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Hepatitis E virus: advances and challenges

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Abstract

At least 20 million hepatitis E virus (HEV) infections occur annually, with >3 million symptomatic cases and ~60,000 fatalities. Hepatitis E is generally self-limiting with a case fatality rate of 0.5–3% in young adults. However it can cause up to 30% mortality in pregnant women in the third trimester, and can become chronic in immunocompromised individuals such as those receiving organ transplants or chemotherapy and individuals with HIV infection. HEV is transmitted primarily via the faecal–oral route, and was previously thought to be a public health concern only in developing countries. It is now also being frequently reported in industrialized countries, where it is transmitted zoonotically, or through organ transplantation or blood transfusions. Although a vaccine for HEV has been developed, it is only licensed in China. Additionally, no effective, non-teratogenic and specific treatments against HEV infections are currently available. Although progress has been made in characterizing HEV biology, the scarcity of adequate experimental platforms has hampered further research. In this review, we focus on providing an update on the HEV lifecycle. We will further discuss existing cell culture and animal models and highlight platforms that have proven to be useful and/or are emerging for studying other hepatotropic (viral) pathogens.

In the late 1970s, a large-scale waterborne epidemic of hepatitis spread through 200 villages in the Kashmir Valley of India, causing 52,000 cases of icteric disease and 1,700 deaths[1]. Although the patients' clinical symptoms resembled hepatitis A, they were seronegative for both hepatitis A virus (HAV) and HBV. This finding led to the proposed existence of a new "enteric non-A non-B hepatitis" (ENANBH)[2,3]. A few years later in 1983, similar symptoms were noticed in an outbreak at a Soviet military camp in Afghanistan. A volunteer ingested pooled stool extracts from nine affected patients, and developed the typical signs and symptoms of acute hepatitis. This finding established that the virus could be transmitted via the faecal–oral route and led to the identification of 27–30 nm spherical virus-like particles

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Author contributions

I.N. and A.P. wrote the article. All authors contributed equally to researching data for the article, discussion of content and reviewing/editing the manuscript before submission

Competing interests statement

A.P. and Q.D. are inventors on a patent application defining an hepatitis E virus transcomplementation system for antiviral drug screening and ORF3's viroporin function as a antiviral drug target.

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in the patient's stool that produced hepatitis when inoculated in cynomolgus monkeys[4]. The first partial cDNA of ENANBH was cloned and sequenced in 1990, and ENANBH was renamed 'hepatitis E virus' (HEV) the same year[5]. HEV, an RNA virus, is now recognized as a global health problem in both developing and industrialized regions including South and East Asia, East Africa, Mexico, Western Europe and the USA[6–24]. In this Review, we provide a summary of current knowledge on HEV, highlight cell culture and animal models that have advanced our understanding of the virus, and discuss areas in which the existing models can be improved.

Epidemiology

The global burden of hepatitis E is high; every year there are an estimated 20 million events of HEV infection, 3.3 million symptomatic cases, and 60,000 deaths attributed to HEV genotypes 1 and 2[24,25]. Genotypes 1 and 2 are limited to humans and mostly affect developing countries, where the virus is transmitted through faecally contaminated drinking water[1]. HEV accounts for 50% of acute hepatitis cases in India, and has caused 17 reported large-scale epidemics in Africa between 1988 and 2013 [18–20,26–39]. Although large outbreaks of HEV are limited to developing countries, an increasing number of autochthonous cases are being identified in the developed world, where the prevalent HEV strains are genotypes 3 and 4[40]. In developed nations, the primary routes of HEV transmission are zoonotic (for example, consumption of undercooked pork) and blood transfusions or organ transplants from infected donors [41–44]. HEV is estimated to have a 6% seroprevalence rate in the USA, with higher prevalence in many European countries: for example, in southern France, HEV seroprevalence is 39.1% among blood donors on average, but ranges 21.9–71.3% depending on the geographical area[40,45]. HEV prevalence is probably underestimated as many practitioners do not routinely test for HEV in the presence of acute hepatitis symptoms, and seroprevalence studies have used serological assays with low sensitivity[46,47]. The assays used to detect anti-HEV IgG concentrations in serum or plasma vary considerably in sensitivity and are not standardized, complicating the interpretation of available seroprevalence data[48]. In Europe, awareness of HEV has been increasing over the past 10 years – studies by bloodbank centers in Denmark, France, Germany, Ireland, Netherlands, Spain, and the UK have found that 0.02–0.14% of blood donations are positive for HEV RNA[49–54]. Currently, blood transfusions are routinely screened for HEV RNA in Ireland and the UK, and the Netherlands have started screening blood transfusions in 2017[55]. Selective screening occurs in France and Germany for high-risk patients, and blood authorities in Greece, Portugal, Spain, and Italy are currently evaluating whether to implement HEV screening[55]. In the United States, only 0.002% of plasma donations were shown to be positive for HEV RNA, suggesting that screening plasma-derived products in the US may not be necessary given the poor utility and low number of donors with positive for HEV RNA[56].

HEV classification and transmission

The HEV strains affecting humans are classified into genotypes 1, 2, 3, 4, and most recently 7, and fall under the species *Orthohepevirus A* (Figure 1) [57]. Outside of *Orthohepevirus A*, there are several species of HEV that infect animals but are not transmissible to humans:

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Orthohepevirus B (chicken), *Orthohepevirus C* (rat, ferret), *Orthohepevirus D* (bat), and the genus *Piscihepevirus A* (trout)[57]. HEV genotypes 1 and 2 of *Orthohepevirus A* are restricted to humans, primarily water-borne and associated with epidemics and sporadic cases in developing countries. Infections with HEV genotypes 1 and 2 are generally self-limiting and not associated with chronic disease, and are endemic to resource-poor regions in many countries in Asia, Africa and Latin America[58]. By contrast, HEV genotypes 3, 4 and 7 of *Orthohepevirus A* are primarily zoonotically transmitted through the consumption of animal products, and are associated with sporadic or cluster cases of hepatitis in industrialized countries[59]. Chronic cases of hepatitis E caused by infections with HEV genotypes 3, 4 and 7 have been reported in immunocompromised individuals, such as organ transplant recipients and individuals infected with HIV[40,60]. Although HEV infects a broad range of species including bats, ferrets, rabbits and chicken, the primary species that are considered reservoirs for transmission to humans are swine, deer and wild boar[61]. Of these, swine are arguably the biggest reservoir of infection and mostly likely source of zoonotic infections, with HEV RNA detected in 73% of swine farms in Sweden (based on measurements of swine faeces), 47% of swine herds in Spain (based on detection of HEV RNA in swine sera), and 24% of pig farms in France (based on presence of HEV RNA in swine liver)[62–64]. Infection in swine is subclinical, causing only mild hepatic lesions, and therefore swine are not routinely tested for HEV infection[65]. Studies measuring the presence of HEV RNA in commercial pork-based food products detected genotype 3 RNA in 47% of pork pâtés (Canada), 22% of pork liver sausages (Germany), and 30% of figatelli (French/Corsican liver sausage) samples (France)[66–68]. HEV can be inactivated by heating at 71°C for 20 min, therefore transmission primarily occurs through the consumption of undercooked food products[69]. HEV genotypes 7 and 8 infect dromedary and Bactrian camels, and there is some limited evidence of genotype 7 transmission to humans from the consumption of camel milk and meat[70–72]. Additional zoonotic hosts have been reported, including moose, rat, ferret, wild boar and dolphin, where it is unknown whether the corresponding HEV strains are transmissible to humans[57]. Little is known about the mechanisms underlying the host ranges of the various HEV genotypes.

