FSA Project FS301014:

A critical review of approaches to assess the infectivity of hepatitis E virus

A report to the United Kingdom Food Standards Agency

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Lay Summary

Hepatitis E disease, caused by the hepatitis E virus (HEV), is an emerging issue within the UK, with a steady rise in the number of cases since 2011. HEV appears common in pigs (although infected pigs do not show symptoms), and evidence suggests that many hepatitis E cases may be associated with the consumption of undercooked pork meat and products. Little information is available on whether cooking can eliminate HEV, since there is currently no robust method for measuring its infectivity. To assist the FSA to identify future potential research, a critical review has been performed on published work on methods, e.g. animal models and cell culture, which have been used to detect infectious HEV.

HEV infectivity can be clearly demonstrated by monitoring for signs of infection in an animal model. However this approach has several disadvantages, such as lack of reproducibility and unsuitability for performing large numbers of tests, and not least ethical considerations.

Growth in cell culture can unambiguously show that a virus is infectious and has the potential for replication, without the disadvantages of using animals. Large numbers of tests can also be performed, which can make the results more amenable to statistical interpretation. However, no HEV cell culture system has been standardised, and few studies have shown that any are useful for measurement of HEV infectivity in food samples.

The main recommendation of this report is that a cell culture-based method for assessing HEV infectivity in pork products should be developed. Systems comprising promising cell lines and HEV strains which can grow well in cell culture should be tested to select an assay for effective and reliable measurement of HEV infectivity over a wide range of virus concentrations. The assay should then be harnessed to a procedure which can extract HEV from pork products, to produce a method suitable for further use. The method can then be used to determine the effect of heat or other elimination processes on HEV in pork meat and products, or to assess whether HEV detected in any surveyed foodstuffs is infectious and therefore poses a risk to public health.

Executive Summary

Hepatitis E, caused by the hepatitis E virus (HEV), is an emerging issue within the UK, with the numbers of confirmed non-travel related hepatitis E cases increasing significantly since 2010. HEV infection appears common in pigs, and epidemiological evidence suggests that many hepatitis E cases may be associated with the consumption of undercooked pork meat and products. Limited information is available regarding the survival or elimination of the virus in food production and / or preparation settings, or the effect of cooking procedures on HEV and as yet there is no robust system for evaluating these aspects, as validated and standardised methods to assess the infectivity of the virus are not available.

There is an urgent need to develop an HEV infectivity assay which would allow the determination of whether HEV detected in foods is infectious or not. To assist the FSA to identify future potential research towards this end, a critical review has been performed on published work on methods which have been used to detect infectious HEV. These methods can be grouped into 3 broad categories: animal models, cell culture, and alternative assays.

The significant advantage of using an animal model is that HEV infectivity can be unequivocally demonstrated by monitoring for signs of infection such as clinical disease, faecal shedding of the virus, viremia or seroconversion of specific antibodies. Pigs and monkeys have been the animal models most often used for study of the human pathogenic HEV genotypes 1-4. Intravenous inoculation of HEV into these models mostly resulted in successful infection, whereas the few reported oral infection studies mostly failed. The distinct virus concentrations needed for successful infection have not been determined. To date, only pigs have been used in studies assessing the infectivity of food preparations. Although rats and mice seem to be less susceptible to HEV gt1-4 infection, gerbils, shrews and especially rabbits may be candidates of small animal models for HEV infectivity assessment; however, the number of studies performed so far is too low to enable substantiated conclusions.

A number of *in vivo* studies have been performed with potential surrogate viruses such as avian HEV, rabbit HEV, rat HEV and ferret HEV. In each of the studies, the ability to generate an infection model has been confirmed in principle. It is not clear, however, how closely these potential viruses would correspond to human pathogenic strains, e.g. in their response to elimination procedures. There are several other disadvantages in using animals to routinely monitor for infectious HEV, and which would preclude using animal models to

rigorously determine whether an inactivation treatment is effective. There are issues with repeatability, and the time taken to conduct experiments can be lengthy. The potential for extensive replication is very low, especially when testing several specific parameters, which will limit the statistical significance of any findings. Finally, use of animals in research carries expense and ethical implications which are likely to outweigh the value of information required.

Many reports are available that describe successful propagation of HEV (including animal HEV strains which could be used as surrogates for human HEV) in cell culture. The cell lines PLC/PRF/5, A549 and HepG2/C3A are most often used. The appearance of a readily visible cytopathic effect is described in some of the early studies, but in later reports, a CPE was rarely observed during HEV replication and the virus growth was mainly detected by amplification of viral nucleic acid or detection of viral antigens. Most of the reports show only limited data on repeatability. Validation studies on HEV cell culture systems are distinctly lacking. To date, only two studies have reported the successful isolation of HEV from food. However, cell culture can unambiguously demonstrate infectivity, and avoids the disadvantages of using animals. Most importantly, the use of replicates and the simultaneous analysis of a higher number of samples can result in a higher degree of confidence of the results. Also, standardisation of cell culture methods should be easier than that of animal experiments. If a CPE can be observed visually, the assay should be relatively simple to perform. If no CPE can be observed, HEV replication in the cells can be monitored by amplification of viral nucleic acid or detection of viral antigens. Generally, most cell culture assays described for HEV are still time-consuming and require further optimization. Although some cell lines (e.g. PLC/PRF/5 and A549) have been used successfully in several independent studies, none has been validated as repeatable and reproducible by interlaboratory trial and the suitability for infection with different virus concentrations has mostly not investigated.

Alternative methods for estimating viral infectivity, such as the capsid integrity assay, or receptor binding assay, are options and could in principle be easier to use and faster to perform as compared to infectivity assays. Such methods have been proposed for evaluating infectivity of other enteric viruses, but currently only one study utilizing a capsid integrity assay has been published for HEV, and the assay has not been validated against actual infectivity assays such as animal models or cell culture.

Summarizing the findings, no method has been shown to be fully effective (i.e. reproducible and validated) for measuring infectivity of HEV in foods, and despite the thorough review of methodologies presented above, it is not possible to definitively recommend any system which can be used currently for this purpose.

It is recommended that for urgent analyses of selected sample types, while a more useful method is under development, the pig inoculation model may be used. This model has been shown to be suitable for food sample analysis, although the limitations of this approach must be recognised.

It is recommended that the development of the use of surrogate viruses or alternative assays should be postponed until an efficient assay for human-pathogenic HEV is available, so that both approaches can be validated against infectivity assays with human HEV.

The main recommendation of this report is that a cell culture-based method for assessing HEV infectivity in pork products should be developed and validated. Systems comprising promising cell lines and cell-culture adapted HEV strains, with detection of viral antigens during infection, should be evaluated in an interlaboratory trial, to select a system which can repeateably and reproducibly allow a quantitative determination of infectious HEV units in a reasonable time. The resulting assay should be harnessed to an efficient sample treatment procedure which can deliver suspended virus particles free of food-derived substances. The developed method (sample treatment + cell culture-based infectivity assay) should be validated as repeatable and reproducible by interlaboratory trial. The final method, demonstrated as robust and reliable, can then be used in studies to determine the effect of heat or other elimination processes on HEV in pork meat and products, or to assess the infectivity of HEV detected in surveyed foodstuffs.

Keywords: Hepatitis E virus, infectivity, detection, method

Glossary

Antigenicity. The capacity to stimulate the production of antibodies or the capacity to react with an antibody.

Capsid. The protein shell of a virus.

Ct value (**Cycle threshold value)**. The relative concentration of a PCR product based on the fluorescence intensity reaching an arbitrarily chosen threshold level during real-time PCR

Cytopathic effect. Morphological changes in cells caused by viral infection.

Elimination. Removal of infectious virus by a deliberately applied procedure.

ELISA. Enzyme-linked immunosorbent assay.

Fomite. Any object or substance capable of carrying infectious organisms, and hence transferring them from one individual to another.

GC (Genome copies). The number of complete virus nucleic acid sequences.

GE (Genome equivalents). The approximated copy number of the RT-(q)PCR target sequence based on standards or the dilution of known positive samples to extinction.

Hepatocyte. The main function cell type in the liver.

IFA (Immunofluorescence assay). A staining technique for virus-infected cells in cell culture.

Inactivation. The decline in virus infectivity effected by a deliberately applied procedure or through natural processes.

Infectivity. The ability of a pathogen to establish an infection in a host organism or cell.

Intragastric inoculation. Administration of a substance directly into the stomach.

Intraperitoneal inoculation: Administration of a substance into the body cavity (peritoneum).

Intravenous inoculation. Administration of a substance into a vein.

Level 3 biohazard. Aa biological agent that can cause severe to fatal disease in humans, but for which vaccines or other treatments exist.

ORF: Open reading frame. The part of a gene that contains no stop codons.

PFU (Plaque-forming units). Number of infectious virus units which can be quantified by the formation of discrete zones of infection on cell culture monolayers.

RT-PCR (**Reverse transcription polymerase chain reaction).** A molecular technique to amplify and subsequently detect the amount of viral or other RNA in a sample.

RT-qPCR (Reverse transcription quantitative polymerase chain reaction). RT-PCR used to estimate the number of original target RNA copies in the reaction, based on calibration using known standards.

Seroconversion. When a specific antibody becomes detectable in the blood.

Specific pathogen free (SPF) animals. Laboratory animals that are guaranteed free of particular pathogens.

Stability. Persistence of the intact virus capsid.

Survival. The persistence of virus infectivity after exposure to natural or man-made conditions.

$TCID_{50}$ (Tissue culture infectious dose$_{50}$). The dilution of a virus suspension which can infect 50% of cultured cell sheets exposed to it (analogous to a most probable number test in bacteriology). $TCID_{50}$ can be related to infectious virus units.

Viremia. The presence of a virus in the blood.

1. Introduction

1.1 Background to the report

Human enteric viruses are known to be a major cause of infectious intestinal disease in the UK, with foodborne transmission an important route of infection. The Food Standards Agency (FSA) funded second study of infectious intestinal disease in the community (referred to as the IID2 Study) provides extensive information on the enteric virus-associated burden of infection (*http://www.foodbase.org.uk/results.php?f_report_id=711*).

Although hepatitis E virus (HEV) can be considered as an emerging enteric viral pathogen, it is not specifically targeted by food safety management systems. Little information is available regarding the survival or elimination of the virus in food production or preparation settings (Cook and van der Poel, 2015), and as yet there is no robust system for evaluating these aspects, as validated and standardised methods to assess the infectivity of HEV are not available.

1.2 HEV

HEV is a small, non-enveloped, single-stranded, positive-sense RNA virus, 35 nm in diameter. The genome of approximately 7.2 Kb in length (Tam *et al.*, 1991) is capped at the 5′ end and polyadenylated at the 3′ end. It contains short stretches of untranslated regions (UTR) at both ends. The HEV genome has three open reading frames (ORFs). ORF1 encodes the non-structural polyprotein (nsp) that contains various functional units: methyltransferase (MeT), papain-like cysteine protease (PCP), RNA helicase (Hel) and RNA dependent RNA polymerase (RdRp) (Chandra *et al.*, 2008). ORF2 encodes the viral capsid protein, the N-terminal signal sequence and glycosylation loci. ORF3 encodes a small regulatory phosphoprotein. HEV was designated in 2004 as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson *et al.*, 2005). HEV is categorised and notifiable in the UK and several other countries as a level 3 biohazard. The mammalian HEV genome was first cloned from cDNA libraries prepared from the bile of macaques experimentally inoculated with stool suspensions from human patients (Reyes *et al.*, 1991). Later PCR amplification products were obtained from the genomes of multiple geographically distinct isolates of HEV (Purcell & Emerson, 2001).

A novel classification system groups HEV as a member of the family *Hepeviridae* into the genus Orthohepevirus (Smith *et al.*, 2014). This genus includes the species Orthohepevirus B which infects birds, Orthepevirus C which infects rodents, soricomorphs and carnivores and Orthohepevirus D which infects bats. The fourth species Orthohepevirus A comprises seven genotypes, out of these the genotypes (gt) 1 to 4 and genotype 7 have been shown to infect humans. Gt 1 viruses are predominantly isolated from outbreaks and sporadic cases in Asia and Africa, whereas gt 2 strains mainly have been observed in outbreaks in Mexico and Africa. Gts 3 and 4 are zoonotic and are observed in different animal species and sporadic human cases, worldwide for HEV gt 3 and mainly in Asia for HEV genotype 4. Gt 7 has been identified in camels and in a man regularly eating camel meat and drinking camel milk (Lee *et al.*, 2015).

Ingestion of HEV particles is assumed to be an important infection route for locally (as opposed to transfusion- or transplant-associated) acquired infections (Pavio & Mansuy, 2010). After entry by the oral route, the virus is passed through the intestinal tract, where it may replicate, although this has not been demonstrated to date. Via the portal vein, HEV can reach the liver (Panda *et al.*, 2007), which is believed to be the main HEV target organ. Hepatocytes most likely are the main replication cell type (Williams *et al.*, 2001). HEV attaches to host cells via specific high affinity receptors and enters the cytoplasm by clathrin-mediated endocytosis (Kapur *et al.*, 2012). After replication in the liver it is released into the bile and blood (Purcell and Emerson, 2001). On the basis of the manifestations and course of the disease it can be assumed that immune mechanisms are involved, and responsible for the processes leading to liver damage (Jameel, 1999). The dose response relationship of HEV is currently unknown.

