

INTRODUCTION

CHAPTER 1

Viruses occupy a unique position in biology; unlike most bacteria, fungi, and parasites, they are obligate intracellular parasites that depend on the biochemical machinery of the host cell for replication, therefore viruses cannot capture and store free energy, and they are not functionally active outside their host cells. Viruses are nonliving infectious entities that can be said to lead a kind of borrow life, becoming part of living system only after they have infected a host cells and their genome become integrated with that of the cells. Looking back to the history, viruses were described as “filterable agents” for their small size allowing them to pass through filters that are designed to retain bacteria. By this principle, Martinus Beijerinck was the first to discover the tobacco mosaic virus in 1899, and since then more than 6,000 types of viruses have been classified in 1,950 species and in more than 391 different higher taxa edited by the International Committee on Taxonomy of viruses (ICTV), a body empowered by the International of Microbiological Societies to have authority on matters of virus classification and nomenclature containing succinct and accurate information about all virus species: their taxonomic position, morphology, genome organization and replication, antigenic properties, and biological properties (Van Regenmortel, 2003; Fauquet *et al*, 2005; Mayo and Ball, 2006). Further, it is available an accessible Genbank system (part of International Nucleotide Sequence Database Collaboration, comprising the DNA Databank of Japan, the European Molecular Biology Laboratory, and Genbank, which is located at the National Centre for biotechnology Information (NCBI) in the U.S. National Institutes of Health) that allows molecular biologist and virologists world-wide to search all viruses and to identify new sequences and new virus names. The Genbank system contains currently 3,142 “species” of viruses not present in the ICTV current master list of 2005, but their sequences are collected in this system (Fauquet and Fargette, 2005).

The past 33 years have seen a rapid acceleration in the impact of scientific and technological progress. This situation, which is unprecedented in the history of mankind, also goes hand in hand with the globalisation process affecting communications, international trade and the economies of different countries and

regions of the world (Annual reports of OIE reference laboratories and collaborating centres, 2006). The world organization for animal health (OIE) was established in 1924 and in 2008 comprised 172 Member Countries and Territories (Manual of diagnostic tests and vaccines for terrestrial animals, 2008), is the international standard-setting scientific organization concerned with matters relating to the health and welfare of terrestrial and aquatic animals, has fully embraced these changes. The aim of OIE is to collect, analyse and disseminate relevant information on the diagnosis, control and surveillance of animal disease through its four Specialist Commission, including Biological Standard Commission, Reference Laboratories and Collaborating Centres and international renowned expert by them reaches its decisions on solid scientific grounds, and incorporates them into its *Codes* and *Manuals* of standards. In order to reach out the target it needs increasing the accuracy, transparency and speed of animal disease, including zoonosis reporting (Annual reports of OIE reference laboratories and collaborating centres, 2006). Disease like Hepatitis E Virus (HEV) to be considered at international level and included in the OIE *List* need at least one 'yes' answer, meaning that the criterion has been met, to the following basic criteria Parameters (**Fig.1**):

- International Spread
- Significant Spread within Naïve Populations
- Zoonotic Potential
- Emerging Diseases

After considering these parameters it is clear that potentially infection by HEV satisfies most of all of these criteria. The other side, epidemiology aspects, pathobiology, real potential infection transmission route, as direct and indirect contact human to human, are not at all clear. It needs understand more deeply our knowledge already acquired in order to analyses risk in the animal and meat trade in front of animal and public health concern in zoonotic way.

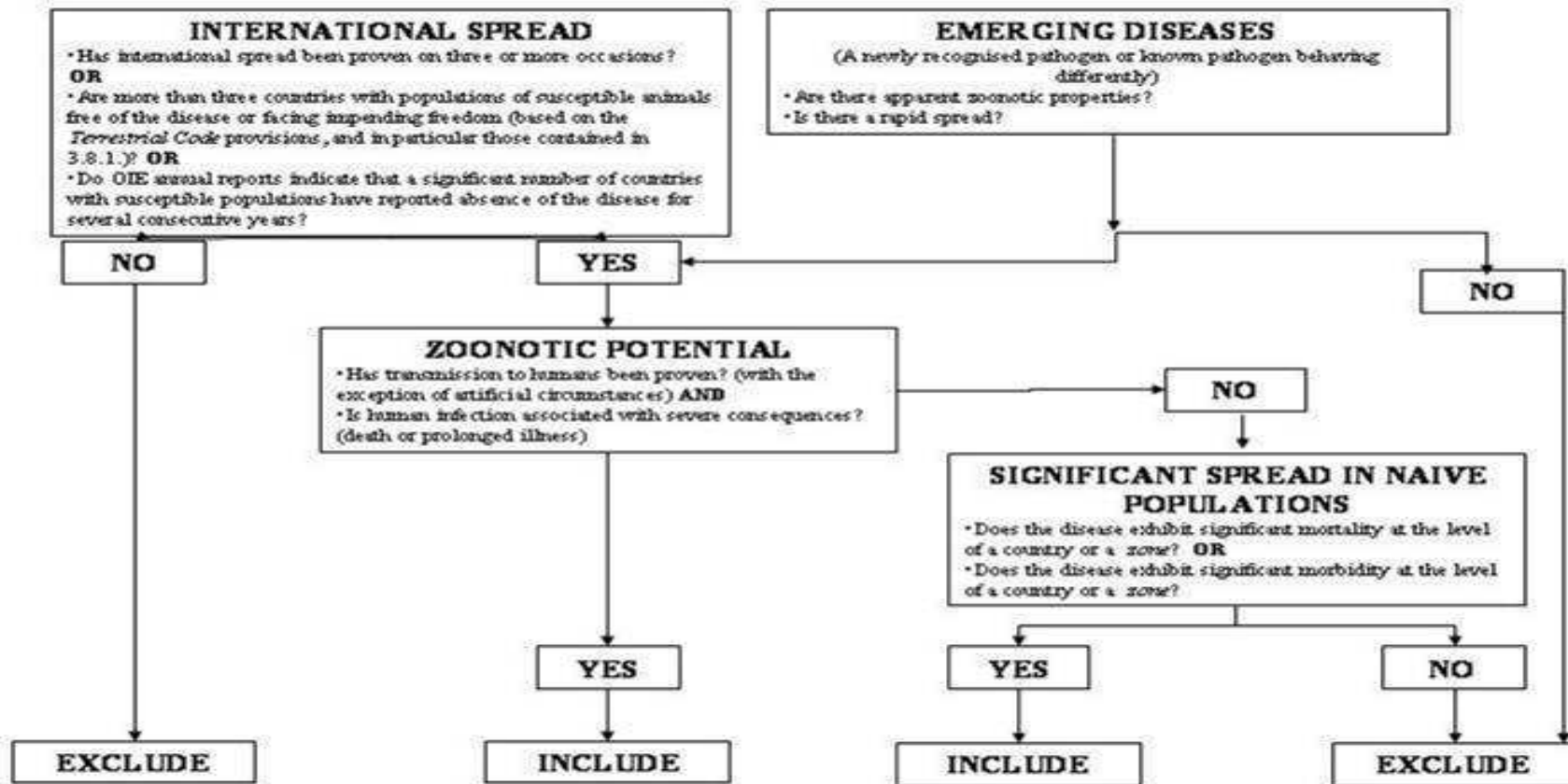


Fig.1. the criteria described above are applied according to the decision-making model shown below: (Article 2.1.1.2. of TAHSC).

INTRODUCTION

CHAPTER 1.1

HISTORY

Hepatitis E is an infectious viral disease with clinical and morphological features of acute hepatitis observed in young, middle-aged adults and among pregnant women, and also recently reported in kids (youngest ever reported case: 7 years old boy in India) (Thapa, 2009). Hepatitis E is typically a self limited, acute viral hepatitis lasting 1-4 weeks; it does not progress to chronic disease. In rare cases, some patients have severe disease, which progresses to fulminant liver failure; the overall case fatality rate for the general population in disease-endemic countries ranges from 0.1 to 4%. Case fatality rates are much higher (up to 25%) among pregnant women infected with HEV during the third trimester (Krawczynski, 2007). The substantial morbidity is associated with large epidemics of hepatitis E disease initially described from the Indian subcontinent where in the winter 1955-56 a large epidemic of acute viral hepatitis, more than 29 000 icteric cases, resulted from contamination of a major water treatment plant with raw sewage in New Delhi, India (Vishwanathan, 1957; Chuttani *et al.*, 1966). The cause was originally considered to be an example of water-borne hepatitis A. Nevertheless, several other epidemics occurred in Europe and United States in the 18th and 19th centuries with epidemiological features similar to acute viral hepatitis (Cockayne, 1912; Blumer, 1923) as reported from Asia Indian subcontinent indicated an epidemiological pattern distinct from that observed for HAV (Khuroo *et al.*, 1980; Khuroo *et al.*, 1983). The disease was first recognized as a distinct clinical entity in the 1980s when retrospective serological tests performed on stored clinical samples collected during water-borne epidemics in New Delhi during 1955-56 and another epidemic in Kashmir, were found to lack serological markers for acute hepatitis A and B (Wong *et al.*, 1980), suggesting that a new viral hepatitis agent was responsible for the epidemic. At the beginning the disease was classified in the group of enteric non-A non-B hepatitis (ET- NANBH) regarding all viral hepatitis

resulting from viruses other than HAV or HBV, other well-characterised viruses, or predisposing conditions referred to collectively as non-A non-B hepatitis.

The first identification of virions by Transmission Electronic Microscopy (TEM) was detected since middle of 70' by Feinstone (Feinstone *et al.*, 1975) like possible cause of ET-NANBH and temporarily classified in *Picornaviridae* family like HAV type II (Panda *et al.*, 2007). The first proof viral etiologic and faecal route of transmission of this form of hepatitis was obtained in 1983 (Balayan *et al.*, 1983) when an investigator deliberately ingested pooled stool extracted from presumed case of ET-NANBH in Russia 1983 and virions were visualized by TEM (27-34 nm of diameter) in stool samples collected in preclinical and early post clinical phases. The disease was subsequently transmitted to *cynomolgus macaques* (*cyno.*) by intravenous inoculation of virus containing stool extract, which excreted similar viral particles in the faeces.

In 1990, Reyes cloned the genome, of 7.6Kb by infected cyno's bile, after inoculation of human faecal material from Burma's patient (Reyes *et al.*, 1990). The genome Burma strain, first identified, sequenced the following year was antigenically and biophysically unrelated to the picornaviruses (Arankalle, 1988). It was classified in the new member of *Caliciviridae* family under the separate genus *Hepevirus*, this name deriving from the sigla of Hepatitis E virus (Tam *et al.*, 1991), and currently it is the sole member of *Hepeviridae* family (Fauquet *et al.*, 2005). After two distinct isolates first recognized and designated as Burma and Mexico strains, others from Sargodha, Pakistan, China etc. have subsequently been sequenced, although the majority of HEV isolates have only been sequenced partially.

The disease became known as hepatitis E and its agent as HEV. The syllable 'E' can describe the tree features of the epidemiology of HEV: 'enteric' (in the gut), 'epidemic' like in tropical or subtropical areas: Asia and Middle East, northern and western parts of Africa and North America (Mexico) (Krawczynski, 1993; Aggarwal and Krawczynski, 2000) transmitted primarily by the faecal-oral route associated with poor sanitation and weak public health infrastructures or 'endemic' in much of Asia and Africa and Latin America, where it causes substantial morbidity and mortality, (1% of general population). Hepatitis E predilection is for older men and women during pregnancy (up to 25%) (Hamid *et al.*, 1996) in developing countries, but it represents a public health concern worldwide (Khuroo *et al.*, 1981; Suzuki *et al.*, 2002; Emerson and Purcell, 2003; Khuroo *et al.*, 2003; Okamoto *et al.*, 2003; Dalton

et al., 2008). Industrialised countries where the sanitation systems are well established are considered traditionally non-endemic for HEV, except possibly rare disease cases travel-associated to countries where HEV is hyperendemic. However, autochthonous cases of sporadic hepatitis E in people with no pre-illness history of recent travel abroad have been reported in many developed regions such as North America, Europe like England and Wales, Spain, France, Netherlands, Germany, Austria, Italy, Greece even in Japan, Taiwan, New Zealand and Australia (Chapman *et al.*, 1993; Psychogiou *et al.*, 1995; Heath *et al.*, 1995; Worm *et al.*, 1998; Hsieh *et al.*, 1998; Zanetti *et al.*, 1999; Tsang *et al.*, 2000; Pina *et al.*, 2000; Teich *et al.*, 2003; Widdowson *et al.*, 2003; Mansuy *et al.*, 2004; Sainokami *et al.*, 2004; Ijaz *et al.*, 2005; Dalton *et al.*, 2007a;). The source of HEV infection in industrialized countries is not known. Serological tests have detected anti-HEV antibodies in healthy individual of developed countries ranging from 0.5% and up to 25% in some areas depending on the geographic location. The higher rates suggested that subclinical or unrecognized infection may be common (Thomas *et al.*, 1997; Zanetti *et al.*, 1994; Aggarwall and Krawcznski, 2000; Dalton *et al.*, 2007b).

Since the 90', the prevalence of anti-HEV antibodies has been detected also in a wide range of domestic and feral mammals including: monkeys, swine, rodents, chickens, dogs, cats, cattle, sheep, goats, horses, donkeys and mice. Increasing evidence supports the hypothesis of a zoonotic infection, as long as the animal can be infected by virus like-HEV and be the source or reservoir of infection for human beings (Arankalle *et al.*, 2001; Banks *et al.*, 2004a; Meng *et al.*, 2002; Hirano *et al.*, 2003b).

In 1997 a novel virus was first identified in pigs, in the Midwestern United States, characterized and designated swine hepatitis E virus (swHEV) to distinguish it from human hepatitis E virus (hHEV) (Meng *et al.*, 1997). However, two cases of acute clinical human hepatitis E - first case (US-1) involved a patient who had not been in endemic countries, second case (US-2) that had travelled to Mexico prior diagnosis of the disease were reported in the same area, caused by virus strains very closely genetically and phylogenetically related to the swine HEV recovered from pigs in the same country and differing extensively from other strains of HEV (Meng *et al.*, 1998). Since then, in many parts of the world several other porcine strains have been identified and characterized, sharing high sequence identity at the nucleotide level and at the aminoacid level with human HEV strains belonging to the same

geographic location (Hsieh *et al.*, 1999; Banks *et al.*, 2004a; Buti *et al.*, 2004; Caprioli *et al.*, 2007; Goens and Perdue, 2004; Peron *et al.*, 2006; Zheng *et al.*, 2006).

Balayan's group first demonstrated that not alone *cynomolgus monkeys* and *rhesus monkeys* resulted successfully infected with a hHEV isolate from adult patient, but also domestic pigs (*Sus scrofa domestica*) were reported to be susceptible to infection with a hHEV strain (Balayan *et al.*, 1990). Experimental infections showed that swine HEV can cross species barriers and infect non-human primates and that US-2 strain of hHEV could infect specific-pathogen free (SPF) pigs (Meng *et al.*, 1998; Halbur *et al.*, 2001; Williams *et al.*, 2001; Meng *et al.*, 2002; Banks *et al.*, 2004a). Following studies assessed the potential risk of infection in swine veterinarians, and pig handlers resulted highly positive for anti-HEV compared to control subjects (Drobeniuc *et al.*, 2001; Meng *et al.*, 2002; Withers *et al.*, 2002; Siochu *et al.*, 2004). The first compelling evidence for zoonotic food-borne transmission was obtained from clusters of cases in Japan related to ingestion of meat at shared meal of raw Sika deer meat (Tei *et al.*, 2003; Takahashi *et al.*, 2004) or undercooked pork liver (Matsuda *et al.*, 2003; Tamada *et al.*, 2004). Of interest, swHEV strain (swJL145) isolated from a packaged pig liver purchased from local grocery stores in Japan was 100% identical to the virus recovered from an 86-year-old patient who had contracted sporadic hepatitis E after ingestion of undercooked pig liver/intestine few weeks before onset of the symptoms of disease. This is a confirmation of potential risk factor for HEV infection (Yazaki *et al.*, 2003). Recently, presence of HEV genome has been reported also in commercial pig livers in the United States (Feagins *et al.*, 2007).

In 2001 another animal strain of HEV, designated as avian HEV (aHEV) to distinguish from mammalian HEV, was firstly discover from bile samples of chickens associated with Hepatitis-Splenomegaly (HS) syndrome in the USA (Hasquenas *et al.*, 2001). Although the aHEV strain is related genetically and antigenically to hHEV and swHEV, it apparently does not infect humans and experimental infection in *rhesus macaques* and mice failed (Huang *et al.*, 2004; Meng *et al.*, 2009), whilst it was able via oronasal route inoculation to infect specific-pathogen-free (SPF) chickens and turkeys (Sun *et al.*, 2004; Billam *et al.*, 2005).

Nowadays HEV disease is considered to be an emerging zoonosis (Péron *et al.*, 2006).

1.2 General Features

Hepatitis E virus consists of a 7.2 Kb positive stranded (+) RNA polyadenylated viral genome packaged within a non-enveloped capsid with icosahedron symmetry varying between 27-34 nm in diameter (Fauquet *et al.*, 2005), and believed to be composed of a single capsid protein (Fig.1.1). The variable size of virions depends from the laboratory where it has been identified, possibly depending on proteolytic digestion in the passage through the gut and on its sensitivity to freeze-thaw cycles or storage of stool preparation. The buoyant density of HEV is 1.35 g/cm^3 in CsCl and 1.29 g/cm^3 in potassium tartrate and glycerol gradient and sedimentation coefficient computed is found $\sim 183\text{S}$; sometimes the HEV particle was found to sediment at 165S (Fauquet *et al.*, 2005; Panda *et al.*, 2007).

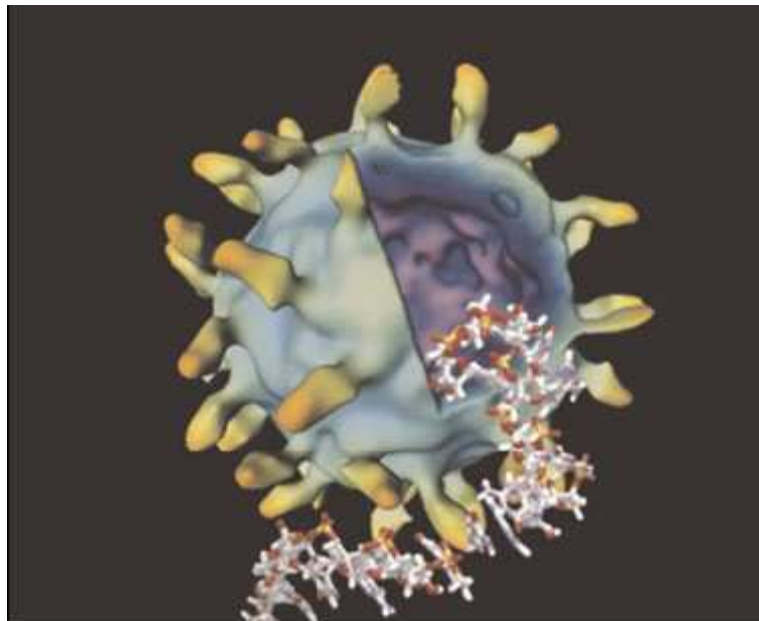


Figure 1.1. Hepatitis E Virus Particle. The three-dimensional structure of a self-assembled, recombinant HEV particle has been solved to 22\AA resolution by cryo-electron microscopy and three-dimensional image reconstruction (adapted from Cheng *et al.* <http://www-ucdmag.ucdavis.edu/current/23:4;2006>) (Panda *et al.*, 2007).

The viral genome encompasses three open reading frames (ORFs: ORF1, ORF2 and ORF3 flanked by short untranslated region) encoding respectively the non-structural polyprotein (186KDa), the major capsid protein (72KDa) and an immunogenic small protein with an unidentified role (~ 13.5KDa) (Zafrullah *et al.*, 1997; Ansari *et al.*, 2000). The ORF2 of HEV has been expressed using various expression systems including *Escherichia coli*, insect cells using baculoviruses, and animal's cells using transfection. Baculovirus expression system revealed multiple forms of pORF2 ranging in size from 72 to 52KDa, of which the 50-53 KDa forms are secreted as virus-like particles (VLPs), which are labile, like Calicivirus, being degraded following high speed pelleting in sucrose.

HEV does not seem to tolerate exposure to high concentration of salts (including caesium chloride), but is almost certainly resistant to changes of pH because it is able to survive in the gastrointestinal environment (Zafrullah *et al.*, 2004; Panda *et al.*, 2007).

In literature, three studies have been reported related to the thermal stability of HEV (Emerson *et al.*, 2005; Feagins *et al.*, 2008), but before them there was another single report of HEV (isolated in Guangzhou, China) being inactivated by heating at 56°C for 30 min, but time course or range of temperature tested was not reported, and the results have not been confirmed (Huang *et al.*, 1999). The first study compares the thermal stability of three HEV strains, belonging to three different genotypes of HEV, between them and toward a strain of HAV. Range of incubation's temperatures used was 0°C to 70°C for 1h before being used for infecting the hepatoblastoma cell line Hep G2/C3a. The first strain tested was Akluj strain, belonging to genotype III and collected from an Indian patient infected with HEV; a 50% of inactivation resulted at a temperature between 45°C and 50°C, and almost all the virus was inactivated at 56°C. To determine the rate of inactivation, the Akluj strain was heated at 56°C for 0, 15, 30, 60 min and 60°C before inoculation onto the cells. The second strain, Mex14, belonged to genotype II and was collected from an experimentally infected *rhesus macaque*, and was not inactivated by incubation at 56°C being almost totally inactivated at 60°C (80%). The last strain, SAR55 collected from a Pakistan patient, belongs to genotype I, and resulted to be inactivated by approximately 50% at 56°C, and 96% at 60°C. The HAV HM175 strain (previously reported to be relatively stable at 60°C) was only 50% inactivated by incubation at 60°C and almost totally inactivated at 66°C.

In the second study (Feagins *et al.*, 2008) Meng's group tried to inactivate by traditional cooking methods a genotype III HEV that contaminated commercial pig livers sold in United States grocery stores. Since a reliably successful cell culture system for HEV propagation is not available, an experimental infection was conducted on five groups of pigs (SPS) involving negative and positive control, inoculated intravenously with pool of homogenates of two HEV-positive livers incubated in three different ways and times: 56°C in a water bath for 1h, stir-fried at 191°C (internal temperature of 71°C) for 5 min or boiled in water for 5 min, respectively. The results demonstrated that incubation of homogenates of the contaminated pig livers at 56°C for 1h did not inactivate the virus confirming *in vitro* results of Purcell's group.

In the third study, HEV (JE03-1760F strain) in faecal specimens, obtained in the acute phase from a 67-year-old Japanese patient with chronic renal failure who contracted domestic infection of genotype III HEV in 2003, was inactivated by heat-treated at following temperatures: 56°C for 30 min, 70°C for 10min, 95°C for 1 min, 95°C for 10 min before infecting 21 different cell lines, including PLC/PRF/5 (human hepatocarcinoma cells) and A549 (human lung carcinoma cells). The results corroborated previous reports: HEV at 56°C HEV was still infectious and could be propagated in two of the 21 cell lines, but for the other temperatures considered HEV was not detectable in the culture medium throughout the observation period of 50 days after inoculation (Tanaka *et al.*, 2007).

1.3 GENOME ORGANISATION

The HEV viral genome consists of a single sense positive stranded RNA genome of approximately 7.2 Kb in length (Tam *et al.*, 1991; Fauquet *et al.*, 2005). The entire molecular sequence shows: a 7-methylguanosine cap (m7G) at the 5' end carrying a short non-coding region (NCR) of 27-35 nucleotides (nt) forms a hairpin structure possibly involved in virus replication; the following encoding region that consists of three open reading frames (ORFs: ORF1, ORF2, ORF3), and the end at 3' with terminal 65-74 nucleotides comprising another NCR that terminates at a polyadenylated tail with approximately 150-200nt long implicated in the initiation of

virus replication (Okamoto *et al.*, 2007). In experimentally infected *cynomolgus macaques* (Tam *et al.*, 1991) and in cell culture (Xia *et al.*, 2000), it has been shown the presence of one genomic RNA (~7.5Kb) and two subgenomic (~3.7Kb and ~2Kb) HEVs RNA (**Fig.1.2**).

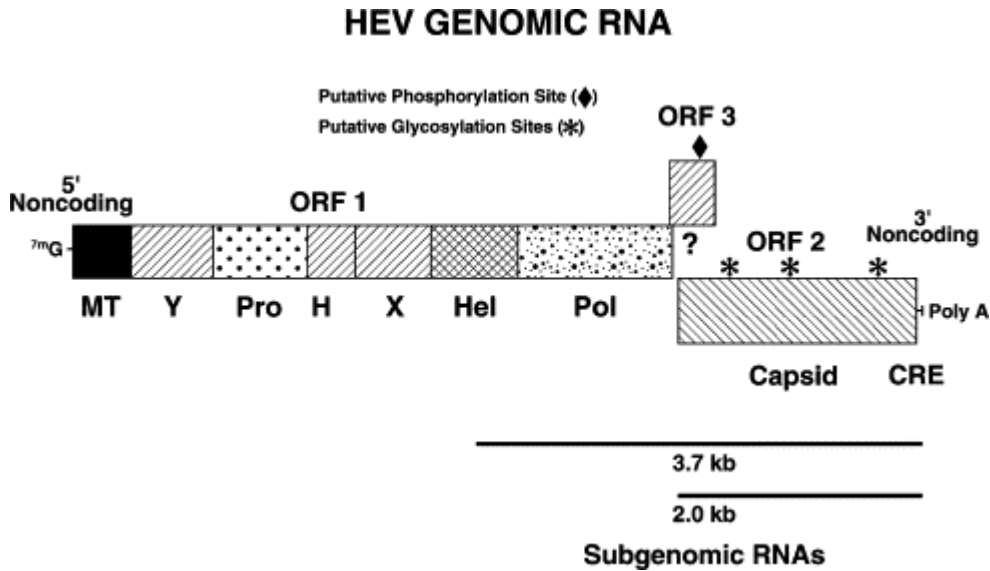


Fig.1.2. The genome of HEV consists of a single-stranded, positive sense RNA with a size of 7.2 kb. There are three open reading frames (ORFs) that encode the non-structural proteins, a small protein of unknown function and the capsid protein, respectively. The genome also encodes putative phosphorylation and glycosylation sites and contains a cis-reactive element (CRE). Two subgenomic RNAs were reported previously; the smaller of the two has been shown to express both ORF2 and ORF3 (Purcell and Emerson, 2008). Humoral immune response has been detected against all three ORFs (Aggarwall *et al.*, 2007).

1.2 ORF1 and viral encoded protein

The open reading frame one (ORF1) is the largest (5079nt) of three ORFs, begins after 28nt down of the 5'NCR of the viral genome and terminates at nucleotide position 5109 and encodes a 1693 aminoacid polyprotein including viral non-structural proteins such as methyltransferase, a papain-like cysteine protease, a

helicase and an RNA-dependent RNA Polimerase (RdRp) (Koonin et al., 1992; Krawczynski, 1993; Aggarwall and Krawczynsky, 2000; Magden et al., 2001).

1. METHYLTRANSFERASE

The Methyltransferase domain has been suggested by computer-assisted assignments to encompass an amino terminal domain between 60 to 240 aminoacids. The viral enzyme presents properties similar to members of the large alpha-virus like superfamily of positive-strand RNA viruses such as alpha-virus nsP1, brome mosaic virus replicase protein 1a, bamboo mosaic virus etc. This suggested that these viruses could have evolved from a common ancestor virus (Koonin and Dolja, 1993; Panda *et al.*, 2007). Downstream of methyltransferase domain there is: Y domain with 200 aminoacids showing similarity to rubella virus, but at the present no particular function is known (Panda *et al.*, 2007).

2. PAPAINE-LIKE CYSTEINE PROTEASE

A Papain-like protease domain follows the Y domain encompassing 440-610 aminoacids, and has been identified in other virus-like alphavirus and rubella virus and others like hepatitis C virus (HCV). It is postulated that this viral protease is involved in either co- or post-translational viral polyprotein processing to yield discrete non-structural gene products (Panda *et al.*, 2007). A conserved “X domain” of unknown function flanks the papaine-like protease domains, preceded by a proline-rich region “P” that might constitute a flexible hinge between the X domain and the upstream domains (Koonin *et al.*, 1992).

3. HELICASE

The Helicase domain, encompassing 960-1204 aminoacids of the full-length polypeptide, belongs to the typical Helicase superfamily and shows the highest overall similarity with the helicase of beet necrotic yellow vein virus. It promotes unwinding of DNA, RNA or DNA-

duplexes required for genome replication, recombination, repair and transcription (Panda *et al.*, 2007).

