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# Cell culture systems for the study of hepatitis E virus

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ABSTRACT

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and is the leading cause of enterically-transmitted viral hepatitis worldwide. Increasing numbers of HEV infections, together with no available specific anti-HEV treatment, contributes to the pathogen's major health burden. A robust cell culture system is required for virologic studies and the development of new antiviral drugs. Unfortunately, like other hepatitis viruses, HEV is difficult to propagate in conventional cell lines. Many different cell culture systems have been tested using various HEV strains, but viral replication usually progresses very slowly, and infection with low virion counts results in non-productive HEV replication. However, recent progress involving generation of cDNA clones and passaging primary patient isolates in distinct cell lines has improved *in vitro* HEV propagation. This review describes various approaches to cultivate HEV in cellular and animal models and how these systems are used to study HEV infections and evaluate anti-HEV drug candidates.

## 1. Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and a member of the genus Orthohepevirus in the family *Hepeviridae*. HEV infections commonly cause acute hepatitis but can also take a chronic course. Ribavirin is the treatment of choice for most patients, although type I interferon (IFN) has been evaluated in infected transplantation patients. However, no effective and specific treatments against HEV infection have been developed, as their development has been challenging due to difficulties in propagating HEV *in vitro*. Before HEV cell culture systems were developed, various heterologous expression systems, such as *Escherichia coli* or insect cells, were used to study HEV protein interactions and the HEV life cycle, but with inconsistent results. Different cell culture systems have also been tested using different HEV strains, again with varying outcomes. Recent breakthroughs have been achieved by identifying compatible cell lines, such as HepG2/C3A and A549, and isolating specific HEV strains (e.g. Kernow-C1/p6). Here, we summarise the key facts of HEV, review existing cell culture systems and surrogate models for analysing HEV's life cycle and evaluating anti-HEV treatments and discuss various primary isolates and cDNA clones tested in primary, cancer cell lines and stem cell-derived hepatocellular systems.

## 2. Hepatitis E virus

Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and is the leading cause of enterically-transmitted viral hepatitis worldwide but can also exhibit extrahepatic manifestations, such as neurological syndromes, renal injury and haematological disorders (Sood et al., 2000; Mishra et al., 2007; Colson et al., 2008; Kamar et al., 2014a; Pischke et al., 2014). The first description of HEV as a novel agent responsible for enterically-transmitted non-A, non-B hepatitis dates back to 1991 (Reyes et al., 1990), whereas the first documented HEV epidemic was retrospectively determined to be caused by faecally-orally transmitted HEV in New Delhi, India in 1955–1956 (Viswanathan and Sidhu, 1957; Viswanathan, 2013). After its recognition during an HEV outbreak in the Kashmir valley region of India in 1978 (Khuroo, 2011), similar incidents in the 1980s were reported in Nepal (Kane et al., 1984), Burma (Hla et al., 1985), Pakistan (Cock et al., 1987), Mexico (Centres for Disease Control and Prevention, 1987) and China (Zhuang, 1991). Since then, persistent HEV infections and high mortality rates have been described in different cohorts (Kane et al., 1984; Hussaini et al., 1997; Kamar et al., 2012; Wedemeyer et al., 2012; Höner zu Siederdissen et al., 2013). Descriptions of chronic HEV infections in solid organ transplant recipients in France (Kamar et al., 2008) and transmission through blood transfusions (Colson et al., 2007; Kamp et al., 2018) increased awareness for a potentially underestimated disease not considered a major clinical problem in developed countries. Following serological and molecular studies, HEV is now recognised as globally distributed. With approximately 20 million people infected with HEV leading to approximately 3.3 million cases of acute illness and 44.000–70.000 deaths per year, this pathogen

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substantially impacts the health care system and must be socio-economically considered (Wedemeyer et al., 2012).

### 2.1. HEV taxonomy and distribution

Initially, HEV was classified into different genotypes (GTs) and subtypes (1a-e, 2a-b, 3a-j and 4a-g) (Meng et al., 1999), but the International Committee on the Taxonomy of Viruses recently reassigned HEV to family *Hepeviridae*, which includes the two genera Orthohepevirus and Piscihepevirus (Smith et al., 2016). The latter genus includes only the cutthroat trout virus (CTV), while Orthohepevirus is comprised of four species (A-D): Orthohepevirus A, with HEV isolates from humans, swine, rabbit, rat and several other animals and Orthohepevirus B, C, and D with HEV isolates from birds and other mammals (Smith et al., 2016). To date, HEV infections in humans have been caused by five different GTs, whose genomic organization is highly conserved, but differ in their worldwide distribution, hosts, route of transmission, and sequence identity. GT1 and 2 viruses are found mainly in developing countries and are transmitted via the faecal-oral route during waterborne outbreaks. They solely infect humans and have shown high epidemic potential in India, North Africa, and Asia, causing up to 120.000 symptomatic cases in a single outbreak (Khuroo et al., 2016). In contrast, animals are reservoirs of HEV GTs 3 and 4 infections, including pigs (Goens and Perdue, 2004; Dong et al., 2011), deer and mongoose (Meng et al., 1999), wild boars (Kaci et al., 2008), shellfish (Said et al., 2009), rodents (Said et al., 2009; Dong et al., 2011), bison, cattle and dogs (Dong et al., 2011) predominantly present in developed countries (Wedemeyer et al., 2012). In 2014, GT7 was newly identified in dromedary camels in the Middle East (Woo et al., 2014) and is widespread in Pakistan, the United Arab Emirates and four African countries (Rasche et al., 2016). HEV GTs 3, 4, and 7 can cross the species barrier, as individuals became infected with HEV after eating infected raw deer meat (Tei et al., 2003; Tomiyama et al., 2009), sausages containing pig liver (Colson et al., 2010; Wenzel et al., 2011) or drinking camel milk (Lee et al., 2016).

### 2.2. Genomic organization and gene products

HEV is an icosahedral virus 27–34 nm in diameter (Balayan et al., 1983; Emerson and Purcell, 2013). The virion contains an approximately 7.2-kb single-stranded RNA genome in positive orientation that encodes three open reading frames (ORF1-3) and an additional ORF (ORF4), exclusively present in GT1 (Nair et al., 2016). The HEV genome resembles eukaryotic mRNA with a 5′-7-methylguanylate cap and a 3′ poly(A) tail (Emerson and Purcell, 2013). During viral replication, two RNA species are generated and transcribed: a full-length RNA and a 2.2-kb subgenomic RNA that allow expression of ORF2 and ORF3 (Graff et al., 2006; Ichiyama et al., 2009). HEV is a quasi-enveloped virus that can be shed as a non-enveloped virus in the faeces or circulate in the blood with a lipid-derived envelope protective against neutralising antibodies; both forms have distinct entry mechanisms (Yin et al., 2016; Chapuy-Regaud et al., 2017).

ORF1 is the largest viral gene product and is comprised of a methyltransferase macro domain, putative papain-like cysteine protease, helicase and an RNA-dependent RNA polymerase (RdRp) connected by a Y-domain and a hypervariable region essential for viral replication (Emerson et al., 2004; Borkakoti et al., 2014; Debing et al., 2016b). Maturation and potential processing of the ORF1 by the encoded protease is not fully understood (Ansari et al., 2000; Ropp et al., 2000; Sehgal et al., 2006; Suppiah et al., 2011; Perttilä et al., 2013; Paliwal et al., 2014). ORF4 was identified in the coding sequence of ORF1 exclusively in HEV GT1 and increases RdRp activity (Nair et al., 2016). ORF2 encodes the 660-amino acid virus capsid and is relatively conserved among the viral genotypes, harbouring a typical signal peptide sequence and three potential glycosylation sites (Jameel et al., 1996). The capsid plays a crucial role during virion assembly and viral attachment to the host cell and is the major target for neutralising antibodies (Li et al., 1997; Kalia et al., 2009; Xing et al., 2011). The ORF3 protein is a 13-kDa protein of 113 (GT3) or 114 amino acids (GTs1, 2 and 4), respectively, found in quasi-enveloped virions that participates in virion morphogenesis and viral egress (Yamada et al., 2009a; Emerson et al., 2010; Nagashima et al., 2011). Recently, an ion channel activity has been reported for the ORF3 protein, which is critical for release of infectious particles (Ding et al., 2017).

### 2.3. Course of infection and treatment options

HEV infections are usually self-limiting and asymptomatic in immunocompetent individuals but can cause arthralgia, flu-like myalgia, vomiting, and symptoms characteristic of hepatitis, such as jaundice and itching (Wedemeyer et al., 2012). In immunocompromised patients, such as HIV-infected patients, solid organ transplantation (SOT) recipients, individuals under immunosuppressive therapy, or those with haematological diseases, HEV can progress to chronicity and cause fulminant hepatitis (Clemente-Casares et al., 2016). Most chronic cases are caused by GTs 3 (Sridhar et al., 2015) and 4 (Geng et al., 2014). For GTs 1 and 2, mortality rates of 25% associated with fulminant hepatic failure have been observed in HEV-infected pregnant women (Kumar et al., 2004; Pérez-Gracia et al., 2017). Observed vertical transmission of the virus (Khuroo et al., 1995) can cause adverse outcomes, especially in the third trimester, such as pre-term delivery, abortion, stillbirth, intrauterine infection and neonatal death (Kumar et al., 2004; Patra et al., 2007; Pérez-Gracia et al., 2017).

