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Molecular Characterization and Prevalence of Hepatitis E Virus in Swedish Wild Animals - A Zoonotic Perspective

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Cover: This dendrogram reflects the phylogenetic relationship of Hepatitis E virus (HEV) isolated from different species. Clockwise description: 12 to 15 o'clock represents genotype 3; 16 o'clock represent genotypes 5-6; 17-18 o'clock represent genotype 4. 18-19 o'clock represent genotypes 1-2 and 20-24 o'clock represent a number of animal HEV, including the novel moose HEV, which is described in this thesis. (photo: Jay Lin)

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MOLECULAR CHARACTERIZATION AND PREVALENCE OF HEPATITIS E VIRUS IN SWEDISH WILD ANIMALS - A ZOONOTIC PERSPECTIVE.

Abstract

Observation of chronic hepatitis E virus (HEV) in immunosuppressed patients, and unexplained high hepatitis E virus (HEV) prevalence in the human population raises public health concern. The aim of this thesis is to molecular characterize and investigate the prevalence of HEV in Swedish wild life and their association with HEV transmission to humans. A novel virus was detected in a sample from a Swedish moose (*Alces alces*). The genome was highly divergent with sequence identity of 30-60% to other HEVs. Genome sequence and phylogenetic analysis showed closest relationship with HEV genotypes1-7 (gt1-7). In addition, three open reading frames (ORFs) was also detected, and all these observed properties suggested the virus as a member of the *Hepeviridae* family. Markers for ongoing (HEV RNA) and/or past HEV infection (anti-HEV) was demonstrated in 67 (29%) of 231 Swedish moose samples collected from various Swedish provinces. Thus, moose are frequently infected with HEV. Its closest similarity with the HEV gt1-7 group, which includes strains that also infects humans, may indicate a potential for zoonotic transmission of this HEV. A survey detected HEV markers in the wild life, which included samples from wild boars (*Sus scrofa*) and different deer species, fallow deer (*Darna darna*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and moose (*Alces alces*). These markers were ongoing and/or past infections, and were found in 53 (22%) out of 245 animal samples. The viral nucleic acid sequences strains were sequenced and compared with autochthonous Swedish human HEV cases, of whom three were found infected with strains similar to wild boar strains. These results indicate that Swedish wild animals are often infected with HEV and may be an important source of HEV transmission to humans who come into contact with wild animals or eat game meat. The introduction of a single amplicon PCR of near complete HEV genomes enabled the identification of possible virulence marker, and the detection of possible recombination events between Swedish swine and wild boar, and that there may have been zoonotic transmission of HEV strains between Spain and France.

Keywords: Hepatitis E virus, wild life, deer, wild boar, moose, swine, zoonosis, recombination, virulence, classification, molecular tracing and epidemiology.

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To My Family

I have not failed. I’ve just found 10,000 ways that won´t work
Thomas A Edison

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Lin J., Norder H., Uhlhorn H., Belák, S. and Widén, F. (2013). Novel Hepatitis E virus found in Swedish moose. *The Journal of General Virology* 95(3), 557-570.

II Lin J., Karlsson, M., Olofson A. S., Belák S., Malmsten J., Dalin A. M., Widén F and Norder H. (2015). High prevalence of Hepatitis E Virus in Swedish moose- a phylogenetic characterization and comparison of the virus from different regions. *PLoS One* 10(4), e0122102.

III Lin J., Norder H., Belák S., and Widén F. (2015). Near complete single PCR amplification of porcine Hepatitis E virus genomes; characterization of genomic regions of potential recombination and markers of zoonosis and virulence elements (manuscript).

IV Roth A., Lin J., Magnius L., Karlsson M., Bélak S., Widén F. and Norder H. (2015) Swedish wild ungulates are commonly infected with hepatitis E virus (manuscript).

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Abbreviations

aa	amino acid
ab	antibody
ag	antigen
AH	Acute Hepatitis
ALT	Alanine Aminotransferase
BLSV	Big liver and spleen disease
CGW7	CLC Genomics Workbench 7 (software)
C_t	Threshold cycle number (used in qPCR)
dPPR	downstream Poly proline region
ELISA	Enzyme-Linked Immune Absorbent Assay
EM	Electron Microscopy
ER	*Endoplasmatic Reticulum*
FH	Fulminant hepatitis
GRP78	Glucose-Regulated protein 78
gt	genotype
HAV	Hepatitis A virus
HEV	Hepatitis E virus
HRP	Horse Radish Peroxidase
HSC70	Heat Shock Cognate protein 70
HSP90	Heat Shock protein 90
HSPG	Heparan sulfate proteoglycans
HSS	Hepatitis-spenomegaly syndrome
HVR	Hypervariable region
ICTV	International Committee on Taxonomy of Viruses
IDR	Intrinsically Disordered Region
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JR	Junction region

MEGA5	Molecular Evolutionary Genetics Analysis 5 (software)
MeT	Methyltransferase
ML	Maximum Likelyhood
mRNA	Messenger RNA
MSA	Multiple sequence alignement
NJ	Neighbor Joining
NS	None structural protein
nt	nucleotide
ORF	Open Reading Frame
P	Protruding
PCP	Papain-like Cysteine Protease
PDB	Protein Database
p-distance	proportion distance
pi	post-infection
PPR	Poly Proline Region
PSAP	Proline-Serine-Alanine-Proline
qPCR	quantitative real-time RT-PCR
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
S	Shell
SPF	Specific Pathogen Free
swHEV	swine hepatitis E virus
T_m	melting temperature of the PCR product
UPGMA	unweight pair-group method using arithmetic averages
uPPR	upstream Poly proline region
UTR	Untranslated region
VLP	Virus Like Particles
WHO	World Health Organization

1 Introduction

1.1 Hepatitis E Virus (HEV): One virus with many faces in different places

The disease previously associated with enterically transmitted none-A to D hepatitis, now known as hepatitis E, is an infectious viral disease caused by the agent hepatitis E virus (HEV). The disease is one of the most common cause of acute viral hepatitis globally. According to WHO, 20 million people are or have been infected with HEV. HEV is a RNA virus with a positive single stranded genome. It has caused several human epidemics in India (Chobe *et al.*, 1997), Pakistan (Rab *et al.*, 1997), China (Zhuang *et al.*, 1991), Africa (Kim *et al.*, 2014) and Mexico (Huang *et al.*, 1992). HEV was first recognized as a new pathogen during the Kashmir water-borne epidemic in 1978, at that time called non-A non-B hepatitis (Khuroo, 1980), but HEV has also retrospectively been traced back as the cause to a large outbreak in New Dehli, India in 1957 (Viswanathan, 1957). In 1981, a similar hepatitis epidemic occurred in a Soviet military camp located in Afghanistan and the infectious agent HEV was isolated for the first time. The discoverer, Dr. Balayan developed acute hepatitis following ingestion of a water phase stool suspensions from the 1981 water-borne epidemic and he sampled his stool and blood during his illness. These samples were used for further characterization of the virus (Balayan *et al.*, 1983). It took almost ten years for the HEV genome to be sequenced after the isolation of HEV cDNA from a HEV infected Cynomolgus monkey bile (Reyes *et al.*, 1990).

The clinical properties of acute HEV hepatitis are indistinguishable from hepatitis caused by the hepatitis A virus (HAV). The disease course is mostly asymptomatic or with mild symptoms, but can also cause severe hepatitis. In infected pregnant women the mortality rate is up to 25% (Kamar et al., 2012a; Aggarwal, 2011). HEV is important from the public health perspective in

developing countries (the Middle East, southeast and central Asia, Africa and the American continent), where it frequently cause large epidemics. HEV is fecal-orally transmitted, and the transmission is favoured by crowded settings with poor hygiene and water sanitation. The spread of the virus is usually through consumption of contaminated water or food (Okamoto, 2007). In developed countries HEV infection have long been considered as a poor hygiene and travel related illness. The situation was not investigated until several studies triggered questions why the general population in many industrialised countries like USA, Japan, Canada and several European countries (including Sweden) had high prevalence of antibodies against HEV (anti-HEV), ranging 5-53% in some regions (Kamar et al., 2012a; Mansuy et al., 2011; Guo et al., 2010; Olsen et al., 2006). This high prevalence indicates widespread asymptomatic HEV infections. The increased numbers of autochthonous (locally acquired) sporadic HEV cases with no history of travelling to HEV endemic countries raised the question if HEV would have other sources than water as viral reservoirs to infect humans (Kamar et al., 2012a).

Since the early 1990s, serological evidence of past HEV infections from several animal species and in some cases virus detection suggested that animals could be infected with HEV-like viruses. The breakthrough came in 1997, when a swine HEV strain was identified in the USA for the first time and named swine hepatitis E virus (swHEV) (Meng *et al.*, 1997). This new HEV variant was also genetically correlated to two human HEV strains isolated in the USA from individuals with no history of travelling to endemic HEV affected areas (Meng *et al.*, 1997). Since then, it has been found that domestic swine and wild boars across the globe are frequently infected by HEV, suggesting porcine as the main reservoir for HEV infections (Meng, 2010; Widén *et al.*, 2010; Meng *et al.*, 1997). The HEV transmission pathway is often unknown in the industrial part of the world with good sanitary conditions. However, there are well documented zoonotic reports by ingestion of HEV contaminated porcine products (swine/wild boar) or from consumption of deer.

Apart from the previously mentioned HEV hosts, additional animal species can be infected with HEV e.g. rabbit, mongoose and camels. The HEV variants infecting these hosts are classified into genotypes 1-7 (gt1-7) and all are members of the recently suggested species *Orthohepevirus* A of the *Orthohepevirus* genus (Smith *et al.*, 2014). Currently, only gt1-4 have been associated with human infections, and gt1-2 exclusively have human as host. These genotypes are associated with large outbreaks in developing countries often due to poor sanitary conditions. Swine and wild boar are possible viral reservoirs for gt3 and gt4 which both have zoonotic properties (Meng, 2013;

Meng, 2010). The discovery of new HEV variants found in a wide range of animal species has led to important HEV classification changes (Smith *et al.*, 2014), that are needed for this, seemingly, ever expanding virus family. Other issues include the poor knowledge of its replication and infection pathways, mainly because the HEV research progress has been hampered by the lack of efficient cell culture and small animal models (Kenney & Meng, 2015). There are improved models with potential, but they still suffer from complicated and expensive setups and are unsuitable for routine labs. A wide range of extrahepatic manifestations and increased incidences of chronic HEV infections in immunosuppressed patients also raises concern (Kamar *et al.*, 2012a). The discovery of HEV in animals, including moose, have broadened the known host range and diversity of HEV, and raised public health concerns for zoonosis and food safety. High HEV seroprevalence in the human population indicate that unidentified sources HEV transmission may exist and it is of importance to find these transmission routes. Thus, HEV may exist in our "backyard", but this awareness can be used for minimising the zoonotic transmission and indicate better preventive measures.

1.2 Etiology- Biology of hepatitis E virus (HEV)

1.2.1 Morphology and genomic organization

HEV was first sequenced in 1990 (Reyes *et al.*, 1990). Its genome consists of a single stranded positive sense RNA, which varies in size from 6.6-7.6kb depending on the virus strain described (Thiry *et al.*, 2015). The HEV genome is contained in a small, non-enveloped icosahedral symmetric virus capsid of about 27-34 nm diameter. HEV was first assigned into the *Picornaviridae* and later the *Caliciviridae* family, based on the first findings of its morphology and other physiochemical properties. Later it was clear that the genomic organization was different from *Caliciviridae* and other existing virus families. HEV was therefore classified into its own genus, *Hepevirus* of the novel family, *Hepeviridae* by the International Committee on Taxonomy of Viruses (ICTV). However, with the recent discoveries of several divergent animal HEVs, including the HEV found in moose (study I-II) have indicated the need for a revised HEV classification. A consensus HEV classification was recently presented (Smith *et al.*, 2014) and will most likely represent the update for the classification of HEV. This update suggest two genera: *Orthohepevirus* with four species (A-D) and *Piscihepevirus* with a single species *Piscihepevirus* A, and there seven genotypes (gt1-7) within the *Orthohepevirus* A species (see chapter 1.2.3 for more information). At least strains belonging to gt1-4 appear to share the same serotype, i.e. infection with one genotype infers immunity

against the other (Emerson & Purcell, 2003). The genome has the features of eukaryotic mRNA (Figure 1), and comprises of a 7-methylguanine cap at its 5′ end followed by three partial overlapping open reading frames 1-3 (ORF1, ORF2 and ORF3) and ends with a poly(A) stretch at the 3′ end (Tam *et al.*, 1991). Although ORF2-3 are encoded in the main HEV genome, its protein expression has been demonstrated to occur through a smaller viral RNA species of 2.2kb subgenomic RNA (Graff *et al.*, 2006). In addition, the viral genome also has short 5´- and 3´untranslated regions (UTRs), and a region covering from the 3´end of ORF1 to the start of ORF2/3, which is homologous to a junction region (JR) found in alphaviruses (Purdy *et al.*, 1993). These elements are likely to form into complex secondary structures containing conserved stem-loops and hairpin structures with properties important for HEV RNA replication, translation and packaging (Ahmad *et al.*, 2011; Reyes *et al.*, 1993). The appearance of the viral genome as mRNA facilitate viral protein translation through the caped 5'-end, disguising the viral genome from the immune response (Ahmad *et al.*, 2011). Studies have confirmed the ORF1-3 expression by detecting antibodies against these proteins in HEV infected humans and experimental animals (Khudyakov Yu *et al.*, 1994). However, the expression kinetics of these proteins during the viral life cycle are still not fully understood.

An investigation showed a 76nt region (at nucleotide position 130 to 206) within the 5' UTR that could bind with the N-terminal end of ORF2, suggesting it to function as a RNA encapsidation signal (Surjit *et al.*, 2004). The end of ORF2 and the 3'UTR are believed to form secondary structures and have been demonstrated to bind to a cloned recombinant HEV RNA dependent RNA polymerase (RdRp), indicating an important role in the HEV replication process (Agrawal *et al.*, 2001; Emerson *et al.*, 2001). Viral proteins have beside their essential function to replicate and encapsidate the viral genome, also displayed additional functions for host cellular protein interaction. All ORF1-3 have shown such interactions, see following section for more information.

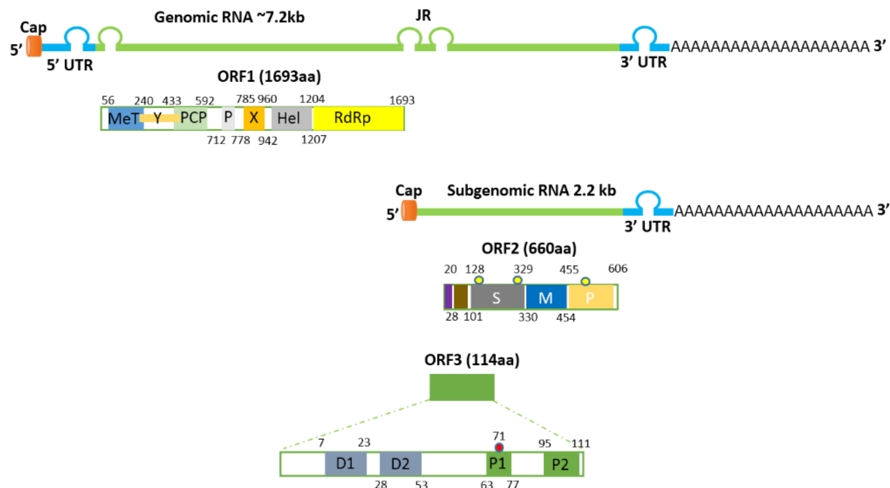

Figure 1. Illustration of the ~7.2kb HEV genome consisting of a cap at its 5’ end and terminates with a poly(A) tail at its 3’ end. Nucleotide (nt) and amino acid (aa) position based on gt1-SAR55. There are also short sections of untranslated regions (UTRs) at 5’ and 3’ ends that folds into stem-loop structures (indicated with blue and green colour). These UTRs are involved in virus replication, translation and packaging. Three open reading frames 1-3 (ORF1-3) are shown. ORF1 encodes a none-structural polyprotein (with the following domains: MeT; Methyltransferase; Y: Y-domain; PCP: papain-like cysteine protease; P: poly proline region; X: X- or Macro-domain Hel; Helicase RdRp: RNA dependent RNA polymerase) and ends within a junction region (JR); Both ORF2-3 proteins are translated from a caped bistronic subgenomic RNA that is produced from viral replication starting in the JR. The ORF2 encodes the viral capsid with the following regions: ER-signal (purple) and viral RNA binding region (brown), S-, M-, P-domain. The accessory protein ORF3 contains the following domains (D1, D2, P1 and P2). All predicted domains and their possible boundaries positiions are illustrated with numbers. The three yellow dots represents glycolysation sites in ORF2 (amino acid position 137, 310 and 562), while the red dot is the phosphorylation site in ORF3 at amino acid position 71.

The ORF1 polyprotein

The ORF1 (~5.0kb) occupies more than a third of the HEV genome and encodes the non-structural poly-protein (pORF1) of about 1693-1704 amino acids (aa), (Figure 1). This poly-protein is involved in the replication of the viral genome and processing of viral proteins (Ahmad *et al.*, 2011). The ORF1 consists of six functional domains including a methyltransferase (MeT), followed by the Y-domain, a papain-like cysteine protease (PCP), a hypervariable region (HVR) with a prolin rich region (PPR), macro domain (X-domain), a RNA helicase and a RNA dependent RNA polymerase (RdRp) at the 3’-ORF1 terminal end (Koonin *et al.*, 1992). The predicted MeT representing residues 56-240 (Koonin *et al.*, 1992), is suspected for the 5’ terminal end capping activities, since both the HEV genomic and subgenomic RNAs (encoding ORF2 and ORF3) are capped (Huang *et al.*, 2005; Chen &

Meng, 2004; Kabrane-Lazizi *et al.*, 1999b). A cDNA corresponding to amino acids 1-979 was expressed in a baculivirus system showing that both metyltransferase and guanyltransferase activities were detected (Magden *et al.*, 2001).

The subsequent downstream Y domain still remains elusive with unknown assigned function. The putative PCP domain between 1300-1779nt/433-592aa of ORF1 has long been suspected to have a role in the ORF1 processing (Koonin *et al.*, 1992). But its presence and polyprotein processing properties still remain controversial and more work has to be performed to clarify if it exist and how it function.

Further downstream is the hypervariable region (HVR), which includes the proline rich region (PPR) corresponding to 2137-2337nt/712-778aa. The PPR is suggested as an intrinsically disordered region (IDR), rich in polar and charged amino acids and may act as a flexible hinge (Purdy, 2012). The mutations within the PPR have shown preference for cytosine in the first and second codon positions leading to increased frequency of proline residues. The PPR of gt1 shows more conservation and less substitution rates compared with the zoonotic gt3-4, which may reflect wider host range flexibility of these latter genotypes (Purdy et al., 2012). Several studies, including study III reported that gt3 can acquire fragment inserted into the PPR regions, but how these insertions occur is still unknown. These fragment insertions have frequently been observed in strains from immunosuppressed patients with chronic HEV infection. Recombinants appears also to have an improved replication capacity when tested on cell lines as model (see chapter 1.7.3 from more information). It has been shown that the inserted sequences could come from the host cell genome or from the virus genome itself, and it is proposed to open new protein-protein interactions with new potential regulation sites (Lhomme et al., 2014a; Purdy, 2012).