The incubation period of hepatitis E ranges from 15-60 days. Virus particles can be found in the bile and faeces of infected persons during the late incubation phase, and subsequently for up to 2 weeks after the onset of clinical disease (Chauhan *et al.*, 1993). The clinical symptoms of the diseases in most cases are very similar to those reported during hepatitis A. During acute HEV genotype 1 infection, the case fatality rate is 0.5% to 4% (Chandra *et al.*, 2008). The most susceptible for infection with HEV genotype 1 are young adults and pregnant women and the case-fatality rate during pregnancy approaches 15 to 25% (Jin *et al.*, 2016). HEV genotype 3, which is most prevalent in Europe, mainly causes hepatitis in the middle-aged and elderly; additionally, men were found approximately 4-fold more

suscusceptible to symptomatic disease than women (Scobie and Dalton, 2013). Infection by HEV gt3 is mainly asymptomatic, with only around 33% of infected persons developing disease symptoms (Scobie and Dalton, 2013).

HEV is a main cause of epidemic hepatitis in developing countries and single cases of hepatitis in higher income countries. According to the World Health Organisation (http://www.who.int/mediacentre/factsheets/fs280/en/) worldwide there are 20 million hepatitis E infections, over three million acute cases of hepatitis E and 57,000 hepatitis E-related deaths every year. Ijaz *et al*. (2014) reported a total number of 2,713 acute cases of hepatitis E diagnosed between 2003 and 2012 in England and Wales, of which 51% were considered to be indigenous infections. There was a sharp increase in confirmed cases in these regions between 2010-2012 (274 in 2010, 456 in 2011 and 579 in 2012)) whilst travel associated hepatitis E remained relatively stable at between 100 and 200 cases per year during the same time period. Data on confirmed HE cases to 2016 is available at *https://www.gov.uk/government/publications/hepatitis-e-symptoms-transmission-prevention-treatment/hepatitis-e-symptoms-transmission-treatment-and-prevention*, but it is not clear what numbers are indigenous or travel-associated. The number of infections in England per year (as indicated by the presence of HEV-antibody via seroepidemiological studies) is estimated to be around 60,000 (Ijaz *et al*., 2014). Laboratory reports of HEV in Scotland have increased from 13 in 2011 to 183 in 2013 (Information from Health Protection Scotland http://www.hps.scot.nhs.uk/giz/wrdetail.aspx?id=67396&wrtype=9).

1.3 HEV in pigs and other animals

HEV can infect several animals including cats (Okamoto et al., 2004) cattle (Arankalle *et al*., 2001), wild boar (Schielke *et al*., 2009), deer (Tei *et al*., 2003) and camels (Lee *et al*., 2015) as determined by demonstration of HEV RNA or HEV-specific antibodies. Judging by antibody prevalence, HEV infection appears common in pigs (Meng *et al*., 1997b; Worm *et al*., 2002; Clemente-Casares *et al*., 2003), although symptoms are usually not apparent (van der Poel *et al*., 2001). HEV strains with very similar RNA sequences have been detected in pigs and humans (Banks *et al*., 2004; van der Poel *et al*., 2001), which prompts concern over the extent of zoonotic transmission of the virus through consumption of contaminated pork products.

1.4 Prevalence of HEV in pork products and other food matrices

HEV has been detected in pork products (liver, sausages) sold at retail, in several countries including the UK (Berto *et al*., 2012, Di Bartolo *et al*., 2012). HEV RNA detection rates of 6.5% (Bouwknegt *et al*., 2007), 4.0% (Wenzel *et al*., 2011) and 6.0% (Di Bartolo *et al*., 2012) have been reported for commercially available pig liver. In pig liver sausages and raw sausages from Germany, HEV RNA detection rates between 20% and 22% have been describes (Szabo *et al*., 2015). Especially high detection rates of 57.1-58.3% have been reported for a local liver sausage from France called "Figatelli" (Colson *et al*., 2010; Martin-Latil *et al*., 2014). HEV RNA has also been detected in fresh produce items such as lettuce (Kokkinos *et al*., 2012) and raspberries (Maunula *et al*., 2013), and in shellfish (Iaconelli *et al*., 2015), however, with lower detection rates.

The tests used for HEV RNA detection are based on the reverse transcription-polymerase chain reaction (RT-PCR), targeting specific sequences of the HEV genome, for example the conserved region of the ORF3 locus (Jothikumar *et al*., 2006). Distinct protocols for the homogenization and virus extraction from pork products prior to RT-PCR analysis have been developed (Martinez-Martinez *et al*., 2011; Martin-Latil *et al*., 2014; Szabo *et al*., 2015). However, there is currently no standard method for detection of HEV in foods. Fera has made a proposal to the European Committee for Standardisation via the British Standards Institute, that the method used by Berto et al. (2012) and Di Bartolo et al. (2012) for detection of HEV in pork products be taken forward for international standardization.

1.5 Zoonotic transmission of HEV

Foodborne transmission of HEV was first demonstrated in clusters of Japanese patients after eating raw or undercooked meat from swine, wild boar or Sika deer (Tei *et al.*, 2003; Takahashi *et al.*, 2004). The genomic sequences of HEVs identified from the infected patients were identical to those recovered from the frozen leftover meat (Tei *et al.*, 2003; Yazaki *et al.*, 2003). Through either detection of HEV sequences and/or epidemiological study, more hepatitis E cases have been linked to the consumption of food products contaminated with the virus. This includes infection via locally produced meat products (Colson *et al.*, 2010) but also from game meat and processed pork (Legrand-Abravanel *et al.*, 2010). Eating raw or undercooked pork products has been identified as a higher risk factor of HEV infection (Colson *et al.*, 2010; Said *et al.*, 2014) with the possibility of several thousand people being infected each year (Hewitt *et al*., 2014). Bivalve molluscs are known transmitters of enteric

viruses and especially oysters are eaten raw worldwide; HEV has been detected in mussels, shellfish and other bivalves (Donia *et al*., 2012). More recently HEV sequences have been detected on soft fruits and vegetables, with irrigation water as the suspected contamination origin (Brassard *et al.*, 2012; Kokkinos *et al.*, 2012).

1.6 Inactivation of HEV

The identification of undercooked pork products as a risk factor for HEV infection raises the question of which cooking regimes will inactivate the virus. A limited number of studies have been performed on HEV inactivation. These indicate that the virus could remain infectious at temperatures used in some cooking regimes, although inactivation by heating at 71^{o}C for 20 min has been demonstrated (Barnaud *et al*., 2012), and that chlorine at concentrations between 0.4 and 11.2 mg/L could effect a 2-log reduction of HEV per min in clean and sewage-contaminated water (Girones *et al*., 2014). There are significant gaps in our knowledge regarding the survival of HEV in foods and the environment (including food contact surfaces), and also regarding the effect of elimination procedures used in food supply chain settings (Cook and van der Poel, 2015). The lack of a reliable infectivity assay has hampered extensive study. It has been recommended (Cook and van der Poel, 2015) that studies be undertaken to develop an efficient propagation system (based on *in vitro* cell culture), to facilitate the acquisition of extensive information on the survival of HEV in food and the environment, and its response to disinfection and elimination procedures.

1.7 Closely related viruses of the family *Hepeviridae*

There have been several viruses identified infecting different animal species, which are related to HEV and classified within the *Hepeviridae* family (Johne *et al*., 2014a). HEV strains closely related to human HEV genotype 3 strains have been repeatedly detected in rabbits (Zhao *et al*., 2009; Cossaboom *et al*., 2011). In one case, a strain very similar to the rabbit HEV strains was found in a human hepatitis E patient thus raising the question of the zoonotic potential of this virus (Izopet *et al*., 2012). However, all of the rabbit strains form a phylogenetic lineage separate from the human viruses indicating that frequent zoonotic transmission is rather unlikely. Other HEV-related viruses have been detected in rats (Johne *et al*., 2010) and ferrets (Raj *et al*., 2012) and some studies have been performed on these viruses in order to establish a small animal hepatitis E model. These viruses are only distantly related to human HEV strains and are grouped into a separate virus species *Orthohepevirus* C (Smith *et al*., 2014). Other viruses even more distantly related are the bat HEV (Drexler *et*

al., 2012) and the avian HEV (Payne *et al*., 1999). Whereas little is known about bat HEV, the avian HEV is a well-known pathogen in chickens worldwide leading to Big Liver and Spleen Disease (Handlinger and Williams, 1988) and hepatitis-splenomegaly syndrome (Ritchie and Riddell, 1991). In fish, the Cutthroat trout virus (CTV) has been shown to have some sequence similarities to HEV (Batts *et al*., 2011). This virus has been grouped into a separate genus because of the low genetic relationship to other hepeviruses. However, CTV can be efficiently propagated in cell cultures (Debing *et al*., 2013). Some of the HEV-related viruses may exhibit similar properties like human HEV and might therefore serve as surrogate viruses in *in vivo* and *in vitro* studies..

1.8 Aim of the study

A key aim of the FSA's Strategic Plan for 2015-2020 is the reduction of foodborne disease to ensure the food supply chain is safe. The FSA's Advisory Committee on the Microbiological Safety of Food (ACMSF) published an extensive review of viruses in the food chain which included HEV (ACMSF, 2015). The ACMSF report recommended further research on the heat inactivation of HEV in pork products, on the effect of curing and fermentation of HEV in pork products, and a survey of HEV contamination in pork products at retail. However, to facilitate this research, there is an urgent need to develop an HEV infectivity assay which would allow the determination of whether HEV detected in foods, both before and after the application of an elimination procedure, is infectious or not. Knowing whether the virus is infectious (and therefore likely to cause illness) will allow better interpretation of future surveillance and heat resistance studies on HEV to inform risk assessment and management. To assist the FSA with identifying suitable methods to allow this interpretation, and to facilitate recommendations on appropriate research and development of an effective method to determine HEV infectivity, particularly in foods, it is timely to review the current information on potentially applicable methodologies.

1.9 Methodology of the critical review

Briefly, the review was performed by performing a literature search using an agreed list of search terms, then screening references from the search to select publications for review. During the preparation of the review, a series of teleconferences was held between the participants to discuss progress. Information from a foodborne viruses workshop, held during the of the review, was also considered for incorporation. Details of how the review was performed are given in Annex 1.

2. Animal models for determining infectivity of HEV genotype 1-

Several types of animal species are susceptible to experimental infection with one or more HEV genotypes, and have been used as models to study the pathogenesis and pathology of hepatitis E disease (Krawczynski *et al*., 2011; Purcell *et al*., 2001; Yugo *et al*., 2014). Descriptions of some animal studies, which illustrate pertinent issues in the use of animals as infectivity models, are given below.

2.1 Gerbils

Mongolian gerbils can be intraperitoneally inoculated with HEV and subsequently show signs of liver damage (Yang *et al*., 2015) and kidney damage (Soomro *et al*., 2016). Li *et al*. (2009) observed faecal shedding of HEV (gt4) in 11 out of 12 gerbils inoculated intraperitoneally, after 14 days post inoculation (dpi). Seven animals were viremic (HEV RNA detected in sera) at 7 dpi, and all twelve animals were viremic at 14 dpi.

2.2 Mice

Balb/c nude mice have been shown to be susceptible to infection with swine HEV after oral or intravenous inoculation (Huang *et al*., 2009). Four animals were inoculated with ~1 x 10^5 genome copies (GC) HEV gt4 isolated from swine faeces, and faeces was collected daily for RT-PCR analysis. HEV was detected in the faeces of all four mice after 4 dpi. After 4, 7, 14 or 21 dpi, one mouse was euthanised and samples of serum and internal organs taken for analysis by RT-PCR and ELISA; HEV RNA was detected in samples taken from the mouse euthanised at 4 dpi, and increased anti-HEV IgG in the mouse euthanised at 14 dpi.

2.3 Pigs

Following the first identification and characterisation of a strain of HEV naturally infecting pigs (Meng *et al*., 1997b), the virus was shown to be capable of experimental infection of pigs. Meng *et al*. (1998) intravenously inoculated four 4-week old specific pathogen-free (SPF) pigs with serum from naturally infected pigs containing 10^3 GE HEV (genotype not identified). The pigs were monitored weekly for anti-HEV IgG in serum, and for HEV RNA in serum and faeces. Anti-HEV IgG was detected in all four pigs by 8 weeks post-inoculation (wpi). HEV viremia (detection of viral RNA in serum) was observed in the pigs between 3 and 6 weeks. Faecal shedding was observed in one pig at 2 wpi, in 2 pigs at 3 wpi, and in 1 pig at 6 wpi. Meng *et al*. (1998) also inoculated SPF pigs with faecal suspensions from

patients infected with 2 HEV strains (genotype not identified), but no signs of infection were observed in the pigs over 14 weeks of monitoring.