4. RNA-DEPENDENT RNA POLIMERASE (RdRP)

The RdRP domain, encompassing 1200-1700 aminoacids of the carboxy terminal part of ORF1, shows a conserved aminoacid motif recognised in all positive strand RNA viruses as the canonical Glicine-Aspartate-Aspartate (GDD). It has been observed that mutations in this motif (GDD to GAD) generate replication-deficient replicone unable to replicate or do so very inefficiently. RdRP spreads out a crucial role in replication binding to the 3'NCR of HEV directing the synthesis of the complementary strand RNA (Panda *et al.*, 2007). Several linear B-cell epitopes have been identified in the ORF1 protein, and appear to be particularly concentrated in the region of the RdRP (Kaur *et al.*, 1992).

1.3.2 ORF2 AND VIRAL ENCODED PROTEIN

The Open reading frame 2 consists of ca. 1980nt beginning downstream of ORF1 from 5147nt to 7124nt. Translation of this region produces the HEV structural polypeptide (pORF2) of 660/599 aminoacids (Okamoto, 2007), highly conserved. The 5' end of ORF2 region presents a range of approximately 350-450nt most conserved among HEV isolates; recently it has been used for classifying different subtypes of genotypes of HEV (Lu *et al.*, 2006). In animal cells, the major capsid protein is expressed in a ~74KDa form (pORF2) and a ~88KDa glycosylated form (gpORF2) that was immunoreactive with sera from chimpanzees infected with HEV (Jameel *et al.*, 1996). pORF2 is synthesized as an 82KDa precursor (ppORF2) cotranslationally translocated via N-terminal signal sequence to the endoplasmic reticulum (ER) membrane. The putative signal peptides consist of three regions an amino terminal region of 22 aminoacids stretch positively charged residues (Arg), a central hydrophobic core with 14-residues and the third region contains a turn-inducing stretch of proline residues, followed by the signal peptidase cleavage site. ppORF2 is processed by cleavage in the endoplasmic reticulum into the mature

polypeptide (pORF2), and then glycosylated (gpORF2) at N-linked glycosylation sites “Asn-X-Ser/Thr” (N-X-S-T) at residues 137, 310 (appear to be the major site of N-Glycan addition) and 561, attached to them as a core unit of oligosaccharides (Glc₃Man₉Glc-NAc₂) while the polypeptide chains are being translocated across the ER membrane (Zafrullah *et al.*, 1999). This process occurs usually for the synthesis of envelope proteins but is rare for capsid proteins. The glycosylation sites are conserved in the ORF2 sequences of all HEV isolates sequenced so far (Tam *et al.*, 1991; Huang *et al.*, 1992; Tsarev *et al.*, 1992) as well as in swHEV (Meng *et al.*, 1997). Mutations in the pORF2 glycosylation sites prevented the formation of infectious virus particles and resulted into low infectivity in macaques (Graff *et al.*, 2008). The 88KDa gpORF2 obtained is transported to the cell surface by a bulk flow mechanism in the absence of any signal of retention in the endoplasmic reticulum. Final assembly occurs at the cytoplasmic membrane with encapsidation of HEV positive-stranded genomic RNA. Expression of gpORF2 in mammalian cells (COS-1 and HepG2) showed that it is expressed intracellularly, as well as on the cell surface and has the potential to form noncovalent homodimers (Pelham and Munro 1993; Jameel *et al.*, 1996; Zafrullah *et al.*, 1999; Panda *et al.*, 2007). Recently, it has been suggested that gpORF2 is an unstable form of protein (Torresi *et al.*, 1999). Although pORF2 is proposed to take part in the capsid assembly, the role of gpORF2 is not clear being possibly involved in apoptotic signalling (Jameel *et al.*, 1999). The ORF-2 has been expressed *in vitro* and characterized by heterologous expression systems including *Escherichia coli* (Panda *et al.*, 1995), mammalian cells using plasmids (Jameel *et al.*, 1996), alphavirus vectors (Torresi *et al.*, 1997; Torresi *et al.*, 1999), baculovirus expression systems (McAtee *et al.*, 1996; Robinson *et al.*, 1998), recombinant vaccinia virus (Carl *et al.*, 1994) and yeast (Tyagi *et al.*, 2001). However, the results from infection of the insect *Spodoptera frugiperda* (Sf-9 cell line) by recombinant baculoviruses appear to be impressive for the multiples forms obtained (of ~72KDa, ~59-62KDa, ~ 50-55 KDa) varying with respect to size and solubility of stable protein products. The full length ORF2 product from insect cells are insoluble, whereas the truncated products, mapping to aminoacids 112-660 assemble into virus-particles, indicating that cleavage and assembly of the capsid protein occur in the system (Tsarev *et al.*, 1993; McAtee *et al.*, 1996; Zhang *et al.*, 1997). Moreover, expression of the truncated 112-660 pORF2 (belonging to a strain from Myanmar) undergoes further processing at the carboxy- terminus in insect *Trichopulsia ni* (Tn-5 cell line),

generating with high efficiency ($1.0 \text{ mg}/10^7 \text{ cells}$) a secretory 50 KDa pORF2, which was capable of self-assembling into empty VLP secreted into the culture medium (Li *et al.*, 1997). However, the VLPs deleted of the N-Terminal region rich in basic residues resulting by this last intracellular process led by protease can be found only in insect cells, but not in vertebrate cells (where glycosylation also occurs). The size of empty VLPs (23.7nm) is smaller than that authentic native HEV virion (27nm), and similar virus particles have not been found in the bile or stools from patients infected with hepatitis E or from experimentally infected monkeys. Expressed VLPs were used as an antigen for enzyme-linked immunosorbent assay (ELISA) against antibodies to HEV, appearing to be specific and sensitive enough to detect anti-HEV IgG as well as IgM in human and experimentally infected monkey sera (Li *et al.*, 1997; Li *et al.*, 2000). Immunodominant epitopes in ORF2 and ORF3 have been included in commercially available diagnostic ELISA for HEV (Yarborough *et al.*, 1991; Dawson *et al.*, 1992; Courasaget *et al.*, 1993; Khudyakov *et al.*, 1993; Khudyakov *et al.*, 1994). The ORF2 epitopes are located at the extreme 3' end of that reading frame (Yarborough *et al.*, 1991). Antibody response to pORF2, highly immunogenic, neutralizes the virus and is protective (Purcell and Emerson, 2008). Currently, a single serotype has been described, with extensive cross-reactivity among circulating human and swine and chickens strains (Fauquet *et al.*, 2005; Okamoto, 2007).

1.3.3 ORF3 AND VIRAL ENCODED PROTEIN

The third and the last open reading frame 3 (ORF3) consists of 369/366 nt, partially overlaps with the first ORF1 by 4 nt, and shares most of the remaining nucleotides of ORF2 at the 3' end (Panda *et al.*, 2007). ORF3 encodes for a 123/122 aminoacid immunogenic phosphoprotein of 13.5 KDa (pORF3) with a not fully defined function (Tam *et al.*, 1991). Recombinant ORF3 protein expressed in eukaryotic cells accumulates in the cytoplasm and is associated with the cytoskeleton in cell fractionation studies, appears non-glycosylated and it does not undergo post-translational modification that would significantly alter its size (Zafrullah *et al.*, 1997). Recent study using a replicon with deleted ORF3 in cell culture showed a normal RNA replication, suggesting that ORF3 is neither required for HEV replication nor for virion assembly or infection of culture cells (Emerson *et al.*, 2006).

Another study (Yamada *et al.*, 2009) provides evidence that the pORF3 is required for virion egress from infected cells. In addition, pORF3 is present on the surface of HEV particles suggesting that the HEV particles released from infected cells are lipid-associated. In its primary sequence, pORF3 contains two large hydrophobic domains in N-terminus of ORF3 rich in polycysteine stretch. Domain 1 may serve as a cytoskeleton anchor at which pORF2 can assemble the viral nucleocapsid, although it was reported that recombinant ORF2 protein assembled into small but typical icosahedrons in the total absence of ORF3 (Zafrullah *et al.*, 1997; Xing *et al.*, 1999) and bound also mitogen-activated protein kinase phosphatase (MAPKP) Kar-Roy *et al.*, 2004). Another smaller hydrophobic domain (Domain 2) follows in the primary sequence, that has been shown to homo-dimerize (43 aminoacids region) in yeast cellular environment (Fields *et al.*, 1989; Chien *et al.*, 1991), and in human hepatoma cells it was demonstrated to interact with another host protein endogenous hemopexin (Hpx), an acute-phase plasma glycoprotein that plays important roles in inflammation. The pORF3-Hpx interactions must have significant importance on viral pathogenesis (Ratra *et al.*, 2008) (**Figure 1.3**).

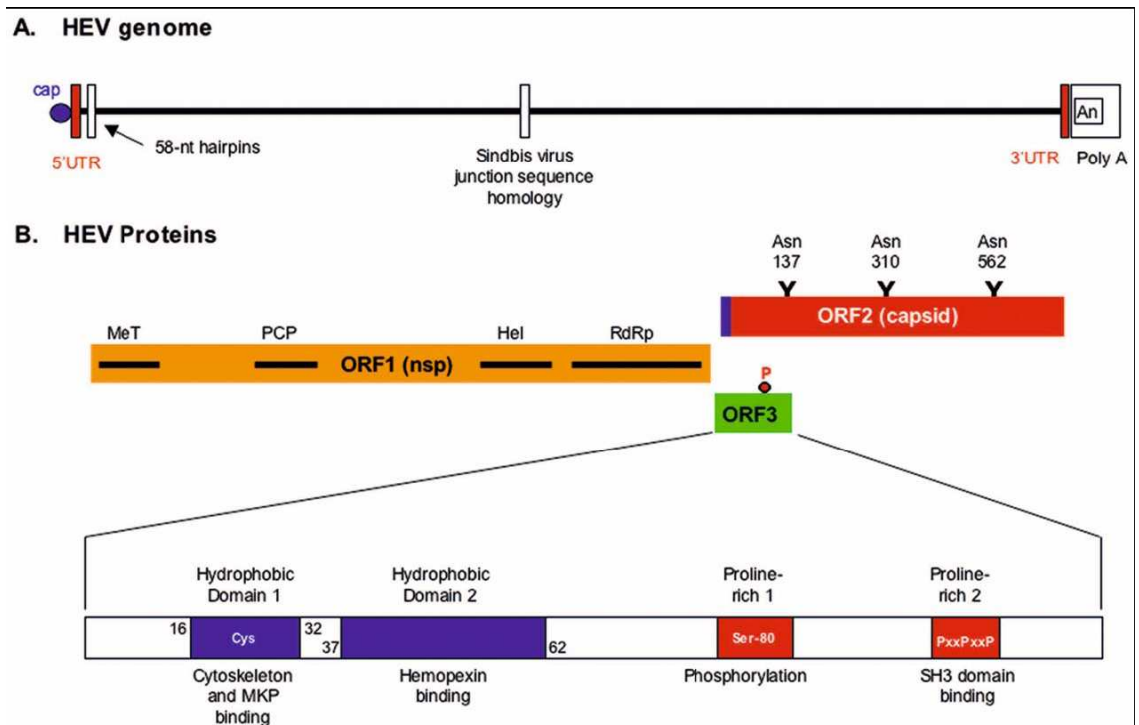


Figure 1.3. Genome organization and proteins of HEV. (A) The ~7.2 kb positive strand RNA genome of HEV is capped at the 5' end and polyadenylated at the 3' end.

It contains short stretches of untranslated regions (UTR) at both ends (red box). Other structural features proposed to be important for replication are also indicated. **(B)** The three open reading frames (ORFs) are shown. ORF1 encodes the nonstructural polyprotein (nsp) that contains various functional units – methyltransferase (MeT), papain-like cysteine protease (PCP), RNA helicase (Hel) and RNA dependent RNA polymerase (RdRp). ORF2 encodes the viral capsid protein; the N-terminal signal sequence (blue box) and glycosylation sites are indicated. ORF3 encodes a small regulatory phosphoprotein. Details of the ORF3 proteins are shown, including two N-terminal hydrophobic domains (blue boxes) and two C-terminal proline-rich regions (red boxes). Functions discovered for these domains are indicated below the illustration (Chandra *et al.*, 2008).

C-terminal end of ORF3 contains two stretches with homology to the polyproline helices (PXXPXXP motif encompassing aminoacids 75 to 86) that binds several proteins containing *src* homology y 3 (SH3) binding domain, as protein tyrosine kinase (PTKs); it also contains a mitogen-activated protein kinase (MAPK) phosphorylation site (Ser-80) conserved in genotype I and III strains but not in genotype II and IV strains of HEV (Tyagi *et al.*, 2001). The overlaps of the dimerisation domain with SH3 binding and phosphorylation site suggested that pORF3 might have a dimerisation-dependent regulatory role to play in signal transduction pathway (Panda *et al.*, 2007). Further, it is possible that the pORF3 protein forms a dimer prior to interacting with full-length ORF2. After dimerization, pORF3 gets phosphorylated, which makes it capable of binding to non-gpORF2 (Tyagy *et al.*, 2002). C-terminal part of the pORF3 contains also several antigenic epitopes located in the most variable positions in the Burmese strain (aminoacids positions 112 to 117) in contrast with the Mexico strain (aminoacids positions 95 to 101) where these are located in the N-terminal part (Kudyakov *et al.*, 1994). The epitopes specifically react with human acute- and convalescent-phase sera of infected patients and were also recognized by sera from experimentally infected *cynomolgus macaques* (Yarborough *et al.*, 1991; Khudyakov *et al.*, 1993). Antibody response to pORF3 is short-lived and does not neutralize the virus (Purcell and Emerson, 2008).

1.4 GENOTYPES

Extensive genomic diversity has been observed among HEV isolates, but a single serotype is recognised (Emerson and Purcell, 2003; Okamoto, 2007). Current classification encompasses V genotypes. Genotypes I and II were isolate only in human beings except one case, i.e. a Cambodian swine strain belonging to genotype I identified by Caron *et al.*, 2006. Genotypes III and IV have been isolated in both human and swine, and also wild boar, deer and mongooses (Meng *et al.*, 1997; Goens and Perdue, 2004; Okamoto, 2007; Panda, 2007).

Genotype I was first identified and subjected to sequencing in 1991 (Tam *et al.*, 1991) from a sample that came from Myanmar (Burma strain) showing more than 88% of nucleotide identity with other genotype I strains isolated in Asia (China, India, Nepal and Pakistan) and Africa (Chad and Morocco) (Okamoto, 2007).

In 1992, a new strain completely different than the Burma strain was sequenced from outbreaks in Mexico (1986) and classified as genotype II. Differently than genotype I, outspread in many geographic regions, genotype II occurs in fewer countries (Lu *et al.*, 2006).

Genotypes III was identified in 1997 in the USA from an autochthonous patient without history of travel abroad sequenced and became the first strain belonging to genotype III (Kwo *et al.*, 1997). Later on, genotype III HEV has been shown to be distributed in many countries worldwide including Asia, Europe, Oceania, North and South America (Pina *et al.*, 2000; Takahashi *et al.*, 2003; Banks *et al.*, 2004a; Peron *et al.*, 2006.)

Genotype IV was identified in 1999 in China, and was different from strains belonging to genotype I from the same areas (Wang *et al.*, 1999). Other isolates were identified exclusively in Asia, particularly in Japan and Taiwan (Takahashi *et al.*, 2002b; Takahashi *et al.*, 2003).

Genotype V encompasses the avian HEV (Huang *et al.* 2004; Purcell and Emerson 2008). Phylogenetic analyses based on the complete genomic sequence of HEV confirmed that avian HEV was segregated into a distinct branch separate from human and swine HEVs of the four known genotypes (Huang *et al.*, 2004, Meng, 2009). Avian HEV was shown to share about 57-61% nucleotide sequence identity

over the entire region with mammalian HEVs, common antigenic epitope(s) in the capsid protein with hHEV and swHEV (Hasquenas *et al.*, 2002) but approximately 80% nucleotide identity with the big liver and spleen disease (BLSV) virus identified in Australian chickens (Payne *et al.* 1999); Hasquenas *et al.*, 2001). Additional studies are needed for classifying avian HEV definitively in the 5th genotype or in a separate genus.

Currently, the four genotypes I-IV are classified into different subtypes, based on approximately 300-450 nucleotides of sequence in the 5' end of ORF2 region which are most conserved among all HEV isolates. The phylogenetic analysis demonstrated that HEV can be divided into total 24 subtypes. Genotype 1 was divided in 5 subtypes (1a, 1b, 1c, 1d, 1e), genotype 2 in two subtypes (2a, 2b), genotypes 3 segregate in 10 subtypes (3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i, 3j) and genotype 4 in 7 subtypes (4a, 4b, 4c, 4d, 4e, 4f and 4g) (Lu *et al.*, 2006) (Fig.1.4) (Meng, 2009).

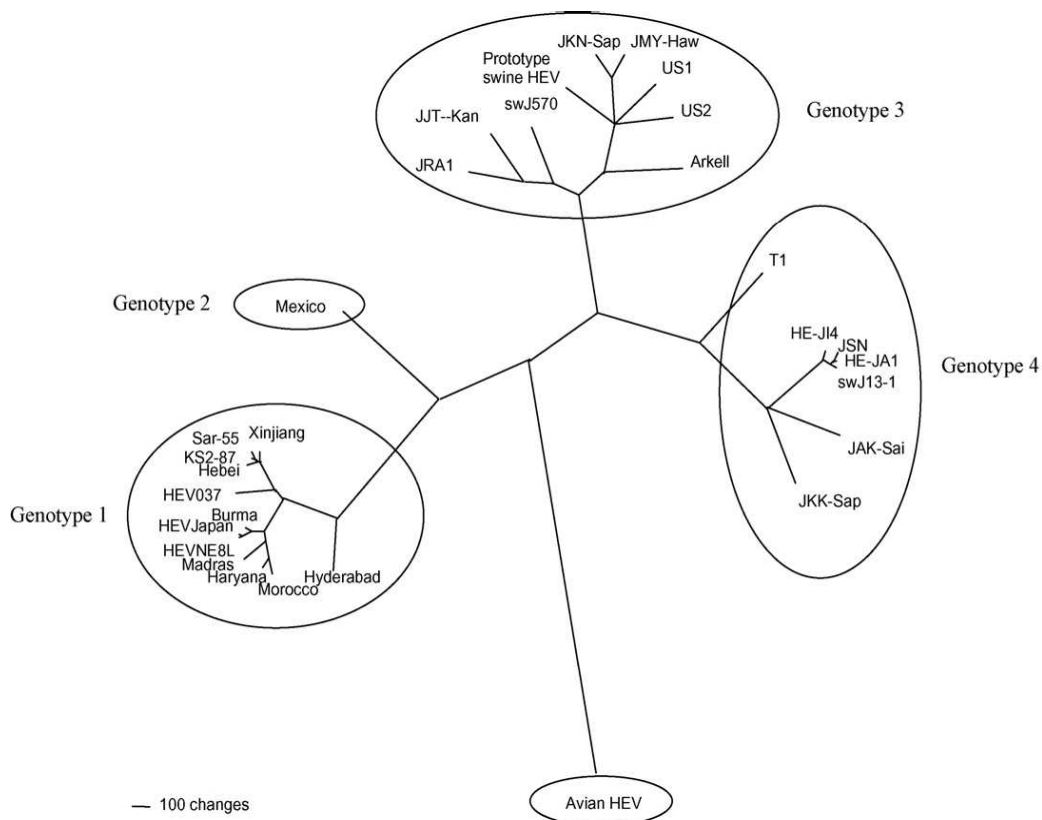


Fig.1.4. A phylogenetic tree based on the complete genomic sequences of 30 human, swine, and avian HEV strains. A scale bar, indicating the number of characters that change is proportional to the genetic distance. Modified with permission by the Society for General Microbiology from Huang (Huang *et al.*, 2004).

1.5 GENETIC VARIABILITY AND QUASISPECIES

Comparing nucleotide sequences of 75 strains (a total of whole or nearly entire genomic sequences of HEV are available for comparison in Gen Bank, as 11th of January 2007) belonging to different genotypes demonstrated that at aminoacid level the difference of ORF2 genomic region was about 6.5%-11.7% among all isolates (Okamoto, 2007). The high degree of conservation of the aminoacid sequences of the capsid structural protein observed among distinct genotypes from different regions in the world is correlated to a little antigenic diversity, which confirms the presence of just one serotype; also, these isolates showed significant degree of nucleic acid variability depending on the high error rate of the viral RNA-dependent RNA polymerase and the absence of proofreading mechanisms (Okamoto, 2007). The high genetic variability among different isolates is a feature of RNA viruses: the mutation frequency of different varieties of RNA virus's ranges from 10^{-4} - 10^{-5} substitutions per base per round of copying (Domingo, 1996). In fact, *quasispecies* is designated as a level of genomic diversity that characterizes RNA viruses in which the non-proofreading polymerase leads to error-prone replication, resulting in better environment adaptability and capacity for rapid evolution during passage from host to host (Schneider and Roossinck, 2001). That implies that we can find diverse nucleotide sequence in an outbreak source represented by one strain, and not just the relation "one outbreak, one strain". The *quasispecies* structure describes an equilibrium status between variants in a replicating virus population under selective pressure such as immunological response (Grandadam *et al.*, 2004). This diversity confers an advantage for survival and evolution as documented for human, animal and plants (Domingo *et al.*, 1998, Schneider and Roossinck, 2001) persistently infected by RNA viruses as HIV type I (Wolinsky *et al.*, 1996), HCV (Farci *et al.*, 2000), and less in acute and self-limited infection like dengue virus (Wang *et al.*, 2002), HAV (Sanchez *et al.*, 2003) and HEV. Large epidemics of HEV are mainly caused by faecal contamination of drinking water resources rather than by person-to-person transmission. Therefore, the diffusion of HEV among humans is assumed to be clonal according to "one outbreak, one strain" (Arankalle *et al.*, 2001). The first proof of the *quasispecies* nature of epidemic HEV was based on retrospective analyses and inter

and intra patient sequence diversity studies, assessed by restriction fragment length polymorphism (RFLP) and by sequencing a 448bp sequence corresponding to ORF2, examining 23 serum samples collected during a water-borne outbreak of HEV genotype 1 in 1986-1987 in Tanefdour (Algeria) (Grandadam *et al.*, 2004). This outbreak was caused by faecal contamination of the water in the rain season in the course of flooding, and was expected to follow the “one outbreak, one strain” scheme; however, the study revealed the contrary, showing an inter-patient heterogeneity by RFLP which divided 23 isolates into three separate profiles (A, B and C). A following molecular epidemiology study on HEV infection on Kathmandu valley (Nepal) (Okamoto, 2007) investigated the genetic changes in HEV strains in the community, analyzing nucleotide sequences of HEV isolates, collected from patients recovered every two years (1997, 1999, 2000, 2002). HEV-viremic samples were typed as genotype I and further as subgenotype Ia (further segregated into five clusters) and Ic (detected only in 1997), highlighting the chance of mixed viral infections. High genetic variability in the community was observed among HEV strains and even among HEV strains of the same subtype obtained each year throughout the observed period, but no significant aminoacid substitutions were recognized in the HEV strains isolates. This fact suggests that genomic mutation of HEV may occur naturally in infected individuals without immunological pressure from the host.

However, among the four genotypes of HEV, less genomic variability appears in HEV strains of genotype I and II than genotypes III and IV. Pairwise comparison of the 75 entire sequences of HEV isolates reveals (Okamoto, 2007) an inter-genotype difference of 23.6-27.7%. In genotype I, the intra-genotype diversity was up to 11.8%, while for genotype 3 and 4 it showed a wider range of up to 19.3% and 17%, respectively. For genotype II, only one entire sequence is available on line (Mexican strain, Mex-14) (Huang *et al.*, 1992), and partial sequences for 16 African strains belong to the same sub-genotype. Phylogenetic analyses suggest that genotype II HEV strains segregate into at least two subgenotypes, i.e. Mexican and African. The 16 African isolates differ from each other by up to 10.3% and from the Mexican isolates by up to 16.8% (Okamoto, 2007). Then the inter-genotype difference is higher in genotype III than genotype IV, corroborating the classification of four genotypes of HEV in total 22 subgenotypes (Lu *et al.*, 2006), with genotype III being divided in 10 subgenotypes (IIIa-IIIg) and genotype 4 in 7 subgenotypes (IVa-IVg).

Hence, genotype I and II appear to be more conserved that may be due to the finding that, except for the Cambodian swine strain case mentioned above (Caron *et al.*,2006), they have so far been identified only in human beings, during large epidemics in developing countries where they are endemic. The geographical distribution is distinct between these two genotypes (**Fig.1.5**). Genotype I is present in Asia (India, Pakistan, Nepal, China, Bangladesh, Uzbekistan, and Kyrgyzstan) and Africa (Central African Republic, Morocco, Algeria, Namibia, Sudan, Egypt, and Chad). Genotype II is present in Mexico and occasionally in Africa (Nigeria, Namibia, Egypt, Central African Republic, and Chad) (Pavio *et al.*, 2008).

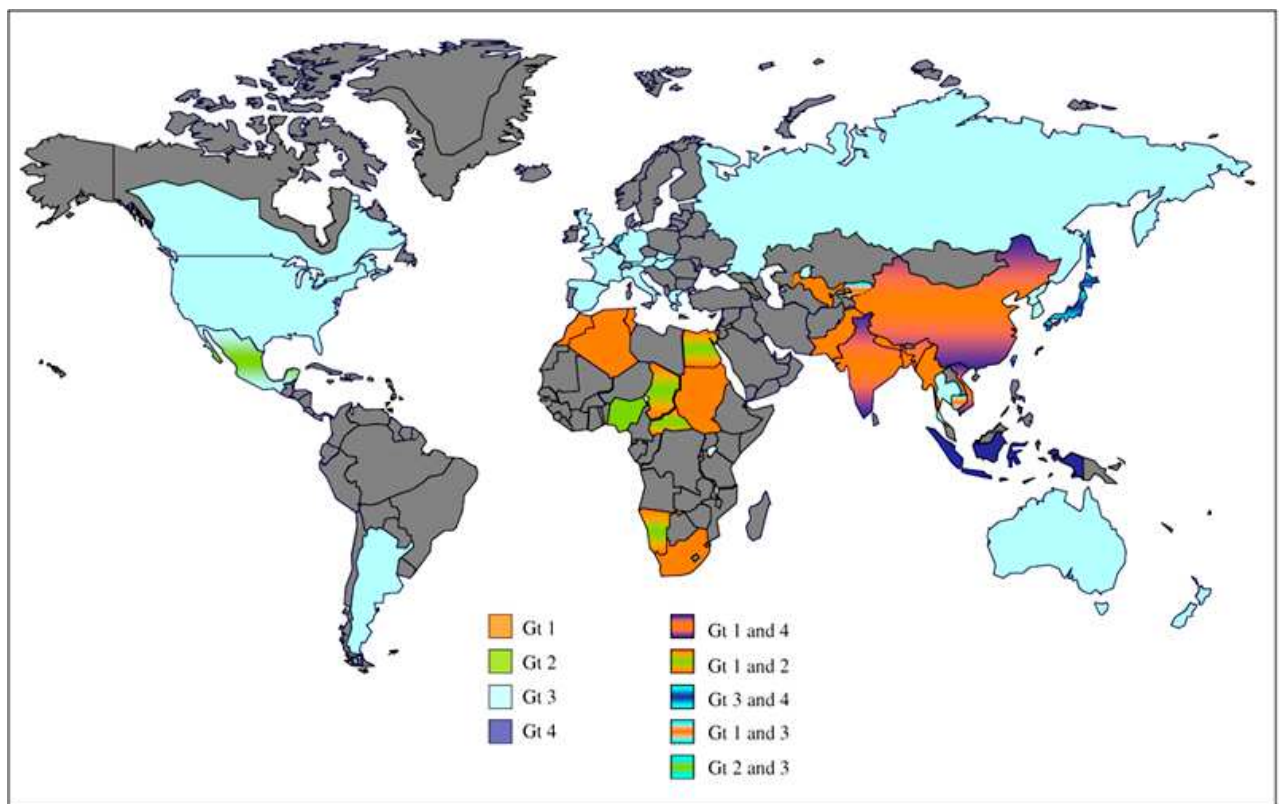


Fig.1.5. Geographical distribution of HEV isolates according to genotypes (Gt). HEV Gt I and II: epidemic strains causing human infection. HEV Gt III and IV zoonotic strains isolated from humans and a variety of animals, particularly pigs. In some countries, different genotypes co-circulate in distinct ecological niches: Gt I and IV in China, India, and Vietnam; Gt I and II in several African countries, including Namibia, Chad, and Sudan; Gt III and IV in Japan; Gt I and III in Cambodia; Gt II and III in Mexico (Pelosi and Clarke, 2008).