The X- or macro-domain, is the downstream flanking region at position 2356-2829nt/785-960aa (Neuvonen & Ahola, 2009; Egloff et al., 2006). Macro domains can be found in a large range of proteins of bacteria, archaea and eukaryotes, and contribute to ADP-ribose metabolism and posttranslational modifications (Han et al., 2011). It is suggested that the viral macro domain may function as poly (ADP-ribose)-binding unit and is also attracting cellular factors for participation in viral RNA replication and/or transcription (Egloff et al., 2006). The increased sequence diversity of the PPR and X domain at the acute phase of an HEV infection was suggested to be associated with persistence of the virus in immunosuppressed solid organ patients (Lhomme et al., 2014b).

The next following domain (2881-3615nt/960-1204aa) of the HEV ORF1 region is encoding the helicase, which is essential for the viral replication. The putative HEV helicase contains seven motifs that participates in the binding and hydrolysis of nucleotides triphosphates (NTPs), and binding of nucleic acids (DNA/RNA), (Koonin *et al.*, 1992).

The RdRp is found in the subsequent flanking region (3546-5106nt/1207-1693aa). This essential enzyme is found in all RNA viruses. Its function is to replicate the genomic RNA. Which most likely occurs through an anti-genomic RNA intermediate, in the case of HEV, through a minus sense RNA genome intermediate. As in the RdRp of other RNA positive-stranded RNA viruses, eight motifs can be found in the HEV RdRp, including GDD amino acid sequences that binds Mg^{2+} required for replicase activity (Koonin *et al.*, 1992). The RdRp activity has been demonstrated in HEV replicon systems (Graff *et al.*, 2005; Agrawal *et al.*, 2001).

It is still unclear whether the pORF1is processed into separate components or remain unprocessed as a large poly-protein. Studies observing ORF1 processing into smaller units have been reported (Parvez, 2013; Karpe & Lole, 2011; Sehgal et al., 2006; Ropp et al., 2000), however contradicting studies showing the opposite have also been reported (Perttila et al., 2013; Suppiah et al., 2011; Ansari et al., 2000).

Junction region

The conserved junction region (JR) including ORF1 stop codon and the start codons of the overlapping reading frames ORF2 and ORF3, is predicted to encode secondary stem-loop RNA structures (Cao *et al.*, 2010; Huang *et al.*, 2007). The authentic start codon (AUG) positions of ORF2-3 in JR has been investigated in different studies: The first study of liver tissue of macaques experimentally infected with HEV, detected three RNA species of 7.2kb, 3.7kb and 2kb designated as the genomic and two subgenomic RNA (Tam *et al.*, 1991). This model suggested that the ORF1 stop codon at position 5105 (gt1 SAR-55 Strain) overlapped with the ORF3 codon at position 5104. The ORF2 was suggested to be translated from the 3.7kb subgenomic RNA, while the ORF3 was translated with the 2kb subgenomic RNA. However, the 3.7kb subgenomic RNA could not be confirmed in other studies. Another challenging model from stable Huh-7 cell lines created with HEV RNA replicons expressing the neomycin resistance gene from ORF2 and ORF3, showed stable expression only of the genomic RNA and 2.2kb subgenomic RNA (Graff *et al.*, 2006). This subgenomic RNA started at 5122 and was bicistronic for the translation of both ORF2 and ORF3. This model also explains the reading frame differences observed for gt4, which contains an extra nucleotide T-insert

between 5116/5117 (SAR-55) resulting in a different reading frame for the ORF3 start codon. The translation of ORF3 from position 5131 (SAR-55) should be the same for all gt1-4. Several other studies supports this model through intrahepatic inoculation of unchanged and mutant gt3 swine HEV replicons into swine and through secondary structure predictions (Huang *et al.*, 2007). Another study confirmed the existence of the 2.2kb subgenomic RNA and its starting position at 5122 through PLC/PRF/5 cells inoculated with fecal suspension containing gt4 or transfected *in vitro* from a cloned cDNA produced from infectious gt3 RNA (Ichiyama *et al.*, 2009). The role of the JR secondary structure in viral replication was demonstrated when Huh7-cells were transfected with unchanged or mutant JR replicons with reporter genes (Cao *et al.*, 2010). The viral negative-strand RNA may act as a template for the positive-strand genome and subgenomic RNA synthesis, the former within the JR in a primer-independent manner. The JR of negative-sense directed RNA is predicted to contain a folded stem-loop structure. Mutations on the predicted loop or part of the stem of the subgenomic RNA start site considerably reduced or stopped reporter activity. The sequence of the JR therefore play important role in HEV replication.

The ORF2 protein – Viral capsid

The ORF2 corresponds to nucleotide positions 5145-7125 in the genome. It encodes the viral capsid of ~660 amino acids depending on the HEV strain. This structural protein is highly immunogenic and is proposed to encapsidate the viral RNA and interact with the host cell e.g. while entering into and exit from the host cell (Xing *et al.*, 2010). The 111aa N-terminal region appears to bind to the 5' region of the viral RNA (Surjit *et al.*, 2004). This region also contains signal sequence, which translocates the ORF2 protein to the *endoplasmatic reticulum* (ER), where it is glycosylated at three conserved asparagine sites (137, 310 and 562), (Zafrullah *et al.*, 1999). This is required to produce infections virus particles and for efficient propagation, as has been shown in cell lines with HEV replicons (Yamada *et al.*, 2009b; Graff *et al.*, 2008). There is a broad antigenic cross-reactivity between ORF2 proteins from known HEV genotypes, which has been demonstrated with western blot and antisera using recombinant capsid from various HEV strains including avian HEV (Haqshenas *et al.*, 2002).

The accessory protein ORF3

There are currently no homologues to the ORF3 in the sequence databases. The protein is located at nucleotide positions 5131-5475nt of the HEV genome. The small 114aa protein contains two N-terminal hydrophobic (D1 and D2) and

two C-terminal proline-rich regions (P1 and P2), (Ahmad *et al.*, 2011). Multiple functions have been proposed for this phosphoprotein including interaction with host cell proteins associated with immune evasion, cell survival promotion (Kar-Roy *et al.*, 2004), acute phase response modulation (Chandra *et al.*, 2010; Chandra *et al.*, 2008; Moin *et al.*, 2007) and immunosuppression (Surjit *et al.*, 2006; Tyagi *et al.*, 2004). This protein appears not to be essential for infection and replication (Emerson *et al.*, 2006), but its presence is needed for the virion release from cells (Nagashima *et al.*, 2011). The phosphorylated form of ORF3 (S71 residue) was shown to interact with the non-glycosylated form of the capsid protein (Tyagi *et al.*, 2002). This post-translational interaction suggested a regulatory role of ORF3 during virion assembly. Substantial sequence diversity of ORF3 has been observed between genotypes and even within genotypes, but also within more divergent HEV strains found in wild animals e.g. moose HEV (Studies I).

Viral particle structure

The capsid protein expressed in mammalian cells was reported as an 74kDa unglycosylated and an 88kDa glycosylated forms (Jameel *et al.*, 1996) and it is still controversial which form/s build the virion. From 3D structure studies of the HEV capsid it is observed that the main structure of the virion shell uses two identical capsid proteins (homodimers) as a base for the construction of the virion shell. The existence of two different forms of the HEV virus like particles (VLPs) have been shown T=1 and T=3 (Figure 2), consisting of 60 and 180 capsid monomers, respectively.

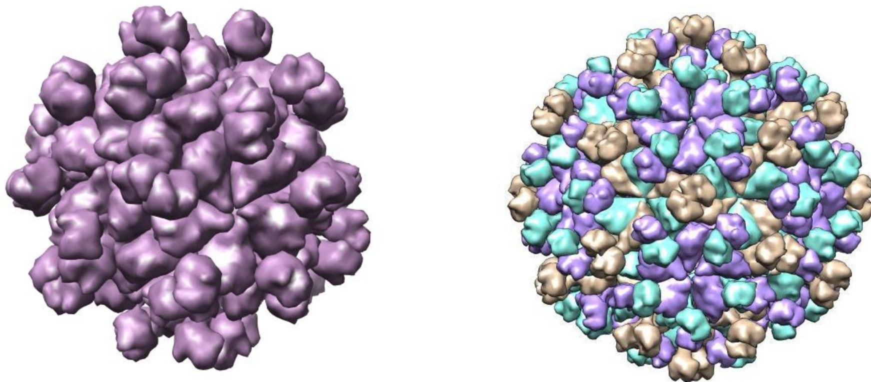

*Figure 2.*Structural representation of T=1 (left), 3HAG (Guu et al., 2009) and T=3 (Right), 3IYO (Xing et al., 2010), adapted from the Protein database (PDB), http://www.rcsb.org/pdb/home/home.do, (accessed 2015-07-15).

These monomers have been shown to form three distinct domains assigned shell (S), middle (M) and a protruding (P) domains (Xing *et al.*, 2010; Yamashita *et al.*, 2009; Li *et al.*, 2005b). The T3 structure is suggested to be the more likely for packaging the HEV virion (Cao & Meng, 2012; Xing *et al.*, 2010). The structure of the wild type virion has not been resolved and therefore still remain unknown (Mori & Matsuura, 2011). The possible existence of two types of HEV virions have been suggested, one nonenveloped viron found in fecal samples and one enveloped-like virion found in serum. The latter is associated with ORF3 and lipids with unknown structure (Yamada *et al.*, 2009a; Takahashi *et al.*, 2008). These two suggested virion types indicate that more studies should be performed.

1.2.2 Viral life cycle overview

The life cycle of HEV is largely unknown due to the lack of efficient culture systems and small animal models (Ahmad *et al.*, 2011). This has hampered the understanding of the HEV pathogenesis and antiviral drug development. HEV most likely enters the body orally and the primary site for viral replication is believed to be the liver, with the hepatocytes being the most likely cell type to be infected (Ahmad *et al.*, 2011). Current knowledge suggest that the structural HEV capsid protein binds to cellular receptor/s to start viral entry and initiate replication (Figure 3A). The specific cellular receptor for HEV is still unknown, but through ORF2 binding studies; the ORF2 C-terminal region was suggested to bind to heat shock cognate protein 70 (HSC70), (Parent *et al.*, 2009), a member of the heat shock proteins acting as chaperons. Heparan sulfate proteoglycans (HSPG) has also been suggested as viral receptor on the cell surface (Kalia et al., 2009). A receptor-dependent clathrin-mediated endocytosis (Figure 3B) has been demonstrated to be involved in the HEV particle entry (Kapur et al., 2012).

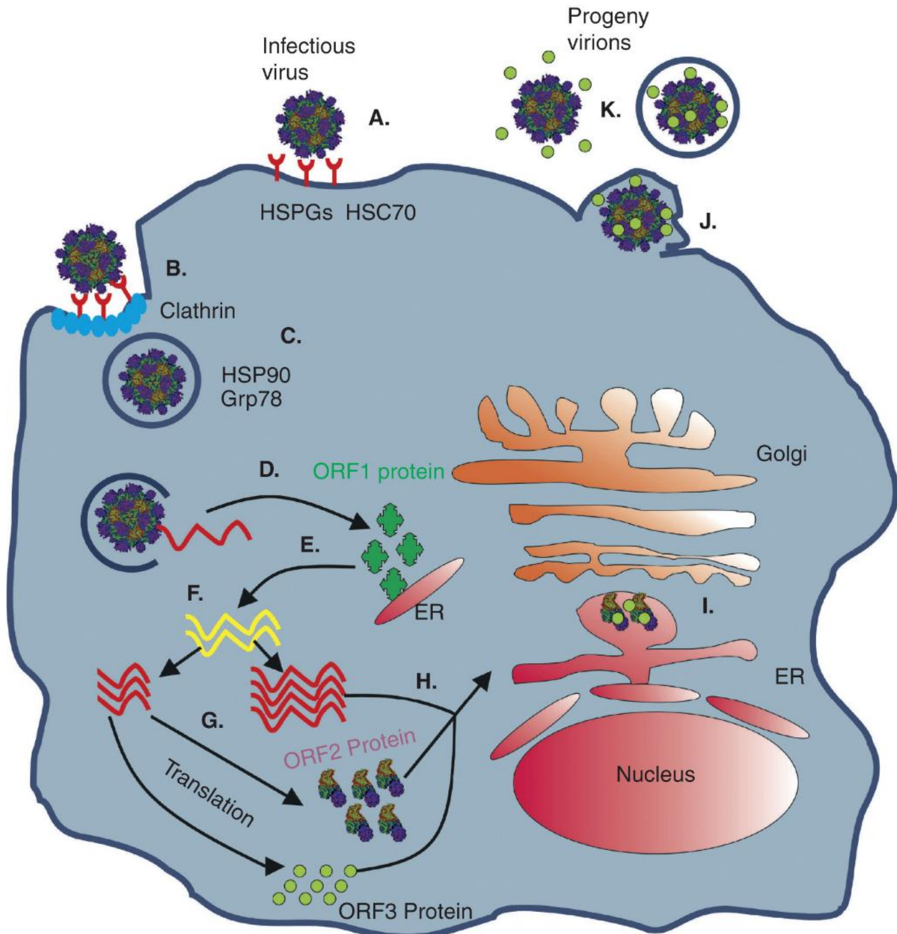

Figure 3. A schematic HEV replication from entry to egress from the host cell. A-C) Binding of HEV virion to putative host receptors. D) Release of HEV genome enabling translation of ORF1 protein, which most likely uses the available positive sense HEV genome as template for negative sense synthesis E). This is in turn a template for the production of new positive sense HEV genomic RNA, including a smaller subgenomic genome, which ORF2 or ORF3 are translated from, see G). H) Particle assemble initiates with the binding of ORF2 to the genomic RNA and interaction with ORF3. (I-J) The virions are transported to the plasma membrane for the release of the membrane-associated HEV particles. K) The virion will lose the membranes when passed through the digestive system, ready for infecting a new host. This is reproduced from (Kenney & Meng, 2015), with permission from the publisher Taylor & Francis.

Once inside the cell, the capsid is thought to interact with heat shock protein 90 (HSP90) and glucose-regulated protein 78 (Grp78) for the intracellular transport and uncoating (Yu *et al.*, 2011; Zheng *et al.*, 2010), (Figure 3C). Like cellular caped mRNA, the cap structure in the 5'UTR terminal of the HEV genome recruits 40S ribosomal subunit to start cap-dependent translation of

NS-polyprotein ORF1 (Figure 3D). It is thought that the viral RdRp of the ORF1 associates with the host ER through a predicted transmembrane domain corresponding to residues 4449-5109 to begin replication of the viral genome (Rehman *et al.*, 2008). The replication process most likely involves synthesis of negative-sense RNA (Figure 3E) which has been detected in tissues from HEV infected animals (Varma *et al.*, 2010; Nanda *et al.*, 1994). This most likely occurs when the RdRp binds to the secondary structure of the viral 3' UTR genome, which initiates the synthesis of negative sense RNA genome. This template is then used for the synthesis of full genome and the 2.2kb SgRNA (Figure 1 and Figure 3F), (Cao & Meng, 2012).

This enables the translation of more ORF1 proteins, and capsid protein from ORF2 and the small ORF3 protein translation from the bicistronic subgenomic RNA (Figure 3G), or the HEV genome is encapsidated with help from ORF2 (Ahmad *et al.*, 2011; Graff *et al.*, 2006), (Figure 3H). Assembly and release of the HEV virions are still poorly characterized. However, it has been shown that the ORF2 protein can bind the viral RNA through a 76-nt region at the 5' end of the HEV genome and most likely package it through the assembly of progeny virions (Surjit *et al.*, 2004), see Figure 3H. The N-terminal end of ORF2 also contain a signal sequence, which translocates the ORF2 protein to the endoplasmatic reticulum (ER), where it is glycosylated at three conserved asparagine sites (137, 310 and 562), (Zafrullah *et al.*, 1999), see Figure 3I. The ORF3 protein is thought to be involved in a later step through an amino acid motif PSAP, associated with protein interactions, important for the release (Figure 3I-J) of the membrane-associated HEV particles from infected cells (Nagashima *et al.*, 2011). It has been shown that this virion form that circulates in blood has stealth properties masking HEV from antibodies targeting virions without membrane, which also can be seen with hepatitis A virus (HAV), (Feng & Lemon, 2014). The potential membrane surrounding the released virions is most likely cleaved/removed in the gut (Figure 3K) when the virus is shed with feces (Okamoto, 2013).

1.2.3 Continous discovery of new HEVs require an updated HEV classification

Recently, several HEV related strains have been detected in addition to gt1-4 (Figure 4 and Figure 5). A common property of these HEV-related strains are the highly divergent genomes as compared to gt1-4, despite the characteristic genomic organisation with three HEV ORFs. Their genetic classification, and cross species and zoonotic potential need to be further investigated.

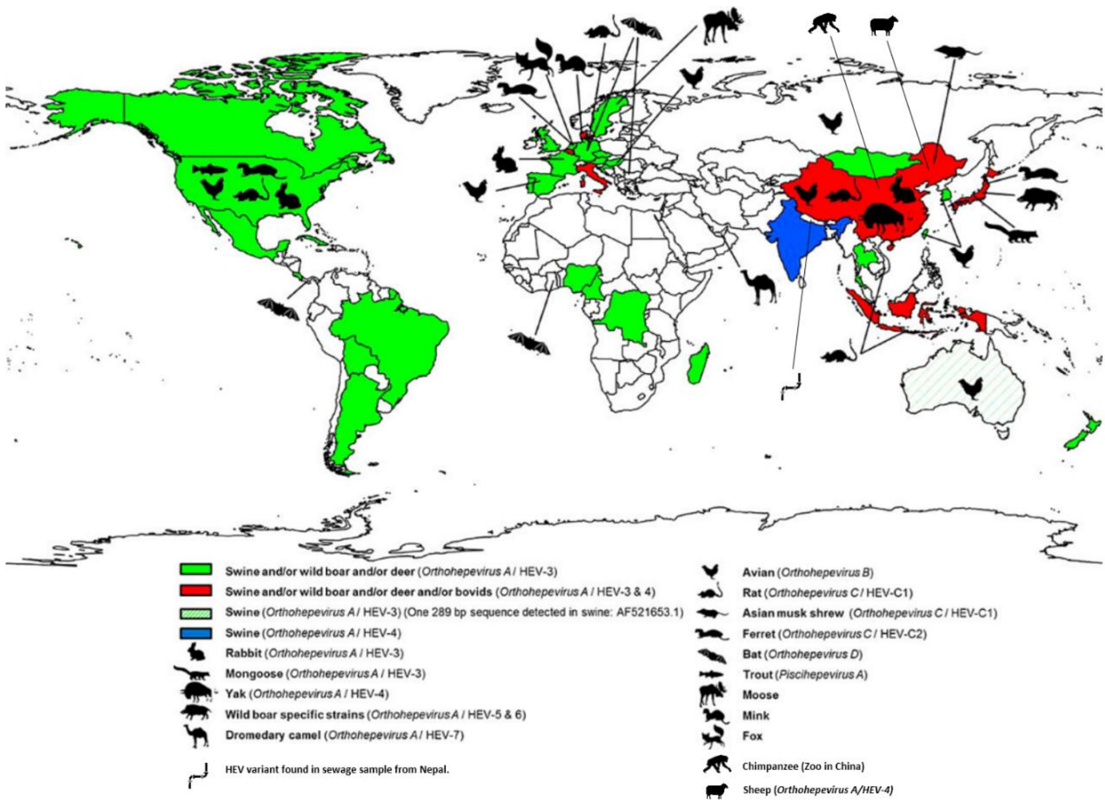

Figure 4. Geographic distribution of anime HEVs and HEV variants with high genome sequence divergence compared with gt1-7 (HEV 1-7). Figure adapted from Thirty *et al*., (2015), updated with HEV from sheep, chimpanzee and sewage sample from Nepal. More information is referred to the main text.