To mimic consumption of undercooked pork meat, Kasondorkbua *et al*. (2002) prepared homogenates of muscle tissue from HEV-infected pigs. The HEV genotype was not determined in this early study. Nine SPF pigs were inoculated with this homogenate orally by stomach tube. The muscle homogenate did not contain detectable HEV RNA however. Concurrently, another group of 9 pigs were inoculated with a purified suspension of virus containing 10^6 GE ml-1, by oral drop (3 pigs), stomach tube (3 pigs), and intravenously (3 pigs) (). Serum samples were taken weekly and analysed for anti-HEV IgG by ELISA and HEV RNA by RT-PCR. Neither HEV RNA nor seroconversion was detected in any pig inoculated with the muscle suspension, or by oral drop or stomach tube with the HEV suspension; all 3 pigs inoculated intravenously with the HEV suspension did seroconvert however. The authors concluded that HEV transmission via the faecal-oral route may require a higher viral dose than an intravenous route. In the same study, Kasorndorkbua *et al*. (2002) intravenously inoculated 6 pigs with homogenates of liver tissue from infected pigs, containing 10^2-10^4 GE HEV ml^{-1}. HEV RNA could could be detected in four of the pigs (by 14 dpi), and five pigs seroconverted (between 3 and 8 wpi).

Bouwknegt *et al*. (2007) used five 7-8 week old domestic pigs (*Sus scrofa domestica*) to determine whether commercially-sold livers in which HEV gt3 RNA was detected contained infectious virus. The pigs were inoculated intravenously (site not given) with 3-4.5 ml of liver homogenates containing 20 GE HEV, and subsequently examined for faecal shedding of the virus. Only in an animal given a high dose of HEV (2 ml of a liver suspension from a previously experimentally inoculated pig, which contained 10^4 GE ml-1) as a control, was shedding observed, at 7 dpi. It is implied in the paper that some or all or the pigs were killed at 21 dpi, and that their liver and bile were analysed for HEV by RT-PCR; no HEV RNA was detected. The authors considered that the number of infectious HEV in the inocula from the commercial livers may have been too low to infect the pigs, but stated that no procedure was available to increase the concentration without increasing the risk of infarction, or obstruction of blood vessels, in the animals.

A swine bioassay was used by Feagins *et al*. (2007) to detect infectious HEV in pig livers sold in grocery stores. The livers had been analysed by end-point PCR, so the HEV GE titer

was not determined. Homogenates of three livers which had tested positive for HEV gt3 RNA were intravenously inoculated into 4-week old pigs (5 per liver sample), which were then monitored for 8 weeks for faecal shedding, seroconversion, and viremia. HEV RNA was detected by 1 wpi in the faeces of all pigs inoculated with two of the liver samples; pigs inoculated with the third sample did not display faecal shedding. Seroconversion and viremia in the inoculated pigs followed the same sample pattern. . The authors suggested that either storage conditions in the grocery stores (e.g. repeated freeze-thaw) inactivated HEV in the third liver sample, or that the infectious virus titre was too low in this sample to initiate infection in the pigs.

Feagins *et al*. (2008a) subsequently used the swine bioassay to study inactivation of HEV gt3 in pig liver by heat. Of the five pigs inoculated with untreated liver homogenate, 4 showed HEV RNA in faeces and one did not. Four out of five pigs inoculated with liver homogenates treated at 56^{o}C for 1 hr developed an active HEV infection, whereas no signs of infection were detected in the pigs inoculated with homogenates of liver stir-fried at 191^{o}C for 5 min or boiled for 5 mins in water.

Feagins *et al*. (2008b) used a similar swine bioassay to demonstrate that an HEV gt4 strain isolate from a human patient could infect pigs. Five SPF pigs were intravenously inoculated with 10^3 monkey infectious dose (MID_{50}) HEV gt4, and faecal and serum samples collected weekly for 8 weeks. HEV RNA was detected in the faeces of 3 pigs at 1 wpi, but in none the following week. At 3 wpi all pigs displayed faecal shedding of the virus, but detection of HEV RNA in subsequent weekly samples was intermittent. Seroconversion was observed in one pig at 2 wpi, in another at 3 wpi, and the rest by 4 wpi.

In a study (Casas *et al*., 2009) to investigate a contact route of exposure of HEV between pigs, 16 three-week old piglets (species not given) were inoculated orally with a 1:10 suspension of HEV gt3 -containing pig bile: physiological saline, administered by syringe. The suspension contained 10^5 GE HEV. Faecal and serum samples were taken twice a week to be analysed by RT-PCR (serum samples were also monitored for anti-HEV IgG by ELISA). Only 4 pigs showed evidence of HEV infection. Faecal shedding was observed in 3 pigs by 22-25 days (one pig died of a bacterial infection after 16 days), but IgG was observable in all 4 pigs. The authors considered that the HEV dose was not sufficient to infect all of the inoculated pigs, at least in a single dose.

Relatively early (at 3 dpi) faecal shedding was observed by Lee *et al*. (2009) in 18/18 pigs intravenously inoculated with HEV gt3. The inoculum was prepared as a 10% suspension of faeces from a previously infected pig which had not received any maternal antibodies; it may be possible that the early shedding in the inoculated pigs was due to a high titre of HEV in the inoculum, although the HEV RNA titre was not stated in this study.

Feagins *et al*. (2011) constructed chimeric viruses containing HEV gt1 and gt3 RNA, gt1 and gt4 RNA, and gt3 and gt4 RNA, by transfecting Huh7 cells with recombinant RNA clones. Cell-free lysates of infected cultures were intravenously inoculated into pigs, and faecal swabs and serum samples taken weekly. Only gt3/gt4 chimeras produced signs of infection, with pigs displaying faecal shedding between 1 and 2 wpi.

Barnaud *et al*. (2012) used SPF pigs to study thermal inactivation of HEV gt3 in infected pig liver. The age or strain of the pigs was not given. The pigs were inoculated intravenously in the ear with a liver homogenate, and subsequently examined for signs of HEV infection, i.e. faecal shedding and seroconversion. Seroconversion of pigs inoculated with homogenates of infected liver was not observed until at least 14 dpi. The earliest faecal shedding observed was 7 dpi. Pigs inoculated with liver homogenates which had been subject to different time-temperature combinations (three pigs for each combination) were housed in different pens, except in one case where pigs inoculated with viral suspensions from liver homogenate treated at a higher temperature were kept in the same pen as animals inoculated with viral suspensions from liver homogenate treated at a lower temperature. The authors stated that this was due to practical reasons, but admitted that it could have resulted in cross-exposure of the pigs.

Wild boar (*Sus scrofa domestica*) can also be experimentally infected with HEV. Schlosser *et al*. (2014) extracted HEV gt3 from the liver of a naturally infected wild boar, and intravenously inoculated 2 ml of the suspension (containing ~4 x 10^7 GC HEV) into 4 experimental animals. Blood and faecal samples were taken from the boars every 3 – 4 dpi. HEV RNA was detected in the faeces of all 4 boars by 5 dpi. Only 2 animals seroconverted, by 17 dpi.

2.4 Rabbits

Ma *et al*. (2010) intravenously inoculated 7-week old rabbits (species not stated) with HEV strains obtained from stools of infected human patients, and monitored the animals for signs of infection. Nine rabbits were inoculated with HEV gt1 (1.1×10^6 GE ml^{-1}) and a further nine with HEV gt4 (1.1×10^7 GE ml^{-1}), and monitored weekly for 14 weeks by analysis of serum for anti-HEV IgG, and analysis of faeces for HEV RNA. Seroconversion was seen in one rabbit inoculated with HEV gt4 by 5 wpi and in one rabbit inoculated with HEV gt1 by 6 wpi. By 14 wpi the number of seropositive rabbits had increased in each group but not all rabbits had seroconverted. No rabbit inoculated with HEV gt1 shed the virus in faeces at any time point. HEV gt4 RNA was detected in the faeces of 2 rabbits at 2 wpi, and one rabbit was intermittently positive up to 12 wpi.

Cheng *et al*. (2012) inoculated 4-5 month old European rabbits (*Oryctolagus cuniculus*) with dilute stool suspensions containing, separately, HEV gt1 (1 strain), HEV gt3 (1 strain) and HEV gt4 (3 strains). Five rabbits were intravenously inoculated in each test. The inocula contained between 3×10^3 and 6×10^5 GE. The animals were monitored weekly for 10 weeks for faecal shedding and seroconversion. No faecal shedding of HEV or seroconversion was observed in rabbits inoculated with gt1. Rabbits inoculated with gt3 were positive for anti-HEV IgG by the end of the experiment, but none displayed faecal shedding of the virus. In all 5 rabbits inoculated with one of the gt4 strains, faecal shedding was observed at 1 wpi and continued for the duration of the study, and all were positive for anti-HEV IgG by 10 wpi; however, with the other two gt4 strains, faecal shedding was only transiently observed in one rabbit, and only some of the rabbits seroconverted. Subsequently rabbits were orally inoculated with a low (3.3×10^3 GE), medium (3.3×10^4 GE) or high (3.3×10^5 GE) dose of the HEV gt4 strain that had productively infected all 5 rabbits tested with it previously. Only rabbits orally inoculated with the high dose displayed faecal shedding and seroconversion (both beginning at 1 wpi).

2.5 Rats

An early report (Maneerat *et al*., 1996) indicated that human HEV can be transmitted to rats. Three rats inoculated with HEV (genotype not determined) derived from faeces of an infected patient displayed histopathological changes in various organs, and faecal shedding of the virus (at 7 dpi). However, Li *et al*. (2013) observed no signs of infection in rats inoculated with HEV gt1, gt3 or gt4.

2.6 Simians

Various simian species have been shown to be able to be infected with HEV.

Common chimpanzee (*Pan troglodytes*)

McCaustland *et al*. (2000) summarised some previous studies of HEV infection using chimpanzees. Animals had been inoculated intravenously and intragastrically with faecal suspensions obtained from infected human patients (no genotype information available) and monitored for signs of infection including seroconversion, viremia and faecal shedding. All animals tested displayed one or more signs of infection, the onset varying depending on the HEV strain with which the chimpanzees had been inoculated. Bi *et al*. (1998) also reported observation of seroconversion of chimpanzees 1 month after inoculation (mpi) with HEV (serotype not identified).

Cynomolgus monkeys (*Macaca fascicularis*)

Cynomolgus monkeys (*Macaca fascicularis*) can be challenged with HEV and show signs of infection. Erker *et al*. (1999) intravenously inoculated 3 monkeys with serum from a patient infected with an HEV strain (genotype not identified at that time but similar to strains which had been identified in pigs). Faecal shedding and viremia were observed in one animal, given 2 ml serum, at 15 days; animals inoculated with 0.4 – 0.625 ml did not display any signs of infection. Li *et al*. (2004) intravenously inoculated a 4-year old monkey with a HEV gt1 strain isolated from a human patient with hepatitis, and could detect HEV RNA 10 days post-inoculation.

de Carvalho *et al*. (2013) infected cynomolgus monkeys with HEV gt3 strains from naturally and experimentally infected pigs, and from two clinically infected humans. The pig strains were inoculated as faecal suspensions, and the strains isolated from humans inoculated as serum suspensions or pooled serum and faeces. Inoculation was intravenous, but the site was not stated. The animals were monitored for 67 dpi, with blood, saliva and liver biopsy samples taken weekly, and faecal samples taken more frequently. RT-PCR was used to monitor for HEV RNA in faeces, serum, and saliva, and ELISA was used to monitor for presence of anti-HEV antibodies (IgA, IgG and IgM). All animals showed signs of infection with HEV but the pattern of signs was not consistent in each animal, with some animals showing no viremia, some not displaying faecal shedding, and others not showing evidence of all any antibody types.

HEV gt4 could infect a cynomolgus monkey when faecal extracts from a viraemic human patient were intravenously inoculated (Geng *et al*., 2016). HEV RNA could be detected in the faeces and urine of the animal at 10 dpi, persisting until 25 dpi.

Rhesus macaque monkey (*Macaca mulatta*)

Pina *et al*. (1998) used Rhesus macaques to determine whether HEV detected in sewage was infectious. Two animals were inoculated intravenously with 25 ml sewage over a 5 day period (5 ml per day); the HEV (genotype not determined) titre of the sewage was not determined. Both animals began to shed the virus in faeces between 1 and 2 wpi (whether this was following the first or the last day of inoculation was not stated).

Arankalle *et al*. (2006) examined the susceptibility of Rhesus macaques to infection by an HEV gt4 strain obtained from a previously infected pig, by monitoring anti-HEV IgG in serum, and HEV RNA in blood and faeces, of two animals inoculated intravenously with a faecal suspension from an HEV-positive pig. The animals were monitored up to 65 dpi. Rising anti-HEV IgG levels could be detected in each macaque after 18 dpi, and HEV RNA could be detected in the blood of each animal from the third to the seventh week after inoculation. Detection of HEV RNA in one animal began at 5 dpi, but was only intermittent after that, and no HEV RNA could be detected in the faeces of the second animal.

2.7 Tree shrew

Yu *et al*. (2016) intravenously inoculated 5 tree shrews with gt4 HEV, and observed HEV RNA in the faeces of three animals by 3 dpi and all five by 5 dpi. Viremia was also observed in the infected tree shrews at 7 dpi.

2.8 Summary

Table 2.1 summarises the information from the studies reviewed above. It is evident from the literature review, that pigs and monkeys are the animal models most often used. Intravenous inoculation of HEV into them mostly resulted in successful infection, whereas the few oral infection studies mostly failed. Only pigs were used in studies assessing the infectivity of food preparations. Rats and mice seem to be less susceptible to HEV gt1-4 infection. Gerbils, shrews and especially rabbits may be candidates of small animal models for HEV infectivity assessment, but the number of studies performed so far is too low to enable substantiated

conclusions, and no studies using foods have been performed. Generally, the virus concentration needed for infection of the animals has not been specified. In most of the studies, only the amount of HEV RNA was determined, which does not necessarily correlate with the amount of infectious virus present in the inoculum. Therefore, the limits of detection of infectious HEV still need to be established for the different animal models.