Genotypes III and IV were not associated with large epidemics but with sporadic cases and identified in human and swine, wild deer and boars that suggests much greater genetic diversity including different species and different geographic areas (Lu *et al.*, 2006). Genotype III is present in Europe (France, Germany, Austria, Greece, Italy, Spain, Great Britain, Netherlands, and Sweden) and also in the United States, Canada, Argentina, Brazil, Chile, Mexico, Australia, New Zealand, Russia, Kurdistan, Korea, Kampuchea, Thailand, Taiwan and South Africa. Genotype IV, the geographical distribution is more limited: Japan, China, Indonesia, South Africa, Taiwan, Vietnam and India (Pavio *et al.*, 2008). Geographic distribution is considered to be an important parameter (Clemente-Casares *et al.*, 2003; Okamoto *et al.*, 2007; Panda *et al.*, 2007) as isolates circulating in the same area show higher sequence similarity with human and animals isolates derived from the same area than to distant area (Banks, 2004a; Clemente- Casares *et al.*, 2003; Peron *et al.*, 2006; Zheng *et al.*, 2006).

A few studies conducted in India (Arankalle *et al.*, 2002; Arankalle *et al.*, 2003) demonstrated that human and swine strains evolved separately. These data are in contrast with to the reports indicated above and others from USA (Meng *et al.*, 1997; Huang *et al.*, 2002) and Taiwan (Wu *et al.*, 2000), where in both humans and swine HEV belonged to the same genotype, i.e., III and IV, respectively. In India, hHEV isolates collected during 1976-2001 from different parts of India were all classified as genotype I, while the swine HEV recovered from Western India (2000) belonged to genotype IV. The swine isolates in 1999 and 1985 from Southern India clearly showed that HEV infection has been highly endemic in this species for a long time. The second study confirms the earlier observation in that none of the pigs resulted to belong at genotype I, except for three pigs imported from USA belonging to genotype III, and some others belonging predominantly to genotype IV, standing for a significantly distinct circulation of genotypes among HEV-infected humans (type I) and pigs (type IV) in the same country. However, a recent study suggests the possible occurrence of an event of recombination between human genotype III and swine genotype III strains (van Cuyck *et al.*, 2005), that implies the simultaneous infection of a single host with both human and animal HEV. Combining HEV *quasispecies* variability with possible co-infection and recombination events, viral variant with increase pathogenicity for humans might be selected (Pavio *et al.*, 2008).

1.6 TAXONOMY OF HEV

Since identification of HEV by electron microscopy in 1983, the agent of ET-NANBH was assumed to be an RNA virus and it was suggested to group this virus into the *Picornaviridae* family as HAV type 2 (Balayan *et al.*, 1983). Further, HEV presented a certain number of homologies in the non-structural polyprotein with Rubella virus of the *Togaviridae* family or plant flavivirus (Koonin *et al.*, 1992). The first HEV strain cloned and sequenced came from a Burma patient (Burma strain), and was shown antigenically and biophysically unrelated to the picornaviruses. The genome organization of HEV and its morphology present similarities with members of the *Caliciviridae* family. Therefore it was provisionally classified in this family as a separate virus cluster in addition to the 4 mainly genera of Norovirus and Sapovirus, affecting humans and animals, and the animal Lagovirus and Vesivirus. Looking more deeply into the coding region for helicase and polymerase that is more conserved than the structural protein region only the latter four groups were confirmed members of the *Caliciviridae* family, while HEV strains were removed from this family (Berke and Matson, 2000).

Comparative analysis of the polymerase for the phylogenetic study of HEV, *Picornaviridae* and *Togaviridae* suggested that HEV was more closely related to the *Togaviridae* family (Emerson and Purcell, 2003) (**Fig. 1.5**). Presently, HEV is now classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (Fauquet, 2005).

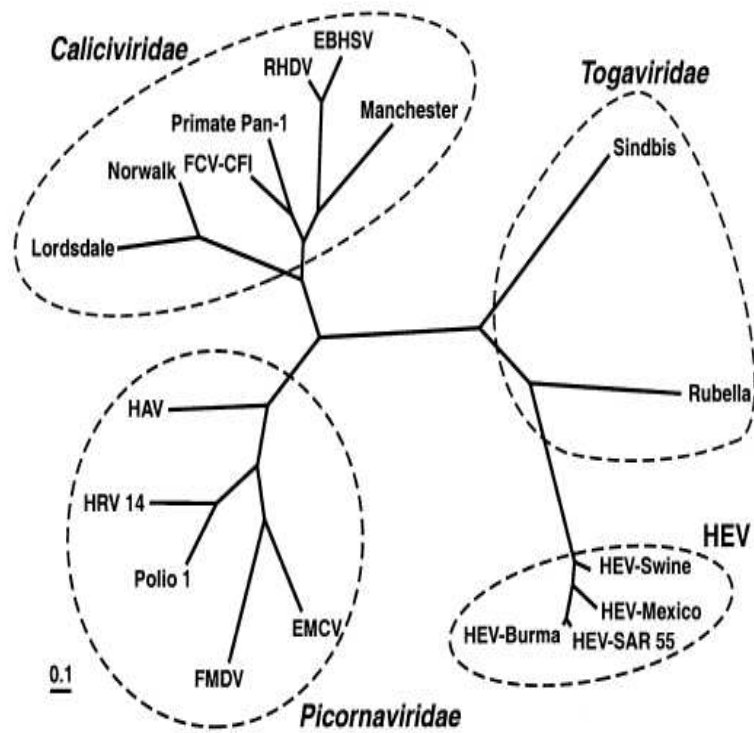


Fig.1.6. Maximum likelihood phylogram of partial polymerase nucleotide sequences showing that HEV segregates away from the *Caliciviridae* and is more closely related to the *Togaviridae* (Berke and Matson, 2000).

1.7 ZOONOSIS

The possibility that HEV infection can be a zoonosis in industrialized countries where HEV is not endemic is based on the isolation and characterisation of virus in pigs, initially in the USA with high nucleotide homology with human strains from the same country (Meng *et al.*, 1997) and subsequently in Taiwan (Hsieh *et al.*, 1999), in Japan (Okamoto *et al.*, 2001). In 2001 (van der Poel *et al.*, 2001) HEV swine strains were identified in The Netherlands, showing close genetic similarity to European human strains. In 2002 field isolates of swine HEV were identified from different geographic area (Huang *et al.*, 2002) demonstrating nucleotide identity between swine (88-100%) and against human strains (89-98%). In 2004 in United Kingdom HEV were identified two UK pig strains with 100% aminoacid sequence identity to one autochthonous human case of HEV in UK (Banks *et al.*, 2004a) (**Fig.1.6**). In Spain, 2006, de Deus *et al.*, (2007) identified in swine affected by various pathology, HEV strains with nucleotide identity (85.7%-100%) between swine and 85-97.6% nucleotide identity against to human strains. In Italy Caprioli *et al.*, (2007) identified swine strains with close nucleotide homology against Spanish human strains. Finally, swine may act as a reservoir for HEV infection in humans, as suggested by the high genetic homology between HEV strains isolated from pigs and human HEV strains from the same geographic area “sympatric”, suggesting cross-species infection of HEV (Meng *et al.*, 1998b; Nishizawa *et al.*, 2003; Okamoto *et al.*, 2004).

The first human strain was identified in 1990 (Balayan *et al.*, 1990) and was subsequently used in the experimental infection of pigs, although the isolate from the infected pig was not sequenced, which would have not enabled differentiation of the inoculum strain from any potential pre-existing infection with swine HEV (Balayan *et al.*, 1990). Two humans strains, genotype 1 (Sar-55) and genotype 2 (Mex-14), were unsuccessfully used in the experimental infection of SPF pigs (Meng *et al.* 1998a), but this may have been due to the genetic diversity between the inoculum strain and those that typically infect swine successfully genotype 3 (75% homology at the nucleotide level). However, SPF pigs were successfully infected with human strain genotype 3 (US-2), and infection was confirmed by sequencing to demonstrate identity with the inoculum strain (Meng *et al.* 1998b). Further, human strain genotypes 1, 2 and 3 have been used in the experimental infection of monkeys (Erker *et al.*, 1999b). A swine

strain isolated from a raw sewage sample from a purification plant located close to an abattoir, in an area where hepatitis E is not endemic (Barcelona, Spain), was shown to infect monkeys (Pina *et al.*, 1998).

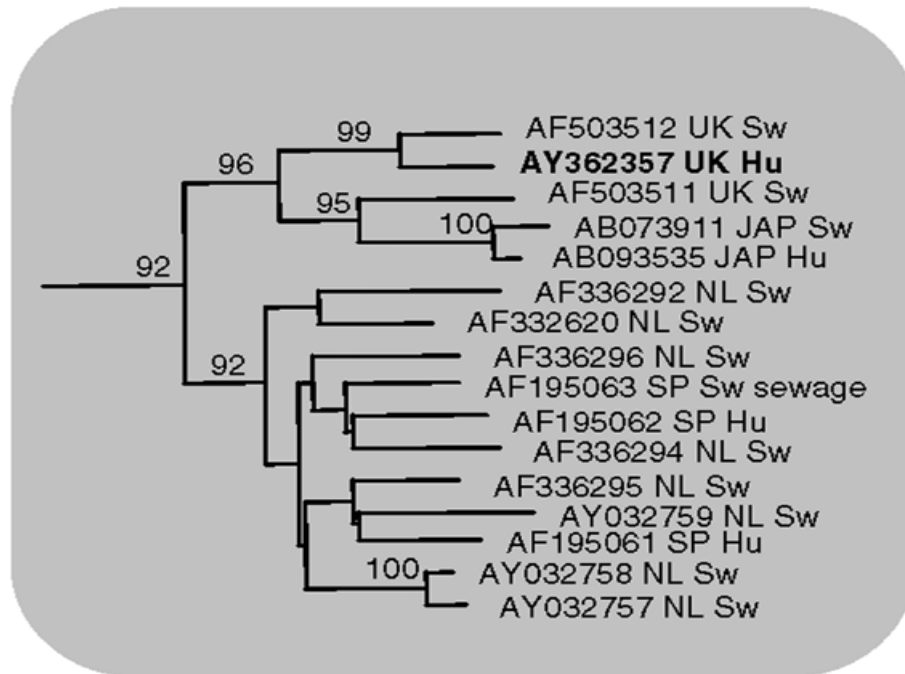


Figure 1.6. Human United Kingdom isolate (AY362357) is shown in bold and compared with closely related swine and human hepatitis E virus isolates (GenBank accession no., country of origin, and host are indicated). Bootstrap values greater than 70% are considered significant and are indicated. (Banks *et al.*, 2004a – used with permission from the authors)

However, HEV replication has been demonstrated to occur in the liver and in intestinal tract (Ha *et al.*, 2004); further, in Hokkaido (Japan) 9 patients (90%) had a history of consuming grilled or undercooked pig liver 2-8 weeks before the disease onset. Packages of raw pig liver sold in grocery stores as food in Hokkaido were tested for the presence of hepatitis E virus (HEV) with 1.9% found positive. One swine HEV isolate (swJL145) from a packaged pig liver had 100% identity with the HE-JA18 isolate recovered from an 86-year-old patient in Hokkaido. Two swine HEV isolates (swJL234 and swJL325) had 98.5-100% identity with the HE-JA4 isolate obtained from a 44-year-old patient in Hokkaido (Yazaki *et al.*, 2003). Further in

USA, recently, (Feagins *et al.*, 2007), found 11% of liver HEV positive in the retail store. Homogenates of these positive livers were used to successfully infect pigs SPF, demonstrating that the HEV in the liver was positive and also infectious (Feagins *et al.*, 2007). Further, the same group (Feagins *et al.*, 2008) determined if the infectious HEV in contaminated commercial pig livers could be inactivated by traditional cooking methods before inoculating the pigs. Liver incubated at 56°C for 1h was still infectious, stir-fried at 191°C (internal temperature of 71°C) for 5 min or boiled in water for 5 min, respectively, appeared inactivated because the pigs did not become infected. So, HEV in contaminated commercial pig livers can be effectively inactivated if cooked properly, although incubation at 56 degrees C for 1 h cannot inactivate the virus. Thus, to reduce the risk of food-borne HEV transmission, pig livers must be thoroughly cooked.

In the muscle there did not appear to be extra-hepatic sites replication of the virus, but probably virus was present during the viremia phase (Teo, 2006).

Recently, a hepatitis E outbreak on board a UK cruise ship in returning from an 80 night world cruise has been investigated. Health Protection Agency (HPA) was informed of four cases of jaundice on board a cruise ship departed from Southampton on 7 January and returned on 28 March 2008, and an epidemiological investigation was launched by HPA to identify any additional case of HEV and potential risk factor for infection. The investigation was a cohort study to include all 2850 UK passengers who were on the cruise at any point. A total of 851 of the 2850 eligible passengers took part in the investigation. Finally, 33 (4%) individuals were identified with recent acute HEV infection, although only 11 of these were symptomatic cases. A common source outbreak was shellfish eaten on board the cruise ship. The causative agent was identified as HEV genotype 3 which was closely related to the other genotype 3 strains isolated in Europe. (Hepatitis A &E Symposium, London, 25-3-09) This habit to eat raw shellfish corroborating the potential risk of HEV infection, as demonstrated by high seroprevalence in people (Cacopardo *et al.*, 1997). Further, swine may be a reservoir of infection because seroepidemiological studies show higher seroprevalence found in people with occupational exposure to swine (farmer, veterinarians, butchers, merchant,) than control people (Drobeniuc *et al.*, 2001; Withers *et al.*, 2002; Meng *et al.*, 2002).

1.8. PATHOGENESIS

Hepatitis study has been facilitated by laboratory determination of HEV replication, immune response, and liver pathologic features in patients and in infected primates with hepatitis E (Balayan *et al.*, 1983; Krawczynski K., *et al.*, 1999). It has been estimated that the infectivity titre of HEV for macaques is 10000-fold higher when inoculated intravenously compared with when it is ingested (Emerson and Purcell, 2003). After ingestion, probably, the virus replicates in the intestinal tract (the primary site of replication has not been identified yet) and reaches the liver, but it is presumably via the portal vein serving the liver (Panda *et al.*, 2007). It replicates in the cytoplasm of hepatocytes (Hussaini *et al.*, 1997) and is released into the bile and bloodstream, by mechanism that are still not understood, and excreted in the faeces. It is not known if the virus replicates in the intestinal tract or if the all virus in the faeces originates in the liver (Emerson and Purcell, 2003). The incubation period (the time from infection to clinical symptoms) based upon limited study of oral infection in human volunteers (Balayan *et al.*, 1983; Chauhan *et al.*, 1993), was found to be 4-5 weeks. Viral excretion in the faeces begins approximately 1 week prior to the onset of illness and persist for 2-4 weeks, in some cases RT-PCR has yielded positive results for as long as 52 days after onset (Nanda *et al.*, 1995). The viremia can be detected in the first 2 weeks after the onset of illness (Clayson *et al.*, 1995), during the late phase of the incubation period, but may also be present transiently and may disappear before the onset of clinical symptoms; prolonged periods of HEV-RNA positivity in serum ranging from 4 to 16 weeks have also been reported (Chauhan *et al.*, 1993; (Nanda *et al.*, 1995). Viral excretion and viremia has been detected by RT-PCR also prior to liver abnormalities which normally appear with a rise in aminotransferase level, by as long as 10 days and reaches a peak by the end of the first week. Simultaneously the humoral immune response appears that is measured as anti-HEV IgM or IgG level, detected by enzyme immunoassay on serum samples (Jameel *et al.*, 1999; Aggarwall *et al.*, 2000). Immunoglobulin M antibody to HEV (anti-HEV) appears first during clinical illness but disappears rapidly over a few months (4-5 months) and is closely followed by Immunoglobulin G anti-HEV that appears a few days later and persists for at least a few years; its titre increases throughout the acute phase into the convalescent phase, and may remain high from 1 to 4.5 years after the acute phase of illness (Dawson *et al.*, 1992; Favorov *et al.*, 1992). The exact duration of persistence

of anti-HEV is not known. In one study, anti-HEV was detected in 47% of persons 14 years after acute HEV infection (Khuroo *et al.*, 1993). Determination of IgM anti-HEV is useful for the diagnosis of acute infection, whereas the presence of IgG anti-HEV indicates HEV infection, not necessarily recent (Aggarwall *et al.*, 2000).

The symptoms of hepatitis E are typical of acute icteric viral hepatitis; the commonest recognizable form of illness has an initial prodromal phase (preicteric phase) lasting a few days, with a variable combination of flu-like symptoms, fever, mild chills, abdominal pain, anorexia, nausea, aversion to smoking, vomiting, clay-coloured stools, dark or tea coloured urine, diarrhoea, arthralgia, asthenia and a transient macular skin rash (Aggarwall *et al.*, 2000). These symptoms are followed in a few days by lightening of the stool colour and the appearance of jaundice. Itching may also occur. With the onset of jaundice, fever and other prodromal symptoms tend to diminish rapidly and soon disappear entirely. Laboratory test abnormalities include bilirubinuria, variable degree of rise in serum bilirubin (predominantly conjugated), marked elevation in serum alanine aminotransferase (ALT), aspartate aminotransferase and gammaglutamyltransferase activities, and a mild rise in serum alkaline phosphatase activity. The magnitude of transaminase rise does not correlate well with the severity of liver injury. The illness is usually self-limiting and typically lasts 1–4 weeks (Aggarwall *et al.*, 2000). No evidence of chronic hepatitis has been reported in general nor has cirrhosis been detected following acute hepatitis E. However, two recent reports present biochemical histological and genetic evidence of chronic HEV infection in transplant patients (Haagsma *et al.*, 2008; Kamar *et al.*, 2008). A few patients, however, have a prolonged clinical illness with marked cholestasis (cholestatic hepatitis), including persistent jaundice and prominent itching. In these cases, laboratory tests show a rise in alkaline phosphatase and a persistent bilirubin rise even after transaminase levels have returned to normal (Aggarwall *et al.*, 2000). The prognosis is good as jaundice finally resolves spontaneously after 2–6 months. Other infected individuals have a milder clinical course and develop only non-specific symptoms that resemble those of an acute viral febrile illness without jaundice (anicteric hepatitis) (Aggarwall *et al.*, 2000). Histological features of hepatitis E may differ from those of other forms of acute viral hepatitis. Nearly half of hepatitis E patients have a cholestatic-type of hepatitis, which is characterized by canalicular bile stasis and gland-like transformation of parenchymal cells. In these patients, degenerative changes in hepatocytes are less marked (Gupta *et al.*, 1957,

Aggarwal *et al.*, 2000) In other patients, changes resemble those of other forms of acute hepatitis, such as the presence of ballooned hepatocytes and acidophilic bodies, and focal or confluent hepatocyte necrosis. No particular zonal distribution of hepatocyte damage is observed. In both forms, lobules contain an inflammatory infiltrate consisting predominantly of macrophages and lymphocytes and, in patients with a cholestatic type of hepatitis, of a few polymorphonuclear leucocytes. The Kupffer cells appear prominent. Portal tracts are enlarged and contain an inflammatory infiltrate consisting of lymphocytes and a few polymorphonuclear leucocytes and eosinophils; polymorphonuclear cells are particularly increased in the cholestatic type of lesion (Gupta *et al.*, 1957, Aggarwal *et al.*, 2000). In cases with severe liver injury, a large proportion of hepatocytes are affected, leading to submassive or massive necrosis with collapse of liver parenchyma (Aggarwal *et al.*, 2000). In its most benign form, HEV infection is entirely unapparent and asymptomatic and passes unnoticed. A small proportion of patients have a more severe disease with fulminant or subacute (or late-onset) hepatic failure. The exact frequencies of asymptomatic infection and of anicteric hepatitis are not known but probably far exceed that of icteric disease as, in disease endemic areas, a large proportion of individuals who test positive for anti-HEV antibodies do not recall having had jaundice (Aggarwall *et al.*, 2000). Hepatitis E has a mortality rate of 0.2-1% in general population (Chandra *et al.*, 2008). In developing regions hepatitis E is most common in young adults (15-40 years of age), and in disease-endemic regions, this infection constitutes an important cause of acute and fulminant hepatic failure. Hepatitis E appears to cause more-severe disease among pregnant women, particularly those in the second and third trimesters; they are more frequently affected during hepatitis E outbreaks and have a worse outcome (Aggarwall *et al.*, 200). It has been shown that HEV commonly causes intrauterine infection as well as substantial prenatal morbidity and mortality (Khuroo *et al.*, 1995), suggesting that the placenta may be a preferred site of viral replication as Lassa fever (McCormick *et al.*, 1986; Hamid *et al.*, 1996). Death is usually due to encephalopathy, haemorrhagic diathesis or renal failure. In a preliminary report (Longer *et al.*, 1993) cynomolgus monkeys infected intravenously with HEV developed acute tubular necrosis with focal haemorrhages suggesting that HEV may replicate in monkey kidneys. In pregnant monkeys, however, no increased mortality has been observed (Arankalle *et al.*, 1993; Krawczynski *et al.* 1989). In endemic countries such as India, the mortality rates of

women with acute genotype 1 hepatitis E in the third trimester of pregnancy are usually fairly high (26-64%) (Navaneethan *et al.*, 2008). Why acute HEV infection in pregnant women causes severe liver disfunction is not known.

2. TECHNIQUES FOR INVESTIGATION OF HEV

Several methods have been available for investigate HEV, differing one another by sensitivity and specificity, as qualitative and quantitative PCR, cell culture, confocal, and transmission and scanning electron, microscopy).

2.1 QUALITATIVE PCR

Qualitative PCR or conventional RT-PCR assays is mostly utilized in direct diagnosis of HEV. The samples collected may be faeces, serum, from animal or human, cultures of infected cells cultivated in 2D and 3D configurations, or necroscopic tissue highly positive as bile and liver (Panda *et al.*, 2007; de Deus *et al.*, 2007). HEV is an RNA virus and needs to be extracted before being subjected to retrotranscription reaction phase to cDNA. This is a limiting step, because cDNA it easily degradable, if in the samples the viral load is so low at initial state, may give rise at the end to false negativity. Various sets of sense and antisense synthetic oligonucleotide primers may be used for the detection of HEV genome, differing based on conservative region target in the genome against middle or terminal part of ORF1, C terminal of ORF2 (Panda *et al.*, 2007). There are reports which indicate universal degenerate primers (Erker *et al.*, 1999a; Inoue *et al.*, 2006b), for identifying positives samples even though the strains belong to the different genotype. In this study e.g. has been used then following primers A1/S1 (Erker *et al.*, 1999a) and 3156/7 primers (Huang *et al.*, 2002) used to amplify the ORF2 region. Most of all the time the first product of PCR amplification it is not sufficient quantity to be visualized on electrophoresis gel. However, if the first product of PCR has been amplified by *nested*- PCR with internal primers: A2S2 (Erker *et al.*, 1999a; Di Bartolo *et al.*, 2008) and 3158/9 (Huang *et al.*, 2002), respectively, the PCR product became clearly visible on the electrophoresis gel through ethidium bromide dye. This absorbs UV light intercalating into DNA and makes it fluoresce orange when visualized under UV

transilluminator. There are hazards risks in the use of ethidium bromide which may be fatal if inhaled, causes irritation to skin, eyes and respiratory tract, and may cause heritable genetic damage. The DNA after the electrophoresis on gel could be quantified after excised from the gel, extracted and concentrations measured by nanodrop Spectrophotometer, nevertheless this are still a longer time procedure.

2.2 QUANTITATIVE PCR

Quantitative real time RT-PCR (qRT-PCR) assay is used to detect HEV genotype 3 with high specificity and sensitivity in the same samples used as mentioned at the initial step to investigate the presence of HEV in the target samples by extraction of RNA, retro transcription reaction and qualitative PCR. But, about the benefits resulted by performing qRT-PCR are simply, it does not need to perform retrotranscription reaction in separate assay and after PCR reaction and *nested*PCR which could give a chance of cross-contamination correlated to the increase of the sensitivity when *nested* PCR is performed . Further, qRT-PCR does not need to run the PCR product on electrophoresis gel and excise the expected band product, extract DNA, quantify the DNA present in the samples by Nanodrop Spectrophotometer and subjected it to sequencing and phylogenetic analyses. With the qRT-PCR by RNA (extracted from the target samples) may directly retrotranscribed and amplified in one step and give the possibility to quantify with accuracy how much DNA molecules of the amplified sequence related to the initial concentration of RNA (copies ml⁻¹) there are present in the samples analysed in “real time” as the assay is called. At the end, a conventional PCR assays require more time and are generally less sensitive than real time PCR. In literature have been reported two real time RT-PCR methods more used for the detection of HEV different by use of the reporters: Taqman or SYBER green I (Kubista *et al.*, 2006). The difference between them is in a presence of non specific label or probe as fluorescent reporter that bind the specific fragment amplified and reports its presence by fluorescence. In SYBER green method, the DNA double strand specific dye (asymmetric cyanine dyes) emits fluorescence in the presence of every DNA at double helix, and then result with less specificity because it emits fluorescence signal in the presence of any double stranded DNA including undesired primer-dimer products interfering with the formation of specific product leading to erroneous readouts (Kubista *et al.*, 2006). Despite of SYBER green methods, in this

study Taqman method has been used that resulted highly specific because it includes the use of molecule label with dye called probe. By the incorporation of probe in the reaction mixture, the amplification leading by Taq Polymerase working in 5' to 3' direction, a probe carrying two dyes (one of them is a fluorescent reporter dye and other is a quencher dye) hybridizes to the amplicon during the PCR reaction. The two fluorescent dyes interact whenever the probe is intact, causing the quencher dye to quench the reporter dye. During the amplification, the Taq polymerase cleaves the 5' end of the probe, releasing the quencher dye and result in an increase in fluorescence. The fluorescence of these dyes increases with the amount of double stranded DNA product formed that can be monitored throughout the reaction. During the initial cycles the signal is weak and cannot be distinguished from the background. As the amount of product accumulates a signal develops that initially increases exponentially. Thereafter the signal levels off and saturates (Kubista *et al.*, 2006) fluorescence based on real-time PCR assay with specific primers and probe annealing to the highly conserved region of HEV as, described in Jothikumar protocol (Jothikumar *et al.*, 2006) for the detection of HEV genotypes 1-4. Jothikumar protocol was optimized properly for swHEV and also Gyarmati protocol (Gyarmati *et al.*, 2007) but they are also used for human diagnostics. The difference among two protocols consists in the first use primers and probes that annealing ORF3 of HEV genome with all reactions in one tube protocol. While the second consists of the use of primers and probes that anneal to ORF2 of HEV genome encompassing two separate steps one for retrotranscription and another for PCR.

To quantify the copy number of the initial state ribonucleic acid (copies ml⁻¹) that are present in the samples product in one reaction real time it is necessary generate a curve in the real time PCR assays. For generation of standard curve in the real time PCR (need to dilute the note plasmid in distilled water from 10⁻¹ to 10⁻¹²) for determining the detection limit of the real time PCR assays is determined as the highest dilution detected and the sensitivity was calculated from the number of genomic equivalents in highest dilution detected (Gyarmati *et al.*, 2007).

2.3 CELL CULTURES

The first experiment of cultivation of HEV *in vitro* started in 1996 (Tam *et al.*, 1996). When chimpanzee hepatocytes experimentally infected by HEV (Burma strain, prototype genotype 1) isolated by liver and subsequently cultivated *in vitro* (Tam *et al.*, 1996) demonstrating that a single-stranded, positive-sense RNA virus. Replication of HEV is presumed to involve the synthesis of a full-length negative-strand intermediate which would in turn serve as template to generate additional viral genomic copies. Recently, (Tanaka *et al.*, 2007) cultivated 21 cell lines. The cells were inoculated for 1h with HEV human faecal suspension, genotype 3, at two different temperatures: 35.5°C and 37.5°C. The HEV RNA was detected in the, inoculum, culture media by qualitative PCR (Mizuo *et al.*, 2002) and quantitative RT-PCR (Jothikumar *et al.*, 2006). Only hepatocarcinoma cell line (PLC/PRF/5) supported the replication of the HEV maintained at 35.5°C with highest viral load throughout the observation period. Tanaka's group performed also a study of thermal stability incubating the inoculum at 56°C for 30 min, 70°C for 10 min, 95°C for 1min or 95°C for 10 min, as control at 25°C for 30 min. HEV incubated at temperature higher than 70°C did not grow in PLC/PRF/5 cells, while HEV incubated at 25°C for 30 min and 56°C for 30 min was still infectious, corroborating the previous report by Emerson *et al.*, 2005.