With the continuous discovery of new HEVs, several proposed classification strategies have been presented (Johne et al., 2014a; Meng, 2013; Smith et al., 2013a). However, a consensus HEV classification was very recently proposed from the members of the ICTV *Hepeviridae* Study Group, and the criteria was based on phylogeny and host range (Smith *et al.*, 2014). It is proposed that all HEVs are placed into two genera instead of one: *Orthohepevirus* with four species (A-D) and *Piscihepevirus* with a single species *Piscihepevirus A* (Figure 5).

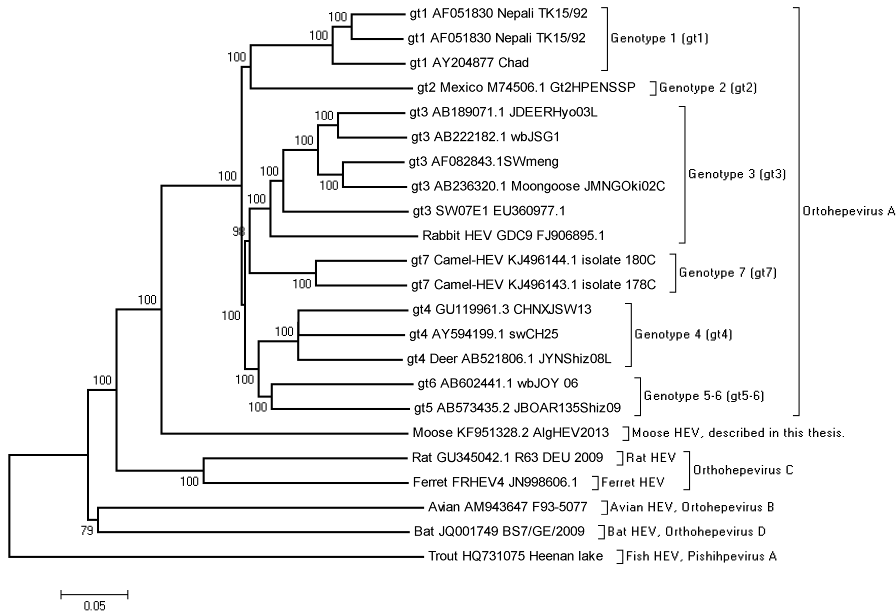

Figure 5 Phylogenetic tree of selected full HEV genomes and their classification according to Smith et al., (2014). The moose HEV is currently unassigned HEV variant and is described in this thesis.

Species like *Orthohepevirus* A and *Orthohepevirus* C are proposed to contain genotypes, seven and two respectively. The members of *Orthohepevirus* A consist of genotypes 1-7 (Smith *et al.*, 2014). The species where these genotypes can be found are human (gt1-4), swine (gt3-4), wild boars (gt3-6), rabbit (gt3), deer (gt3-4), mongoose (gt3), camel (gt7), and rat and ferret, (Figure 5). The zoonotic potential is still unknown for the more divergent gt5-7. *Orthohepevirus* B contain viruses found in chickens and was the first highly divergent genome detected. Since then it has been associated with the avian disease hepatitis-spenomegaly syndrome (HSS) in USA (Haqshenas *et al.*,

2001) or big liver and spleen disease (BLSV) in Australia, (Payne *et al.*, 1999) which can have an important economic impact for the affected breeder. About 80% nucleotide sequence identity was found between HSS and BLSV HEV strains, showing that they are variants of the same virus (Guo *et al.*, 2006; Huang *et al.*, 2004). *Orthohepevirus* C has two members found in rat (HEV-C1) and ferret (HEV-C2), and *Orthohepevirus* D contain bat virus (Figure 5). Partial sequences from other possible members of the family was recently identified in mink (Krog *et al.*, 2013) with similarity to HEV in ferret, fox and rat (Thiry *et al.*, 2015). The HEV identified in moose is still unclassified. Studies I-II of this thesis discuss the classification issues, describe the investigation of the HEV prevalence in Swedish moose, and discuss the zoonotic perspective of moose HEV, which is still unclear. *Hepeviridae* is a dynamic and expanding family of vertebrate viruses and a flexible consensus classification is therefore needed.

1.2.4 HEV subtype classification and genotypes 1-4

Although not officially recognized by ICTV, the most widely used gt1-4 subtyping classification suggest a total of 24 subtypes (1a-e, 2a-b, 3a-j, and 4a-g), (Lu *et al.*, 2006). This was based on 49 complete genomes and different subgenomic sequences, and individual subgrouping was assigned to nucleotide differences of 12-18% for e.g. gt3 and gt4. But recent studies, have found inconsistencies mainly concerning the reliability of the subtype separation and the inability to support newly detected and highly divergent HEV variants found in animals (Oliveira-Filho *et al.*, 2013; Smith *et al.*, 2013a). Despite these limitations, classification under genotype level is still very useful and important for both clinical and epidemiological studies e.g. tracing currently circulating strains in the population (Widén *et al.*, 2010; Norder *et al.*, 2009), including from studies II and III.

Genotype 1 and 2 (gt1-2)

Genotype 1 (gt1) dominates in Asia and Africa, genotype 2 (gt2) includes strains from Mexico and Africa, and both of these genotypes have only been found in humans (Kamar *et al.*, 2012a). Both genotypes are responsible for large outbreaks and epidemics in developing countries or overcrowded areas such as in refugee camps with poor sanitary conditions and drinking water contaminated with fecal matter (Kamar *et al.*, 2012a).

Genotype 3 (gt3)

Swine HEV assigned as genotype 3 (gt3) was first discovered in the USA, 1997. Further studies showed that this agent was highly prevalent in swine and

raised public health concerns for zoonotic infections (Meng *et al.*, 1997). It turned out later that gt3 was not a coincidental discovery, and that it was widely prevalent in the world, especially associated with swine and wild boar. This genotype is also responsible for most autochthonous HEV infections in Europe, USA, and Japan (Kamar *et al.*, 2012a). Gt3 has been detected in many animal species reflecting its cross-species infection ability. Evidence of zoonotic transmission like highly similar gt3 genomic sequences from the patient and left overs of food products made from swine and deer, have been demonstrated (Li *et al.*, 2005a; Tei *et al.*, 2003). From a phylogenetic perspective, gt3 is divided into two main groups assigned as 3.I and 3.II (Widén *et al.*, 2010; Norder *et al.*, 2009). Ten subtypes (3a-j) are further proposed (Lu *et al.*, 2006) and are distributed within 3.I or 3.II group. It appears that gt3c, gt3e and gt3f are the most frequent subtypes both in humans and porcine in European countries (Widén *et al.*, 2010; Legrand-Abravanel *et al.*, 2009; Norder *et al.*, 2009). Previous studies have shown the subtype gt3f appears to dominate in Sweden (Widén *et al.*, 2010; Norder *et al.*, 2009). Currently, only one complete genome of a Swedish swine gt3f has been published (Xia *et al.*, 2008), but study III expand this list with six near complete gt3f genomes from porcine HEV, characterised from a zoonotic, recombinant and virulence perspective. Swedish deer species, including the largest deer, the moose have never been investigated for HEV infection before and the zoonotic risk from consumption of deer indicated by other studies motivated the work presented in papers I-II and IV.

Rabbit HEV a distant member of gt3

In 2009, a novel HEV was found in rabbits (Zhao *et al.*, 2009) and since then it has been discussed whether the rabbit HEV belong to gt3 or not. With 85% sequence identity with each other and ~73-79% identity with gt1-4, raised question whether if it should be placed in its own genotype or as a distantly related gt3 member. However, it is becoming more acceptable to place the rabbit HEV in the gt3, since it according to the phylogenetic studies, forms a distant third gt3 subgroup (Smith *et al.*, 2014).

Genotype 4 (gt4)

Genotype 4 (gt4) was discovered in 1999 from patients in China. The first whole genome was sequenced in the year 2000. This genotype is mostly prevalent in Asia, where it has been recovered from both in pigs and humans with high genetic similarity indicating zoonotic transmission (Liu et al., 2012; Wang et al., 2000; Wang et al., 1999). However, gt4 have also been found in

wild boar (Sato et al., 2011) and some cases associated with zoonosis (Kim et al., 2011).

The dominance of gt1 infections in China appears to have shifted over the last 25 years, where gt4 has overtaken the gt1 in number of isolated strains (Liu *et al.*, 2012). This may result from the fact that sanitary conditions have improved and food-borne replaced water-borne transmission generating a genotype switch. However, it is important to remember that the actual gt4 status before 1999 was not investigated because molecular studies was focused on large epidemics and not on sporadic cases caused by gt4 (Liu *et al.*, 2012). Gt4 has recently also been observed in Europe (France, Belgium and Denmark), both in humans and swine, raising the question if gt4 was introduced to European domestic swine through imported swine meat from Asia and suggest that gt4 is already established in Europe (Bouamra *et al.*, 2014; Colson *et al.*, 2012). Like for gt3, other animal species besides swine have been observed to be gt4-infected, like sheep and yak (Wu *et al.*, 2015; Midgley *et al.*, 2014; Xu *et al.*, 2014; Wang & Ma, 2010), but these results need to be confirmed by other laboratories as well.

1.3 Clinical outcome

This section is divided into several sub sections, starting with the introduction of the clinical manifestations of HEV in humans and animals. The pathogenesis of HEV and other clinical manifestations of HEV infection is only partially understood.

1.3.1 Clinical manifestation in humans

Whether infected through larger epidemics or autochthonous sporadic transmission, clinical symptoms can occur, which is important for the diagnostic identification of HEV. The clinical hepatitis E infection in humans is often near undistinguishable from hepatitis A virus (HAV) infection. It may cause self-limiting acute infection (AH), asymptomatic infection with non-existent and mild symptoms, or fulminant hepatitis (FH). The most typical clinical signs are: elevated transaminases, jaundice, abdominal pain, headache, fever, nausea/vomiting, anorexia, pruritus and hepatomegaly (enlarged liver), (Aggarwal, 2011). The incubation period from the exposure of HEV to development of clinical signs of infection ranges from two weeks to two months (Purcell & Emerson, 2008). It is believed that the liver is the main target organ, but how HEV reaches the liver is unknown and other extra-hepatic sites where HEV replication occurs is still being investigated. But once it has reached the liver, the virus replicates in the cytoplasm of hepatocytes,

passed in the bile and then being shed in feces. Both viremia and fecal shedding are detected before the onset of disease. This is mainly indicated with elevated alanine aminotransferase (ALT), with peak levels during the acute phase, thereafter the ALT levels will gradually return to normal levels. The humoral response makes its presence in parallel with elevated ALT in form of increased anti-HEV IgM followed IgG, which may remain circulating in the body up to 14 years (Emerson & Purcell, 2003). A general summarized overview of the course of HEV infection is shown in Figure 6.

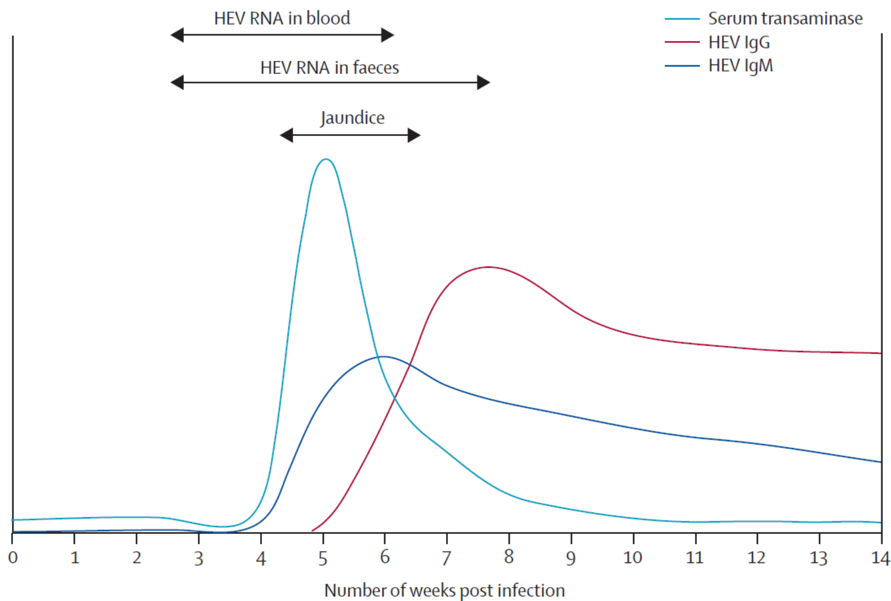

Figure 6. An overview of the HEV infection through time, showing the virus presence at different locations and serological response. This is reproduced (Dalton *et al.*, 2008a) with permission by Elsavier.

Although most HEV infection are self-limiting, there is still mortality rates up to 0,5-4% during outbreaks (Aggarwal, 2011). however, the mortality rate is increased when it comes to certain patients groups such as pregnant women and patients with other liver diseases (Aggarwal, 2011). The comparable few reported human clinical HEV cases is contradictory to the in general high HEV seroprevalence 5-53% observed in many countries (Kamar *et al.*, 2012a; Mansuy *et al.*, 2011; Guo *et al.*, 2010; Olsen *et al.*, 2006).

This is especially true for developed countries with good sanitary standard, where the high seroprevalence corresponds only to a small fraction of the total

reported HEV infections, and this may be due to low infectious dose causing subclinical HEV infections (Kamar *et al.*, 2012a).

Pregnancy and HEV infection

Both epidemic and sporadic forms of hepatitis E, especially in hyperendemic areas with gt1-2, are characteristically associated with an increased mortality rate up to 25% in the third trimester in infected pregnant women (Kamar et al., 2012a). This appears not be common for gt3-4 although there has been some documentation with acute hepatitis (Anty et al., 2012), but not with mortality as outcome. The actual mechanism of the high mortality of HEV infection during pregnancy is still unclear and is constantly under debate. The pregnancy status itself is characterized with maternal immune tolerance toward the fetus, so it can survive but still being able to fight threatening infections. One theory of the pathogenesis associated with HEV is that the hormonal changes in the pregnant woman shift immune response profile during the trimester period from Th1 to Th2 profile (Navaneethan et al., 2008; Pal et al., 2005). Higher viral loads were also observed in pregnant women compared to women with no pregnancy (Borkakoti et al., 2013). Other FH cases caused by gt1 in India, have been shown infected with strains with amino acid mutations, suggesting the existence of different virulent HEV strains (Mishra et al., 2013). The genetic composition of the host may also be involved in disease outcome. HEV can also be transmitted vertically, from mother-to-foetus and may cause high mortality in young infant for unknown reasons. This is exemplified from a report from India, where 15 of 19 infants born from HEV infected mothers, and six of the 19 infants died whereas 9 managed the infection (Khuroo & Kamili, 2009).

Chronic hepatitis E and HEV infection with pre-existing liver disease

A growing number of studies highlight that HEV can cause chronic infection in immunosuppressed patients, who can rapidly develop fibrosis and cirrhosis and subsequent liver failure if not treated (Fujiwara *et al.*, 2014). Chronic HEV infection has therefore often been observed in organ transplant recipients (e.g. liver, heart and kidney), (Fujiwara *et al.*, 2014) and in HIV positive individuals (Hajji *et al.*, 2013; Dalton *et al.*, 2009). Common with these studies is that it has been reported from developed countries in Europe and USA and all infecting strains have been gt3 (Fujiwara *et al.*, 2014). Several approaches are available to treat chronic infection successfully; as dose reductions of immunosuppressive therapy, treatment with the antiviral drug ribavirin and administrated pegylated interferon alpha (Kamar *et al.*, 2011b; Haagsma *et al.*, 2010; Mallet *et al.*, 2010). Individuals with pre-existing liver disease, may also

develop higher frequency of clinical manifestations and liver damage when HEV infected (Radha Krishna *et al.*, 2009; Ramachandran *et al.*, 2004; Hamid *et al.*, 2002).

Extra-hepatic manifestations

During the HEV infection, extra-hepatic manifestations could occur which may case diagnostic difficulties for the clinician. It is therefore important to highlight these manifestations because HEV is most probably an under-diagnosed pathogen.

A recent review identified 25 studies of HEV infections associated with neurological problems (Cheung et al., 2012). The most frequent were Guillain-Barré syndrome and brachial neuritis. Another study found that 5.5% of patients with locally acquired HEV infection developed neurogical symptoms (Kamar et al., 2011a). One renal transplant patient with chronic HEV infection was diagnosed with complication associated with both the central and peripheral nervous system (Kamar et al., 2010). A gt3 was isolated from the cerebrospinal fluid and its genomic sequence was different from the gt3 variants in the serum. Other less frequent extra-hepatic manifestations are renal complications, thrombocytopenia and pancreatitis (Kamar et al., 2012b; Aggarwal, 2011).

1.3.2 Clinical outcome in animals

The pathogenesis of HEV has been studied only in swine. Domestic swine worldwide are commonly infected by HEV, with gt3 and/or gt4, and are most frequently detected in piglets 2-4 months of age, whereas younger or older are less frequently infected (Widén *et al.*, 2010; Meng *et al.*, 1997). This is due to the protection caused by the maternal immunity in very young piglets (de Deus *et al.*, 2008a; Meng *et al.*, 1997), while older swine have already established HEV immunity (Williams *et al.*, 2001; Yoo *et al.*, 2001; Hsieh *et al.*, 1999). It is still unclear how the virus enters the swine and reach the liver, which is suspected to be the primary replication site (Williams *et al.*, 2001; Meng *et al.*, 1997). Swine appear not to show any signs of clinical illness during the HEV infection, however mild liver lesions have been reported (Halbur *et al.*, 2001; Meng *et al.*, 1997). In one experimentally HEV infected swine, the negative HEV RNA strand as an indicator of HEV replication was detected in extra-hepatic tissues such as: tonsils, lymph nodes, spleen, stomach, kidneys, lungs, both small and large intestine and salivary glands up to 20-27 days post-infection (pi) (Williams *et al.*, 2001). Similar extra-hepatic sites for HEV replication have also been reported in other studies (de Deus *et al.*, 2008a; Choi & Chae, 2003). During experimental HEV infection, viral RNA has been

detected in feces earlier than in the bile in about tenfold higher quantities. This finding suggested pre-amplification of HEV taking place first in the gastrointestinal tract followed by spread to liver and then followed by viremia (Meng *et al.*, 1998a; Meng *et al.*, 1998b). The viremia phase may last for about 2 weeks, but the virus can be detected in feces for additional 3-50 days pi and seroconversion occurs 2-3 weeks pi (Lee *et al.*, 2009; Halbur *et al.*, 2001; Williams *et al.*, 2001). Wild boars are also frequently infected with HEV and are consider as an additional HEV reservoir, see study IV, (Widén *et al.*, 2010). Like domestic swine, infection of wild boar also appear to have an asymptomatic outcome (Schlosser *et al.*, 2014).

