10^5 copies per well [65]. In sharp contrast, no progenies were detectable in the culture supernatant upon the inoculation of swine and boar HEV strains at an HEV load of $<1.8 \times 10^4$ copies per well. These results indicate that swine HEV strains of genotypes 3 and 4 from domestic pigs and wild boar can replicate as efficiently as human HEV strains in human cultured cells, thereby supporting the zoonotic nature of HEV in the culture systems for HEV as well.

Construction of infectious cDNA clones of wild-type and mutated HEV genomes and analysis of the function of the ORF3 protein in virion egress

A full-length infectious cDNA clone (pJE03-1760F/wt) of the genotype 3 JE03-1760F strain was developed [66].

The inoculation of the pJE03-1760F/wild-type (wt) virus into PLC/PRF/5 or A549 cells in six-well plates at 1.0×10^5 copies per well allowed the cDNA-derived virus to grow as efficiently as the original feces-derived virus in both PLC/PRF/5 and A549 cells, thus reaching 10^6 copies/ml on 30 dpi in the respective cells. Therefore, the reverse genetics system for HEV is utilizable in a robust cell culture system to elucidate the mechanism of HEV replication and the functional roles of HEV proteins.

Previous studies suggested the expression of the intact ORF3 protein to be essential for the infection of animals [67, 68], but this protein is not required for infection and virion morphogenesis in vitro [69]. However, an immuno-capture reverse transcription-polymerase chain reaction (RT-PCR) assay using an anti-ORF3 mAb suggested the presence of ORF3 protein on the surfaces of cell culture-generated HEV particles and those in circulating blood [61, 70]. Therefore, to examine whether ORF3 protein is responsible for virion morphogenesis and viral release from infected cells [19], a full-length cDNA clone of an ORF3-deficient mutant (pJE03-1760F/ΔORF3; hereafter, ΔORF3 for simplicity) was constructed by mutating the initiation codon of the ORF3 gene (ATG to GCA) on the pJE03-1760F/wt cDNA clone (Fig. 4a).

The culture supernatant of cells transfected with RNA transcripts of the ΔORF3 cDNA clone was inoculated onto A549 cells to determine whether the ΔORF3 mutant virus in the culture medium of the transfected cells was infectious and replication-competent and, if so, whether HEV virions were released from the ΔORF3 mutant-infected cultured cells. Viral RNA was nearly undetectable in the culture supernatant of A549 cells inoculated with the ΔORF3 mutant (Fig. 4b). Despite the significantly lower levels of HEV RNA in the culture supernatant of ΔORF3 mutant-inoculated A549 cells, the intracellular HEV RNA was nearly equal to that observed in cultured cells inoculated with the pJE03-1760F/wt (Fig. 4b). When applied to sucrose density ultracentrifugation, the ΔORF3 mutant in the culture supernatant of the transfected cells banded at 1.27–1.28 g/ml, in contrast to the pJE03-1760F/wt virus, which peaked at 1.15–1.16 g/ml (Fig. 4c) [19].

The advent of an infectious cDNA clone of genotype 1 HEV (the Sar55 strain), allowed Emerson et al. [20] to confirm the findings of the previous study conducted by using a genotype 3 infectious cDNA clone of the JE03-1760F strain, which reported the ORF3 protein to be essential for virion egress from infected cells, the ORF3 protein to be present on the surfaces of HEV particles released from infected cells, and the HEV particles released from infected cells to be lipid-associated [19]. Genotypes 2 and 4 HEVs have not yet been examined for the function of the ORF3 protein in virion egress using infectious cDNA clones. However, given its conservation,