Clinical manifestations

Hepatitis E most commonly manifests as self-limited acute hepatitis, causing symptoms of anorexia, nausea, vomiting, malaise, abdominal pain and jaundice typically lasting 1 month. HEV infection is clinically indistinguishable from HAV infection, and is associated with a 1–2% mortality in immunocompetent patients[24]. A poorly understood clinical consequence of HEV is its severe effect in pregnant women, in which HEV infection can cause acute liver failure, haemorrhage and stillbirth, and result in up to 25% mortality in the third trimester[73–75]. The mechanisms underlying increased HEV virulence in pregnant women are unknown, but could be related to hormonal and/or immunological changes during pregnancy[75]. Acute infection has also been associated with high mortality among children under 2 years of age, and is more severe among patients with pre-existing liver disease[39,76]. HEV genotypes 3, 4 and 7 can become chronic in immunocompromised patients, such as organ transplant recipients and individuals infected with HIV[73,77–79]. These patients are at higher risk of developing chronic infection and rapid progression to

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cirrhosis[60,80,81]. Notably, evidence has emerged that commonly used immunosuppressive drugs such as tacrolimus can increase the risk of developing chronic HEV in solid organ transplant (SOT) recipients[82]. For example, it has been reported that the odds ratio of developing chronic HEV when using tacrolimus is 1.87 (CI: 1.49–1.97, p<0.004) as compared to when using cyclosporine A for immunosuppression[82]. Additionally, SOT recipients who were seropositive at the time of transplantation can become reinfected upon taking immunosuppressive therapy, and the infection can progress to chronicity[83]. A recent case study reported on a patient presenting with primary hepatocellular carcinoma (HCC) who was positive for HEV but not other chronic hepatitis viruses including HBV and HCV, which are commonly associated with HCC[84]. While these data do not prove any causal relationship, they may warrant further analysis on whether persistent HEV can culminate in HCC.

HEV primarily replicates in the liver but it has been associated with a number of extrahepatic symptoms. Correlations have been found between HEV and pancreatitis, neurological symptoms (most commonly neuralgic amyotrophy and Guillain–Barré syndrome), haematological disorders (including severe thrombocytopenia), glomerulonephritis and mixed cryoglobulinaemia, and cutaneous T cell lymphoproliferative disorders[85–90]. Direct causation between HEV and neurological symptoms remains to be proven, but 12 cases have been reported in which HEV RNA was detected in the cerebrospinal fluid of patients demonstrating neurological symptoms such as Guillain-Barre syndrome and neuralgic amyotrophy, and HEV has additionally been shown to replicate in neurons *in vitro*[79,88,91,92]. HEV has also been shown to replicate in intestine, lymph nodes, spleen and kidney in a swine model[93]. These findings reveal that hepatitis E is a complex disease whose pathogenesis and clinical progression needs to be characterized more thoroughly.

Treatment

There is currently no direct-acting treatment for HEV infection, and it remains a major public health concern particularly among immunocompromised patients and pregnant women. If possible, in transplant recipients or other immunocompromised patients, reduction of immune suppression is attempted first, which results in a sustained virologic response in 30% of patients (defined as undetectable HEV RNA in serum for 4 weeks)[60,82,94]. The current treatment of choice for HEV infection in chronically infected patients is monotherapy with the nucleoside analogue ribavirin; however, ribavirin is specifically contraindicated in pregnant women, who disproportionately have adverse effects as a result of HEV infection[95]. No established treatment for HEV is available for pregnant women, so only supportive care is provided (also known as symptomatic treatment), resulting in up to 30% maternal mortality associated with fulminant hepatic failure, spontaneous abortion and stillbirth[96]. Additionally, ribavirin-resistant HEV strains; for example, a genotype 3 HEV strain with a mutation in the C-terminal of the viral polymerase (encoding the G1634A protein variant) are being reported with increasing frequency in nonpregnant, chronically infected patients[97]. Some mutations that have been associated with ribavirin treatment failure clinically – such as the G1634R and Y1320H variants – have not resulted in ribavirin sensitivity *in vitro*, but have led to increased

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viral replication[97,98]. Alternative treatments for ribavirin failures, which include pegylated interferon, have met with limited success and have not been systematically evaluated[99–101]. Finally, preliminary studies suggest that sofosbuvir, an HCV-specific direct-acting antiviral agent, inhibits replication of genotype 3 HEV *in vitro;* however, this finding remains to be independently and clinically verified[102,103]. Ultimately, there is a great need for novel therapies against HEV[97,98,104,105].

Prevention efforts have focused on sanitation, as the primary route of HEV transmission worldwide is contaminated water, and on vaccination. Hecolin (Innovax, China), a protein-based HEV vaccine eliciting anti-capsid antibodies and inducing a vigorous T cell response, is the only option for vaccination but is currently only licensed in China[106–108]. A second protein-based vaccine, rHEV, also contains amino acids from the capsid protein (ORF2) and was tested in phase II clinical trials but despite good safety and efficacy profile was not further developed due to the cost of clinical trials and development [109,110]. Vaccine efficacy for Hecolin was shown to be 100% against genotype 1 HEV after three doses in a phase III trial in China, and cross-protects against genotype 4[111]. Notably, Hecolin has not been tested in pregnant women, but immunogenicity has been confirmed in pregnant mice[111,112]. More studies are needed to confirm whether Hecolin is effective against other genotypes including genotype 3, and to evaluate its efficacy in pregnant women and also to test efficacy in other patient populations beyond China.

An HEV vaccine holds great promise for preventing disease among residents and travellers to endemic regions such as Southeast Asia, and for reducing the alarming HEV-associated mortality in pregnant women. However, the vaccine will need to be made available outside of China, a move that is hindered by HEV not being on the WHO's prequalification vaccine priority list[113]. Furthermore, distribution of the vaccine in high-endemicity regions, where health care access is often limited, will be an obstacle. Still, the vaccine holds potential to benefit high-risk patients in developed nations as well, such as SOT recipients who could be administered the vaccine as a preventative measure. Ultimately, widespread distribution of the HEV vaccine will most likely depend on public and private sector partnerships.

The molecular virology of HEV

The HEV virion is icosahedral in shape and measures 27–32nm in diameter. The capsid consists of a single, self-assembling protein whose crystal structure has previously been elucidated[114]. HEV was declared to be a 'quasi-enveloped' virus in 2016, existing in both non-enveloped and enveloped ('eHEV') forms, similar to HAV[115]. HEV is shed in faeces as a non-enveloped virus, but HEV produced in cell culture contains a lipid envelope[116]. The quasi-enveloped nature of HEV affords protection from neutralizing antibodies against the ORF2 and ORF3 proteins in the 'eHEV' form; however, attachment and entry of eHEV particles is far less efficient than that of non-enveloped HEV particles[117–119]. Non-enveloped HEV and eHEV are believed to have distinct cellular entry mechanisms, and further studies are required to characterize these processes[117].

The HEV virion contains a positive-sense, single-stranded RNA genome of ~7.2kB in length, and is classified in the *Hepeviridae* family. The viral genome is organized into

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three open reading frames (ORFs 1–3), and contains three short untranslated regions (UTRs)[120,121]. A fourth open reading frame (ORF4) has been described only in HEV genotype 1, and is translated into a protein that enhances activity of the RNA-dependent RNA polymerase (RdRp)[122] (Figure 2). ORF1 is the largest viral gene product of HEV, and encodes the non-structural proteins of the virus including the RdRp, RNA helicase and methyltransferase[123–125]. HEV also contains several other, less well-characterized domains including the 'X' and 'Y' domains, the hypervariable region (HVR), and a putative papain-like cysteine protease (PCP). HEV plausibly contains a PCP based on bioinformatic comparison that identified a domain in HEV distantly related to the protease of rubella virus[126]. Most positive-strand RNA animal viruses, including alphaviruses, togaviruses and picornaviruses, contain a protease that mediates polyprotein processing; however, for HEV, experiments attempting to show protease function and ORF1 polyprotein processing have yielded conflicting data [127–129]. The fate of the ORF1 polyprotein and the function of the PCP-like domain of HEV both remain rich areas for further research. ORF2, the second largest viral gene, is located downstream of ORF1 and encodes the viral capsid protein of HEV[130]. The ORF2 capsid is highly immunogenic and is the basis for the Hecolin HEV vaccine, however in the quasi-enveloped state eHEV virions are resistant to anti-ORF2[117]. Although no clinical data currently suggest that the presence of eHEV limits vaccine efficacy, it could have an effect on viral spread once an infection is already established. Finally, ORF3, which is only 360bp in length, almost entirely overlaps with ORF2 and encodes a functional ion channel that is critical for release of infectious viral particles[131]. ORF3 has additionally been shown to interact with a variety of host proteins including tumor susceptibility gene 101 protein (TSG101), a key component of the endosomal sorting complexes required for transport (ESCRT) pathway that is used by a number of viruses (including HIV) for budding of progeny virions[132,133].