Table 2.1. Summarised information from studies involving animal models for HEV genotypes 1-4

Animal species	HEV genotypes tested	Route of inoculation	Evidence of infection	Parameter measured	Used to test HEV infectivity in food	Used to test inacti-vation of HEV
Gerbils	gt4	Intraperitoneal	Faecal shedding, viremia	Viral RNA	No	No
Mice	gt4	Intravenous, oral	Faecal shedding, seroconversion viremia	Viral RNA, anti-HEV IgG	No	No
Pigs	gt3, gt4	Intravenous, oral	Faecal shedding, seroconversion viremia	Viral RNA, anti-HEV IgG	Yes	Yes
Rabbits	gt1, gt3, gt4	Intravenous	Faecal shedding, seroconversion	Viral RNA, anti-HEV IgG	No	No
Rats	"Human HEV", gt1, gt3, gt4	Intravenous	Faecal shedding	Viral RNA	No	No
Simians	gt1, gt3, gt4	Intravenous	Faecal shedding, seroconversion viremia	Viral RNA, anti-HEV IgG	No	No
Tree Shrew	gt4	Intravenous	Faecal shedding, viremia	Viral RNA	No	No

2.9 Advantages and disadvantages of animal models

The significant advantage of using an animal model is that HEV infectivity can be unequivocally demonstrated by monitoring for signs of infection such as clinical disease,

faecal shedding of the virus, viremia or seroconversion of specific antibodies. The pig model has also been demonstrated to be suitable for assessment of HEV infectivity in food samples, albeit in a limited number of studies. Therefore, the pig model can be used immediately for investigation of a low number of specific food samples without the need for extensive method development.

However, one disadvantage of the pig model is that infection is observed indirectly: no visual symptoms are apparent in infected animals, and relatively complex methods e.g. RT-PCR / ELISA must be used to detect the presence of HEV or antibodies against the virus. There are several other disadvantages in using animals to routinely monitor for infectious HEV, and which would preclude using animal models to rigorously determine whether an inactivation treatment is effective. There are issues with repeatability, in that not all individual animals inoculated with an HEV suspension have shown signs of infection. High doses may be necessary to produce infection, which may limit the level of reduction of infectivity which can be detected. The time taken for the signs of infection to manifest can be lengthy, taking several days or weeks, and may not be the same for each individual animal inoculated. The potential for extensive replication is very low, especially when testing several specific parameters, as animals require housing and care. This will limit the statistical significance of the findings. Finally, use of animals in research carries ethical implications which are likely to outweigh the value of information required. The advantages and disadvantages of animal models to assess HEV infectivity are summarised in Table 2.2.

Table 2.2. The advantages and disadvantages of animal models to assess HEV infectivity

Advantages	**Disadvantages**
Can demonstrate actual HEV infectivity Pig model can immediately be used for HEV analysis of food samples	Reproducubility has not been investigated systematically Time-consuming Low potential for replication Low statistical significance Ethical approval required

3. Animal models for potential surrogate viruses

A variety of animal species can be infected by specific HEV strains (Johne *et al*., 2014a), and these strain / host combinations have the possibility to be used as models for human / zoonotic HEV. The studies reviewed below have been grouped by viral surrogate.

3.1 Avian HEV

Avian HEV was first isolated from chickens with hepatitis–splenomegaly syndrome (Haqshenas *et al*., 2001). Sun *et al*. (2004) obtained an infectious stock of avian HEV by inoculating 1 week-old SPF chickens with serum from birds with hepatitis–splenomegaly syndrome; at 28 dpi an HEV-positive faecal sample was collected which was used to make the stock. Dilutions of this stock were inoculated into 1 week-old SPF chickens (2 birds per dilution). All chickens inoculated with low dilutions (10^{-2} – 10^{-4}) seroconverted and displayed faecal shedding by 2 wpi. The HEV in the stock suspension was not quantified however.

Billam *et al*. (2005) examined the potential for the virus to infect adult chickens by the faecal-oral route. They inoculated four 60-week old chickens both nasally and orally with an infectious stock of avian HEV and monitored the birds over 10 wpi for various signs of infection including faecal shedding. One bird displayed faecal shedding by 1 wpi, and all four birds by 3 wpi. One bird became viraemic by 1 wpi, but not more than 2/4 birds were viraemic at any one time during the course of the study.

Guo *et al*. (2007b) intravenously inoculated twenty 15-week old specific pathogen free (SPF) chickens with 1 x 10^4 GE avian HEV in a 10% suspension of chicken faeces, and monitored them weekly for HEV RNA in serum and faeces. At 1 wpi, all 20 birds had developed viremia, but only 14 displayed faecal shedding of HEV. At 2 wpi, 16 out of 16 birds (four had been sacrificed for necropsy) had viremia and faecal shedding. Some HEV-inoculated birds laid eggs during the course of this experiment, and Guo *et al*. (2007a) subsequently found that these eggs contained infectious HEV, by inoculating the egg-whites into chickens and observing viremia and faecal shedding in some of the birds beginning at 3 wpi.

Billam *et al*. (2009) inoculated 18 6-week old SPF chickens with 5 x $10^{2.5}$ chicken infectious dose (CID_{50}) ml^{-1} avian HEV and collected samples of blood and faeces weekly. At 1 wpi, 15

birds were shedding HEV in their faeces and all birds were shedding the virus at 2 wpi. 11 birds had seroconverted by 1 wpi, and 17 by 2 wpi.

Pigs could also serve as a model to study avian HEV. Kasondorkbua *et al*. (2005) intravenously inoculated nine SPF pigs (species not given) with avian HEV (suspending material not stated), and monitored for signs of infection (faecal shedding, seroconversion). At 7 dpi, HEV RNA could be detected in the faeces of 6 pigs, and anti-HEV antibodies (type not stated) could be detected in 5 pigs. Huang *et al*. (2004) attempted to infect Rhesus macaques with avian HEV but no sign of infection (seroconversion, viraemia, faecal virus shedding or elevation of serum liver enzymes) was observed.

3.2 Ferret HEV

HEV was first detected in ferrets by Raj *et al*. (2012), who isolated it from the faeces of infected animals. Li *et al*. (2015) showed that rats and monkeys could not be infected with ferret HEV. The intravenous inoculation of two ferrets with ferret HEV from cell culture indicated successful infection by HEV shedding, seroconversion and elevation of liver enzymes (Li *et al*., 2016a).

3.3 Rabbit HEV

Ma *et al*. (2010) inoculated ten SPF rabbits with serum samples from HEV-infected rabbits bred in rabbit farms. The inocula contained between 1 x 10^3 and 7 x 10^4 viral GC. Blood samples and faeces were collected weekly thereafter, and tested for HEV RNA by RT-PCR, and for seroconversion. Seroconversion was observed between 4 and 9 wpi, but not in all animals. Faecal shedding began sporadically among the rabbits after 2 weeks, but at no time could HEV be detected in all the rabbits' faeces.

Cheng *et al*. (2012) inoculated 4-5 month old European rabbits (*Oryctolagus cuniculus*) with suspensions containing 2 strains of HEV previously obtained from infected rabbits. Groups of five rabbits were intravenously inoculated in each test. The inocula contained between 3 x 10^5 and 4 x 10^5 GE ml^{-1}. The animals were monitored weekly for 10 weeks for faecal shedding and seroconversion. Seroconversion was observed at 1 wpi in rabbits inoculated with one HEV strain, and at 4 wpi in the rabbits inoculated with the second strain. Faecal shedding was observed beginning at 1 wpi in some of the rabbits. However, even at 10 wpi, not all rabbits were shedding HEV (4 shedders in each group).

Pigs can be infected with rabbit HEV. Cossaboom *et al*. (2012) intravenously inoculated 2 groups of four pigs each with a different strain of rabbit HEV. The inocula contained between 2×10^5 and 2×10^6 GE. The animals were monitored weekly for 10 weeks for faecal shedding and viremia. In one group, faecal shedding was observed in 2 out of 4 pigs after 6 wpi, but viremia was not observed in any pig at any sampling time. In the other group, only 1 pig shed HEV, after 5 wpi, and viremia only sporadically detected in 2 pigs after 2 and 9 wpi respectively.

3.4 Rat HEV

HEV was first detected in rats by Johne *et al*. (2010). Subsequently, Purcell *et al*. (2011) inoculated 13 rats with serum samples from HEV-infected rats, and monitored them for seroconversion over 3 weeks. At the end of the experiment only 3 rats had seroconverted.

Li *et al*. (2013) confirmed that laboratory rats can be infected intravenously with rat HEV. In addition, the rats could be infected orally by rat HEV present in rat faeces. When contaminated faeces were placed in a cage containing 2 rats, one animal's faeces was HEV RNA-positive after 13 days and the other after 36 days. Immunosuppressed nude rats were demonstrated to be highly susceptible to rat HEV shedding large amounts of the virus for prolonged times.

Cossaboom *et al*. (2012) reported that pigs inoculated with rat HEV displayed no signs of infection. No HEV could be detected in any of 9 pigs inoculated with rat faeces throughout 35 days of monitoring (Kasondorkbua *et al*., 2005). However, as the distinct HEV strain present in the rat-derived inoculum was not characterized further, it is not clear whether human HEV or rat HEV was used for inoculation.

3.5 Summary

Table 3.1 summarises the information from the studies reviewed above. It is evident from the literature review that a relatively large number of studies have been performed with avian HEV, whereas infection with rabbit HEV, rat HEV and ferret HEV has only been tested in a few experiments. In each of the studies, the ability to generate an infection model has been confirmed in principle.

Table 3.1. Summarised information from studies involving animal models for potential surrogate viruses

Surrogate	Animal species	Route of inoculation	Evidence of infection	Parameter measured	Used to test HEV infectivity in food	Used to test inactivation of HEV
Avian HEV	Chickens	Intravenous, nasal, oral	Faecal shedding, seroconversion viremia	Viral RNA, anti-HEV IgG	No	No
Avian HEV	Pigs	Intravenous	Faecal shedding, seroconversion	Viral RNA, anti-HEV IgG	No	No
Ferret HEV	Ferrets	Intravenous	Faecal shedding, seroconversion	Viral RNA, anti-HEV IgG	No	No
Rabbit HEV	Pigs	Intravenous	Faecal shedding, viremia	Viral RNA	No	No
Rabbit HEV	Rabbits	Intravenous	Faecal shedding, seroconversion	Viral RNA, anti-HEV IgG	No	No
Rat HEV	Rats	Intravenous	Faecal shedding, seroconversion	Viral RNA, anti-HEV IgG	No	No

3.6 Advantages and disadvantages of animal models for potential surrogate viruses

A major advantage of using a surrogate virus would be that no human-pathogenic virus has to be handled. In addition, as several rodent viruses have been identified as potential surrogate viruses, the establishment of a rodent model, which would be easier to handle than a pig or monkey model, may be possible.

However, it is not known whether the surrogate viruses accurately reflect the characteristics of human HEV. Most of the surrogate models are less developed than the human HEV models.

The general advantages and disadvantages for the animal models for the surrogate viruses are the same as those described in Section 2.9 above, i.e. repeatability, lengthy time for the signs of infection to become manifest, low potential for extensive replication, and negative ethical implications.

Table 3.2. The advantages and disadvantages of animal models for potential surrogate viruses to assess HEV infectivity

Advantages	Disadvantages
Use of viruses which are non-pathogenic for humans Rodent model easier to handle than pig model	Similarity with human HEV questionable Most of the surrogate models are less developed than the human HEV models Poorly repeatable Time-consuming Low potential for replication Low statistical significance Ethical approval required

4. Cell culture for HEV genotype 1-4

There have been several attempts to produce an efficient cell culture system for HEV (Okamoto *et al*., 2011), with varying degrees of success. HEV replication in cultured cells has been monitored by a range of techniques, including formation of cytopathic effect (CPE), detection of viral RNA and detection of viral proteins. Infection of cultured cells has been used in some studies to evaluate the response of HEV to elimination procedures (Cook and van der Poel, 2015). Below, some examples are given of the use of cell culture for detection of infectious HEV.

4.1 Studies using cell culture for detection of infectious HEV gt 1-4

Huang *et al*. (1992) described one of the first attempts to culture HEV from a Chinese patient infected with HEV. The authors do not specify which genotype it was, but designate the strain "87a". The cell lines used were a human embryo lung diploid cell strain "2BS" and the LLC-MK2 continuous cell line. Inoculated tissue cultures (with strain 87a) were incubated at 35°C and were examined for CPE daily for 1 week. Two additional passages were performed on the cultures. Virus-containing cultures were serially passaged as before. Before analysis of the tissue culture was performed, the viruses were purified by precipitation and ultracentrifugation. This purified virus suspension was then analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The authors report presence of a CPE after the third day of the second passage in the 2BS cell line. The cytopathic effects were however not observed by the third passage in the LLC-MK2 cells or in normal cells. They also report that the positive results obtained from the 2BS cells were repeatable. No mention is made of how long they monitored the LLC-MK2 cells.