2.4 MICROSCOPY

Microscopy is the technical field of using microscopes to view samples or objects. There are three well-known branches of microscopy, optical, electron and scanning microscopy. Optical and electronic microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/ electron beam interacting with the subject of study, and the subsequent collection of this scattered radiation in order to build up an image. This process may be carried out by wide-field irradiation of the sample (e.g. standard light microscopy and transmission electron microscopy) or by scanning of a fine beam over the sample (e.g. confocal laser scanning microscopy) and scanning electron microscopy (Abramowitz and Davidson, 2007).

2.4.1 CONFOCAL MICROSCOPY

There has been a tremendous explosion in the popularity of confocal microscopy in recent years (Batsumoto *et al.*, 2002). The technique of laser scanning confocal microscopy has become an invaluable tool for a wide range of investigations in the biological and medical sciences for imaging thin optical section in living and fixed specimens ranging in thickness up to 100 micrometers (Nathan *et al.*, 2006). The basic key to the confocal approach is the use of spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus. Confocal Microscopy offers several advantages over conventional wide field optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the capability to collect serial optical section thin (0.5 to 1.5 μm) to fluorescence specimens from thick specimens (Sandison and Webb *et al.*, 1994).

The choice of fluorescent probes for confocal microscopy must address the specific capabilities of the instrument to excite and detect fluorescence emission in the wavelength regions made available by the laser system and detectors. Many of the classical fluorescent probes that have been successfully utilized for many years in widefield fluorescence (Johnson, 1998; Kasten, 1999) including fluorescein isotiocianato, Lissamine rhodamine, and Texas red, are also useful for confocal microscope. Another popular fluorescent dye useful for confocal microscopy is DAPI and Alexa fluor 488. DAPI (4', 6-diamidino-2-phenylindole), blue fluorescent, that bind externally to the double helix stained DNA and also to chromatin. Alexa fluor dyes are sulfonate rhodamine derivatives and exhibit higher quantum yields for more intense fluorescence emission than spectrally similar probes, and have several additional improved features, including enhanced photostability, adsorption spectra matched to common laser lines, pH intensivity, and high degree of water solubility (Nathan *et al.*, 2006). This last feature enables the water soluble Alexa fluor probes to be readily utilized for both live-cell and tissue section investigations, as well as in traditional fixed preparations.

Alexa fluor dyes are available in a broad range of fluorescence excitation and emission wavelength maxima, ranging from the ultraviolet and deep blue to the near-infrared regions. Alphanumeric names of the individual dyes are associated with the

specific excitation laser for which the probes are intended. For example, Alexa fluor 488; green fluorescent, is designed for excitation by the blue 488 nanometer line of the argon or krypton-argon ion lasers (Nathan *et al.*, 2006). The inconvenience in the use Alexa fluor is the resistance to photobleaching appearing so dramatic (Berlier *et al.*, 2003) that even when subjected to irradiation by high-intensity laser sources, fluorescence intensity remains stable for relatively long periods of time in absence of antifade (also termed antiphotobleaching) reagents.

Most of the software packages accompany commercial confocal instruments are capable of generating composite and multidimensional views of optical section data acquired from z-series images stack (Nathan *et al.*, 2006). The three-dimensional software packages can be employed to create either a single three-dimensional representation of the specimen or a video (movie) sequence compiled from different views of the specimen volume (Nathan *et al.*, 2006).

2.4.2 ELECTRONIC MICROSCOPY AND SCANSION MICROSCOPY

The electronic transmission electron microscopy (TEM) technique is so specific, labour, expensive, but was essential precursors for understanding the natural history of HEV being a successful tool to detect the viral particle creating hepatitis non-A non-B non C in 1975 (Feinstone *et al.*, 1975). The virus particle of 27-34 nm appeared unenveloped, was detected in stool samples collected during preicteric and early icteric phases and to determine antibody titres in the sera (Balayan *et al.*, 1983). In general, TEM technique does not serve as a diagnostic tool since it usually requires of large amounts of antigen and high antibody titre; and further, virions are shed degraded form in faeces (Panda *et al.*, 2007).

The scanning electron microscope (SEM) produce very high-resolution of a sample surface, revealing details about 1-to 5 nm in size. Due to the way these images are created, SEM micrographs have a large depth of field yielding a characteristics three-dimensional appearance useful for understanding the surface structure sample composition (Wiegemann and Lehmann, 2009).

OBJECTIVE OF THE RESEARCH

This experimental section describes the results of the work performed in the first half period of PhD-ship in Italy and in United Kingdom.

- 1st part of the work represents the first extended survey on swine HEV in Italy. The pilot investigation of HEV was conducted on 6 different swine farms in Northern Italy. Stool specimens tested belonged to swine of different age and stage of production, and in particular sow (young and old), usually excluded in prevalence studies, were considered. The aim of this project was to understand more about the prevalence and spread of HEV virus variants/strains circulating in this area, based on phylogenetic analyses.
- 2nd part of the work consisted of a stage period at the Veterinary Laboratories Agency (VLA) in Weybridge (UK) and involved the cultivation of HEV *in vitro* using different cell types in 2D and 3D configuration. The study aimed to evaluate and establish a culture system allowing *in vitro* replication of the virus from faecal suspensions and livers from pigs with high viral load. A wider scope of the study was to understand more about the pathobiology of HEV, regarding: i. How can ingested virus reach the liver? ii. Is it possible that HEV might replicate in tissues and organs such as intestine or kidney in addition to the liver? iii. Which and where are the extra-hepatic sites of replication in the human or in the pig?

PREVALENCE OF HEV IN SWINE HERDS IN NORTHERN ITALY

CHAPTER 3

3.1 INTRODUCTION

The human pathogen HEV is the major cause of viral hepatitis in the world. Recently, it has been demonstrated that the virus can infect animals, and pigs are considered to be the reservoir of the infection (Meng *et al.*, 1997). SwHEV was shown to be very closely related genetically to sympatric human HEV strains in developed regions (Meng *et al.*, 1998). Direct food-borne transmission to humans has been reported following consumption of raw or undercooked deer, wild boar meat and pig liver. The infection is asymptomatic in pigs and is widely distributed in the swine population worldwide. The presence of infectious HEV has been demonstrated in sewage and slurry lagoon samples (Pina *et al.* 1998; Pina *et al.*, 2000; Kasondorkobua *et al.*, 2005). The aim of this study was to investigate the prevalence of HEV excretion in 274 randomly selected asymptomatic pigs of different age classes from six different swine farms of Emilia Romagna region located in Northern Italy, to know which variants/strains were circulating in Italian pig farms, which genotype they belong to and the phylogenetic correlation with swine HEV strains and human strains already known in Italy (Caprioli *et al.*, 2007) and worldwide.

3.2 MATERIAL AND METHODS

3.2.1 SAMPLING

Six farms were visited from January 2006 to June 2006. The farm size ranged from 500 to 1100 sows and herd typologies analyzed were farrow-to-finish (close cycle) and farrow-to-weaning (open cycle). From each farm, at least 10 animals were sampled for each production stage, except for the two farrow to weaning farms where fatteners were not sampled being dislocated far away in different areas. In total, two hundred seventy-four samples belonging to 6 farms were collected from clinically healthy selected pigs randomly chosen from different age groups:

- weaners (3-4 months)

- fatteners (8-9 months)
- young sow (1-2 parities)
- old sow (>2 parities)

This scheme allows systematic sampling of different pig classes within the herds, estimating with a 95% probability, the prevalence of HEV-positive animals with an expected prevalence of 30% and an accepted error of 25%. For each animal, faeces were collected from rectal swabs suspended in 10% (Diethylpirocarbonate) DEPC water and stored at -70°C until processing. Each stool sample has been suspended in DEPC water following protocol described below for prepares one faecal suspension sample:

- Weight 1g of stool and introduce it in one sterile eppendorf tube of 1.5ml filled with 1ml of water.
- Vortex 1 min
- Centrifuge the tube at 1000 x g x 30 min at 4°C.
- Transfer the supernatant into sterile eppendorf of 1.5 ml and save the faecal suspension at -80°C.

3.2.2 RNA EXTRACTION AND RT-*nested* PCR

RNA was extracted from 170µl of faecal suspension into a 40µl elution volume, using QIAamp Viral Kit (Qiagen, Hilden, Germany).

An HEV-specific RT-nested-PCR was performed using SuperScript One-step RT-PCR with Platinum Taq (Invitrogen; Carlsbad, CA, USA), and two degenerate primer sets targeting the ORF2 region. RT-PCR primers were HEVORF2con-S1-5'GACAGAATTRATTTTCGTCGGCTGG-3' and HEVORF2con-A1-5'CTTGTTTCRTGYTGGTTRTCATAATC-3'. For the *nested*-PCR step, HEVORF2con-S2 – 5'GTYGTCTCRGCCAATGGCGAGC-3' and HEVORF2con-A2-5'GTTTCRTGYTGGTTRTCATAATCCTG-3' primers were used, yielding a final product of 145bp (Erker *et al.*, 1999) (**Table 3.2.2**). Four microliters of RNA were added to 2X Buffer RT-PCR Master Mix (Invitrogen), dNTP mix (0.2mM each nucleotide) and 0.3µM of primers HEVORF2con-A1 and HEVORF2con-S1, in a final

volume of 15µl. RT-PCR was performed by: reverse transcription cycle at 45°C for 30 min, followed by 94°C for 2 min, and 40 cycles at 94°C for 45sec, 49°C for 45 sec, and 72°C for 1min, followed by a 7 min at 72°C.

Samples were then subjected to *nested*-PCR using internal primers HEVORF2con-S2 (Erker *et al.*, 1999). PCR mix was set-up with the following reagents (final concentration): 1X PCR buffer (Applied Biosystem, ABI, Foster City, CA, USA), 2.5mM MgCl₂, 0.2µM of each primer, 2 units of AmpliTaq DNA Polymerase. The PCR was performed as follows: 94° for 9 min, followed by 35 cycles at 94° for 45 sec, 49° for 45 sec, and 72° for 1min. Negative and positive swine stools were included as control in each assay. Amplified products were stained with ethidium bromide in a 2% agarose gel.

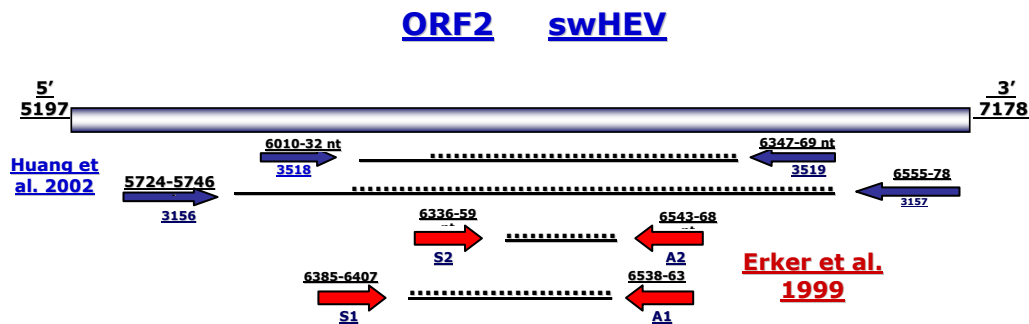


Table 3.3.2 Full length HEV ORF2 showing where the set of primers: S1/A1; S2/A2 (Erker *et al.*, 1999); 3156/7; 3158/9 (Huang *et al.*, 2002) anneals to the DNA sequence in PCR and *nested*-PCR.

3.2.3 SEQUENCING AND PHYLOGENETIC ANALYSIS

Sixteen HEV positive *nested* RT-PCR products (band expected 145bp) selected randomly from different farms were excised from agarose gel. DNA was purified with a Qiaquick gel extraction kit (Qiagen, Hilden, Germany), and sequenced (ABI Prism 310 DNA sequencer, Applied Biosystem Foster City, CA, USA) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) using PCR primers.

The sequences obtained were aligned with others swine and human sequences saved in the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>), and edited using the DNASIS Max Software (Hitachisoft). Phylogenetic analyses were carried out with the Bionumerics software packages (Applied Maths, Kotrijk, Belgium), and the dendrogram was obtained with the UPGMA method. For phylogenetic analyses human and swine strains belonging to all known genotypes were included, as described below:

- genotype 1 (HuHEV, Mya86-Li strain, accession no. DQ079624);
- genotype 2 (HuHEV, Mexico strain, accession no. M74506);
- genotype 3 (SwHEV and HuHEV) (United States, accession no. AF082843 and AF060669), SwHEV swJ570 (Japan, accession no. AB073912), SwHEV NLSw15 and HuHEV NLSw20 (The Netherland, accession no. AF332620 and DQ200292), HuHEV (Hungary, accession no. AY940427.1), SwHEV (Hungary, accession no. EF530672), SwHEV P354 (United Kingdom, accession no. AF503511), SwHEV NLSW28 (The Netherlands, accession no. AF336292), sewage sample HEV BCN13 (Spagna, AF490994), swine Italian strains accession no.: MO/9_3/06/IT, EF681107; MO/36_4/06/IT, EF682083; HEVBO/01, EF681109; HEVPI/01, EF681110; Italian human strains accession no. AF110390;
- genotype 4 (HuHEV, JKK-SAP strain, accession no. AB074917 e swHEV, swCH31 strain, accession no. DQ450072)
- avian HEV (Stati Uniti, accession no. AY535004) (Huang *et al.*, 2004).

3.2.4 STATISTICAL ANALYSES

To evaluate the effect of herd size, farms were arbitrarily divided into two categories based on the medium of sows present: large farms (>1000), and small farms (<1000 sows). To analyse the difference in HEV prevalence against the animal production stage and the herd dimension, a binary logistic regression was performed. Before this, the proportion of positive animals was evaluated using X^2 test. The factors screening with p value <0.15 were then evaluated using binary logistic regression. The model was based on the simultaneous entry of all variables, and its efficacy was assessed based on the likelihood-ratio and the Hosmer-Lemeshow statistic. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated from the final binary logistic model.

All statistical analyses were performed using the software SPSS 12.0.0 (SPSS Inc., Chicago, IL, USA)

3.3 RESULTS

3.3.1 RT-nested PCR

One hundred fifteen out of 274 pigs tested (42%) resulted HEV positives. All six farms tested resulted HEV positives, with mild prevalence ranging between 12.8% and 72.5% (**Table 3.3.1**). All age groups and production stage of pigs tested were shown to be HEV-positive (**Table 3.3.2**). The higher prevalence was observed, in the fattening stage between weaners (90-120-day-old), with 27 positives out of 64 tested (42.2%), while the prevalence was 27% at fattener age (> 120-day-old). Concerning gilts among breeding animals, 43.1% of gilts tested resulted positive. The prevalence decreased slightly in young sow (38.6%), being higher in old sow (53.4%). Logistic regression analysis was performed to investigate the risk factors for HEV infection, as reported in **Table 3.3.2**. The farm size is important to investigate the risk factor for HEV (HEV prevalence was significantly higher in herds with >1000 sows), and the probability to acquire the infection from pig to pig is 5 times more than found in herds with < 1000 sow (OR=4.98; 95% CI 2.73-9.09; p=0.000). About the age of the sows, the odds of virus shedding was also 2.54 times higher in old sows (> 2 parities), close to statistical significance (95% CI 0.98-6.55, p=0.054).

3.3.2 SEQUENCING AND PHYLOGENETIC ANALYSIS

A phylogenetic analysis was performed on the nucleotide sequence of 16 isolates selected randomly from each farm, out of 115 positive PCR products. The Italian swine strains HEV isolated showed a sequence homology lower than 79% with respect to genotypes 1, 2, 4, and between 87.6% and 96.2% with the genotype 3 reference strains (**Fig. 3.3.4**). The strains identified in this study belong to genotype 3 as long as swine HEV and indigenous European strains circulating worldwide identified so far. Among 16 swine HEV strains, nucleotide sequence identity ranged from 90.5% to 100%, whereas the aminoacid sequence was identical in all cases, due to silent mutations. To determine the similarity between swine strains identified in this study to others strain circulating worldwide, the target sequences were compared with sequences from the NCBI database representing human and swine HEV strains circulating worldwide and belonging to the four known genotypes. In particular, one group of seven strains clustered close (91.6%-96.2% identity) to a human HEV strain reported in The Netherlands in 2005 (DQ200292). Nine other swine strains appeared to be related (93.1%) to both a swine HEV strain from The Netherlands (AF490994). The latter two HEV strains were classified into genotype 3 subtype f (Lu *et al.*, 2006).

Table 3.3.1: Detection of swine HEV RNA in stools, by type of herd and production stage (Di Bartolo *et al.*, 2007)

Type of farm ^a	Farm n°	Pigs positive/tested by production stage (%)										Total Prevalence (%)	
		Gilts (0 parities ^b)		Young sows (1-2 parities ^c)		Old sows (> 2 parities ^d)		weaners (< 120 days old)		Fatteners (> 120 days old)			
F-W	2	7/10	(70.0)	7/10	(70.0)	7/10	(70.0)	8/10	(80.0)	-	-	29/40	(72.5)
F-W	6	4/9	(44.4)	4/10	(40.0)	8/10	(80.0)	7/9	(77.8)	-	-	23/38	(60.5)
total F-W		11/19	(57.9)	11/20	(55.0)	15/20	(75.0)	15/19	(78.9)	-	-	52/78	(66.7)
F-F	1	3/10	(30.0)	5/10	(50.0)	9/10	(90.0)	1/10	(10.0)	6/10	(60.0)	24/50	(48.0)
F-F	3	4/9	(44.4)	2/10	(20.0)	2/9	(22.2)	2/10	(20.0)	2/10	(20.0)	12/48	(25.0)
F-F	4	2/10	(20.0)	2/10	(20.0)	1/10	(10.0)	1/7	(14.3)	0/10	(0.0)	6/47	(12.8)
F-F	5	5/10	(50.0)	2/7	(28.6)	4/9	(44.4)	8/18	(44.4)	2/7	(28.6)	21/51	(41.2)
total F-F		14/39	(35.9)	11/37	(29.7)	16/38	(42.1)	12/45	(26.7)	10/37	(27.0)	63/196	(32.1)
All farms		25/58	(43.1)	22/57	(38.6)	31/58	(53.4)	27/64	(42.2)	-	-	115/274	(42.0)

^a F-W: open cycle (farrow-to-weaning); F-F: close cycle (farrow-to-finish)

^b Age range: 7-10 months

^c Age range: 11-15 months

^d Age range: 1-5 years

Figura 3.3.1: Total Prevalence for farm

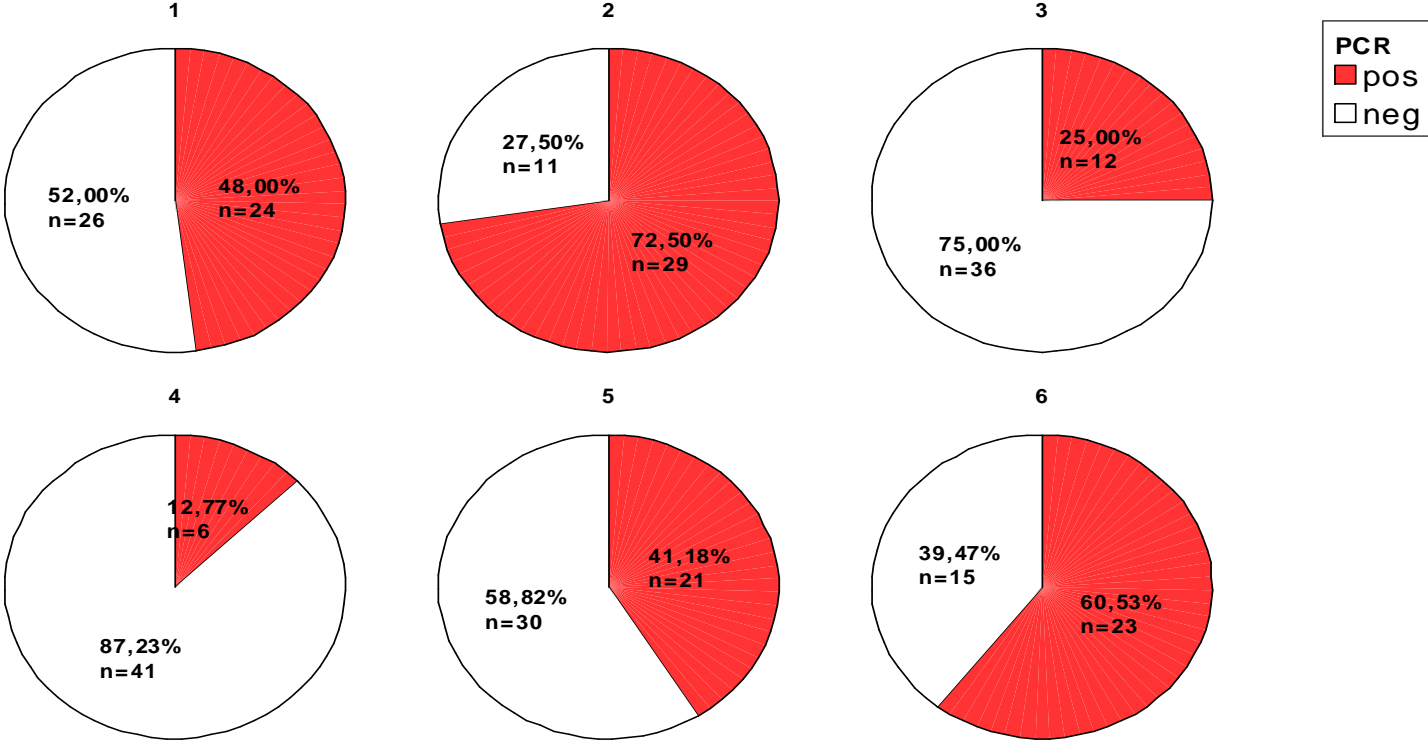


Table 3.3.2: Logistic regression analyses of HEV shedding according to swine age, production stage and herd size.

Production stage	HEV-positive pigs/total (%)	Odds ratio	95.0% C.I.for OR	<i>P</i>
Gilts (0 parities ^a)	25/58 (43.1)	1.58	0.61-4.09	0.345
Young sows (1-2 parities ^b)	22/57 (38.6)	1.33	0.51-3.47	0.559
Old sows (> 2 parities ^c)	31/58 (53.4)	2.54	0.98-6.55	0.054
Weaners (< 120 days old)	27/64 (42.2)	1.38	0.54-3.51	0.504
Fatteners (> 120 days old)	10/37 (27.0)	-	-	-
Herd size				
> 1000 sows	97/179 (54.2)	4.98	2.73-9.09	0.000
< 1000 sows	18/95 (18.9)	-	-	-
Costant	-	0.15		0.000

^a Age range:7-10 months

^b Age range: 11-15 months

^c Age range: 1-5 years

Figure 3.3.3: Total prevalence per production stage.

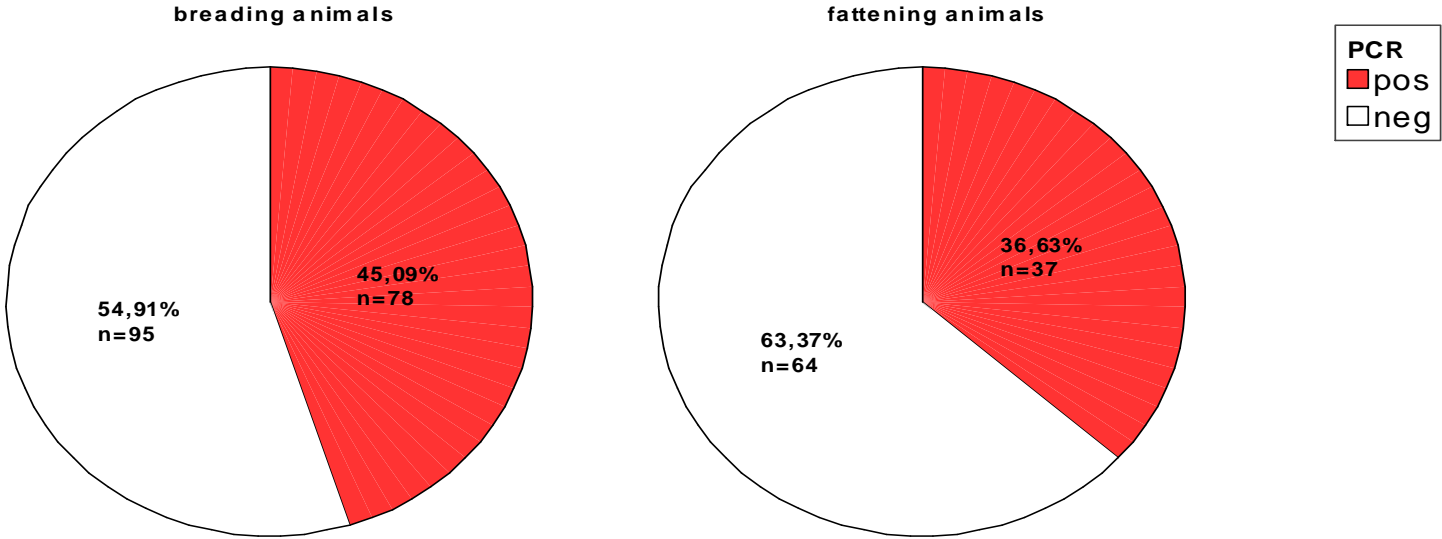


Figure 3.3.4: Weaning prevalence per farm

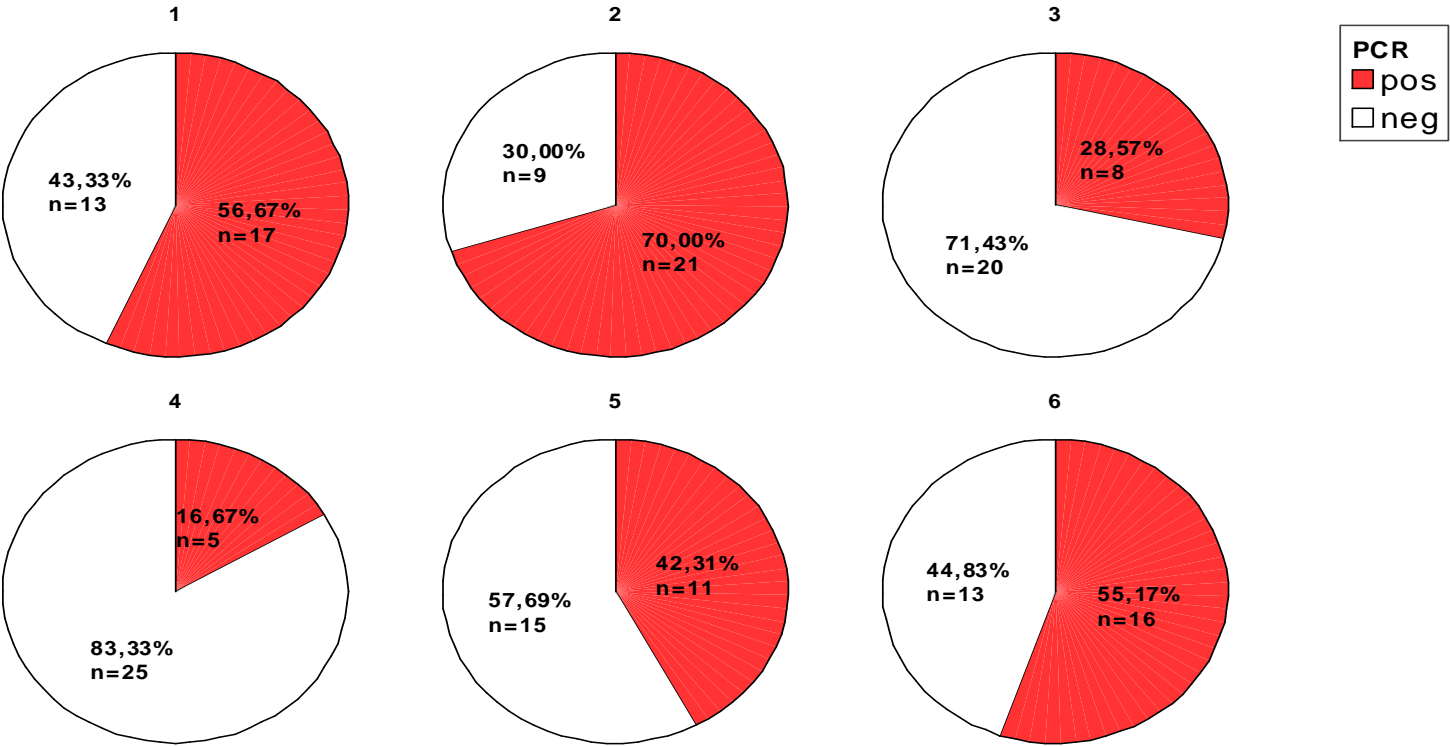
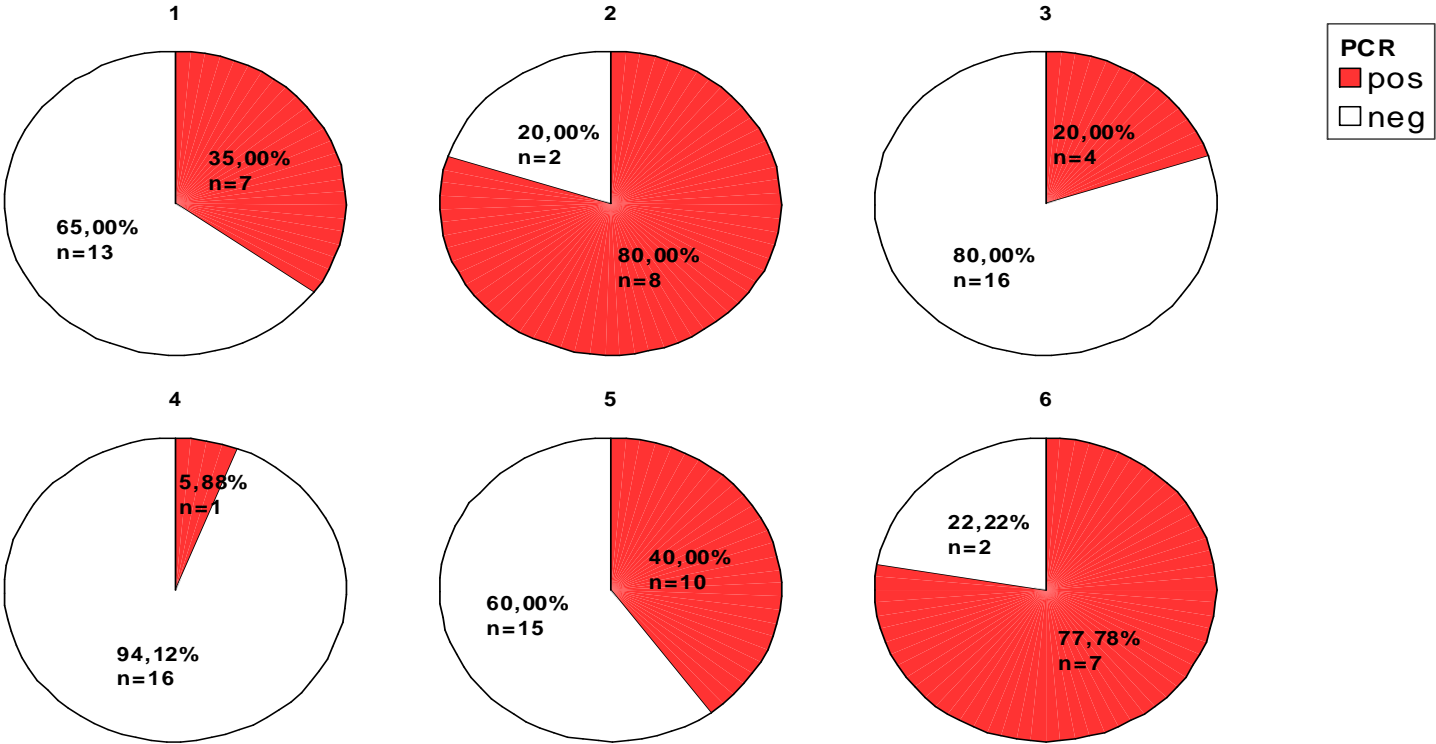