HEV infections are usually subclinical in immunocompetent individuals and do not require medications in general. However, antiviral treatment of severe acute infections should be considered in patients with risk factors for fulminant liver failure (European Association for the Study of the Liver, 2018). Ribavirin monotherapy is currently the first treatment of choice for patients with chronic HEV infection (Dalton et al., 2014; European Association for the Study of the Liver, 2018). Several modes of action have been described in the setting of HCV infection, including depletion of cellular guanosine triphosphate pools, upregulation of IFN-stimulated genes, direct inhibition of viral polymerase, inhibition of viral RNA capping, viral RNA mutagenesis or modulation of adaptive immunity by restoring T-cell balance (Paeshuyse et al., 2011). However, the exact mechanism on how ribavirin clears HEV infection remains elusive. While the majority of patients clear HEV after 3–5 months of ribavirin treatment, cases of on-therapy failures or post-treatment relapses have been reported (Pischke et al., 2013; Debing et al., 2014; Kamar et al., 2014b). Moreover, ribavirin therapy can lead to adverse side effects, such as severe anaemia, that requires dose reduction. Renal impairment may also prevent optimal dosing and possibly impair treatment responses. Furthermore, recently identified viral isolates with lower ribavirin sensitivity demonstrate higher treatment failure rates (Debing et al., 2014, 2016b; Todt et al., 2016b, 2016c). In China and Nepal, a HEV vaccine (“Hecolin” or HEV 239) comprised of amino acids 368–606 of GT1's ORF2-encoded capsid, has been approved and tested in a completed phase 2 clinical trial (Shrestha et al., 2007; Zhu et al., 2010). Although very immunogenic and potentially efficacious, the vaccine's known protective capacity against HEV GT3 infection is pending (World Health Organisation, 2016).

Based on HEV's genotypic and phenotypic diversity, newer and safer antiviral treatments against HEV are urgently needed. Unfortunately, HEV has been very difficult to propagate *in vitro*, although more efficient cell culture systems for HEV study have recently become available that can potentially facilitate the development of new drug targets and thus novel antiviral compounds.

## 3. Cell culture systems for studying human HEV

### 3.1. HEV patient isolates

A prerequisite for establishing a virus-cell culture system is a primary viral isolate that efficiently infects cell lines. The first attempts to

detect HEV particles in patient samples via cell culture methods were performed in 1987 by Pillot et al., who inoculated liver cell lines with faecal specimens from enterically-transmitted non-A, non-B (ENANB) hepatitis patients from France and the Ivory Coast (Pillot et al., 1987). The release of viral antigen from infected cells demonstrated that HEV particles can be found naturally in stool, as well as isolated *in vitro* using liver cell lines. Similar approaches were applied one year later with samples from an ENANB hepatitis outbreak from India; monkey kidney cells were inoculated with the ENANB stool samples Ahm-84 and Kol-81 (GT1). As no cytopathogenic effects (CPE) were observed, and HEV-specific tests were not yet available, no further work was performed on these two isolates (Arankalle et al., 1988).

Identification of a molecular clone in 1990 that hybridised to DNA obtained from ENANB-infected patients from Somalia, Borneo, Pakistan and the Soviet Union suggested a single virus or class was responsible for the majority of clinical disease cases (Bradley et al., 1988), which was later named hepatitis E virus (Bradley, 1990; Reyes et al., 1990). The recovered virus strains include isolates still used today, such as Sar-55 (GT1) from a Pakistani patient (Iqbal et al., 1989; Tsarev et al., 1992), Mexican isolate MEX-14 (GT2) (Huang et al., 1992a), strain 87A (GT1), which was recovered from a hepatitis E epidemic in China (Huang et al., 1992b) and F23 (GT1) from Morocco (Meng et al., 1997a) (Fig. 1).

In the following years, these strains were used to establish cell culture systems and develop serological tests and neutralisation assays. In addition, several studies optimised isolation and cultivation of HEV from stool specimens. For example, precipitation of virus extracted from faecal extracts was improved using PEG and the infection efficiency of extracted viruses was enhanced by the addition of 30 mM $MgCl_2$ to the culture medium in order to protect the virions from inactivating factors (Huang et al., 1999). Some of the first infection experiments after the discovery of HEV were performed by Huang et al. (1992b), who visibly observed infection of human fetal lung diploid fibroblast cells with strain 87A (Huang et al., 1992b) and later successfully infected a human lung cell line with this strain (Huang et al., 1995). Propagation of HEV in these cells was analysed by PCR, and a HEV-specific band was detected after the fourth passage, which was confirmed to be 87A by genomic sequencing. This group has also shown strain G93 (GT1) to replicate in the same human lung cell line (Huang et al., 1999), similar to what was observed by Wei et al. (2000). Moreover, Meng et al. (1997a) successfully inoculated HEV strains Sar-55 (Pakistan, GT1) and F23 (Morocco, GT1) from stool extracts into liver cells. Although these and other early experiments provided valuable information regarding *in vitro* HEV replication, they were limited by a lack of virologic methods and knowledge about HEV itself.

A significant advancement occurred in 2007 when Okamoto and colleagues established a more robust HEV culture system using a faecal specimen from a Japanese patient with acute hepatitis E (strain JE03-1760F, GT3) with a very high viral load of $2.0 \times 10^7$ copies/ml (Tanaka et al., 2007; Okamoto, 2011). The faecal suspension was used as an inoculum, and replication capacity studies of HEV in 21 cell lines (see also section 3.3) yielded efficient propagation in two cell lines: a hepatoma cell line and a lung cancer cell line, both already known to support HEV infection according to findings of Pillot et al. (1987) and Huang et al. (1995). Similarly, strain HE-JF5/15F (GT4), isolated from a fulminant hepatitis E patient with a high viral titre ($1.3 \times 10^7$ copies/ml), was efficiently cultivated *in vitro* (Tanaka et al., 2009; Fig. 1). Interestingly, upon serial passage, neither stain caused CPE in infected cells (Tanaka et al., 2009). Shukla and colleagues subsequently isolated and adapted the Kernow-C1 strain (GT3) in cell culture (Shukla et al., 2011; Fig. 1) using faecal samples containing a high viral load of approximately $10^{10}$ viral genomes per gram obtained from an HIV patient chronically co-infected with HEV. The virus was semi-purified from the faeces and used to inoculate several cell lines. After 7 days, the cells were stained for viral antigens, which yielded infected foci in all cultures. After six passages, an adapted virus that very efficiently infected a hepatoma cell line was isolated and termed Kernow-C1/p6. This virus differs strikingly from strains JE03-1760F and HE-JF5/15F due to an additional genomic insertion in its hypervariable region that confers enhanced replication as seen in several other RNA viruses (Steinhauer, 1999; Becher and Tautz, 2014). Of note, a minority of viral genomes containing the S17 insertion (from human ribosomal protein S17) was identified in faecal samples from which the original Kernow-C1 strain was isolated, indicating that the insertion was obtained from the infected host and was not a cell culture artefact (Shukla et al., 2012). Several groups showed that serum samples can also be used to isolate HEV and to infect human cell lines (Takahashi et al., 2010b; Johne et al., 2014), demonstrating that viral shedding is not limited to faeces and that many sources of HEV virions from primary isolates can be used in cell culture. For example, HEV strain 47832 (GT3) was successfully isolated from serum of a chronically infected transplant patient (Johne et al., 2014).

In summary, research has shown successful isolation of patient-derived HEV particles from stool and serum samples and that subsequent infection experiments are possible using lung- or liver-derived cell lines. A high viral load is beneficial for initial virus isolation and propagation. Recent advances have enabled the in-depth characterisation of individual patient isolates and persistently infected cell cultures to allow future investigations of patient-derived HEV isolates, including viral responses to treatment approaches.

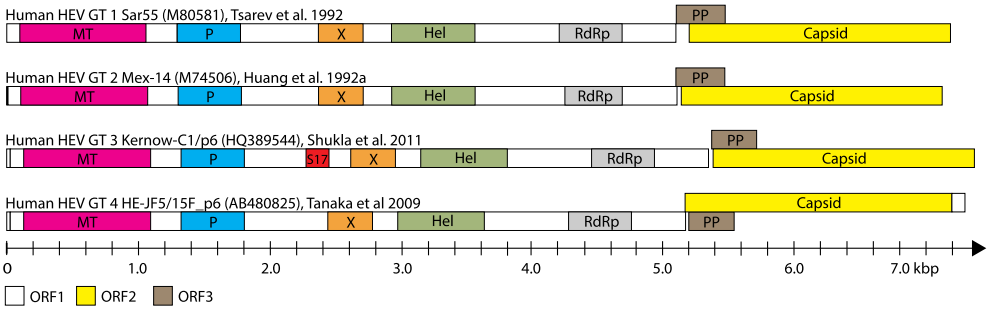


**Fig. 1.** Representative genomic organization of human HEV GT1-4., consisting of ORF1 (polyprotein, white), ORF2 (capsid protein, yellow) and ORF3 (phosphoprotein, brown). Predicted gene length (colour coded) according to GenBank information are depicted relative to genome size (Factor 0.02). MT = Methyltransferase; P = Papain-like cysteine peptidase; S17 = Fragment of the human S17 ribosomal protein; X = X-domain (putative macro domain protein/Appr-1-pase catalytic site, including ADP-ribose binding site, critical for viral RNA replication); Hel = Helicase; RdRp = RNA-dependent RNA polymerase; PP = Open reading frame 3 encoding for a phosphoprotein. The number behind the name displays the accession number in GenBank.

### 3.2. Complementary DNA (cDNA) clones

While HEV patient isolates can be used for *in vitro* and *in vivo* infection studies, their genomes cannot be modified, limiting their utility in investigating the function of viral proteins and non-coding regions. To overcome the reproducibility issues of handling patient isolates and propagating viruses from them with low replication efficiency, infectious cDNA clones were developed. One of the first full-length HEV cDNA clones, pSGI-HEV(I) (GT1), was constructed from subgenomic PCR amplification of hepatitis E outbreak isolates from India (Panda et al., 2000). RNA resulting from the *in vitro* transcribed cDNA was successfully transfected into the human hepatoma cell line HepG2, and viral particles produced by the cells were infectious in monkeys. However, *in vitro*-produced RNA alone did not lead to an infection of rhesus monkeys.