Huang *et al*. (1995) described another study using the same strain (87a) as described in their 1992 manuscript. On this occasion, they describe the use of A549 cells to propagate HEV, along with other cell lines (LLC-MK2, Vero, BHK-21 and 2BS cells). The authors do not state how long the cells were incubated or how much confluence was obtained before inoculation. The cell monolayer was inoculated with the virus suspension and incubated for 10 days. Observations for CPE were performed daily. Results showed a strong CPE after passage 1, 2 and 3 for both A549 cell lines and for the 2BS cell line, but no CPE for the others. Detection was also performed by PCR.

Tam *et al*. (1996) developed a serum-free cell culture system to determine if the propagation of primary hepatocytes from the livers of experimentally infected macaque monkeys remained susceptible to virus infection and replication. The authors adapted a previously described serum free medium (SFM) cell culture system in order to replicate HEV *in vitro.* To detect replication of HEV, a highly specific RT-PCR assay was used to detect the presence of positive and negative strand RNA. Two cynomolgus macaques monkeys were intravenously inoculated with HEV (Burma strain, GenBank acc.-no. M73218). Hepatocytes were isolated from the livers at the time of biopsy. RNA from infected hepatocytes and from cell-free culture media was analysed on 1, 3, 7, 9, 14 and 24 dpi. Positive strand signals increased and reached a maximum 14 dpi. HEV negative strand RNA signals were present throughout the study. The authors concluded that the *in-vivo* infected hepatocytes were able to be cultured and support virus replication over a 9-10 week period. There is no indication that CPE was observed or that the system was reproducible.

Dzagurov *et al*. (1997) observed a cytopathic effect after day 7 of a 25th passage of HEV-infected FRhk-4 cells. RT-PCR showed viral replication began 4 dpi.

Meng *et al*. (1997a) reported using a HEV cell culture system for the purposes of developing a PCR-based seroneutralisation assay. The authors used a cell culturing system previously described by Pillot *et al*. (1987). The human hepatocarcinoma cell line PLC/PRF/5 and the Pakistani virus strain gt1 SAR-55 HEV were used (faecal specimen from an experimentally infected rhesus monkey at 21 dpi). In addition, a human HEV strain F23 obtained from a faecal specimen was used. They report that the system allowed them the opportunity to develop a neutralisation assay; however they noted that the lack of a cytopathic effect and the low level of replication meant they had to use RT-PCR to detect the presence of the HEV.

Divizia *et al*. (1999) examined the susceptibility of the PLC/PLF/5 cells using the gt1 SAR-55 HEV strain, and demonstrated HEV replication. Seventy-five stool samples obtained from acute hospitalised patients were tested for presence of the HEV genome by RT-PCR. Four of these samples tested positive (3 IgM and one IgG positive) by RT-PCR. These were used to infect 2 day old monolayers of PLC/PLF/5 cells. Only one sample out of the four positives extracted from the cell culture showed HEV replication using RT-PCR applied to positive and negative replicative strands of HEV genomic RNA after 10 days post infection. No cytopathic effect was observed.

Huang *et al*. (1999) describes the culture of sporadic hepatitis E virus in A549 cells. Four virus strains, G93-1, G93-2, G93-3, and G93-4 (genotypes not identified), were originally isolated from A549 cells associated with the faeces of 4 patients with HEV infection. HEV was inoculated onto a 2D monolayer of A549 cells. 2BS cells (a diploid strain of human foetal lung fibroblasts) were used for passages 26 to 33 in the study. The inoculated cell monolayer was incubated at 37°C and observed for CPE. CPE was observed after 2 d. Specific important conditions to enable replication in the cell lines were noted. Passage 4 of HEV strain G93-2 was inoculated into A549 cell monolayers and after release of the virus into the medium and a clean-up procedure, immunoelectron microscopy was performed which successfully identified virus particles of strain G93-2.

Wei *et al*. (2000) used the method of Huang *et al*. (1992, 1995) to isolate an infectious HEV from the faeces of a patient. After 24 hours CPE was observed in A549 cells, which became round and gradually lysed until the cell monolayer was destroyed. The HEV strain, designated 93G (genotype not identified), could be passaged at least 4 times.

Le *et al*. (2001) performed experiments of propagation of HEV using several cell lines. The genotype or strain of HEV was not reported. HEV was obtained from monkeys which had been inoculated with supernatants from patients with acute HE. The samples from the monkeys were ultra-centrifuged prior to use. Human derived cell lines (KMB17; A549; BEL7402 and Hela) and primate cells (Vero) were inoculated with HEV. Sensitivity was analysed using CPE observation, RT-PCR and immunofluorescence. A CPE was observed in KMB17, A549 and BEL7402 cells between 7 and 9 dpi. The HEV genome could be detected by RT-PCR after ten passages. Neither CPE nor amplification of the HEV genome RNA by RT-PCR could be detected in the Hela or Vero cells after the second to fourth passage.

Emerson *et al*. (2005) performed a heat stability study of wild type HEV. The HEV gt1 strains SAR-55 and Akluj were isolated from the faeces of patients from Pakistan and India respectively. Both of these HEV strains had a 50% monkey infectious dose (MID_{50}) of $10^{6.5}/0.5\ ml^{-1}$ of 10% faecal suspension. In addition a Mex 14 (genotype 2) virus stock was obtained from a rhesus macaque monkey which had been experimentally infected. An infectivity assay was performed using HepG2/C3A cells (CRL-10741) obtained from the American Type Culture Collection (ATCC). After culturing the cells for 5-6 days at 34.5°C (no CPE recorded), convalescent-phase serum collected from a chimpanzee infected with

HEV was incubated with air-dried fixed cells at room temperature for 20 min. After staining, the cells were examined using a fluorescent microscope, and the number of infected cells quantified. Virus suspensions were incubated at various temperatures for 1 h, inoculated onto HepG2/C3A cells and the number of infected cells counted and compared to the number observed when untreated suspension was used as inoculum. The Akluj strain had a 50% inactivation temperature between 45 and 50^{o}C, with almost complete inactivation being achieved at 56^{o}C. The Mex 14 strain appeared to be more stable than the Akluj strain. It was 80% inactivated at 60^{o}C. The Sar55 strain displayed 50% inactivation at 56^{o}C and 96% at 60^{o}C, being slightly more resistant to heating than the Akluj strain. Inactivation of the Akluj strain was determined to be rapid at 56^{o}C, since, after 15 min incubation, 95% inactivation was observed. 1% of the virus was still infectious after heating for 60 min. The authors suggested that in order to inactivate HEV in a slab of meat, the temperature/time combination would necessarily have to be higher than that described in this paper.

Takahashi *et al*. (2007) investigated faecal shedding durations and load changes of HEV using faeces and serum samples from patients with acute HEV infection. Informed consent was required to collect the serum and faecal samples from 11 patients. Two of the samples were identified as gt1, two as gt3 and the remaining seven as gt4. Detection and quantification of the HEV RNA was performed. Firstly, extraction of the RNA was performed using a detergent-based reagent, then a nested RT-PCR targeting the ORF2/ORF3 overlapping region of the HEV genome was employed. For cell culture, a PLC/PRF/5 cell line was used. After confluence was reached (no information on time taken to confluence) the cells were trypsinised to separate and disperse them, and diluted 1:4 in medium and added to 6 well microplates 1-2 days before virus infection. Every 2 days thereafter, 1 ml of the 2 mls medium was removed and stored prior to virus titrations. HEV RNA was detected in all 11 patient samples. One of the patient's faecal samples (gt3) was used for evaluation of the cell culture system. The HEV viral load was measured by RT-PCR in infected culture medium up to 50 dpi. HEV RNA was first detected at 12 -14 dpi with increasing copy numbers being found at 50 dpi. The full length genomic sequence of the isolate used in the cell culture (designated JE03-1760F, wild-type HEV) was determined and 29 nucleotide substitutions found. The authors cautiously speculate that theses substitutions could be responsible for the efficient replication efficiency in the cell culture system, but acknowledge that further studies are required to confirm this. They suggest that a mutagenized infectious cDNA clone may

provide the mechanism by which nucleotide substitutions lead to replication and protracted faecal shedding of HEV.

Tanaka *et al*. (2007) describe the development and evaluation of a cell culture system for a gt3 HEV. A total of 21 cell lines were used including PLC/PRF/5 and A549 cells, these being the only ones which could be used to successfully propagate HEV. Detection of HEV was by RT-PCR and HEV RNA could be detected in the culture medium of both cell lines 12-14 dpi up to 38 dpi. No CPE was evident during virus replication. Different viral loads were also used to see if this had an effect and it was found that 6.0×10^4 GC per well gave the most efficient results using this system. The authors speculate that efficiency of the propagation depends on an initial high viral load (2.0×10^7 GC ml^{-1}faecal suspension). This theory was tested by using suspensions with lower viral loads with results showing no propagation.

Lorenzo *et al*. (2008) developed a cell culture system for gt3 HEV (strain JE03-1760F), in PLC/PRF/5 and A549 cells. A faecal suspension of HEV was used to inoculate the cells and the system was able to yield 10^8 GC ml^{-1} in the culture supernatant. The authors set out to quantify HEV RNA in the culture supernatants of the PLC/PRF/5 and A549 cells that had been inoculated with the JE03-1760F strain. Faecal supernatant containing the gt3 JE03-1760F HEV strain was inoculated on fresh monolayers of PLC/PRF/5 and A549 cells. Cytopathic effect was not observed during the culture of either of the cell lines. After performing qRT-PCR, HEV RNA appeared in the culture media of both PLC/PRF/5 and A549 cells after 12 dpi and persisted in the supernatants of the PLC/PRF/5 and A549 cells up to the end of the 118^{th} or 114^{th} day of observation respectively. In addition, the JE03-1760F strain was successively propagated in PLC/PRF/5 cells up to thirteen generations of serial passages with the highest HEV load of 10^8 GC ml^{-1}.

Tanaka *et al*. (2009) developed a cell culture system for gt4 HEV. The authors report this system is more efficient for HEV propagation using PLC/PRF/5 and A549 cells. This was dependent on a high viral load faecal suspension (1.3×10^7 GC ml^{-1}). HEV RNA was detectable in the culture medium of the primary propagation passage of the PLC/PRF/5 cells 16 dpi and the viral load increased up to 60 dpi. Six passages were performed on both the PLC/PRF/5 and A549 cells. The RNA from HEV progenies was detected in the first passage of the PLC/PRF/5 cells between 6 dpi and 42 dpi. HEV progenies from this passage were subsequently inoculated into PLC/PRF/5 and A549 cells, and the authors report that the A549

cell line supported a more efficient propagation of HEV due to a larger number of HEV RNA GC ml^{-1} being reported after 24 dpi. No CPE was reported.

Zaki *et al*. (2009) investigated the use of cell culture and nested RT-PCR to detect HEV in serum and stool samples from human patients. For culturing the HEV, the cell line HPG11 was used in monolayer format. The viral load of the suspension used to inoculate the cells was not provided. The infected cell cultures were incubated for 21 days. The infected culture plates were examined on a daily basis for CPE. Those displaying a CPE were freeze – thawed three times to extract the virus from the cells, after which RT-PCR was conducted, presumably to verify the presence of the virus. The authors stated that the culturing was successful, but gave no details of how long it took before CPE was observed, or of the results of RT_PCR confirmation.

Shukla *et al*. (2011) developed a cell culture system using a gt3 Kernow-C1 strain of HEV derived from the faeces of an HIV-1 patient chronically infected for 2 years with HEV. The faeces contained approximately 10^{10} viral genomes per gram. The virus was inoculated onto five human and one rhesus cell lines and 7 d later the cells were stained for immunofluorescence microscopy with antibodies to ORF2 capsid protein and to ORF3 protein. Due to the fact that these proteins are translated from a subgenomic mRNA, their presence indicates viral RNA synthesis has occurred. All six cultures contained infectious foci, the number being more than 7.5-fold higher in HepG2/C3A cells than in Huh7.5 or PLC/PRF/5 cells, A549 cells, Caco-2 cells, or rhesus kidney cells, which suggests that HepG2/C3A cells were the most permissive. The Kernow-C1 strain was shown to contain a genome insertion derived from human ribosomal RNA, which was speculated to be responsible for more efficient cell culture replication.

After their development of a cell culture system for HEV using faecal samples, Takahashi *et al*. (2010) further investigated the ability of PLC/PRF/5 and A549 cells to support the replication of HEV derived from serum samples obtained from 23 patients. The authors aimed to determine the ability to culture HEV from these serum samples. Detection and sequencing of HEV RNA was performed as described by Takahashi *et al*. (2007). RNA from three serum samples containing gt3 or gt4 HEV inoculated on monolayers of PLC/PRF/5 cells were detected on the 4^{th} to 6^{th} dpi, and an increase in viral load continued until 30 dpi. Thirteen cell cultures with an inoculum load of $3.5 \times 10^4 – 3.0 \times 10^6$ GC per well and 5 out of

16 cell cultures with an inoculum load of 2.0 x 10^4 GC per well produced detectable HEV without showing a CPE. The number of GC ml^{-1} was then calculated after 30 dpi with the median being 5.8 x 10^5 GC ml^{-1} upon inoculation at 10^6 GC per well, a median of 1.3 x 10^5 GC ml^{-1} upon inoculation at 10^5 GC per well and a median of 3.4 x 10^4 GC ml^{-1} upon inoculation at 4 x 10^4 GC per well. For the A549 cells, 6 HEV antibody positive serum samples and 3 HEV negative antibody samples were inoculated, with all culture mediums samples testing positive for HEV, regardless of the presence or absence of HEV antibodies. No CPE was observed in either the PLC/PRF/5 or A549 cells, regardless of the titre of the initial inoculum and HEV load. The authors summarise that this study reveals that various gt1, gt3 and gt4 strains in serum samples can replicate efficiently in both PLC/PRF/5 and A549 cells. To avoid cell damage due to sample toxicity, lso of note is that a 5-25 fold dilution was required when a faecal specimen was used to inoculate this cell culture system, whilst a dilution of only 1:2 was required for serum samples.