As for many other viruses heparan sulfate proteoglycans (HSPG) are required for the attachment of HEV virions to their target cells, and eHEV enters cells through a process that involves receptor-dependent clathrin-mediated endocytosis, the Rab5 and Rab7 GTPases and lysosomal lipid degradation[117,134–136]. Non-enveloped HEV and eHEV are believed to have distinct entry mechanisms, and little is known about entry mechanisms for non-enveloped HEV specifically. A cell surface receptor mediating HEV entry also remains to be identified. Upon viral entry of eHEV, the quasi-envelope is believed to undergo lysosomal lipid degradation to expose the capsid protein[117]. The virion then uncoats in a poorly understood process, and the positive-sense HEV RNA is translated by host factors to produce the ORF1 polyprotein containing the RdRp. The RdRp then transcribes complementary full-length negative-sense viral RNA, which serves as a template for transcribing positive-strand full-length HEV RNA, and a 2kB subgenomic transcript encoding ORF2 and ORF3[137–140] (Figure 3). Limited evidence suggests that an additional 3.7kB subgenomic RNA might be transcribed from the negative-sense template[137,141,142]. Both the full-length and subgenomic transcripts are capped and polyadenylated[137]. Host ribosomes are then thought to use *leaky scanning* to translate the ORF2 protein (pORF2) and ORF3 protein (pORF3) from the subgenomic RNA in the endoplasmic reticulum[143]. Leaky scanning refers to a phenomenon in which a ribosome will occasionally skip a 'weaker' initiation codon – possibly an 'ATG' triplet in a weak Kozak consensus sequence – and instead use a second,

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downstream initiation codon; in this way the ribosome can translate two proteins from the same transcript. The regulation and relative levels of translation of these proteins are not well understood. The pORF2 capsid is then processed in the endoplasmic reticulum and glycosylated at three sites, a modification necessary for the formation of infectious virions[130,144,145]. pORF2 binds the 5' end of HEV, an interaction that could mediate encapsidation; however, a complete picture of the role of pORF2 in the life cycle remains to be determined[146]. Different studies show that pORF2 localizes to the Golgi, cytoplasm and even the nucleus, suggesting that the protein could have multiple, hitherto unknown functions[147,148]. pORF3, a 113 amino acid phosphoprotein, is another poorly understood HEV product that is required for release of virions and was shown to exhibit ion channel activity in a paper published in 2017[131]. The ORF3 ion channel does not seem to have any discernible preference for specific anions or cations, and the mechanisms underlying viroporin-mediated viral release remains to be determined. Cell lysis and the subsequent release of infectious virions can be triggered by increased membrane permeability as a result of viroporin accumulation [149] but this process does not seem to have a role in HEV egress as HEV is thought to be a non-cytolytic virus. Viroporin insertion in cellular membranes have been proposed to disrupt the chemoelectrical barrier by facilitating flux across membranes, therefore dissipating the membrane potential of internal vesicles or the plasma membrane and stimulating viral budding [150].

In addition, pORF3 interacts with a broad range of host cellular proteins including 3IP, microtubules, SH3 and Pyst1 (both leading to activation of MAPK), bikunin (serine protease inhibitor), TSG101, hemopexin, fibrinogen, HIF1A, CIN85, HNF4 and hepsin[130,151–162]. These diverse interactions suggest that pORF3 might modulate the host environment in multiple ways to create favourable conditions for the viral life cycle, in addition to its role in viral release[163]. Notably, the role of pORF3 in virion release is dependent on a highly conserved PSAP motif which enables the interaction with TSG101, a cellular factor involved in the budding of viruses and a member of ESCRT pathway[132,164–171 79]. The ESCRT pathway is used by a number of other RNA viruses (for example, HIV) during viral release, and involves budding of the virus through the cellular membrane, leading to the acquisition of a lipid envelope[172]. Thus, the ESCRT pathway would explain how eHEV particles are formed; however, this aspect suggests that non-enveloped HEV uses a different mechanism for release that is thus far unknown, or that non-enveloped HEV is formed through shedding of the lipid envelope following release. Ultimately, more studies are needed to close the numerous gaps in our understanding of the HEV life cycle.

Cell culture models

HEV has historically been extremely difficult to culture *in vitro*, replicating at very low titers. Early experiments developing *in vitro* infection systems with full-length virus used a variety of cell types including primary hepatocytes from macaques, human HepG2 hepatoma cells, A549 lung adenocarcinoma cells and simian primary kidney cells (Table 1)[173–176]. However, amplification was required to detect HEV in the medium of these cells, and this lack of an efficient cell culture study hampered efforts to study the HEV life cycle. Breakthroughs in developing robust *in vitro* systems to study HEV have been achieved not

only through identifying compatible cell lines, but also through the isolation of specific strains with improved replication efficiency *in vitro*.

Cell lines.

Generally, genotype 1 HEV has been more difficult to culture *in vitro* than genotypes 3 and 4, so the available cell-culture-adapted strains are mostly derived from the latter. In 2009, the genotype 3 JE03–1760F and genotype 4 HE-JF5/15F HEV strains were isolated from infected patients and found to have increased replication efficiency in A549 cells and PLC/PRF/5 liver hepatoma cells[177,178]. These strains accumulated mutations after being serially passaged in cell culture, that presumably enhanced their ability to replicate in these systems. In 2011, the genotype 3 Kernow-C1 strain of HEV was isolated from a chronically infected patient who was co-infected with HIV and found to efficiently infect human, deer and pig cell lines after being serially passaged six times in culture[179]. The increased ability of the Kernow-C1/p6 strain to replicate *in vitro* was due to a 57-amino acid insertion from the human S17 ribosomal RNA into the ORF1 HVR domain. The S17-containing recombinant strain was present in the original faecal sample from the patient, who had become host to multiple quasi-species of HEV as the virus mutated during the length of his chronic infection. Over six serial passages in HepG2/C3A human hepatoma cells, the S17 insertion-containing strain was found to propagate more efficiently and become the dominant quasi-species. Introduction of the S17 insertion into a different, genotype 1 strain of HEV markedly enhanced its ability to transfect hamster BHK-21 cells[180]. Similarly, another genotype 3 HEV strain, LBPR-0379, was identified to contain an insertion in its HVR region from the S19 human ribosomal protein that conferred a growth advantage in cell culture[181]. The mechanisms whereby these insertions improve replication of HEV *in vitro* and broaden the virus' host range are unknown and the subject of great interest. Furthermore, they demonstrate the ability of the virus to mutate into quasi-species and acquire novel capabilities during chronic infection.

In vitro studies of HEV have used the HepG2, HepG2/C3A, HepaRG, Huh7, Huh7.5 and S10–3 hepatoma cell lines to study viral replication[118,131,182–184]. HepG2/C3A, a subclone of the HepG2 hepatoma cell line, is a popular model for *in vitro* drug testing and exhibits an improved hepatic phenotype over HepG2 cells[185]. Replication of the Kernow-C1/p6 strain of HEV was reported to be ~7.5-fold higher in HepG2/C3A cells than in Huh7.5, PLC/PRF/5, and A549 cells, making the combination of the Kernow-C1/p6 strain with HepG2/C3A a powerful tool for studying HEV[179]. That said, hepatoma and other tumour-derived cell lines do not adequately reproduce the physiological environment of primary cells (hepatocytes) because of their abnormal cell proliferation and aberrant gene expression and regulation[186].

Primary hepatocytes.