The only HEV type to cause more severe symptoms in animals is avian HEV associated with hepatitis-spenomegaly syndrome (HSS) and big liver spleen disease (BLS) (Billam et al., 2005). The disease in chickens is characterized by enlarged liver and spleen with histological changes in form of hepatic necrosis and haemorrhages leading to increased mortality among egg laying chickens and broilers, and 20% reduction of egg production (Sun et al., 2004). This may cause substantial economic loss. Except for avian HEV, no other serious hepatitis E related disease in animals have been reported. The clinical outcome of HEV infection in moose is discussed in study II.

1.4 Epidemiology

1.4.1 General epidemiology

WHO estimates that there are globally around 20 million people infected annually with HEV resulting in approximately 56,600 deaths, however the numbers are most likely to be much higher. HEV have from the past to present haunted the human population with large outbreaks. About 70 outbreaks from the year 1955 have been documented (Perez-Gracia et al., 2014), and the largest and most recent outbreaks can be found in Table 1.

The HEV infection pattern can roughly can be divided into three geographically degrees of HEV endemicities: hyperendemic, endemic and not endemic/lack of data, (Figure 7). The geographical distribution of HEV genotypes is complex and constantly changing (Figure 7). Gt1-2 only infects humans and causes both infections and large waterborne outbreaks, mostly occurring in developing countries located in tropical and subtropical areas, assigned as hyperendemic HEV regions (Ruggeri et al., 2013). Large HEV outbreaks have also occurred in the past and gt1-2 are the most likely genotypes behind these events, Table 1.

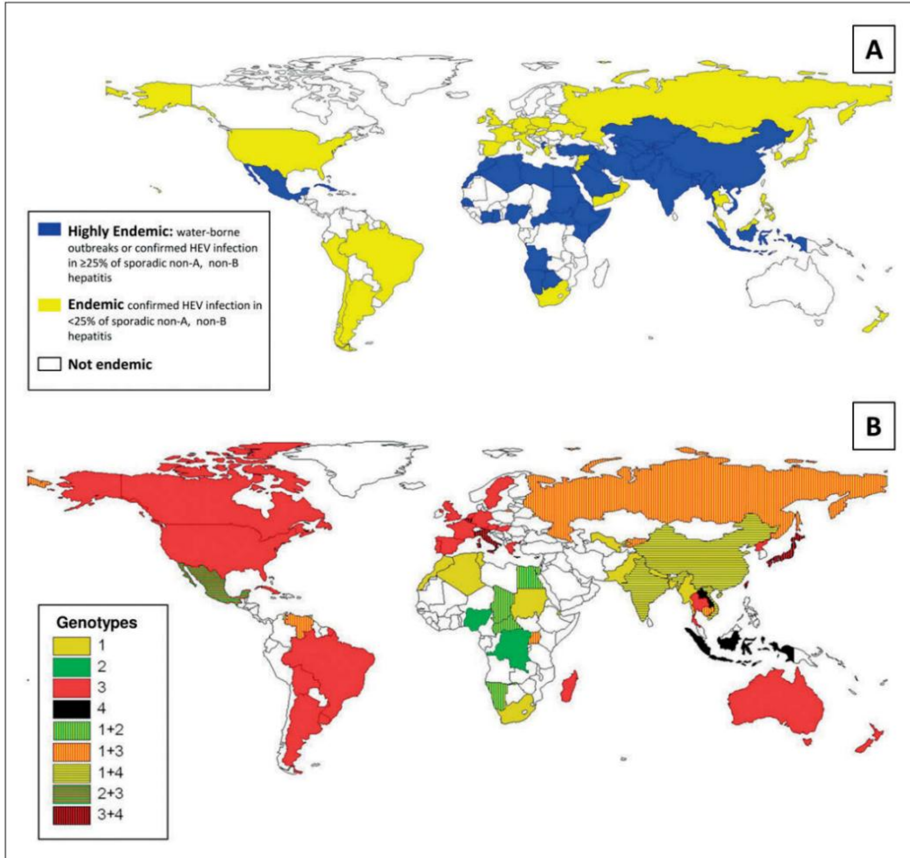

Figure 7. The worldwide HEV infection distribution illustrating A) the locations of the three endemic grade of the infection in different colours. B) from a genotype perspective. The colours for each country represent the most frequent HEV genotypes from human and animals (frequently from swine). The image was adapted from (Ruggeri *et al.*, 2013) with permission from Professor Fabio Ostanello.

Gt3-4 not only infects human but also a wide range of animals that therefore could possible act as virus reservoirs for human infection. These genotypes are frequently behind autochthonous sporadic HEV cases in developed countries in America, Europe, Oceania and Asia (Ruggeri *et al.*, 2013). Gt4 is common to Asia, but now appears to be spreading in Europe (Midgley *et al.*, 2014; Jeblaoui *et al.*, 2013; Colson *et al.*, 2012).

Table 1. List of some of the largest and most recent outbreaks of hepatitis E and number of cases in each outbreak.

CONTINENT	YEAR	COUNTRY	CASES	REFERENCE
Africa				
	1988–1989	Somalia	11,413	(Bile *et al.*, 1994)
	2007–2008	Uganda	>10,535	(Teshale *et al.*, 2010a)
	2012	Kenya	223	(UNHCR, 2012a)
	2013	Sudan	3991	(UNHCR, 2012b)
	2014-15	Ethiopia	1117	(Browne *et al.*, 2015)
Asia				
	1955	India	29300	(Arankalle *et al.*, 1994)
	1973–1974	Nepal	10,000	(Khuroo, 1991)
	1976–1977	Myanmar/Burma	20,000	(Khuroo, 1991)
	1978–1979	India	20,000	(Khuroo, 1991)
	1979–1980	India	6000	(Khuroo, 1991)
	1981–1982	Nepal	4337	(Khuroo, 1991)
	1981–1982	India	15000	(Khuroo, 1991)
	1985	Turkmenistan	16,175	Albetkova et al., 2007
	1986-1988	China	120,000	(Zhuang *et al.*, 1991)
	1987	Nepal	7405	(Shrestha, 2006)
	1990	India	>3000	(Arankalle *et al.*, 1994)
	2004	Indonesia	49	(World Health, 2005)
	2012	India	>4000	(News, 2012)

It should be noted that the HEV prevalence varies between and within countries and may reflect the population studied, the time when the study was performed and the sensitivity of the assay used for the study. The actual HEV seroprevalence reported from many studies may differ.

1.4.2 Epidemiology of Human HEV infection

Hyperendemic regions- with high disease prevalence

In the hyperendemic regions, the disease is caused by gt1-2, usually associated with contaminated waterborne outbreaks transmitted via the classical fecal-oral route that may affect large a part of the population simultaneously, and are often reoccurring. In some regions the outbreaks are seasonal because of monsoon or flooding events e.g. Nepal (Shrestha, 2006). From a subtype perspective, subtype gt1a, gt1b and gt1c are prevalent in Asia, while gt1d and gt1e are localised in Africa (Lu *et al.*, 2006). Large gt1-2 outbreaks are signified with mortality rate up to 25% in the third trimester of pregnant women (Kamar *et al.*, 2012a). An age depended HEV seroprevalence in developing countries shows that most children under the age of 10 years have a low seroprevalence opposite to hepatitis A, where children over 10 years frequently have antibodies against this virus (Emerson & Purcell, 2003; Arankalle *et al.*, 1995). The seroprevalence increases dramatically (up to 40%) between the ages of 15-30 years old (Kamar *et al.*, 2014; Emerson & Purcell, 2003). Unlike several other infections with fecal-oral transmission, person-to-person transmission of HEV is considered uncommon (Aggarwal & Jameel, 2011). Sporadic cases caused by gt1-2 observed in travellers/guest worker returning from hyperendemic regions are well documented in the literature (Norder *et al.*, 2009).

Regions with low HEV disease endemicity

Numerous studies have demonstrated autochthonous hepatitis E detected in patients who had never travelled to foreign countries in Europe, North America, New Zeeland and Japan (Drobeniuc *et al.*, 2013; Dalton *et al.*, 2008b; Dalton *et al.*, 2007a; Dalton *et al.*, 2007b; Mansuy *et al.*, 2004; Mizuo *et al.*, 2002) and study IV. This has changed the view regarding HEV as a disease limited to developing countries or to travellers returning from such areas. Several studies have reported high HEV seroprevalence (5-53%) (Kamar *et al.*, 2012a; Mansuy *et al.*, 2011; Guo *et al.*, 2010; Olsen *et al.*, 2006). HEV RNA has been identified in one out of 4,500 German and one out of almost 8,000 Swedish healthy blood donors (Baylis *et al.*, 2012). It seems that wide spread HEV infections are occurring silently as subclinical infections, while clinical disease associated with HEV only constitute the top of an iceberg (Kamar *et al.*, 2012a; Emerson & Purcell, 2003). There can also be a wide geographic variation in seroprevalence and incidence within a country e.g. the seroprevalence is about four times higher in southern France compared to northern France (Boutrouille *et al.*, 2007). Similar north-south pattern was also

seen in the UK, with seroprevalence of 16% in southwest England while only 4.6% in Edinburgh and 12% in the rest of England (Cleland *et al.*, 2013; Beale *et al.*, 2011; Dalton *et al.*, 2008b). The cause for this seroprevalence variation is unclear and may reflect the use of different assays or different regional habits. In contrast with developing countries, autochthonous hepatitis E is associated with the zoonotic gt3-4 and the transmission route for many HEV cases is unknown. The mortality rate seen in gt1-2 is higher than with gt3-4 for pregnant women, although there have been documented cases with acute hepatitis caused by gt3 (Anty *et al.*, 2012). Another dissimilarity is that elderly males (mean age of ~60 years; male/female ratio, >3:1) are overrepresented among clinical hepatitis E cases (Drobeniuc *et al.*, 2013; Dalton *et al.*, 2008b; Dalton *et al.*, 2007a; Mansuy *et al.*, 2004). The reason for this finding remain largely unsolved, but professional and/or life style and gender-related physiological factors may contribute. So what causes the general high seroprevalence in the population? The high prevalence indicate that other sources of infection than food may play a role.

1.4.3 Epidemiology of animal HEV infection

As stated by WHO, about 75% of the new diseases that has affected humans over the past ten years have been caused by pathogens originating from animals or from products of animal origin (WHO, 2014). The HIV, SARS and now MERS, all with animal origin are good examples of threats to the public health. From a one health perspective, the continuous screening and characterization of viruses with epidemic potential and detection of new viruses should therefore be prioritized and early detection may contribute to stop new outbreaks in an early stage. The discovery of HEV in animals have significantly broaden our concept of the host range and diversity of HEV. The detection of HEV antibodies in several animal species (Thiry *et al.*, 2015; Pavio *et al.*, 2010) indicate that new HEV like viruses still remain to be discovered. The zoonotic potential of many of the recently discovered novel animal HEVs still remains unknown and their prevalence is under investigation.

Domestic swine, wild boar and rabbit

The gt3-4 have been found to be highly prevalent in swine and wild boar worldwide. Factors affecting the prevalence rates may be geographical region, sample type, living pattern, age of the animals and the use of different assay brands.

HEV subtypes gt3a and 3b dominates in the US and Japan and is clearly distinguished from 3f, 3c and 3e which mainly circulates in Europe in both

humans and swine (Izopet et al., 2012; Luciano et al., 2012; Bouquet et al., 2011; Renou et al., 2011; Widén et al., 2010; Legrand-Abravanel et al., 2009; Norder et al., 2009; Lu et al., 2006). However, a molecular tracing study based on numerous full-length of gt3 from acute hepatitis E patients, swine and wild boar suggested that subtype gt3e was introduced from Europe to Japan through importation of swine in the 1960s (Nakano et al., 2013). The same study also proposed that a movement of subtype 3e from swine to wild boar had occurred in Japan. Close geographically specific genetic relatedness among gt3 and gt4 HEV strains from humans and swine have been observed in Europe and Japan (Forgach et al., 2010; Widén et al., 2010; Fogeda et al., 2009; Norder et al., 2009; Reuter et al., 2009; Dalton et al., 2007b; Yazaki et al., 2003; Schlauder et al., 1998). An interesting study from Sweden on HEV strains recovered from piglets in twelve farms, wild boars from nine counties and infected humans showed that piglets in Swedish farms were infected with strains distinct for each farm (Widén et al., 2010). Most HEV strains from Swedish swine and wild boars belong to subtype 3f, which is the most common subtype circulating in France, the Netherlands and Spain (Kaba et al., 2009; Legrand-Abravanel et al., 2009; Peralta et al., 2009; Rutjes et al., 2009; van der Poel et al., 2001); All Swedish HEV strains seemed to form clades in the phylogenetically tree according to geographical origin, country- and even county-specific and allowed for the identification of the geographical origin of HEV strains (Widén et al., 2010).

Beside swine, wild boar also act as HEV reservoir. The subspecies *Sus scrofa* is prevalent in Europe, while the two phylogenetically different subspecies Sus scrofa leucomyxtas and Sus scrofa riukiuanus are prevalent in Japan (Watanobe et al., 1999). The HEV prevalence in wild boar have been evaluated in several European countries and Japan, with seroprevalence ranging from 8 to 43% and HEV RNA prevalence between 2 and 68%, see study IV, (Widén et al., 2010), Germany (Schielke et al., 2009), France (Carpentier et al., 2012), Spain (Boadella et al., 2012; de Deus et al., 2008b), the Netherlands and Italy (Martelli et al., 2008), but also in Japan (Li et al., 2005a; Masuda et al., 2005; Nishizawa et al., 2005; Sonoda et al., 2004; Takahashi et al., 2004a; Tamada et al., 2004; Matsuda et al., 2003) and Australia (Chandler et al., 1999). In contrast to HEV infection usually occurring in 2-4 months old piglets with short asymptomatic self-limiting course (Widén et al., 2010; Meng et al., 1997), HEV positive wild boars were shown to be infected in several age classes from 4 months up to 2 years old or even older, study IV, (de Deus et al., 2008b; Nishizawa et al., 2005; Sonoda et al., 2004). The difference in HEV prevalence profile in relation to age may be that wild boar live in smaller family groups in larger territories and not as

swine with a large population confined to a limited area facilitating HEV transmission. Additional explanation indicates that the HEV infection in the animals may become chronic or they may be re-infected, if they have not developed protecting immunity, maybe due to co-infection with other agents. Lack of anti-HEV responses and prolonged HEV persistence were detected in some wild boar experimental infected with HEV (Schlosser et al., 2014). Most of the conducted HEV prevalence studies have observed that infected wild boars appeared to be healthy, as has been shown for infected domestic swine (Schlosser et al., 2014; Meng et al., 1998a; Meng et al., 1997). The high HEV prevalence and subclinical nature of HEV in the animals are worrying because of the possible risk of transmission either through direct contact with wild boar or by consumption of undercooked meat or organs. Evidence for such zoonotic pathway have been reported on several occasions, especially from Japan (Li et al., 2005a; Masuda et al., 2005; Takahashi et al., 2004a; Tamada et al., 2004; Matsuda et al., 2003). Recent studies from Japan also found unrecognized novel HEV genotypes in wild boars, suggested as gt5 and gt6 (Sato et al., 2011; Takahashi et al., 2011). Their zoonotic ability still remains unclear and further studies are needed.

Moongoose, rabbit and Deer

Molecular and serological evidence of HEV infection in the family of *Cervidae* (deer) has been reported for several deer species like Sika deer (*Cervus nippon*), Red deer (*Cervus elaphus*) and Roe deer (*Capreolus capreolus*) (Di Bartolo *et al.*, 2015; Choi *et al.*, 2013; Boadella *et al.*, 2010; Forgach *et al.*, 2010; Rutjes *et al.*, 2010; Reuter *et al.*, 2009; Tomiyama *et al.*, 2009; Matsuura *et al.*, 2007; Takahashi *et al.*, 2004a). The HEV seroprevalence can be estimated to 2-30% and HEV RNA prevalence around 30%. Both gt3 and gt4 have been reported, however the HEV strain detected in moose is not a gt1-4 HEV variant and it is further investigated in studies I-II & IV. The high HEV prevalence in the *Cervidae* family suggest an additional important HEV reservoir linked to consumption of deer meat or dear related products, and transmissions to humans have also been documented (Choi *et al.*, 2013; Takahashi *et al.*, 2004a; Tei *et al.*, 2003).

The prevalence of HEV infected moongoose in Japan is estimated to 8-21% and a full genome of this strain demonstrated that it belong to gt3, related to a swine HEV strain (Li *et al.*, 2006; Nakamura *et al.*, 2006). Cross-species transmission to other animals or humans has not been documented, but it is highly suspected that mongoose HEV is zoonotic due to its genetic similarity to other gt3 strains.

The HEV strains found in rabbit are considered distantly related to gt3 (Smith *et al.*, 2014) and appear to be prevalent in farmed rabbits with seroprevalence of 7-57% and HEV RNA is found in 7-16% (Cossaboom *et al.*, 2011; Geng *et al.*, 2011; Zhao *et al.*, 2009). A study from France has shown sequences closely related to rabbit HEV in humans, which suggests rabbits as a possible viral reservoir for human infections as well (Izopet *et al.*, 2012).

Fox, Rats, Ferret, mink, fish and bats

Rats and bats are known to carry several pathogens, which can be transmitted to humans, but although HEV was detected in these animals, they exhibited a highly divergent genome sequence compared to gt1-4. Antibodies to HEV have been detected with seroprevalence up to 80% among wild rats, like black rat (*Rattus rattus*), cotton rat (*Sigmodon hispidus*) and Norwegian rat (*Rattus norwegicus*), (Hirano *et al.*, 2003; Arankalle *et al.*, 2001; Favorov *et al.*, 2000; Kabrane-Lazizi *et al.*, 1999a). However, none of the studies recovered the viral genome and it was not until 2010 that the cause of seropositivity was detected using nested broad spectrum RT-PCR. A highly divergent HEV with 50-60% similarity to avian and human HEV strains respectively, was recovered from fecal samples of wild Norwegian rats from Germany and later a full genome was achieved (Johne *et al.*, 2010a; Johne *et al.*, 2010b). Subsequently, several rat HEV strains have been isolated from wild rats from other regions of Germany, but also from France, USA, China, Indonesia, Denmark and Vietnam (Mulyanto *et al.*, 2014; Widen *et al.*, 2014; Li *et al.*, 2013a; Li *et al.*, 2013b; Wolf *et al.*, 2013; Johne *et al.*, 2012). This divergent Rat HEV is suggested to be classified in the species *Orthohepevirus* C (Smith *et al.*, 2014). HEV sequences related to Rat HEV was detected in Asian musk shrews which also shared the environment with wild rats in China (Guan *et al.*, 2013). A recent study used several rat liver tissues and detected a gt3 like HEV (Lack *et al.*, 2012). The same year, HEV was also detected in feces in 9.3% in pet ferrets that appeared to be clinically healthy. The genome was related to rat HEV (Raj *et al.*, 2012). Recently in 2013, partial genome sequence of HEV variant genetically related to ferret was found in four farmed mink from Denmark (Krog *et al.*, 2013). The same year, additional genomic HEV related fragments related to the *Orthohepevirus* C species were found in feces of two foxes, but this finding may have been due to consumption of rodents (Bodewes *et al.*, 2013).

