Molecular Studies on Hepatitis E viruses

Edmilson Ferreira de Oliveira Filho

INAUGURAL DISSERTATION
submitted to the Faculty of Veterinary Medicine
in partial fulfilment of the requirements
for the PhD-Degree
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by

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Declaration

“I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.”

Edmilson Ferreira de Oliveira Filho

Table of Contents

I List of abbreviations

aa	Amino acids
A549	Human alveolar epithelia lcell line
aHEV	Avian Hepatitis E virus
ALF	Acute liver failure
ALT	alanine aminotransferases
AST	aspartate aminotransferases
BSA	bovine serum albumin
°C	degree celcius
CD	cluster of differentiation
cDNA	complementary DNA
cm	centimetre
CTV	cutthroat trout virus
ddH_2O	deionized distilled water
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ET-NANBH	enterically transmitted non-A and non-B hepatitis
FCS	fetal calf serum
FHF	fulminant hepatic failure
g	gram
GT	Genotype
GTT	gamma-glutamyltransferases
h	hour(s)
HA	hemagglutinin
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HEPES	N-2-hydroxyethylpiperazine
HEV	Hepatitis E virus
ICTV	International Committee on Taxonomy of Viruses
IEM	immune electron microscopy
IFN	interferon
Ig	Immunoglobulin
M	molar
mAbs	monoclonal antibodies
mg	milligram
min	minute(s)
ml	milliliter
mM	millimolar
mRNA	messenger RNA
NCR	non-coding region
NK	natural killer cells
ng	nanogram
nt	nucleotide(s)

ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV2	Porcine Circovirus 2
P.I.	post infection
pmol	picomolar
PolyA	polyadenylic acid
qRT-PCR	quantitative RT-PCR
RdRp	RNA-dependent RNA-polymerase
RNA	ribonucleic acid
RPM	rotations per minute
RT-PCR	reverse transcriptase PCR
s	second(s)
SAP	serum alkaline phosphatase
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	tumor necrosis factor
Tris	tris-hydroxymethylaminomethane
Tween	20 polyoxyethylenesorbiten monolaurate
TTV	Teno Torque Virus
UV	ultraviolet
V	volt
VLP	virus-like particles
Vol	volume
µg	microgram
µl	microliter
µM	micromolar

1 Introduction

Hepatitis E is an emerging infectious disease distributed worldwide which affects humans. The causative agent Hepatitis E virus (HEV) also occurs in animals such as domestic swine and wild boar. HEV was first associated with acute hepatitis in humans on basis of clinical and epidemiological observations. The disease is self-limiting in the majority of the patients, however, high morbidity and mortality rates have been described in pregnant women. In contrast no clinical disease has been associated with HEV in animals.

The objectives of this work were:

- Detection of HEV in different animal populations;
- Study of the genetic variability of HEV;
- Expression of the capsid protein;
- Cultivation of HEV in cell lines and primary cells

2 Literature review

2.1 Taxonomy and phylogeny of *hepeviridae*

2.1.1 Taxonomy and phylogeny

Due to clinical and epidemiological characteristics Hepatitis E virus (HEV) was initially thought to belong to the same family as Hepatitis A virus (HAV), namely the *Picornaviridae* (Sreenivasan et al., 1984a). According to morphological features and similarities to Noroviruses with regard to genome organization (Bradley et al., 1988), HEV was then repositioned as a member of the *Caliciviridae* in a separate genus *Hepevirus*. Based on molecular analyses HEV was later placed as a single species of the family *Hepeviridae*, genus Hepevirus (Emerson et al., 2005; Meng et al., 2011). In the meantime three distinct avian hepatitis E viruses (avian HEV) were considered as genotypes within an unassigned species in the family *Hepeviridae* (Meng et al., 2011). The recently reported rat hepatitis E virus is a related virus which may represent a new genotype (Meng et al., 2011).

Another potential member of the family recently identified in cutthroat trout shows similarities to HEV in genome organization and size of 7269 nt. Phylogenetic analyses

suggested that the cutthroat trout virus (CTV) is also a new member of the family *Hepeviridae* (Batts et al., 2011).

According to the commonly accepted classification HEV found in mammals can be grouped into four major genotypes (1-4) with 24 proposed subtypes (Lu et al., 2006). This classification was confirmed by the ninth ICTV report which lists the four genotypes Burma (1), Mexico (2), Meng (3) and T1 (4) within the HEV species (Figure 1) (Meng et al., 2011).

The criterion adopted for definition of genotypes is a divergence of nucleotide sequences in the ORF 2 region of more than 20 %, similar to the criteria used for Noroviruses (Worm et al., 2002). Genotypes 1 and 2 were found only in humans. Genotypes 3 and 4 have been reported in humans and in different animal species and are connected to zoonotic cases (Panda et al., 2007; Pavio et al., 2010).

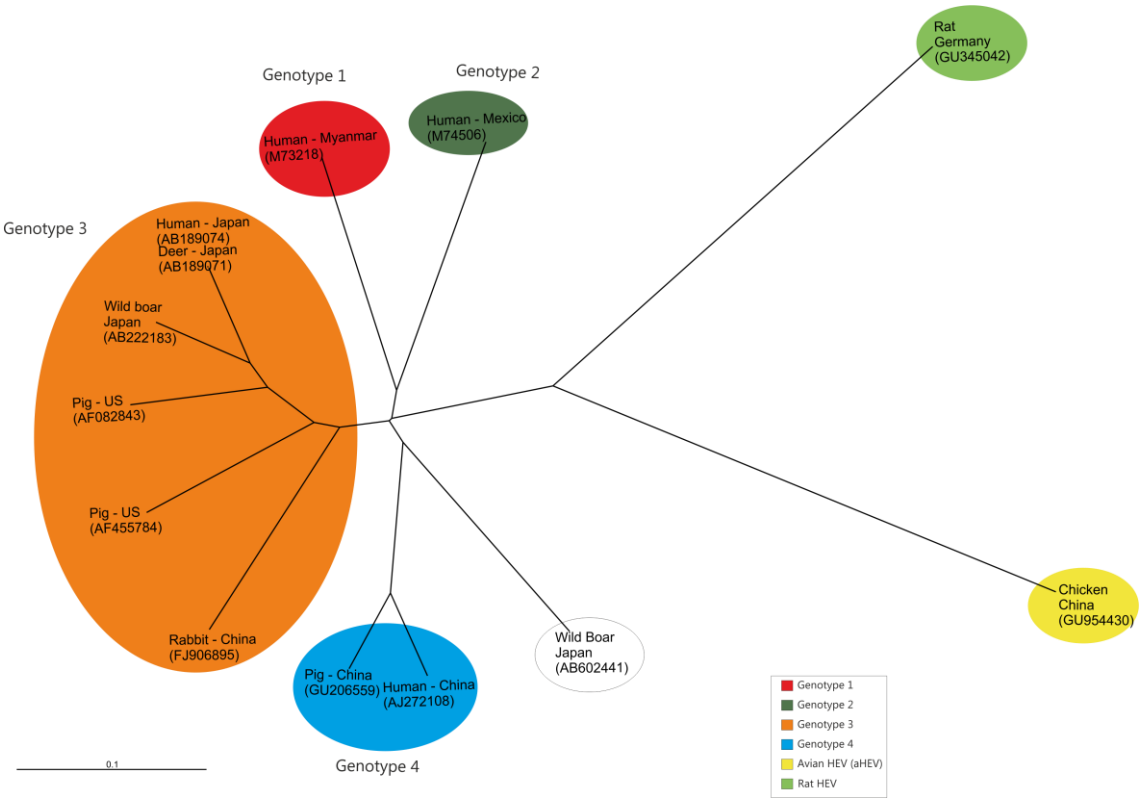

Figure 1: Phylogenetic tree based on complete capsid sequences showing the four major genotypes, the new wild boar genotypes and the rat and chicken viruses. Tree was calculated by the neighbor-joining methods. Branches are proportional to the genetic distances.

Subtype classification is controversial and not accepted by all researchers in the field. For instance, there are a number of publications including partial and complete genomic sequences of HEV with no differentiation into subtypes (Sonoda et al., 2004; Takahashi et al., 2003; Tei et al., 2003; Wibawa et al., 2004). Based on this mismatch, a part of this thesis deals with the classification of HEV (Oliveira-Filho et al., 2013).

2.2 Early history

The first epidemiological study about hepatitis E came from India in the early Fifties. The infectious acute hepatitis outbreak in Delhi was extensively described. In the peak of the outbreak the incidence was almost 190 cases per day. During more than 6 weeks about 29,300 cases were reported; it has been estimated that approximately 68 % of the population of Delhi was infected (Viswanathan, 1957). Without knowing the infectious agent a very detailed study was performed; some epidemiological data differed from hepatitis caused by HAV. The fatality-rate showed that the pathogen was of low virulence. However, when “infectious hepatitis” occurred during pregnancy there were reports of complications such as still-birth, neonatal death and a high case-fatality ratio. The study pointed to water borne infection due to sewage contamination of the Jumna River, the main water source. Nevertheless the unusual pathogen was not identified (Naidu and Viswanathan, 1957). More than 15 years after the outbreak a group of researchers analyzed patient samples from the Delhi outbreak 1955-56 and two more infectious hepatitis outbreaks in India (Ahmedabad 1975-76 and Pune 1978-79). No evidence for infection with either HAV or HBV was found and it was suggested that an unrecognized agent had been responsible for the outbreaks (Wong et al., 1980). Previous studies suggested the presence of unknown non-A and non-B viral agent(s) linked to hepatitis in different countries and designated non-A and non-B hepatitis (Francis and Maynard, 1979; Stakhanova et al., 1979). The unknown agent was named “enterically transmitted non-A and non-B hepatitis” (ET-NANBH) (Jameel, 1999; Sreenivasan et al., 1984a).

In 1983 a scientist infected himself ingesting fecal suspension from an ET-NANBH patient. Spherical 27 to 30 nanometers virus-like particles (VLP) were observed in his feces and characterized using immune electron microscopy (IEM). The volunteer had previously been exposed to HAV and had no antibodies against HBV, but developed antibodies against the VLPs recovered in his feces. Afterwards cynomologus monkeys were inoculated with the virus-containing stool and hepatitis was confirmed by liver enzymatic profile, specific antibody response and excretion of VLPs (Balayan et al., 1983).

Later the ET-NANBH virus from a Burmese (Myanmar) patient was inoculated in cynomologus monkeys and HEV cDNA was isolated for the first time. In the same study it was also demonstrated that the viral genome had a plus strand RNA genome and was polyadenylated; the name hepatitis E virus (HEV) was proposed (Reyes et al., 1990;

Zuckerman, 1990). Afterwards the first full-length HEV genome was cloned and sequenced (Tam et al., 1991) and the structural proteins expressed, which allowed the development of serological diagnostic tests (He et al., 1995). Since then the number of reports of HEV in the human population has increased progressively showing that HEV was present in different continents and countries such as Pakistan (Tsarev et al., 1992), Mexico (Huang et al., 1992) and China (Aye et al., 1992; Yin et al., 1994).

2.3 Morphology and molecular biology of HEV

2.3.1 Morphology and genome organization

HEV virions are non-enveloped spherical particles with a size of 27 to 32 nm in diameter. They possess a positive strand RNA genome with a size of approximately 7.2 kb with three partly overlapping open reading frames (ORFs), a capped 5' end and polyadenylated 3' end (Mushahwar, 2008). The genome organization is the same for genotypes 1, 2 and 3 and only differs regarding the position of ORF3 in genotype 4 (Fig 2A and 2B) (Panda et al., 2007). In addition subgenomic viral RNA is also present (Graff et al., 2006).

The 5' end of the genome contains a short non-coding region (NCR) with 26 to 28 nucleotides in length. ORF1 has a size of approximately 5.1 kb. This region encodes a polyprotein which is cleaved into the viral nonstructural proteins as methyltransferase, papain-like cysteine protease, helicase and RNA dependent RNA polymerase (RdRp); these enzymes are involved in viral replication, transcription and polyprotein cleavage (Kaur et al., 1992; Koonin et al., 1992; Reyes et al., 1990).

ORF 2 encodes the structural capsid protein and has a size of approximately 1983 nt for members of the genotypes 1, 2 and 3 and 2025 nt for members of genotype 4. This protein is highly immunogenic and is responsible for the functions such as assembly and host interaction. It has a high nucleotide heterogeneity and has been subject of both diagnostic tests and vaccine development (Engle et al., 2002; Koff, 2007; Panda et al., 2007; Tsarev et al., 1997; Zhang et al., 2001b).

ORF 3 has a size of 369 nt and encodes a small phosphorylated protein which binds to the hepatocellular cytoskeleton and forms a complex together with the capsid protein. Other possible ORF 3 functions are related to the regulation of cellular signs (Jiménez de Oya et al., 2007; Khuroo, 2008; Panda et al., 2007).

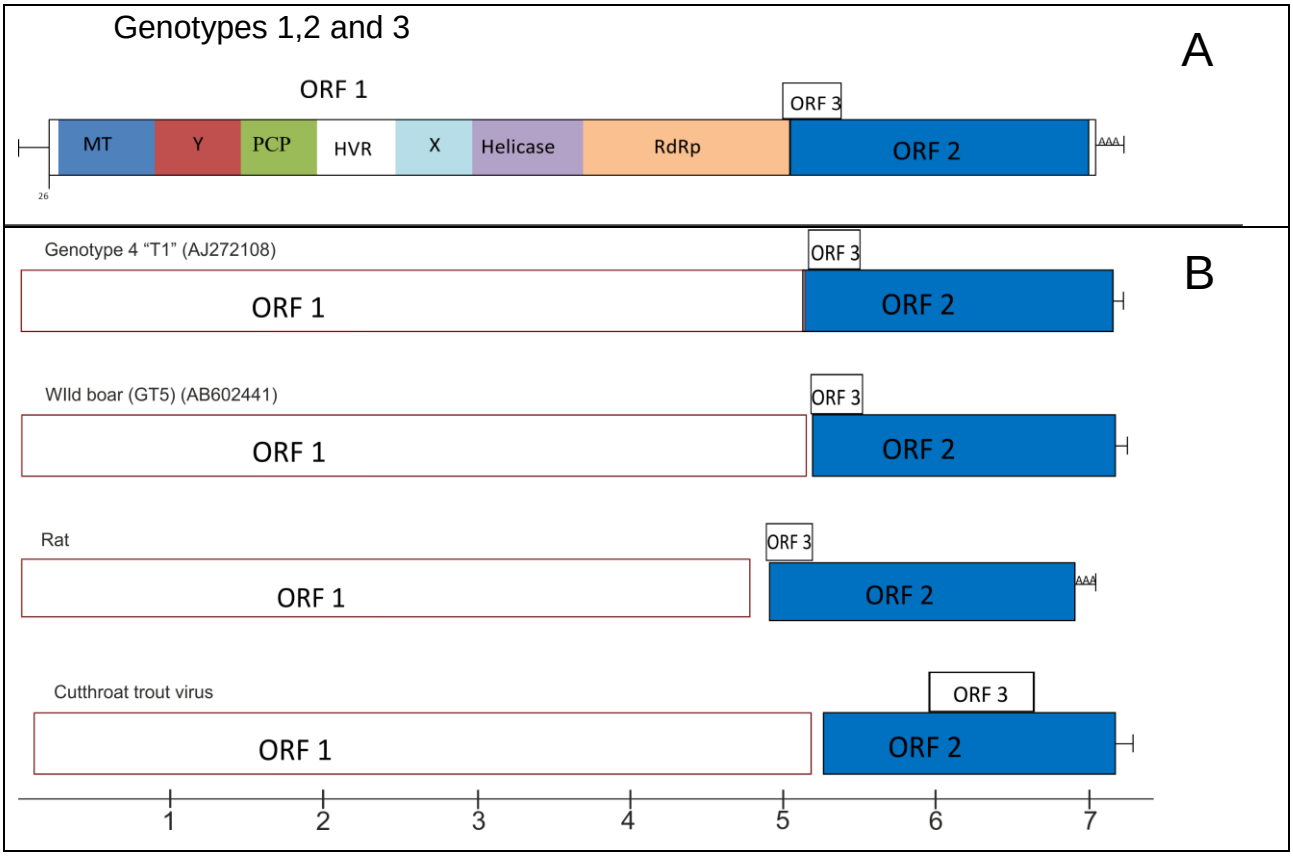

Figure 2: Genome organization of GT 1-3 (A) and GT 4 and HEV like viruses from wild boar, rat and Cutthroat trout virus (B). Scale from 1 to 7 shows genome size in kilo bases (Kb).