Fig. 3 Quantification of HEV RNA in the culture supernatants of A549 cells inoculated with homogenate samples of swine (*sw*) liver tissues (**a**) and wild boar (*wb*) liver tissues (**b**). Modified from reference [65]
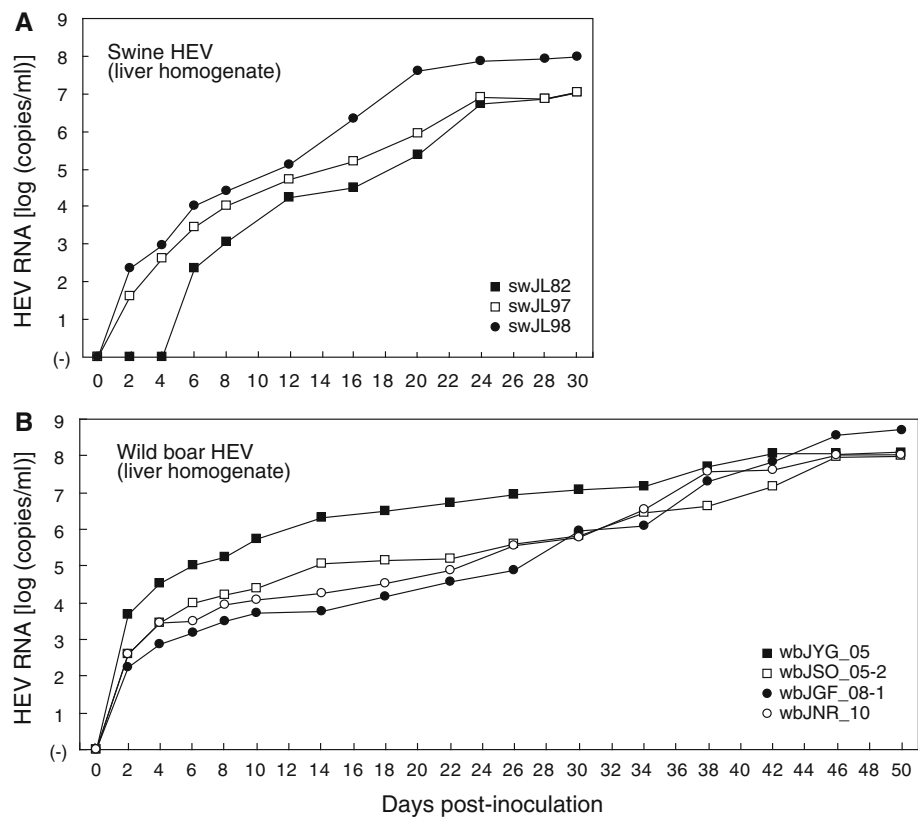
and because the association of HEV virions of genotype 1, 3, or 4 in the blood circulation with ORF3 protein and lipids on the surface has been noted [61], it is very likely that the function of the ORF3 protein related to HEV morphogenesis is common to all HEV strains, irrespective of genotype.

A Pro-rich sequence is present in the C-terminal region of the ORF3 protein in all mammalian and avian HEV strains, and the Pro, Ser, Ala, and Pro (PSAP) motif between amino acid residues 95 and 98 of the ORF3 protein (Fig. 4a) is conserved among all known HEV strains, including avian HEV strains [71]. The introduction of amino acid substitutions (two Pro-to-Leu replacements) in the PSAP motif (mutLSAL) (Fig. 4a) significantly reduced the virus yield in the culture supernatant, similar to an ORF3-deficient variant, ΔORF3 (Fig. 4b). Notably, no significant difference in the intracellular HEV RNA level was observed upon inoculation of the wild-type virus and its ORF3 variants (Fig. 4b). The particles generated in the culture supernatant of the mutLSAL RNA-transfected cells banded at 1.26–1.27 g/ml, similar to the particles in the ΔORF3 RNA-transfected cells (Fig. 4c), thus suggesting that the PSAP motif in the ORF3 protein is indispensable for the formation of membrane-associated HEV particles. Viral particles in the culture supernatant of the mutLSAL RNA-transfected cells were efficiently captured by an anti-ORF2 mAb, but not by an anti-ORF3 mAb, which was

similar to the findings observed in the supernatant of the ΔORF3 RNA-transfected cells, with or without prior treatment with 0.1 % sodium deoxycholic acid. These results indicate that an intact PSAP motif in the ORF3 protein plays a pivotal role in the release of HEV particles having lipid-associated membranes and ORF3 protein [71].