A virus infecting and causing disease in trout was identified in 1988 and named cutthroat trout virus, CTV (Hedrick et al., 1991). About twenty years later, the whole genome was sequenced and its genomic organization was found similar to HEV (Batts et al., 2011). Due to the high sequence diversity

compared to other current HEVs it was suggested as a sole member in the genus Pischihepevirus in the Hepeviridae family (Smith et al., 2014).

A bat HEV study was performed in 2012 on 3,869 bat stool and serum samples from 85 bat species across five continents. HEV was detected in three bat families (Hipposideridae, Vespertilionidae and Phyllostimidae) (Drexler et al., 2012). The same study also screened for bat HEV in 90,000 pooled human sera from blood donors/patient sera from Cameroon and Germany with negative result indicating that there is still no evidence for bat HEV transmission to humans.

Other recently identified HEVs

The HEV seroprevalence in sheep from eight Chinese counties was 29% (Wu *et al.*, 2010). A subsequent study for the identification of HEV like virus in sheep revealed a HEV seroprevalence of 35% and partial sequencing of ORF2 revealed a HEV RNA prevalence of 5.3%. The sequencing of the strains identified a gt4 variant (Wu *et al.*, 2015). The HEV seroprevalence of 58% in butchers was also studied and was suggested that sheep may be a source for HEV transmissions in humans. Several studies have demonstrated HEV prevalence in cattle (6.3%), (Zhang *et al.*, 2008). However, only one study from China has genetic information of the infecting strains, of which a 189nt ORF2 fragment was sequenced from eight cow feces samples, and shown to belong to the gt4 group (Hu & Ma, 2010). Additional studies have to confirm the presence of viruses in these animals. A recent study recovered and sequenced full genome HEV sequences closely related to gt4 from yak in China (Xu *et al.*, 2014). A HEV-related virus was recovered in droppings from three dromedary camels in Dubai. These isolates were more than 20% divergent from other HEV types on the nucleotide level (Woo *et al.*, 2014). They are most similar to viruses in the genus *Orthohepivirus* A, and assigned as gt7 (Smith *et al.*, 2014). Sequencing partial HEV sequences revealed a HEV like virus in chimpanzee from a zoo in China (Zhou *et al.*, 2014). Another novel HEV related virus was identified through partial sequencing using high throughput sequencing (HTS) on sewage samples from Nepal. This virus was shown to be highly divergent that it was suggested to be classified in a new genus (Ng *et al.*, 2012). But several of these studies need to be confirmed by others and full genome is required for appropriate HEV classification.

Other potential animal HEV reservoirs

HEV antibodies have been detected in both cats and dogs (McElroy *et al.*, 2015; Liang *et al.*, 2014), but still no HEV RNA has been isolated. Despite the suggested presence of anti-HEV, it has been difficult to detect and sequence

HEV-related virus RNA from these species. The serological results may be due to false positivity in the assays. This may also be due to low virus concentration in the sample or to high genome sequence divergence requiring an updated PCR assay, and which is discussed in studies I-II.

1.5 Diagnosis and detection of HEV

1.5.1 Detection of HEV RNA - a marker for active HEV infection

The diagnosis of ongoing HEV infection can be obtained either by anti-HEV IgM detection or by direct detection of HEV. The latter can be done through molecular techniques that detect viral nucleic acid (HEV RNA) or through electron microscopy (EM) for finding of viral particles which provided the first evidence for the existence of HEV (Bradley *et al.*, 1987). EM is unsuitable for clinical routine diagnostics because of the requirement of expensive equipment, low sensitivity, maintenance and skilled personnel. The preferred detection of HEV is therefore detection of viral RNA in feces and/or serum and if possible from liver samples. The viral RNA is present in the blood and/or feces for some weeks. The commonly used methods for detecting viral RNA is gel based- or quantitative real-time based- reverse transcription-polymerase chain reaction (RT-PCR)

Conventional gel based RT-PCR

For avoiding confusion with gel based RT-PCR, the term quantitative real-time RT-PCR is just abbreviated as qPCR throughout the thesis. The conventional gel based RT-PCR allows determination of genotype through sequencing, but there is increased risk for contamination due to several separate sample handling steps. The RT-PCR are used in this thesis for HEV typing by sequencing and amplification of larger HEV genomic fragments for sequencing near complete HEV genomes (Studies I-IV).

Quantitative real-time RT-PCR (qPCR)

The qPCR approach is now widely used, because it enables the detection and quantification of the viral nucleic acid, with high sensitivity and specificity for a reasonable cost, and is well suited for routine work. The qPCR requires no electrophoresis thereby avoiding contamination of the laboratory. However, the electrophoresis approach can sometimes not be avoided and is especially useful for confirming unclear positive qPCR results and for strain typing through sequencing.

This thesis used two Taqman based qPCR HEV protocols, which are targeting the ORF1 terminal end or the ORF2/ORF3 overlapping region of gt1-

4 (Gyarmati *et al.*, 2007; Jothikumar *et al.*, 2006). Both Taqman protocols have proven to be robust when compared with other qPCR protocols (Vasickova *et al.*, 2012) and are therefore used for HEV screening followed with HEV typing by RT-PCR. The Taqman chemistry consist of a primer set that direct the sequence targeting and a dual labelled probe with a fluorophore and a quencher at each terminal primer end. The probe binding to target sequence, followed by activity of DNA polymerase (with 3' exonuclease activity) will release the fluorophore and quencher through the elongation step of the PCR cycle (Kubista *et al.*, 2006). The recording of light signals released by the fluorophore are interpreted with a machine and its software plots the fluorescence against the number of cycle on a logarithmic scale. The number of cycles where the fluorescence surpass a certain threshold level, which is set above background, and is designated the threshold cycle (C_t). This can be done automatically by the software or manually. In theory, the amount of amplicons doubles in every cycle leading to an exponential increase of fluorescence. However, in reality the efficiency of the amplification also depends on primers, templates and presence of potential inhibitors. This can be evaluated in parallel during the original experiment with serial dilution of DNA template as a positive control sample in separate tubes. A standard curve of the change in the C_t with each dilution can be plotted and its slope of linear regression is then used for determining the qPCR efficiency.

1.5.2 Serological detection of anti-HEV markers for past/recent HEV infection

Past infections leaves traces in form of antibodies, at least for some time. The antibody IgM is suggested as first indicator of recent or ongoing infection, and IgA can also be detected during acute HEV infection (Chau *et al.*, 1993). However, not all strains/genotypes induce IgA antibodies and therefore more data are needed to clarify the diagnostic significance of these antibodies. The serological appearance of IgM and IgA is soon succeeded by IgG production which remain and may be detectable for up to 14 years (Emerson & Purcell, 2003; Bryan *et al.*, 1994; Khuroo *et al.*, 1993; Dawson *et al.*, 1992). The detection of anti-HEV antibodies using Enzyme-linked immune absorbent assay (ELISA) is a useful and very frequently used standard method for estimating the HEV prevalence. HEV specific antibodies remain detectable over more extended period of time compared to viral RNA. This enables diagnosis over a longer detection window. Despite the existence of gt1-4 and that gt3-4 infections in both humans and animals, only one serotype has been described until now. Thus, the same antigen of any gt1-4 can be used for detecting previous gt1-4 infections (Emerson & Purcell, 2003). All three HEV ORFs have been shown to have antigenic properties (Khudyakov Yu *et al.*,

1994; Purdy *et al.*, 1992). ORF2 has been shown to be more immunogenic, probably because this structural protein contain a variety of antigenic domains exposed to the immune system (Tsarev *et al.*, 1993). This antigen is used in most serological assays.

The detection of anti-HEV in humans or animals usually requires specific designed assays for each host. However, an approach that allows for the host-independent detection is the double-antigen sandwich ELISA, which is useful for epidemiological investigations and this ELISA is used in study II and study IV. The antigen (ag) for e.g. HEV attached to solid area is used to capture specific antibodies from serum. The detection of the antibody is attained with the same antigen labelled with e.g. horseradish peroxidase (HRP) which is added and bound to the second variable domain of the bound antibody. When unbound labelled antigen has been removed, chromogen with hydrolysing agents are added and hydrolysed by the bound Ag-HRP of the antigen-antibody-antigen sandwich complex, resulting in colour change (Figure 8).

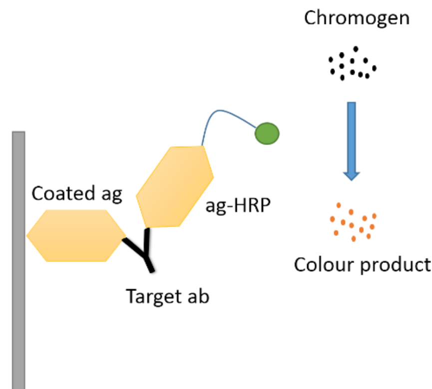

Figure 8. The general principle of the host independent the double-antigen sandwich ELISA. Abbreviation: ag: antigen, ab: antibody, HRP: Horse radish peroxidase. Pre-coated ag capture target ab from serum and detection of the ab is done with the same ag coupled with HRP which in present with chromogen and hydrolysing agents will result in colour change that can be detected.

The amount of the colour intensity can be measured and is proportional to the amount of antibody captured in the sample and from the included positive control with standardized antigen. A useful feature of this assay is the lack of discrimination between classes of antibody, thus detecting total anti-HEV. An assay based on this was developed for detection of anti-HEV in humans and animal samples (Hu *et al.*, 2008). It should be noted that there are currently no guidelines from WHO and no established standards when designing an assay for detecting anti-HEV antibodies. A wide selection of assays are commercially available as well as in house assays. The absence of guidelines

of HEV assays requires cautious interpretation and comparisons of seroprevalence data, because of different sensitivity and specificity for each assay used in the laboratories. As an example, a recent study demonstrated high variation from three commercial assays when analysing sera from 200 apparently healthy healthcare workers and found 4.5%, 18% and 29.5% of the samples positive for anti-HEV IgG (Wenzel *et al.*, 2013). Thus, continuously improved serological assays leading to a standardization are needed for providing more reliable and comparative serological data. For now, samples that are positive in anti-HEV assay may need supplemental test detecting HEV RNA to better estimate HEV prevalence.

1.5.3 Cell culture and other animal model systems

In general, the use of cell cultures for rapid detection of viruses for diagnostic purposes is limited as newer and faster methods of detection (quantitative real time PCR) has replaced cell cultures, however cell culture is very sensitive and is still the preferred methods for test of infectivity of a given sample, but also inoculation into susceptible laboratory animals can be used for such studies (Leland & Ginocchio, 2007). However, the absence of an efficient cell culture and small animal model for HEV have hampered the research on HEV. Additionally, the infectivity of HEV samples positive in qPCR e.g. food has not been easily demonstrated. Common approaches to the determination of the infectivity in swine or small animals have been used as infection models. However, this is not an optimal detection method for several reasons because of costs for labour and maintaining the animals as well as for ethical reasons.

Cell culture models

Replication of HEV in cell cultures is very challenging and difficult. This is reflected by the fact that only a small number research groups have access to cell culturing system that support growth of adapted strains of HEV. A cell line that is permissive to all strains is still not available. Some factors that are suspected for contributing to the troublesome HEV cell culturing are:

a) the HEV positive samples normally have low viral loads. Higher virus titers increase the possibility of infecting cells and replication.

b) additional unidentified receptors or other host factors required for optimal HEV replication may be missing in the 2D cell culture environment, which is available within the 3D-environment of the infected host.

c) the HEV disintegrates when stored despite sample storage at low temperature, therefore if the cell culture is not performed soon after the collection of the sample, replication is less probable to occur (Huang *et al.*, 1999).

It is only in recent years that researchers have tried to culture different HEV strains *in vitro* on cell lines of human and animal origin (Okamoto, 2013). The first successful isolation of a human HEV strain was done on the human lung cancer cell line A549 (adenocarcinom human alveolar basal epithelial cells) (Huang *et al.*, 1999). The first efficient HEV cell culturing system was the hepatocarcinoma cell line PLC/PRF/5 using a fecal samples containing gt3 with 10^7 virus copies/ml from a Japanese patient with chronic renal failure (Tanaka *et al.*, 2007). The same study also demonstrated the potential of the A549 cell line and observed that viral appearance and titer in the cell culture was largely dependent the initial original titer of the tested virus inoculum. Both PLC/PRF/5 and A549 cell lines have since then been used in several studies demonstrating repeated passages of replication of gt1, gt3-4 of both human and animal origin including strains from deer, wild boar and swine (Takahashi *et al.*, 2012; Takahashi *et al.*, 2010; Tanaka *et al.*, 2009).

Instead of culturing a monolayer of PLC/PRF/5, a 3D cell culturing system with this cell line supported by microspheres also demonstrated successful HEV replication (Berto *et al.*, 2013b). This culturing system would possible give a more natural tissue-like dispersal of receptors on the cells, thus mimicking the *in vivo* environment. HEV replication was demonstrated on this 3D culture system with samples from contaminated French pork liver sausages (Berto *et al.*, 2013a). A recent study has further demonstrated HEV replication in the human liver cell line HepaRG and the porcine embryonic PICM-19, but the titers of the progeny virions were lower compared to PLC/PRF/5 and A549 (Rogee *et al.*, 2013). However, there are some issues that have to be taken into consideration when using the latter cell lines: The PLC/PRF/5 cell line has the hepatitis B virus genes incorporated in the cell genome which can unexpectedly affect the HEV infection; the A549 is not a hepatic derived cell line and therefore may not represent the replication in the liver (Rogee *et al.*, 2013).

A special gt3 strain named Kernow-C3, isolated from a HIV patient with persistent HEV infection (Dalton *et al.*, 2009) was shown to infect 10 cell lines from different species, as human, chicken, pig and deer (Shukla *et al.*, 2011). This shows the potential cross-species infection capacity of HEV. In addition to PLC/PRF/5 and A549, the human cell line HepG2/C3A was shown to support the most efficient propagation of HEV compared to other cell lines. The detection of virions associated with lipids and ORF3 from cell culture supernatant and serum have been reported (Shukla *et al.*, 2011; Takahashi *et al.*, 2010) (Shukla *et al.*, 2011), but their role and occurrence in natural infection have to be further studied.

The rabbit HEV, a distant gt3 related HEV, has been shown to replicate efficiently in both A549 and PRF/PLC/5 cell lines, suggesting a potential zoonotic risk of rabbit HEV (Jirintai *et al.*, 2012). Another recent study showed interesting results when liver homogenates containing rat HEV, which was inoculated into human hepatocarcinoma cell lines, PLC/PRF/5, Huh7 and HepG2 cells, but no replication occurred in the A549 cell line. The rat HEV replicated in human PLC/PRF/5, HuH-7 and HepG2 cell lines, and the cells released progeny virion particles with lipid-associated membranes (Jirintai *et al.*, 2014). This HEV infection across species barriers demonstrate that the zoonotic properties of the novel animal HEVs found in e.g. rat, moose (Study I, II and IV), ferret etc. cannot be ignored and should be further investigated. The continuous improvements of HEV cell culture systems is anticipated to contribute to the diagnosis of HEV infections and may offer ways of evaluating antiviral treatments.

Animal model systems

None-human primates (Chimpanzee and macaques) have been used as experimental models for all gt1-4. Other animal e.g. pigs, rabbit and rat could to certain extent be used for infection studies for gt-3-4 (Purcell & Emerson, 2001). However, animal models have limitations in reproducing clinical aspects of human hepatitis, with minimal elevation in serum levels of liver enzymes and moderate present pathological liver lesions (Meng *et al.*, 1997). Although HEV is believed to be fecal-oral transmitted in humans, most experimental infections with none-human primates have used intravenous route of HEV inoculation because a much higher dose is required for infection through the oral route (Arankalle *et al.*, 1994).

The swine model systems have been used to give a better understanding on the relationship of HEV genes, replication and cross-species/zoonotic abilities. The model supported replication of the first infectious clone of gt3 and helped in the identification of the authentic initiation start codon site for HEV ORF3 (Huang *et al.*, 2007; Huang *et al.*, 2005). Moreover, studies of gt3 mutants in the HVR of ORF1 have shown that this variable region was non-essential for the infection and may play a role for the virus attenuation (Pudupakam *et al.*, 2009). The main drawback of this model is that it does not reproduce a hepatic disease with obvious clinical signs which makes it less suitable for pathogenicity studies.

Other potential animals as HEV model system are chicken, rats and rabbits. As model for HEV infection in humans, avian HEV appears to be limited in host range, however they offer a unique hepatic disease model (HSS/BLS) that can be used to study some aspects of human hepatitis E disease. As with HEV

infection in swine, experimental infection in rabbits has a subclinical course (Ma *et al.*, 2010). However, two recent studies may indicate new ways for using rabbit to better understand HEV pathogenesis in humans. The first study used specific pathogen free (SPF) rabbits, which led to the development of chronic HEV infection (>6 months) and associated liver fibrosis with a rabbit HEV strain and extrahepatic HEV replication e.g. in brain was also observed (Han *et al.*, 2014). Interestingly, the chronic disease pathology was not seen when rabbits were infected with gt4. The pathogenesis of HEV in SPF rabbits is unclear, but may contribute to a better understanding of chronic HEV infection seen in e.g. immunosuppressed patients. The second study used rabbit as model system to increase our understanding of HEV pathogenesis during pregnancy. This study provided experimental result of adverse effects and poor outcome of HEV infection during pregnancy, including high maternal mortality, infertility and miscarriage. Vertical transmission associated with HEV replication in the placenta was also suggested (Xia *et al.*, 2015).

Thus, the expanding host range of HEV offers possibilities to identify potential new animal HEV strains that could lead to better development of animal model/s for HEV. Genetic identification and characterization of novel animal HEV strains are therefore desired together with an efficient *in vitro* cell culture. The possibility to propagate multiple strains of HEV will significantly contribute to improved cost-effective treatments and vaccine against HEV.

1.6 Routes of transmission

Several transmission pathways have been reported: mainly through contaminated water and food, but vertical transmission and person-to person transmission can also occur (Kamar *et al.*, 2012a) Possible transmission routes are illustrated in Figure 9.