Similarly, Emerson et al. (2001) used cDNA fragments produced by RT-PCR to construct full-length cDNA clones of HEV isolate Sar-55 (GT1; see also Section 3.3). Intriguingly, pSK-HEV-2-derived viruses exhibited the same phenotype as wild-type Sar-55 from clinical samples, indicating that HEV cDNA clones can be appropriate surrogates to study the virus. Furthermore, Emerson's experiments and other studies showed that a 5′-cap is essential for *in vivo* infectiousness of HEV RNA, explaining why earlier infection assays with uncapped pSGI-HEV(I) failed (Emerson et al., 2001; Zhang et al., 2001). Lack of a 5′-cap caused significantly reduced viral protein expression and viral progeny infection due to decreased HEV RNA stability and diminished binding capacity to translation initiation complexes (Emerson et al., 2001, 2004).

Based on these results, full-length cDNA clones of several HEV GTs were subsequently generated in the following years (Fig. 2). Huang et al. (2005b) designed a system to generate swine HEV-derived cDNA clones pSHEV-1 to −3 by the amplification of eight overlapping fragments covering the entire genome ligated into the pGEM-9zf(-) vector (Fig. 2). All three cDNA clones were replication competent in Huh-7 cells. Analogous to the work of Huang et al. (2005b), Yamada et al. (2009b) synthesised a cDNA clone corresponding to GT3 strain JE03-1760F (pJE03-1760F/wt; Fig. 2) by amplifying three fragments covering the entire genome ligated into the pUC19ΔAatIISapI vector. When transfected into PLC/PRF/5 and A549 cells, increased levels of pORF2 were measured by immunofluorescence staining, indicating successful spread of *in vitro* HEV infection (Yamada et al., 2009b). Similarly, a GT4 clone (pHEV-4TW; Fig. 2) derived from strain TW6196E (Feagins et al., 2011) was constructed by Córdoba et al. (2012) from three overlapping genomic fragments. The construct was transfected into a hepatocellular carcinoma cell line. ORF2 expression indicated the clone's replication competence and ability of its progeny to infect HepG2/C3A cells. Interestingly, the GT3 and 4 clones are not only infectious *in vitro* but also retain their *in vivo* infectivity of pigs, their natural hosts.

A cDNA clone derived from the wild type and adapted GT3 strain Kernow-C1 was generated by Shukla et al. (2012). They observed increased replication efficiency after six serial passages (P6; Fig. 2) compared to the early passage (P1), due to the acquisition of several point mutations and a 58 aa insertion into the hypervariable region of ORF1 identified as a fragment of the human S17 ribosomal protein. Removal of the S17 insertion (Kernow-C1/p6-delS17), while maintaining the point mutations, decreased the replicative ability of the cDNA clone. Interestingly, the S17 insertion did not only lead to increased replication of Kernow-C1/p1 but also enhanced *in vitro* viral fitness of GT1 strain Sar-55 (Shukla et al., 2012; Nguyen et al., 2014). Similarly, Nguyen et al. (2012) detected a S19 sequence insertion within the same region of GT3 strain LBPR-0379 (Fig. 2), and Johne et al. (2014) observed comparable cell culture-adaptive mutations resulting in up to eight amino acid exchanges and a 186-nucleotide insertion when A549 cells were persistently infected with GT3 strain 47832 (Fig. 2). The position is equivalent to that of the Kernow-C1 strain after six serial passages and the LBPR-0379 strain. However, the inserted sequence originated from the adjacent genome region and parts of ORF1. While these results indicated that the insertions could be used as a tool to enhance virus replication, Shiota et al. (2013) constructed a full-length cDNA clone based on the strain G3-HEV83-2-27 (GT3, Fig. 2), which does not acquire any insertions when passaged under cell culture conditions.

After the successful generation of competent HEV cDNA clones, genome editing was applied to examine the function of HEV proteins. For example, induced mutations and deletions have revealed the importance of ORF3 for HEV infectivity *in vivo*, especially for virus release (Graff et al., 2005a, 2005b; Huang et al., 2007; Yamada et al., 2009a), as well as the significance of the non-coding intergenic junction region between ORF1 and ORF2/ORF3 for viral replication (Cao et al., 2010). cDNA clones have also been used to construct intergenotypic chimeras between GTs 1 (human, pSK-HEV-2), 3 (swine, pSHEV-3) and 4 (human, pHEV-4TW) and between GTs 1 (Sar-55, pSK-E2) and 3 (Kernow-C1/p6) to study HEV's species tropism (Córdoba et al., 2012; Nguyen et al., 2014). Additional studies analysing HEV genome modifications are reviewed by van Tong et al. (2016).

Another approach to advance HEV research involves generation of subgenomic cDNA replicons and introduction of reporter genes into cDNA clones. Several groups successfully generated cDNA clones of Sar-55 and Kernow-C1/p6 in which the ORF2/ORF3 genes were partially recombined with (enhanced) green fluorescent protein (GFP/EGFP) (Emerson et al., 2004; Pudupakam et al., 2009) or a luciferase reporter

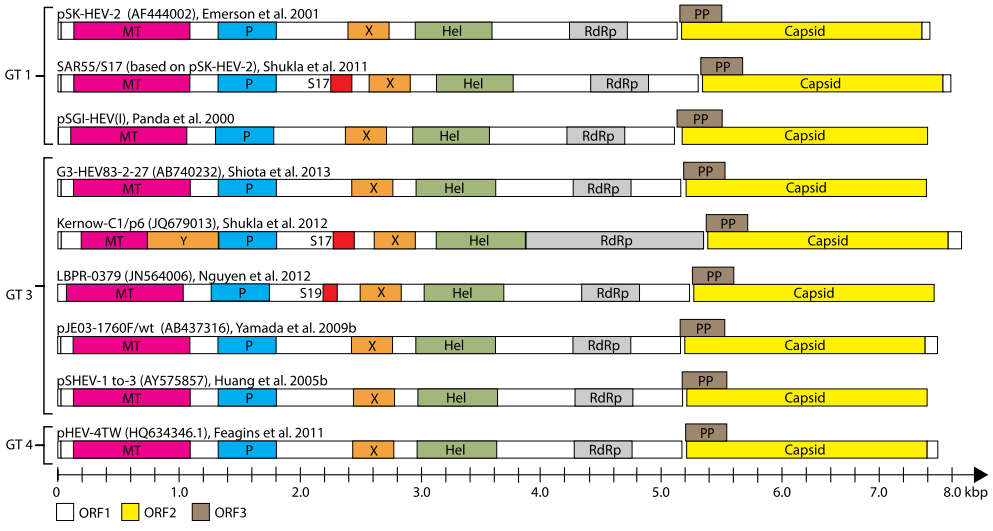


**Fig. 2.** Genomic organization of exemplary full-length HEV cDNA clones. Predicted gene length (colour coded) according to GenBank information are depicted relative to genome size (Factor 0.02). ORF1 (polyprotein, white), ORF2 (capsid protein, yellow) and ORF3 (phosphoprotein, brown). MT = Methyltransferase; Y = Y-domain; P = Papain-like cysteine peptidase; S17 = Fragment of the human S17 ribosomal protein; S19 = Fragment of the human S19 ribosomal protein; X = X-domain (putative macro domain protein/Appr-1-pase catalytic site, including ADP-ribose binding site, critical for viral RNA replication); Hel = Helicase; RdRp = RNA-dependent RNA polymerase; PP = Open reading frame 3 encoding for a phosphoprotein. The number behind the name displays the accession number in GenBank.

gene (Graff et al., 2005b; Cao et al., 2010; Shukla et al., 2012; Shiota et al., 2013). Due to the lack of viral capsid proteins, these clones replicate but do not produce infectious particles, making research safer and easier and allowing monitoring of replication with GFP or luciferase. In summary, the construction of cDNA clones has enabled genome editing of HEV, functionality studies, and the generation of adapted or tagged viral clones. However, low to moderate viral titres are still the main limitation in these approaches.

### 3.3. Cancer-derived cell lines

HEV is a zoonotic pathogen with a broad host and tissue tropism that should be able to infect various cell lines. Indeed, HEV infects various immortalised cell lines derived from colon, kidney, liver, lung, neuronal and placental tissue of human and non-human origin (Table 1). First attempts to propagate HEV *in vitro* began before the virus was identified when sporadic outbreaks of ENANB hepatitis were observed in the 1980s. Pillot and colleagues used faecal extracts from ENANB hepatitis patients to inoculate different human cell lines (Pillot et al., 1987) and observed that, upon incubation, viral antigens were released from the hepatoma cell line PLC/PRF/5, while no antigens were detected in the hepatoma cell line HepG2 or in the lung fibroblast cell line MRC-5. One year later, another group found that ENANB hepatitis stool specimens from India did not cause a visible CPE or detectable infection in monkey kidney cells (Arankalle et al., 1988). In a similar approach, Huang and co-workers used stool samples from an ENANB hepatitis epidemic in China to inoculate human fetal lung diploid fibroblast cells (2BS) and a monkey kidney cell line (LLC-M2K) (Huang et al., 1992b). Consistent with previous studies, they did not observe CPE in the kidney cells but did detect continuous and reproducible CPE in the 2BS cells. Electron microscopy confirmed that the viral particles produced by 2BS cells (termed strain 87A and later

**Table 1**
Immortalised cell lines used to study human HEV. Unless otherwise indicated, the cell line originates from human tissue.