Cellular factors involved in the release of hepatitis E virions

Recent studies have revealed that viral matrix proteins play critical roles during the later stages of virus budding in many enveloped RNA viruses, including retroviruses, orthomyxoviruses, rhabdoviruses, and filoviruses: these viral proteins possess a so-called late (L)-domain containing P(T/S)AP, PPxY, and YxxL, which are critical motifs for the efficient release of an enveloped virus [72–76], and they hijack host proteins in the vacuolar protein sorting pathway [75, 77]. This pathway gives rise to multivesicular bodies (MVBs), which are topologically identical to virus budding [78]. The PTAP motif was first identified in the Gag protein of human immunodeficiency virus (HIV) and has been reported to bind to the tumor susceptibility gene 101 (Tsg101), which is identified as a critical cellular protein required for the budding of enveloped viruses, e.g., HIV and Ebola virus, from the plasma membrane [79, 80].
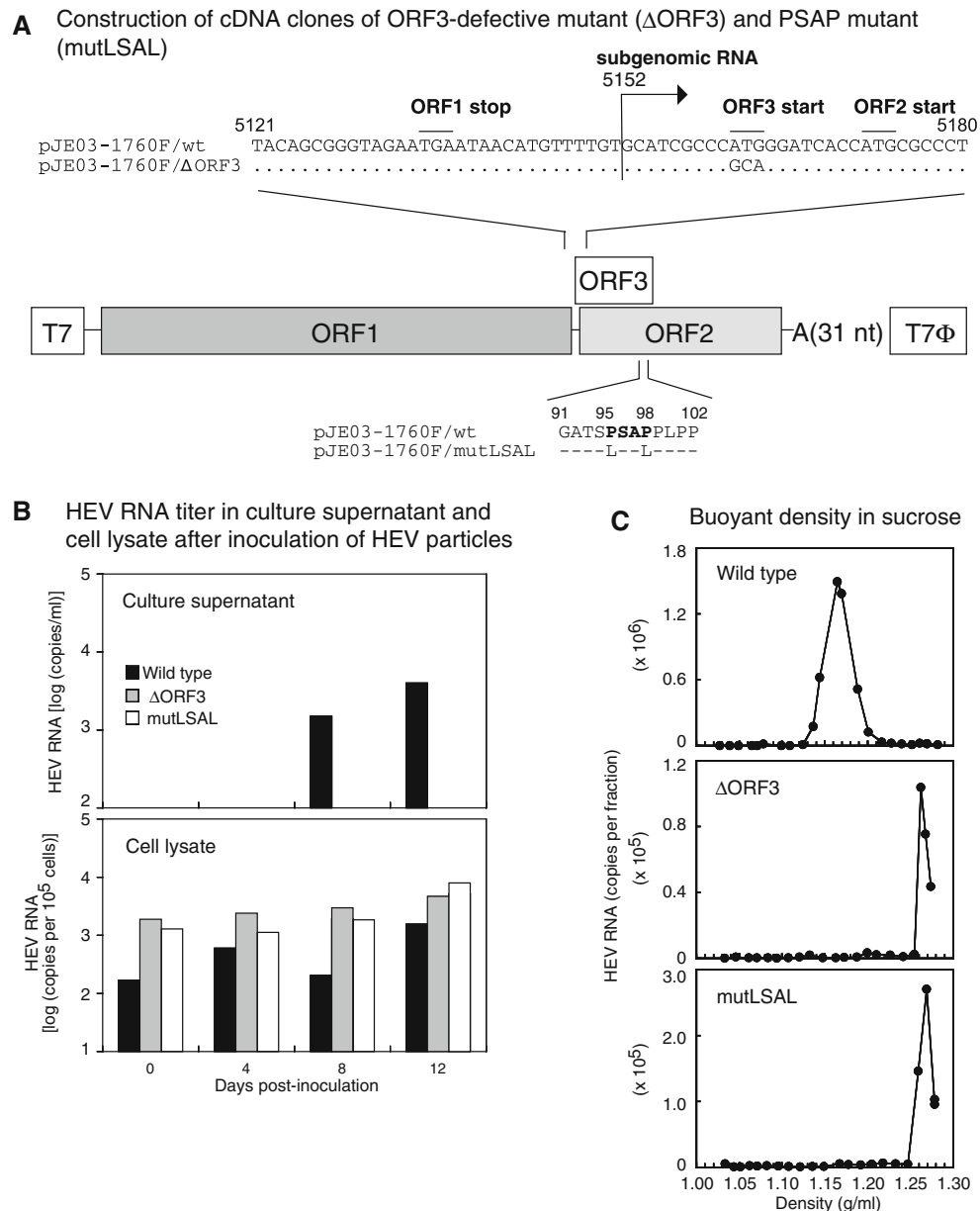
A Construction of cDNA clones of ORF3-defective mutant (ΔORF3) and PSAP mutant (mutLSAL)