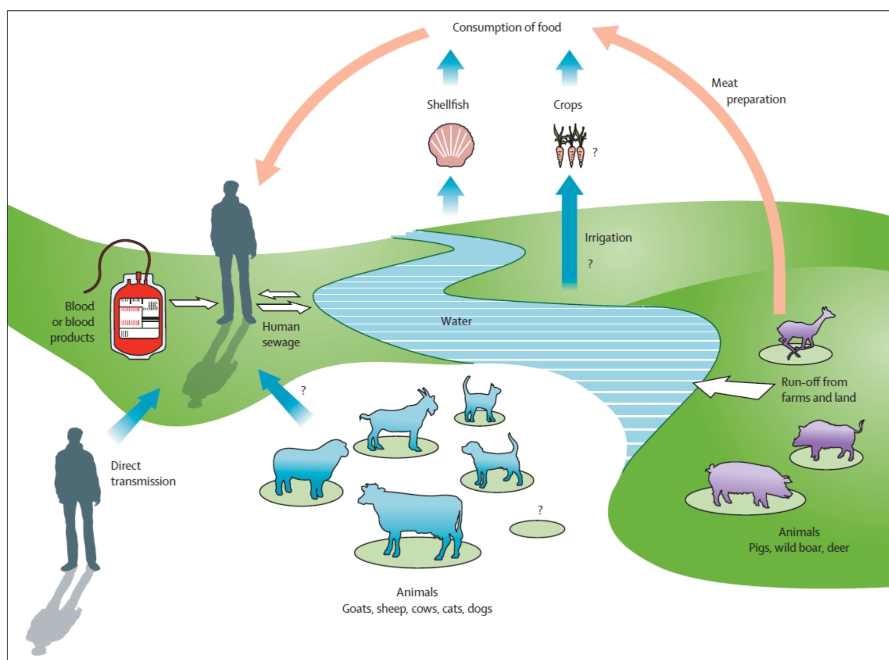

Figure 9. Possible and confirmed HEV transmission routes from reservoirs to humans. This is reproduced (Kamar *et al.*, 2012a) with permission by Elsavier.

1.6.1 Waterborne

The water borne HEV epidemics are caused by gt1 and gt2 and most likely by fecal contamination of drinking water supplies (Kamar *et al.*, 2012a). Several HEV epidemics are known to have occurred during in recent times and the risk for epidemics increases with natural disasters e.g. flooding, earth quakes and overcrowding in refugee camps (Table 1). The outbreaks are characterized by high morbidity and mortality among pregnant women and young children (Teshale *et al.*, 2010b).

1.6.2 Foodborne zoonosis

The identification of the source of HEV infections caused by gt3-4 infections are not always straightforward because of the long incubation period from the moment of infection to the appearance of clinical symptoms. During this period, the food has usually been thrown away. However, several studies demonstrate that HEV may be present in the food chain (Berto *et al.*, 2012; Di Bartolo *et al.*, 2011; Leblanc *et al.*, 2010). Many studies have confirmed that HEV may be prevalent in meat/livers bought from the grocery store in different countries e.g. Netherlands and Southern Germany with a HEV RNA prevalence of 4% with 0.8% in India and 2% in Japan respectively (Wenzel *et*

al., 2011; Kulkarni & Arankalle, 2008; Bouwknegt *et al.*, 2007; Yazaki *et al.*, 2003). In addition, there are studies that have documented zoonotic incidents associated with consumption of HEV infected food, where the retrieved HEV strain from the patients was identical or near identical with the strain recovered from the consumed food. Some examples of zoonotic transmission are reported from Europe and Japan. In southern France three out of five family members who consumed Figatelli, a liver sausage that is traditionally eaten raw, got hepatitis E (Colson et al., 2010). Virus isolated from the Figatelli has also been shown to be infectious in 3D culture systems (Berto *et al.*, 2013a). A Japanese study collected a series of HEV infection among people who consumed uncooked dear meat and testing of both left over deer meat and patient, resulted in identical HEV sequences. Family members who consumed none or little of the meat remained uninfected (Tei *et al.*, 2003). Another study from Japan reported two men, who independently from each other were admitted to hospital with severe hepatitis. The investigation showed later that the men were acquaintance and had consumed uncooked wild boar liver (Matsuda *et al.*, 2003). Consequently, consumers of pork products are exposed for potential HEV infections.

In addition to consumption of infected pork products, the use of human or animal waste contaminated water for irrigation, may pose a risk for HEV transmission. The crop irrigation may also further distribute HEV contaminated water; into rivers, coastal waters and shellfish, which may cause disease in consumers (Crossan *et al.*, 2012; Kamar *et al.*, 2012a). However, data on possible association of HEV with vegetables and fruits are very limited. HEV RNA have been found on strawberries with high partial sequence similarity with another HEV strain detected on a swine farm in Quebec, but its infectious capacity has not been evaluated (Brassard *et al.*, 2012). An outbreak involving passengers in a cruise ship returning from a journey in 2008 resulted in jaundice in four patients and out of 789 tested persons, 25% were seropositive, consisting of 4% IgM and 21% IgG seroconversion indicating recent/past HEV infections (Said *et al.*, 2009). The genotype detected in the patients was gt3 similar to strains circulating in Europe suggesting a shared source of infection and consumption of seafood was considered a risk factor.

1.6.3 Other routes

HEV RNA and antibodies against HEV have been detected in healthy individuals and blood donors (Cleland *et al.*, 2013; Baylis *et al.*, 2012). HEV transmission by blood transfusion have been documented. (Matsubayashi *et al.*, 2008; Colson *et al.*, 2007; Boxall *et al.*, 2006). It is estimated that the majority (75%) of donated blood was given to immunosuppressed patients in the UK

(Bihl & Negro, 2009) and chronic HEV infection have been found in this patient group. Studies have shown an increase in the risk for extensive liver damage if chronic infection is left untreated, which could result in the need for liver transplantation (see chapter 1.3.1 regarding Chronic HEV infection). At the moment there is no obligatory screening of HEV markers in blood/organ donors. This may be changed in the future.

Vertical transmission from mother to infant have been reported (Khuroo & Kamili, 2009). Several professions have also been associated with higher HEV infection risks, e.g. veterinarians, slaughter house staff, forestry workers etc., (Yugo & Meng, 2013; Carpentier *et al.*, 2012; Dremsek *et al.*, 2012). Markers of HEV have also been detected in pets like dogs and cats, and rabbit (Caruso *et al.*, 2015; McElroy *et al.*, 2015; Liang *et al.*, 2014), suggesting pets as a potential source for human HEV infections. Observation of a human HEV strain that is genetic closely related to rabbit HEV strains and its ability to replicate in human cell lines and cause cross species infections in pigs reinforce the potential of HEV transmission from rabbits to humans (Cossaboom *et al.*, 2012; Izopet *et al.*, 2012; Jirintai *et al.*, 2012).

1.7 Adaptation and evolution of HEV

1.7.1 General overview

The genetic differences between HEV genotypes 1-2 and 3-4 may depend on different transmission pattern reflecting the host specificity. Several novel HEV types not related to gt1-7 have recently been discovered, including the moose HEV from studies I-II & IV. However, their cross-species abilities and their contribution to the general HEV evolution still remain unclear.

Gt1 has previously been the dominating HEV type in China, however, there are signs that there is a decrease of Gt1 infections and an increase of gt4 instead. The cause for this is unclear, but may reflect a changing lifestyle and improved water sanitation, which may favour HEV strains with zoonotic properties (Liu *et al.*, 2012). Another perspective is that HEV may have taken advantage of the predator and prey relationship, which may be reflected by the hepatitis variants found in ferret, mink and fox, which have closest genetic relationship with rat HEV (Thiry *et al.*, 2015). These hypothesis are some of the challenges for future HEV research to confirm.

1.7.2 Genomic variability of genotypes 1-4 and quasispecies

Although differences exist between the genotypes 1 to 4, the genomic variability still remains low, especially on the amino acid level. When 75 HEV isolates of gt1-4 with full/near complete genomes were compared, the inter-

genotype difference was only 6.5-11.7% amino acid difference of ORF2. This high degree of conservation correlates to the reduced antigenic diversity and to the single serotype of HEV (Okamoto, 2007). Despite this limitation, a significant sequence diversity on the nucleotide level has been observed from different regions of the world, not to mention the high sequence diversity observed in recent novel animal HEVs. One of the factors for creating such diversity may be high error rate of the viral RdRp with absence of proofreading properties. The mutation frequency of a wide different RNA viruses ranges from 10^{-4} to 10^{-5} substitutions per base per round of copying (Domingo, 1996). The HEV mutation rate was estimated to $1.40–1.72 \times 10^{-3}$ base substitution per site per year based on closely related isolates from Japan sampled 7.5 year apart (Takahashi *et al.*, 2004b). The high error rate of viral RdRp contributes to an increased diversity of the replication virus strains within the infected host. In fact, this results in a mixture of closely related variants termed quasispecies for each virus strain (Grandadam *et al.*, 2004). The quasispecies effect may contribute for the evasion from the immune system, and only some strains in the swarm of viruses, may have the ability to efficiently infect host cells. This could explain why high viral load is needed in cell culture and animal models, because only a fraction of the swarm is infectious. This may also contribute to the observed extrahepatic replication sites in swine and wild boar (Schlosser *et al.*, 2014; Choi & Chae, 2003; Williams *et al.*, 2001). For humans, this may possible manifest as neurological, hematologic and autoimmune syndromes as has been observed during hepatitis E virus infection (Aggarwal, 2011).

1.7.3 HEV recombinants emerging from uptake of additional nucleic acid fragments

Several reports have shown that HEV recombination can occur by exchanging part of the genome or by additional fragments into the PPR, which is part of the HVR. The recombination could be the result of co-infection of different strains/genotypes (Smith *et al.*, 2013b; Moal *et al.*, 2012; Takahashi *et al.*, 2002). The uptake of fragments have been detected in gt3 isolated from chronic infected patients. The fragments could originate from the same infecting strain or from different stain due to co-infection of the host with several strains (Johne *et al.*, 2014b; Lhomme *et al.*, 2014a; Nguyen *et al.*, 2011). Examples of acquirement of genetic material from the host have also been described. Segments of human genes were identified in the PPR of HEV RNA taken from a cell culture system (partial S17 ribosomal genes), (Shukla *et al.*, 2012; Shukla *et al.*, 2011) and from a chronic infected patient (partial S19 ribosomal gene), (Nguyen *et al.*, 2011). The mechanism behind the insertion is still unclear. The fragment insertion into the PPR is suggested to have a host

adaptation effect, but it may also have a virulence property. Similar inserts have been observed in strains isolated from swine and patients indicating previously possible zoonotic events (Study III). The usefulness of the PPR as phylogenetic marker for tracing and comparing strains have been shown, which is demonstrated in study III.

1.7.4 HEV origin hypothesis

Did HEV appear first in animals or in humans? The answer to this question is still unknown. If the ancestors of gt1-4 is hypothesised to have been transmitted from humans to animals, then animals would act as viral reservoirs for maintenance of infection and reinfection HEV of mammals including humans. However, many of the animal species have an older evolutionary history than the human species, this could be interpreted that HEV have an animal origin instead. The roots of sequences from gt1-4 and rat HEV were estimated and suggested to have animal origin (Purdy & Khudyakov, 2010). Hosts of *Orthohepervirus* C have been detected in *Carnivora*, *Rodentia* and *Soricomorpha*, while members of *Orthohepervirus* A have recovered from *Artiodatyla*, *Carnivora*, *Lagomorpha* and primates. Such diversification of hosts indicate absence of co-evolution with its hosts, and suggest that HEV is an opportunistic pathogen (Smith *et al.*, 2014). The future discovery of other HEV related viruses may not give the whole picture of the HEV origin, but at least progress the understanding of *Hepeviridae* evolution.

1.8 HEV Prevention and control

1.8.1 Virus survival and inactivation

Information regarding the survival of HEV under various conditions in food and the environment as well as the effect of elimination procedures e.g. heating are important to better estimate the risk associated with the pathogen. However, in the case of HEV, there is currently no validated cell culture system. To study HEV inactivation, the majority of current studies rely on qPCR. Others have used *in vivo* testing to determine infectivity after the inactivation treatment, but these methods are not standardized and relative expensive (Cook & van der Poel, 2015). The few studies performed indicate that HEV remain infectious at temperatures used for cooking. The results showed that HEV could survive refrigeration (4°C), freezing (-20°C), (Cook & van der Poel, 2015), and heating to 71°C for at least 5 minutes and that liver tissue may have a protective effect on the virus (Barnaud *et al.*, 2012).

There are a signifant gaps in our knowledge regarding the survival of HEV in food and in the environment as well as the effects of elimination procedures

used in the food chain. The necessary trials are dependent on the development of sensitive, cost effective and reproducible cell culture system, which will allow identification of HEV infectivity to be readily performed. This would further improve our understanding of HEV transmissions routes, develop control and prevention strategies of HEV contamination and transmission.

Here are some points, partially adapted from Cock and Van der Poel (2015), that could contribute to the progress knowledge of survival and inactivation of HEV:

- A robust cell culture for HEV is needed and else the recommended studies below cannot be easily done.
- Additional studies are required to determine the heat effects on HEV, in free suspension and in food products, especially in swine meat.
- The survival on surfaces should be determined, especially in in the food production facilities. The data generated should give a better knowledge and better understanding of the risk for cross-contamination in the food chain.
- The effects of disinfectants on HEV needs to be fully investigated. This is useful for selecting the most appropriate disinfectant for cleaning swine pens to reduce or eliminate risk for HEV transmission to swine and farmers.
- Freeze-thawing may have a negative effect on HEV and this should be investigated as well.
- Experimental inactivation studies comparing HEV with other microorganisms such as hepatitis A virus (HAV) and the highly resistant bacteriophages as PhiX174 should be performed to evaluate inactivation in absence of an efficient cell culture system.

1.8.2 HEV therapeutics and vaccine

The key strategy for preventing HEV infection (gt1-2) in developing countries is improving the sanitary infrastructure and vaccination with the newly HEV vaccine. However, vaccination would probably not be possible due to the cost.

Therapeutics

At the current stage, there is no established diagnostics and therapeutics for treatment of HEV. Antiviral treatment for patients with acute hepatitis E is not considered, only for patients developing fulminant hepatitis E (Izopet *et al.*, 2015). The identification of chronic HEV infections in immunosuppressed patients has led to different procedures to treat their infections, because if the infection is left untreated it may led to progressive liver damage (Fujiwara et

al., 2014). The first step is to reduce the immunosuppressive therapy, leading to clearance of one third of the patients (Kamar *et al.*, 2011b). If the approach remains unsuccessful, then interferon and ribavirin treatment can be used (Izopet *et al.*, 2015). Ribavirin is now the reference treatment for HEV infections (Izopet *et al.*, 2015), but the mechanism on how it inhibits HEV replication is still unclear and requires investigation. More research of treating serious clinical outcome with HEV infection in pregnant women is also needed.

Vaccine

Vaccines can be efficient medical means to prevent viral infections. There are currently two vaccines based on the HEV gt1 and developed with recombinant technology, the rHEV (expressed in baculovirus) and the HEV239 vaccine expressed in *E. coli* that successfully passed phase II/III trials. The rHEV vaccine was developed GlaxoSmithKline and consists of a 56kDa recombinant protein from ORF2. The vaccine was 95.5% effective with three doses, when evaluating the safety and immunogenicity in a phase II trial on Nepalese Army volunteer in the Kathmandu Valley (Shrestha *et al.*, 2007). Despite the success and potential of this vaccine, it is still not known if it will be marketed. The vaccine HEV239 is based on 26kDa protein translated in *E. coli* from ORF2 which produces a 23nm VLP. It has gone through phase III trial and was conducted on 112,604 Chinese persons of which about 48,000 persons were administrated tree doses of the vaccine. The negative control group of similar size was administrated hepatitis B virus vaccine. The vaccine was 94-100% effective in preventing symptomatic cases of hepatitis E (Zhu *et al.*, 2010). During the trials, pregnant women were also vaccinated and had no adverse effects of the vaccination (Wu *et al.*, 2012). Unfortunately, vaccination would not be possible for the people who needs it, especially in the most poorest part of the world due to the economic reasons (Zhang et al., 2013).

2 Aims of the thesis

The aims of this thesis were to provide understanding of the biology of HEV, its prevalence in different animal species, in the environment and the molecular epidemiology of hepatitis E virus (HEV). This will be useful to increase our understanding on how transmission across the species barrier occur, and if virulence can be predicted. The aim was also to increase our understanding of important risk factors for transmission of HEV from animals to humans in Sweden and elsewhere.

The specific aims were:

- to identify and characterize the genome of a new virus in moose and proving its relationship to the *Hepeviridae* family (Study I-II & IV).

- to investigate the prevalence of HEV in moose in Sweden and its importance as HEV reservoir (Study II and IV).

- to investigate HEV markers in the wild life of Sweden for the identification of additional HEV reservoirs. (Study IV)

- to characterize and compare sequenced HEV strains from animals and humans regarding zoonotic transmissions by molecular and phylogenetic means. (Study I-IV)

- to use a single HEV amplicon approach to amplify porcine HEV genomes and investigate their properties from a potential recombination, zoonotic and virulence perspective (Study III).

3 Material and methods

This section summarizes the material and methods (M&M) used in the four studies of the thesis. Common basic sample storage and nucleic acid isolation approaches for all studies are summarized in section 3.1, followed by specific M&M for studies I-IV (3.2-3.5).

3.1 General Material and methods approaches

3.1.1 Sample types and storage

Samples from swine were mostly in form of droppings and collected from several Swedish farms. In collaboration with certified hunters, samples from wild boar and different members of the deer family e.g. moose, were collected. From some animals, liver, bile, and kidney were also obtained. Multiple samples from the same individual were sometimes received. All samples were stored in -70ºC for long time storage and -20ºC respectively 4ºC degrees for short time storage.

3.1.2 RNA isolation and cDNA synthesis

Total RNA isolation from organs were performed according to the manufacturers' instructions by homogenization in 2ml grinding tubes (Eppendorf) containing 2 mm zirconia beads (BioSpec Products) and 600µl buffer RLT from an RNeasy Mini kit (Qiagen). RNA in serum and feces was extracted with QIAamp Viral RNA mini kit (Qiagen, Germany) according to the manufacturers' instructions. Alternatively, nucleic acid extraction was done in the EasyMag instrument (Biomerieux, Marcy l´Etoile, France) according to the manufacturers' instructions. The concentration and quality of RNA was determined by NanoDrop (NanoDrop Technologies). The 20µl cDNA synthesis mix consisted of 1µl Oligo $dT_{(20)}$ (Invitrogen) or 1µl GeneRACER Oligo $dT_{(24)}$ (Invitrogen), used for priming cDNA synthesis with 3µl RNA, 1µl

(40 U) of RNaseOUT (Invitrogen) and 1µl (200 U) of Superscript III, RNase H^{-} reverse transcriptase (Invitrogen). One microliter (end concentration 5%) polymerase GC melt from an Advantage GC 2 polymerase mix kit (Clontech) was also added to facilitate amplification of high GC-content regions and reduce secondary structure formation in the HEV genome (Xia et al., 2008). The cDNA reaction was kept at 50°C for 60 min, followed by 15min incubation at 70°C. The reaction was finalized with 2U of E. coli RNase H (Invitrogen) for 20min at 37°C.