Figure 3.3.5: Prevalence of fatteners per farm



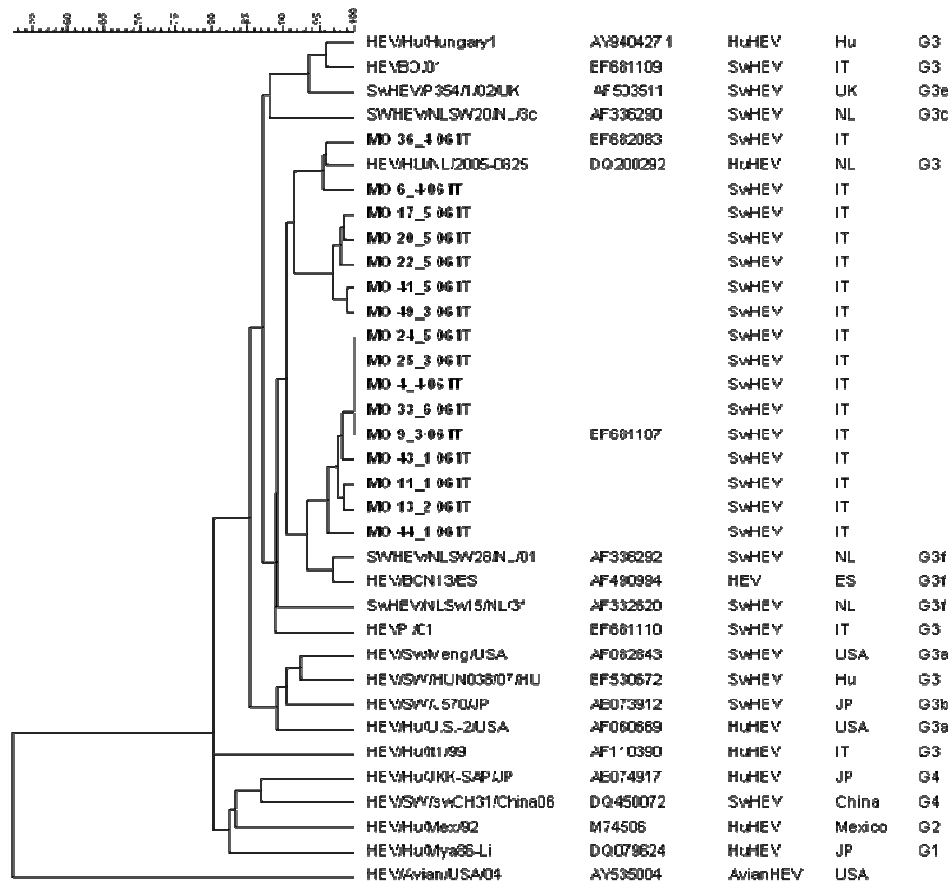


Figura3.3.7: Dendrogram, drawn using UPGMA and an avian HEV strain (United States, accession no. AY535004) as outgroup, based on 80 bp of the ORF2 fragment. GenBank accession no., origin, country, and genotype are reported. Animals are indicated as: YS (0 parities), young sow 7–10 months; YS (1–2 parities), young sow 11–15 months; OS (>2 parities), old sows 1–5 years; weaners, <120 days; fatteners, >120 days. Strains identified in this study are indicated with code MO/individual no./farm no./06/IT (boldface type). Phylogenetic tree of identified sequences (Di Bartolo *et al.*, 2007).

3.4 DISCUSSION

This is the first extended pilot investigation on swine HEV prevalence in Italian swine herds. A preliminary work regarding the same aspect, in the same area, was published in 2007 (Caprioli *et al.*, 2007), but the difference was in the numbers of the samples tested (34 faecal pool and 22 sera) and the rate of virus shedding observed was markedly lower (5.9%, only two faecal pools resulted HEV positives) than 42% (115 stools out of 274 tested) in this current study. However, the preliminary report published (Caprioli *et al.*, 2007) underestimated the rate of virus shedding, probably due to the numbers and sampling or better optimization of the diagnostic technique used (Rutjes *et al.*, 2007). However, the rate of virus shedding is in line with other European reports recently published: Fernandez-Barredo *et al.*, 2006 (23.29%; 34/146); Rutjes *et al.*, 2007 (55%; 53/97); Seminati *et al.*, 2008 (18/66 in the sera, 27.3%; and 7/41 in the stools, 17.1%). The current study is the second similar report in Europe, after a Dutch investigation (van der Poel *et al.*, 2001). Altogether, these data suggest that HEV infection is probably more frequent in European intensively reared swine herds than previously thought, and confirm that pigs represent an important reservoir of HEV infection (van der Poel *et al.*, 2001; Banks *et al.*, 2004; Fernandez-Barredo *et al.*, 2007; Seminati *et al.*, 2008).

The HEV prevalence was higher in herds with a number of sows >1000, independent of farm typology (farrow-to weaning or farrow-to-finish), ranging from 12.8% to 72.5%. However, the prevalence was also higher in farms with a farrow-to-weaning (66.7%) than farrow-to-finish (32.1%) typology, but the low number of farrow-to-weaning farms examined is a limit to final conclusions. Further, it is needed to consider that the higher prevalence and virus spread of HEV in bigger herds of farrow-to-weaning farms could be related to other variables, common for most swine diseases, as the number of pigs in the herd, frequency of pigs introduction, the numbers of suppliers etc. The virus shedding was observed in pigs of all age categories within the herd, with pattern of infection similar among the farms. Previous reports (Meng *et al.*, 1997; Meng *et al.*, 1998b) conducted on serum and faecal samples demonstrates that HEV RNA can be primarily detected in pigs of 2-5 months of age, while animals younger than 2 months are generally negative. These data are in line with the knowledge that maternal immunity protects the piglets from HEV

infection for approximately 2-3 months (Nakai *et al.*, 2006; Satou and Nishiura, 2007). In this study, the prevalence data (42.2%) in pigs of 90-120 days of age is in accordance with this infection pattern. Most of studies published confirm that viremia lasts 1-2 weeks after infection and that virus excretion in the faeces lasts 3-4 weeks followed by seroconversion of IgM in IgG and elimination of the infection by the immune system (Meng *et al.*, 1998a; Nakai *et al.*, 2006). Hence, it was not expected to detect the virus in pigs of 6-8 months of age. This study demonstrated that HEV could be detected in the faeces of all age categories, from weaners of 3-4 months (42.2%) to old sow (53.4%) well beyond 6-8 months of age. Similar results has been recently published in USA, where 11% of commercial pig livers from grocery store were found positive for HEV RNA (Feagins *et al.*, 2007), and in Spain (Fernandez-Barredo *et al.*, 2006, Fernandez-Barredo *et al.*, 2007; de Deus *et al.*, 2008); Japan (Nakai *et al.*, 2006) and Canada (Leblanc *et al.*, 2007), where HEV has been found in up to 41.2% of slaughtered swine at 22-29 weeks of age. In these reports HEV was detected in breeding or in old pigs (6-8 months), suggesting that the swine may retain its susceptibility to HEV infection at any age, possibly sustained by an incomplete protective immunity, and that the infection in pigs could be more prolonged than previously thought or might become chronic. However, the pigs may be incompletely protected against the new virus continuously introduced by new animal and new suppliers into the farms. This hypothesis could be justified by the high prevalence of infection in old sows found in Italy. In this study, all samples were randomly selected from pigs looking clinically healthy, as reported in the literature (Clemente-Casares *et al.*, 2003; Banks *et al.*, 2004; Fernandez-Barredo *et al.*, 2006; Zheng *et al.*, 2006).

Phylogenetic analyses in this study was performed on 16 swine HEV genomes sequenced, within the 80bp ORF2 fragment analyzed, showing that all HEV strains belonged to genotype 3, sharing 90.5%-100% of nucleotide identity and a 100% of amino acid identity. The 16 swine HEV strains, representing the different farms tested, and five strains from four farms proved identical. The analysis results suggested that at least 12 different virus variants/strains were circulating in the pig farms of Northern Italy at that time of investigation, that more strains can be present in a same farm, and that at same strain of HEV can be spread in farms separated geographically, implying a common source or origin of infecting virus. These Italian swine strains presented closer homology with human or swine HEV circulating in Europe genotype 3 than with other strains belonging to the same genotype but from

more distant geographic areas (van der poel *et al.*, 2001; Banks *et al.*, 2004). The finding of HEV prevalence in pigs at slaughtering age raises public health concern for persons with occupational exposure to swine (e.g. farmers, veterinarians, and butchers) by contact with either infected pigs or with environment and working instruments contaminated with pig faeces. But the public health concern affects everyone, and particularly the consumer, because there is a potential risk of animal-to-human transmission HEV by direct food-borne transmission via ingestion of contaminated undercooked meat.

CULTIVATION OF HEV *IN VITRO*

CHAPTER 4

4.1 INTRODUCTION

There has been a growing interest in recent years in the development and use of three-dimensional (3D) cell culture systems in order to replace the classical two-dimensional (2D) monolayer system. Tissues and organs are three dimensional (3D). However, our ability to understand their formation, function, and pathology has often depended on 2D cell culture studies or animal model system (Yamada and Cukierman, 2007). The pathobiology of hepatitis E virus (HEV) has been difficult to study because of the absence of a reliable cell culture system that can provide high-titre virus, or that would enable the investigation of virus viability in PCR positive animals and environmental samples. HEV has been propagated through 2D cell culture system, in 2BS (human embryo lung diploid cell strain), A549 (human lung carcinoma cell line), PLC/PRF/5 (hepatocarcinoma cell line) HepG2/C3a, primary hepatocytes from non human primates (chimpanzees, cynomolgus macaques, tamarins and African green monkeys) and FRhK (female rhesus kidney cells) in a limited number of laboratories (Huang *et al.*, 1992, Kazachov *et al.*, 1992; Tsarev *et al.*, 1994; Huang *et al.*, 1995; Tam *et al.*, 1996 Tam *et al.*, 1997 Huang *et al.*, 1999; Wei *et al.*, 2000; Emerson *et al.*, 2006 ; Tanaka *et al.*, 2007). However, the reproducibility of these systems still needs to be established.

Three dimensional (3D) culture has shown promise in the facilitation of viral replication of fastidious viruses, for example, Norovirus (Straub *et al.*, 2007), in the culture of several enteric bacterial pathogens *in-vitro* (Nickerson *et al.*, 2001; Chopra *et al.*, 2006; Carterson *et al.*, 2005; Crabbé *et al.*, 2008).

New methodology is required to investigate whether HEV replicates in tissues and organs other than the liver, for example in the intestine or kidney. We investigated the Rotary Cell Culture System (RCCS) as a tool for the *in-vitro* cultivation of HEV. RCCS, developed by NASA, was originally designed to protect cell cultures from the high shear forces generated during the launch and landing of the space shuttle. During development cells in suspension were observed to aggregate and form structures resembling tissues, suggesting that this methodology may also be

useful in the study of co-cultures of multiple cell types and associations between proliferation and differentiation during the early steps of tissue formation. Therefore, the RCCS (**Fig.4.1**) is a new technology that offers the potential for growing anchorage dependent or suspension cells to acquire structure and function similar to *in vivo* cells and tissues.

The objective of this study was to investigate the potential of RCCS in the cultivation of HEV, and to compare with 2D cultivation in the same cell types.

A

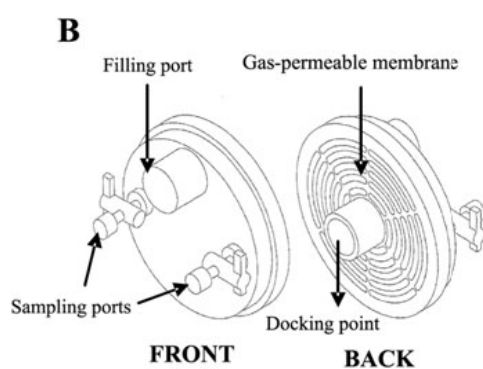


Fig. 4.1. The Rotary cell culture system (RCCS-4DQ, Synthecon).

A: The RCCS is available as a one, two, four or eight station rotator base. The system depicted consists of a four Station Rotator Base, along with a power Supply with Tachometer. Each station is capable of rotation at independent speeds, enabling four experimental conditions and/ or experiments to be run simultaneously. The system is supplied with four Rotary wall vessels (RWV). (B): The cylindrical RWVs is completely filled with culture medium, cells and micro carrier beads through the filling port on the face of the vessel. All bubbles are removed from the

RWV through the sampling ports to reduce shear. The vessel is attached to the rotator base by docking point and rotated on its axis that is parallel to the ground creating a solid body rotation. Cell-beads aggregates in the RWV are maintained in a gentle fluid orbit and do not collide with the walls or any others parts of the vessel (i.e., suspension culture). As 3D tissues grow in size, the rotation speed is adjusted to compensate for the increased settling rates of the larger particles. The cells and/or tissue particles join to form larger tissue particles that continue the differentiation process. Oxygen supply and Carbon dioxide removal are achieved through a gas-permeable silicone rubber membrane that covers the back of the RWV bioreactors. (Nickerson *et al.*, 2001; Crabbé *et al.*, 2008).

4.2 MATERIAL AND METHODS

4.2.1. CELL TYPES

A range of human (Hep G2/C3a, Int 407, PLC/PRF/5) and animal cell types (PK-15) were investigated, including a number in which limited replication of HEV had been reported by other laboratories. In addition several interferon knocked-out (IFN KO) cell lines were investigated: Int-V-11c and Int-V-7E intestinal cell lines, and PK-15 IFN- KO. The human hepatoblastoma cell Hep G2/C3a has the following properties; strong contact inhibition of growth, high albumin production, high production of alpha-fetoprotein (AFP) and an ability to grow in glucose-deficient medium, does not express endogenous CDO (Cysteine dioxygenase) protein (this enzyme catalyzes the oxidation of L-cystein sulfonic acid which is the first major step in cysteine catabolism in mammalian tissues (Dominy *et al.*, 2007)). This reaction is the rate-limiting step in the eukaryotic taurine biosynthetic pathway, and taurine levels are directly correlated to cysteine dioxygenase activity. Taurine is an important antioxidant and low levels of taurine have been linked to a variety of cardiac, neural, and autoimmune disorders (Imnsand and Ellis, 2008). Hep G2/C3a cell line was obtained from the American Type Culture Collection (ATCC).

The human embryonic intestinal epithelial cell line Int-407 was obtained from Ken-Mellits (Nottingham University; Collaborator), in addition to Int-407 subclones (Int-V-11c and Int-V-7E IFN KO) that had been engineered to constitutively express the V-protein of the paramyxovirus, simian virus 5 (SV-5): The V protein degrades the signal transducer and activation of transcription 1 (STAT-1), preventing the

STAT-1 mediated interferon response. IFN-KO phenotype was preserved in these cell lines with the addition of puromycin. The Alexander hepatocarcinoma cell line (PLC/PRF/5) was obtained from the American Type Culture Collection (ATCC). This cell line synthesises hepatitis B surface antigen (HBsAg) and has been used for culture of HBV *in vitro*. PLC/PRF/5 has been used for the cultivation of HEV in 2D format (Tanaka *et al*, 2007).

Porcine Kidney cells (PK15; wild type (wt)) and PK15 IFN-KO were kindly supplied by Severina Anna La Rocca (CSFV group, VLA). The PK15 IFN-KO cells had been engineered to constitutively express the protein N^{Pro} (N terminal cysteine-like autoprotease) which inhibits the production of type I interferon. The expression of N^{Pro} was activated with the addition of Tetracycline (TET) 5-6 hrs prior to cell infection, to avoid activation of a signal-transduction pathway that triggers the transcription of a diverse set of genes that, in total, establish an antiviral response in target cells. IFN-KO phenotype was preserved in this cell line with the addition of Blasticidin and Hygromycin.

4.2.2 PREPARATION AND SERIAL PASSAGE OF 2D CULTURES

Prior to use, all cells types used in this study were stored in 1ml volumes in vials in liquid nitrogen (-196°C). Cells were thawed by hand or by incubation at 37°C in a water bath for approximately 2 min. Cells were then transferred to an appropriately sized culture flasks containing the recommended medium (**Table 4.2.2.1**) and incubated at 37°C in 5% CO₂ until cells attached to the bottom of the flask. Following cell attachment fresh medium was added to the flask to remove the cryoprotective agent (DMSO), which inhibits cells growth.

Cell line	Int 407	Int-V-7e IFN KO	Int-V-11c IFN KO	Hep G2/C3a	PK-15	PK-15 IFN KO	PLC/PRF/5
MEDIUM	D-MEM	D-MEM	D-MEM	H-MEM	E-MEM	E-MEM	E-MEM
FBS	10%	10%	10%	10%	10% TET free	10% TET free	10%
NEAA	0.1mM	0.1mM	0.1mM	0.1mM	0.1mM	0.1mM	0.1mM
NaHCO₃	/	/	/	1.5g/l	/	/	1.5g/l
L-Gln	/	/	/	/	/	/	2mM
C₃H₃NaO₃	/	/	/	1.0mM	/	/	1.0mM
Puromycin hydrochloride	/	5µg/ml	5µ/ml	/	/	/	/
Blasticidin	/	/	/	/	/	1 µg/ml	/
Hygromicin	/	/	/	/	/	50 µg/ml	/
PEN/Strep/ Myco	Standard	Standard	Standard	Standard	Standard	Standard	Standard

Table 4.2.2.1 . Overview of the components of the complete growth medium used with each cell line. The abbreviations indicate: FBS (Fetal Bovine Serum); NEAA (Non essential aminoacids); NaHCO₃ (Sodium Bicarbonate); L-Gln (L-Glutammine); C₃H₃NaO₃ (Sodium pyruvate); PEN/Strep/Myco (Penicillin/Streptomycin/ Mycostatin) in standard concentration: 100U/ml, 100U/ml, 20U/ml, respectively.

SUBCULTURING PROCEDURE

Volumes used in this protocol are for 75cm² flask, and can be proportionally reduced or increased for culture vessels of other sizes.

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with PBS.
3. Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.03% (w/v) EDTA solution, to remove all traces of serum which contains trypsin inhibitor.
2. Add 0.5 ml of Trypsin-EDTA solution to flask place at 37° to facilitate dispersal (usually within 3-5min). Cells are observed under an inverted microscope until cell layer is dispersed.
3. Add 5.5ml of complete growth medium and aspirate cells by gently pipetting.
4. Add appropriate aliquots of the cells suspension in according to subculture ratio into new culture vessel.
5. Incubate cultures at 37°C. and 5% CO₂ in air atmosphere.

Cell cultures were initially grown as a standard monolayer in Nunclone Surface T25 cm² or T75 cm² flasks with the appropriate complete growth medium (**Table 4.2.2.1**), according to the subculture procedure protocol described above. Cells were subsequently seeded in 24 or 96 well plates (4x 10⁴ cells/well in 24 well plates and 1x 10⁴ cells/well in 96 well plates) or in T25 flasks (used for serial passages procedure, described below) 24-48h prior to infection. Cell cultures were infected with HEV upon reaching 70-80% confluency.

INOCULUM

Three sources of HEV isolates were used as inoculum in this study. HEV-positive faecal samples collected during a herd prevalence study in the UK (McCreary *et al.*, 2008) were subsequently tested by qPCR and samples found to contain the highest viral loads of HEV were selected (Refs.:1/10; 2/1; 6/109; 10/189; 3.8; 5.8; 7.21). Two HEV-positive liver samples were also used as inoculum, one that had been collected from a retail outlet and the second from a pig that had been experimentally infected with swHEV (Bouwknegt *et al.*, 2009) (kindly supplied by

Wim H. M. van der Poel, Central Veterinary Institute Wageningen University and Research Centre, Collaborator) (Refs. L28 and L3 respectively). These HEV Real Time PCR-positive samples (Ref. 1/10; 2/1; 6/109; 10/189) either separately or as a pool of four were chosen for the first experiment as reported in McCreary *et al.*, (2008) work. These inoculums were used for infect cell cultures according to the following protocol:

Protocol for one 24 well microplate:

1. Grow cell culture in the T25 flasks.
2. Confluent cells trypsinized off and seeded (4×10^4 cells/ml) in one 24 well microplate.
3. Set up the plate:

Well	1	2	3	4	5
A	V-ve	NI	V+ve <u>2/1</u>	V+ve <u>6/109</u>	V+ve <u>10/189</u>
B	V-ve	NI	<u>2/1</u>	<u>6/109</u>	<u>10/189</u>
C	V-ve	NI	<u>2/1</u>	<u>6/109</u>	<u>10/189</u>
D	V-ve	NI	<u>2/1</u>	<u>6/109</u>	<u>10/189</u>

Abbreviations:

V-ve: negative inoculum from HEV RT-PCR negative sow.

NI: Not Infected (Cell control)

V+ve: positive inoculum (the underlined represents the inoculum code)

4. After 24-48h at 70% of confluence infect the cells in the plate with 100 μ l of each inoculum (dil.1.10) for 1h at 37°C
5. Wash 1x with PBS
6. Replace fresh medium
7. Collect 140 μ l of medium for RNA extraction; represents T0
8. Incubate the plate at 37 °C throughout observation period

9. Collect medium every 2 days for RNA extraction from half plate and add 560 μ l of Viral Lyses Buffer for 10 min on the cells in apposite well. Follow QIAamp Viral RNA mini kit (Qiagen) procedure (for more details see section: 4.4 Inoculum preparation) and perform real time PCR
10. Fix the other half plate with 80% Acetone for 15' and store the plate in – 20°C for IPX staining.

For PK-15 the folloing protocol was adopted except for IPX staining (where 96 well microplates were used), performing the experiment in duplicate.

Serial passages procedure for one flask

1. Grow cell culture in T25 flasks.
2. Confluent cells trypsinized off and split 1:2 in other T25 flasks.
3. At 70% of confluence, infect fresh cells with 600 μ l of culture supernatant from previous passage (6-7 days), subjected 2X to Freeze thaw cycles and following filtration with spin-X 0.22 μ m (Costar) centrifuge tube filters at 10,000 g at 4°C for 15-25 min before infecting fresh cells.
4. Save aliquots of medium and cells every 3DPI and test it by real time PCR for HEV.

Six serial passages were performed for Int-V-7E and Int-V-11c cell lines; while for PK-15 IFN five serial passages of culture supernatant were performed following the same “serial passages procedure for one flask”, except for step.3 which was performed without purification of the culture supernatant and with just one freeze-thaw cycle before inoculation for passages 1, 2, 3, 4 and 5 on fresh cells PK- 15 IFN KO grown in T25 flasks (6-7 days).

4.2.3 PREPARATION OF 3D CULTURES

All cells types were initially grown as a standard monolayer with the appropriate complete growth medium (**Table 4.2.2.1**). Confluent cells were subsequently split 1:2 in a new basal complex cell culture medium, GTSF-2. In addition to traditional growth-factors, GTSF-2 contains a blend of three sugars (glucose, galactose, and fructose), minimal essential medium L-15 base supplemented with 6% fetal bovine serum and other supplements (Goodwin *et al*, 1993) (**Table 4.3**). Cells were grown in a Cell Coat Collagen type I T-G Flask T-75 cm² (2×10^5 cells/ml) at 37°C in a 5% CO₂ environment in preparation for seeding into the RWV.

After 4 days, cells were trypsinized at 95% confluency, and resuspended in fresh medium at a density of 2×10^5 cells/ml. Cells were then introduced into a RWV (Synthecon, Inc, Houston TX, USA.) containing 5 mg/mL (for Int 407 and Hep G2/C3A cell lines) or 10 mg/mL (for Int-V-7E and Int-V-11c IFN KO, PK-15 IFN KO, PLC/PRF/5 cell lines) of porous Cytodex-3 microcarrier beads (collagen type-I-coated porous microspheres, average size 175 µm in diameter (Sigma). Cells were cultured in the RWV bioreactor in GTSF-2 at 37°C and 5% CO₂, with a rotation speed appropriate to maintain the cell aggregates in suspension during the entire culture duration (approximately 17-25 rotations/min initially with subsequent increase to 27–35 rotations/min, depending on the size of the aggregates). Cell growth was monitored daily and fresh medium was replenished (90% of the total vessel volume) on the 3rd day following seeding, then every 24 hours depending on the growth and metabolic activity of the cell cultures (as monitored by tissue culture media color change). 3-D cells were harvested 34 days after seeding into the RWV. Mature 3-D aggregates were placed into flat bottom, ultra low attachment Costar 24-well plates, ($\sim 1-3 \times 10^5$ cells/well for Int 407 cells and $\sim 6-14 \times 10^5$ cells/well for Hep G2/C3A) containing a coverslip covered with collagen type I (Sigma) for observation by confocal microscopy, and into standard non tissue culture treated 24 well plates for collection of medium for RNA extraction and analysis by real time PCR. The cells were infected with swHEV virus (Refs. 1/10 and 2/1) on the 1st day after seeding cells in the microplates.

Table 4.4. GTSF-2 complex medium with relative supplements ¹.