2.3.2 Genome replication

Due to the lack of an efficient cell culture system or animal model the mechanisms of HEV replication are not well known. A replication model has been proposed based on analogy to other single stranded RNA viruses and some knowledge of HEV (Fig. 3)(Ahmad et al., 2011). It is believed that HEV particle uptake occurs by receptor-mediated endocytosis using a not yet identified receptor at the cell surface. After uncoating, RNA is translated into the non-structural polyprotein by host ribosomes; it is assumed that the papain-like protease cleaves the ORF 1 encoded polyprotein. The RdRp replicates (alone or with aid of cellular proteins) the positive RNA into negative RNA strands (Agrawal et al., 2001), which will serve as template for synthesis of the positive sense RNA strand by the viral RNA polymerase. In parallel the subgenomic RNA is translated by the structural proteins in the ORF 2 and ORF 3. The capsid protein packages the genome probably with the aid of the cytoskeleton phosphoprotein (ORF 3) and the virions are assembled and released by a mechanism not yet identified.

Three potential N-glycosylation sites have been identified within the capsid protein sequence (Asn137, Asn310 and Asn562), however the ORF 2 protein is probably not glycosylated (Mori and Matsuura, 2011).

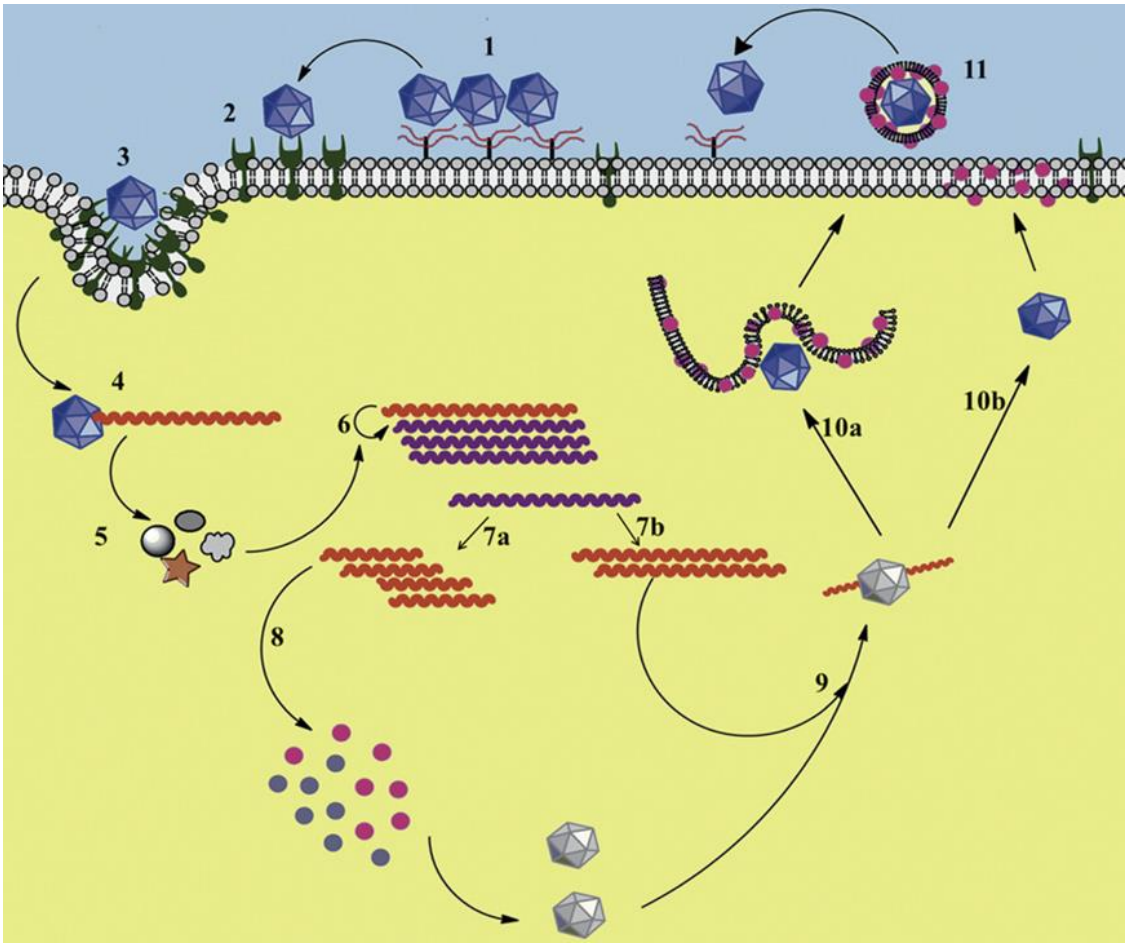

Figure 3: Proposed replication of HEV (Ahmad et al., 2011). Attachment (1), binding to cellular receptor (2), and particle internalization (3); uncoating (4), RNA translated into nonstructural proteins (5); positive sense RNA replicated into negative strands (6); synthesis of subgenomic (7a) and full-length positive sense RNA (7b); subgenomic RNA translated into ORF2 and ORF3 proteins (8); genomic RNA packaged by capsid protein (9); ORF 3 associated with endomembranes (10a) or plasma membranes (10b); mature virions associated with ORF3 proteins and lipids released (11). Reprinted from Virus Research, Vol. 161, Imran Anmad, R. Prasida Holla and Shahid Jameel, Molecular Virology of hepatitis E virus, Pages No. 47-58, Copyright (2011) with permission from Elsevier.

2.3.3 Viral particle structure

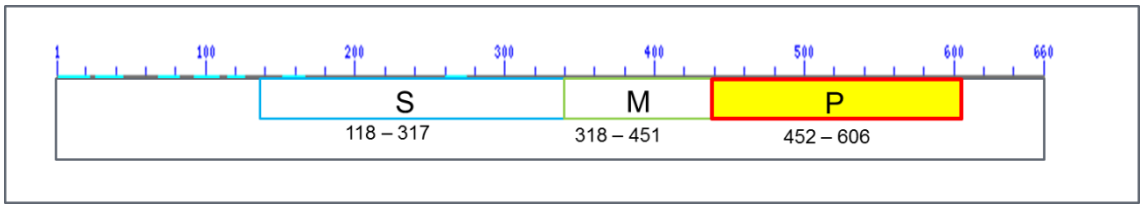

Figure 4: Structural domains of the HEV capsid protein according to Xing el al., 2010. Shell (S) from aa 118-317, middle (aa 318-451) and protruding (aa 452-606) domains

The HEV capsid subunits are formed by two identical molecules (homodimers), which represent the main structure responsible for the virion shell (Xing et al 1999). The capsid protein comprises about 660 amino acids with a molecular size of approximately 70 kda and can be divided into three different domains: S (shell), M (middle) and P (protruding). These domains are located in position 118-317, 318-451 and 452-606,

respectively (Fig. 4) (Xing et al., 2010). Another study has called the M and P domain P1 and P2, respectively (Guu et al., 2009).

The S domain forms the internal skeleton of the particle, forming a continuous capsid shell. It contains an anti-parallel jelly roll-like containing eight ß-strands with four short α-helices (Guu et al., 2009; Yamashita et al., 2009). The M domain has a twisted anti-parallel ß-barrel structure with six ß-strands and four α-helices. It is tightly associated to the S domain and linked to the P domain by a long proline-rich hinge (Yamashita et al., 2009). The association of these two domains makes it possible for the capsid protein dimer to change its conformation, allowing a very unique topology (Mori and Matsuura, 2011). The P domain is a single individual domain forming a twisted anti-parallel ß-sheet structure. It forms dimeric spikes stabilizing protein interactions across the two-folds (two-fold like spikes) (Guu et al., 2009; Mori and Matsuura, 2011; Yamashita et al., 2009).

2.4 HEV infection

2.4.1 Mode of transmission

The main route of human HEV transmission is fecal-oral. The first reported outbreak pointed already towards an association between ingestion of water or food contaminated with HEV (Aye et al., 1992; Huang et al., 1992; Skovgaard, 2007; Sreenivasan et al., 1984b; Wong et al., 1980). Other less common routes are vertical transmission (transplacental) as well as horizontal via blood transfusion or organ transplantation (Halac et al., 2011; Hosseini Moghaddam, 2011; Khuroo and Kamili, 2009; Kumar et al., 2001; Panda et al., 2007; Rostamzadeh Khameneh et al., 2011; Tamura et al., 2007a).

In swine different routes of transmission have been tested and it was evident that the main route of transmission is again fecal-oral. After becoming infected animals shed viral particles in feces without showing clinical symptoms (Kasorndorkbua et al., 2004). It has also been suggested that HEV can be transmitted from one farm to another by fecal contamination or the movement of people and animals (Yan et al., 2008). For instance, a common HEV strain has been reported among two distinct farms who shared piglets (Vasickova et al., 2009).

Another study suggested that the major route of transmission in Europe is related to consumption of offal, wild boar or food contaminated during preparation (Wichmann et

al., 2008). As a foodborne pathogen HEV particles can actually be ingested via water, undercooked meat from swine or wild animals such as deer, crops, ingestion of mollusks from contaminated water or sewage (Li et al., 2007; Meng, 2011).

2.4.2 Blood transfusion

Positive serum samples were detected by ELISA in American and German blood donors (Dawson et al., 1992). Another study in Germany with samples from three different groups (blood donors, patient with history of acute hepatitis and patients positive for antibodies against other hepatitis viruses) showed that 37 % of the HEV seropositives had received a blood transfusion before. The authors raised the question of the possible transmission route (Wang et al., 1993). Afterwards many studies reported HEV antibodies in other European countries such as Switzerland (Lavanchy et al., 1994), Italy (Zanetti and Dawson, 1994), Australia (Moaven et al., 1995) and Brazil (Parana et al., 1997).

These findings have raised concern about the risk of transmission via blood transfusion. The first molecular evidence for transfusion-transmitted HEV came in 2004 from a 67-year-old Japanese patient. The HEV sequence was highly similar to that of one donor sample (Matsubayashi et al., 2004). Another report is of a 21-year-old Japanese patient who was receiving chemotherapy to treat T-cell lymphoma and was diagnosed with hepatitis E after receiving multiple transfusions from at least 84 donors. The transfused blood aliquots were screened and HEV RNA was detected on the product transfused on day 26. Complete genomic sequences were identical, evidencing the transmission (Tamura et al., 2007a).

2.4.3 Clinical disease (humans)

HEV infection can cause acute liver disease which is mild and self-limited in the majority of cases. However, in some cases it can induce the so-called “Fulminant Hepatic Failure” (FHF) which is a severe acute hepatic disease with low chances of recovery. The non-specificity and diversity of the clinical symptoms may lead to misdiagnosed cases. For example it has been suggested that acute hepatitis may be frequently diagnosed as an unknown cause and the patient receives symptomatic treatment (Sherman, 2011). In addition Hepatitis E can be misdiagnosed in drug induced acute liver injury cases (Davern et al., 2011).

HEV infection often manifests as subclinical disease. Usually the patients show typical signs and symptoms of acute liver disease, very similar to HAV infection. The

course can be completely asymptomatic or accompanied by fever. Clinical signs and symptoms including the incubation period can range from 15 to 60 days. According to studies in volunteers incubation periods of 36 (Balayan et al., 1983) and 30 days (Chauhan et al., 1993) were observed. The classical symptomatic infection can be divided into three phases: pre-icteric from 1-10 days, icteric from 12-15 days up to one month and post-icteric which is characterized by normalization of liver enzyme levels (Aggarwal, 2011; Panda et al., 2007).

The pre-icteric phase is characterized by unspecific gastrointestinal symptoms such as nausea, vomiting and epigastric pain. The icteric phase starts suddenly as result of high levels of bilirubin in the tissues. It can be evidenced by jaundice, dark urine, clay colored feces and frequently by fever and arthralgia. Within this phase the liver functions are transformed and the alteration of laboratory findings such as alanine aminotransferases (ALT), aspartate aminotransferases (AST), gamma-glutamyltransferases (GGT), bilirubin and prothrombin levels and serum alkaline phosphatase (SAP) may be noted (Srivastava et al., 2011).

2.4.3.1 Fulminant hepatic failure

Fulminant hepatic failure (FHF) or acute liver failure (ALF) is an acute hepatitis followed by encephalopathy within four weeks of the first symptoms. There is a loss of function of 80-90 % of the liver cells. The outcome can be classified regarding the appearance of encephalopathy until 24 weeks after the onset of symptoms. The prognosis is poor to moderate and the survival rate may range according to the course of the disease (O'Grady et al., 1993; Trey and Davidson, 1970; Vaquero and Blei, 2003).

The mechanism of how HEV is related to FHF pathogenicity is not completely understood. The complications associated with FHF are hepatic encephalopathy, cerebral edema, coagulopathy, hepatic parenchyma necrosis, renal failure, pulmonary edema, cardiovascular disorders and coma (Acharya et al., 1996; Alam et al., 2009; Harry et al., 2003; Trewby et al., 1978). Once FHF is diagnosed the patient should be moved to an intensive care unit and the possibility of transplantation should be considered (Vaquero and Blei, 2003).

2.4.3.2 HEV infection during pregnancy

Hepatitis E in pregnant women is an explosive disease with elevated case-fatality rates (Khuroo and Kamili, 2003). In comparison with other hepatitis viruses, HEV is most frequently associated with severe complications in pregnant women (Beniwal et al.,

2003; Jaiswal et al., 2001; Khuroo and Kamili, 2003). A study with pregnant patients suffering of acute viral hepatitis has shown that HEV was associated with almost half of the patients. In addition, vertical transmission can occur: it has been reported that all HEV RNA positive women have delivered HEV positive babies (Kumar et al., 2001). The reported outcome or complications regarding vertical transmission were miscarriage, abortion, mother death, neonatal death, premature delivery and self-limiting disease in the babies (Khuroo and Kamili, 2009).