3.2 Past and active HEV detection assays, gel-based PCRs and Sanger sequencing

3.2.1 ELISA for detecting past HEV infections

For detection of total HEV-specific antibodies representing past HEV infections (studies II and IV), sera were tested by double antigen sandwich ELISA (HEV Ab EIA, Axiom Diagnostics, Worms, Germany), performed according to the manufacturer's instructions and the optical density (OD) was measured at 450nm. This assay was selected since it commonly used in European surveys, because it as good specificity and sensitivity and it is host animal independent for detection of total HEV antibodies

3.2.2 Quantitative PCR for detecting active HEV infections

Detection of HEV RNA representing active HEV infection was performed with a wide spectrum gt1-4 quantitative PCR (qPCR) assay (studies I, III and IV) with the following protocol: Three microliters of extracted and purified RNA were analysed using an Ag-Path-ID one-step RT-PCR kit (Applied Biosystems), with a total volume of 12.5µl containing 250nM JVHEV forward respective reverse primers and 100nM Cy5 based probe targeting the overlapping ORF2/3 region (Jothikumar *et al.*, 2006) and 0.4×enzyme mix, or with primers targeting a sequence downstream of the ORF2 region with 500nM forward and 250nM reverse primers, 250nM, 260nM FAM based probe (Gyarmati *et al.*, 2007) and 1×enzyme mix. Both methods have been shown to be more sensitive in comparison with other HEV detection methods (Vasickova *et al.*, 2012) and using both methods increase the detection of clinical HEV positive samples. Although the qPCR assays was able to detect a more divergent moose HEV variant, probably with cross reactions, a modified one step TaqMan qPCR assay was constructed. It also targeted the ORF2/3 overlapping region using the One step RT-PCR kit (Qiagen) (study II). The 12.5µl PCR-mix contained 3µl of purified RNA, 600 nM each of primers HEV F8, HEV R8, and FAM-based probe P8, 1X PCR buffer, and 1X enzyme mix.

A 50µl PCR mix was used in study IV due to nucleic acid extraction in the EasyMag instrument. All qPCR samples were analyzed on a Rotor-Gene 3000 instrument (Corbett Research, UK) with the following settings: 50°C for 30 min, 94°C for 15 min, cycled 55 times between 94°C 15s and 60°C 60s. Fluorescence was monitored during the annealing step of each cycle. The diluted plasmid containing the full HEV genome of gt3 SWX07-E1 (Xia *et al.*, 2008) or with a plasmid containing a cloned 2.1kb moose HEV fragment (Lin *et al.*, 2013) were used as a control and for the generation of standard curves.

3.2.3 Gel-based PCR, terminal amplification and Sanger sequencing

To acquire additional viral genomic sequence information, short fragment and long fragment PCR were performed:

a) a commonly used PCR targeting the RdRp (Zhai *et al.*, 2006) was set up (Study I, III and IV): a total PCR mix of 30 µl with 6µl of synthesized HEV positive cDNA template, 1.2U Platinum *Taq* polymerase (Invitrogen), 1×PCR RXN buffer, 1mM $MgCl_2$, 0.2µM of each ESP and EAP primer, 5% DMSO and 0.2mM dNTP. The cycling parameters were 95 °C 3 min, cycled 40 times 94 °C 1min, 55 °C 1min, 72°C 1min and finishing with 72°C for 10min.

b) the previous PCR protocol was modified for improved amplification of partial moose HEV RdRp (Study II): In the first PCR, the total reaction volume was 15µl containing 1X buffer, 0.2µM dNTP, 1mM $MgCl_2$, 0.6U of platinum *Taq* polymerase, 1µl cDNA/PCR template and three pooled modified forward primers (Pool 1: ISP-4232A, ISP-4232B and ISP-4232E) and two pooled modified reverse primers (Pool 2: EAP-4576E and EAP-4576F) with a total concentration of 0.2µM for each pool. The semi-nested PCR contained the same reagents as the first PCR except that the reverse primer was replaced with three modified pooled primers (Pool 3: IAP-4561E, IAP-4561F and IAP-4561M). In study IV, the semi-nested PCR was performed in 50µl reaction mix with 5µl cDNA, 31.9µl of RNase-free H2O (Sigma), 1xTaq buffer (Applied Biosystems), 2.25mM $MgCl_2$ (Applied Biosystems), 0.2mM dNTP (Roche), 0.3mM of each primer, and 1U of Taq polymerase (Roche). Primers ISP4232-Pool0-H and EAP4576- Pool0-H were used for the first round PCR. Five µl of the first round product were used as template in the second amplification round with primers ISP4232-Pool 0-H and IAP4561-Pool 0-G. PCR reactions started with 94°C for 3min, followed by cycling for 40 times between 94°C for 40s, 56°C for 30s and 72°C for 65s and finally 72°C for 10min. A double nested-PCR with the same primers as in the second PCR was used for two purified PCR products with low amplicon concentration.

c) with phusion PCR, longer amplicons could be amplified (studies I and III). The PCR mix contained 1µl cDNA as template with 0.15µl Phusion Hot

Start High-Fidelity DNA polymerase (Thermo Scientific, Finnzyme) with provided 1×GC buffer, 0.3µl 0.2mM dNTP, 0.5µM of forward primer, 0.5µM reverse primer and 0.45 µl DMSO (final 3%) were also added in a total PCR volume of 15µl. The PCR program had the following profile: 98°C for 2min, then cycled 40 times 98°C 20s, T_m: 65 or 70°C 30s, 72°C 2-3min depending of amplicon size, and terminated with 72°C for 10min. Sometimes a Semi-nested/nested PCR is needed for increasing the fragment concentration from the first PCR and specific downstream primers from both 5' and 3' were used instead. Depending of the aim of the PCR assay, different positive controls were used: Full genome of gt3 cloned within a vector (SWX07-E1), (Xia *et al.*, 2008). Clinical HEV positive from feces/liver samples e.g. gt3f strains Spanish SW8a24 from liver and SW46_8-Dalarna from swine feces were also used as positive HEV controls. Depending on the amplicon size, PCR products were verified in 0.8-2.0% agaros gel with GelRED (Biotium). Amplicons were purified with QIAquick PCR Purification Kit (Qiagen, Germany) or Wizard SV gel and PCR clean-up system (Promega) or PureLink Quick gel extraction kit (Invitrogen). The phusion PCR generated amplicons lacking the 3'terminal end overhangs required for TOPO XL cloning (Invitrogen). Therefore, poly(A) overhangs were synthesized before the cloning procedure in a 10µl reaction mix with final concentration of 0.2mM dATP, 1XPCR RXN buffer, 2.4mM $MgCl_2$, 0.5U platinium Taq polymerase (Invitrogen) and 8.22µl of purified PCR product. The reaction was incubated in 72°C for 15 min and put on ice and cloning procedure was performed according to the manufacturer's instructions.

An approach called rapid amplification of cDNA ends (RACE) opens the possibilities to amplify the HEV terminal ends. Only the 3'UTR terminal end was amplified using a RACE kit (Invitrogen), and resulted in a 1.3kb overlapping PCR product according to PCR program profile 98°C 2min, 98°C 10s, 65°C 30s, 72°C 2min and 72°C 10min. Sanger sequencing reactions were carried out with a Big Dye Terminator Cycle Sequencing Ready reaction kit version 3.1 (Applied Biosystems) with program profile 95°C 15s, 50°C 10s, 60°C 4min cycling 25 times. The sequencing primers for moose HEV are found in Studies I-II and for porcine gt3 HEV in study III and for other HEV found in wild animals (Study IV).

3.3 Sequence-, phylogenetic-, and statistical analysis

3.3.1 General sequence analysis tools

Different tools were used for sequencing analysis: Assembling and analysis of overlapping sequence regions into a consensus sequence was done with

Seqman within the DNAstar program package version 8 or 10. Detection of known and other possible open reading frame/s (ORF/s) were done in the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Verified sequenced consensus HEV sequences (Study I-IV) were deposited to the the NCBI Genbank database (http://www.ncbi.nlm.nih.gov/genbank/) with assigned unique Genbank numbers. Some studies were used as supportive guidance for identifying ORF1, ORF2 and ORF3 domain/motif regions in the moose HEV genome (Ahmad et al., 2011; Xing et al., 2010; Koonin et al., 1992), (Study I-III). HEV ORF1, ORF2 and ORF3 codon based multiple sequence alignment (MSA) of selected HEV regions/HEV genomes and phylogenetic analysis were performed with MEGA5 (Tamura et al., 2011) or CLC Genomic Workbench 7 (CGW7). Nucleotide (nt) and amino acid (aa) sequence identity comparisons were done in CGW7. Region specific or more unspecific primers with nucleotide ambiguities were designed from MSA of several HEV genomes/genomic regions. Primers were ordered from Thermo Scientific Webshop (http://www.thermohybaid.de/). The MSAs often act as the backbone for other post-analysis and should be constructed with high consideration e.g. through codons.

3.3.2 Phylogenetic relationship analysis of HEV

The phylogenetic tree analysis is a useful method to better understand the phylogenetic relationship between newly sequenced genomic materials (Study I-IV). In terms of virus perspective, it is possible to apply the phylogenetic analysis e.g. molecular typing and tracing, detecting possible recombinations, geographical clustering, evolution and classification, especially on unclassified novel HEV variants to shed more light on the increasingly complex *Hepeviridae* family. The phylogenetic trees were codon based MSAs of various regions of different sizes e.g. on regions commonly used for typing in RdRp (Zhai *et al.*, 2006) and ORF1 concatenated in frame with ORF2 representing the full HEV genome. Maximum likelihood (ML) or neighbour joining (NJ) or unweight pair-group method using arithmetic averages (UPGMA) based approaches with 1,000 bootstrap were used for generating the trees in MEGA5 or CGW7 or PHYLIP package version 3.65.

An alternative method for investigating genetic HEV relationship especially for new unclassified divergent HEVs was through calculating the amino acid (aa) p-distances (Smith *et al.*, 2013a). The p-distance is the proportion of amino acid sites at which the two sequences to be compared are different. It is obtained by dividing the number of amino acid differences by the total number of sites compared. The p-distance separating the HEV genotypes was

suggested with a p-distance value of at least 0.06 (Smith *et al.*, 2013a). A p-distance/aa sequence divergent percentage matrix was generated with MEGA5 using an in frame concatenated ORF-ORF2 MSA of different HEVs including the moose HEV (Study I).

3.3.3 Possible recombination and identification of the moose HEV ORF2-3 start codons

Genetic material exchange through recombination is a common phenomenon within all organisms and viruses, and act as a factor for driving evolution. However, the detection of such events are not straight forward, but tools like the bootscan within the Simplot 3.5.1 tool (http://sray.med.som.jhmi.edu/SCRoftware/simplot/) can be used for detecting possible recombination events (Study III). The bootscan employed a 200bp window, 20 step with a ML based F84 as distance model with 1,000 replicates.

The junction region (JR) with partial overlapping region covering the ORF1-end and the true ORF2 and ORF3 start codons were not easy to determine in the moose HEV. Therefore, a similar approach as in (Huang *et al.*, 2007) was performed to detect the possible "true ORF2 and ORF3 start codons". A MSA of the gt1-4 JR containing putative cis-reactive element and putative start codons for ORF2-3 were analysed. The supportive secondary structure analysis with Mfold (http://mfold.rna.albany.edu/?q=mfold) for the JR of gt3 and the moose HEV were performed for supporting the MSA JR analysis (Study I).

3.3.4 Statistical analysis

To estimate apparent prevalence confidence intervals (Cis), one sample Z-test for proportions was used. A χ^2-test for equality of two proportions was used in study II for analysis of significant difference ($p<0.05$) within age classes, sex and Swedish counties. The Fisher´s exact test was used in study IV. The statistical analysis were performed in R, version 3.0.2.

3.4 High throughput sequencing (HTS)

New and unknown viruses always pose a challenge to be detected, amplified and sequenced with traditional methods due to high sequence divergence in their genomes, or with low sample concentration. Therefore a high throughput sequencing (HTS) was performed for obtaining complete or near complete moose HEV genome with the Illumina MiSeq platform sequencing (Study II) as follows:

Synthesized dsDNA triplicates of the liver sample positive for moose HEV RNA (Study I) were diluted and prepared with Qubit dsDNA HS assay kit (Life technologies, USA) according to manufacturer's protocol and the concentration was measured with Qubit 2.0 Fluorometer (Life technologies, USA). A 1ng sample (0.2ng/µl) was index library tagged with index 5 and 7 (I5 and I7) primers and fragmented at the same time (tagmented) through a 5-cycle PCR amplification using the Illumina Nextera XT kit, according to Illumina MiSec protocol. The samples were loaded on a chip and analyzed on a Bioanalyzer (Agilent Genomics, Germany) for DNA concentration, size, and size distribution. The DNA samples were diluted, pooled and a total input of 1 ng DNA was loaded into a cartridge containing Technologies MiSeq v2 Reagent 300 cycle kit, according to MiSec protocol. The assembled contigs from reads generated through de novo assembly with default settings in the CLC Genomics Workbench 6.0 were BLAST-searched for HEV and all putative HEV contigs were subsequently assembled into several larger consensus sequences. All remaining non-related HEV consensus sequences were removed. The 5kb algSWE2013 (KF951328.1) sequence (Study I) was compared with the MiSeq assembled HEV sequence, and the putative 5'-UTR terminal start position was identified through a MSA of Gt1 (AY230202), Gt3 (EU360977) and Gt4 (HQ634346) genomes as templates. The Gt2 excluded, because the complete 5'-UTR sequence was absent. Identification of putative HEV domains was done according to (Koonin *et al.*, 1992) and the "NCBI ORF Finder" was used for exploring new possible ORFs (Study II).

4 Result and discussion

4.1 Detection, amplification, characterization and prevalence of hepatitis E virus found in Swedish moose

4.1.1 Unclear HEV status in moose and collection of wild life samples

Previous studies have shown that deer can be infected with HEV, cases with both gt3 and gt4 have been identified, and evidence for zoonotic transmission have also been documented (Choi *et al.*, 2013; Tamada *et al.*, 2004; Tei *et al.*, 2003). This is an emerging public health concern. The largest deer, the moose (*Alces alces*), which is regularly hunted for consumption in Scandinavia has not previously been investigated for HEV. This prompted us to screen markers of HEV in moose to see if this species also could act as a HEV reservoir for potential human transmissions. This could possible increase our understanding on why the HEV seroprevalence is unusual high (~9%) in the general population in Sweden (Olsen *et al.*, 2006). This is not reflected in the reported clinical cases, because only up to 22 hepatitis E cases are reported yearly in Sweden, indicating that most HEV infections are asymptomatic, or not diagnosed. The proportion of zoonotic HEV transmission compared to the total numbers of HEV infections is still unknown. To get samples from wild animals is not a simple task, although the National Veterinary Institute (SVA) receive such samples on a regular basis, for screening for other pathogens. However the sample numbers were still not enough for serious studies. Therefore the Swedish hunters were asked to help with sending in samples of wild boar, moose and deer to both SVA and the Sahlgrenska Hospital.

4.1.2 New HEV like virus found in a moose

In Study I, two gt1-4 qPCR assays targeting the overlapping region of ORF2-3 (Jothikumar *et al.*, 2006) and ORF2 (Gyarmati *et al.*, 2007), gave positive signal for HEV RNA in one out of six moose liver samples with C_t-values of

34 and 35 respectively. A kidney sample from the same individual was also found HEV positive with similar C_t-values. A partial HEV RdRp fragment frequently used for typing (Zhai *et al.*, 2006) was obtained and sequenced, which showed surprisingly divergent HEV like sequence. With assistance of three overlapping PCR assays, a 5.1 kb partial moose HEV genome starting from the proline hinge region (PPR) of ORF1 covering both ORF2 and 3 to the 3' terminal end with the poly-A tail was acquired and sequenced. Additional upstream PCR fragment amplifications towards the 5' end of the genome were unsuccessful, probably due to extensive sequence diversity. The sequence identity was 37-63% compared with existing HEVs and phylogenetic analysis showed that moose HEV formed its own separate branch between gt1-4 including gt5-6. It separated from the other divergent animal HEVs like those found e.g. in rat, ferret, bat and fish. The ORF2 and ORF3 start codons were difficult to identify in the moose HEV genome. A secondary structure analysis with Mfold for the junction region (Huang *et al.*, 2007) was therefore applied, which suggested that the ORF2 and ORF3 start codons were located in the stem forming the putative second loop structure of the junction region, when compared with gt3.

These observation and the existence of three ORFs characteristic for HEV, clearly supported the classification within the *Hepeviridae* family. However, further subsequent classification was unclear due to absence of classification support in the current gt1-4 HEV grouping. At this time no new consensus HEV has been presented in the literature. Therefore, an alternative method for investigating genetic relationships with highly divergent HEVs was demonstrated with help of aa p-distances (Smith et al., 2013a). This approach was tested on the moose HEV and suggested it as a new species in the *Hepevirus* genus. Several nucleotide substitutions, some resulting in unique amino acid substitutions were detected in the 3'UTR, several motifs were also observed across the sequenced partial ORF1, ORF2 and ORF3. These mutations and the different secondary structures in the junction region may contribute to host specificity and possible virulence factors. An update of the current PCR assays was also required for optimal detection and amplification of moose HEV, due to its high divergence from other HEV types.

4.1.3 HEV prevalence in Swedish moose

To rule out that this novel HEV identified in the moose was not a single event, the prevalence of this virus was investigated in moose from an age group, gender and geographical (Swedish province) perspective (Study II). The HEV RNA prevalence was determined by an updated Taqman based qPCR (marker for active infection) with increased sensitivity from Ct=34-35, (Gyarmati *et al.*,

2007; Jothikumar *et al.*, 2006) to Ct=25 when tested on the same moose HEV positive sample from study I. This high titre of HEV RNA explains why the HEV positive moose sample in study I could be amplified with less optimal primers. No standard HEV serology method exists, especially not for moose HEV. Therefore, a host independent double sandwich ELISA assay to identify markers of past infection was used. The assays were tested on sera and 51 fecal samples from 231 Swedish moose shared with another study for investigating *Anaplasma phagocytophilum* infection in moose (Malmsten *et al.*, 2013). Like with wild boar that is commonly infected with HEV (Widén *et al.*, 2010; de Deus *et al.*, 2008b), moose was also found frequently infected as indicated with markers of active and past infection found in 67 (29%) animals. While 34 (15%) were positive for HEV RNA, 43 (19%) were seropositive for anti-HEV antibodies, and 10 (4%) had both markers. The detection of HEV RNA in serum and/or feces or both in some moose most likely mirrors stages in the infection cycle. As with swine (Yugo & Meng, 2013), the fecal-oral route is suggested as the main HEV transmission route between moose, because HEV RNA was detected in feces of some animals, but other routes cannot be overlocked.