Component	Concentration ¹	Source/order number of designation
MEM – α supplemented with 2.25 g/liter	1200 ml (40%)	Sigma
L-15	1800 ml (60%)	GIBCO
NaHCO ₃	4.05 g/L	Sigma/S-5761
HEPES	9.0g	Research Organics/6003H-2
Folic Acid 67ug/ml	300ul	SIGMA/F-8758
0.5% Nicotinic Acid	2ml	Sigma/ N-4126
Bactopeptone	1.8 g	Difco/0118-01
I-inositol	0.072g	Sigma/I-5125
Fructose	0.39g	Sigma/F-3510
Galactose	0.75g	Sigma/ G-5388
D-Glucose	3.0g	Sigma/G-5250
200mM L-Gln [2Mm]f	45ml	Sigma/G-5763
Gentamycin	3ml	Gibco/600-5750AD
Fungizone or Amphotericin B solution	6ml	Sigma A 2942
<i>Insulin-Transferrin-Sodium-Selenite</i> (ITSS)	15ml	Sigma/I-1884
FBS	6%	Autogenbioclear

¹ Concentrations are provided for the preparation of a 3 Litre volume of medium

The cells grown in the RWVs were infected 26-28 days after seeding. These cells lines were inoculated with HEV real time PCR-positive swine faecal suspensions (Refs: KM: 3.8; 5.9; 5.10; 7.21; 10/189) and two livers sample: L28 and L3 used to infect in parallel 2D format as well as subjected to filtration before infection of the cells. One hour after inoculation at 35°C 47.5 ml of maintenance medium were added into the 3D vessel and 900 μ l into 2D plates. Small quantities of medium plus few aggregates of cells (140 μ l) were collected according to the Qiagen Kit, for

performing RNA extraction and subsequent real time PCR, one hour after infection (representing T0) and every 2-4 days up to 58 DPI before changing 50% of the medium. The medium collected was stored at -80°C. The maintenance medium used for virus culturing consisted of GTSF-2 medium complex containing 2% (v/v) heat-inactivated FBS and 30mM MgCl₂ at final concentration; other supplements were the same as in the growth medium. The rotation speed of the bioreactor after infection was kept constant at 25 rpm until the end of the experiment.

4.2.4. INOCULUM PREPARATION

The faecal samples, liver tissues and liver culture samples were stored at -20°C until use and were subjected to different preparation protocols.

Faecal samples were defrosted and 0.2 g of faeces was suspended in 1.8 ml PBS (0.01M, pH 7.2) (Dil.1:10), six 5 mm glass balls were added and the suspension was mixed by vortexing. The sample was then centrifuged at 8,000 g for three minutes, and the supernatant was aliquoted and stored at - 80°C until use. On the day of virus infection the supernatant was filtered using a sterile spin-X centrifuge tube filter (0.22µm; Costar) at 10,000 g at 4°C for 15-25 min.

Liver tissues were defrosted and 0.3g liver was suspended in 2.7ml of phosphate-buffered saline (0.01M, pH 7.2) (Dil.1:10) using a pestle and mortar, and homogenized using a needle and syringe. The homogenate was transferred into a 2ml tube and centrifuged at 8,000 g for 3 min. The supernatant was filtered using a sterile spin-X centrifuge tube filter (0.22µm; Costar) at 10,000 g at 4°C for 15-25min.

Liver culture sample was diluted 1:5 in the growth medium and centrifuged at 10,000 g for 3 min. The supernatant was filtered using a sterile spin-X centrifuge tube filter (0.22µm; Costar) at 10,000 g at 4°C for 15-25 min.

The 3D cell aggregates were infected in the plates (Hep G2/c3a and Int 407 cell line) initially with 500µl of inoculum (Ref. 1/10 and 2/1) diluted with medium 1:5, for Int 407, and 1:10, for HepG2/C3a. In the following experiments the 3D cell aggregates were infected directly in the vessels with 1.4 to 2.5 ml of inoculums for 3D and 50µl to 100µl on the cells in 2D configuration of 24 or 96 well plates, respectively. The inoculums were diluted always with medium 1:5 for liver culture sample and 1:10 for the other inoculums, and incubated for 1h at 35.5°C with 5% CO₂.

4.2.4.1 CONVENTIONAL PCR AND REAL TIME PCR

Quantification of HEV RNA was performed by real-time RT-PCR.

RNA was extracted from 50 µl of sample, faecal supernatant or culture medium using the QIAamp Viral RNA mini kit according to the manufacturer's extraction (Qiagen). For both livers and animal cells the RNeasy mini kit (Qiagen) was used, according to manufacturer's instructions as described below. Negative controls (HPLC water) were inserted between every two samples to check for contamination.

Protocol: Purification of Viral RNA (QIAamp Viral RNA mini kit, Qiagen):

1. Pipet 560µl of prepared buffer AVL containing carrier RNA into a 1.5ml microcentrifuge tube.
2. Add 140 µl of plasma, serum, urine, faecal supernatant, cell culture supernatant, or cell-free body fluid to the buffer AVL-carrier RNA in the micro centrifuge tube. Mix by pulse-vortexing for 15 s.
3. Incubate at room temperature (15-25 °C) for 10 min.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 560 µl of ethanol (96%-100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
6. Carefully applied 630 µl of the solution from step 5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1min. Place the QIAamp spin column into a clean 2ml collection tube, and discard the tube containing the filtrate.
7. Carefully open the QIAamp Mini spin column, and repeat step 6.
8. Carefully open the QIAamp Mini spin column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing filtrate.
9. Carefully open the QIAamp Mini spin column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for

- 3 min. Continue directly with step 11, or - to eliminate any chance of possible buffer AW2 carryover - perform step 10, and then continue with step 11.
10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
 11. Place the QIAamp mini spin column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and add 50 μ l of Buffer AVE equilibrated to room temperature. Close the cup, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min. Repeat this step with the same eluate to increase viral RNA yield by up to 10%.
 12. Store RNA in -80 °C.

Protocol: Purification of total RNA from animal tissue (RNeasy mini kit, Qiagen)

Before starting add β -mercaptoethanol (β -ME) to lysis buffer (RLT) before use: add 10 μ l per 1ml Buffer RLT.

1. Thaw frozen tissue.
2. Weigh 30mg.
3. Place it directly into a suitably size glass mortar with 600 μ l of lysis Buffer RLT. Disrupt the tissue with pestle and homogenize it using a needle and syringe.
4. Centrifuge the lysate for 3 min in a microcentrifuge at full speed. Carefully remove the supernatant by pipetting, and transfer it to a new microcentrifuge tube. Use only this supernatant (lysate) in subsequent steps.
5. Add 1 volume of 70% ethanol to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.
6. Transfer up to 700 μ l of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at 8000 x g. Discard the flow-through.

7. Add 700µl of Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g to wash the spin column membrane. Discard the flow-through.
8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g to wash the spin column membrane. Discard the flow-through.
9. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g to wash the spin column membrane.
10. Optional: Place the RNeasy spin column in a new 2ml collection tube (supplied), and discard the old collection tube with flow-through. Close the lid gently, and centrifuge at full speed for 1 min.
11. Place the RNeasy column in a new 1.5ml collection tube. Add 50 µl of RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1min at 8000 x g to elute RNA
12. Repeat step 11 using the same 50 µl for concentrate yield RNA.
13. Store RNA in -80 °C.

Protocol: Purification of total RNA from animal cells (RNeasy mini kit, Qiagen)

Before starting, add β-mercaptoethanol (β-ME) to lysis buffer (RLT): add 10µl per 1ml Buffer RLT.

1. Harvest cells according to step 1a.
 - 1a. Determine the number of cells (do not use more than 1×10^7 cell/ml). Trypsinize the cells and count them with hemocytometer (i.e. liver culture sample from The Netherlands are approximately 4×10^4 cell/ml). Pellet the cells by centrifuging for 5 min at 300g in a centrifuge tube. Carefully remove all supernatant by aspiration, and proceed to step. 2.
2. Disrupt the cells by adding 350 µl of Buffer RLT. Vortex or pipet to mix and proceed to step.3.
3. Homogenize the lysate according to step 3c.
 - 3c. Pass the lysate at least 5 times through a blunt 20-gauge needle (0.9mm diameter) fitted to an RNase-free syringe. Proceed to step 4.

4. Proceed forward like Protocol: Purification of total RNA from animal tissue, described above (RNeasy mini kit, Qiagen).

PCR for Differentiations markers:

For cDNA synthesis 5 µl of RNA was mixed with 8 µl of HPLC water and 1 µl random hexamers (500 µg/ml; Promega). The reaction was incubated at 65°C for 10 min to allow the primers to attach to the RNA, and placed on ice. Four µl of buffer MMLV (Promega) (5X), 1.0 µl dNTPs (10mM), 0.5 µl RNAsin Inhibitor RNAase (40U/µl; Promega) and 0.5 µl RTMMLV reverse transcriptase (200 U/µl; Promega) was then added in a final volume of 20µl. The RT reaction was incubated at 42°C for 60 min, followed by 94°C for 5 min to denature the enzyme, and placed on ice.

The human genes examined were: Albumin (Alb) Cytokeratins (CK) 8, 18, 19, Cyt P450 (Sharma *et al.*, 2005) and Interleukin-6 (IL-6) (Steensberg *et al.*, 2001). Primers sequences used were (Alb) Albumin Forward : 5'- CCA GGA AGA CAT CCT TTG-3', Albumin Rev: 5'- CCT GAG CCA GAG ATT TCC-3'); CK8 forward: 5'- TGA GGT CAA GGC ACA GTA CG-3', CK8 reverse: 5'- TGA TGT TCC GGT TCA TCT CA-3'; CK-18 forward: 5'- TGG TCA CCA CAC AGT CTG CT-3', CK18 reverse: 5'-CCA AGG CAT CAC CAA GAT TA-3'; CK19 forward: 5'- AGG TGG ATT CCG CTC CGG GCA-3'; CK19 reverse: 5'- ATC TTC CTG TCC CTC GAG CA-3'; CytP450 Forward: 5'- CAG AGA TGG AGA AGG CCA AG-3' CytP450 reverse: 5'- CCC TAT CAC GTC GTC GATCT-3'; IL-6 forward 5'-GGTACATCCTCGACGGATCT-3' and reverse 5'-GTGCCTCTTTGCTGCTTTTAC-3'. Ten µl of QPCR mix (Qiagen), 10µM primer forward, 10 µM primer reverse, and 8 µl HPLC water was added to 2µl of DNA in a final volume of 20µl. The reaction conditions were 94°C for 4 min, and 35 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 45sec, 72°C for 10 min and performed on Perkin Elmer Thermocycler). Negative and positive controls for IL-6 were included in each run.

Viral RNA was quantified using a real time RT-PCR that targets ORF-3 of HEV (Juthikumar *et al.*, 2006). Primers and probe were JHEVF (forward): 5'-

GGTGGTTTCTGGGGTGAC-3', JHEVR (reverse): 5'-
 AGGGGTTGGTTGGATGAA-3' and JVHEVP (probe): 5'-
 TGATTCTCAGCCCTTCGC-3') with a 5'-6 carboxy-fluorescein fluorophore
 reporter dye (FAM) and a 3' black hole quencher (BHQ) dye. This assay was run
 using 12.5 µl of Superscript III Platinum one step Quantitative RT PCR reaction mix
 (Invitrogen), 5 µl water HPLC, 1µl Mg SO₄ 50µM, 0.15 µl RNAsin Inhibitor RNAasi
 (40U/µl; Promega) 0.05µl Rox Reference Dye (25 µM; Invitrogen), 0.5 µl JVHEVF
 primer (10 µM), 2.1 µl JVHEVR primer (10 µM), 0.7 µl JVHEVP probe (10 µM)
 and 0.5µl Taq Superscript III (100U/µl) (Invitrogen). The reaction conditions were
 50°C for 30 min, 95°C for 2 min and 45 cycles of 95 °C for 15 sec, 55°C for 30 sec
 and performed on an MX4000 Thermocycler (Stratagene). Negative and positive
 extraction controls were included in each run. Fluorescence was monitored during
 the annealing step of each cycle. At the end of the reaction, Rox Reference Dye was
 used for normalisation of the fluorescent reporter signal. The reproducibility of the
 quantitative assay was assessed by testing each sample in duplicate and the mean
 value was reported. A quantitative PCR uses a standard so that copy number could be
 extrapolated.

4.2.4.1.1 PRODUCTION OF STANDARD FOR QUANTITATIVE PCR FOR HEV

Cloning of PCR amplicon into pGEM® - T Easy Vector

Primers JVHEVF AND JVHEVR were used to amplify a 70 nucleotide fragment of
 ORF-2 of the HEV genome. The PCR amplicon was visualised by agarose gel
 electrophoresis, and purified from the agarose gel using a QIAquick gel extraction kit
 (Qiagen), according to manufacturer's instructions. Sequencing was used to confirm
 that the purified product was HEV specific. Purified PCR product was ligated into
 the cloning vector, pGEM-T Easy Vector System (Promega). Ligation reactions
 were prepared in a sterile 1.5 polypropylene ml tube (Eppendorf) to a final volume of
 10 µl, containing 1 x Rapid Ligation Buffer (Promega), 1 µl pGEM-T Easy Vector
 (Promega), and 1 µl T4 DNA ligase and 3 µl of purified PCR product. The cloning
 vector (pGEM – PCV-2₁₆₀) was transformed into DH5αTM Competent *E.coli* cells
 (Max EfficiencyTM; Gibco BRL), according to manufacturer's
 instructions. Transformed DH5αTM Competent *E.coli* cells were cultured on Luria-
 Bertami (LB) agar plates and bulked up in LB broth. Plasmid DNA was purified

using the the Plasmid Maxi kit (Qiagen), performed according to manufacturer's instructions. A restriction digest was performed to demonstrate that the 70 nucleotide fragment of ORF-2 of the HEV genome had been successfully ligated into the plasmid. Purified plasmid DNA was digested using the restriction endonuclease *EcoRI* (G[▼]AATTC / CTTAA[▲]G). Reactions were prepared in 0.5 ml polypropylene tubes (Eppendorf) to a final volume of 20 µl, containing 1 x Buffer D (Promega), 24U *EcoRI* (Promega) and 8 µl plasmid, and incubated at 37 °C for 2 hours. The digest was visualised by agarose gel electrophoresis to confirm that the insert was of the correct size.

TRANSCRIPTION OF THE INSERT WITH MAXIScript T7 KIT (AMBION)

The plasmid has been first linearized with the restriction enzyme SalI (Promega). Linearization reactions were prepared to a final volume of 20 µl, containing 16.3 µl of sterile water, 2 µl of 10x Buffer, 0.2 µl of Acetylated BSA (10 µg/ µl), 0.5 µl of SalI enzyme (10 u/ µl) and 1 µl of DNA (1 µg/ µl). To confirm that the linearization was efficient, both the digested and undigested products have been run on a 1.5% agarose gel. The digested product have been cut out of the gel and purified using the QIAquick gel extraction kit (Qiagen), and eluted in 10 µl. The digested product has been transcribed into RNA using the Maxiscript T7 kit (Ambion), following the manufacturer's instruction. To eliminate the residual DNA present in the transcript, a DNase digestion reaction have been set up using the Deoxyribonuclease I, amplification grade (Invitrogen), following the manufacturer's instructions. To confirm that the elimination of the residual DNA has been complete a PCR reaction without retrotranscription has been set up, and the PCR product has been run on a 1.5 % agarose gel. No bands were detectable on the gel, confirming that the DNA has been completely transcribed into RNA. The RNA has been subsequently purified with a Ethanol purification protocol. One µl of Glycogen (Invitrogen) have been added to 100 µl of RNA. After mixing, 0.1 volume of Sodium Acetate and 3 volumes of ice cold 100% ethanol have been added to the sample, and the reaction incubated overnight at -20 C. After a spin at 8000 x g for 30 minutes, 2.5 µl of oce cold 75% ethanol have been added too the sample. This has been subsequently centrifuged at 10000 x g for 2 minutes, and the supernatant have been discarded. The pellet has been resuspended in 40 µl of sterile water.

4.2.4.1.2 CALCULATION OF COPY NUMBERS

To calculate the copy number of the RNA transcript and create working solutions to use in the PCR reaction and on line calculator have been used:

www.edu/research/gsc/resources/cndna.html

The amount of RNA has been calculated with Nanodrop (39.5 ng/ μ l), and the length of the template was 164 bp. The resulting number of copies was 1.82×10^{11} . To prepare a working solution stock of 0.2×10^9 , a dilution ratio of 910 has been calculated (909 μ l of water + 1 μ l of RNA). The working solution have been stored at -20 C, and diluted by 10 fold until 10 copies reaction for every PCR reaction.

4.2.4.2 VIRUS INFECTION IN 2D AND 3D CONFIGURATION

The cells grown in 2D configuration were infected in the 24 well microplates, 96 well microplates or in the flask with 100-120 μ l, 40-50 μ l, and 600-1000 μ l of inoculum respectively. Cells were inoculated 24-48h after splitting at 70% confluency, for 1h at 35.5°C (Tanaka *et al.*, 2007). The inoculum was then removed and the medium was added. At this point an aliquot of medium was collected (T0) 1 hour after infection, for analysis by real time RT-PCR, and then alternatively every 2-4 days. The plates were then fixed for analysis by Immunoperoxidase staining.

For the cells grown in 3D configuration: the day before virus infection, prepare three 24-well micro plates with lid flat bottom, ultra low attachment (Costar) with cover slip covered with fifty-fifty of Collagen solution type I from rat tail (Sigma) plus 100% ethanol subjected to filtration with 0.20 μ m filter To stop the infection at 24h, 48h, and 72h, cells were fixed and visualized under a confocal microscope.

Harvest mature 3D aggregates of the Int 407 and Hep G2/C3a cells from rotating wall vessel (RWV) bioreactor, seeding the cells randomly in 4 microplates for each cell line. Three 24-well microplates are used for Microscopy. One standard 24 well microplate is used to collect the medium for extraction RNA and perform real time PCR. The cells were washed three times with Hank's balanced salt solution, removing supernatant and adding 1ml of 1x non enzymatic dissociation solution (Sigma) and using cell strainer 70 μ m nylon (BD) to separate the beads from the cells, counting the cells with a haemocytometer.

The HEV RNA titre of the virus stocks was estimated to be 3.0×10^7 copies ml^{-1} by real time PCR. Prior to inoculating the cells with the swine faecal suspension, the virus stock was subjected to purification by passage through a series of microfilters with a pore size of 1.2 μ m, 0.80 μ m, 0.45 μ m and final filter at 0.20 μ m (Minisart), or to filtration with spin-X centrifuge tube filter sterilized 0.22 μ m (Costar) at 10,000 x g at 4°C for 15-25min.

Four microplates of 3D cell culture from each cell line were infected with 0.5ml of the filtered virus stock that had been previously diluted with GTSF-2 medium (1:5 for Int 407 and 1:10 for Hep G2/C3a). One hour after inoculation at 37°C in a humidified 5% CO₂ atmosphere, the solution was removed and 1 ml of GTSF-2 complex medium was added into each well. Virus culturing was performed at 37°C in

a humidified 5% CO₂ atmosphere. One, two and three days after inoculation, the cells were fixed with 0.5ml of 4% Paraformaldehyde (PFA) and left at 4°C o.n.

4.2.5 IMMUNO PEROXIDISE (IPX) STAINING

Negative and positive polyclonal antibodies for HEV to be used in the Immunoperoxidase (IPX) protocol were selected from 56 swine serum samples that had been tested using an Anti-HEV enzyme-linked immunoassay (ELISA) for pig IgG (Tubigen). The ELISA was performed according to manufacturer's instructions.

Cells were fixed with 4% paraformaldehyde (PFA) for 30 min or with 80% acetone for 15 min, stained by antibodies diluted in Immuno Peroxidase (IPX) serum conjugated diluents* following the procedure described below:

- Pooled pig anti-HEV polyclonal:
Positive (+ve) and negative (-ve) pig serum selected by ELISA TEST described above (Dil. 1:100)
- Mouse monoclonal antibody against ORF2 (Ascitis fluid, 2B1H1, from ISS) (Dil. 1:500)
- An irrelevant mouse Mab raised against pestivirus (Ascitis, supplied by CSF group) (Dil. 1:500)

Secondary Antibody (2nd Ab) tested were:

- Swine Immunoglobulins HRP **DAKO** A/S Denmark
Horseradish peroxidase-conjugated IgG fraction of polyclonal rabbit (Dil. 1:300)
- Alternatively, Polyclonal rabbit anti-mouse Igs/HRP 1.3 g/L (P0164) **DAKO** A/S Denmark (Dil. 1:2000).

Staining Procedure for 2D cells:

- Fix microplates with cold Acetone 80% in PBS for 15' at RT or 4% PFA in PBS.
- Add 50µl of 1stAb diluted in IPX serum conjugate diluent
- Incubate x 35' at 37°C
- Wash 3x with washing Buffer IPX**
- Add 50µl 2nd Ab diluted in IPX serum conjugate diluent
- Incubate 35' at 37 °C
- Wash 3x with washing Buffer IPX
- Wash 1x with H₂O to neutralize pH
- Freshly prepare the substrate; Ethyl carbazol made by mixing: 2.5ml of acetic buffer, 2.5uL hydrogen peroxide and 150uL ethyl carbazol. Subject to filtration with 0.20µm filters. Flood the wells with 50µl of ethyl carbazol in every well and leave the plate at 37 °C for 25min monitoring the staining every 10 min under microscope.
- Wash with Buffer IPX (2x)
- Add 250µl of PBS to every well
- Observe under light microscope and take pictures

*IPX serum conjugate diluent was prepared according to the following SOP (Standard Operating Procedure): add 21g NaCl and 10ml Tween 80 to the 1L of PBS 0.01M pH 7.6. Stir with magnet until it is mixed thoroughly. Store at room temperature and prepare fresh every 2 weeks.

**IPX washing buffer was prepared as follows: add 25ml Tween 80 to the 5L of PBS 0.01M pH 7.6. Stir with magnet until mixed thoroughly. Store at room temperature and prepare fresh every month.

Staining Procedure for 3D cells:

1. Harvest 200µl of the 3D aggregates PLC/PRF/5, infected and non Infected (NI), from 4 vessels.
2. Use tube of 1.5 ml for washing cells 1x with 1ml of PBS, centrifuging at 600 x g for 2.5 min to remove supernatant, repeat this step other 2 times.

3. Add 1ml of trypsin-EDTA, incubate for 15' in shaking, again centrifuge and add 1ml of trypsin-EDTA for other 15 minutes to separate the cells. Repeat this step again monitoring under microscope.
4. Fix the cells with 50 μ l of 4% of PFA in PBS for 5-15' at RT.
5. Use BD Falcon cell strainer, 70 μ M nylon, to astray the beads from the 3D cells and centrifuge for 3 minutes at 600 x g.
6. Set up 50 μ l of cells fixed in PFA and use Cytospin3 centrifuge (Shandon) 300 x g for 5 minutes with cytoclip mounting slide clips and disposable cytofunnel chambers to stick the cells fixed on the coverslip.
7. Use Super Pop Pen (Zymed Laboratories Invitrogen Corporation) to delimit the spherical area where the cells are disposed, leave 15 min to dry up.
8. Endogenous cell peroxides can beremoved by adding a solution of 0.1% of Sodium Azide plus 3% of H₂O₂ onto the cells fixed on the coverslip. Incubate for 15 min.
9. Wash 3x with PBS
10. Add 50 μ l of 1stAb diluted in IPX serum conjugate diluent
11. Incubate o.n. at 4 °C
12. Wash 3x with washing Buffer IPX
13. Add 50 μ l 2ndAb diluted in IPX serum conjugate diluent
14. Incubate 1h at 37°C
15. Wash 3x with washing Buffer IPX
16. Wash 1x with H₂O to neutralize pH
17. Freshly prepare the substrate; Ethyl carbazol made by mixing: 2.5ml of acetic buffer, 2.5 μ L hydrogen peroxide and 150 μ L ethyl carbazol. Incubate at 37°C for 25-70min, monitor the staining under microscope.
18. Wash with Buffer IPX (2x)
19. Add 50 μ l of PBS 1X
20. Observe under microscope and take pictures
21. Save cover slips in the fridge.

4.2.6 MICROSCOPY

4.2.6.1 CONFOCAL MICROSCOPY FOR OBSERVATION OF DIFFERENTIATION/ INFECTION MARKERS

Infected and uninfected 3D cell aggregates were stained with the differentiation markers Zo-1, Cytokeratin 18 and Villin according to the following procedure.

Protocol:

1. Place 100µl of infected and uninfected 3D aggregates in 1.5ml tube
2. Take off the medium
3. Add 1ml of 4% paraphormaldehyde in PBS incubate at room temperature for 30 min.
4. Wash cells 1x with PBS.
5. Permeabilize cells: with 1.0ml of Triton 0.01% made by diluting 100% triton with 0.1M PBS 7.2 pH for 30 min.
6. Wash cells with PBS 3x
7. Prepare Zo-1 (raised in rabbit) (Ab-Cam), Cytokeratin 18 and Villin (Ab-cam) (produced in mouse) antibodies dilution in PBS (Dil.1:100)
8. Adding 150 µl of each markers in apposite tube and incubate at room temperature 45'
9. Wash 1x with PBS
10. Add 150ul of 1:100 secondary antibodies (anti-mouse produced in goat Alex fluor 488 Ig (H+L) (Invitrogen) for 20' at 37°C) in infected and uninfected 3D cell aggregates and incubate for 45 minutes in dark.
11. Transfer 3D cell aggregates on microscope slide
12. Blot away any excess PBS
13. Stain nuclei with one-drop Vecta shield Mounting medium with DAPI (H-1200 Lot P1203 Vector Store 2-8°C) that stain nuclei
14. Lay cover slip down on the dot of mounting medium.
15. After 10', seal coverslip with nail varnish by applying it around the cover slip.

16. Visualize under confocal microscope (LEICA TCS SP2), keeping shielded from direct light to prevent photobleaching, save the pictures
17. Store coverslip at 4°C for up to 6 months.

For infection marker the same protocol described above was used but with the following antibodies:

- 1st Ab : mouse Mab (Wang) produced in goat diluted Immuno Peroxidase (IPX) serum conjugated Diluent (Dil. 1:1000)
1. 2nd Ab: Anti-mouse produced in goat Alex flour 488 Ig (H+L) (Invitrogen) in (Dil.1:200).

4.2.6.2 TRANSMISSION ELECTRON MICROSCOPY (TEM) AND SCANSION MICROSCOPY (SEM)

Infected and uninfected intestinal cell aggregates and infected and uninfected hepatocyte cell aggregates were fixed in 1ml of 3% glutaraldehyde and sent to the Molecular Pathogenesis and Genetics Department, Veterinary Laboratories Agency for analysis by TEM and SEM.

4.3 RESULTS

4.3.1 RESULTS 2D CONFIGURATION

4.3.1.1 REAL TIME RESULTS

At the beginning of this work the following cell lines were cultivated in 2D configurations: the human hepatoblastoma cell line Hep G2/C3a, two recombinant human embryonic intestinal cell lines, Int 407 IFN KO (Int-V-7E and Int-V-11c); the porcine kidney cell lines, PK-15, wt and IFN KO. In the first experiment, Hep G2/C3a cells were grown 9 days before infection with the pooled faecal suspension (1/10; 2/1; 6/109; 10/189). Conventional RT *nested*-PCR 3158-3159 gave a negative result throughout the post-infection period of 35 days. The following table (**Table 4.3.1.1**) summarizes the remaining cell types cultivated in 2D configurations.

Experiments with the remainder cells types cultivated in 2D format inoculated with HEV were in most part unsatisfactory. Seven HEV-PCR positive faecal suspensions were used to inoculate the cells in 2D configurations but just 1 inoculum out of 7 (10/189) resulted positive, enabling to infect the Int-V-7e intestinal recombinant cell line as well as PK-15 cells. Real Time RT-PCR results on the time points and estimated copy numbers are reported below (T0-T6):

T0: 2.5×10^3 copies ml⁻¹

T2: 5.2×10^4 copies ml⁻¹

T4: 2.5×10^4 copies ml⁻¹

T6: 3.0×10^4 copies ml⁻¹

Of note, real time RT-PCR did not detect virus replication throughout the 6 serial passages, resulting negative for HEV. This was also reflected in the fact that cytopathic effect (CPE) did neither occur in recombinant intestinal cell lines used in the single passage nor in 6 serial passages.