On the other hand, different studies have questioned the statements and epidemiological designs of the previous studies. Following cases during 1986 to 2006 it was demonstrated that the mortality and the outcome in ALF pregnant patients were not different than in non-pregnant women, girls, boys and men and should not be considered as a poor prognostic variable (Bhatia et al., 2008). Seroprevalence rates reported in pregnant women are similar to the general population suggesting that they are not more susceptible to HEV than other population groups (Cevrioglu et al., 2004; Oncu et al., 2006).

2.5 HEV in animals

In the mid-nineties there was a search for an animal reservoir of HEV. After experimental infection swine excreted HEV particles in the feces (Balayan et al., 1990). Another study found HEV IgG and also RT-PCR positive swines (Clayson et al., 1995). In 1997, partial genomic HEV RNA fragments infecting swine were reported for the first time and phylogenetic analysis confirmed that both swine and human sequences were closely related (Meng et al., 1997b). This discovery opened a new door in HEV research; swine hepatitis E viruses began to be reported from different countries.

Domestic pigs and wild boars are now considered as the main reservoir for HEV genotypes 3 and 4 (Meng, 2010). However HEV RNA has been found in other animal species such as deer, mongoose, rabbit, rat and chicken (aHEV). In addition, anti-HEV antibodies have been found in various other animal species such as wild rodents, dogs, cats, cattle, sheep, goats and horses (see table 3) (Arankalle et al., 2001; Li et al., 2006a; Mochizuki et al., 2006; Peralta et al., 2009; Vitral et al., 2005; Zhang et al., 2008).

2.5.1 Domestic pigs

A number of studies have reported both anti-HEV antibodies and the presence of HEV RNA, showing that the virus is endemic in swine herds in different countries and continents (Table 1).

The prevalence of anti-HEV antibodies in swine has been shown to be age dependent. Antibodies against HEV in swine arise around twelve to 15 weeks of age and high seroprevalence rates can be observed already in two to four month-old piglets (Jinshan et al., 2010). However, prevalence of anti-HEV antibodies in adults are usually higher than in young swine in a given population (Chang et al., 2009). The IgG antibodies remain detectable until slaughter age (de Deus et al., 2008a; Meng et al., 1997b) and IgM remains for five to seven weeks and is, as in humans, related to viremia (de Deus et al., 2008a).

The detection rates of genomic HEV range according to age as well but seem to be higher in young animals, in contrast to antibody detection. Several studies from different countries reported that higher prevalence rates of HEV RNA have been detected in swine between two and four months of age (McCreary et al., 2008; Siripanyaphinyo et al., 2009; Ward et al., 2008; Yu et al., 2008). Although unusual, HEV can also be detected in older animals. For instance few studies have reported high prevalence rates of HEV RNA in adult and old sows in different farms in Northern Italy, England and Thailand (Di Bartolo et al., 2008; McCreary et al., 2008).

Almost all subtypes from genotypes 3 and 4 have been found in swine herds around the world. A high viral heterogeneity can be found in the same population or region (Di Bartolo et al., 2008). For instance, different subtypes of genotype 4 HEV have been detected in swine feces from farms in the same region in Shanghai (Yan et al., 2008).

Table 1: Prevalence of HEV RNA (feces and/or blood) and seroprevalence found in swine in different studies. Genotypes are shown in parenthesis and "-" means not found/in the study.

Country	Seroprevalence	HEV RNA	Reference
Asia			
China	26.8 %	-	(Meng et al., 1999b)
	-	7.2 % (G4)	(Zheng et al., 2006)
	-	5 % (G4)	(Yan et al., 2008)
	68.3 %	5.8 %(G4)	(Li et al., 2008)
	67 %	4.6 % (G3)	(Zhang et al., 2008)
	-	22.3 %(G3, 4)	(Li et al., 2009b)
	52.2 %	8.4 % (G4)	(Jinshan et al., 2010)

	82.2 %	0.8 % (G4)	(Geng et al., 2010)
	81.2 %	47.9 % (G4)	(Geng et al., 2011)
	-	6.7 % (G4)	(Geng et al., 2011)
	78.8 %	1.9 % (G4)	(Wang et al., 2002)
	-	23.1 % (G3)	(Ning et al., 2007)
	82.3 %	22.9 % (G4)	(Chang et al., 2009)
Japan	57.9 %	10.1 % (G3, G4)	(Takahashi et al., 2003)
	13.2 %	14.5 % (G3)	(Tanaka et al., 2004)
	55.7 %	3.9 % (G3, G4)	(Takahashi et al., 2005)
	74.6 %	1.8 % (G3)	(Sakano et al., 2009)
Taiwan	37.1 %	2.63 % (g 3)	(Hsieh et al., 1999)
	-	1.3 % (G3)	(Wu et al., 2000)
India	66.5 %	-	(Arankalle et al., 2001)
	94.7 %	12.3 % (G4)	(Arankalle et al., 2003)
	-	2 % (G4)	(Vivek and Kang, 2011)
Korea	-	17 % (G3)	(Yu et al., 2008)
	39.5 %	1.9 %(G3)	(Lee et al., 2009a)
	40.7 %	-	(Meng et al., 1999b)
Thailand	30.7 %	-	(Meng et al., 1999b)
	64.7 %	7.75 % (G3)	(Siripanyaphinyo et al., 2009)
Mongolia	91.8 %	36.6 % (G3)	(Lorenzo et al., 2007)
Oceania			
Indonesia	73.6 %	1 % (G3)	(Utsumi et al., 2011)
New Caledonia	-	6.5 % (G3)	(Kaba et al., 2011)
Bali	71.7 %	1 % (G4)	(Wibawa et al., 2004)
New Zeeland	75 %	37.8 % (G3)	(Garkavenko et al., 2001)
Americas			
US	-	Genotype 3	(Meng et al., 1997b)
	-	35.4 % (G3)	(Huang et al., 2002)
Canada	18.2 %	-	(Meng et al., 1999b)
	-	34.3 % (G3)	(Ward et al., 2008)
Argentina	22.7 %	88.9 %(G3)	(Munné et al., 2006)
Brazil	24.3 %	-	(Vitral et al., 2005)
	-	9.6 % (G3)	(dos Santos et al., 2011)
Bolivia	-	31.8 % (G3)	(Dell'Amico et al., 2011)
Africa			
Congo	-	2.5 % (G3)	(Kaba et al., 2010a)
Europe			
Belgium	-	7 % (G4 and G3)	(Hakze-van der Honing et al., 2011)
Czech Republic	-	36.7 % (G3)	(Vasickova et al., 2009)
England	-	21.5 % (G3)	(McCreary et al., 2008)
France	40.5 %	31.2 % (G3)	(Kaba et al., 2009)
	16.3 %	3.4 % (G3)	(Rose et al., 2011)
Germany	49.8 %		(Baechlein et al., 2010)
Hungary	-	27.3 % (G3)	(Reuter et al., 2009)
	-	21.0 % (G3)	(Forgách et al., 2010)

Italy	-	42 % (G3)	(Di Bartolo et al., 2008)
	-	29.9 % (G3)	(Martelli et al., 2010)
	87 %	64.6 % (G3)	(Di Bartolo et al., 2011)
The Netherlands	-	15 %(G3)	(Hakze-van der Honing et al., 2011)
Spain	25 %	negative	(Pina et al., 2000)
	20.4 %	18.8 % (G3)	(Jiménez de Oya et al., 2011)
	71.4 %		(Peralta et al., 2009)
	-	23.3 % (G3)	(Fernández-Barredo et al., 2006)
	-	37.7 % (G3)	(de Deus et al., 2007)
Sweden	-	29.6 % (G3)	(Widén et al., 2011)

2.5.2 Wild boar and deer

The first report of HEV RNA in wild boar came from Japan and came only a few years after discovery of HEV in swine. During an HEV outbreak investigation in Japan in 2003 a series of human cases was linked by epidemiological investigation to the consumption of uncooked wild boar liver and Sika deer meat. Nevertheless it could only be evidenced in deer since there were no wild boar liver left to be tested (Matsuda et al., 2003; Tei et al., 2003). After this report wild boar samples were screened; HEV RNA has been detected for the first time in wild boar from Japan (Sonoda et al., 2004).

Since then HEV has been detected in wild boar herds from different countries. For instance, in free-living wild boar from Japan (Nishizawa et al., 2005; Sakano et al., 2009; Sonoda et al., 2004) and from several European countries such as Spain, Germany, Hungary, Italy, the Netherlands and Sweden (Table 2) (Adlhoch et al., 2009b; de Deus et al., 2008b; Kaba et al., 2010b; Kaci et al., 2008; Martelli et al., 2008; Reuter et al., 2009; Rutjes et al., 2010; Widén et al., 2011). In contrast, only a few studies have found HEV positive deer since the first report. HEV was reported in wild Sika deer in Japan, in Roe deer (*Capreolus capreolus* and *C. rufus*) in Hungary and red deer (*Cervus elaphus*) in the Netherlands (Reuter et al., 2009; Rutjes et al., 2010).

Different from domestic swine, high detection rates of HEV RNA have been reported not only in young animals but also in adult wild boar (de Deus et al., 2008b; Martelli et al., 2008). In addition, it seems that the viral heterogeneity is higher in wild boar populations. Different subtypes have been reported within the same populations in Germany and Sweden (Adlhoch et al., 2009b; Widén et al., 2011). In Japan genotypes 3, 4 and another lately proposed new genotype were found in wild boar (Sato et al., 2011; Takahashi et al., 2011). So far only genotype 3 viruses have been reported in deer.

Table 2: Prevalence of HEV RNA and seroprevalence of HEV antibodies in wild boar reported in different studies.

Country	Seroprevalence	HEV RNA	References
Japan	8.1%	3,3% (G3 and 4)	(Sato et al., 2011)
	8.6%	2.9% (G3)	(Sonoda et al., 2004)
	-	2.3% (G3)	(Nishizawa et al., 2005)
	4.5%	1.1% (G3)	(Sakano et al., 2009)
France		2.5% (G3)	(Kaba et al., 2010b)
Germany	24.3%	68.2% (G3)	(Adlhoch et al., 2009b)
	-	5.3% (G3)	(Kaci et al., 2008)
Hungary		12.2%(G3)	(Reuter et al., 2009)
		10.7%	(Forgách et al., 2010)
Italy		25% (G3)	(Martelli et al., 2008)
Spain	28%	19.6% (G3)	(de Deus et al., 2008b)
Sweden		8.2% (G3)	(Widén et al., 2011)

2.5.3 Other animals species

Both anti-HEV IgG and IgM antibodies as well as HEV RNA have been found in mongoose specimens from Japan; mongoose HEV clusters in genotype 3 (Li et al., 2006a; Nakamura et al., 2006). A recent study in China has reported HEV RNA in rabbits. Phylogenetic analysis has shown that HEV found in Chinese Rex rabbits might represent a novel genotype of HEV closely related to genotype 3 (Zhao et al., 2009).

The presence of anti-HEV antibodies was reported in rats and other rodent species (Favorov et al., 2000; Kabrane-Lazizi et al., 1999). Later on HEV-like viruses have been detected in Norwegian rats (*Rattus norvegicus*) from Germany (Johne et al., 2010a; Johne et al., 2010b).

A number of serological studies have reported anti-HEV antibodies in several other animal species such as dogs, cows, horses, goats and wild rodents (table 3) (Arankalle et al., 2001; Chang et al., 2009; Geng et al., 2011; Geng et al., 2010; Mochizuki et al., 2006; Okamoto et al., 2004; Peralta et al., 2009; Vitral et al., 2005; Wang et al., 2002; Zhang et al., 2008). The meaning of presence of the antibodies in these species is not completely clear. For instance, in rabbits, chicken and Norwegian rats new viruses have been sequenced and are related to the other viral strains detected in humans, pigs and wild boars. This explain the presence of antibodies in these species, for instance the rat HEV are closely related and can react to HEV antibodies in humans (Dremsek et al., 2011). Regarding the other species in which only antibodies have been detected it is still not clear whether HEV can infect these species or some other viruses cross reacting with the HEV are present.

Table 3: Seroprevalence of HEV in different species.

Family	Species	Country	Sero-prevalence	References
Artiodactyl	Cattle	India	6.1%	(Arankalle et al., 2001)
		Brazil	1.5%	(Vitral et al., 2005)
		China	6.3%	(Wang et al., 2002)
			6%	(Zhang et al., 2008)
			29.3%	(Chang et al., 2009)
			10.4%	(Geng et al., 2010)
			25.3%	(Geng et al., 2011)
			14.9%	(Geng et al., 2011)
	Sheep	China	9.3%	(Geng et al., 2011)
			9.8%	(Chang et al., 2009)
	Sheep	Spain	1.9%	(Peralta et al., 2009)
	Goat	China	24%	(Zhang et al., 2008)
			28.2%	(Geng et al., 2010)
	Goat	Spain	0.6%	(Peralta et al., 2009)
	Horse	China	16.3%	(Zhang et al., 2008)
			14.3%	(Geng et al., 2011)
Carnivores	Mongoose	Japan	8.3%	(Li et al., 2006a)
	Dog	India	22.7%	(Arankalle et al., 2001)
		China	17.8%	(Zhang et al., 2008)
		Brazil	6.97%	(Vitral et al., 2005)
		Japan	2.4%	(Mochizuki et al., 2006)
	Cat	Japan	4%	(Mochizuki et al., 2006)
		Japan	32.6%	(Okamoto et al., 2004)
		Spain	11.1%	(Peralta et al., 2009)
Rodents	Rodent	India	11.2%	(Arankalle et al., 2001)
	Wild rodents	Brazil	50%	(Vitral et al., 2005)
Avian	Chicken	Brazil	20%	(Vitral et al., 2005)
		China	1.9%	(Zhang et al., 2008)
			2.5%	(Geng et al., 2011)
	Duck	China	12.8%	(Zhang et al., 2008)
			3%	(Geng et al., 2011)
	Pigeon	China	4.4%	(Zhang et al., 2008)

2.6 Pathogenesis and immune response

In experimental infection with animals, viral RNA has been detected in the liver and a number of other tissues (bile, kidney, gallbladder, spleen, large and small intestines, lymph nodes and tonsils) (Bouwknegt et al., 2009; dos Santos et al., 2009; Leblanc et al., 2010; Lee et al., 2009b). It has been shown that HEV replicates in the hepatocytes (Tam et al., 1996), however there is evidence of extrahepatic replication sites e.g. in lymph nodes and intestinal tract tissues (Williams et al., 2001). Even if it is not completely clear where HEV replicates, it is feasible to postulate that the liver plays an important role in the disease.