Cultures of primary hepatocytes are more desirable for *in vitro* experiments for HEV infection, but their use has several practical limitations. Indeed, it was shown that HEV can infect primary hepatocytes of cynomolgus monkeys, but infection of primary human hepatocytes has not been reported yet[173]. Once isolated, primary hepatocytes do not proliferate or undergo limited proliferation, which is a challenge. Furthermore, the phenotype of these primary hepatocytes is unstable, as they tend to de-differentiate within

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days in conventional culture systems, thereby precluding longer-term studies of HEV infections[187]. Primary human hepatocyte dedifferentiation can be delayed or prevented in collagen sandwich cultures, by aggregation in spheroids or in co-culture with non-parenchymal cells[188–192]. Primary human hepatocytes aggregated into spheroids have been infected with HCV[193]. For the latter approach, both self-assembling (SACC) and micro-patterned primary human hepatocyte co-cultures (MPCC) are effective formats to stabilize hepatic function, especially if oxidative stress is reduced during the initiation of the culture[194 195,196]. MPCC and SACC primary human hepatocytes have been infected with HBV, HCV and *Plasmodium falciparum* and *P. vivax* [197–200] and therefore might prove useful to establish longer-term HEV infections, possibly even with non-cell culture-adapted patient isolates. Infections in primary human hepatocytes are frequently hampered by considerable variability in the susceptibility between different hepatocyte donors although the underlying etiology of this variability is unknown.

Stem cell derived hepatocyte like cells and tissue organoids.

In 2016, induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (HLCs) were shown to support HEV replication for the Kernow-C1/p6 strain, although at lower levels than HepG2/C3A cells[201]. iPSCs carry the advantages of being indefinitely self-renewing, more easily amenable to genetic manipulations than fully differentiated cells, capable of differentiating into numerous cell types and able to produce patient- and tissue-specific primary — they are a promising *in vitro* model for studying HEV in a more physiologically relevant context than hepatoma-derived cells[202–204]. To better approximate the cellular complexity of the liver, efforts are under way to create more complex cultures, incorporating additional liver resident non-parenchymal cell populations including endothelial cells, cholangiocytes, Kupffer cells, fibroblasts, and stellate cells[205,206]. (Figure 4). Combining these cell types, preferably in physiologically relevant ratios, will be particularly helpful to more accurately mimic the inflammatory environment of the liver during HEV infection. As an additional layer of complexity, incorporating primary hepatocytes in a 3D architecture will be important to closely mimic the transcriptional heterogeneity of hepatocytes in the liver that is influenced by a variety of environmental cues, including oxygen and nutrient gradients. Differences in transcriptional activity have clearly been documented for genes involved in metabolism but could conceivably also affect the susceptibility and host response to (viral) pathogens, such as HEV[207]. Tremendous advances have been made in engineering very sophisticated (primary) cell culture platforms, which have already been successfully used to study other hepatotropic viruses such as HBV[200,208]. Undoubtedly, the HEV field will benefit from these advances and might even become a driver in refining them.

Tissue tropism.

It is important to note that in contrast to HBV, HCV and hepatitis delta virus (HDV), which are thought to productively infect only hepatocytes *in vivo*, HEV has a broader tissue tropism. HEV RNA and/or antigens have been detected in small intestine, colon, lymph nodes, placenta, dermal microvascular endothelial cells, and neurons based on studies in swine and humans[90,209–212]. Using negative-strand-specific PCR, HEV replication has been detected in human placenta[209]. This finding raises the possibility that non liver-derived cells could have potential as *in vitro* systems to study HEV. Indeed, HEV is capable of

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replicating in a number of non-liver cell types. Notably, the lung adenocarcinoma-derived A549 cell line was one of the earliest cell lines used to culture HEV, and is permissive to the genotype 3 JE03–1760F and genotype 4 HE-JF5/15F HEV strains, as well as swine, wild boar and rabbit-derived strains of HEV [177,178,213–215]. HEV has also been shown to infect porcine embryonic stem cell-derived hepatic cells[184]. It should be clearly noted, though, that the ability of a given virus, such as HEV, to infect and replicate in a cell line, does not necessarily imply that this cell type would be a natural reservoir for the virus. Exemplary for this point is that despite replicating in A549 cells *in vitro,* HEV has not been correlated with pulmonary HEV-related symptoms in patients.

Animal models

Even with these advancements, tissue cultures have limited utility in studying virally induced immune responses and disease. Thus, creating suitable animal models for HEV will remain a priority to study the pathogenesis of HEV, for example, during both fulminant acute and chronic hepatitis E, and to test novel antiviral therapeutics. The optimal model should be fully immunocompetent, susceptible to genetically diverse HEV strains causing disease in humans and recapitulating clinically apparent disease. From a practical perspective, the model should be cheap, easy to propagate, amenable to genetic manipulations and optimally a plethora of reagents should be available to monitor host responses to the infection. Such a model does not exist – yet.

To bridge this gap, (at least) three alternative and certainly not necessarily mutually exclusive approaches could be taken: conceivably, surrogate models, that is species which naturally support HEV infection to some extent, could be used; the host environment of a usually resistant species could be engineered to render it more conducive to HEV infection; HEV could possibly be adapted genetically to enable the virus to overcome species barriers.

Primates.

In contrast to the other hepatitis viruses, which exhibit a narrow host range largely limited to humans and closely related great apes (HBV, HCV, HDV) or at least smaller primates (HAV), certain HEV genotypes have been found in a variety of species. These include swine, deer, and rabbit that serve as reservoirs for HEV in industrialized nations [59 216]. Additional zoonotic hosts include moose, rat, wild boar (genotype 5,6), camel (genotype 7,8), and dolphin (genotype 3), but it is unknown whether the corresponding HEV strains are transmissible to humans [57]. Despite the broad host range of HEV, infection is subclinical in most zoonotic hosts, and tractable small animal models to study the virus *in vivo* are lacking. Non-human primates have been among the few animals successfully used to study genotypes 1 and 2 of HEV, in particular rhesus monkeys, cynomolgus macaques and chimpanzees (Figure 5)[217–219]. Numerous experiments have been done in chimpanzees using genotype 1 HEV, including analyses of transcriptomic changes associated with infection, measurement of the duration of faecal shedding and viraemia, and the discovery that capping of HEV is required for infectivity[220–223]. Chimpanzees and rhesus monkeys have additionally been used to show that swine HEV can cross the species barrier and infect non-human primates[224]. Infected primates develop clinical responses to HEV that mimic

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some features of human disease, including focal hepatocyte necrosis with accumulations of macrophages and activated Kupffer cells, and the anti-HEV immune response in primates is similar to that observed in humans [221,225]. Thus, nonhuman primates have been useful models for studying hepatitis E clinical progression and for immunological studies[220,226–228]. Both cynomolgus and rhesus monkeys have been used to test the efficacy of ORF2 capsid protein-derived anti-HEV vaccines[229,230 231]. However, there are limitations to the use of non-human primates in modelling HEV *in vivo.* Attempts to reproduce HEV-associated mortality in the context of pregnancy were unsuccessful in pregnant rhesus monkeys, who also do not exhibit vertical transmission of the virus[232 233]. Furthermore, primates are expensive to maintain, their use raises ethical concerns, and hepatitis E infection in primates does not accurately mimic some aspects of disease progression in humans, in particular HEV-associated liver injury in the context of pregnancy[232,233].

Swine.

Swine were discovered to be natural hosts to HEV in 1995, and are now known to be the primary route of HEV transmission to humans in developed countries[234,235]. Unlike rhesus monkeys, which can be infected with HEV genotypes 1–3, swine are only susceptible to genotypes 3 and 4[236,237]. Infection in swine is largely subclinical, but pigs develop relatively more severe hepatic lesions when infected with human genotype 3 HEV than swine genotype 3 HEV[65,235,238–241]. Swine have been used to demonstrate extrahepatic sites of HEV replication, including small intestine, lymph nodes and colon, and for studies on cross-species infection[211,242]. In 2017, experimental pigs treated with immunosuppressive drugs were used to successfully establish chronic HEV infection with genotype 3, the same genotype responsible for chronic infection in humans[243]. Thus, swine have proven to be useful models for studying genotypes 3 and 4 of HEV, but are not susceptible to genotype 1, which accounts for the majority of clinical cases in humans worldwide.

Rabbits.