Zhang *et al*. (2011) developed cell culture systems for a swine HEV gt4. Swine cells (IBRS-2) and human cells (A549) were used. Detection was confirmed by HEV RNA as well as CPE. CPE was observed after the 8^{th} passage of IBRS-2 cells and after the 22^{nd} passage of A549 cells. The CPE appeared to be very strong (characterized by the destruction of the monolayer), with the culturing of the cells being terminated at passage 12 of the IBRS-2 and at passage 24 of the A549 cells. Each passage was performed after 3 days from the previous, so although the CPE was strong, the time to CPE was quite lengthy.

A short communication from Nguyen *et al*. (2012) report that colleagues in Japan successfully adapted gt3 and gt4 HEV strains to grow efficiently in cell culture without the appearance of a CPE. Both of the strains had been isolated from patients with acute hepatitis E and it was noted that the successful culture of these strains was dependent on starting the culture with a high titre inoculum. The authors point out that these two cell-culture adapted strains were similar to other strains in their respective genotypes, but in contrast, another adapted gt3 strain (Kernow C1, the only isolate from a patient up to that point which had been adapted to grow in cell culture), differed significantly from other gt3 viruses. This particular strain was isolated from a HEV / HIV co-infected patient (after 2 years of infection). The strain was culturable using HepG2/C3A hepatoma cells and the unexpected outcome was that passaging in these cells selected for a very rare recombinant genome. This raised the question of whether the insertion of a foreign sequence and cell culture selection of

the recombinant was unique to this isolate, or an anomaly, or whether it was due to the long period of infection which had allowed mutations and alterations to take place, and whether this was true with other cases of long HEV infections. To attempt to answer this question, the authors characterised a gt3 strain (LBPR-0379) from a patient with chronic HEV. This strain also contained a genome insertion derived from another human ribosomal RNA gene. They cultured faeces and serum (10 months into the infection) using HepG2/C3A cells and found that infectious virus was released into the medium after 46-51 days of continuous culture without showing CPE. Virus titre was quantified using an immunofocus-forming assay. The paper goes on to describe the sequences obtained after further passages of the strain, but the conclusion obtained for the purpose of this review is that the culturing takes a long period of time.

To determine whether HEV detected in pig livers sold at retail was infectious, Takahashi *et al*. (2012) homogenised 200 mg samples of seven livers (six containing gt3 and one containing gt4) in PBS, centrifuged the homogenate, and used the supernatant to inoculate cultures of A549 cells. Every 2 days, aliquots of culture media were tested for HEV RNA by RT-PCR. HEV RNA could be detected at 2-4 days in three cultures inoculated with liver homogenates containing gt3 at titres of 2×10^5 and above; however no HEV RNA was detected in cultures inoculated with HEV strains at 3×10^3 or less. CPE was not described.

Berto *et al*. (2013b) developed a 3D cell culture system for the propagation of HEV. PLC/PRF/5 cells were grown as a monolayer to 95% confluence, then the cell sheet was trypsinised and the cell suspension added to porous microspheres coated with collagen, in a rotating wall vessel (RWV) bioreactor. In this system, cells attach to the microspheres and the complex is kept in suspension by rotation of the vessel. The culture was incubated for at least 28 d prior to inoculation with HEV gt3. The virus was obtained from an experimentally infected pig's liver; the liver tissue had been homogenised and filtered and the filtrate (containing ~1 x 106 HEV GC ml^{-1}) used for inoculation. Post-inoculation, samples of the culture were taken every 3 days up to 49 dpi and thereafter weekly until 175 dpi. The samples were subjected to RNA extraction and qRT-PCR. Subsequently, the presence of infective virus was inferred by the observation of an increase in the number of viral RNA copies and hence the assumption of virus replication in the cells. An increase in the number of viral RNA copies began at 24 dpi and peaked at 39 dpi with another increase between 85 dpi and 155 dpi. A further indication of the infectivity of the extracted HEV was that infected cell

extracts could be used to inoculate a fresh 3D culture, and virus replication detected as previously. The authors did not define any criteria regarding the point at which infectivity could be conclusively inferred (e.g. a defined increase in viral copy number /sustained time of increase in viral copy number).

Subsequently, Berto *et al*. (2013a) used this 3D cell culture system to evaluate the infectivity of HEV gt3 extracted from 4 samples of pork liver sausages obtained from retail outlets,. The presence of HEV in these sausages had previously been determined by RT-PCR. The virus was extracted from the sausage by homogenisation in cell culture medium, followed by filtration to remove debris. The RT-PCR had not been calibrated in this study, therefore signal intensity was measured by the Ct value. The method was comparatively reproducible in 2 laboratories; in one laboratory a continued increase in RT-PCR signal was observed up to ~45 dpi, and in the other up to ~60 dpi. Immediately after inoculation, and at irregular intervals thereafter up to 80 dpi, samples of cells and supernatant were taken for RNA extraction and RT-PCR. The presence of infective virus was only detected in one out of the four samples.

Devhare *et al*. (2013) analysed an induced antiviral response after infecting A549 cells with HEV gt1. Viruses were obtained from the stool of a confirmed hepatitis E patient (anti-HEV IgM positive). A 10% stool suspension was centrifuged and the supernatant filtered. Purification was then carried out using sucrose step gradient centrifugation and the HEV RNA genome copy number determined by RT-PCR (1.4×10^6 copies ml^{-1}). A screen of S10-3 cell lines, hepatoma cells (PLC/PRF5, Huh7 and HepG2) and non-hepatoma cells (caco2 and A549) was performed using RT-PCR and based on the results of the screen, A549 cells were chosen for further work. After 50-60% confluence was achieved the cells were infected with the virus and appropriate dilutions thereof. The authors report that due to HEV not showing a cytopathic effect and the fact that a small increase in copy number would not produce any significant increase in signal by RT-qPCR, that a different approach should be used to demonstrate replication. Negative strand RNA detection of the virus was performed using negative strand-specific primer based RT-PCR. RT-PCR signals were obtained (using gel electrophoresis) up to 96 h post infection. An immunofluorescence assay to detect the HEV ORF2 protein was also performed using infected A549 cells stained with ORF2 specific monoclonal antibodies and positive results showing 15-20% IFA positivity were obtained up to 6 days post infection. It was also observed that exposure of the virus to UV for 30 minutes

resulted in complete inactivation, as demonstrated by negative results being obtained for both the negative sense RNA and IFA up to 12 days post infection.

Rogée *et al*. (2013) presented the development of two cell culture systems for HEV replication. Cell lines used for this study were a human hepatoma-derived cell line (HepaRG, obtained from BIOPREDIC International), and a porcine embryonic stem cell-derived cell line (PICM-19, from pig embryonic stem cells). It is noted that these particular cell lines have similarities to primary hepatocytes, with regard to their morphological and functional properties. The authors suggest that cell lines used in other studies may not be as effective as previously thought. It is pointed out that PLC/PRF/5 contain several integrated genes belonging to hepatitis B virus which could affect replication, and that A549 cells are not hepatic in origin. Gt3 HEV (subtype 3f, GenBank accession no. JN906976) was obtained from swine faecal samples and quantified using qRT-PCR. 3D Matrigel-embedded cultures of cell lines were also used. The HepaRG and the PICM-19 cells were infected with HEV at a multiplicity of infection (m.o.i.) of 0.5. Detection of negative strand HEV RNA was performed by nested RT-PCR. Immunofluorescence staining was performed on HepaRG and PICM-19 cells grown on glass coverslips. These were infected with HEV at an m.o.i. of 10 and the infection assay performed 21 dpi. HEV detection was estimated after heat treatments. Viral suspensions were heated to 56 ^{o}C for 60 mins and 95 ^{o}C for 5 mins and were used to inoculate the cell lines. HEV incubated at room temperature for 60 mins was used as a control. Results showed that at 4 dpi, both these treatments inactivated HEV, whilst in contrast, the control was detected throughout the infection period in the supernatant of HepaRG, with the growing phase starting at 8 dpi. The same was found using the PICM-19 cells. No reports of reproducibility.

Oshiro *et al*. (2014) investigated the infection (and replication) of swine-derived HEV using a primary human hepatocyte cell line, cultured from livers of patients with metastatic tumours. The use of the hepatocytes required patient consent and approval from a research ethics committee. HEV was obtained from the faeces of naturally infected swine. The isolates used were gt3 and gt4, namely G3JP, G3US, G3SP and G4JP. Passages were not performed due to prior knowledge that hepatocytes do not proliferate in culture. The cells were cultured in rat-tail-collagen-coated six-well plates, on rat-tail-collagen-coated four-well chamber glass slides or on 15 mm rat-tail-collagen-coated glass coverslips in 35 mm Petri dishes at a density of 1.26×10^{6} viable cells cm^{-2}. HEV RNA was extracted from cells and medium and quantified

using qRT-PCR. The amounts of virus RNA decreased at 2 dpi and then increased until the end of the experiment. This decrease may have been due to degradation of virus which had not infected the cells. Immunofluorescence staining was also used to observe infected cell cultures on the collagen coated coverslips. Numbers of infected cells increased with time after infection, resulting in a hypothesis that HEV infected cells were the result of cell-to-cell transmission through the cell membrane, rather than via the culture media. A comparison was also made of the HEV genome by amplifying the full length of HEV in nested RT-PCR before and after inoculation then sequencing, demonstrating that there were no differences in the bases between inoculated and propagated HEVs, which suggest that HEV gt3 can replicate in cultured human hepatocytes without accruing changes in nucleotide sequence.

Owada *et al*. (2014) established a cell culture system used for propagating blood-derived specimens of gt3 and gt4 HEV. The authors set out to evaluate a commercial pathogen reduction system. This system was applied to contaminated platelet samples which had been spiked with HEV gt3 or gt4. The platelet samples were subsequently examined before and after the treatment. The strains used were obtained from plasma and serum from blood donors and patients confirmed to be HEV RNA positive. Ten plasma derived gt3strains and 4 serum derived (3 x gt4 strains and one – SA1 not determined) were used to establish the culture system. PLC/PRF/5 and A549 cells were infected for 2 h before being cultured. The maintenance medium was recovered on a weekly basis and HEV RNA copy numbers quantified using qRT-PCR. Confirmation of HEV infectivity was performed by detection of the virus in the cell culture supernatant. Using A549 cells, only two of the strains were found to be infectious – one gt3 and one gt4 strain. The gt3 strain took 42 dpi to reach a plateau whilst the gt4 strain took 60 dpi. The HEV gt3 strain was also cultured in PLC/PRF/5 cells at a high concentration ($10^{5.5}$ copies ml^{-1}). The load recovered from the culture medium 175 dpi was $10^{7.9}$ copies ml^{-1}. Although the gt3 strain could infect both the PLC/PRF/5 cells and the A549 cells, the production efficiencies differed. Using a higher load of the gt3 strain in the PLC/PRF/5 cells, a higher concentration of virus progeny was confirmed, whilst using the A549 cells, a lower amount of progeny was obtained. It was concluded that because the PLC/PRF/5 cells are derived from human hepatoma, this cell line could be more suited to the production of HEV than A549 cells. No appearance of a CPE was described.

Johne *et al*. (2014b) inoculated serum samples from two patients showing acute hepatitis E, one patient showing chronic hepatitis E and a liver homogenate from an HEV-infected wild boar onto A549 cells. Only the strain from the chronically infected patient showed signs of

replication as demonstrated by increasing genome amounts beginning with day 35 after inoculation. The strain, designated 47832c (gt 3) could be passaged more than two times on A549 cells showing increased genome copies at 7 dpi. Viral proteins and virus particles were demonstrated in the cells and the culture supernatant. A persistently infected cell line continuously shedding HEV into the culture supernatant was produced and passaged several times. A CPE was evident in only two passages of this cell line, but not in earlier or later passages. Strain 45632c was shown to contain a special genome insertion in its ORF1 derived from another part of the HEV genome. It was speculated that this insertion is responsible for efficient cell culture replication.

Addition of the immunosuppressant calcineurin inhibitor tacrolimus might stimulate replication of HEV in cultured cells. Wang at al. (2014) found that addition of 5 µg ml^{-1} tacrolimus to HEV-infected Huh7 cells for 48 h increased viral RNA in the cells by 35%. They did not report whether CPE could be observed.