Clinical symptoms or disease have not been associated with the presence of HEV in animals. Nevertheless the infection can induce a mild to moderate subclinical hepatitis (Martín et al., 2007). Some studies have attempted to associate the presence of HEV with clinical disease in animals. For example, HEV was related to hepatitis and liver lesions in naturally infected pigs (de Deus et al., 2008a). In addition HEVs have been detected in non-healthy swine: some of the positive animals showed mild to moderate liver lesions but have been diagnosed with other diseases such postweaning multysystemic wasting syndrome (de Deus et al., 2007).

Co-infection with HEV and other viruses may induce immune system dysfunction in domestic swine (Savic et al., 2010). For instance, it has been shown that pigs infected with HEV and Porcine Circovirus 2 (PCV2) are more likely to be infected with Teno Torque Viruses (TTVs) (Savic et al., 2010). In addition transplacental HEV infection has been evidenced in aborted fetuses and suggested that the co-infection with PCV2 may be responsible for reproductive disturbance (Hosmillo et al., 2010).

In humans HEV will induce mild or self-limiting disease in most cases. However in some cases infection might induce FHF or evolve to chronic hepatitis. The mechanism of liver/hepatocyte damage is still poorly understood. Accordingly it is not yet clear whether cell damage is caused directly by the presence of the virus in host cells or by host immune responses as reported for other hepatitis viruses (Rehermann and Nascimbeni, 2005).

Uncomplicated or mild disease has been associated with an increase of IFN-ɣ and TNF-α-secreting T cells (Srivastava et al., 2011). Regarding to the innate immune response it has been suggested that NK and NKT cells are activated during acute hepatitis E (Srivastava et al., 2008). CD4+ and CD8+ seem not to be activated in the peripheral blood (Srivastava et al., 2007; Tripathy et al., 2012), however the presence of CD8+ was reported in the liver of a FHF HEV infected patient and may be involved in hepatitis E pathogenesis (Prabhu et al., 2011). T-cell response also seems to be involved in the pathogenesis of chronic hepatitis E (Suneetha et al., 2012).

2.7 Diagnosis

Due to its clinical and epidemiological characteristics the diagnosis of HEV may be challenging. It is difficult to distinguish hepatitis E from other causes of acute viral hepatitis and HEV may not be detected even if the correct tools are employed. The first assay for detection of HEV was based on immune electron microscopy (Balayan et al.,

1983). Afterwards different serological and molecular assays (RT-PCR and qRT-PCR) were developed (Jothikumar et al., 2006; Li et al., 2006b).

In general the diagnosis includes the detection of IgG and IgM antibodies against HEV as well as HEV RNA in serum and feces (Teshale and Hu, 2011). Recently some cell lines were shown to be permissive for HEV infection (Okamoto, 2011b; Tanaka et al., 2007), however this has not been validated so far as a diagnostic test for HEV.

A proper diagnose of hepatitis E in humans should combine markers for liver function, the appropriate serological test and molecular detection. The results from serological tests should consider the epidemiological situation. For instance a positive antibody titre in an endemic region may be meaningless. The detection of HEV in animals indicates contact with HEV and can be useful for epidemiological surveys and risk analysis studies.

2.7.1 Serological assays

The production of the first cDNA HEV clone allowed the expression of recombinant proteins (Tam et al., 1991). This led to a number of commercial and in-house assays based on different recombinant proteins and synthetic peptides from animal and human origin (Goldsmith et al., 1992; Meng et al., 1997a; Meng et al., 1998a). All three HEV ORFs have shown different antigenic regions (He et al., 1995; Khudyakov et al., 1994; Purdy et al., 1992). However, ORF 2 is more immunogenic and definitely contains a great number of antigenic domains which were target for most serological assays (Table 4). Currently there are a number of commercial and in-house tests including ELISA and Western blot-based techniques (table 5).

Table 4: Different genomic regions and expression systems for expression of the HEV capsid protein.

Systems used to express HEV proteins		
Expression System	**Protein/Region**	**References**
Baculovirus **(pupae of silkworm, SF9, Trichoplusia ni larvae)**	ORF 2: 55 kda based on Sar55 isolate	(Arankalle et al., 2003; Arankalle et al., 2001; de Deus et al., 2008b; Hsieh et al., 1999; Meng et al., 1999a; Pina et al., 2000)
	ORF 2: 111 - 660aa (G4 HE-J1 strain)	(Lorenzo et al., 2007; Mizuo et al., 2002; Sonoda et al., 2004; Takahashi et al., 2003; Wibawa et al., 2004)
	ORF 2: 111 - 660aa (G3)	(Jiménez de Oya et al., 2011; Jiménez de Oya et al., 2009)
E .Coli **(GST)**	ORF 2: 394 - 604 (G1)	(Wang et al., 2002)
	ORF 2: 394 - 604	(Chang et al., 2009)
	ORF 2: 452 - 617	(Obriadina et al., 2002; Vitral et

		al., 2005)

Table 5: Commercial kits for detection of anti-HEV antibodies.

Company	References
Abott	(Munné et al., 2006; Pina et al., 2000)
Genelabs Diagnostic	(Adlhoch et al., 2009b; Lee et al., 2009a; Wu et al., 2000)
Genelabs Inc., Singapore	(Wang et al., 2002)
MP Biomedicals Asia Pacific previously Genelab® Diagnostics, Singapore	(Leblanc et al., 2007)
Viragent HEV-Ab kit, Cosmic Corporation	(Siripanyaphinyo et al., 2009)
Institute of Immunology, Tokyo, Japan	(Utsumi et al., 2011)
Wan Tai Pharmaceutica	(Chang et al., 2009; Zhang et al., 2008; Zheng et al., 2006)
recomWell HEV and recomLine HEV IgG, Mikrogen	(Adlhoch et al., 2009b)
Adaltis EIAgen kits, Adaltis Italia	(Kaba et al., 2009)
ELISA (IgG and IgM) kit BioChain	(Di Bartolo et al., 2011)

2.7.2 RT-PCR and qRT-PCR

RT-PCR has been employed for diagnosis of HEV. Other techniques for detection of genomic HEV have been successfully used such as Southern Blot hybridization combined with reverse transcription (van der Poel et al., 2001). The first amplification of a HEV genome has taken place together with the first isolation of HEV cDNA from bile of an experimentally infected macaque using a random primer strategy (Reyes et al., 1990). Afterwards different RT-PCR setups with a number of primers were used in order to detect different regions of the HEV genome.

In swine and other animals the detection of HEV in both serum and feces is rather difficult in comparison with humans as animals do not present clinical symptoms. Prevalence rates can range according to the material used for diagnosis and factors related to the primers such as specificity, location and size of target genomic region. Prevalence can increase when more than one kind of sample (e.g. liver, bile, serum, feces) is used (Di Bartolo et al., 2011); for instance it has been reported that the detection rate of HEV RNA is higher in bile than in other organs, feces and serum (de Deus et al., 2007). Amplification using different genomic regions based primers show differences in sensitivity and may produce false negative results when different

genotypes are involved (Arankalle et al., 2003; Fogeda et al., 2009). It has been suggested that smaller PCR products may be amplified easier due to RNA degradation (Kaci et al., 2008).

2.8 Epidemiology

2.8.1 The virtual epidemiological transition

Since its discovery hepatitis E virus has been associated with infectious hepatitis outbreaks in Asia, Africa, the Middle East and Central America. The occurrence of hepatitis E has been linked to poor sanitary conditions and was considered a disease of developing countries for the last twenty years (Aggarwal and Naik, 2009; Viswanathan, 1957). At the end of the eighties until the early nineties it was unthinkable that hepatitis E would be diagnosed in developed countries (Scharschmidt, 1995). Nevertheless antibodies against HEV in healthy individuals and blood donors from Europe and North America could not be explained. In addition to traveler associated sporadic cases in Europe and in North America (Skaug et al., 1994) several autochthonous cases were reported in patients without travel history in the US, Europe, Australia and New Zealand (Mast et al., 1996; Preiss et al., 2006).

The first detection of HEV in domestic swine has added an important feature to disease epidemiology. The genetic proximity with human viruses raised the possibility of an animal reservoir. The viruses were revealed to be present in domestic swine and wild boar populations in both developing and developed countries (Meng, 2010; Meng et al., 1997b).

It became clear that autochthonous cases were more frequent than previously recognized in developed countries (Clemente-Casares et al., 2003). Nowadays HEV is considered endemic in countries such as Belgium, England, France, Germany, Italy, the Netherlands, Spain, the US (Borgen et al., 2008; Dalton et al., 2008; Hakze-van der Honing et al., 2011; Meng, 2011; Romanò et al., 2011; van der Poel et al., 2001; Wichmann et al., 2008).

2.8.2 Geographical distribution

The four HEV genotypes are distributed worldwide and prevalence ranges between the different continents and between different socioeconomic situations (Dalton et al., 2008). The Genotype 1 was initially found in Asian countries such as Bangladesh and Myanmar (Sugitani et al., 2009; Tam et al., 1991; Yin et al., 1994) and in African

countries such as Chad and Morocco (van Cuyck et al., 2003). Genotype 2 sequences have been detected in Mexico and Nigeria (Huang et al., 1992; Lu et al., 2006), genotype 3 in the US, Japan, Argentina, Brazil and in European countries such as Belgium, France, Germany, Hungary, Italy, the Nederlands, the United Kingdom (Banks et al., 2004; dos Santos et al., 2009; Fukuda et al., 2007; Reuter et al., 2009), Genotype 4 sequences in China, Taiwan and Japan (Inoue et al., 2009; Liu et al., 2012).

Genotypes 1 and 4 have been reported in a recent study from Germany. A GT 1 patient had been traveling to outside Europe but a GT 4 patient was confirmed as an autochthonous case (Wichmann et al., 2008). Recently, genotype 4 has been also detected in swine from Belgium being the first report of GT 4 in pigs in Europe; however it remains unclear how the GT 4 strain was introduced into the European swine population (Hakze-van der Honing et al., 2011).

Multiple genotypes might occur in the same country, population or even in the same individual (human or animal) (Li et al., 2009b). The distribution of the various HEV genotypes in both human and animal populations in China (where genotypes 1, 3 and 4 are present) is a very good example of how complex the geographical distribution can be (figure 5). Accordingly it has been suggested that the incidence of infection has decreased with genotype 3 and increased with genotype 4 in the swine population in Shanghai.

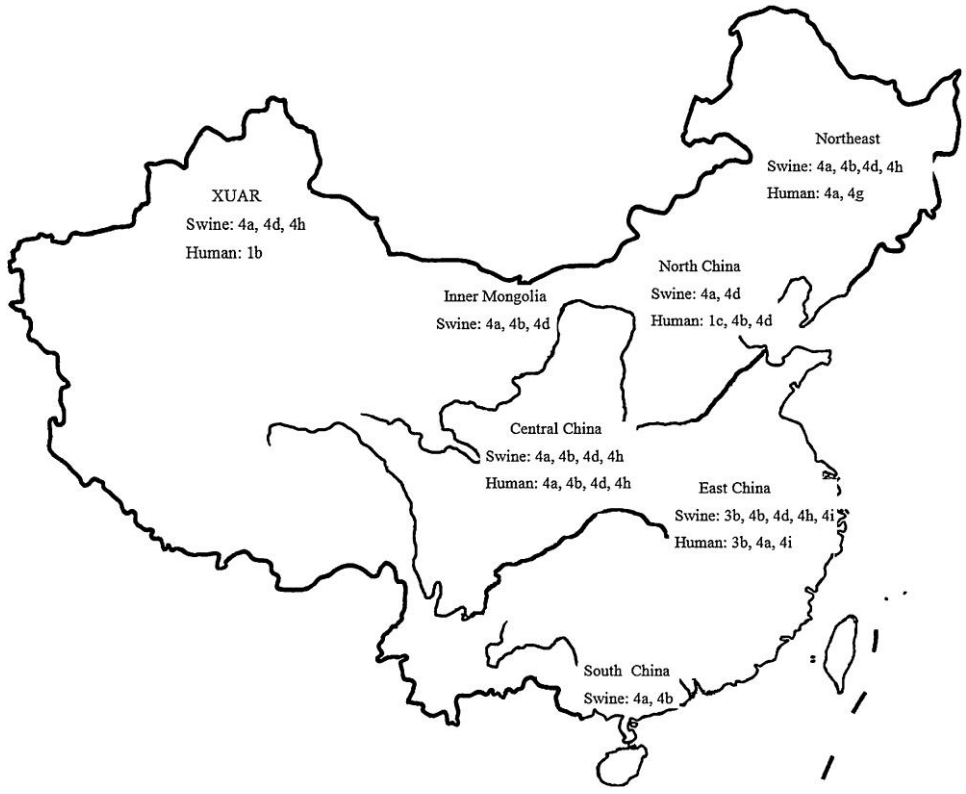

Figure 5: Map of China showing the different HEV strains (Genotypes and subtypes) found in both human and animal population (from Zhu et al., 2011 with permission[2]). Reprinted from Journal of Clinical Virology, Vol. 52, Yu-Min Zhu,Shi-Juan Dong,Fu-Sheng Si,Rui-Song Yu,Zhen Li,Xiao-Ming Yu,Si-Xiang Zou, Swine and human hepatitis E virus (HEV) infection in China, Pages No. 155-157, Copyright (2011) with permission from Elsevier.

2.8.3 Zoonotic aspects of HEV infection

The first evidence for zoonotic transmission of HEV was reported in association with the ingestion of deer meat. Genomic sequences of the viruses found in frozen deer meat matched 100 % to the ones recovered from HEV patients (Tei et al., 2003). Similar results have been reported in other cases which involved wild boar and pork meet from Japan (Li et al., 2005b; Masuda et al., 2005; Miyashita et al., 2012). In addition HEV RNA has been detected in commercial pig livers bought in local groceries in the US, Japan, France and the Netherlands (Bouwknegt et al., 2007; Colson et al., 2010; Yazaki et al., 2003).

HEV sequences from genotypes 3 and 4 found in swine and wild boar are closely related to those reported from humans (Siripanyaphinyo et al., 2009); (Zheng et al., 2006). In a study including 42 patients with hepatitis E it was shown that the viral strains were closely related to European swine strains (Legrand-Abravanel et al., 2009).

Genotypes 1 and 2 could not be found in swine in regions where they were prevalent in the human population. For instance, in India, where genotype 1 HEV is endemic in humans, it was shown that only genotype 4 is endemic in the swine population (Arankalle et al., 2003). Similar results were found in study in Thailand and Mexico where HEV genotypes 1 and 2 were detected in the human population but only HEV genotype 3 has been found in pigs (Cooper et al., 2005). Experimental studies led to similar results. Domestic pigs were inoculated with both swine GT 3 and human GT 1 viruses, but only the swine viruses could be recovered (Meng et al., 1998b). In another experimental study intergenotype chimeric viruses were inoculated into swine. The two chimeras with recombinant viruses from GT 1 replaced with GT 3 and GT 4 capsid protein were not infective to swine; in contrast, a recombinant GT 3 infectious clone with GT 4 capsid was able to infect domestic pigs (Feagins et al., 2011).

2.9 Prevention and control

2.9.1 Prevention and prophylaxis

In the developing countries good sanitation conditions such as access to clean water and sewerage systems are fundamental in the control of hepatitis E outbreaks.