Rabbits are another natural host of HEV who could serve as useful models for HEV studies[244]. Rabbit strains of HEV have been experimentally shown to infect swine and cynomolgus macaques, demonstrating a high potential for cross-species transmission[245,246]. Rabbits show limited clinical symptoms from HEV, but notably studies from one group suggest that rabbits support chronic infection and extrahepatic replication of HEV, and that pregnant rabbits have high HEV-associated mortality[247,248]. Given the relatively small size of rabbits and the potential for transmission of rabbit HEV strains to humans, rabbits might be an interesting model in which to explore the pathogenesis of hepatitis E.

Small animals.

There has been limited success infecting naive mice with HEV, who are not natural hosts for the virus. One study reported that Balb/c nude mice were susceptible to genotype 4 HEV isolated from swine, however these findings have not been confirmed independently[249]. Conceivably, mouse orthologues of certain yet-to-be-identified host factors only inefficiently support different aspects of the viral life-cycle. Greater understanding of the different aspects of the viral life-cycle and essential cellularly encoded co-factors would potentially

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allow us to overcome the species barrier of HEV genetically and to create mouse models that robustly support HEV infection.

Alternatively, tissue humanization approaches have been effective in establishing infections with other human hepatotropic pathogens. Mice growing a partially human liver can support infections with hepatotropic pathogens *in vivo*, including HBV, HCV, HDV and liver stages of parasites causing malaria in humans[250–260]. Humanized mice are usually generated through transplantation of human hepatocytes into immunocompromised liver injury recipients. The resulting human liver chimeric mice have been used to successfully establish infection with HEV genotypes 1 and 3 in the past few years[261–264]. These xenotransplanted mice are a tractable and valuable model for drug testing and for studying long-term viral persistence within the 3D context of the liver. However, a considerable shortcoming of singly engrafted human liver chimeric mouse models is their inability to mount cellular and humoral immune responses due to their highly immunocompromised status that is necessary to prevent graft rejection. To study HEV-specific immune responses, which counteract the infection but are also thought to contribute to the progression of liver pathogenesis, xenorecipients co-engrafted with both human hepatocytes and components of a human immune system in a single recipient might prove useful. Such dually engrafted mice have been used in to study human immune responses to HBV and HCV for example[265–267]. However, given that the human immune response is generally weak in such humanized mouse models, continued refinements of the xenorecipients strains and engraftment procedures remain critical.

Rats are another potential rodent model for HEV, and unlike mice, are natural hosts for HEV[268]. However, the HEV strains infecting rats are classified under *Orthohepevirus C*, and are only distantly related to the human-tropic strains of HEV, which are classified in *Orthohepevirus A*. One group successfully infected athymic nude rats with rat HEV, however rats are not susceptible genotypes 1, 2, or 3 of human HEV, and furthermore rhesus monkeys are not susceptible to rat HEV[269,270]. Although it is conceivable to generate HEV chimeras between strains of *Orthohepeviruses A* and *C*, this approach could prove difficult because of genetic incompatibilities that could compromise the fitness of these genomes. Finally, gerbils are another rodent model that warrant further investigation for their potential as a tool to study HEV. Several groups have reported successful infection of Mongolian gerbils with genotype 4 HEV[271–273].

Conclusions

Although HEV is becoming increasingly recognized, much work remains to be done in understanding its pathogenesis and molecular mechanisms (Box 1). Little is known about key aspects of the viral life cycle – for example, the cellular (co-)factors involved in different steps in the viral life-cycle, the most prominent receptor(s) mediating viral entry, whether and how polyprotein processing occurs for ORF1, and the role of ORF3 in viral release. A better understanding of these mechanisms could hold the key to developing direct-acting antiviral therapeutics. The clinical pathogenesis of hepatitis E disease contains many mysteries as well, from the high mortality rate in pregnant women caused by specific strains of the virus, to the many extrahepatic symptoms that are being reported in association

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with infection. Ultimately, more clinical data and better cell culture and animal models are needed to understand interactions between the virus and its host. Finally, as we encroach into new ecological spaces, more accurate epidemiological data is needed to understand the transmissibility of HEV from foods, and to characterize the full extent of the host range of HEV.

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Box 1 |

Key questions or challenges in HEV research

- Gaining a thorough mechanistic understanding of crucial aspects of the viral lifecycle: entry, genome replication, assembly and release
- Biophysical analysis and biochemical composition of hepatitis E virus (HEV) particles
- (noneveloped HEV versus enveloped HEV (eHEV))
- Obtaining high-resolution structures of pORF1 and pORF3
- Identification of essential host factors governing different aspects of the HEV lifecycle
- Defining the HEV host and tissue tropism
- Mechanism of HEV-mediated pathogenesis in clinically relevant settings (e.g. acute liver failure during pregnancy and extrahepatic manifestations)
- Effect of HEV co-infection in patients with underlying liver disease, such as chronic viral hepatitis
- Creating robust cell culture models supporting infection with all HEV genotypes
- Developing tractable (small) animal models that adequately recapitulate disease symptoms observed in patients
- Collecting epidemiological data on the prevalence and transmissibility of different HEV genotypes in humans and animals
- Mechanisms of innate immune recognition and correlates of immunological protection
- Developing direct-acting or host-targeting antiviral agents that can effectively cure HEV infection and can be administered to all patient populations

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Key points

- HEV causes varying disease severity among patient subpopulations: it is self-limiting in most young adults, but causes ~30% mortality in pregnant women, and lead to chronicity in immunocompromised patients.
- HEV has a broad but poorly characterized host range, and in industrialized countries it is primarily transmitted zoonotically through the consumption of undercooked meat.
- A prophylactic vaccine against HEV exists but is currently only licensed in China.
- There is currently no direct-acting therapy available against HEV, and no non-teratogenic treatment options for pregnant women, creating a need for development of new therapeutics.
- The molecular biology of HEV remains incompletely understood.
- New model systems are emerging to study HEV, but more refined models are needed to gain insights in the interactions of HEV with its host including mechanisms of HEV pathogenesis.

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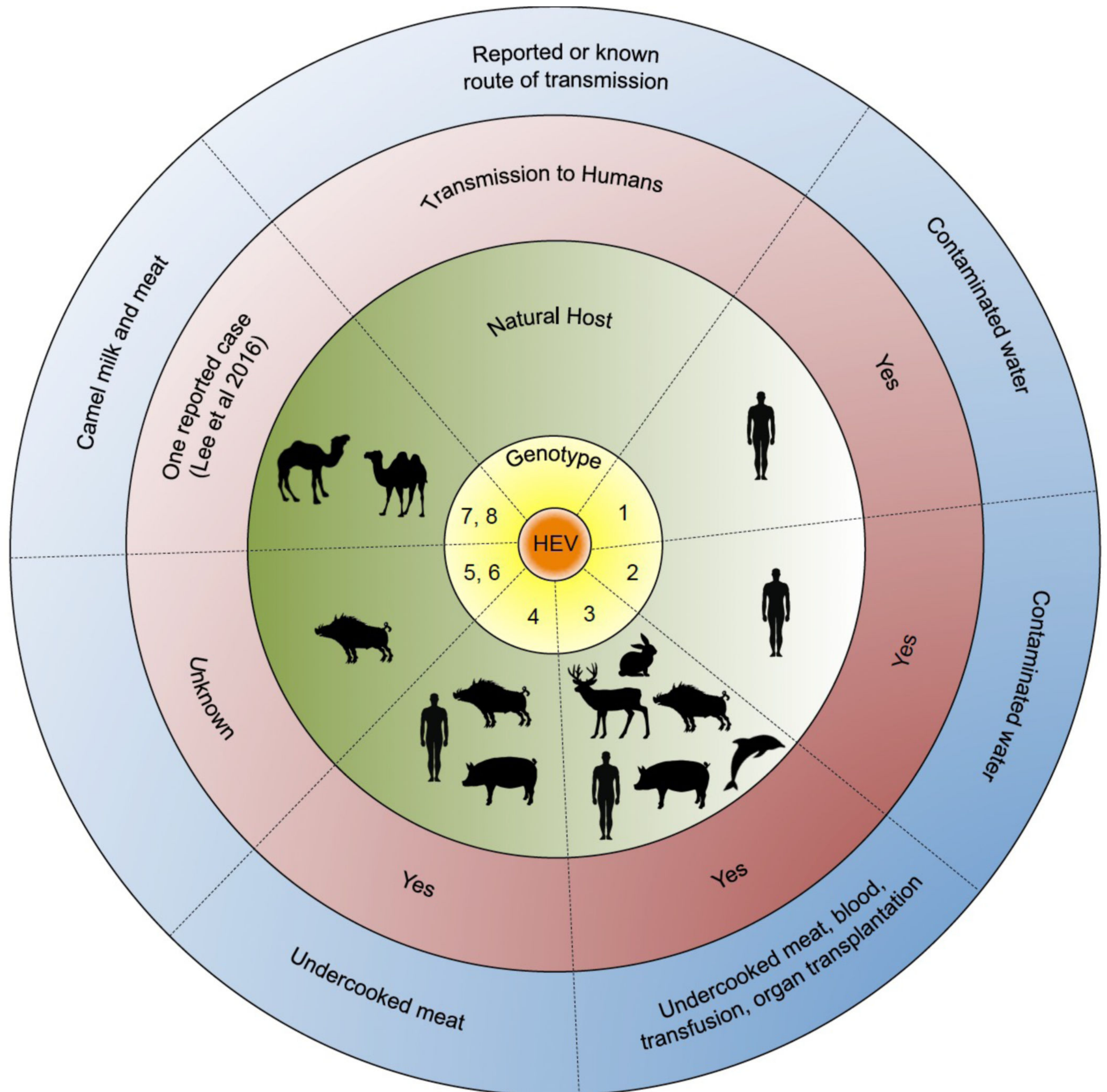