B HEV RNA titer in culture supernatant and cell lysate after inoculation of HEV particles

C Buoyant density in sucrose

Fig. 4 Characterization of the ORF3 mutants of HEV. **a** Schematic illustration of the full-length cDNA clone of the HEV JE03-1760F strain (pJE03-1760F/wild-type [wt]) and its derivative mutants. The nucleotide (*nt*) sequence of nt 5121–5180 of the full-length cDNA clones of pJE03-1760F/wt and pJE03-1760F/ΔORF3 (ΔORF3, for simplicity) and the amino acid sequence of amino acid (aa) 91–102 of the ORF3 protein of the full-length cDNA clones of pJE03-1760F/wt and its Pro, Ser, Ala, and Pro (*PSAP*) mutant mutLSAL (pJE03-1760F/mutLSAL; mutLSAL, for simplicity) are aligned. Although not illustrated in the Fig., a Ser-to-Leu mutation at aa 87, which is identical to the majority of reported genotype 3 HEV strains, was also introduced in the mutLSAL. The stop codon of the ORF1 gene and the proposed initiation codons of the ORF2 and ORF3 genes are indicated by *lines above the nucleotides*. The *dots* represent

nucleotides identical to those at the top (wild-type). The initiation site of subgenomic mRNA transcription [92] is depicted by a *vertical line with an arrow facing right*. The ΔORF3 mutant was generated by mutating ATG (Met) to GCA (Ala) at the start codon of the ORF3 gene. The mutLSAL mutant was produced by converting CCC (Pro) to CTC (Leu) at the 95th codon and CCT (Pro) to CTT (Leu) at the 98th codon of the ORF3 gene. **b** HEV RNA in the culture supernatant (*upper panel*) and cell lysate (*lower panel*) of A549 cells inoculated with the culture supernatant of wild-type, ΔORF3, or mutLSAL RNA-transfected PLC/PRF/5 cells was quantified by measuring the RNA titer by real-time reverse transcription-polymerase chain reaction (RT-PCR). **c** Sucrose density-gradient fractionation of HEV in culture supernatants from PLC/PRF/5 cells transfected with RNA transcripts of wild-type, ΔORF3, or mutLSAL. Modified from reference [71]

Tsg101 binds to the PSAP motif of HEV located within the ORF3 protein [81]. A co-immunoprecipitation procedure also showed the direct interaction of ORF3 protein with Tsg101 and the loss thereof with the PSAP mutations in PLC/PRF/5 cells transfected with a full-length wild-type or mutant ORF3 expression plasmid [71, 82]. These results

indicate the requirement of an intact PSAP motif in the ORF3 protein for the formation and release of membrane-associated HEV particles possessing ORF3 proteins on the surface, in agreement with the observation by Emerson et al. [20], who reported the PxxP motif of the ORF3 protein to play an important role in virus egress and infection.