For instance, the use of chlorination reduces the amount of fecal coliforms and contributes to the control of hepatitis E (Naik et al., 1992). In developed countries the consumption of raw or undercooked meat and meat products from swine, wild boar and deer should be avoided.

Few measures can be applied in order to prevent vertical transmission of HEV. The presence of HEV RNA and Anti-HEV IgG has been reported in colostrum, but HEV infected mothers can safely breastfeed. Close contact (mother-baby) should be avoided only if acute disease (with viremia) is present (Chibber et al., 2004; Kumar et al., 2001).

2.9.2 Vaccines

At least two distinct recombinant HEV vaccines went to clinical trials (Li et al., 2005a; Shrestha et al., 2007; Zhu et al., 2010).

One vaccine is based on a recombinant capsid protein expressed via the baculovirus system using *Spodoptera frugiperda* (Fall armyworm) cells and produced by GlaxoSmithKline®. The vaccine seems to be efficient in preventing hepatitis E (Shrestha et al., 2007), but it has been stated that there were no plans for further development or commercial use of the vaccine (Holmberg, 2010). In addition the design of the clinical trial has been a subject of criticism due to the bias such as predominance of young males, absence of children, pregnant women and patients with chronic liver disease (Goel and Aggarwal, 2011).

The apparently most promising vaccine is called “HEV 239” and is based on a recombinant peptide corresponding to aa 368 to 606 of the capsid protein of a genotype 1 isolate. It is expressed in bacterial cells (E. coli) and produced by Wantai Biological Pharmaceutical®, China (Li et al., 2005a). The vaccine has passed the clinical trials phase 2 and has been deemed safe and immunogenic in humans (Zhang et al., 2009). Recently the vaccine has undergone the phase 3 clinical trial and only a few mild adverse reactions were observed. According, “HEV 239” was well tolerated and efficient to prevent hepatitis E in the general adult population (Zhu et al., 2010). Later the vaccine was reported to be safe even for pregnant women and the fetus (Wu et al., 2011). It is expected that this vaccine will be available on the market soon.

3 Materials and Methods

3.1 Materials

3.1.1 Cells

	Origin
A549 (adenocarcinom human alveolar basal epithelial cells)	Institute of Virology, FB 10, JLU Gießen
***E. coli* TOP 10 (chemically competent cells)**	Invitrogen
***E. coli* K12 JM109**	competent *E. coli* cells, Institute of Virology, Gießen
Rosetta cells	Institute of Virology, Gießen
QIAGEN EZ Competent Cells	Qiagen

3.1.2 Virus and antibodies

	Origin
Hepatis E Virus (infected liver fragment)	Central Veterinary Institute of Wageningen University and Research Centre, The Netherlands, kindly provided by from Prof Dr. Wim van der Poel

	(Bouwknegt et al 2008)
Peroxidase Goat anti-Swine IgG	Dianova
HEV infected human serum	Virus diagnostic, UKGM, Gießen
Peroxidase Goat anti-Human IgG	Dianova
Anti-His Antibody	Institute of Virology, Gießen
Anti-Ubiquitin mAb	Institute of Virology, Gießen

3.1.3 Samples

3.1.3.1 Sera and fecal samples

A total of 105 fecal and 600 serum samples were collected between 2003 and 2006 in a previous survey in pigs throughout Germany (table 7). Additionally, 124 wild boar sera collected in 2008 for the Classical Swine Fever Virus survey from Hesse State were kindly provided by "Landesbetrieb Hessisches Landeslabor, Gießen" (table 6). Further 145 sera samples from semi-intensive wild boars from Morroco were collected. Information about the sample collection is shown in table 8.

3.1.3.2 Samples origin

Table 6: List of wild boar sera samples from Hesse State.

HEV Wild Boars		
Sample Identification		
Number	*Reference*	*Origin*
WB 1	U-445/1	Rheingau-Taunus-Kreis
WB 2	U-445/2	Rheingau-Taunus-Kreis
WB 3	U-445/3	Rheingau-Taunus-Kreis
WB 4	U-447/1	Rheingau-Taunus-Kreis
WB 5	U-447/2	Rheingau-Taunus-Kreis
WB 6	U-447/4	Rheingau-Taunus-Kreis
WB 7	U-447/7	Rheingau-Taunus-Kreis
WB 8	U-447/9	Rheingau-Taunus-Kreis
WB 9	U-447/10	Rheingau-Taunus-Kreis
WB 10	U-448/1	Rheingau-Taunus-Kreis
WB 11	U-448/2	Rheingau-Taunus-Kreis
WB 12	U-448/5	Rheingau-Taunus-Kreis
WB 13	U-471/1	Groß-Gerau
WB 14	U-471/2	Groß-Gerau
WB 15	U-471/3	Groß-Gerau
WB 16	U-472/2	Heppenheim
WB 17	U-472/4	Heppenheim
WB 18	U-472/5	Heppenheim
WB 19	U-515/1	Lahn-Dill-Kreis/Wetzlar
WB 20	U-515/2	Lahn-Dill-Kreis/Wetzlar
WB 21	U-601/3	Limburg

WB 22	U-682/3	Wiesbaden
WB 23	U-682/6	Wiesbaden
WB 24	U-686/3	Wiesbaden
WB 25	U-690/8	Lahn-Dill-Kreis/Wetzlar
WB 26	U-544/2	Frankfurt
WB 27	U-544/1	Frankfurt
WB 28	U-517/1	Frankfurt
WB 29	U-515/4	Lahn-Dill-Kreis/Wetzlar
WB 30	U-515/3	Lahn-Dill-Kreis/Wetzlar
WB 31	U-544/3	Frankfurt
WB 32	U-544/4	Frankfurt
WB 33	U-544/5	Frankfurt
WB 34	U-571/4	Marburg-Biedenkopf/Lahn
WB 35	U-571/5	Marburg-Biedenkopf/Lahn
WB 36	U-571/6	Marburg-Biedenkopf/Lahn
WB 37	U-601/1	Limburg
WB 38	U-601/2	Limburg
WB 39	U-654/5	Frankenberg
WB 40	U-738	Marburg
WB 41	U-739/2	Marburg
WB 42	U-690/9	Lahn-Dill-Kreis/Wetzlar
WB 43	U-690/13	Lahn-Dill-Kreis/Wetzlar
WB 44	U-691/2	Lahn-Dill-Kreis/Wetzlar
WB 45	U-692/6	Lahn-Dill-Kreis/Wetzlar
WB 46	U-692/11	Lahn-Dill-Kreis/Wetzlar
WB 47	U-654/1	Frankenberg
WB 48	U-654/2	Frankenberg
WB 49	U-654/3	Frankenberg
WB 50	U-654/4	Frankenberg
WB 51	U-474/2	
WB 52	U-747/10	Waldeck-Frankenberg
WB 53	U-766/3	Hochtaunuskreis
WB 54	U-766/7	Hochtaunuskreis
WB 55	U-766/12	Hochtaunuskreis
WB 56	U-766/15	Hochtaunuskreis
WB 57	U-804/2	Offenbach am Main
WB 58	U-804/4	Offenbach am Main
WB 59	U-747/11	Frankenberg
WB 60	U-766/1	Hochtaunuskreis
WB 61	U-739/6	Marburg
WB 62	U-740/2	Lahn-Dill-Kreis/Wetzlar
WB 63	U-740/3	Lahn-Dill-Kreis/Wetzlar
WB 64	U-1138/29	Hochtaunuskreis
WB 65	U-1138/33	Hochtaunuskreis
WB 66	U-1138/26	Hochtaunuskreis
WB 67	U-1138/15	Hochtaunuskreis
WB 68	U-1138/13	Hochtaunuskreis
WB 69	U-1138/10	Hochtaunuskreis

WB 70	U-1114/10	Frankenberg
WB 71	U-1114/13	Frankenberg
WB 72	U-1114/7	Frankenberg
WB 73	U-1114/5	Frankenberg
WB 74	U-1114/3	Frankenberg
WB 75	U-1087/43	Lahn-Dill-Kreis/Wetzlar
WB 76	U-1087/36	Lahn-Dill-Kreis/Wetzlar
WB 77	U-1087/21	Lahn-Dill-Kreis/Wetzlar
WB 78	U-1087/11	Lahn-Dill-Kreis/Wetzlar
WB 79	U-1087/10	Lahn-Dill-Kreis/Wetzlar
WB 80	U-950/2	Giessen
WB 81	U-950/1	Giessen
WB 82	U-971/14	Hochtaunuskreis
WB 83	U-971/3	Hochtaunuskreis
WB 84	U-971/15	Hochtaunuskreis
WB 85	U-971/16	Hochtaunuskreis
WB 86	U-971/19	Hochtaunuskreis
WB 87	U-971/18	Hochtaunuskreis
WB 88	U-971/22	Hochtaunuskreis
WB 89	U-1087/4	Lahn-Dill-Kreis/Wetzlar
WB 90	U-949/9	Lahn-Dill-Kreis/Wetzlar
WB 91	U-925/6	Marburg-Biedenkopf/Lahn
WB 92	U-925/1	Marburg-Biedenkopf/Lahn
WB 93	U-806/2	Groß-Gerau
WB 94	U-804/8	Offenbach am Main
WB 95	U-804/7	Offenbach am Main
WB 96	U-804/5	Offenbach am Main
WB 97	U-1022/4	Marburg
WB 98	U-1022/3	Marburg
WB 99	U-1022/2	Marburg
WB 100	U-1022/1	Marburg
WB 101	U-1114/1	Limburg
WB 102	U-1114/3	Limburg
WB 103	U-949/19	Lahn-Dill-Kreis/Wetzlar
WB 104	U-949/15	Lahn-Dill-Kreis/Wetzlar
WB 105	U-949/13	Lahn-Dill-Kreis/Wetzlar
WB 106	U-1088/3	Frankenberg
WB 107	U-1088/1	Frankenberg
WB 108	U-1034/22	Limburg
WB 109	U-1034/20	Limburg
WB 110	U-1034/16	Limburg
WB 111	U-1034/15	Limburg
WB 112	U-1034/12	Limburg
WB 113	U-1034/5	Limburg
WB 114	U-1041/9	Lahn-Dill-Kreis/Wetzlar
WB 115	U-1056/25	Rheingau-Taunus-Kreis
WB 116	U-1056/2	Rheingau-Taunus-Kreis
WB 117	U-1041/1	Lahn-Dill-Kreis/Wetzlar

WB 118	U-1036/2	Limburg
WB 119	U-1041/5	Lahn-Dill-Kreis/Wetzlar
WB 120	U-1036/1	Limburg
WB 121	U-1056/1	Rheingau-Taunus-Kreis
WB 122	U-1022/7	Marburg-Biedenkopf/Lahn
WB 123	U-1041/2	Lahn-Dill-Kreis/Wetzlar
WB 124	U-1041/15	Lahn-Dill-Kreis/Wetzlar

Table 7: List of domestic swine feces samples collected in Germany.

HEV Swine	
Sample Identification	
Number	*Reference*
1	KP63SW/M 10/04
2	KP105 - 5SW
3	KP72 SW-M5 3/1/04
4	KP103 SW-3 23.08.04
5	KP42 SW-JV 7891 - 1.10.03
6	KP92 SW-1 10.08.04
7	KP100 SW-5 16.08.04
8	KP108 SW-8 23.8.04
9	KP126SW
10	KP 34SW-JV243 1.3.08
11	KP 36SW JC376
12	KP37 SWJV377/01.10.03
13	KP54SW My5/11.02.04
14	KP98SW 3/16.8.04
15	KP106SW 23.8.04
16	KP112SW Schwarzweisschen
17	KP113SW Rosarot
18	KP122SW
19	KP23SW My 14/16.8.03
20	KP33SW JV186 1.10.03
21	KP43SW JV7892/1.10.03
22	KP52SW My3/11.2.04
23	KP55SW My6/11.2.04
24	KP60SW M552/03
25	KP97SW 2/16.8.04
26	KP117SW
27	KP58SW M626/03
28	KP124SW
29	KP75SW M53/2/04
30	KP24SW My 15/16.08.03
31	#18483/03 KP9SW
32	KP48SW 230/03 5
33	KP65SW M15/04
34	KP127SW
35	KP95 Sw 4/10.08.04
36	KP87SW Kw 2/2.6.04

37	KP120 SW
38	KP50SW My 1/11.2.04
39	KP12SW My 3/6.8.03 Kot sw
40	KP38SW JV378/1.10.03
41	KP32SW JV181/1.10.03
42	KP73SW M54/1/04
43	KP57SW M633/03
44	KP39SW JV379/1.10.03
45	KP101SW 1 Schwein 23.08.04
46	KP78SW K100/04
47	KP69 SW M24/04
48	KP67 SW 21/04
49	KP11SW My 2/6.8.03 Kot sw
50	KP21SW My12/4.8.03
51	KP102SW
52	KP81SW
53	KP56SW
54	KP35SW JV175/1.10.03
55	KP41SW JV7802/1.10.03
56	KP93
57	KP123
58	KP90
59	KP31
60	KP119
61	KP94SW
62	KP19SW
63	KP28SW
64	KP51SW
65	KP107
66	KP18
67	KP89
68	KP46
69	KP91
70	KP16
71	KP79
72	KP121
73	KP25
74	KP76SW
75	KP86SW
76	KP26SW
77	KP10SW
78	KP59SW
79	KP80SW
80	KP45SW
81	KP68SW
82	KP30SW
83	KP77SW
84	KP15SW

85	KP29SW
86	KP88SW
87	KP61SW
88	KP74SW
89	KP14SW
90	KP99SW
91	KP13SW
92	KP40SW
93	KP125SW
94	KP17
95	KP22
96	KP96
97	KP27
98	KPSw98
99	KPSw99
100	KPSw100
101	KPSw101
102	KPSw102
103	KPSw103
104	KPSw104
105	KPSw105

Table 8: List of serum samples of semi-intensive wild boar from Morocco.