Fig 1. Host range of hepatitis E virus.

The *Orthohepevirus A* genus is classified into hepatitis E virus (HEV) genotype 1–8. Genotypes 1 and 2 are limited to human hosts and are transmitted via the faecal–oral route, primarily through contaminated water. Genotypes 3 and 4 have multiple hosts, and can be transmitted to humans through the consumption of undercooked meats, including pork. Genotypes 5 and 6 are known to infect wild boar; however, it is unknown whether these genotypes can be transmitted to humans (although there have been reports of wild boar genotype 3 HEV transmission to humans). Finally, genotypes 7 and 8 infect dromedary

and bactrian camels, respectively. There has been one case reported of genotype 7 HEV transmission to a liver transplant patient who consumed camel meat and milk.

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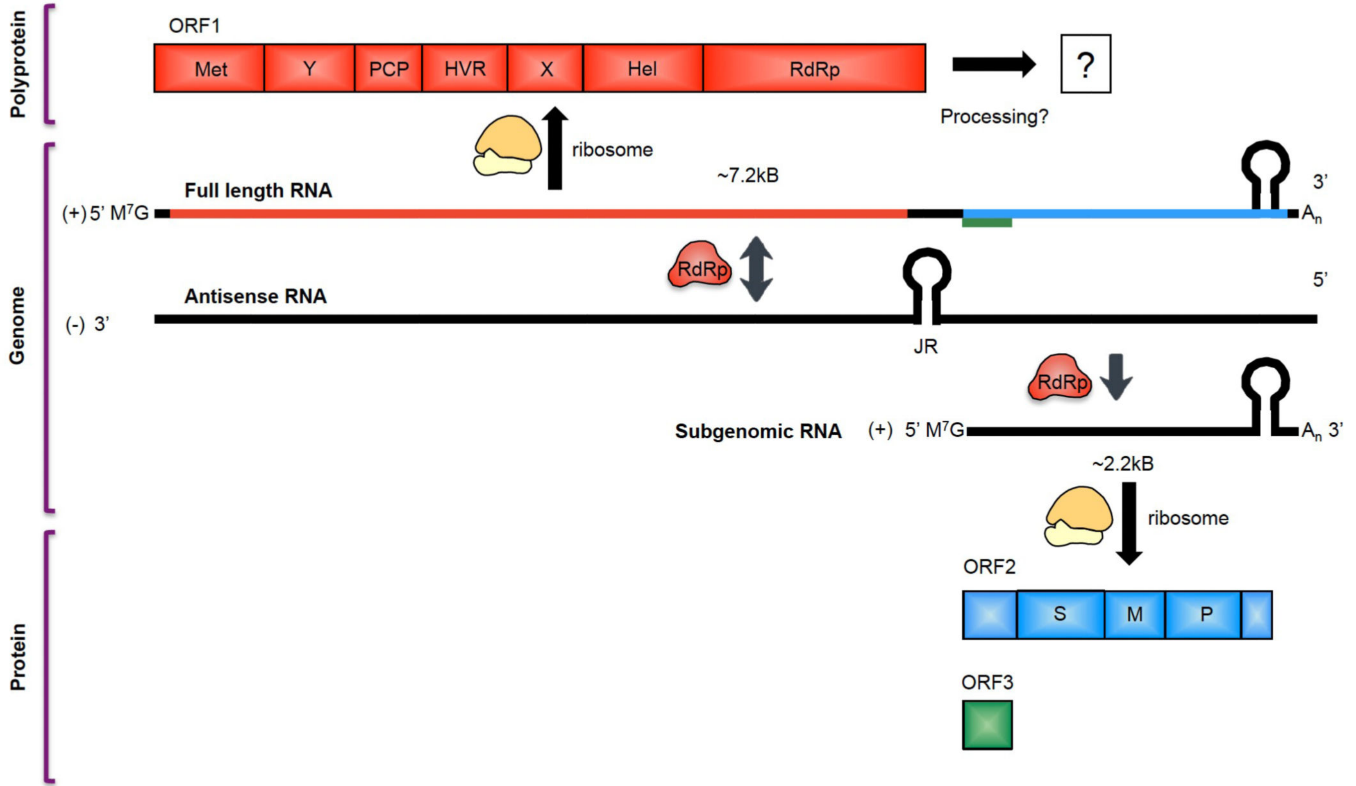

Fig 2. Genetic organization and translation of hepatitis E virus.

Hepatitis E virus (HEV) is a ~7.2kB, positive (+)-sense single-stranded RNA virus. The mRNA is capped at the 5' end, polyadenylated at the 3' end, and the junctional region (JR) between ORF1 and ORF2/3 contains a stem-loop structure that is critical for HEV replication. After viral entry and uncoating, the (+)-sense full-length viral genome is translated by host ribosomes to produce the ORF1 polyprotein, which contains the non-structural replication machinery of the virus including the methyltransferase (Met), RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp), as well as several non-enzymatic regions essential for efficient viral replication (the 'Y', 'X', and 'hypervariable' (HVR) regions). Additionally, ORF1 contains a putative papain-like cysteine protease (PCP) based on sequence similarity to the protease of rubella virus, though data showing protease activity for this region have been conflicting. It is unclear whether the ORF1 polyprotein undergoes processing into smaller units. HEV genotype 1 is thought to contain an additional open reading frame, ORF4, that is translated into a viral protein enhancing RdRp activity. After translation of the ORF1 polyprotein, the RdRp from ORF1 transcribes an antisense (–)-stranded intermediate RNA from the (+)-sense strand. The (–)-sense strand then serves as a template for the transcription of more (+)-sense full-length RNA for packaging into new progeny virions, as well as a shorter, ~2.2kB subgenomic RNA (sgRNA) encoding ORF2 and ORF3. These viral genes are ~2.2kB and ~360bp in length, respectively, and ORF3 entirely overlaps with ORF2 except for one leading base pair. The sgRNA, which is capped at the 5' end and polyadenylated at the 3' end, is then translated into the ORF2 capsid protein and the ORF3 viroporin based on a leaky scanning mechanism by host ribosomes. Regulation of transcription of the sgRNA is poorly understood.