When the intracellular localization of the ORF3 protein and Tsg101 was examined by immunofluorescence confocal microscopy, a high degree of co-localization of wild-type ORF3 protein and Tsg101 was observed in the cytoplasm. In contrast, the mutLSAL ORF3 protein showed essentially no signal of co-localization with Tsg101, despite the fact that the mutated ORF3 protein showed an intracellular localization similar to that of the wild-type ORF3 protein. These results support the notion that the ORF3 protein interacts with Tsg101 through its PSAP motif in infected cells [82]. The effect on virion release from infected cells was examined by utilizing small interfering (si) RNA against Tsg101 to examine whether Tsg101 was functionally involved in HEV budding through its interaction with the ORF3 protein. The depletion of endogenous Tsg101 by siRNA led to a significant reduction of HEV release in cultured cells, although the HEV RNA replication was not affected by the transfection of siRNA against Tsg101. These results indicate that Tsg101 plays a pivotal role specifically in the release of HEV virions, thus corroborating the role attributed to Tsg101 in the budding of other known enveloped viruses such as retroviruses [80], although HEV is known to be a non-enveloped virus. It was also strongly suggested that HEV utilizes the mechanism of cellular MVB sorting, because Tsg101 is a component of the endosomal complex required for transport (ESCRT)-I complex which is involved in MVB sorting.

The vacuolar protein sorting-associated protein 4 (Vps4) ATPase is one of the final effectors in the MVB pathway and functions downstream of Tsg101, and therefore it was hypothesized that dominant negative (DN) mutants of Vps4 might disrupt virion release. In fact, DN mutants of Vps4 inhibit the budding of infectious HIV particles in co-transfection experiments [76]. The overexpression of DN mutants of Vps4 inhibited the release of HEV substantially. The reduction in the release of HEV particles in the presence of DN mutants of Vps4 appears to be due to a specific block in the formation of membrane-associated virions. It is likely that Vps4 is required for the final step in the formation of membrane-associated HEV particles. These results suggest that HEV utilizes the MVB pathway for virion egress, and that the enzymatic activities of Vps4 are involved in the virus release [82].

Whether the membrane-associated HEV particles are generated intracellularly or at the cell surface remains unclear. HEV particles with lipid membranes and the ORF3 protein on their surfaces were found abundantly in the lysates of cells infected with wild-type HEV (unpublished observations), and the ORF3 protein and Tsg101 were co-localized in the cytoplasm, as described above, thus suggesting that mature membrane-associated HEV particles are generated before their release from the surfaces of infected cells. An immunofluorescence assay using anti-ORF3 mAb and antibody against CD63, another MVB marker protein, revealed that the ORF3 protein was co-localized with CD63 in the HEV-infected cells [82]. Taken together, these findings indicate that HEV likely utilizes the cellular ESCRT mechanism in the cytoplasm, but not at the cell surface, to induce the release from infected cells. Further studies are needed to clarify whether membrane-associated HEV particles really bud into the intracellular vesicles formed in infected cells, and whether they are derived from the Golgi apparatus, trans-Golgi network (TGN), or endosomes, and also whether the mature virions are released into the extracellular environment by the exosomal secretion pathway, similar to the pathway in known enveloped viruses such as the hepatitis C virus (HCV) [83, 84].

Although it has been reported that monotherapy using interferon or ribavirin inhibits the viral replication in immunocompromised patients with chronic HEV infection following solid-organ transplantation [85, 86], no approved antiviral drugs are yet available to prevent or treat HEV-associated diseases. A conserved virus release mechanism would therefore be likely to be an excellent target for antiviral drugs, and human tetherin/BST-2 is reported to inhibit the release of HIV, filoviruses, and herpesviruses [87–91]. It may therefore be interesting to analyze whether tetherin/BST-2 inhibits the virion release of HEV. A better understanding of the mechanisms whereby viruses recruit cellular factors should therefore help greatly in the development of new therapeutic strategies.

Conclusion

This article has described the development of the first efficient cell culture systems for a wide variety of HEV strains from humans, pigs, and wild boar. The unique characteristics of cell-culture-generated HEV particles and those in the circulating blood were reviewed, and the current knowledge on virion egress obtained by the established cell culture models, reinforced by a reverse genetics system, was introduced. These cell culture systems, with the aid of reverse genetics systems, will solve many mysteries and answer numerous questions surrounding the epidemiology of HEV infection, and will be useful for the further understanding of the HEV life cycle and the investigation of potential antiviral drugs, as well as

for launching new preventive strategies against this zoonotic pathogen.

Conflict of interest The author declares that he has no conflict of interest.

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