HEV Wild boar (Morocco)			
Reference	*Sex*	*Date of birth*	*Date of collection*
A (9F01)	Female	Jan 2009	21.03.2010
B (9F02)	Female	Jan 2009	21.03.2010
C (9F03)	Female	Jan 2009	21.03.2010
D (9F04)	Female	Jan 2009	21.03.2010
E (9F05)	Female	Jan 2009	21.03.2010
F (9F06)	Female	Jan 2009	21.03.2010
G (9F07)	Female	Jan 2009	21.03.2010
H (9F08)	Female	Jan 2009	21.03.2010
I (9F09)	Female	Jan 2009	21.03.2010
J (9F10)	Female	Jan 2009	21.03.2010
K (9F11)	Female	Jan 2009	21.03.2010
L (9F12)	Female	Jan 2009	21.03.2010
M (9F13)	Female	Jan 2009	21.03.2010
N (9F14)	Female	Jan 2009	21.03.2010
O (9F15)	Female	Jan 2009	21.03.2010
P (9F16)	Female	Jan 2009	21.03.2010
Q (9F17)	Female	Jan 2009	21.03.2010
R (9F18)	Female	Jan 2009	21.03.2010
S (9F19)	Female	Jan 2009	21.03.2010
T (9F20)	Female	Jan 2009	21.03.2010
U (9F21)	Female	Jan 2009	21.03.2010

V (9F22)	Female	Jan 2009	21.03.2010
W (9F23)	Female	Jan 2009	21.03.2010
X (9F24)	Female	Jan 2009	21.03.2010
Y (9F25)	Female	Jan 2009	21.03.2010
Z (9F26)	Female	Jan 2009	21.03.2010
AA (9F27)	Female	Jan 2009	21.03.2010
AB (9F28)	Female	Jan 2009	21.03.2010
AC (9F29)	Female	Jan 2009	21.03.2010
AD (9F30)	Female	Jan 2009	21.03.2010
AE (9F31)	Female	Jan 2009	21.03.2010
AF (9F32)	Female	Jan 2009	21.03.2010
AG (9F33)	Female	Jan 2009	21.03.2010
AH (9F34)	Female	Jan 2009	21.03.2010
AI (8F01)	Female	Apr 2008	21.03.2010
AJ (8F02)	Female	Apr 2008	21.03.2010
AK (8F03)	Female	Apr 2008	21.03.2010
AL (8F04)	Female	Apr 2008	21.03.2010
AM (8F05)	Female	Apr 2008	21.03.2010
AN (8F06)	Female	Apr 2008	21.03.2010
AO (8F07)	Female	Apr 2008	21.03.2010
AP (8F08)	Female	Apr 2008	21.03.2010
AQ (8F09)	Female	Apr 2008	21.03.2010
AR (8F10)	Female	Apr 2008	21.03.2010
AS (8F11)	Female	Apr 2008	21.03.2010
AT (8F12)	Female	Apr 2008	21.03.2010
AU (8F13)	Female	Apr 2008	21.03.2010
AV (8F14)	Female	Apr 2008	21.03.2010
AW (8F15)	Female	Apr 2008	21.03.2010
AX (8F16)	Female	Apr 2008	21.03.2010
AY (8F17)	Female	Apr 2008	21.03.2010
AZ (8F18)	Female	Apr 2008	21.03.2010
BA (8F19)	Female	Apr 2008	21.03.2010
BB (8F20)	Female	Apr 2008	21.03.2010
BC (8F21)	Female	Apr 2008	21.03.2010
BD (8F22)	Female	Apr 2008	21.03.2010
BE (8F23)	Female	Apr 2008	21.03.2010
BF (8F24)	Female	Apr 2008	21.03.2010
BG (8F25)	Female	Apr 2008	21.03.2010
BH (9F01)	Female	Jan 2009	21.03.2010
BI (9F02)	Female	Jan 2009	21.03.2010
BJ (9F03)	Female	Jan 2009	21.03.2010
BK (9F04)	Female	Jan 2009	21.03.2010
BL (9F05)	Female	Jan 2009	21.03.2010
BM (9F06)	Female	Jan 2009	21.03.2010

BN (9F07)	Female	Jan 2009	21.03.2010
BO (9F08)	Female	Jan 2009	21.03.2010
BP (9F09)	Female	Jan 2009	21.03.2010
BQ (9F10)	Female	Jan 2009	21.03.2010
BR (9F11)	Female	Jan 2009	21.03.2010
BS (9F12)	Female	Jan 2009	21.03.2010
BT (9F13)	Female	Jan 2009	21.03.2010
BU (9F14)	Female	Jan 2009	21.03.2010
BV (9F15)	Female	Jan 2009	21.03.2010
BW (9F16)	Female	Jan 2009	21.03.2010
BX (9F17)	Female	Jan 2009	21.03.2010
BY (9F18)	Female	Jan 2009	21.03.2010
BZ (9F19)	Female	Jan 2009	21.03.2010
CA (9F20)	Female	Jan 2009	21.03.2010
CB (9F21)	Female	Jan 2009	21.03.2010
CC (9F22)	Female	Jan 2009	21.03.2010
CD (9F23)	Female	Jan 2009	21.03.2010
CE (9F24)	Female	Jan 2009	21.03.2010
CF (9F25)	Female	Jan 2009	21.03.2010
CG (9F26)	Female	Jan 2009	21.03.2010
CH (9F27)	Female	Jan 2009	21.03.2010
CI (9F28)	Female	Jan 2009	21.03.2010
CJ (9F29)	Female	Jan 2009	21.03.2010
CK (9F30)	Female	Jan 2009	21.03.2010
CL (9F31)	Female	Jan 2009	21.03.2010
CM (8F01)	Female	Oct 2008	22.03.2010
CN (7F01)	Female	2007	22.03.2010
CO (8F02)	Female	Oct 2008	22.03.2010
CQ (7F01)	Female	Apr 2007	22.03.2010
CR (7F02)	Female	Apr 2007	22.03.2010
CS (7F03)	Female	Apr 2007	22.03.2010
CU (8F12)	Female	Apr 2008	22.03.2010
CV (7F05)	Female	Apr 2008	22.03.2010
CW (8F01)	Female	Apr 2008	22.03.2010
CX (9F01)	Female	Jan 2009	22.03.2010
CY (8F02)	Female	Apr 2008	22.03.2010
CZ (8F03)	Female	Apr 2008	22.03.2010
DA (8F04)	Female	Apr 2008	22.03.2010
DB (8F05)	Female	Apr 2008	22.03.2010
DC (9F02)	Female	Jan 2009	22.03.2010
DD (8F06)	Female	Apr 2008	22.03.2010
DE (8F07)	Female	Apr 2008	22.03.2010
DG (9F03)	Female	Jan 2009	22.03.2010
DH (8F09)	Female	Apr 2008	22.03.2010

DI (8F10)	Female	Apr 2008	22.03.2010
DJ (8F11)	Female	Apr 2008	22.03.2010
DK (9F04)	Female	Jan 2009	22.03.2010
DL a (9F05)	Female	Jan 2009	22.03.2010
DM (7F02)	Female	Apr 2007	22.03.2010
DN (7F04)	Female	Apr 2007	22.03.2010
DO (7F01)	Female	2007	22.03.2010
DP (7F02)	Female	2007	22.03.2010
DR (7F03)	Female	2007	22.03.2010
DS (9F02)	Female	Jan 2009	22.03.2010
DT (9F01)	Female	Aug 2009	22.03.2010
DU (9F02)	Female	Aug 2009	22.03.2010
DV (9F03)	Female	Aug 2009	22.03.2010
DW (9F04)	Female	Aug 2009	22.03.2010
DX (9F05)	Female	Oct 2009	22.03.2010
DY (9F06)	Female	Oct 2009	22.03.2010
DZ (9F07)	Female	Oct 2009	22.03.2010
EB (5317)	Male	Jan 2009	22.03.2010
EC (3089)	Male	Jan 2009	22.03.2010
EE (9283)	Male	Jan 2009	22.03.2010
EF (6252)	Male	Jan 2009	22.03.2010
EG (4366)	Male	Jan 2009	22.03.2010
EH (5463)	Male	Jan 2009	22.03.2010
EI (5661)	Male	Dec 2008	22.03.2010
EJ (1981)	Male	Jan 2009	22.03.2010
EL (3067)	Male	Mar 2009	22.03.2010
EM (0270)	Male	Jan 2009	22.03.2010
EN (5389)	Male	Jan 2009	22.03.2010
EO (5914)	Male	Jan 2009	22.03.2010
EP (1940)	Male	Apr 2008	22.03.2010
ET (5519)	Male	Apr 2008	22.03.2010
EV (5676)	Male	Apr 2008	22.03.2010
EZ (6313)	Male	Apr 2008	22.03.2010
FD (9748)	Male	Apr 2007	22.03.2010

3.1.3.3 Liver samples (hepatocytes)

Liver samples were collected in the slaughterhouse in Giessen.

3.1.4 Enzymes and enzyme buffers

Buffer 1 New England Biolabs

Buffer 2 New England Biolabs

Buffer 3 New England Biolabs

Buffer 4 New England Biolabs

Buffer ECO RI	New England Biolabs
ECO RI	New England Biolabs
ECO RV	New England Biolabs
Spe I	New England Biolabs
AlwN1	New England Biolabs
BamHI	New England Biolabs
SacII	New England Biolabs
AleI	New England Biolabs
T4-DNA-Ligase	New England Biolabs
T4-Ligase Puffer	New England Biolabs
Superscript II RNAse H reverse Transkriptase 200 U/µl	Invitrogen

3.1.5 Prefabricated media and kits

QIAamp Viral RNA Mini Kit	Qiagen
Rneasy Mini Kit	Qiagen
One Step RT-PCR Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAex II Extraction Kit	Qiagen
TOPO TA Cloning Kit	Invitrogen
NucleoBond® Xtra Midi Kit (Midipred)	Macherey-Nagel

3.1.6 Reagents and chemicals

1 Kb DNA ladder	Invitrogen
100 bp DNA ladder	Invitrogen
Acrylamide	Fluka
Agar-Agar	Difco
Agarose NEEO ultra-quality	Roth
AgarPlaquePlus®Agarose	BD Biosciences
	Pharmingen
Amonium persulfate	AmpliChen
Ampicillin	Serva
Bacto Tryptone	Difco
Bovine Serum Albumin (BSA)	Gibco-BRL

Red Bromophenol	Sigma
Dimethyl sulfide (DMSO)	Sigma
Deoxynucleotide triphosphastes (dNTPs)	Roth
Ethidium bromide	Roth
Ethanol	Roth
Ethylenediaminetetraacetic acid (EDTA)	Roth
Glycine	Roth
Isopropanol	Roth
Methylamine wolfram	Plano
β-mercaptoethanol	Merk
Octyl glucoside	Fluka
Penicillin/Streptomycin	Sigma
Phenol red	Riedel-de Haen AG
Sodium dodecyl sulfate (SDS)	ICN
Tetramethylethylenediamine (TEMED)	Boehringer Mannheim
Tris(hydroxymethyl)aminomethane (TRIS)	Boehringer Mannheim
Triton X-100	Fluka
Trypan blue	Serva
Polysorbate 20 (Tween 20)	Fluka
Ultra Pure Sequa Gel Complete Buffer	National Diagnostics
Urea	United States Biochemical

3.1.7 All-purpose medium and buffers

Ca/Mg Solution	0,1 g/l CaCl2 x H2O, 1,16 g/l MgCl2 x 6H2O, 1,15 g/l Na2HPO4 x 2H2O in 950 ml dissolved in dd Water, autoclaved, stored at 4°C
Deficient PBS (without Ca and Mg Chloride)	0,8 g/l NaCl, 0,2 g/l KCl, 0,2 g/l KH2PO4 x H2O, 1,15 g/l Na2HPO4 x H2O in 950 ml dissolved in dd Water, autoclaved, stored at 4°C
PBS++	950 ml deficient PBS with addition of 50 ml Ca/Mg solution

3.1.8 Media and buffers for cell culture

Freezing medium for	10% (v/v) DMSO in FBS

cryopreservation of cells	
Fetal Bovine Serum (FBS)	PAA Laboratories
Penicillin/Streptomycin 500X	50.000 UI/ml Penicillin G, 50 mg/ml Streptomycinsulfat; dissolved in Aqua dd; sterile filtered (0,2 µm), and stored in 10 ml aliquots at -20°C
Amphotericin 500X	1.25 mg/ml (100 mg / 80 ml) dissolved in Aqua dd; sterile filtered (0,2 µm) and stored in 10 ml aliquots at -20°C
Trypan blue	0,25% (w/v) Trypan blue, 0,15 M NaCl in Aqua dd, filtered (0,2 µm), and stored at 4°C
Trypsin Solution	8 g/l NaCl, 0,2 g/l KCl, 1,44 g/l Na2HPO4 x 2H2O, 2,5 g/l Trypsin, 1:300, 0,16 g/l Red Phenol dissolved in dd water, sterile filtered (0,1 Mm), and stored at 4°C
Cell Culture Medium CCM-34	4,5 g/l DMEM-Powder, 200 µM L-Alanin, 225 µM L-Aspartat, 933 µM Glycin, 510 µM L-Glutamat, 217 µM L-Prolin, 184 µM Hypoxanthin, 0,1 mg/l Biotin, 44 mM $NaHCO_3$, sterile filtered, store at 4°C
Maintenance Medium	Cell Culture Medium, 10% FCS, 1 ml Penicillin (500X), 1ml Streptomycin (500X) and 1 ml Amphotericin (500X)

3.1.9 Buffer for reverse transcription (RT) and polymerase chain reaction (PCR)

PCR Water (Roth)	double de-ionized water
Buffer VIIIA (2,5 x)	125 mM Tris (pH 8,3), 187,5 mM KCl, 7,5 mM MgCl2, 25 mM DTT, 1,25 mM dNTPs; -20°C
Buffer VIIIB (5 x)	25 mM Tris (pH 8,3), 100 mM KCl, 6,5 mM $MgCl_2$, 1,25 mM dNTPs, 0,5% Triton X 100, 0,1% BSA; -20°C
TE-Buffer	10 mM Tris-HCl, 1 mM EDTA, in Aqua dd diluted, pH 7,6
10 x PCR-Puffer	NatuTec

Gel loading solution	Orange G: (0,25%) 2,5 ml Orange G 1%, 3,0 ml Glycerin, 4,5 ml H_2O DNA loading buffer for agarose gels: 0,1% (w/v) Orange G, 5% (w/v) Ficoll 400 in 5 x TAE buffer, 4°C
100bp DNA ladder	Invitrogen
1kb DNA ladder	Invitrogen
Rothiphorese® 50x TAE Buffer	Roth
Deoxynucleoside Triphosphate Set PCR	Roche

3.1.10 Solutions for DNA cloning

LB Medium	10 g/l Bacto-Trypton, 5 g/l Hefeextrakt, 8 g/l NaCl, dissolved in Aqua dd , pH 7,5 (with NaOH), autoclaved, stored at 4°C
LB Medium Ampicillin	500 ml LB-Medium, 100µg/ml Ampicillin
LB Medium Kanamycin	500 ml LB-Medium, 100µg/ml Kanamycin
SOC Medium	2% Trypton, 0,5% yeast extract, 10 mM NaCl, 2,5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose
X-Gal Solution	40 mg/ml X-Gal (5-bromo-4-chloro-indolyl-galactopyranoside), dissolved in DMF (Dimethylformamid)

3.1.11 Solutions for SDS PAGE and PAGE-staining

Semi Dry Blot Buffer	14 µg Glycine, 3.7µg Tris, 200 ml methanol (for 1 litre)
4 x Protein Loading buffer	250 mM Tris-HCl pH 6,8, 8% (w/v) SDS, 6 M Urea, 0,004% (w/v) Blue Bromphenol, 0,004% (w/v) Red Phenol, 40% (v/v) Glycerin, filtered, stored in 4 ml Aliquots at -20°C . for reducing conditions: add 5% (v/v) 2-Mercaptoethanol or 10mM DTT.
10% Jagow-Mini Gel separating gel	2,5 ml acrylamide, 3,3 ml Jagow-gel buffer, 3,6 ml Aqua dd, 0,5 ml Glycerine 87%, 50 µl APS 10%, 5 µl TEMED

4% Jagow-Mini Gel separating gel	1 ml acrylamide, 2,5 ml Jagow gel buffer, 6,4 ml Aqua dd, 80 µl APS 10%, 10 µl TEMED
10 x Anode buffer	2 M Tris-HCl, pH 8,9
10 x Cathode buffer	1 M Tris-HCl, 1 M tricine, 1% SDS, pH 8,25
Coomassie staining solution	2,5 g Serva Blue, 454 ml methanol, 92 ml glacial acetic acid, fill up to 900 ml, then add 100 g of Trichloroacetic acid (TCA)
Coomassie destaining solution	10% (v/v) acetic acid, 30% (v/v) methanol in Aqua dd
1 M NaCl in PBS++	58,44 g NaCl in 1l PBS++
SeeBlue® Plus2 Pre-Stained Standard	Invitrogen
Western Lightning® Chemiluminescense Reagent Plus	Perkin Elmer

3.1.12 Buffers for protein purification (500 ml)

Table 9: Preparation for the different buffers (FPLCA, FPLCB, FPLCA-urea, FPLCB-urea and FPLC lyse) used for protein purification. The values in () indicates the amount which should be add on the buffer to produce 500 ml.