CELL TYPE	Int-V-7e and Int-V-11c	Int-V-11c (6 Serial Passages)	Int-V-7e (6 Serial Passages)	PK-15 Wt and IFN KO	PK-15 KO (5 Serial passages)
Cultured before infection (Days)	6	6	6	6	6
Infection period (Days)	8	7	7	6	6
Inoculums Code positive in Real Time PCR for HEV expressed in copy numbers:	2/1, (3.0x10 ⁷) 6/109(3.0x10 ⁷) 10/189(3.0x10 ⁷)	3.8 (4.8 x 10 ⁷) 5.8 (3x 10 ⁷) 7.21 (2.8 x 10 ⁷) Pool of three Faecal samples Used above	3.8 (4.8 x 10 ⁷) 5.8 (3x 10 ⁷) 7.21 (2.8 x 10 ⁷) Pool of three faecal samples used above	Pool of: 1/10; 2/1; 6/109; 10/189 3.0 x10 ⁷	Pool of: 1/10; 2/1; 6/109; 10/189 3.0 x10 ⁷
HEV Real Time RT-PCR post infection	Just one Pos.: 10/189 in Int-V-7e	All negatives	All negatives	Positive (T0-T6DPI)	All negatives

Table 4.3.1.1 Results of infection attempts in cell lines in 2D configurations, indicating details about days cultured before infection (up to 6 days), ‘Infection duration’ (range between 6-8 days), inoculums used to infect the cells and results of HEV Real Time RT-PCR performed post infection.

Interesting findings came up after infection with HEV inoculum of PK-15 cells, when these cells were inoculated with the same pooled inoculum (1/10; 2/1; 6/109; 10/189) used to infect Hep G2/C3a mentioned above. Differently, in this porcine kidney cells line a CPE was observed, in both wt and IFN KO cells, initially in both a single passage and also in 5 serial passages with and without antibiotics (TET), which activate the antiviral response in target cells. Of note, Real Time RT-PCR results HEV negative for all six serial passages, but resulted positive throughout the observation period of infection just on a single passage for cells infected with pooled inoculum (Table 4.3.1.2). Further testing, by microarray technology and conventional PCR, identified the cause of the CPE as porcine teschovirus (PTV-9) that was present in all of the pooled faecal samples.

CELL TYPE	0DPI	1DPI	2DPI	3DPI	4DPI	5DPI	6DPI
PK-15 +TET WT	1.3.0 X 10²	2.0 X 10¹	NO CT	1.5 X 10⁴	4.0 X 10³	1.5.0 X 10¹	NO CT
PK-15 +TET IFN KO	6.5X 10²	3.0 X 10²	2.5 X 10²	4.1 X 10³	2.0 X 10¹	1.2 X 10¹	4.0 X 10²

Table 4.3.1.2 Results of Real-Time PCR for PK-15 wt and IFN KO in 2D configuration.

The table shows HEV RNA titre (copies ml⁻¹) released in the culture medium (0DPI-6DPI) of PK-15 wt and IFN KO cells. In addition, real time PCR HEV at 3DPI detected RNA titre (expressed in copies ml⁻¹) also in PK-15 wt (8.9 x 10³) and IFN KO without TET (1.7x 10³) (data not shown in the table).

4.3.1.2 IMMUNOPEROXIDASE (IPX) STAINING

In preparation for IPX staining with polyclonal antibodies to swine HEV (Anti-swHEV) (**Fig 4.3.1.2**) in 2D format, an ELISA assay (Tuebingen, Germany) was performed to select adequate negative and positive monoclonal antibodies. Fifty-six pig sera were tested together with the positive and negative controls, and the cut-off values and equivocal ranges were calculated according to the manufacturers' instructions (Tuebingen, Germany) Of 56 pig sera tested, just 5 sera yielded a negative value with an absorbance ≤ 0.024 . Four sera with low absorbance value were chosen and pooled to be used as negative control serum in following IPX staining. As the positive reference serum, a pool of two high OD value serum samples, with an absorbance ≥ 0.530 , was used for IPX staining.

An example of best IPX staining, corresponding to the best results of real time PCR, is shown in the figure below, at 3DPI (**Fig. 4.3.1.2**). Both infected and uninfected PK-15 wt and IFN KO cells at T3DPI were fixed with acetone 80%. IPX staining positive results (**Fig. 4.3.1.2, row E**) in PK-15 wt and PK-15 IFN KO infected with HEV RT-PCR positive inoculum and stained with positive polyclonal against HEV revealed the cytoplasmic accumulations of antigens exhibiting a typically red reaction, consistent with HEV, with lower observable background staining. Conversely, negative results were obtained if cells were stained with negative polyclonal serum (**Fig. 4.3.1.2, row F**). In uninfected cells or cells infected with negative inoculum of HEV, stained with positive or negative polyclonal antibody, no specific cellular staining was seen in the cells that exhibited low background staining (**Fig. 4.3.1.2, row A, B,C, D**). Results of IPX staining in Hep G2/C3a and intestinal cell lines resulted negative (data not shown).

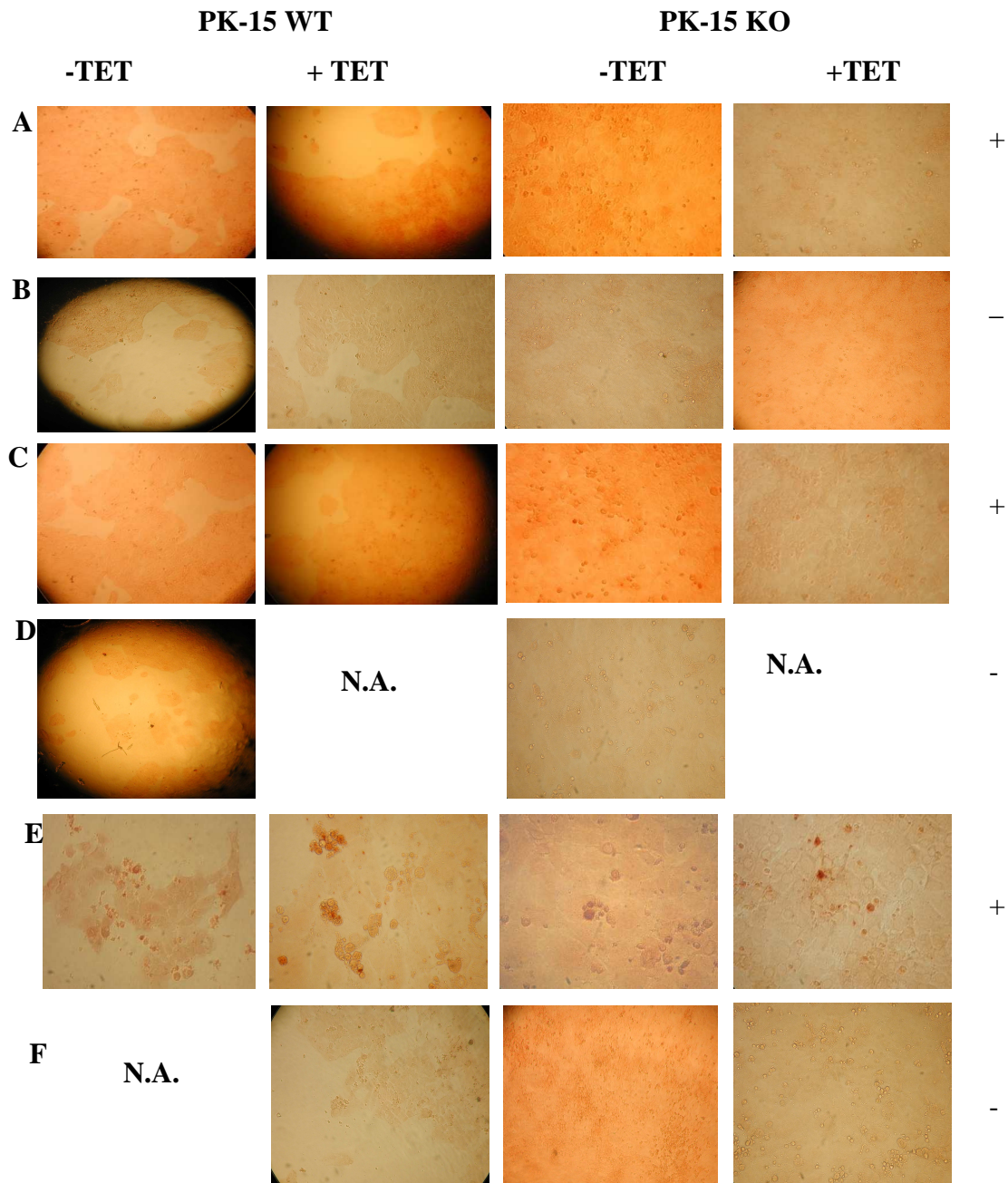


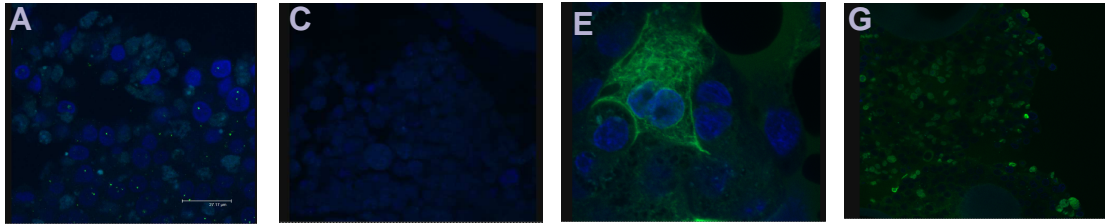
Fig. 4.3.1.2. Results of IPX staining in PK-15 (wt) and PK-15 IFN KO uninfected or infected with swHEV, in the presence or absence of TET, with positive polyclonal against ORF-2 of HEV. HEV positive Poly Ab against ORF-2 of HEV and negative poly Ab at a dilution of 1:100, as 1st Ab; 2nd Ab 1:300 poly anti-swine (DAKO) were used. Magnification 10, 20 X. Row **A** and **B** show uninfected cells stained with positive and negative polyclonal, respectively. Row **C** and **D** show cells infected with negative HEV inoculum, positively and negatively stained with polyclonal antibodies, respectively. Row **E** and **F**. The cells were infected with positive HEV inoculum and stained with positive and negative polyclonal antibodies, respectively. **NA:** not available.

4.3.2 RESULTS 3D CONFIGURATION

4.3.2.1 MARKERS OF INFECTION/DIFFERENTIATION

The RCCS with disposable vessels (**Fig. 4.1**) is a new technology for growing suspension cells that enables the culturing of many types of cells to high densities providing adequate nutrition and oxygenation to acquire structure and function similar to *in vivo* cells and tissues permitting the generation of 3D tissue-like assemblies, modelling many aspects of *in vivo* human tissue (Carterson *et al.*, 2005). At the beginning, to characterize the *in vivo* epithelial expression characteristics of the intestinal and hepatocyte 3D aggregates, the following infection/differentiation markers were used: mouse monoclonal antibodies against ORF2 of HEV, (Cytokeratin-18), CK18, and villin, respectively. Cells stained by mouse monoclonal antibodies demonstrated viral infectivity. CK-18 was used as identifiers of carcinoma (Carterson *et al.*, 2005), while villin, an actin-binding protein marker for differentiated villi, was used to investigate whether the actin filaments in the cells are rearranged or not. In fact, actin changes would confirm entry of the virus in the cells because viral entry leads to cell actin rearrangement (Nickerson *et al.*, 2001). 3D aggregates infected with HEV 24 DPI were stained with antibodies against ORF2 region of HEV, cytokeratin 18 and villin, as demonstrated in confocal images (**Fig. 4.3.2.1**). The positive stainings shown in Hep G2/C3a but not in Int 407 is probably related to different dilutions of the inoculum used to infect the cells (dil.1:5 and 1:10, respectively) (**Fig. 4.3.2.1A.B**). In the past, problems were found due to the secondary antibody attaching to cells and giving false positives. Therefore, the infected cells were tested by just the secondary antibody, showing that this event did not happen in these experiments (**Fig. 4.3.2.1C.D**). In particular, the confocal image (**Fig. 4.3.2.1E**) showed clearly that the nucleus in epithelial cells was held within the cells by a network of intermediate filaments made of keratins. Villin protein also expressed in both cell lines was shown (**Fig. 4.3.2.1.G, H**).

Hep G2/C3A



Int 407

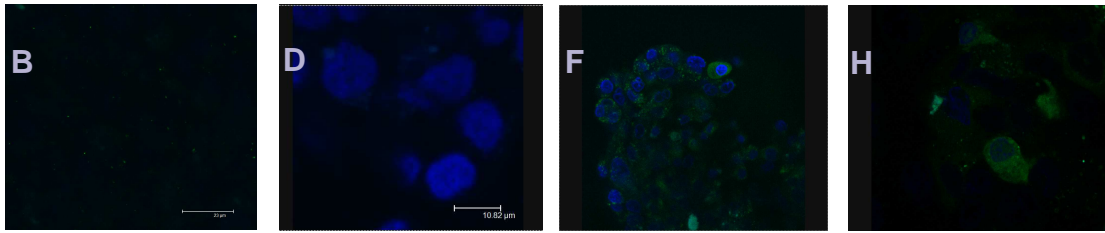


Fig 4.3.2.1. Confocal images of antibody-stained 3D aggregates Hep G2/C3a (A, C, E, G,) and Int 407 (B, D, F and H) demonstrating viral infectivity of genogroup 3 HEV virus at 1DPI. A and B: infected cells stained with mouse monoclonal antibodies against ORF2 region of HEV. Negative controls (C and D) stained with anti-mouse 2nd Ab Alexa fluor 488 alone show that secondary antibody did not attach non-specifically to protein in the cells, while infected cells showed specific staining for cytokeratin 18 (E and F), and villin (G and H).

In parallel, it has also been evaluated by PCR, both the expression of CK-18 and other differentiation markers such as: Alb, CK-8, CK19, Cyt P450, and Il-6, in both cell lines in 3D and 2D configurations (**Fig. 4.3.2.2**). Albumin is the marker of differentiation for mature hepatocytes, is the most abundant blood plasma protein, is produced in the liver and forms a large proportion of all plasma protein (Krishna *et al.*, 1997). The three cytokeratin CK8, CK18, CK19, as mentioned above, are all correlated as markers for epithelial lineage, tumoral and undifferentiated cells. Among the various intermediate filament proteins, cytokeratin 8 (CK8) is especially remarkable as it is produced early in embryogenesis. Cytokeratin 8 and 18 (CK8/18) is the major keratin pair in simple-type epithelia, as in the liver, pancreas, and intestine, and are essential for maintaining structural integrity. CK19 expressed heterogeneously in the basal cell layers of the stratified squamous epithelium that forms the major cavities of the body. Cytochrome P450 is a membrane-associated protein; in the liver it metabolizes multiple substrates including drugs and toxic compounds as well as metabolic products such as bilirubin. In general, in response to viral infections, the concentration of many cytokines in blood is increased (Ramshaw *et al.*, 1997). IL-6 is a pro-inflammatory cytokine secreted by T-cells and macrophages to stimulate immune response to trauma, especially tissue damage leading to inflammation.

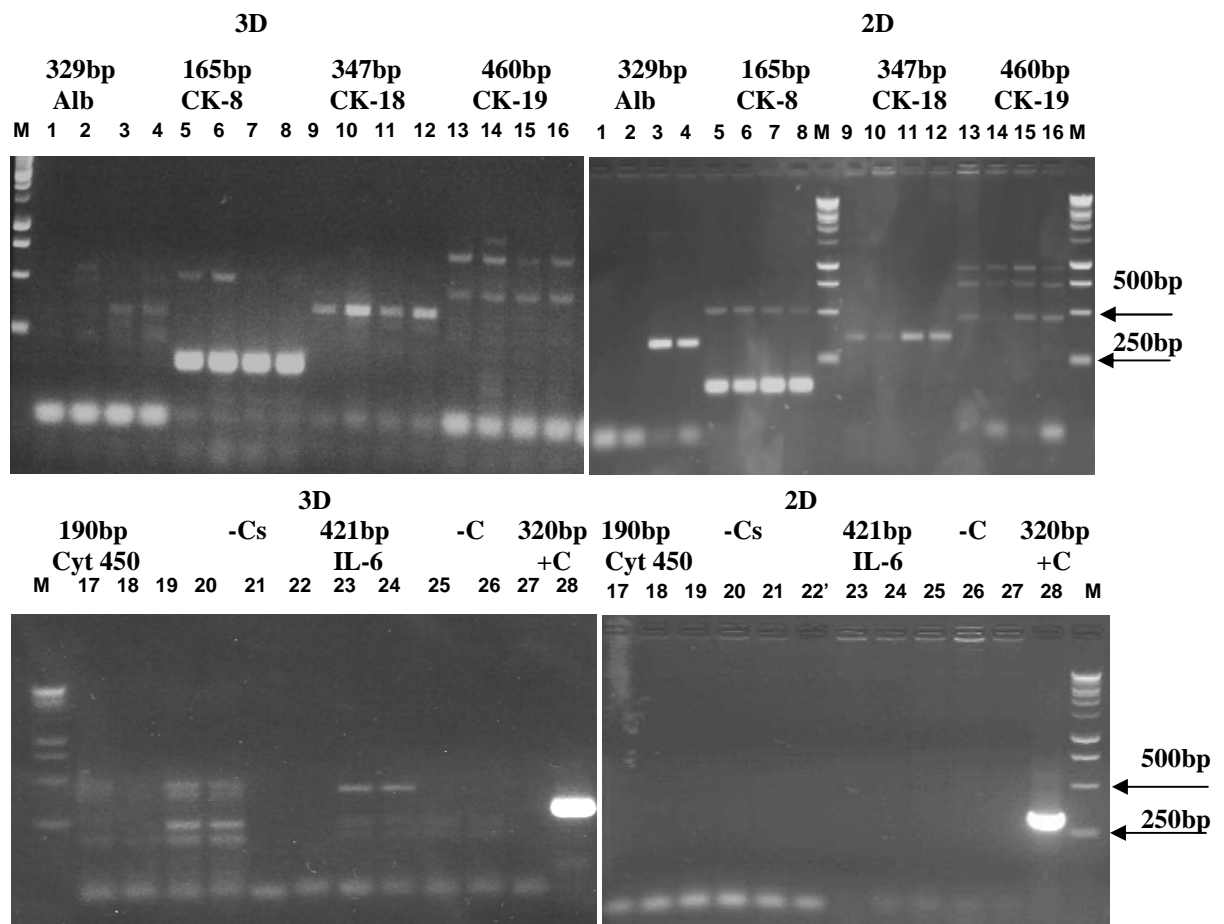


Fig.4.3.2.2 PCR Products of various differentiation markers expressed in 3D and 2D configurations. Left side: gels related to 3D cells; right side :the 2D cultures of intestinal and liver cells. Lane M: 1Kb DNA ladder. Lanes 1,5,9,13,17,23 NI Int (Not infected Int 407); 2,6,10,14,18,24: LV Int (Live Virus Int 407) 3,7,11,15,19,25: NI Hep (Not Infected Hep G2/C3a); 4,8,12,16,20,26: LV Hep (Live Virus Hep G2/C3a). Lanes 1-4: 329bp Alb; lanes 5-8: 165bp CK8; lanes 9-12: 347bp CK-18; lanes 13-16 460bp CK-19; lanes 17-20: CytP450 190bp; lane 21: negative Control of Extraction; lane 22: negative Control of RT-PCR; 22': negative control PCR; lane 23-26: IL-6 421bp; lane 27: negative Control of PCR; lane 28: positive control of IL-6 320bp.

Specifically, 3D aggregates of hepatocytes exhibit albumin expression with an expected band is of 329bp (Sharma *et al.*, 2005). The marker of differentiation for mature hepatocytes (Krishna *et al.*, 1997) was shown in both 2D and 3D configurations, whereas it did not express in intestinal aggregates in both configurations. Albumin in HepG2/C3a cell line is expressed more in 2D than 3D configurations, appearing down regulated in the 3D cells compared to the 2D cell line, but it is known (Stein *et al.* 1998) that when the serum albumin level is low it indicates liver disease, and expression is abnormal in tumoral cells. CK8, 18, 19 are expressed in both configurations with expected bands of 165bp, 347bp, 460bp. All three cytokeratins are correlated as marker for epithelial lineage, tumoral and undifferentiated cells and their expression confirmed the nature of the epithelial cells and Hep G2/C3a tumoral cells used. What this cytokeratin modulation means with respect to the functional specificity of the 3D constructs is yet not sure, but this finding can be considered to be indicative of large-scale phenotypic alteration associated with 3D culture (Nickerson *et al.*, 2001). Cytochrome P450 expression is level dependent on interleukins (IL) when levels of Cyt P450 increase, then IL decreases. This occurred to Hep G2/C3a cell line and Int 407 cell line in 3D configuration, and failed in 2D cell lines, because they are not differentiated enough to express these proteins in standard monolayers. In general in response to viral infections, the concentration of many cytokines in blood is increased (Ramshaw *et al.*, 1997). IL-6 is expressed in 3D intestinal cell line at higher levels than in the respective monolayers and this does suggest that they are mature enough to produce IL-6 as a marker for intestinal differentiation cells. Conversely, IL-6 has been not expressed in hepatocyte aggregates, as expected. It has been assumed that *in vivo* cytokines are responsible for P450 depression because *in vitro* cytokines can depress multiple hepatic P450 isoforms and their mRNAs (Bleau *et al.*, 2001).

4.3.2.2 TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

Characterization of the *in vivo* epithelial cell expression characteristics of the 3D intestinal and hepatocytes aggregates was further examined by transmission and scanning electron microscopy. The 3D intestinal aggregates exhibited: tight junctions, microvilli, rough endoplasmic reticulum formation. A tight junction is an element of epithelial and endothelial junctional complex that consists of the tight junction, adherens junction, and desmosome. Tight junction seals are adjacent to epithelial cells in a narrow band just beneath their apical surface. This creates selective barriers regulating paracellular transport of solutes, immune cells and drugs by diffusion or active transport (Tsukita *et al.*, 1993). Microvilli are finger-like plasma membrane projections present in the free surface of the columnar epithelial cells, which further increase the total area for absorption of nutrients moved in the circulation by blood and digestion. The microvilli also occur on the surface of liver cells facing the bile canaliculus (Motta *et al.*, 1990). The following micrographs show well-developed microvilli, tight junction formation, and rough endoplasmic reticulum in 3D intestinal and 3D hepatocyte aggregates (**Fig. 4.3.3.2** and **Fig 4.3.3.3**). Hepatocytes, in general, are polyhedral in shape, therefore having no set shape or design. They have surfaces facing the sinusoids (called sinusoidal faces) and surfaces which contact other hepatocytes (called lateral faces). Sinusoid-like structures, as a type of sinusoidal blood vessel (with fenestrated, discontinuous endothelium), serve as a location for the oxygen-rich blood from the hepatic artery and the nutrient-rich blood from the portal vein. The 3D hepatocytes aggregates exhibited differentiated hepatocyte cells with the nucleus and many nucleoli (**Fig 4.3.3.4**). Bile Canaliculi formed by grooves on some of the lateral faces of these hepatocytes, as shown in scanning electron micrograph, were identified in uninfected 3D hepatocytes aggregates of 35 days old (**Fig 4.3.3.5**).

Fig. 4.3.3.2

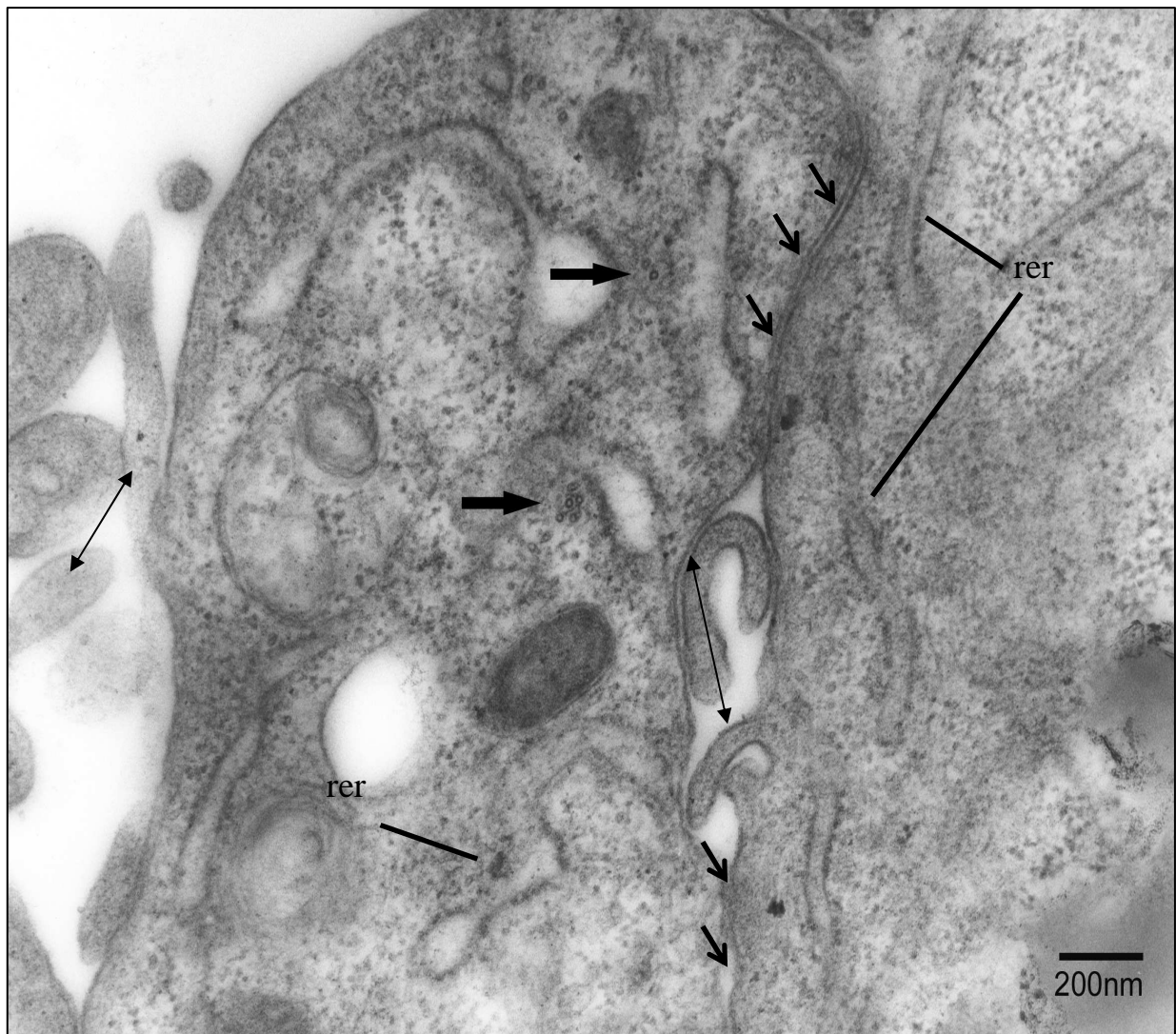
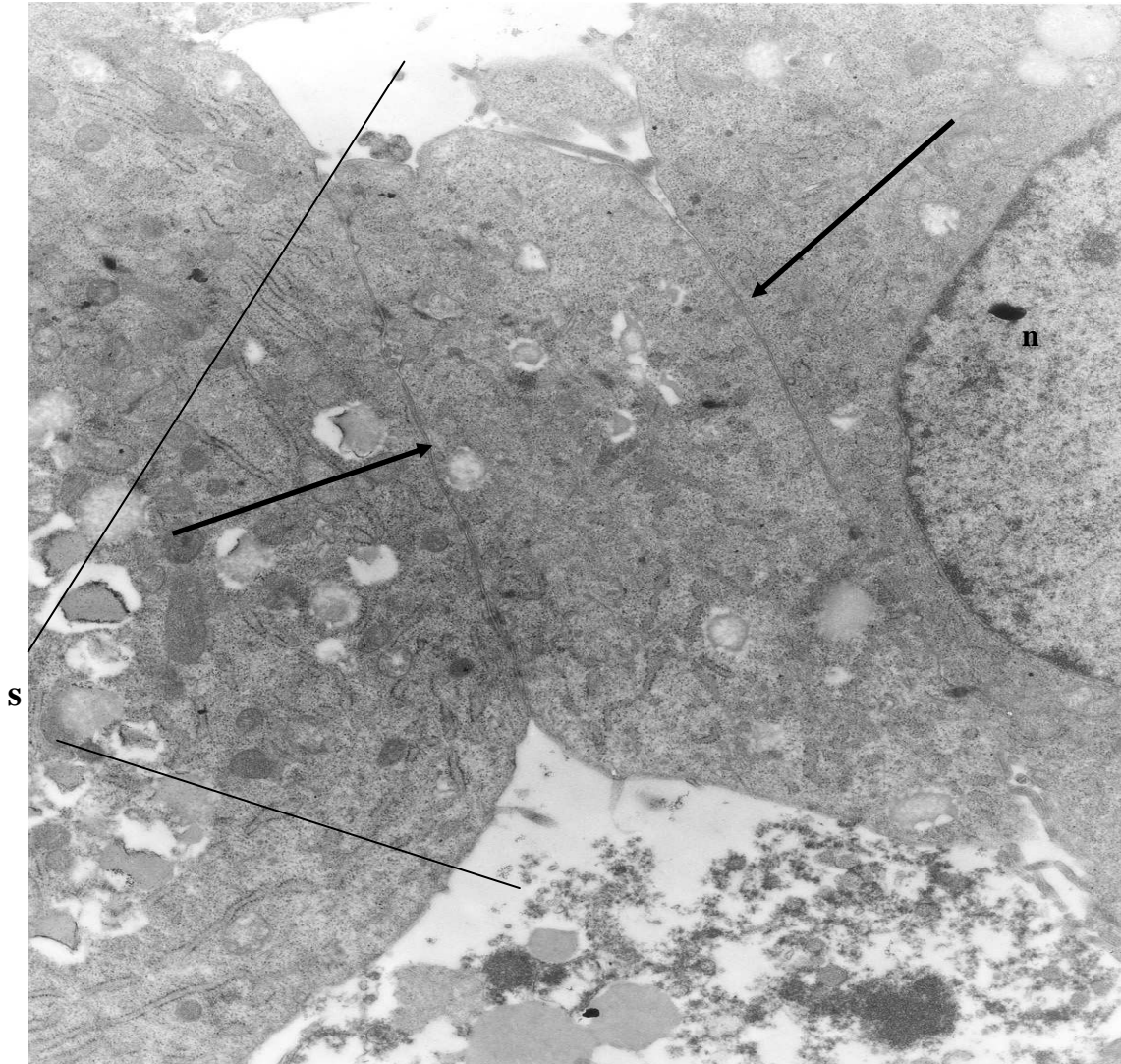


Fig. 4.3.3.2. Transmission electron micrograph of 3D Int-407 aggregates demonstrate (bold arrows) the presence of virus like particle (24nm of diameter) 1DPI, developed and sparse microvilli (double arrow), rough endoplasmic reticulum (rer) (straight line), and standard arrows indicate the presence of formation of tight junction which runs nearly the length of the margin between two 3D cells. Bar: 200 nm. (Kindly supplied by W.A. Cooley, of THE Molecular Pathogenesis and Genetics Department, Veterinary Laboratories Agency, Weybridge (UK).