| Cell line | Origin | GT | Strain/cDNA clone | Reference |
|---|---|---|---|---|
| Caco-2 | Colon | 1 | Sar-55[a] | Emerson et al. (2004) |
| | | 3 | Kernow-C1 | Shukla et al. (2011) |
| Caco-2 subclone C25j | Colon | 1 | Sar-55[a] | Emerson et al. (2010) |
| BHK-21 (golden hamster) | Kidney | 1 | Sar-55/S17[a] | Shukla et al. (2012) |
| FRhK-4 (rhesus macaque) | Kidney | 1 | Sar-55/S17[a] | Nguyen et al. (2014) |
| | | 3 | Kernow-C1/p6[a] | Nguyen et al. (2014) |
| FRhK-4 (rhesus macaque) in co-culture with infected primary monkey kidney cells | Kidney | 1 | 2598 Osh | Kazachkov et al. (1992) |
| LLC-PK1 (pig) | Kidney | 1 | Sar-55 and Akluj | Shukla et al. (2011) |
| | | 1 | Sar-55/S17[a] | Nguyen et al. (2014) |
| | | 3 | US-2; Kernow-C1/p1 or p6 | Shukla et al. (2011) |
| 2BS | Kidney | 1 | 87A and 93G | Huang et al. (1992b, 1999) |
| Hepa-RG | Liver | 3 | Kernow-C1/p6[a] | Xu et al. (2017) |
| HepG2/C3A | Liver | 1 | Sar-55 and Akluj | Emerson et al. (2005, 2010) |
| | | 1 | Sar-55/S17[a] | Nguyen et al. (2014) |
| | | 2 | Mex-14 | Emerson et al. (2005) |
| | | 3 | US-2; Kernow-C1/p1 or p6 | Shukla et al. (2011) |
| | | 3 | LBPR-0379 | Nguyen et al. (2012) |
| Huh-7 | Liver | 1 | Sar-55[a] | Emerson et al. (2004) |
| | | 4 | pHEV-4TW[a] | Córdoba et al. (2012) |
| Huh-7 subclone S10-3 | Liver | 1 | Sar-55[a] | Emerson et al. (2006) |
| | | 1 | Sar-55/S17[a] | Shukla et al. (2012) |
| | | 3 | Kernow-C1/p1-S17[a] | Shukla et al. (2012) |
| | | 3 | Kernow-C1/p6-delS17[a] | Shukla et al. (2012) |
| | | 3 | Kernow-C1/p6[a] | Nguyen et al. (2014) |
| Huh-7.5 | Liver | 3 | Kernow-C1 | Shukla et al. (2011) |
| OHH1.Li (deer) | Liver | 1 | Sar-55 and Akluj | Shukla et al. (2011) |
| | | 3 | Kernow-C1/p1 or p6 | Shukla et al. (2011) |
| PLC/PRF/5 | Liver | 1 | Sar-55 and F23 | Meng et al. (1997a) |
| | | 1 | S5_p0 | Takahashi et al. (2010b) |
| | | 3 | JE03-1760F | Tanaka et al. (2007) |
| | | 3 | pJE03-1760F/wt[a] | Yamada et al. (2009b) |
| | | 3 | Kernow-C1 | Shukla et al. (2011) |
| | | 3 | G3-HEV83-2-27[a] | Shiota et al. (2013) |
| | | 3 | 47832c | Schemmerer et al. (2016) |
| | | 4 | HE-JF5/15F | Tanaka et al. (2009) |
| | | – | ENANB hepatitis | Pillot et al. (1987) |
| A549 | Lung | 1 | 87A and 93G | Huang et al. (1995, 1999) |
| | | 1 | S5_p0 | Takahashi et al. (2010b) |
| | | 3 | JE03-1760F | Tanaka et al. (2007) |
| | | 3 | pJE03-1760F/wt[a] | Yamada et al. (2009b) |
| | | 3 | Kernow-C1 | Shukla et al. (2011) |
| | | 3 | 47832c | Johne et al. (2014) |
| | | 4 | HE-JF5/15F | Tanaka et al. (2009) |
| A549/D3 | Lung | 3 | 47832c | Johne et al. (2016) |
| M03.13, [b]DBRTG, SK-N-MC, DAOY | Neuron | 1 | Sar-55/S17-Luc[a] | Drave et al. (2016) |
| | | 3 | Kernow-C1/p6[a] | Drave et al. (2016) |
| | | 3 | Kernow-C1/p6-Luc[a] | Drave et al. (2016) |
| U87 | Neuron | 3 | Kernow-C1/p6[a] | Zhou et al. (2017) |
| | | 3 | Kernow-C1/p6-Luc[a] | Zhou et al. (2017) |
| JEG-3, [b]BeWo | Placenta | 1 | Sar-55/S17[a] | Knegendorf et al. (2018) |
| | | 3 | Kernow-C1/p6[a] | Knegendorf et al. (2018) |

[a] cDNA clone.
[b] No active infection possible; GT, genotype of HEV.

classified as GT1) were the same as those found in the original stool sample, indicating a successful infection of the cell line.

When the causative agent of the ENANB hepatitis was later discovered to be HEV (see also Lemon and Walker, 2018a, 2018b), more research was performed on the virus utilising existing ENANB hepatitis cell culture systems, such as PLC/PRF/5, for developing a neutralisation assay against HEV strains Sar-55 (Pakistan, GT1) and F23 (Morocco, GT1) (Meng et al., 1997a). Nevertheless, the limitations of these models surfaced very quickly: HEV propagation in PLC/PRF/5 cells only resulted in low titres and no visible CPE (Meng et al., 1997a), while obtaining sufficient 2BS cells for culturing the virus remained difficult. These challenges necessitated the optimisation of other cell line systems. Accordingly, HEV strains 87A and G93 successfully replicated in the human lung cancer cell line A549 (Huang et al., 1995, 1999), with the highest G93 titres reached when A549 cells were maintained in 30 mM $MgCl_2$ at pH 7.2 (Huang et al., 1999). This cell culture system was also successfully used to isolate viral particles from stool samples from infected Chinese patients (Wei et al., 2000).

To find a more robust and efficient *in vitro* model for HEV propagation, several studies investigated the ability of different cell lines to support the HEV life cycle. The first study in this context was published by Tanaka and colleagues, who inoculated 21 established cell lines (PLC/PRF/5, A549, HepG2, Huh-7, IEC-6, NUGC-4, MDCK, MDBK, P19, LLC-MK2, BC3H1, C2C12, HEK293, L929, HT-1080, SK-N-MC, GOTO, C6, CV1, HeLa and MCF) with faecal extracts from two Japanese fulminant hepatitis patients (strain JE03-1760F, GT3 and strain HE-JF5, GT4) (Tanaka et al., 2007, 2009). In accordance with previous studies, HEV RNA was detected exclusively in PLC/PRF/5 and A549 cell supernatants. Surprisingly, at 10–14 days post-inoculation, high genome copy numbers were detected in PLC/PRF/5 cells, probably due to the high viral load of the inocula used. Moreover, this cell culture system enabled serial passaging of HEV, a faster release of progeny virus, increased viral titre and higher infectiousness, indicating adaption of HEV to the culture conditions (Tanaka et al., 2007, 2009; Lorenzo et al., 2008). Later studies by the same group confirmed the growth of several GT3 and GT4 isolates in the PLC/PRF/5 and A549 cells and showed that GT1 specimens can also be passaged in them (Takahashi et al., 2010b). Applying a similar strategy, Shukla et al. (2011) inoculated several cell lines with a highly viraemic stool sample from a chronically infected HIV-HEV patient (strain Kernow-C1, GT3). Immunofluorescence revealed the hepatoma cell line HepG2/C3A to be the most permissive for HEV 7 days post-inoculation, and to a lesser extent, Caco-2, Huh-7.5, PLC/PRF/5 and A549 cells to be permissive as well.

Intriguingly, Tanaka et al. (2007, 2009) did not detect viral RNA in HepG2 cells upon inoculation with GT3 or GT4 samples, while Emerson et al. (2005, 2010) reported infection of HepG2/C3 with HEV GT1 specimens. In their study, Shukla et al. (2011) showed that GT1 (strains Sar-55 and Akluj) and GT3 (strains Kernow-C1 and US-2) infected the hepatoma cell line. Similar to what Tanaka et al. (2007, 2009) showed in PLC/PRF/5 cells, Shukla and colleagues serially passaged the Kernow-C1 strain in HepG2/C3A (resulting in strain Kernow-C1/p6), while their attempts in A549 or PLC/PRF/5 were not successful (Shukla et al., 2011, 2012).

Although many studies used the PLC/PRF/5 and A549 systems by Tanaka et al. (2007, 2009) and the HepG2/C3A system by Shukla et al. (2012), major limitations of these systems involve relatively slow virus replication and unsuccessful propagation of HEV from low titre samples. Recently, Schemmerer et al. (2016) tried to optimise HEV cell culture systems by inoculating various cell lines with a viral isolate from a chronically infected transplant patient (strain 47832c, GT3c). The highest HEV genome copy number ($10^4$ FFU/ml) were detected 14 days post-inoculation in A549 cells, followed by PLC/PRF/5 cells. HepG2/C3A, Huh-7 Lunet BLR and MRC-5 cells only weakly supported replication of the isolate. These findings further demonstrated that existing HEV cell culture systems are not universal and that the respective cell line(s) for each viral isolate study must be carefully chosen.

Once, full-length HEV cDNA clones and subgenomic replicons (Reyes et al., 1990, see also Section 3.2) have been used to evaluate existing or new HEV *in vitro* models. Propagation of HEV was confirmed in the human hepatoma cell line PLC/PRF/5 using clone G3-HEV83-2-27 (Shiota et al., 2013) and in Huh-7.5, Hepa-RG and A549 using clone Kernow-C1/p6 (Xu et al., 2017). Furthermore, Emerson and colleagues transfected Huh-7 and PLC/PRF/5 cells and the intestinal cell line Caco-2 with full-length HEV clones of GT1 strain Sar-55 (Emerson et al., 2004, 2006). Even though infectious viral particles were produced, only 10% of the transfected cells became HEV-positive in the absence of cell-to-cell spread. While adaption of Sar-55 to cell culture growth failed (Shukla et al., 2012), subclones of Huh-7 and Caco-2 cells were selected which appeared to have higher transfection and infection efficiencies and thus supported the full length HEV GT1 life cycle (Emerson et al., 2006, 2010). In addition, other studies showed that subcloning of the permissive cell lines A549 and PLC/PRF/5 supported enhanced HEV replication (Shiota et al., 2015; Schemmerer et al., 2016). While promising, these collective results indicate that, similar to primary HEV isolates, no universal cell culture system is available for the study of HEV cDNA clones.