The only significant difference in prevalence of infection was found in the HEV seroprevalence of the 2-4.5 year old age group compared to 0-1.5 year age group ($p<0.05$), indicating that HEV immunity increase with age. A trend was detected with the largest proportion of active HEV infection presenting in the 0-1.5 year old age group, which was also found with the tick-borne pathogen *Anaplasma* (Malmsten et al., 2013). The decline of passive immunity may explain why this age group is infected with the pathogens for the first time. *Anaplasma* is known to have immunosuppressive properties and frequently infect moose (Malmsten et al., 2013; Rikihisa, 2011), which may make the moose more prone for HEV infections and prolong the infection time period. This has been observed in swine experimentally co-infected with porcine reproductive and respiratory syndrome virus and in immunosuppressed humans (Salines et al., 2015; Aggarwal, 2011). A hypothesis is that ticks may act as a vector for potential HEV transmissions, but more studies are required for confirming that statement.

Near complete moose HEV genome sequencing

A near complete moose HEV genome was high throughput sequenced (HTS). The moose HEV genome appears to be a middle sized HEV genome of around 7kb with 35-60% nucleotide sequence identity to other HEVs. Three main ORFs characteristic of HEV were also identified, but also additional putative ORFs were detected, distinguished from the potential new ORFs found in 5’-

terminal end in rat and ferret (Raj *et al.*, 2012; Johne *et al.*, 2010a). However, the existence of these potentially new HEV ORF's have to be confirmed in future work.

Phylogenetic and relationship classification

The partial RdRp sequence of the thirteen moose HEV sequences and in frame ORF1 concatenated with ORF2 representing complete moose HEV genome, demonstrated a separate monophyletic clade formation with a common ancestor to gt1-6. Closer inspection of the 13 sequenced partial HEV RdRp sequences showed a high similarity to each other resulting in lack of:

a) apparent distinct geographical clustering, contrary to what was observed with HEV strains isolated from rat and Swedish swine/wild boars in previous studies (Johne et al., 2012; Widén et al., 2010; Norder et al., 2009).

b) extensive polyphyletic divergent strains, as previously observed for other animal HEVs like rat HEV and avian HEV (Mulyanto et al., 2014; Bányai et al., 2012; Bilic et al., 2009). These observations may be due to that the virus has recently been introduced into the moose population. The unsuccessful sequencing of all 34 qPCR HEV positive samples could be due to a degradation of the HEV genome during RNA extraction procedure and several freeze-thawing cycles of the RNA; or low virus concentration in the samples (the average C_t-value of 34.5); or lower sensitivity of the conventional PCR assay compared to qPCR. Unsuccessful sequencing of HEV RNA positive samples was described in other studies as well. It may reflect a higher sequence diversity than expected (Kukielka *et al.*, 2015) or a combination of all above mentioned factors.

A gt1-4 qPCR assay was also applied for screening the moose samples for the genotypes with known zoonotic properties, but with negative results. Thus, the question if moose is susceptible to gt3-4 infection or if other deer species are infected with moose HEV still remains unclear. The phylogenetic relationship of moose HEV to the other HEV types showed the highest sequence identity (~60%) with gt1-6, suggesting moose HEV being a new member of the recently proposed species *Orthohepevirus* A (Smith *et al.*, 2014). This classification can be confirmed with identification of new animal HEV like viruses. But, the close genetic similarity to strains with zoonotic properties makes the moose HEV significant and its potential zoonotic properties can therefore not be ignored, because moose is regularly hunted and consumed in whole Scandinavia and other parts of Europe.

4.2 Genomic characterization of potential recombination, zoonotic and virulence elements in porcine HEV genomes

4.2.1 Genomic characterization of the frequently found subtype 3f

For the moment only one complete genome of a Swedish swine HEV of subtype gt3f is available, SWX07-E01 (Xia *et al.*, 2008). Previous studies have shown that this subtype is frequently found in clinical human cases and in swine/wild boar in several European countries e.g. Sweden, France and Spain. The reason is unclear why specifically 3f is commonly spread, but may be related to improved adaptability and virulence properties. Typing of HEV is usually done by partial sequencing. Although sequencing of the complete HEV genome would give a broader and more correct phylogenetic relationship with other HEVs, it is not always feasible because it requires time consuming overlapping PCRs. In study III, a single amplicon PCR approach was applied using cDNA synthesised from RNA extracted from either sera or feces from swine and wild boar. This resulted in amplification of near complete porcine HEV genomes of ~7kb. All three ORFs characteristic for HEV were identified in all six near complete porcine hepatitis E virus genomes that were of subtype 3f. One of the strains, 8a24, was from a Spanish slaughterhouse, the others were of Swedish origin, two from wild boar and three from domestic swine in different Swedish pig farms. All Swedish strains were similar to each other and to the existing Swedish strain SWX07-E1 with 89-92% sequence identity throughout their genomes, and with lower genomic identity (86-87%) to the Spanish HEV strain 8a24. This Spanish HEV strain also had a larger genome (7.3 vs 7.2 kb) than the Swedish strains.

The increase of size of the Spanish HEV genome was identified in the PPR, which was shown to have a duplication, upstream PPR (uPPR) and downstream PPR (dPPR). Due to the nature of a duplication, it is difficult determine which of the uPPR or dPPR was the original fragment. The sequence similarity and the phylogenetic clade formation implied that a past co-infection of different HEV strains could have occurred, possible within a Spanish swine. Thus, the high similarity of the Spanish swine 8a24 strain with a French human HEV strains indicated that possible past zoonotic transmissions cannot be overlooked. The PPR region has a high genetic variability especially in the zoonotic gt3 and is suggested to have a role in HEV adaption to a new host/organ tropism, in modulating the host immune response and in virulence of the strain, possibly by opening new protein-protein interaction sites (Lhomme *et al.*, 2014a; Lhomme *et al.*, 2014b; Purdy *et al.*, 2012).

4.2.2 Phylogenetic relationship and potential HEV recombinations

The separate Swedish branch of HEV strains belonging to subgroup 3.II, within the subtype 3f shown by analysis of partial HEV sequences (Widén *et al.*, 2010), was verified the phylogenetic relationship of the near complete HEV genomes. The 8a24 strain was found forming a cluster with French/Spanish 3f strains from both human and swine. The geographical clustering based on partial genomic sequences isolated from swine, wild boars and to some part from patients (Widén *et al.*, 2010; Norder *et al.*, 2009) was not found when complete genomes were phylogenetically analyzed. This indicates that sequence divergence along the near complete genomes may have been caused by other factors than just introduction of mutations by the viral RdRp. Recombination was therefore analyzed by the bootstrap program of the porcine HEV strains, which suggested a potential exchange of genetic information. This may have occurred between Swedish swine and wild boar HEV strains, between swine from different herds in the same province, and between the Spanish pig strain 8a24 and French human strains. Possible inter/intra genotype recombinations have been described between swine and human HEV strains, especially for gt3-4 (Chen *et al.*, 2012; Wang *et al.*, 2010; Fan, 2009; van Cuyck *et al.*, 2005). Recombination events may be the result of either co-infection or superinfection of the same host with multiple strains as has been shown an immunocompetent hepatitis E virus infected individual and in a patient with acute hepatitis E (Smith *et al.*, 2013b; Takahashi *et al.*, 2002).

Today´s global swine trading may be one of several factors facilitating the geographical spread of HEV strains and their interaction with each other, which may lead to a dilution of the geographic clustering pattern in the complete HEV genomes observed here both from a local and international perspective. Suspected recombination events involving different host species need to be more studied, as they may have implications for the evolution of HEV, especially it's potential to emerge and adapt to new host, which may change/reflect its virulence.

4.2.3 Zoonotic and virulence elements

Specific markers of virulence in HEV is still not known. The immunological reactions are important for the outcome in acute/fulminant cases and high mortality rate in pregnant women (see chapter 1.3.1). Induction of more aggressive immune response may be related to some certain HEV strains with particular mutations across the genome (Bu *et al.*, 2013; Mishra *et al.*, 2013; Fu *et al.*, 2011; Inoue *et al.*, 2009; Takahashi *et al.*, 2009). The subtype 3f is frequently found in swine herds and humans in Europe (Widén *et al.*, 2010; Legrand-Abravanel *et al.*, 2009; Norder *et al.*, 2009) for unclear reason. One

possible explanation could be increased virulence properties. The presence of potential zoonotic and virulence elements throughout the amplified gt3 porcine HEV genomes were therefore investigated in the sequenced strains. Although all porcine 3f strains partially share mutations associated with virulence elements from patients with acute and fulminant hepatitis E (study II), they are still suggested as low virulence strains, with the SWX07-E1 strain being most virulent among the studied strains. If segment inserts in the PPR are marker for virulence, the order of virulence of the studies strains would probably change and put the SW8a24-Spain as the highest virulent strain. Even if most Swedish porcine HEV genotype 3f strains appear to have a low virulence profile, their significance for future public health should not be disregarded. The zoonotic ability of these strains has shown a remarkable dynamic adaptation for cross-species transmission and persistence in immune suppressed patients, perhaps due to fragment insertion/s in PPR, as exemplified with the SW8a24-Spain strain. The PPR and its possible insert may therefore be useful as a phylogenetic tracing-, virulent marker and perhaps also as a medical target for developing drugs binding to this region.

4.3 Wildlife as potential HEV reservoir

The HEV seroprevalence in several developed countries is high and most sources of infection need to be identified. Wildlife has long been suspected to be a source for viral infections. Studies have shown that several species of deer, including moose (studies I-II, IV) and wild boar can be infected with HEV (Widén *et al.*, 2010). In fact, zoonotic cases have been reported on individuals having consumed wild game meat of both deer and wild boar (Choi *et al.*, 2013; Tamada *et al.*, 2004; Tei *et al.*, 2003). Swedes often go into the forest to pick berries and mushrooms, and it is also customary to consume wild game. With these fact in hand, the study IV was therefore performed to investigate if the Swedish wild life may constitute one route of HEV transmission to humans.

4.3.1 Prevalence of HEV markers in wild life

HEV markers were found in about 53 (22%) of the 245 animals from a total of 466 samples consisting of serum and fecal samples. The HEV seroprevalence among the animals was estimated between 5-14%. No anti-HEV was detected from the fallow deer samples, most likely due to low numbers of samples. Although indication of regional difference and spread of specific HEV strains were observed previously (Widén *et al.*, 2010), it could not be detected in study IV. Regional differences in HEV prevalence, between 5% and 88%,

related to animal density, have been shown for wild boar in Poland, Germany and Italy (Larska et al., 2015; Martinelli et al., 2015; Montagnaro et al., 2015; Adlhoch et al., 2009; Martelli et al., 2008). The uneven sampling from both different species and provinces may have contributed to the absence of regional HEV differences in this study IV.

Wild boar

The overall HEV prevalence (marker of both active and past HEV infection) is estimated to 19% (study IV). No significant difference in age between the younger and older animals with anti-HEV, although a trend was towards higher anti-HEV prevalence in older wild boars than in younger animals 5% vs 13%, compared to the total anti-HEV prevalence of 8%. There was no significant difference between HEV RNA detection and age of the animals. However significant HEV RNA difference was found, since moose showed more often active HEV infection than wild boar. It is known that wild boar are frequently infected by other pathogens and some have immunosuppressing properties as experimental tested or observed in swine (Salines *et al.*, 2015; Schlosser *et al.*, 2014), which may result in partial HEV immunity. This could lead to multiple HEV infections during their lifetime, which is reflected in the absent of age differences of both active and past HEV infections. Although the HEV RNA prevalence apparently remain constant of 8-9% during almost 10 years when compared to wild boar samples collect during 2005-2007 (Widén *et al.*, 2010) to 2015 (study IV). However, study IV also investigated HEV RNA prevalence in wild boar fecal and serum samples, which adjusted the total HEV RNA prevalence to 13%. This clearly shows the underestimation of active HEV infection and the need for both fecal and serum samples, which has been suggested in study II. Nevertheless, the HEV prevalence and similar frequency of animals with active and past HEV markers in serum and feces have also been observed in other European countries as well (Martinelli *et al.*, 2015; Rutjes *et al.*, 2010; de Deus *et al.*, 2008b). It can therefore be concluded that this animal species is a possible natural reservoir for HEV in the environment in many countries.

Different deer species (moose, red deer, fallow deer and roe deer)

Additional near 30 moose of a total of 67 animals resulted in with HEV-seroprevalence of 14%, RNA prevalence of 27%, and total HEV prevalence of 37%. This confirmed our previous finding that HEV is as prevalent in moose as in wild boar (study II). There was no significant difference of HEV RNA and anti-HEV between ages of the animal, although a trend was detected towards increased anti-HEV with age. In study II this trend resulted in

significant difference, which may be due to larger sample size. HEV was excreted in feces slightly more often by younger than older animals, but was found in blood at equal frequency regardless of age of the animal. The prevalence of HEV markers was equal between moose, younger and older than one year. As with study II, the zoonotic gt3 could not be found in moose, and larger sample size may be required for its detection.

Although having only 39 samples from three other deer species excluding moose, HEV seroprevalence could be detected in 3 (7%) of the animals. However, no HEV seroprevalence was detected for the five fallow deer, most likely due to a small number of samples. A weak qPCR reactivity was detected from two red deer and one fallow deer. Difficulties with HEV detection in deer samples have been shown to be common in other studies in Europe (Larska et al., 2015; Serracca et al., 2015). On some occasions, gt3 strains have been identified in some of the qPCR HEV positive samples from red deer originated from Spain and Italy, and this genotype also infects swine and wild boar in these countries (Di Bartolo et al., 2015; Kukielka et al., 2015). Usually, most of the HEV qPCR positive samples cannot be sequenced, which could be due to low virus concentration or to a divergent HEV like strain, which probably require another assay for its detection and characterization. The moose HEV from studies I and II, and wild boar gt5-6 (Sato *et al.*, 2011; Takahashi *et al.*, 2011) are example divergent animal HEVs.
Nonetheless, the detection of HEV markers in the deer family indicated that these animals may be potential sources for HEV transmission, also in Sweden.

4.3.2 Wild life HEV transmission routes to humans

Both wild boar and deer share habitats and have been shown to transmit gt3 and gt4 through consumption of undercooked or raw food products taken from infected animals both in Japan and Korea, (Tamada *et al.*, 2004; Tei *et al.*, 2003). If European roe deer and/or wild boars also are infected with genotype 4 strains, it may explain the few sporadic genotype 4 human cases in Europeans without any travel history before their hepatitis E infection (Colson *et al.*, 2015; Bouamra *et al.*, 2014; Midgley *et al.*, 2014; Choi *et al.*, 2013).

Autochthonous Swedish human HEV cases

Out of 14 Swedish autochthonous HEV cases investigated in study IV, at least three persons were infected with HEV strains similar or identical to strains infecting wild boar. Since some of the infected persons are vegetarians or have not consumed meat before falling ill in hepatitis E, an alternative route than consumption of infected wild game or swine meat must exist. One pathway may be through irrigation of vegetables or berries with contaminated water as

has been shown for strawberries (Maunula *et al.*, 2013). Since most of the surveyed infected animals excreted HEV in feces (study IV) and a previous study reported that urine as well as feces may contain HEV RNA (Bouwknegt *et al.*, 2009). An alternative transmission may occur through contaminated berries and mushrooms.

The overall results suggest that certain Swedish wild animals are frequently infected with HEV and may be a significant source for human HEV transmissions, either by direct contact or through consumption of game meat, to which attention should be given by the future public health in Sweden.

5 Concluding remarks

- High prevalence of HEV markers (~22%) in Swedish wild life suggest wild boar and deer family as viral reservoirs and may result in human HEV transmission.

- An absence of HEV prevalence differences between young and old animals in wild life indicate that the animals may have partial HEV immunity. Possible caused by co-infection with other pathogens, and thereby be susceptible to re-infection with HEV.

- Human transmission routes of HEV may be through undercooked or raw meat/products from wild life and domestic swine. An alternative HEV pathway may be through consumption of berries and mushrooms, contaminated with urine or droppings from HEV infected wild animals.

- Surveying for markers of ongoing or past HEV infection (HEV RNA/anti-HEV antibody serology) in the human and animal population gives important information on circulating strains. Such analysis may indicate on animal-virus relationship which may relate to zoonotic transmissions and may be used for reducing/prevention of virus transmission. However, extensive collaboration over the different professional fields is required, resulting in one health perspective.

- Surveying markers of HEV in both human and animals may lead to the discovery of new divergent HEV like viruses, like the discovery of moose HEV. Screening for HEV in fecal and/or serum samples is feasible, and are easier accessible compared to obtain liver and bile

samples. If several sample types from the same individual could be obtained, the estimation of the HEV prevalence will be more accurate.

- The moose is just as commonly infected with HEV as Swedish wild boars. The moose HEV has closest similarity to members of the newly species of *Orthohepevirus* A, encompassing HEV gt1-7. This genetic relationship to the zoonotic gt3-4 indicates that moose HEV cannot be ignored as having zoonotic potential.

- High throughput sequencing opens the possibility to sequence samples containing highly divergent HEV variants, which would be very useful in combination with traditional approaches to identify and characterize new viruses.

- The cross-species properties of moose HEV to infect humans and other deer species are still unclear. It is also not known if moose can become infected with HEV gt3.

- Current results based on partial HEV moose genomes suggest that the moose HEV strains are all genetic closely related, without geographical clustering or other regional differences, indicating either that the virus has recently been introduced into moose, or that there are other constraints against diversification of this virus. There may also be limitations of the used assay for determining more divergent strains.

- Phylogenetic analysis based on partial genome sequence is still useful for epidemiological studies for comparing circulating strains in animals and humans and for tracing source of infection.

- The single HEV amplicon PCR approach opens the possibilities to amplify and clone near complete HEV genome for characterization of the genome from a phylogenetic perspective as well as for studying recombination events and possible evolution of quasispecies.

- Complete genome sequencing enabled detection of possible recombination events between Swedish swine/swine herds/wild boars, and that there may have been zoonotic transmission of HEV strains between Spain and France.

- Investigation of possible virulence markers in porcine HEV of this study suggested low virulence of the subtype 3f strains. But the very high adaptability of the gt3 strains can quickly lead to a more virulent strain in a short time, which was observed in immunocompromised patients.

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- The nucleic acid insertion in the HEV ORF1 PPR may have given the virus adaptation properties to the host and potential virulence. This PPR with and without insertion has a potential to be used for tracing the spread of a strain by phylogenetic analysis and for identification of virulence marker.

6 Future perspectives

- Highly robust, cost-efficient and simple cell culture accepting all HEV strains are desired to better understand the general lifecycle and cross-species properties of HEV. This is also needed for developing improved therapeutic HEV agents and vaccine.

- Screening for moose HEV with assay targeting this HEV variant should be performed with other deer species and on human samples for investigating the possible spread of moose HEV infections.

- More complete moose HEV genomes are needed to better understand the genomic variations of this HEV variant.

- More HEV screening of wild life samples is required to confirm if gt3 is present in Swedish deer or other wild life species, besides wild boar and domestic swine.

- Host range adaption of HEV from a predator-prey HEV relationship still needs to be investigated.

- The continuous identification and characterization of new HEV variants are needed to better understand the origin and evolution of HEV. This would also give more knowledge about how HEV would be classified in the future.

- Inactivation of HEV in food and food products is still poorly understood, and further work and surveillance are needed to prevent the spread of HEV through the food chain.

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