Buffer	Protocol
FPLCA	300 mM NaCl (8.77 g), 50 mM Na_2HPO_4 (4.45 g)
FPLCB	300 mM NaCl (8.77 g), 50 mM Na_2HPO_4 (4.45 g), 500 mM Imidazol (17.02 g)
FPLCA urea	8M Urea (240.2 g), 300 mM NaCl (8.77 g), 50 mM Na_2HPO_4 (4.45 g)
FPLCB urea	8M Urea (240.2 g), 300 mM NaCl (8.77 g), 50 mM Na_2HPO_4 (4.45 g), 500 mM Imidazol (17.02 g)
FPLC lyse	300 mM NaCl (8.77 g), 50 mM Na_2HPO_4 (4.45 g), 1% Triton X-100 (5 ml)

3.1.13 Consumables

Filter paper	Whatman
Cell culture plates	Falcon
Cell culture bottles	Falcon
Gloves (Rotiprotect® Latex und Nitril)	Roth
Pipette Tips	Biozym
RNAse free pipet tips	Kisker Biotech
Polypropylene tubes	Eppendorf
X-films BioMaxMR	Kodak

0.22 µl and 0.45 µl sterile filters	Fisher
500 ml 0.22 µl filter	Nalgene – Thermo Scientific

3.1.14 Instruments and equipment

Analytical balance	Sartorius
Bacteria Shaker	Heraeus
Cell Culture Incubator (with CO_2)	Forma Scientific
Digital printer	Sony
X-ray developer machine	Protec
Freezers	Liebherr, Bosch
Refrigerators	Liebherr
One channel pipette 2 µ, 10 µl, 20 µl, 200 µl, 1000 µl	Biohit, Gilson
Gel eletrophoresis chamber	Institute of Virology, Gießen/Bio-Rad
Gel Chambers	Institute of Virology, Gießen
Combs	Institute of Virology, Gießen
Gel documentation printer	Mitsubishi – Intas
Nanovue plus	GE Healthcare Life Sciences
Centrifuge Kendro Megafuge 1,0R	Heraeus
Centrifuge (4°C) Biofuge Fresco	Heraeus
Centrifuge (without cooling) Biofuge 13	Heraeus
PCR cabinet Lamin Air/PCR Mini Typ HVPCR	Holten
Cell Culture Microscope	Zeiss
Heat block	Institute of Virology, Gießen
Minishaker MS1 (Vortex)	IKA
Water bath	Memmert GmbH & Co. KG
Termocycler	Applyed Biosystems
Realtime	Applyed Biosystems

Laminar Flow	Thermo Scientific
UV light box	Bachofer

3.1.15 Oligonucleotides

Primers were both collected from selected publications or designed using PrimerExpress® (ABI). For design primers were based on an alignment of complete HEV sequences containing all different HEV genotypes and subtypes available at that time. Degenerated primers were designed using IUPAC symbols (table 10). Primer storage concentration was 50 pmol/µl.

Table 10: IUPAC Symbols for degenerated bases.

Description	Bases	Description	Bases
R=	A+G	**Y=**	C+T
M=	A+C	**K=**	G+T
W=	A+T	**S=**	G+C
H=	A+T+C	**D=**	G+A+T
B=	G+T+C	**V=**	G+A+C
N=	A+T+G+C		

3.1.15.1 Diagnostic

For the molecular diagnostic of HEV different primers were employed, either self-designed or from literature. Primers F1, R1, F2 and R2 amplify regions of 404 b and 266 b, respectively, of the ORF2 region. (Lee et al., 2007; Meng et al., 1998). All primers used are described in the tables below.

3.1.15.2 Literature

Table 11: Primer used for HEV detection extract from the literature.

Primer Name	Sequence	Reference
	1st round: 404 bp; 2nd round:266 bp	(Lee et al., 2007; Meng et al., 1998c)
F1	AGCTCCTGTACCTGATGTTGACTC	
R1	CTACAGAGCGCCAGCCTTGATTGC	
F2	GCTCACGTCATCTGTCGCTGCTGG	
R2	GGGCTGAACCAAAATCCTGACATC	
	1st round: 730 bp; 2nd round:347 bp	(Meng et al., 1997b)
3156-EF	AAYTATGCMCAGTACCGGGTTG	
3157-ER	CCCTTATCCTGCTGAGCATTCTC	
3158-EF	GTYATGYTYYGCATACATGGCT	
3159-IRS	AGCCGACGAAATYAATTCTGTC	
	1st round: 197 bp; 2nd round:145 bp	(Erker et al., 1999)
HEVORF2con-s1	GACAGAATTRATTTCGTCGGCTGG	
HEVORF2con-a1	CTTGTTCRTGYTGGTTRTCATAATC	

HEVORF2con-s2	GTYGTCTCRGCCAATGGCGAGC	
HEVORF2con-a2	GTTCRTGYTGGTTRTCATAATCCTG	

3.1.15.3 Self-designed

Table 12: Self-designed primers for HEV detection.

Primer name	Sequence
	1st round: 142 bp; 2nd round: 90 bp
HEV01F	TATG**Y**TGCCCGCGCCA
HEV01R	AAAGGGGTTGGTTGGATGAA
HEV02F	CCGG**Y**CAGTCGCCTGG
HEV02R	GCGAAGGGCTGAGAATCAAC
	1st round: 191 bp; 2nd round: 143 bp
Avian-1F	GCTCATGCTTGCWATGTGCTGC
Avian-1R	TCTACATCTGGTACCGTGCGAGT
Avian-2F	GTGTCAAGGGGCTCCCAAAC
Avian-2R	ACCTGCCGCGGTGACAAC

3.1.15.4 Sequencing

Table 13: Primers designed for amplify the complete ORF2 and ORF3 for sequencing.

Primer name	Sequence	Amplicon size
	ORF2/3	
HEVcF1	GTTGCGCAGGTTTGTGTTGA	591 bp
HEVcR1	CCACGTGAATCTACATCAGGTACAG	
HEVcF2	CGGTCCTGCTCRTGTTGGTT	551 bp
HEVcR2	AGAAGCCCCAGTGCACCA	
HEV7F	TGAGACCTCTGGTGTGGCBG	570 bp
HEV7R	CGGTCCTGCTCRTGTTGGTT	
HEV1306F	TCCCGCGTGGTTATTCAG	678 bp
HEV1983R	TTAAGACTCCCGGGTYTTACCTA	

Table 14: Self-designed primers for amplify the complete ORF 1.

Primer name	Sequence	Amplicon size
	ORF1	
orf1HEV-1F	ATGGTGGAGAAGGGHCAGGA	871 bp
orf1HEV-1R	ATCAACACAAACCTGCGCAACA	
ORF1-2F	TGGTGGCACGTTACACACC	831 bp
ORF1-2R	TCTCCACCATGGCCTCAAC	
ORF1-3F	ATGGAGGCCCATCAGTTCATTAA	974 bp
ORF1-3R	AGCATGAGCCGATCCCA	
ORF1-4F	AAGGTGTATGCGGGGTCATTG	919 bp
ORF1-4R	AGCTCACACACATCAGCCGG	
ORF1-5F	CTATATTTGGTCCTGGCGGC	842 bp

ORF1-5R	GTCTTATTACCRAGCACAGTRCGGCACTC	
ORF1-6F	ACTGTTGAACTCGTTGCAGG	938 or 820 bp
ORF1-6R	TGGTAAAAAGCATGGCAGAG	
HEV ORF1f2-R	AGGTGTAGAGGAGACGACGA	
ORF1_2340F	CGTAAGCCGTCAACACCCC	157 bp
ORF1_2496R	GACAGAGACCACCCCCGG	

3.1.15.5 Primers for sequencing

Table 15: Primers for sequencing targeting M13 and T7 regions found into plasmid vectors (pCR2.1, pdrive and pET 26b).

Name	Sequence
M13 Forward	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
T7 Prom	TAATACGACTCACTA
T7 Term	GCTAGTTATTGCTCA

3.1.15.6 Expression

Table 16: Primers designed for assembling selected regions of HEV ORF2 into the pET 26b+ vector.

Primer name	Sequence
CapForBamHI	**GGATCC**CGCCCTAGGGCTGTTCT
Cap22ForBamHI	**GGATCC**CACTATCGTAATCAGGGCTG
CapRevHindIII	**AAGCTT**GACTCCCGGGTTTTACC**TACCTT**
expR4F	GATCGACCGCGGAATGCTACTCCGTCACCTGCCC
expR4R	CTGCATGGATCCCGGGGTCGTCAGCAGTAT
HEVexp-1180-F	GATCGACCGCGGACAACTGTTTTACTCCCGCC
HEVexp-827R	CTGCATGGATCCAAGACTCCCGGGTTTTACCTA
Cap21EXP-F	GATCGACCGCGGTATGCGCCCTAGGGCTGTT
Cap21EXP-R	CTGCATGGATCCCAGAGCATTACCAGACCAGAAG
Cap22EXP-F	GATCGACCGCGGTATGCACTATCGTAATCAG
Cap22EXP-R	CTGCATGGATCCAGACTCCCGGGTTTTACCTA

3.1.16 Real-time

For the PCR quantification a real-time assay was developed only for a specific strain (DQ996399) in order to evaluate the cell culture infection experiments. Probe (5'-CCCGCCCGGTCGTCTCAGC-3') and primers HEV-RT01F (5'-GGCGAGCCGACTGTCAAGT-3') and HEV-RT02R (5'-CGTGTGGTATAGCAATGCCCTTA-3') (Figure 6).

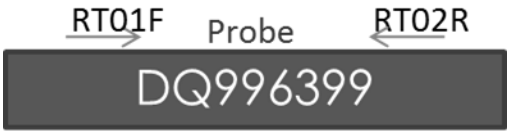

Figure 6: Specific primers and probes for sequence DQ996399.

Another real-time assay was employed in order to evaluate the infection of both cell culture and primary hepatocytes with a different viral strain. Primers JVHEVF (5'-GGTGGTTTCTGGGGTGAC-3') JVHEVR (5'-AGGGGTTGGTTGGATGAA-3') and probe JVHEVP (5'-TGATTCT CAGCCCTTCGC-3') were extracted from the literature (Jothikumar et al., 2006). This was performed in CVI Lelystad, the Netherlands.

3.2 Methods

3.2.1 Nucleic acid extraction

3.2.1.1 Sera, cell supernatants and fecal suspensions

Fecal samples were individually diluted at 1:10 PBS (1 g in 10 ml) and shaken for 15 minutes at room temperature. After that samples were centrifuged at 4000 RPM (2772 x g) for 10 minutes at 4 °C the supernatants were collected and stored at -20 °C. RNA was extracted from 140 µl of fecal suspension (swine), sera and cell supernatant using the QIAamp Viral RNA Mini Kit (Qiagen®) following the manufacturer's instructions.

This kit is based on the principle of the RNA binding to the silica membrane. 140 µl of serum and cell or fecal suspension were added to 560 µl lysis buffer (AVL) containing 5.6 µl RNA carrier and incubated for 10 minutes at room temperature. This step is necessary in order to inactivate the RNAses and to ensure the isolation of pure RNA. The presence of the RNA carrier improves the binding of the RNA to the membrane, which is especially important when dealing with samples with low amount of RNA. After the incubation time 560 µl of ethanol was added, the mixture carefully pipetted and applied to the columns containing the silica membrane (QIAamp mini spin Column, Qiagen®). Afterwards a centrifugation at 6000 x g (8000 RPM) for 1 minute was performed, followed by two washing steps with buffers AW1 and AW2 (8000 RPM for 1 minute and 14000 RPM for 3 minutes, respectively). For elution 50 µl of AVE buffer was added and incubated for at least 1 minute. Columns were centrifuged for 6000 x g for 1 minute and RNA stored at -20°C until use.

3.2.1.2 RNA extraction from cultivated cells

RNA was extracted using RNeasy kit (Qiagen) following manufacturer's instructions. Cell supernatants (cell culture medium) were removed from each well of the plate and cells were re-suspended directly with 600 µl of lysis buffer (RTL). The lysate were shortly whirled with a vortex and displaced in the QIAshredder column and centrifuged for 2 min at full speed. One volume of ethanol 70% (600 µl) was added to each homogenized lysate cells solution and mixed with pipette. It was than displaced in the RNeasy spin column (up to 700 µl) and centrifuged for 15 seconds at 10000 RPM. The flow-through was discarded and each column was washed with 700 µl of RW1 buffer, again followed by centrifugation for 15 s at 10000 RPM. The columns were washed two times with 500 µl of RPE buffer and centrifuged for 15 s and 2 min, respectively, at 10000 RPM. Column was disposed in a fresh 1.5 polypropylene tube (Eppendorf®) and RNA was eluted with 50 µl of RNAse free water placed directly to the spin membrane. After incubation of 1 min column was centrifuged for 1 min at 10000 RPM. RNA was stored at -20°C.

3.2.1.3 DNA extraction/purification

3.2.1.3.1 Phenol-chloroform method.

Before starting it is important to emphasize that with this technique it is desirable to work with large amount of DNA (> 5 µg). In addition the safety recommendation to work with phenol and chloroform should be followed.

One volume of Phenol (100-400 µl) was added to the sample and mixed by carefully inverting the tube several times followed by centrifugation for 4 minutes at 13000 RPM. The formation of two phases should be observed and the upper phase collected and pipetted in a fresh tube.

One volume of Chloroform was added and centrifuged (4 min/13000 RPM). Afterwards the upper phase was collected and disposed in a new tube. 1 ml of 100% Ethanol was added and centrifuged. Carefully the supernatant was discarded, 70% Ethanol added and the tube centrifuged. Lastly the supernatant was discarded and the DNA carefully eluted using 50 µl of RNAse free water.