As infection with HEV primarily causes hepatitis, the majority of established HEV cell culture systems are based on liver cell lines, with the exception of adenocarcinoma-derived A549 and Caco-2 cells. However, the extrahepatic manifestations observed during HEV infections suggest the virus should be able to infect cells of various origins. Using full-length and subgenomic HEV replicons, Drave and co-workers elucidated the viral life cycle of HEV GT1 (strain Sar-55/S17) and GT3 (strain Kernow-C1/p6) in five cell lines derived from the nervous system (Drave et al., 2016). This was the first report showing that neuronal cells support human HEV replication, assembly, and release. Virus entry, however, was only supported by the oligodendrocyte cell line M03.13. Similarly, Zhou et al. (2017) successfully infected different neural cell lines and human inducible pluripotent stem cell-derived neuronal cultures with Kernow-C1/p6 (Zhou et al., 2017). Moreover, the authors showed that the human glioblastoma cell line U87 supported long-term replication and production of HEV. Applying a similar approach, Knegendorf et al. (2018) developed a placental-derived cell culture system in JEG-3 cells for HEV GT1 and GT3 strains. These cell culture systems are extremely valuable for studying extrahepatic HEV infections, identifying factors that lead to severe neurological symptoms or pathogenicity during pregnancy, viral tissue tropism and to develop new antiviral drugs.

Although HEV naturally exhibits broad host tropism, most *in vitro* HEV isolates show a restricted species range and grow in only a few human cell lines, as is observed in other hepatitis viruses. Shukla et al. (2011) demonstrated for the first time that the faecal-isolated Kernow-C1 strain could not only propagate in human cells but also led to minimal infections of cow, mouse, chicken, cat, dog, and rabbit cells, a moderate infection of deer cells (OHH1.Li) and a strong infection of a pig kidney cell line (LLC-PK1) (Shukla et al., 2011). Additionally, Nguyen et al. (2014) successfully infected macaque kidney cells (FRhK-4) with Kernow-C1/p6, whereas Kazachkov et al. (1992) had to co-culture FRhK-4 cells with infected primary monkey cells to successfully propagate GT1 strain 2598 Osh. Reflecting their natural host tropism, GT1 strains Sar-55 and Akluj infected HepG2/C3A much more efficiently than LLC-PK1 or OHH1.Li cells, while the GT3 strains US-2 and Kernow-C1 more strongly infected pig cells than human hepatoma cells. Differences in cell line-usage of HEV specimens may be due to isolate-specific requirements regarding various steps of the viral life cycle (Nguyen et al., 2014). Efficient growth in cell culture and a broad cross-tissue or -species range can be observed for strains derived from high-titre samples of immunocompromised chronically infected HEV patients where complex viral quasi-species exist. As a result, these

heterogeneous viral populations carry mutations allowing them to replicate *in vitro* (e.g. GT3 strain 47832 in A549 cells) (Johne et al., 2014) that can be enriched upon serial passage, as demonstrated for GT3 strain LBPR-0379 in HepG2/C3A cells (Nguyen et al., 2012), (see also Sections 3.1 and 3.2).

Though many groups have successfully propagated various HEV strains in different cell lines, viral replication remains low and can be only detected by PCR. Further, none of the developed culture systems results in high-titre HEV in cell supernatants and thus do not support virus propagation (Okamoto, 2011). Another limitation relates to the distinct phenotypic characteristics of the cell lines utilised, including non-hepatic lineages (e.g. A549), recombinant manipulations (e.g. PLC/PRF/5 expressing hepatitis B viral genes) or the lack of a proper immune response (e.g. Huh-7). Currently, the most frequently used cell culture systems include cell lines A549 or HepG2/C3A transfected with the adapted HEV strains Sar-55/S17 or Kernow-C1/p6, but several studies have shown that subclones generated from the same cell line exhibit different sensitivities to HEV (Emerson et al., 2006; Shiota et al., 2015), further illustrating that correctly pairing virus strains together with suitable cell lines is only one of multiple factors that must be considered when developing and/or evaluating HEV cell culture systems.

### 3.4. Primary human hepatocytes and induced pluripotent stem cells

Cancer cell lines can be advantageous for culturing HEV due to easily handling, robustness, and availability, although tumour-derived cell lines may not faithfully recapitulate some cellular pathways as primary cells. Thus, stem cell-derived cellular systems or primary cells are a more authentic system for studying HEV (as reviewed in Dao Thi et al., 2018). Kazachkov et al. and Tam et al. performed the first studies analysing HEV growth in primary cells. After HEV infection of cynomolgus monkeys, primary monkey kidney cells or hepatocytes were isolated and cultured *in vitro* (Kazachkov et al., 1992; Tam et al., 1996, 1997). Positive genomic and negative replicative RNA strands were detected in the cells, as well as in cell culture supernatants, indicating HEV replication. Later studies showed that primary human hepatocytes (PHHs) and mouse embryonic fibroblasts (MEFs) also support infection with HEV strain Kernow-C1/p6 (Zhou et al., 2015b; Yin et al., 2017). Furthermore, Gouilly et al. (2018) have successfully infected primary stromal cells and decidual and placental explants with GT1 and 3 isolates. Similarly, Bose et al. (2014) found evidence of HEV replication in human placenta *ex vivo*. However, the limited availability of primary cells, and genome editing combinations, donor-to-donor variability and a short life-span often restrict the extensive use of primary cells to study HEV.

Hepatocyte-like cells (HLCs) derived from induced pluripotent stem cell (iPSC) or embryonic stem cells (ESC) have numerous advantages over PHHs, including their capabilities of self-renewal and generation of patient-specific disease models. In 2016, Helsen et al. and Dao Thi et al. successfully demonstrated that the complete replication cycle of Kernow-C1/p6 is supported by iPSC-derived HLCs (Dao Thi et al., 2016; Helsen et al., 2016). Characteristics of the infection were similar to those in the established HEV cell culture system HepG2/C3A, except HEV RNA levels plateaued in HLCs cells but continued to increase in HepG2/C3A cells, likely due of Kernow-C1/p6's previous adaption to the cell line (Helsen et al., 2016). The fitness mutation G1634R, however, resulted in a replication advantage in both human hepatoma cells and HLCs (Debing et al., 2014; Helsen et al., 2016), and treatment of infected HLCs with ribavirin, IFN or sofosbuvir (SOF) significantly inhibited HEV replication (Dao Thi et al., 2016; Helsen et al., 2016), suggesting the HLC system can be used to evaluate potential anti-HEV drugs. Another benefit of HLCs involves their infectivity with non-adapted, primary HEV isolates of all four human GTs, which only grow poorly in cancer-derived cell lines (Dao Thi et al., 2018; Wu et al., 2018). Therefore, HLCs do not only allow the direct analysis of patient isolates but are also suitable for elucidating mechanisms of cell culture adaption and GT-specific differences in HEV biology.

In this context, Wu and colleagues observed that replication of Kernow-C1/p6 is attenuated in HLCs but enhanced in cell lines compared to wild-type Kernow-C1 (see also Sections 3.1 and 3.2) (Wu et al., 2018). Thus, similar to other hepatitis viruses, cell culture adaptions inhibit HEV growth in a physiologically relevant cellular environment. Furthermore, this finding may explain why only a minority of viruses from the original faecal samples carried the S17 insertion that later became dominant in cell culture (Shukla et al., 2011, 2012). Not only replication, but also viral host factor usage, and responsiveness to drugs appear to differ between established cell lines and HLCs. For example, in contrast to cell culture-adapted strains, disruption of the peptidyl-prolyl isomerase A gene or exposure to a cyclophilin A inhibitor does not influence the growth of primary isolates (Wu et al., 2018).

In addition to HLCs, iPSCs or ESCs can differentiate into many other cell types and can thus be used to study extrahepatic HEV infections (Table 2). Helsen et al. (2016) showed that PSC-derived mesodermal and neural progenitor cells only supported replication of HEV subgenomic replicons. In contrast, Zhou et al. (2017) demonstrated efficient infection of these cells, as well as iPSC-derived differentiated human neural cells and primary mouse neurons. These results indicate that the success of HEV infections with iPSC- or ESC-derived cells varies, likely due to their complex differentiation and handling requirements. In conclusion, although pilot studies with PHHs are still limited, they provide a hepatically authentic cellular background for

**Table 2**
Primary cell lines used to study human HEV. Unless otherwise indicated, the cell line originates from human tissue.

| Cell line | Origin | GT | HEV strain/cDNA clone | Reference |
|---|---|---|---|---|
| iPSC-derived HLCs | Germ line | 1 | Sar-55 | Wu et al. (2018) |
| | | 2 | Mexico-14 | Wu et al. (2018) |
| | | 3 | Kernow-C1/p1 | Wu et al. (2018) |
| | | 3 | Kernow-C1/p6[a] | Dao Thi et al. (2016); Helsen et al. (2016) |
| | | 3 | US-2 | Wu et al. (2018) |
| | | 4 | TW6196E | Wu et al. (2018) |
| ESC-derived neural progenitor cells; iPSC-derived iCell neurons; primary mouse neurons | Neuron | 3 | Kernow-C1/p6[a] | Zhou et al. (2017) |
| Fibroblast-like stromal cells | Decidual and placental tissue | 1 | HEV (India) | Gouilly et al. (2018) |
| | | 3 | HEV (India) | Gouilly et al. (2018) |
| PMKC (monkey) | Kidney | 3 | 2598 Osh | Kazachkov et al. (1992) |
| MEF cells (mouse) | Heart | 3 | Kernow-C1/p6[a] | Zhou et al. (2015b) |
| PHH | Liver | 3 | Kernow-C1/p6[a] | Yin et al. (2017) |
| PMH (monkey) | Liver | 3 | HEV-B | Tam et al. (1996) |

[a] cDNA clone; iPSC, induced pluripotent stem cells; HLC, hepatocyte-like cells; GT, genotype of HEV.