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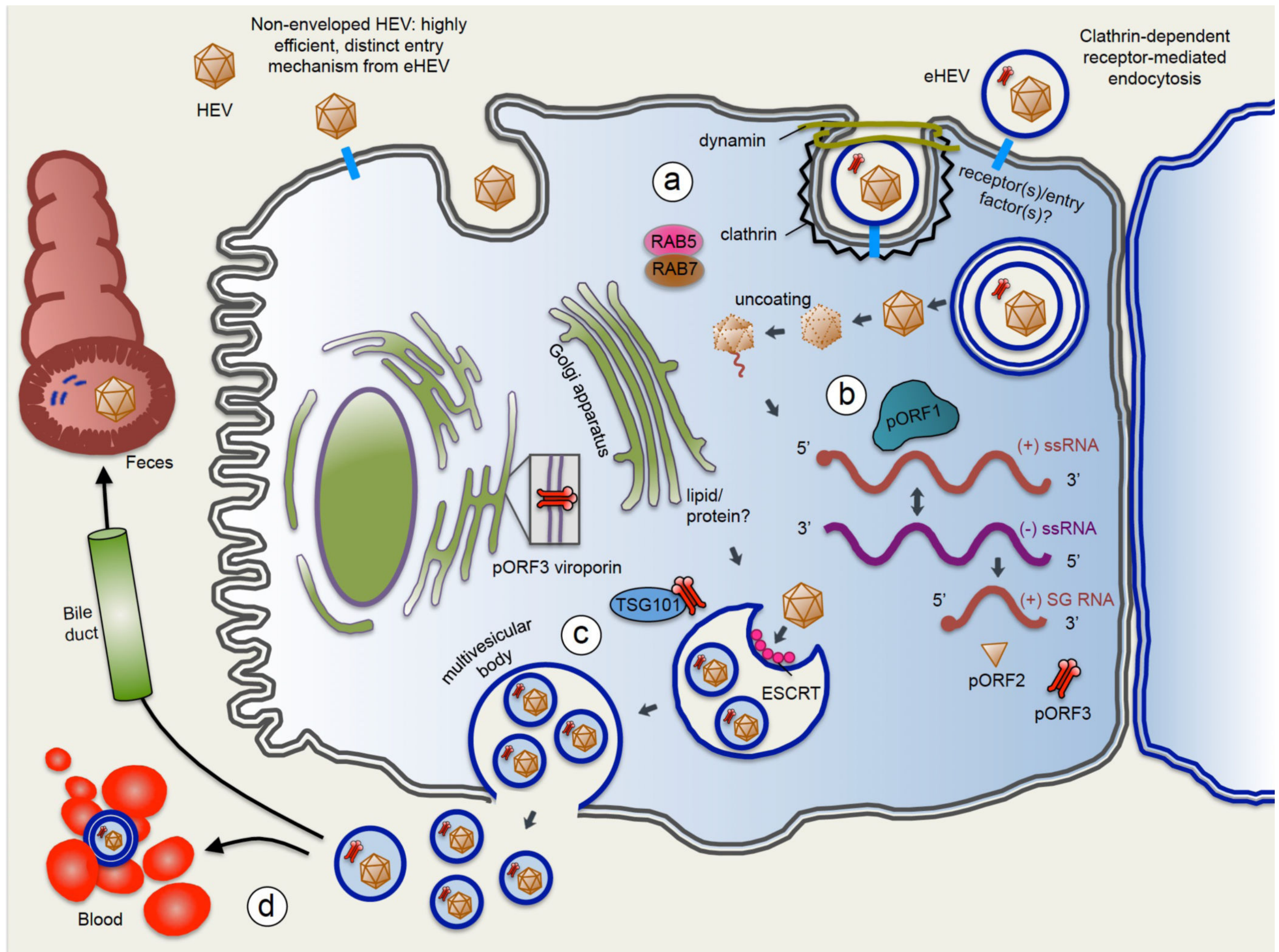

Fig 3. Life cycle of hepatitis E virus.

(1) Viral entry: hepatitis E virus (HEV) is a quasi-enveloped virus, meaning it can exist in the non-enveloped state (HEV) or can be coated in a lipid-derived membrane (eHEV). HEV and eHEV have distinct entry mechanisms. Little is known about entry mechanisms for HEV. For eHEV, the virus enters the cell through clathrin-dependent and dynamin-dependent, receptor-mediated endocytosis. A specific cell surface receptor mediating eHEV entry remains to be identified, but the GTPases Rab5 and Rab7 are known to have a role in eHEV entry. Upon entering the cell, the envelope of eHEV undergoes lysosome-mediated lipid degradation, and uncoats in a poorly understood process to expose the viral mRNA. (2) ORF1 polyprotein (pORF1) containing the RdRp is translated from the (+)-strand, and the RdRp then transcribes full-length (–)-sense RNA. The (–)-sense RNA serves as a template for transcribing more full-length (+)-sense RNA to be packaged into progeny virions, as well as a shorter subgenomic RNA (sgRNA) which encodes ORF2 and ORF3. The ORF2 capsid protein (pORF2), and the ORF3 protein (pORF3), a viroporin essential for viral release, are translated from the sgRNA. (3) pORF3 binds to TSG101, a member of the endosomal sorting complexes required for transport (ESCRT) pathway that is used by several other RNA viruses to bud from cell membranes. The interaction of pORF3 with TSG101 probably

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promotes budding of progeny virions into multivesicular bodies (MVBs), which then fuse with the plasma membrane to release virions from the cell. The lipid envelope of eHEV is thought to be derived from the trans-Golgi network, and viral particles contained in eHEV have been shown to be associated with pORF3. pORF3 has additionally been shown to exhibit viroporin activity, and it is possible that pORF3 exists in multiple forms to perform distinct functions. (4) eHEV released from the apical membrane enters the bile duct, where the lipid envelope is thought to be degraded by detergents and proteases in the bile. This feature would explain why HEV in the faeces is non-enveloped. On the other hand, eHEV released from the basal membrane of hepatocytes enters the serum in its quasi-enveloped form, where it is protected from neutralizing antibodies against pORF2 and pORF3, but is less efficient at infecting cells.

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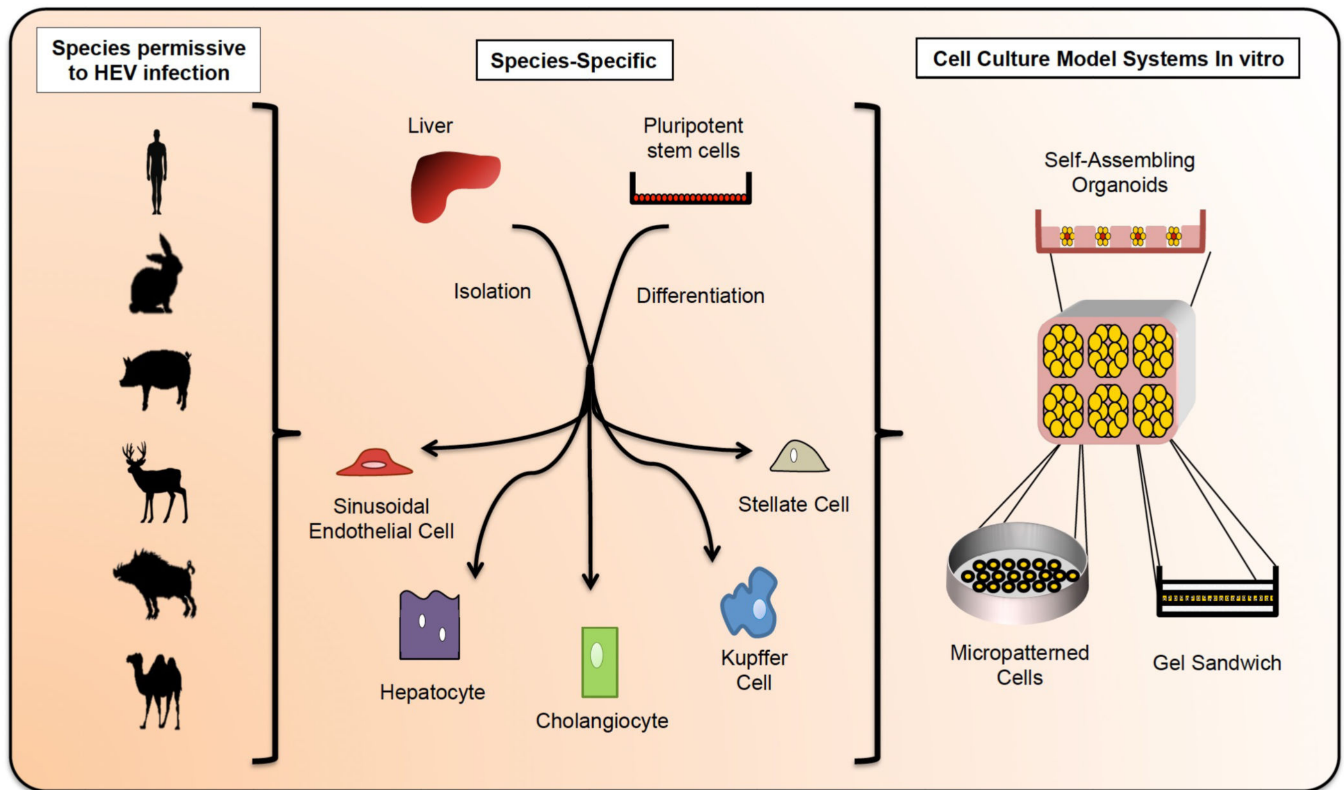