Fig. 4.3.3.3



The **Fig. 4.3.3.3** micrograph of the 3D hepatocytes aggregates show formations of tight junction (standards arrows) that seal adjacent epithelial cells run nearly the length of the margin between three 3D cells and sinusoids like structure (s) (straight lines). (Kindly supplied by W.A. Cooley of The Molecular Pathogenesis and Genetics Department, Veterinary Laboratories Agency, Weybridge (UK)-collaboration).

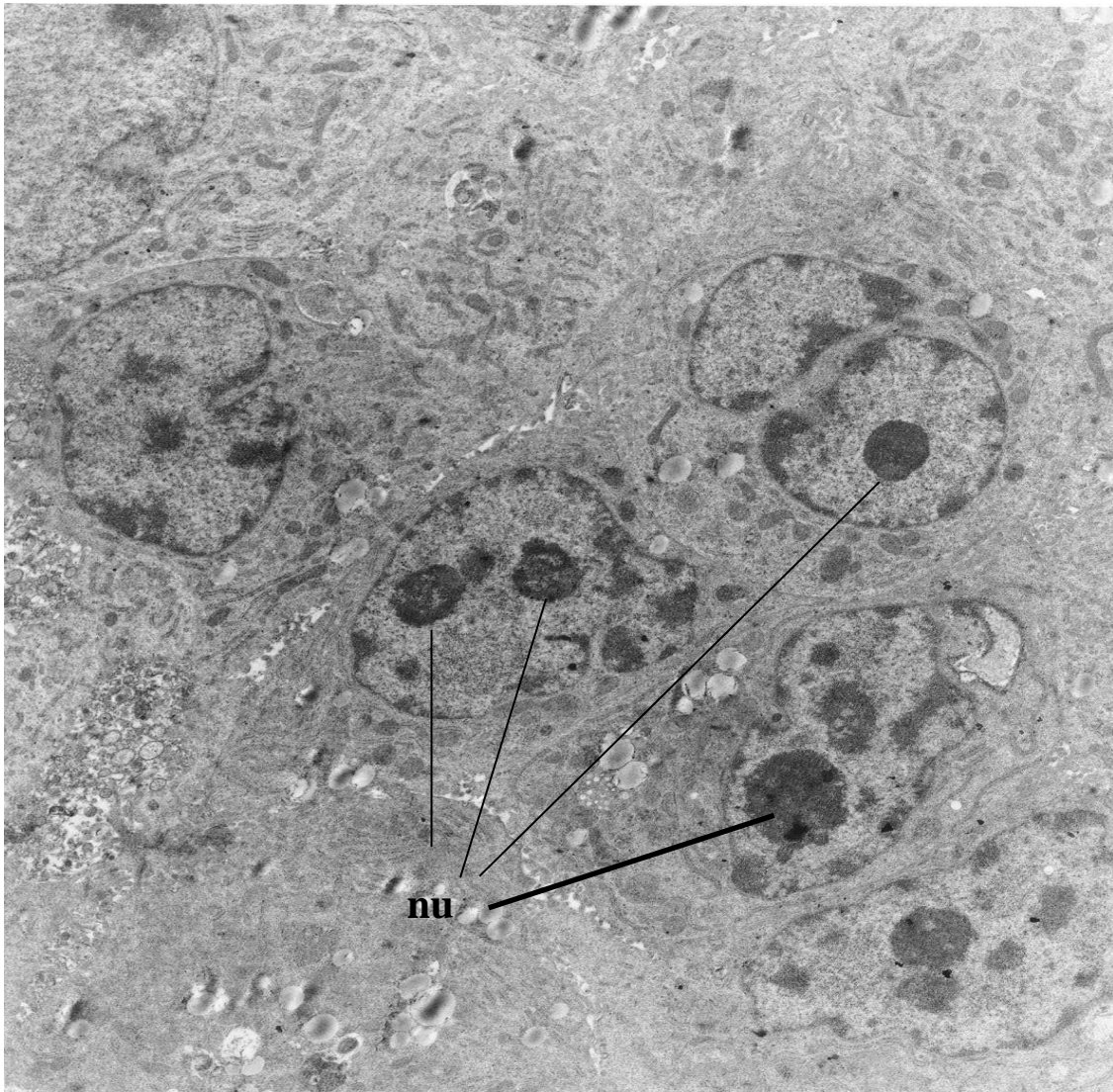


FIG. 4.3.3.4 Transmission electron micrograph of 3-D Hep G2/C3a aggregates shows the presence of nuclei with nucleoli (nu). (Kindly supplied by W.A. Cooley of The Molecular Pathogenesis and Genetics Department, Veterinary Laboratories Agency, Weybridge (UK)- collaboration).

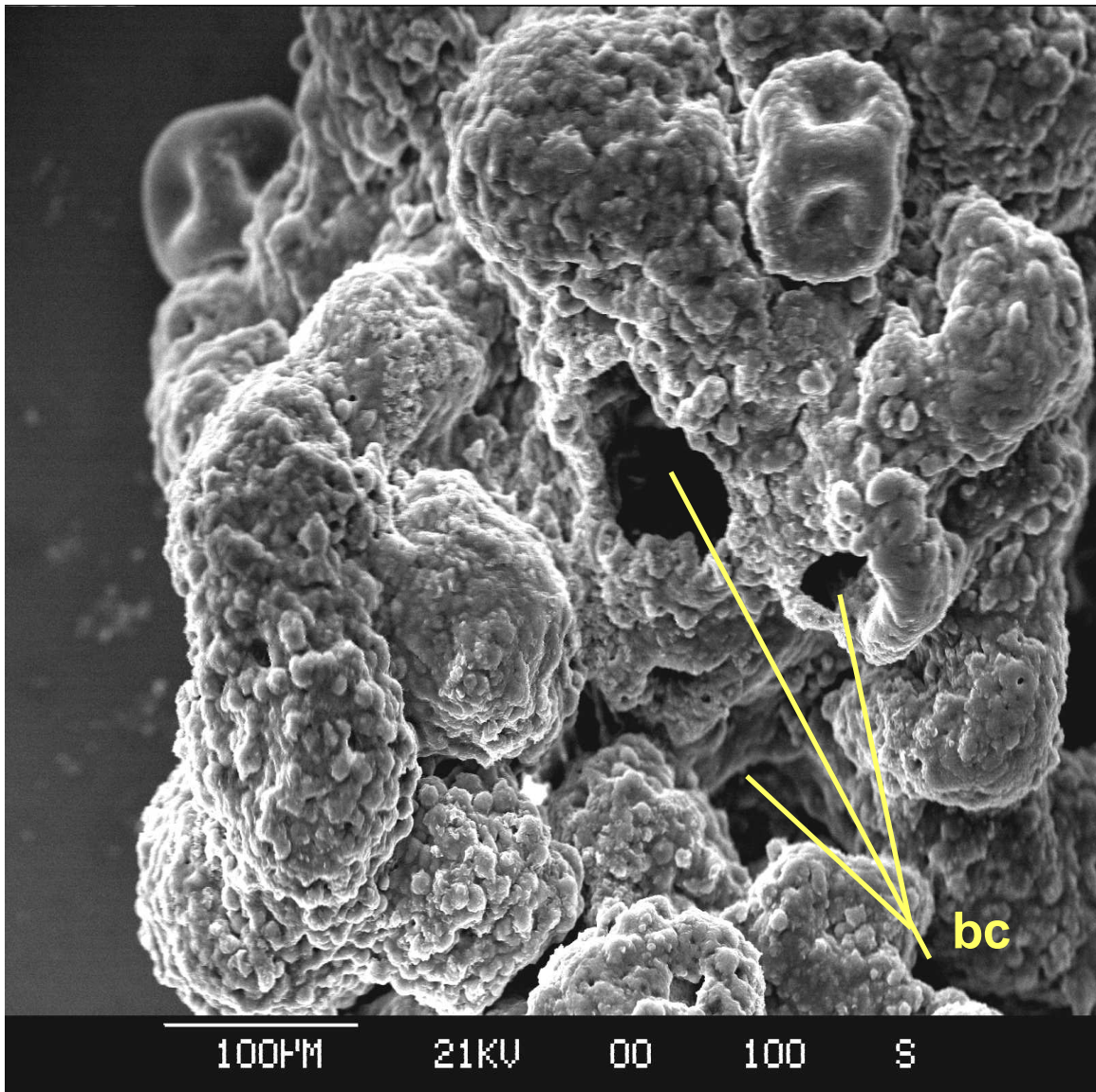


FIG. 4.3.3.5 Scanning electron micrograph of 3-D hepatocyte aggregates shows the (bc) bile canaliculi like structure, as thin tube collecting bile secreted by hepatocytes. Bar. 100µm. (Kindly supplied by J. Collins, of The Food & Environmental Safety Department, Veterinary Laboratories Agency, Weybridge (UK)- collaboration)

4.3.2.3 PK-15 AND 3D CONFIGURATION

4.3.2.3.1 REAL TIME PCR

The real time PCR results were negative throughout the observation period.

4.3.2.3.2 IPX STAINING

The results of HEV infection in PK-15 were negative and for this reason IPX was not performed.

4.3.2.4 INT-407 3D CONFIGURATION

4.3.2.4.1 REAL TIME

The presence of HEV in cell culture supernatant of human embryonic intestinal cell line IFN KO (Int-V-7e and Int-V-11c) cultivated in 3D configurations was checked by real time qPCR. The HEV virus was detected in cells infected with one inoculum (Ref 10/189), resulting positive just in T0 and T5 in Int-V-7e and Int-V-11c T0 and T1 DPI. Other inocula with high RNA titre (1.4×10^9 copies ml^{-1}), such as a liver sample from English retail (L28), were also used to inoculate cells. However no further inoculum proved able to infect the cells or propagate in the Int 407 IFN KO as well as in PK-15 KO. The following table summarizes the results obtained infecting the two cell lines cultivated in 3D configurations, indicating the inoculum used. The high HEV load was expressed in copy numbers, but there was no confirmation that these cell lines do support the replication of the virus *in vitro* or that the virus used was viable (**Table 4.3.2.4.1**).

CELL TYPE	PK-15 KO	Int-V-7E	Int-V-11c
Culture before infection (Days)	26	28	28
Infection Duration (Days)	9	20	20
Inoculums Code positive in Real Time PCR for HEV Expressed in copy numbers	5.9 (9x 10⁶) 5.10 (2 x 10⁵) L28 (1.4x 10⁹)	3.8 (4.8 x 10⁷) 7.21(2.8 x 10⁷) 10/189 (1.9 x10⁷)	3.8 (4.8 x 10⁷) 7.21(2.8 x 10⁷) 10/189 (1.9 x10⁷)
HEV Real Time RT-PCR post infection	negatives	Just one Pos. : 10/189 T0, T5	Just one Pos.: 10/189. T0, T1

Table 4.3.2.4.1 The table's reports a summary of the results of experiments performed in 3D. 'Culture days before infection' referring at the period of growth in the rotary wall vessel (range between 26-28 days), 'Infection duration days' occurred in the vessel (range between 9-20), inoculums used for infects the cells, and results of HEV Real Time RT-PCR post infection are indicated.

4.3.2.4.2 IPX STAINING

The only inoculum 10/189 appeared to infect the PK-15 KO cells, but did not propagate in them. This inoculum was however shown to be real time positive for HEV as well as positive for PTV-9, which causes CPE in this cells line and lead to cell death in few days. For this reason IPX staining was not performed on these cell lines.

4.3.2.5 PLC/PRF/5 IN 3D CONFIGURATION

4.3.2.5.1 REAL TIME PCR

The HEV Ct values in the inoculum after filtration used for PLC/PRF/5 cells were 30, 22, and 24, related to liver culture sample (Hep), extract from experimentally infected pig liver (L3), and liver from retail outlet (L28), respectively. A marked reduction in the Ct values was observed by real time on the day 15 P.I. onwards in the 3D vessels which were seeded with PLC/PRF/5 and inoculated with extract from an experimentally infected pig liver (L3: Ct 32.71) on the day 18. For liver cultures sample (Hep) on the same day, the obtained Ct was 35.42; the Ct values continued to decrease until T58 for both samples (Ct 20.63; 21.14, respectively) (**Fig. 4.3.2.5.1.1**).

These results are indicative of abundant target RNA in the supernatant culture, and suggest that HEV was alive and replicating in the human hepatocarcinoma cells line. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample) (**Table. 4.3.2.5.1.2**). Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent a steady infection state. Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid. Cts ≤ 29 are strong positive reactions indicative of abundant target nucleic acid in the sample.

The PLC/PRF/5 cells line cultivated in 2D configuration were infected just by sample L3, and the Ct value did not appear to diminish indicating minimal amount of target nucleic acid in the cells for no later than 6DPI (**Table 4.3.2.5.2**); that probably means that HEV initially infected the PLC/PRF/5 cell line but did not propagate in 2D configuration. These results suggested that these cells in 2D may not produce the receptor at all, or produce the receptor in a wrong density and localisation not allowing the virus to enter the cells and start the replication cycle.

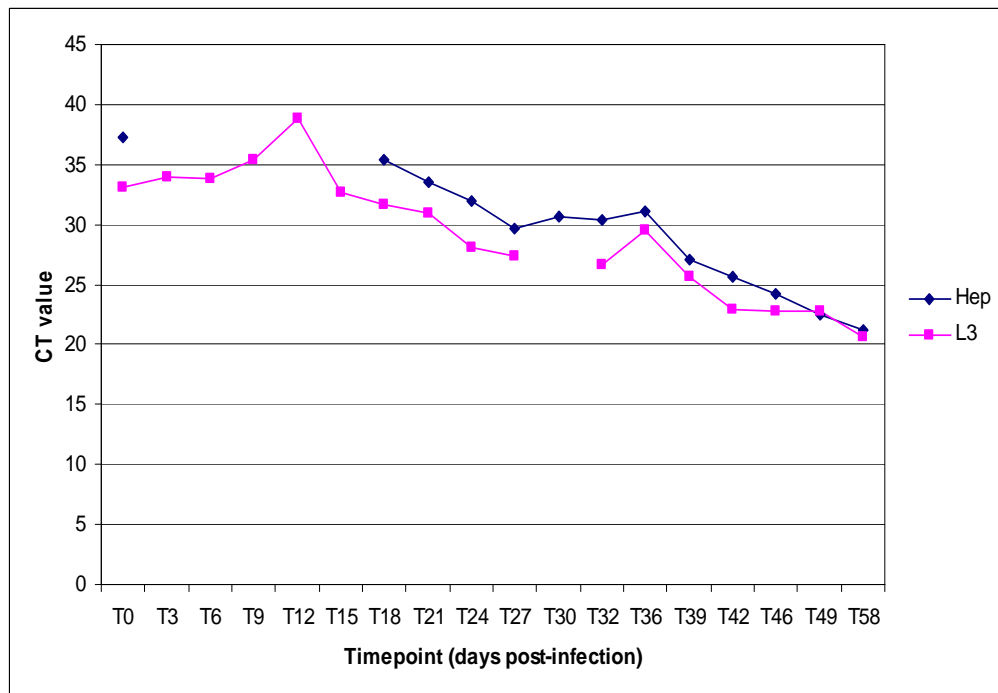


Fig. 4.3.2.5.1.1. Quantification of HEV RNA in culture supernatant of 3D PLC/PRF/5 cells inoculated with liver culture sample (Hep), blue curve, and an extract from experimentally infected pig liver (L3), pink curve. The x-axis is the time point (day post infection), T0-T58, whereas the y-axis reports the Ct values obtained measured by real time RT-PCR. The gaps in the curves indicate “No Ct value” detected.

	T inocula	T0	T3	T6	T9	T12 to T58
Hep	30	No Ct	No Ct	No Ct	No Ct	No Ct
NI	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
L3	22	37.06	38.50	40.05	No Ct	No Ct
L28	24	No Ct	No Ct	No Ct	No Ct	No Ct

Table4.3.2.5.1.2. The table summarizes the Ct values throughout the observation period of infection of PLC/PRF/5 in 2D configuration T0-T58 DPI. **T inocula** indicate the Ct value of the corresponding inoculum estimated before infecting the cells. **Hep** and **L3** indicate cells inoculated respectively with HEV-positive Hepatocytes and liver belonging to an experimentally infected pig from the Netherlands. **NI** (Not infected) indicates uninfected cells. **L28** indicate cells infected with liver from retail outlet from England. **No Ct**: Not Cycle threshold value.

4.3.2.5.2 IPX STAINING

After cell fixation, HEV was detected in cell culture by IPX staining using mouse monoclonal antibodies on the PLC/PRF/5 cells line. **Fig.4.3.2.5.2.** shows stained 3D PLC/PRF/5 cells (with **decreasing CT**) inoculated with liver (L3, T58DPI) , stained with positive Mab against HEV ORF2 (from ISS). Polyclonal rabbit anti-mouse Igs/HRP DAKO was used as 2nd Ab. Positive PLC/PRF/5 cells typically exhibited a red reaction product without any observable background staining. There was neither specific cellular staining nor background using a negative monoclonal antibody against Pestivirus in either infected or uninfected cells (**Fig. 4.3.2.5.3**).

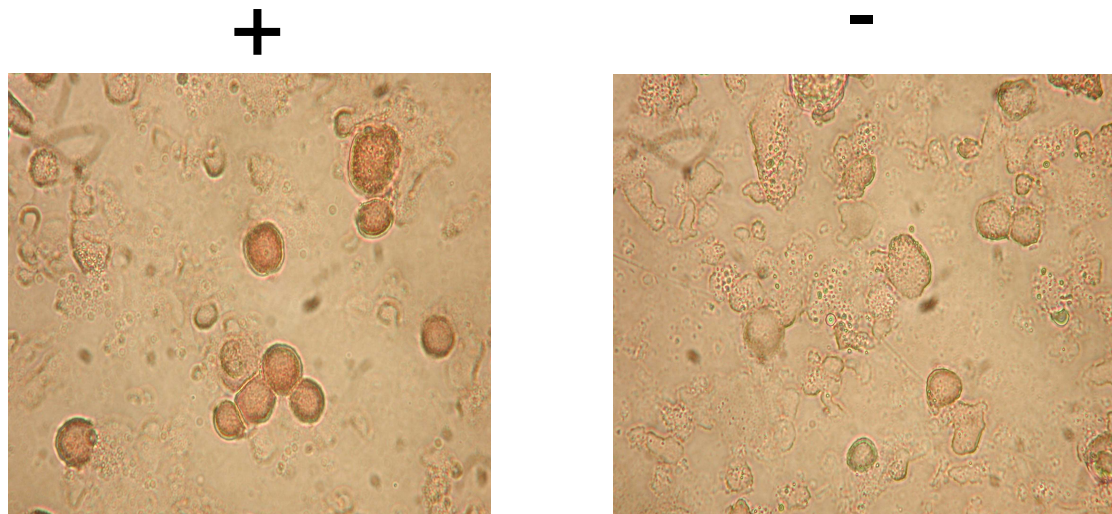


Fig. 4.3.2.5.2 IPX staining of PLC/PRF/5 cell line, T58DPI, infected with liver from a HEV experimentally infected pig ; cells were fixed in 4% Paraformaldheide, and stained with positive (+) and negative (-) monoclonal antibody to HEV. Magnifications 40X.

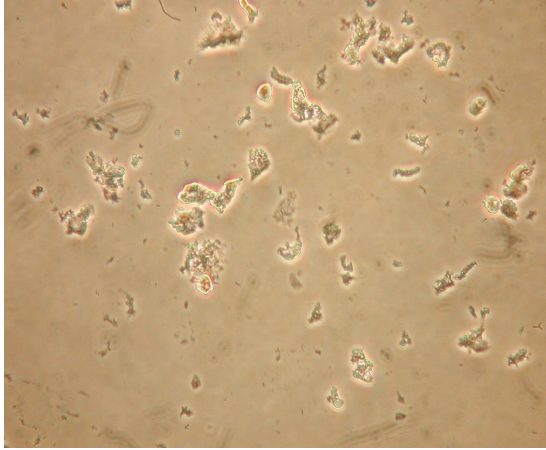


Fig. 4.3.2.5.3 IPX staining of uninfected PLC/PRF/5 cells, T50DPI, after fixation in 4% Paraphormaldheide and staining with positive (+) monoclonal antibody to HEV. Magnifications 20X.

4.4 DISCUSSION AND CONCLUSION

Investigation of the pathobiology of HEV has been constrained because of a lack of reliable cell culture system for viral *in vitro* culture, inhibiting studies into the replication properties of the virus, and vaccine research. A recent study (Tanaka *et al.*, 2007) investigated the potential of 21 cell lines for supporting the replication of HEV in *in vitro* culture, including A549 (human lung carcinoma cell line) (Huang *et al.*, 1995; Huang *et al.*, 1999), Hep G2 (human hepatoblastoma cell line) (Emerson *et al.*, 2005, 2006) PLC/PRF/5 (human hepatocarcinoma cell line) (Emerson *et al.*, 2004). PLC/PRF/5 cell line was reported to support *in vitro* replication of HEV, yielding a high titer of HEV (8.6×10^7 copies ml⁻¹ at day 60 post- infection) when maintained at 35.5°C throughout the observation period (Tanaka *et al.*, 2007). This cell line had been inoculated with a genotype 3 strain (JE03-1760F) which had been obtained from a fecal specimen from a Japanese Hepatitis E patient (Takahashi *et al.*, 2007; Lorenzo *et al.*, 2008; Yamada *et al.*, 2009).

Methodology described by Tanaka *et al.* (2007) was incorporated into this study, using the same medium (including supplements) and incubation temperature for growth and maintenance of cells in 3D configuration. A reduction in *real-time* RT-PCR Ct value was observed in infected PLC/PRF/5 cells in 3D configuration, from 15 days post infection (DPI) (**Chapter 4.3/Fig.:4.3.2.5.1**), indicating an increase in HEV genome copy number, i.e. viral replication. In 2D configuration cells (**Chapter 4.3/Table 4.3.2.5.2**), inoculated with liver from experimentally infected pig, the increase observed in the Ct from the inoculum prior to inoculation to 1 hour after inoculation (T₀) was due to the extra dilution of the inoculum in the cell medium after infection and not related to the minimal infection state. Replication of virus was not detected in the respective parallel 2D cultures. These findings are consistent with the timeframe of viral replication observed by Tanaka *et al.*, (2007), where high viral loads were detected from 12-14 dpi, and which continued through to the end of the observation period (30 DPI). In this study, viral replication was detected in PLC/PRF/5 cells in 3D configuration through 58 DPI.

Viral replication was detected in PLC/PRF/5 cells in 3D configuration using an inoculum derived from a liver sample from an experimentally infected pig, as well as a culture sample from the same liver sample. Fecal sample inoculums had also been

tested in each of the cell lines, and although viral RNA was detected immediately following infection (first few days) and indicating infection of the cells, virus replication was not detected through the remainder of the observation period. The high viral titers of the fecal sample inoculums used in this study (e.g. 1.4×10^9 copies ml^{-1} for sample L28) and the lack of evidence of viral replication in cells in 3D configuration would suggest that the virus in these inoculums was not infectious. It is possible that exposure of the fecal sample to UV sunlight inactivated the virus.

An important question in the pathobiology of HEV is whether there are extra-hepatic sites of viral replication, for example, in the intestine or kidney. In an attempt to investigate this question we included a human intestinal cell line and a porcine kidney cell line to investigate whether they would support replication of HEV. Both cell types were successfully infected with HEV (10/189) but there was no evidence of viral replication.

HEV propagation has been described in 2BS (human embryo lung diploid cell) and A549 (human lung carcinoma cell line), based on the observation of CPE (Huang *et al.*, 1992;; Huang *et al.*, 1999). Lung-derived cell lines were not investigated in this study. However, no CPE was observed in infected Hep G2/c3a, PLC/PRF/5, Int 407 wt or and Int 407 IFN KO cells: an absence of CPE in infected PLC/PRF/5 cells had been reported by Tanaka *et al.* (2007) and Lorenzo *et al.* (2008). CPE was observed in infected PK-15 wt and IFN KO cells. However, this was subsequently shown to be caused by a second virus, PTV-9, a contaminant in the original inoculums.

In a recent study conducted in Italy (Di Bartolo *et al.*, 2007), representing the second specific investigation published in Europe, all farms tested resulted positive for HEV considering animals from all age groups and production stages. Phylogenetic analyses showed that the strains circulating in pig herds investigated in Northern Italy belong to genotype 3.

HEV infection is widely spread in the swine herds. High HEV prevalence was found among weaners and gilts decreasing to some extent in fatteners and young sows, being higher in old sows than in others age groups. However, the observation that HEV infection affects animals at slaughtering age implies a potential risk of HEV infection for the consumers of pork meat, raising high concern for public health in Italy and other industrialized countries. Further, swine HEV is a potential risk first of all for people working in the pig herds by direct animal-human transmission pathways or via indirect passage of virus from the environment and working instruments

contaminated with pig faeces, as may be the case for the butchers and veterinarians. That poses the need to implement on one side bio security measures in animal and food handling and processing, and specific control of animal import on the other, as tools that may result important to prevent new virus introduction.

HEV infection was not found related to other concomitant diseases that could be present in animals. The animals selected for this study looked clinically healthy without rebound negatively in the weaning and health aspects, or on food chain or meat trade in the country. Further, a potentially large number of HEV positive animals could be considered healthy, slaughtered and introduced in the pork food chain representing a reservoir of human infection and a potential source of direct and indirect infection by cross-contamination for the consumer.

Four cell lines were tested in both a 2D and 3D configuration: Int 407/ Int 407 IFN KO (Int 407, IV-7e IV-11c), PK-15 wt/ PK-15 IFN KO, Hep G2/C3a and PLC/PRF/5. All cell lines were successfully infected with HEV, but only the PLC/PRF/5 cell line was shown to support viral replication. CPE was only observed in the PK-15 cell lines, although this was subsequently shown to be caused by a viral contaminant of the inoculums (PTV-9).

Viral replication was not detected in any of the cell lines grown in the traditional 2D configuration. Other studies have demonstrated the 3D cell-culture system as a useful tool in the cultivation of specific bacteria and a single virus, namely Norovirus. The demonstration of viral replication in PLC/PRF/5 cells grown in 3D configuration in this study indicates that the 3D system may be a useful investigative tool in the elucidation of the pathobiology of HEV and may facilitate the opportunity for vaccine research and monitoring of HEV contamination and survival to processing.

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