HEV studies. In addition, stem cell-derived cellular models enable studies of pan-genotype HEV biology and could serve as a platform for testing anti-HEV treatments.

## 4. Cell culture systems for animal-derived HEV

As HEV displays broad species tropism, tissue culture systems from animal-derived HEV (Table 3) were also developed to study viral replication, pathogenesis, and HEV-induced immune responses *in vitro*. The first description of an animal-derived virus closely related to human HEV was published in 1997, when Meng and colleagues identified a novel virus in swine that represented a related, but distinct, new clade of HEV. Swine HEV ORF2 and 3 shared approximately 90–92% and 77–82% amino acid sequence identity with human HEV strains, respectively. The fact, that the majority of adult pigs in the Midwestern United States were seropositive in their study, as well as the recognition of various additional animal HEV strains to date, raises concerns of a potential zoonotic reservoir of this virus family (Meng et al., 1997b). Several groups found individual viruses in pigs (Hsieh et al., 1999), chickens (Payne et al., 1999; Haqshenas et al., 2001), deer (Tei et al., 2003), wild boars (Sonoda et al., 2004), mongoose (Nakamura et al., 2006), rabbits (Zhao et al., 2009), rats (Johne et al., 2010b), cutthroat trout (Batts et al., 2011), bats (Drexler et al., 2012) and ferrets (Raj et al., 2012; Li et al., 2016a). The existence of a bovine virus is still controversial. In China, GT4-positive animals were identified that excrete infectious particles into milk (Huang et al., 2016), and an agent was recently identified in the U.S. from the sera of dairy cows that cross-reacts with human HEV GT3 (Yugo et al., 2018). In contrast, in Germany, no evidence for the prevalence of HEV in cows has been found (Baechlein and Becher, 2017; Geng et al., 2018; Vercouter et al., 2018). This broad host diversity prompted the Hepeviridae Study Group of the International Committee on Taxonomy of Viruses to reorganise its classification (Smith et al., 2013; see also Section 2.1). In the new taxonomic scheme, the family is divided into the genera Orthohepevirus (all mammalian and avian HEV isolates) and Piscihepevirus (CTV). Isolates from humans, pigs, wild boar, deer, mongoose, rabbits, and camels are classified as Orthohepevirus A, whereas isolates from chickens are designated Orthohepevirus B. Orthohepevirus C encompasses isolates from greater bandicoot rats, Asian musk shrew, ferrets, and mink, while Orthohepevirus D includes isolates from bats (Smith et al., 2013; Li and Wakita, 2018) (Fig. 3).

At least three groups have described swine HEV prototypes with highly similar sequences to human GTs 3 and 4 (Meng et al., 1997b; Wu et al., 2000; Nishizawa et al., 2003). Williams et al. (2001) additionally reported extrahepatic manifestations in experimentally infected pigs, a phenomenon also observed in human pathogenic HEV (Amarapurkar and Amarapurkar, 2002; Kamar et al., 2014a). The first evidence that swine HEV could be propagated in both human and pig cell lines was presented by Zhang et al. (2011), who used swine liver homogenate (designated as HEV HB-1, 3) and anal swabs (designated as HEV HB-2, 4) of GT4-infected animals to inoculate porcine kidney IB-RS-2 cells and the human lung cell line A549. Infected cells were passaged serially every three days, and HEV RNA levels and CPE were monitored continuously. Although HEV replicated faster in the porcine cells, the virus successfully infected both types of cells *in vitro* (Zhang et al., 2011). Similar to that of human isolates, this group also detected two new mutations when swine HEV was passaged *in vitro*. Moreover, swine HEV replicated maximum loads of $10^8$ copies/ml in the supernatants of PLC/PRF/5 cells (Takahashi et al., 2012), and human HEV infected the swine kidney cell line LLC-PK1 (Shukla et al., 2011). Other groups determined that animal products sold as food were sources of HEV transmission to humans (Tei et al., 2003; Feagins et al., 2007; Colson et al., 2010; Wenzel et al., 2011; Takahashi et al., 2012), an alarming finding considering the possible zoonotic nature of HEV. In the late 1990s, Meng and colleagues successfully infected rhesus monkeys and a chimpanzee with swine HEV that shared 97% amino acid identity in ORFs 1 and 2 with two U.S. strains GT3 (US-1 and US-2) recovered from patients with clinical hepatitis E. In a reciprocal experiment, specific-pathogen-free pigs were infected with US-2 (Meng et al., 1998b). In contrast, pigs did not show signs of infection when inoculated intravenously with approximately $10^5$ monkey infectious doses of the human pathogenic strains Sar-55 and Mex-14 (Meng et al., 1998a), further corroborating the previously described differences in pathogenicity of human faecal-orally transmitted GTs 1 and 2 *vs.* zoonotic GTs 3 and 4. Potential viral reservoirs were expanded after the identification of at least three genetic lineages of wild boar-indigenous HEV strains in Japan (designated JBOAR135-Shiz09 and wbJOY_06) (Takahashi et al., 2010a, 2011, 2012), in which only one has been successfully cultured in A549 and PLC/PRF/5 cells (Takahashi et al., 2012). For isolates circulating in Germany, Johne et al. (2014) observed high nucleotide sequence similarities of up to 96.5% between human HEV 47832 and wbGER27 isolated from wild boar.

Although being a member of the Orthohepevirus C species with sequence identities ranging only between 55% and 60%, rat HEV harbours

**Table 3**
Cell lines used to study animal-derived HEV. Unless otherwise indicated, the cell line originates from human tissue.

| Type | Cell line | Origin | HEV strain | Reference |
|---|---|---|---|---|
| Immortalised | IBRS-2 (pig) | Kidney | Swine (HEV HB-1, 3) | Zhang et al. (2011) |
| | | | Swine (HEV HB-2, 4) | Zhang et al. (2011) |
| | HEK293 | Kidney | DcHEV | Wang et al. (2018) |
| | A549 | Lung | Swine (HEV HB-1, 3) | Zhang et al. (2011) |
| | | | Swine (HEV HB-2, 4) | Zhang et al. (2011) |
| | | | Rabbit HEV | Jirintai et al. (2012) |
| | | | Wild boar HEV | Takahashi et al. (2012) |
| | | | Rat HEV | Jirintai et al. (2014) |
| | Huh-7 | Liver | Rat HEV | Jirintai et al. (2014) |
| | Hepa-RG | Liver | Swine HEV 3f | Rogée et al. (2013) |
| | HepG2 | Liver | Rat HEV | Jirintai et al. (2014) |
| | LMH (chicken) | Liver | Avian HEV | Huang et al. (2005a) |
| | PLC/PRF/5 | Liver | Swine HEV | Takahashi et al. (2012) |
| | | | Wild boar HEV | Takahashi et al. (2012) |
| | | | Rabbit HEV | Jirintai et al. (2012) |
| | | | Rat HEV | Jirintai et al. (2014) |
| | | | Rat HEV (R63/DEU/2009)* | Li et al. (2015) |
| | | | Ferret HEV (sF4370) | Li et al. (2016a) |
| | | | DcHEV | Li et al. (2016b) |
| | Sf9; Tn5 | Insect cells | DcHEV | Zhou et al. (2015a) |
| | SOB-15; RTG-2; RTGill-W1; RTL-W1 | Rainbow trout | CTV | Nordheim et al. (2016) |
| | CHSE-214 | Salmon | CTV | Batts et al. (2011) |
| | ZF4 | Zebrafish | CTV | Nordheim et al. (2016) |
| Primary cells | Porcine embryonic stem cell-derived PICM-19 cells | | Swine HEV (HEV 3f) | Rogée et al. (2013) |