Qi *et al*. (2015) examined the structure and protein composition of HEV by means of a PLC/PRF/5 cell culture system to propagate two gt4 HEV strains (AJ272108, 2.54×10^{6} copies ml^{-1}, and JQ655736, 2.58×10^{6} copies ml^{-1}). HEV RNA was quantified using qRT-PCR. Monolayers of PLC/PRF/5 cells were used to evaluate the infectivity of gradient fractions. At three dpi, the culture was maintained with 2% FBS and collected every three days and stored at -80°C until virus titrations were performed. Different fractions were tested to determine the infection and replication ability. As an example, one of the fractions tested containing progeny HEV titre reached 5×10^{2} copies ml^{-1} at 27 days and 34 dpi when the titre of inoculated HEV RNA was 3.4×10^{3}copies ml^{-1} and 3.4×10^{4}copies ml^{-1}, respectively. The progeny HEV titre reached 1×10^{6} copies ml^{-1} at 43 days and 47 days. It was found that HEV continued to be detected at 60 dpi. The appearance of a CPE was not reported.

Shiota *et al*. (2015) obtained subclones of PLC/PRF/5 cells by seeding microtitre plate wells with a low density (10 cells ml^{-1}) cell suspension. The subclones were tested for their ability to propagate HEV by inoculating with G3-HEVB3-2-27, and monitoring for increase in viral antigen by ELISA. Highly permissive subclones allowing infection to be detected by 21 dpi were obtained (also less permissive subclones). The appearance of a CPE was not reported.

A cell culture system for the measurement of HEV infectivity has been developed by Johne *et al.* (2016). In this system, the cell-culture-adapted gt3 strain 47832c isolated from a chronically infected patient (Johne *et al.*, 2014b) was used. An A549 cell line persistently infected with this strain, which continuously released HEV particles into the culture supernatant, was used for production of virus stocks. A clonal cell line (A549/D3) generated by seeding single non-infected A549 cells and growing out to a cellular clone was shown to be more susceptible to HEV strain 47832c infection than the parent A549 line. This clonal cell line was used for titration of the HEV strain. Because infection did not produce a CPE, immunofluorescence staining was applied for quantitative detection of infected cells, which was possible over a 4 log dilution range of HEV suspensions. This system was used to examine the effect of heat, and long-term storage, upon HEV present in cell culture supernatant. Johne (unpublished) also preliminary tested the system with HEV extracted from pork meat products; this however was unsuccessful as the cells were deleteriously affected by substances co-extracted from the meat with the virus.

4.2 Summary

Table 4.1 summarises the information on cell cultures which have been reported to detect infectious HEV. It can be concluded from the literature review that many reports are available that describe successful propagation of HEV in cell culture. The cell lines PLC/PRF/5, A549 and HepG2/C3A are most often used. The appearance of a CPE is described in some of the studies, which are mostly published before 2005. In later studies, a CPE was rarely observed during HEV replication and the virus growth was mainly detected by RT-PCR or immunofluorescence staining; the reasons for this trend are not clear. Most of the reports show only limited data on repeated passaging of the isolated viruses and on repeatability of the infection experiments. Validation studies on HEV cell culture systems are lacking. Also, the virus concentration needed for infection of the cell cultures has not been specified in most of the studies as the determined amount of HEV RNA does not necessarily correlate with the amount of infectious virus present in the inoculum. Therefore, the limits of detection of infectious HEV still need to be established for most of the cell culture systems. Using specific cell lines and virus strains, virus titrations over a maximum of 4 log dilutions of inocula have been described.

Only two studies reported the successful isolation of HEV from food. One of these studies inoculated pig livers sold at retail and one used a liver sausage sample for inoculation. The latter study applied a sophisticated 3D cell culture system, and tested it in two laboratories.

Table 4.1. Cell cultures which have been reported to detect infectious HEV

Cell line	Origin of cells	2D or 3D cell culture system	HEV genotypes tested	Parameter(s) measured	Used to test HEV infectivity in food	Used to test inactivation of HEV
PLC/PRF/5	Human hepatocarcinoma	2D, 3D	1, 3, 4	HEV RNA, viral antigen	Yes	Yes
A549	Lung carcinoma	2D	"human strains", 3, 4	CPE, HEV RNA, viral antigen	No	No
A549/D3	Clone of A549	2D	3	Viral antigen	No	Yes
HepG2/C3A	Human hepatocarcinoma	2D	2, 3	viral antigen	No	Yes
BHK-21	Hamster	2D	"human strain"	HEV RNA	No	No
HepaRG	Human hepatocarcinoma	2D, 3D	3	HEV RNA, viral antigen	No	Yes
PICM-19	Porcine	2D, 3D	3	HEV RNA, viral antigen	No	Yes
KMB17	Human embryo lung	2D	"human strains"	CPE, HEV RNA, viral antigen	No	No
BEL7402	Human hepatocellular carcinoma	2D	"human strains"	CPE, HEV RNA, viral antigen	No	No
Hela	Human adenocarcinoma	2D	"human strains"	HEV RNA, viral antigen	No	No
Vero	Primate kidney	2D	"human strains"	HEV RNA, viral antigen	No	No
Caco-2	Human intestinal	2D	3	viral antigen	No	No
Rhesus kidney	Primate	2D	3	viral antigen	No	No
2BS	Human embryo lung	2D	"human strains"	CPE, viral antigen	No	No
Huh7	Human hepatoma	2D	1, 3, 4	viral antigen		
HPG11	Not stated	2D	"human strains"	CPE, HEV RNA	No	No
IBRS-2	Porcine	2D	4	CPE, HEV RNA	No	No

4.3 Advantages and disadvantages of cell culture for testing infectivity of HEV genotypes 1-4

Cell culture appears to be the most promising approach for evaluation of HEV infectivity. It can unambiguously demonstrate infectivity and avoids the disadvantages of using animals. Most importantly, the use of replicates and the simultaneous analysis of a higher number of samples can result in a higher degree of confidence of the results. Also, standardisation of

cell culture methods should be easier than that of animal experiments. If a CPE can be observed visually, the assay should be relatively simple to perform.

However, in some cell lines, CPE is either not produced or can take extensive time to develop. As most of the studies reporting a strong CPE during HEV replication are of older date, this effect should be carefully re-examined. If no CPE is present, HEV replication in the cells has to be monitored by e.g. RT-qPCR or immunofluorescence staining, which is more laborious. Generally, most cell culture assays described for HEV are still time-consuming and have therefore to be optimized. Although some cell lines (e.g. PLC/PRF/5 and A549) have been used successfully in several independent studies, none has been validated as repeatable and reproducible by extensive interlaboratory trial. In addition, only very few studies have shown applicability of cell culture techniques for measurement of infectivity in food samples.

Table 4.2 summarises the advantages and disadvantages of cell culture for testing infectivity of HEV genotypes 1-4.

Table 4.2. The advantages and disadvantages of cell culture for testing infectivity of HEV genotypes 1-4

Advantages	Disadvantages
Can demonstrate actual infection High potential for replication and for use with large sample numbers Higher statistical significance Relatively easy to standardize No ethical approval required	Further development necessary for more rapid assays Adaption for use with food samples necessary Interlaboratory reproducibility to be demonstrated

5. Cell culture for potential surrogate viruses

There are several potential surrogate viruses for human pathogenic HEV (Johne *et al*., 2014a), including HEV strains from animal species, and porcine teschovirus. Some of these potential surrogates can be propagated using cell culture, and might be used to assess HEV survival and elimination. The validity of using surrogates in e.g. Norovirus research has been challenged however (Knight *et al.*, 2016; Richards),

5.1 Cutthroat trout HEV

An HEV strain infecting cutthroat trout was first identified by Batts *et al*. (2011). The virus had previously been isolated from farmed trout, and could be grown on the Chinook salmon embryo (CHSE-214) cell line, producing a strong CPE after ~14 dpi. Debing *et al*. (2013) subsequently passaged cutthroat trout HEV nine times, and obtained a strain which could produce CPE at 9 dpi. The assay was used for testing sensitivity of the virus against hormones.

5.2 Ferret HEV

Li *et al*. (2016b) found that ferret HEV isolated from ferret stool samples could infect PLC/PRF/5 cells, with viral RNA being detected in the culture supernatant 32 dpi. However, no cytopathic effect was observed even after 225 dpi.

5.3 Rabbit HEV

Rabbit HEV strains could be propagated on A549 and PLC/PRF/5 cells (Jirintai *et al*., 2012). Virus replication was monitored using RT-PCR. On both cell lines, HEV RNA could be detected in the culture medium after 2 dpi after inoculation with HEV-infected rabbit liver homogenates containing ~$3 \times 10^6 – 2 \times 10^7$ HEV GC ml^{-1}. No CPE was evident.

5.4 Rat HEV

Jirintai *et al*. (2014) established cell culture systems for rat HEV strains. Liver homogenates containing rat HEV strains (~$2 \times 10^5 – 3 \times 10^7$ GC per culture well) were used to inoculate A549, HepG2, HuH-7, and PLC/PRF/5 monolayers. The cells examined daily for CPE, and the culture supernatants monitored for HEV RNA, over 32 days. In HepG2 cultures, HEV RNA was detected in the supernatant after 2 dpi. In Huh-7 and PLC/PRF/5 cultures, HEV RNA was detected in the supernatant after 2 – 8 dpi, depending on the virus strain. No HEV

RNA was detected in the supernatant of A549 cultures. No CPE was observed in any of the cultured cells at any time.

5.5 Porcine Teschovirus

Porcine teschovirus (PTV) is a picornavirus, which is excreted abundantly within swine herds (Jiménez-Clavero *et al*., 2003). The virus can readily be propagated on swine primary kidney (PK-15) cells showing a clear CPE. Jones and Mulhauser (2015) proposed its use as a surrogate for HEV studies. They modelled the survival of HEV on pork meat, by inoculating pork chops with PTV and storing the meat at 2°C over 8 weeks. They monitored infectious virus using PH-15 plaque assay and PTV RNA by qRT-PCR. They did not however simultaneously test HEV under the same conditions, and therefore the use of PTV as an HEV surrogate was not validated, i.e. by demonstrating similar survival patterns.

5.6 Summary

Table 5.1 summarises the information from the use of cell culture to detect potential surrogate viruses. As evident from the literature review, some of the HEV-like viruses could be propagated in cell culture systems. However, the data are based on very few experiments and the replication seems to be in the same range or slower than human HEV. The only two viruses producing a marked CPE are the fish hepevirus and porcine teschovirus, both of them are only very distantly related to human HEV.

Table 5.1. Summary of information from the use of cell culture to detect potential surrogate viruses

Surrogate tested	Cell line*	Parameter measured
Cutthroat trout HEV	Chinook salmon embryo	CPE
Ferret HEV	PLC/PRF/5	HEV RNA
Rabbit HEV	A549, PLC/PRF/5	HEV RNA
Rat HEV	A549, HepG2, HuH-7, PLC/PRF/5	HEV RNA
Porcine teschovirus	PK-15	CPE

***All cultures were 2D.**

5.7 Advantages and disadvantages of cell culture for testing infectivity of HEV surrogates

An advantage for the use of surrogate viruses would be that they are not pathogenic for humans, and therefore easier to handle than human HEV. However, it is not known how the potential surrogate viruses reflect the characteristics of human HEV. This is especially the case for the two viruses (fish HEV and porcine Teschovirus), which are very distantly related to human HEV. None of the more human HEV-related viruses could be grown as efficiently as human HEV. Also, no cell culture / surrogate system has been validated as repeatable and reproducible by interlaboratory trial.

Table 5.2 summarises the advantages and disadvantages of cell culture for testing infectivity of HEV surrogates.

Table 5.2. The advantages and disadvantages of cell culture for testing infectivity of HEV surrogates

Advantages	Disadvantages
Use of non-human-pathogenic viruses Probably rapid systems after further development	Similarity with human HEV questionable Efficiency of cell culture systems needs to be improved Standardisation necessary

6. Alternative methods for estimating HEV infectivity

An alternative method for estimating infectivity of a virus is the capsid integrity assay. In this assay, the virus preparation is treated with nucleases prior to nucleic acid extraction and PCR detection. By this, only nucleic acid which is protected by an intact virus capsid is detected, whereas free nucleic acid derived from damaged viruses is degraded and not detected (Knight et al., 2013). In the publication by Schielke *et al.* (2011), application of this technique to HEV is described. Prior to extracting HEV from suspensions prepared from a naturally infected wild boar liver, the authors incubated the suspension with RNase A for 1 h at 37^{o}C. They found that detectable HEV Gt3 RNA genome copies decreased by 74% (as compared with an untreated control suspension) after heating of the liver suspension at 56^{o}C 15 min, and by >99% after 30 min at 56^{o}C. They concurrently tested the suspensions using previously published cell culture systems (Huang *et al*., 1999; Emerson *et al*., 2005, and Tanaka *et al*., 2007), but virus replication in the cultures could not be shown even if the untreated samples were used. However, comparing the data with information obtained from similar experiments in the previous studies (Huang *et al*., 1999; Emerson *et al*., 2005, and Tanaka *et al*., 2007) indicated that the results could be comparable. Schielke *et al*. (2011) also investigated the effect of other temperatures, and long-term storage, on HEV survival using the capsid integrity assay; they considered that the correlation of capsid integrity with infectivity may not have been exact and thus HEV survival would have been overestimated, but considered that this would allow a conservative estimate of survival and elimination.