Fig 4. Towards more physiologically relevant 2D and 3D cell culture models for studying HEV.
The species permissive to HEV infection include (but are not limited to) humans, rabbits, swine, deer, wild boar, and camel. In order to better study HEV infection in physiologically relevant *in vitro* models, it will be desirable to generate co-cultures that recapitulate the complexity of the liver including endothelial, stellate, cholangiocytic, Kupffer, and hepatic cells in the appropriate ratios. These cells can be harvested from primary tissue or differentiated from stem cells, and could be derived from the aforementioned species to explore viral host tropism. Primary cultures have the disadvantage of limited durability; this issue can be overcome by differentiating the various cell types from indefinitely self-renewing stem cells. The latter, however, is technically challenging and requires advances in current hepatic differentiation protocols. Incorporating primary and stem-cell derived tissues into a 3D architecture will also be important to more closely mimic the physiological hepatic environment and preserve cell morphology. The architecture of the liver leads to heterogeneous environmental cues (e.g. nutrients, oxygen, inflammatory factors, etc) reaching individual cells, and 3D cultures can better capture this phenomenon. Furthermore it was previously shown that primary human hepatocyte dedifferentiation can be delayed or prevented in collagen sandwich cultures, by aggregation in spheroids, or in co-culture with non-parenchymal cells.

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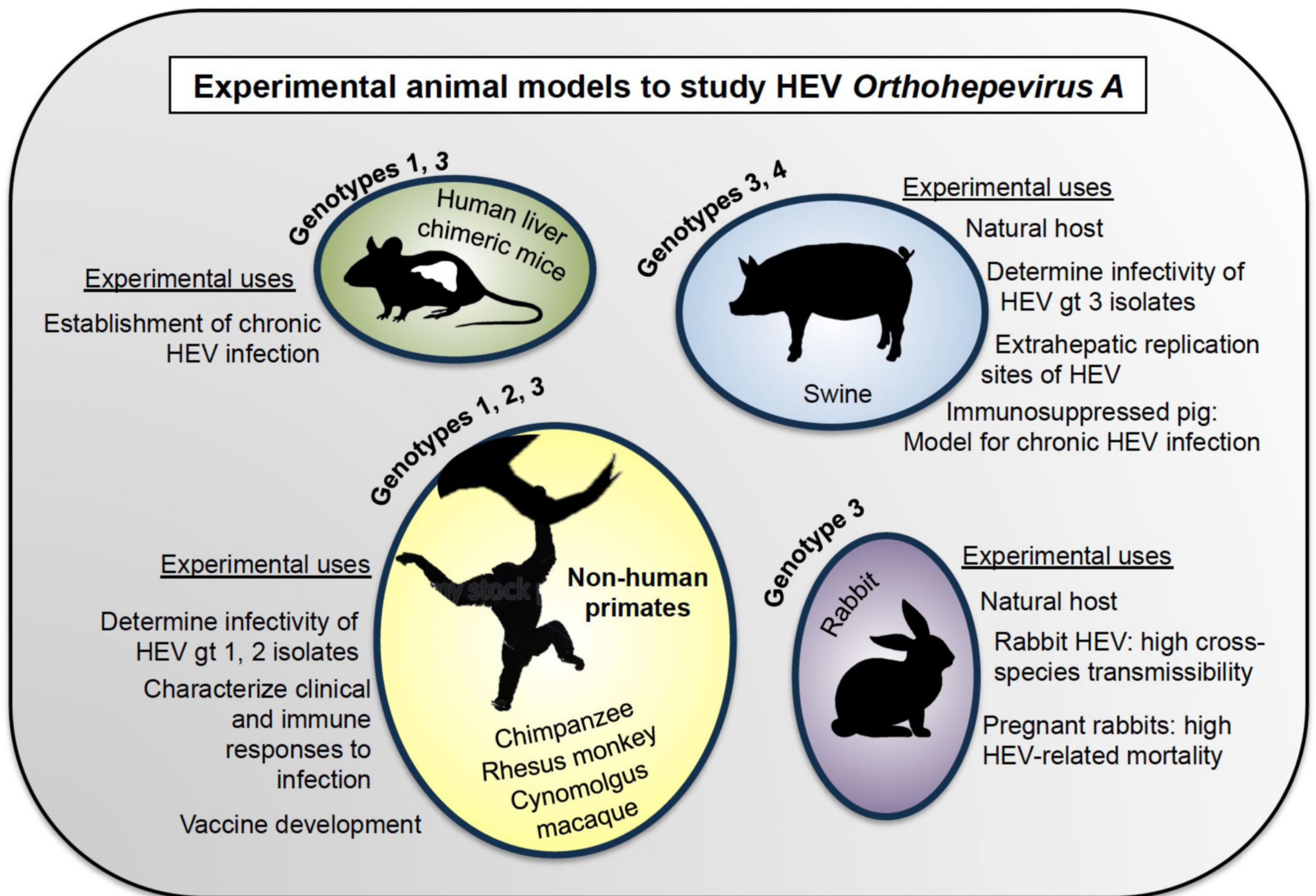

Fig 5. Experimental animal models to study HEV *Orthohepevirus A*.

Experimental animal models that have been used to study HEV include non-human primates, swine, rabbits, and human liver chimeric mice. Chimpanzees, rhesus monkeys, and Cynomolgus macaques were the earliest animal models in HEV research, and have been used to study HEV pathogenesis and vaccine efficacy. Swine, which are naturally infected with gts 3 and 4 of HEV and can transmit these strains to humans, have been used to determine the infectivity of gt 3 isolates, and to show extrahepatic replication sites of HEV. Recently, an iatrogenically immunosuppressed swine model was shown to support chronic infection with gt 3, and similarly, human liver chimeric mice can support chronic infection with gt1 and gt3 HEV. Gt, genotype.

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Table 1.

In vitro models to study HEV *Orthohepevirus A.*

Type	Cell line	Tissue	Species	HEVgenotype	Strainstested
Immortalized	LLC-PK1	Kidney epithelial	Swine	3	KernowC1/p6
	FRhK-4	Kidney epithelial	Rhesusmacaque	1	Sar55
	HepG2, HepG2/C3A	Liver hepatoma	Human	3	KernowC1/p6
	HepaRG	Liver hepatoma	Human	3	KernowC1/p6
	PLC/PRF/5	Liver hepatoma	Human	3,4	JE03-1760F, HEJF5/15F
	Huh7, Huh7.5, S10-3	Liver hepatoma	Human	1,3	Sar55, KernowC1/p6 (Note: S10-3 is a subclone of Huh7 cells selected for its ability to produce infectious Sar55 virus)
	A549	Lung adenocarcinoma	Human	3,4	JE03-1760F, HEJF5/15F
	Caco-2	Colon adenocarcinoma	Human	1	Sar55
Primary cells	Primary tissue	Liver	Hepatocyte-like	1	Hepatocyte-like
	iPSC- derived hepatocyte like cells	Hepatocyte-like	Human	3	KernowC1/p6
	Porcine embryonic stem cells	Embryonic	Porcine	3	Swine HEV genotype 3f

HEV, hepatitis E virus; iPSC, induced pluripotent stem cells.