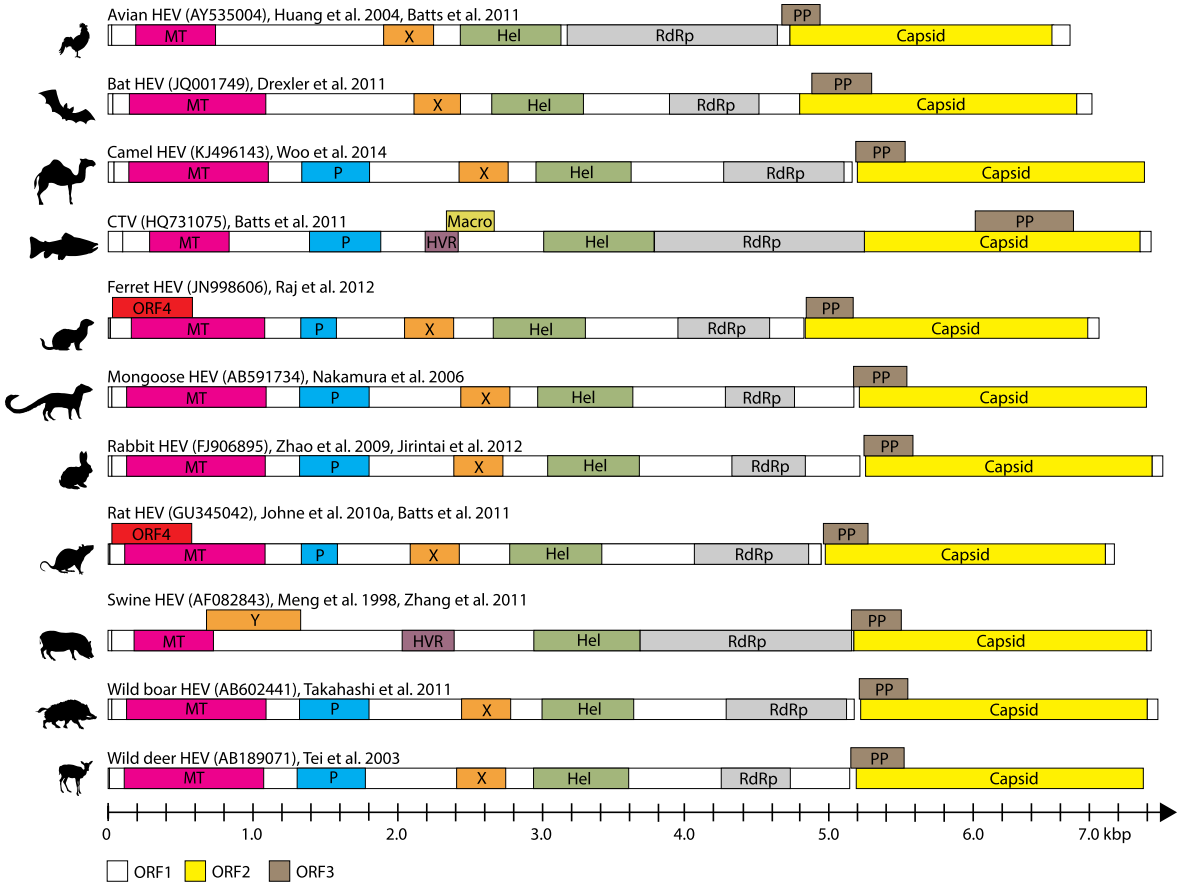


**Fig. 3.** Genomic organization of animal-derived HEV strains, consisting of ORF1 (polyprotein, white), ORF2 (capsid, yellow), ORF3 (phosphoprotein, brown) and ORF 4 (red). Predicted gene length (colour coded) according to GenBank information are depicted relative to genome size (Factor 0.02). ORF4 = Open reading frame 4; MT = Methyltransferase; Y = Y-domain; P = Papain-like cysteine peptidase; S17 = Fragment of the human S17 ribosomal protein; X = X-domain (putative macro domain protein/Appr-1-pase catalytic site, including ADP-ribose binding site, critical for viral RNA replication); Hel = Helicase; RdRp = RNA-dependent RNA polymerase; PP = Open reading frame 3 encoding for a phosphoprotein. The number behind the name displays the accession number in GenBank.

several highly conserved motifs in key viral genes and is a hepatotropic virus (Johne et al., 2010a; Batts et al., 2011; Debing et al., 2016a). Its quasi-enveloped nature is similar to that of human HEV (Jirintai et al., 2014; Kobayashi et al., 2016), although its genome carries an additional ORF (ORF4) (Johne et al., 2010a). Following the identification of HEV-reactive antibodies in several rat species and non-commensal rodents (Kabrane-Lazizi et al., 1999; Favorov et al., 2000; Arankalle et al., 2001; Easterbrook et al., 2007), Johne at al. detected HEV RNA and antigens in the liver cells of infected rats in Norway (Johne et al., 2010a). Li et al. soon generated an infectious cDNA clone of rat HEV (R63/DEU/2009) (Li et al., 2015), of which *in vitro* transcribed RNA intrahepatically injected into rats resulted in RNA positivity in blood and faeces, seroconversion and the isolated progenies' ability to efficiently replicate in human PLC/PRF/5 cells. Similar to what Zhang et al. (2011) observed for swine HEV, Li et al. (2015) found nine additional mutations in the rat HEV isolate recovered from cell culture than in the inoculated primary isolate. In addition to PLC/PRF/5 cells, Jirintai et al. infected human hepatoma cell lines Huh-7 and HepG2 and A549 cells with all three rat HEV genotypes (Jirintai et al., 2014) but detected rat HEV only at the RNA level. In contrast, Johne et al. (2010a) showed the production of full-length infectious virions by passaging infected PLC/PRF/5 cells whose supernatants caused an infection in nude rats. In line with the susceptibility of human cells towards rat HEV, Sridhar et al. (2018) recently detected rat HEV RNA and antigen in clinical samples. Debing et al. (2016a) introduced a possible *in vivo* model for subsequent transfer of these *in vitro* findings by inoculating athymic nude rats with HEV strain LA-B350 (Debing et al., 2016a). The rats subsequently developed a chronic infection comparable to immunosuppressed humans infected with HEV. Furthermore, this group constructed the first rat HEV subgenomic replicon (pLA-B350/luc) for large-scale molecular analysis *in vitro* (Debing et al., 2016a).

In addition to the establishment of swine and rat HEV cell culture models, numerous pilot *in vivo* and *in vitro* studies with other animal HEV strains have yielded promising results. Jirintai et al. (2012) propagated rabbit HEV (Zhao et al., 2009) in human A549 and PLC/PRF/5 cells, while Li et al. (2016a) produced infectious ferret HEV (sF4370; Raj et al., 2012) in PLC/PRF/5 cells and observed an *in vitro* adaption of passaged ferret HEV. Huang et al. (2005a) successfully transfected LMH chicken liver cells with avian HEV (Huang et al., 2004; Batts et al., 2011) and infected chickens with recovered progeny virus, thus demonstrating that LMH cells support viral replication and produce infectious progenitors. In addition, Li et al. (2016b) developed a dromedary camel HEV (DcHEV; Woo et al., 2014) cell culture system in PLC/PRF/5 cells and found accumulating nucleotide changes when DcHEV was serially passaged, whereas its genome was more stable when replicating *in vivo*. More recently, Wang et al. (2018) induced an antiviral response in HEK293 cells upon DcHEV RNA entry into target cells.

Contrary to other animal-derived HEV strains, CTV does not carry human biohazard risk, providing a relatively safe alternative for studying HEV infectivity. Several groups have successfully cultured CTV in cell lines originating from trout: Hedrick et al. (1994), Batts et al. (2011) and Debing et al. (2013) successfully cultured CTV in the Chinook salmon embryo (CHSE-214) cell line. Nordheim et al. (2016) and Bochud et al. (2019) propagated CTV in cells of zebrafish embryos (ZF4) and rainbow trout livers (SOB-15 and RTL-W1), gonads (RTG-2) and gills (RTGill-W1). Although cell culture systems for various animal-derived HEV strains have been developed, none for bats, mongoose, or deer are available.

Li and Wakita (2018) recently discussed available animal models to study acute and chronic HEV infections in humans and Cook et al. (2017) nicely reviewed advantages and disadvantage of surrogate models. For example, rabbits can serve as a pregnancy model due to a similar course of disease in humans, whereas ferrets are suitable to study factors of persistency and protective immune responses. Rats have been considered for antiviral drug screenings but are limited by their resistance to human HEV, although susceptible murine xenograft models (human liver chimeric mice) have been developed to circumvent this problem. However, because these animal lack a functional immune system, host responses cannot be reliably studied when challenged experimentally. Since 1997, pigs have been used to study gene function and mechanisms of HEV replication (Meng et al., 1997b; Huang et al., 2005b, 2007), and tree shrews may be a suitable infection model because of their close relationship to and similar course of disease in primates when infected with human HEV.

In summary, the ideal animal model for HEV infection should combine reproducible viral shedding in faeces, anti-HEV antibody production, elevated serum alanine aminotransferase (ALT) levels, detectable virus RNA in liver and extrahepatic tissue (e.g. kidney, spleen and small intestine), higher mortality rates during pregnancy, development of persistent infection in immunocompromised animals and susceptibility to human HEV (Li and Wakita, 2018). The ability of most animal-derived HEV isolates to infect human cell lines and cause chronic infections in patients underlies the zoonotic capacity of HEV. Thus, insights from animal-derived cell culture models are likely transferrable to HEV transmission, replication, and pathogenesis in humans and potentially can be used to elucidate crucial HEV life cycle steps.

## 5. Antiviral treatment

### 5.1. Current anti-HEV treatment

To date, no specific anti-HEV infection treatment has been developed. HEV normally leads to acute, self-limiting infections but can become fulminant in pregnant women and in the immunocompromised, such as SOT patients and HIV-infected individuals. In one-third of transplant recipients, reversal of immunosuppression leads to clearance of HEV infections (Kamar et al., 2010b, 2011) and is the first line treatment in patients who are viraemic for more than 3 months (European Association for the Study of the Liver, 2018). Off-label use of the broad-range antiviral ribavirin is the only recommended treatment option for patients in whom reversal of immunosuppression is not successful and for other patients (except pregnant women) suffering from severe acute hepatitis E or liver failure (European Association for the Study of the Liver, 2018). Although a retrospective study showed that a 3- to 6-month course of ribavirin monotherapy is effective in most SOT recipients infected with HEV (Kamar et al., 2014b), its efficiency has not been verified in controlled clinical trials. Similarly, treatment failure and poor long-term clinical outcomes have been reported (Pischke et al., 2013; Debing et al., 2014; Kamar et al., 2014b) linked to ribavirin's mutagenic properties (as reviewed by Todt et al., 2016c), which leads to a reversible increase in viral heterogeneity and selection of variants with enhanced replication fitness (Debing et al., 2014; Todt et al., 2016b, 2018a).