Methods for predicting infectivity where putatively infectious virus particles selectively bind to ligand "receptors" mimicking actual cellular binding sites for the virus, followed by detection of viral nucleic acid, have been proposed by several researchers, for use with viruses such as human Norovirus (see review by Cook *et al*., 2016). Such an approach has not yet been used for HEV, for which the cell receptor is not yet known, but the search for putative receptors (Yu *et al*., 2011) and receptor binding sites on the HEV capsid (He *et al*., 2008; Guu *et al*., 2009; Shuizhen et al., 2008; Yamashita *et al*., 2009) may allow its feasibility in future. Another technique which has not yet been reported as being developed for HEV is long-range RT-PCR. Here, the site of PCR amplification is set several kb along the genome from the site of reverse transcription priming. The hypothesis is that the further away the primer binding sites are from each other, the more likely it is that nucleic acid

strand breaks will occur between them, and this will prevent amplification from occurring. If amplification is successful, there are no breaks in the RNA strand, indicating that the viral genome may be intact and therefore the virus particle may be infectious.

6.1 Summary

Currently, only one study utilizing a capsid integrity assay has been published for HEV. It has not been validated against infectivity assays. Other infectivity proxies, such as binding to receptor ligands, or amplification of long regions of the HEV genome by long-range RT-PCR, have not yet been reported.

6.2 Advantages and disadvantages of alternative methods to detect infectious HEV

An advantage of alternative assays would be that they may be easier and faster to perform as compared to infectivity assays. However, all of these assays have to be validated against assays that truly test for infectivity, which has not been done so far.

Table 6.1. The advantages and disadvantages of alternative methods to detect infectious HEV

Advantages	Disadvantages
easier and faster to perform than animal models or cell culture	Provides only an indication of the potential of detected virus to be infective Requires validation against actual infectivity assays to be fully reliable

7. Conclusions

The successful experimental infection of animals with HEV has been repeatedly described. In this model, infectivity is determined by measurement of HEV shedding, viremia or seroconversion of antibodies. The most effective of the animal models appears to be the intravenous inoculation of pigs, as it has been shown to work with preparations derived from food. The advantage of the system is its successful demonstration of its use in HEV stability studies with food samples. However, as the pig model is very laborious, time-consuming and expensive, the potential for extensive replication, especially when testing several specific parameters, is very low. This will limit the statistical significance of the findings, but , the system could be readily applied for studies requiring only low sample numbers and where a quick answer regarding whether infectious HEV is present is a sample is required.. Finally, use of animals in research carries ethical implications.

Many scientific publications report the successful use of cell culture models for isolation and propagation of HEV. Cell lines PLC/PRF/5, A549 and HepG2/C3A have been most often used. Cultures have been used in 2D or 3D format, but there is not enough current information to determine which format is most effective. The occurrence of a CPE, which would simplify the performance of the method, is controversially described in the literature. Only in two cases, cell cultures have been successfully used for analysis of HEV infectivity in food samples so far, and the sample treatment procedures based on homogenisation and filtration are crude and may not be fully reproducible. However, cell culture methods avoid most of the disadvantages described for the animal inoculation experiments. Most importantly, the possibility to use replicates and to do simultaneous analysis of a higher number of samples can result in a higher degree of confidence of the results. Also, standardisation of cell culture methods should be easier than that of animal experiments. However, most of them have not been tested with regard to reproducibility and have not been validated in other laboratories so far. In addition, assay optimization, and definition of sensitivity and specificity, may be necessary before application for testing of food samples.
There are a number of HEV-related viruses which have the potential to be used as surrogates for HEV. The use of these viruses would make analyses easier, because no human pathogenicity would be present, and therefore no high biological containment (i.e. Biosafety Level 3) would be necessary. In addition, the rodent viruses may enable the development of suitable rodent infection models, which are easier and less expensive than pig inoculations.

However, only limited studies have been performed on animal inoculations and cell culture propagation of those viruses so far. Most of the developed techniques do not perform better than that developed for human HEV. The major disadvantage of the surrogate viruses is that it is not known how well they resemble the distinct characteristics of human HEV.

Only one, recent, publication reports the use of a capsid integrity assay as an alternative method for infectivity estimation. Although alternative methods may allow a more easy and rapid analysis of samples, they have to be validated against real infectivity assays first. This has not been done so far.

No study has directly compared the various models against each other, to validate or determine any agreement between them. Also, the suitability of the assays for infection with a range of virus suspension dilutions has not been demonstrated in most cases, which may limit their immediate use for studies on kinetics of virus inactivation.

8. Recommendations

No specific method has been shown to be fully effective for measuring infectivity of HEV in foods, and despite the thorough review of methodologies presented above, it is not possible to definitively recommend any specific system for this purpose.

For urgent analyses of selected sample types, the pig inoculation model may be used. This model has been shown to be suitable for food sample analysis but only a few samples can be analysed, thus limiting this approach.

For surrogate viruses, no clear advantage in comparison to the use of human-pathogenic HEV could be demonstrated so far. Alternative approaches such as capsid integrity offer as yet only indicative assessment of viral infectivity. Before they can be adopted, both of these approaches need first to be validated against infectivity assays with human HEV. Therefore, the development of those techniques should be postponed until an efficient assay for human-pathogenic HEV is available.

By comparison of the published methods for assessing HEV infectivity, cell culture seems to be the most promising technique. However, before a broad application of cell culture for systematic analysis of food samples, several efforts should be made in order to optimize and standardize the system. This should include selection of optimal cell lines and HEV strains. A cell line / cell culture-adapted HEV strain which produced a CPE would be the ideal, but pragmatically, detection of multiplying virus will need to be achieved by more technical methods. RT-qPCR can be used to monitor increase in viral genomes after infection (but is not suitable for quantitative determination of the starting number of infectious viral units). The recommended technique is immunofluorescence staining of infected cells – the focus-forming units (ffu) can be counted visually.

It is therefore recommended that a cell culture-based method for assessing HEV infectivity in pork products is developed and validated. This recommendation mirrors that made as the top research priority ("Development and validation of direct and indirect methods for assessment of HEV infectivity") for HEV during the FSA-EFSA workshop on foodborne viruses, February 2016. This method development and validation should be carried out as follows:

Promising cell lines e.g. PLC/PRF/5, A549/D3 and HepG2/C3A, with cell-culture adapted HEV strains such as gt3 47832c (GenBank acc.-no. KC618403) or gt 3 Kernow-C1, (GenBank acc.-no. JQ679013), should be evaluated in an interlaboratory trial, to select a system which can repeatably and reproducibly allow a quantitative determination of HEV infectivity over a range of virus concentrations.

The assay should be harnessed to an efficient sample treatment procedure which can deliver intact and infectious HEV to the cell culture. Sample treatments which have been previously reported for such use are basic, involving only homogenisation and filtration, resulting in crude extracts which may not be reproducibly effective in all instances. A more effective procedure should be developed involving extraction stages, perhaps similar to the approach used to extract Norovirus and hepatitis A virus from berry fruits (Anonymous, 2013). It will be essential to have a recovery rate allowing at least a 3-log range of HEV to be studied, and to produce an extract containing suspended virus particles free of food-derived substances which can inhibit cell culture.

The developed method (sample treatment + cell culture-base infectivity assay) should be validated as repeatable and reproducible by interlaboratory trial.

The final method can then be used in studies to determine the effect of heat or other elimination processes on HEV in pork meat and products, or to assess the infectivity of HEV detected in surveyed foodstuffs.

Annex 1: How the review was performed

The project commenced on 28th January 2016 with an inception teleconference involving all partners and the FSA. The list of proposed keywords and search terms to be used in the literature search were discussed and agreed; a few amendments to this list were made following on from this meeting. The timescale of papers to be included was proposed to be from 1991 up until current publication (spanning 25 years of literature).

The databases searched on Web of Science were:

- WoS Core Collection
- BIOSIS
- CABI
- Current Contents
- FSTA
- KCI-Korean
- MEDLINE
- Zoological Record

Science Direct: All sciences

Additional Grey Literature sites:

- Royal Society of Chemistry
- USA National Technical Information Services (NTIS)
- OpenGrey
- SIGLE (European grey lit)
- Copac
- CORDIS
- OAIster (worldcat)
- Defra
- Wageningen Library

The search terms used in Web of Science were grouped into:

“hepatitis E virus”, HEV excluding “hendra virus” and "hemorrhagic enteritis" with: “Avian” or “Ferret” or “Rabbit” or “Rat” or “cattle” or “cow” or “sheep” or “lamb” and “Infectivity” or “Infectious”

“hepatitis E virus”, “HEV” excluding “hendra virus” and "hemorrhagic enteritis" with: “In vivo testing” or “Experimental infection” or “Animal model” or “HEV bioassay” or “Long Range PCR” or “Capsid Integrity” or “Receptor binding” or “Persistence” or “Stability” or “Food”

“hepatitis E virus”, HEV excluding “hendra virus” and "hemorrhagic enteritis" with: “Pork products” or “Swine” or “Hogs” or “Cooked pork products” or “Raw pork products” or “Sausages” or “Liver” or “Cured pork products” or “Game” or “Deer meat” or “Wild boar meat” all with “survival” or “detection”

“hepatitis E virus”, HEV excluding “hendra virus” and "hemorrhagic enteritis" with: “Shellfish” or “Bivalves” or “Oysters” or “Mussels” or “Clams” all with “survival” or “detection”

“hepatitis E virus”, HEV excluding “hendra virus” and "hemorrhagic enteritis" with: “Berries” or “Lettuce” or “Vegetables” or “Fomites” or “Surfaces” all with “survival” or “detection”

“hepatitis E virus”, HEV excluding “hendra virus” and "hemorrhagic enteritis" with: “Tissue culture” or “Cell culture”

NOTE the following terms were not used as they would show up under “tissue culture” and “cell culture” search term:

“Mammalian cell culture”

“In vitro cell culture”

“2D tissue culture”

“3D tissue culture”

Total number of references found: 1002

The Science Direct search terms were similar but also had the term “battery” excluded, producing 263 results once duplicates were removed.

The grey literature search produced 82 additional references.

Screening of the abstracts in the database led to the rejection of 1154 papers which described work that was not relevant, for example studies with a purely clinical focus, general reviews, and studies using transfection of cultured cells with HEV RNA. Papers were considered eligible for inclusion if they directly described development of HEV infectivity assays, or studies where HEV infectivity assays were utilised.

4 references were unavailable.

43 additional papers were identified as relevant (mostly background information or publications mentioned in the selected papers but which were not identified in the literature search), and included in the draft review..

A presentation containing summarised initial information from the ongoing review was given at the FSA-EFSA workshop on foodborne viruses, February 2016. At this meeting, the experts present were asked to vote on a list of research priorities. “Development and validation of direct and indirect methods for assessment of HEV infectivity” received the most votes, and was thus identified asthe primary research need forfoodborne viruses. It was agreed that this would be used more for research into survival and elimination of the virus, e.g. effect of heat and disinfectants, rather than as a routine analytical tool, e.g. for food testing. Animal models for HEV infectivity were deemed to be both costly and liable to ethical concerns. It was agreed at the meeting that cell culture was the most promising approach for measuring HEV infectivity. It was also considered that proxy infectivity assays such as capsid integrity would require validation against cell culture before adoption. This consensus was reflected within the current critical review, and consequently the recommendation that cell culture would be the most effective approach for an HEV infectivity assay.

A second teleconference was held on 29th February 2016, to discuss the findings of the review to date, and to make a summary identification of technology gaps. These were discussed by approach, and summarised as:

Animal models

- No large sample size / numbers can be handled
- Statistical issues
- Not known why oral inoculation is ineffective, and animals must be inoculated

Cell culture

- Validation of existing / promising cell culture systems needs to be done
- Sensitivity of cell cultures needs to be determined

- No existing cell culture system for HEV shows a reproducible cytopathic effect (CPE) after infection with the agent, and techniques such as immunofluorescence must be used to show the presence of the multiplying virus. This makes the assay more complicated.
- Existing cell culture assays are slow, taking up to three weeks to result, and should be optimised to allow more rapid results

Proxy assays

- No validation against cell culture, which could lead to overestimation of number of infectious virus

A third teleconference was held on 19th April 2016, to review project progress, and discuss a possible recommendation of a feasible approach to application of an HEV infectivity assay suitable for use on food samples. It was agreed that a cell culture-based method will be ideal, if it is repeatable and reproducible, and is harnessed to an effective procedure for extracting infectious HEV from food. It was discussed that the final report would be likely to recommend that promising cell lines and HEV strains are evaluated in an interlaboratory trial, to try to identify a repeatable and reproducible HEV infectivity assay. Likewise, an extraction method for delivering the virus to the infectivity assay should also be elaborated and ring-trialled. Meanwhile, the use of pigs as an animal model should be kept under consideration for small-scale studies.

Subsequent to the third teleconference, the initial draft of the review was prepared by Fera, Nigel Cook drafting Sections 2, 3, 5 and 6, and Martin D'Agostino drafting Sections 4 and 6. The draft was then completed by Professor Johne. It was then sent to the Food Standards Agency, for comment. When the comments were received, the project partners held a teleconference to discuss response to the comments, and to plan the completion of the review.

During the final revision, 3 further additional publications were added.

Finally, 148 publications were reviewed for the Report.

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