The IFN system is a natural host mechanism to combat infections that has been utilised to treat viral diseases and was part of the standard therapy against hepatitis C before virus-specific drugs were developed. Despite IFN's low success rate and severe side effects, it has been evaluated as a potential hepatitis E treatment. For example, in a kidney transplant patient, a 3-month course of pegylated (PEG)-IFN-α2a eradicated serum HEV RNA, and after 2 months, no virus was detected in the patient's stool (Kamar et al., 2010a). Similarly, PEG-IFN-α2a and PEG-IFN- α2b, together with reduced immunosuppression, resolved chronic hepatitis E in liver transplant patients (Haagsma et al., 2010; Kamar et al., 2010c). In addition to SOT recipients, a HIV-co-infected patient cleared serum HEV RNA after a 6 months of PEG-IFN-α treatment (Dalton et al., 2011). However, only after a subsequent 12-week course of combined PEG-IFN-α/ribavirin treatment HEV was undetectable in the patient's faeces; clearance of HEV in cerebrospinal fluid was not noted until 3 months after completion of the treatment regimen. *In vitro* experiments with HEV Kernow-C1/p6 subgenomic replicons and hepatoma cells confirmed these observations and revealed that, although all tested IFN types acted against HEV, IFN-α2a and IFN-α2b most efficiently inhibited the virus and acted synergistically with ribavirin (Todt et al., 2016a). However, because HEV counteracts the IFN system (Dong et al., 2012; Nan et al., 2014; Todt et al., 2016a; Kang et al., 2018) and IFN is generally contraindicated after transplantation of most organs, the application of exogenous IFN should be considered carefully and is only recommended in liver transplant patients who do not respond to ribavirin (European Association for the Study of the Liver, 2018). Although HEV can be treated using ribavirin and/or IFN in immunosuppressed and HIV co-infected patients, the necessary long treatment duration, severe side effects, and high rate of treatment failure remain problematic and highlight the need for a safe, efficient, and specific anti-HEV drug. Moreover, HEV's disease severity and widespread distribution, especially in immunocompromised patients, emphasises the urgency of anti-HEV treatment research.

### 5.2. Development of new anti-HEV drugs using HEV cell culture systems

In recent years, several labs have identified promising HEV inhibitor candidates (as reviewed by Anang et al., 2018). One relatively rapid and cost-effective approach to find new treatment options is drug repurposing. For example, although HCV and HEV are very distinct viruses belonging to different families, they both cause hepatitis that can be resolved by ribavirin with IFN; thus, the anti-HEV potential of the HCV polymerase inhibitor SOF was evaluated. Interestingly, an *in vitro* study by Dao Thi and colleagues indicated that SOF exhibits antiviral effects against GT3 strain Kernow-C1/p6 and acts additively with ribavirin (Dao Thi et al., 2016). The clinical use of SOF against HEV, however, gave contradicting results. In one case study, a transplant patient in whom reduced immunosuppression and ribavirin monotreatment did not clear HEV was treated with ribavirin combined with SOF (van der Valk et al., 2017). This regimen led to declined levels of HEV RNA, but the infection relapsed after treatment ended. Also, an HCV-HEV co-infected post-transplant patient on an immunosuppression protocol being treated with the two anti-HCV drugs SOF and daclatasvir, did not clear the HEV infection (Donnelly et al., 2017). In contrast, a case of an immunocompetent patient with acute-on-chronic liver failure due to HEV was successfully treated with SOF plus ribavirin (Biliotti et al., 2018). A phase 2 multicentre clinical trial evaluating the treatment of hepatitis E with SOF is currently ongoing (ClinicalTrials.gov Identifier: NCT03282474) and will clarify whether SOF is effective against HEV in patients.

In addition to drug repurposing, the development of drugs from natural sources has continually increased in recent years. One study showed that zinc salts, which are essential micronutrients, inhibit expansion of HEV replicons in hepatoma cells (Kaushik et al., 2017), while two others discovered the natural compound silvestrol as a pan-genotypic HEV inhibitor (Glitscher et al., 2018; Todt et al., 2018b). Using the hepatoma cell system in combination with subgenomic reporter constructs, Todt et al. (2018b) found that silvestrol efficiently blocked the replication of different HEV subgenomic replicons at low nanomolar concentrations. This observation was confirmed with primary HEV isolates and ESC- and iPSC-derived HLCs, as well as in human liver chimeric mice infected with GT1 strain Sar-55. Intriguingly, silvestrol also acted against a virus isolate with enhanced viral fitness and decreased ribavirin sensitivity and exhibited an additive antiviral effect with ribavirin. Glitscher et al. (2018) also showed that silvestrol inhibited the spread of GT3 strain 47832c in A549 cells.

Early research of anti-HEV drug candidates are promising, but further studies and clinical trials are necessary to evaluate their anti-HEV potential *in vivo*. Development of a robust HEV cell culture system that reflects human HEV infection could enable the high-throughput screening of compound libraries in the search for new anti-HEV drugs.

## 6. Conclusion and future perspectives in HEV research

HEV was previously considered to be a “traveller's disease” that caused

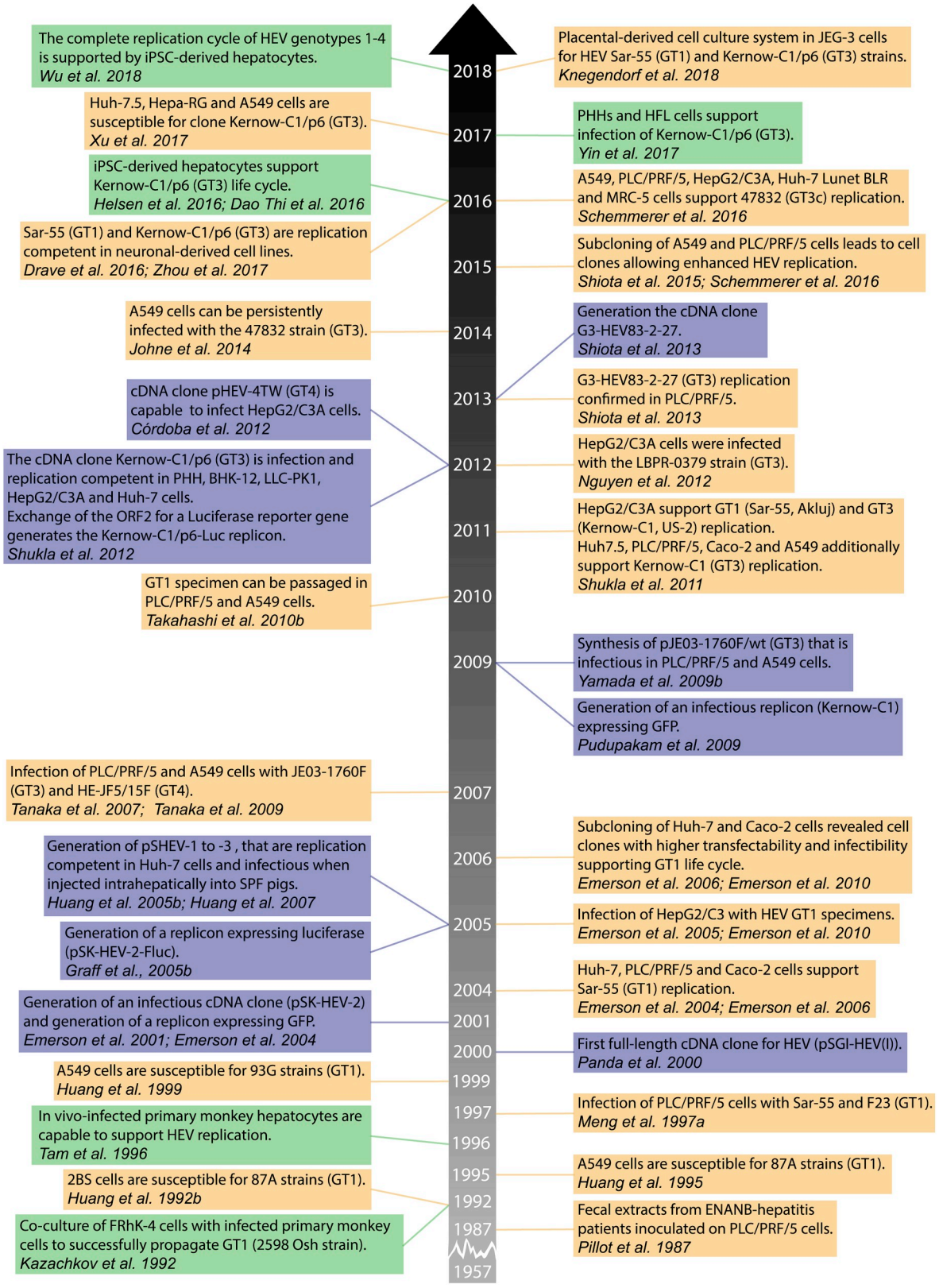


**Fig. 4.** Timeline: Progress in HEV cell culture models. Orange boxes: cancer-derived cell lines which are replication competent for various HEV strains. Green boxes: primary cells and stem cells which were shown to be susceptible for HEV. Blue boxes: cDNA clones generated by various groups.

sporadic outbreaks in developing countries. However, the virus is now recognised as the leading cause of acute viral hepatitis in industrialised countries and is especially problematic in immunocompromised patients. Due to this raised awareness of HEV's public health burden, several groups have worked to establish cellular model systems to study its life cycle and pathogenesis (Fig. 4). However, many virological regarding the HEV

replication cycle question remain open. Future research should continue to focus on robust cell culture models that produce HEV particles to high viral titers. This should ideally be the case for HEV isolates of all genotypes including animal-derived strains. In addition, *in vitro* systems should be developed that allow an efficient infection with primary HEV isolates. Such systems would not only aid in identifying HEV host targets, such as specific cellular receptors, but would also significantly support efforts for developing and/or evaluating new anti-HEV therapeutic options by simplifying drug screening assays and enable functional testing of drug candidates.

## Declarations of interest

None.

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