3.2.2 Nucleic acid amplification

3.2.2.1 Reverse transcription polymerase chain reaction (RT-PCR)

3.2.2.1.1 Reverse transcription

In the reverse transcription the RNA will be transformed into DNA with the use of the enzyme reverse transcriptase. First a master mix was prepared using RNAse free water and reverse primer in a 0.2ml tube. RNA template (2.5 µl) was added to 9 µl of the start mix and denaturized for 3 min at 94º. After cooling down to 4°C and 8.5 µl reverse transcriptase mix was added and incubated for 30 minutes at 45ºC and 80ºC for 2 minutes (table 17). At the end of the reaction cycle the mixture was chilled at 4° C. Following the RT-PCR program described on table 19.

Table 17: Reagents and protocol for reverse transcription.

Start mix	Reverse transcription mix
8.5 µl H2O	8 µl Buffer A
0.5 µl reverse primer	0.25 µl Reverse transcriptase
	0.25 µl RNAse inhibitor
9 µl	8.5 µl

3.2.2.1.2 Polymerase chain reaction (PCR)

The PCR is a molecular technique to amplify copies of a certain DNA fragment. It consists in several cycles of denaturation, annealing and elongation. After DNA denaturation to separate the double strand, the primers will bind or anneal to a specific region in the DNA at a determined temperature. Afterwards with aid of taq polymerase enzyme the second DNA strand will be synthesized complementary to the first strand. These three steps will be repeated several times (cycles) in the interest of increase the amount of DNA molecules.

After the reverse transcription 30 µl of the PCR mix were added to the 0.2ml tube containing the cDNA. Following, a nested PCR was performed using another PCR mix (table 18). For the nested PCR 2.5µl of the HEV PCR reaction was added into 47.5µl of the nested PCR reaction mix (table 18) and following the HEV nested program described on table 19.

Table 18: Protocols for PCR after reverse transcription and nested-PCR.

Amount	Reagent
PCR	
0.5	µl x ***n*** Primer **HEV-F1**
10	µl x ***n*** Buffer VIIIb
19.3	µl x ***n*** Aq dd

0.2	µl x ***n*** Polymerase
Nested	
0.5	µl x ***n*** Primer **HEV-F2**
0.5	µl x ***n*** Primer **HEV-R2**
5	µl x n Buffer 10X
0.4	µl x n dNTP-Mix
0.2	µl x ***n*** Polymerase
40.9	µl x ***n*** Aq dd

3.2.2.1.2.1 PCR programs

Table 19: PCR programs employed for amplification of HEV RNA.

Name	Denaturation	Annealing	elongation	Cycles
HEV PCR	94°C /30 sec	55°C/30 sec	72°C/30 sec	40
HEV NESTED	94°C /30 sec	55°C/30 sec	72°C/30 sec	40

3.2.2.2 OneStep RT-PCR

For the OneStep RT-PCR (Qiagen) a ready to use PRC setup was perfomed according to the manufacturer instructions adding 0.25µl of RNase inhibitor in each reaction (table 20) plus 0.6 µl of each primer (50pmol). Each setup has been performed according to the PCR programs described on table 21.

Table 20: Protocol for OneStep RT-PCR.

Amount	Reagent
10.0	µl x 5x QIAGEN OneStep RT-PCR Buffer
2.0	µl x dNTP Mix
2.0	µl x QIAGEN OneStep RT-PCR Enzyme Mix
0.25	µl x RNase inhibitor
29.55	µl x RNAse free water

Table 21: Different PCR programs employed in the amplification of HEV RNA using the OneStep kit.

Region	Denaturation	Annealing	elongation	Cycles	Amplicon size
Capsid (ORF 2 and ORF 3)					
R1	94°C /1 min	57°C/30 sec	72°C/1 min	40-45	590
R2	94°C /30 sec	56°C/30 sec	72°C/1 min	40	550
R3	94°C /30 sec	55°C/30 sec	72°C/1 min	40	570
R4	94°C /30 sec	55°C/1 min	72°C/1 min	40	677
ORF2.1	94°C /30 sec	54°C/1 min	72°C/1 min	40	843
ORF2.2	94°C /30 sec	55°C/1 min	72°C/1.3 min	40	1230
ORF 1					
ORF1-A	94°C /30 sec	54°C/30 sec	72°C/1 min	40	871
ORF1-B	94°C /30 sec	54°C/30 sec	72°C/1 min	40	831
ORF1-C	94°C /30 sec	54°C/30 sec	72°C/1 min	40	974
~~**ORF1-D**~~	~~94°C /30 sec~~	~~55°C/1 min~~	~~72°C/1 min~~	~~40~~	~~919~~
ORF1-D2	94°C /30 sec	55°C/1 min	72°C/1 min	40	862

ORF1-E	94°C /30 sec	56°C/30 sec	72°C/1 min	40	842
~~**ORF1-F**~~	~~94°C /30 sec~~	~~53°C/1 min~~	~~72°C/1 min~~	~~40~~	~~938~~
ORF1-F2	94°C /30 sec	54°C/1 min	72°C/1 min	40	820
ORF1-F3	94°C /30 sec	58°C/30 sec	72°C/30 sec	40	157

3.2.2.3 Real time PCR

The reverse transcriptase real-time PCR (qRT-PCR) was performed in two steps. First a HEV cDNA was produced and then the qPCR was completed (table 22). 5 µl RNA was added to 14 µl of A-mix aliquot then it was heated to 94 °C for 3 min. The tubes were rapidly chilled on ice and stayed there for at least 2 min. Afterwards 19 µl (RNA + A-Mix) were added to 81µl of RT-mix (B-Mix) and incubate for 30 min at 45 °C, followed by 2 min at 94 °C. After that the tubes were directly placed on ice. For the real-time 2 µl of cDNA was added to 18 µl of the real-time mix samples (table 22). First samples were denatured for 20 s at 95°C, than 40 cycles (1 s 95°C, 20s 60°C, 15 s 72°C). Samples were performed in duplicate or triplicate wells.

Table 22: Reagents mix used in the real-time assay.

Amount	Reagent
A-Mix	
1	Primer RT02R
13	RNAse free water
B-Mix	
40	2.5x PCR buffer 8a
0.5	RNAse Inhibitor
0.5	Superscript RT
40	aq dest
Real-time mix	
10	TaqMan Fast Master Mix
0.4	Primer RT-01F
0.4	Primer RT02R
1	HEV SondeTaq (Sonde 5 µM)
6.2	aq dest

3.2.3 Analysis of PCR products by agarose gel electrophoresis

For gel preparation 1.5% gels were used (agarose powder dissolved in TAE buffer). For a more accurate visualization the concentration of agarose was adjusted according to size of the expected fragment (table 23).

Table 23: Scheme for load different DNA fragment sizes into agarose gels.

Size	Agarose concentration (%)	Voltage	Duration
Small (<100 b)	2.5 -3 %	100	35 min
Standard (100 b – 1 kb)	1.5%	100	35 min

Large (>1 kb)	0.8 – 1%	60-90	40-60 min

Electrophoresis chamber was filled with TAE buffer containing 0.01% etidium bromide. 8 µl of PCR product was mixed with 2 µl of loading buffer and 8 µl added in each slot. Electrophoresis was performed for 35 min at 100 V using 400 mA (for other voltages and durations see table 23). Gel fluorescence was photo-documented through an UV light transilluminator.

3.2.4 Gel extraction

PCR bands and enzymatic reactions were excised in the desired size from the 1, 1.5 or 2% agarose gels. They were weighted and purified using QIAquick or QIAEX Gel extraction kits (Qiagen) following the manufacturer`s recommendations.

3.2.5 Cloning

3.2.5.1 Ligation and transformation

3.2.5.1.1 PCR products

PCR products were ligated to the vectors pCR®2.1-TOPO® (Invitrogen) or pDrive (Qiagen®) and transformed into *E. coli* TOP 10 (Invitrogen) or Qiagen EZ (Qiagen®) chemically competent cells. Cells were plated in LB medium with Ampicillin with addition of 40 µl X-Gal in order to perform blue/white selection. The plates were incubated overnight (at least 16 hours). For each sample five to seven white colonies were picked and grown overnight in tubes containing 3 ml LB medium with ampicillin.

3.2.5.1.2 pET 26b (+) vector

First the pET26b (+) vector with ubiquitin was linearized using the BamHI and SacII restriction sites. After the control digestion the linearized vector ends were zipped using alkaline phosphatase at 37° for 30 min. The reaction was placed on an agarose gel and the linear vector (band) excised from the gel and purified using the Qiaquick extraction kit (Qiagen).

For the ligation approximately 100 ng of vector and 0.2 pmol of insert were used in a 20µl reaction mix.

3.2.6 Plasmid preparation

DNA was extracted from plasmids using the QIAprep Spin Miniprep kit (Qiagen). Two ml from each of the bacterial culture were centrifuged with 5000 RPM for 10

minutes at 4°C. Bacteria pellets were re-suspended gently using 250 µl P1 buffer. Afterwards 250 µl buffer P2 was added and the tubes inverted thoroughly but gently for approximately 10 times. The neutralization buffer (N3) was added and the tubes inverted again 10 times followed by centrifugation at 13000 for 10 minutes. The supernatants were displaced in the silica columns and centrifuged for 1 minute. Silica membranes were washed with 500 µl and 750 µl of buffers PB and PE, respectively. DNA was eluted with 50 µl of elution buffer (EB), incubated for 1 minute and centrifuged for 8000 RPM.

Plasmid DNA samples were immediately digested using restriction enzymes in order to control the presence and the size of the insert. Control digestion was performed using Eco RI in both ends of the insert for both pDrive and pCR®2.1-TOPO. 2 µl of plasmid DNA was added to 8 µl of the digestion mix (Table 24) and samples incubated for 37°C during 90 minutes. After that 4 µl of the loading buffer was added to the restriction mix and placed on the agarose gel.

Table 24: PCR-Clonning-vector digestion using restriction sites.

Reagent	µl
H2O	5.8
Eco RI Buffer	1.0
BSA	1.0
Eco RI	0.2
Total	8.0

3.2.7 Sequencing

Table 25: Several bioinformatic softwares used for different purpose.

Software	Description/Use	Manufactured/ Developed	Available at
Primer Express 2.0 ®	Primer design	Applied biosystems	www.appliedbiosystems.com
HUSAR	Bioinformatic package	DKFZ Heidelberg	http://genome.dkfz-heidelberg.de/
MEGA 4(Tamura et al., 2007b) MEGA 5(Tamura et al., 2011)	Molecular Evolucionary and genetic analysis	Koichiro Tamura, Daniel Peterson, Nicholas Peterson, Glen Stecher, Masatoshi Nei and Sudhir Kumar	http://www.megasoftware.net/
TreeView 1.6.6	Phylogenetic tree drawing	University of Glasgow	*www.taxonomy.zoology.gla.ac.uk/rod/**treeview**.html*
RDP 3(Martin et al., 2010)	Recombination detection	Darren Martin	http://darwin.uvigo.es/rdp/rdp.html

Splits Tree 4(Huson and Bryant, 2006)	Phylogenetic Analysis	Daniel Huson and David Bryant	http://www.splitstree.org/
SimPlot (Lole et al., 1999)	Recombination detection	Stuart Ray	http://sray.med.som.jhmi.edu/SCRoftware/simplot/
IEDB Analysis Resource	Antibody epitope prediction		http://tools.immuneepitope.org/tools/bcell/iedb_input

After fragments were checked to be in the expected size, plasmid DNA samples were sent for sequencing to Qiagen (Hilden, Germany) or Seqlab (Göttingen).

3.2.8 Phylogenetic analysis

Phylogenetic analysis was performed using the HUSAR package (DKFZ, Heidelberg). Sequences were compared to GenBank entries and phylogenetic analyses performed using the HUSAR package. Phylogenetic distances were calculated (Kimura-2-parameter method) and trees generated based on the neighbor-joining or the maximum likehood methods. A bootstrap analysis with 1000 replicates was included. Branch lengths are proportional to genetic distances. Additional analyses such as detection of viral recombination and antigenicity prediction were performed using different softwares (or bioinformatics packages) which can be seen in table 25.

3.2.9 Protein Purification

3.2.9.1 Test expression

Bacterial colonies were inoculated in 1 ml LB-KAN medium and let grown overnight (16 h) at 37°C. In next morning 0.5 ml of the bacterial culture was placed in a fresh tube and induced with IPTG (2 µl) for 4-5 hours at 37°C (shaking); together with the non-inducted cells.

After the incubation 1 volume of non-inducted and 2 volumes of inducted cells were collected and centrifuged at 4000 RPM for 15 minutes. Supernatants were discarded and the pellets dissolved in approximately 15 µl of water each. 5 µl of 4xVD buffer was added to the samples and incubated for 5 minutes at 95°C.

3.2.9.2 Expression for purification

1ml of bacterial culture was added in 250 ml LB-KAN medium and incubated overnight at 37°. One aliquot was collected and the cells were inducted with IPTG (250 µl) during 4-5 hours at 37°C. Bacterial cultures were centrifuged at 3000-4000 RPM for approximately 15 minutes. Supernatants were discarded and bacterial pellets were lysed using lysis buffer and three times freeze in liquid nitrogen and thaw with ultrasound.

Lysed bacteria were transferred to an ultracentrifuge (UC) tube, carefully closed and weighted. UC tubes were centrifuged at 30.000 RPM for 1 hour at 4°C using Rotor TIC60.

3.2.9.3 Purification using Columns

3.2.9.3.1 Western blot was performed using both supernatants and pellets:

(a) Protein detected in the supernatant: protein is soluble and can be directly purified using Ni-Column (using buffer without urea: FPLCA + FPLCB)

(b) Protein can only be detected in the pellet: protein is insoluble and should be solubilized overnight using in FPLCA + Urea (8 M). After that it is (ultra-) centrifuged or sterile filtered (0.45 µm filter) one more time. Subsequently it can be purified using Ni-column (using buffers with urea: FPLCA urea + FPLCB urea).

3.2.9.3.2 Running through the column:

Before starting, it is important to prepare the peristaltic pump and test it properly. Note that air bubbles may break the column.

(1) Wash the column with FPLCB (approximately 10 ml).

(2) Equilibrate the column with FPLCA buffer (approximately 10 ml).

(3) Load up the column with the protein slowly and continuously; approximately 0.75-1 ml/min. This step can be repeated 2-5 times.

(4) Wash the column with 1x FPLCA buffer (approximately 10 ml).

(5) Elute the protein using FPLCB buffer with different imidazole concentration, each aliquot of about 5ml.

   a. 50 mM imidazole
   b. 100 mM imidazole
   c. 500 mM imidazole (3 times)

(6) Wash the column one more time with buffer FPLCB (approximately 10 ml)

(7) Wash column with ddH_2O (approximately 10 ml)

(8) Load the column with ethanol 20%, close it and store at 4°C. It can be further used to purify other aliquots from the same protein.

(9) Clean the pump tubes with water or ethanol 20%.

Finally all protein eluted aliquot with different imidazole concentrations (E1–5) should be placed in an acrylamide gel. After electrophoresis the gel must be stained with commassie and it should be possible the see the band in the expected size and to choose which of the imidazole concentration is more suitable to be used further.

3.2.10 Working with cell culture

3.2.10.1 Storage

Cell lines (master seed or working seed cultures) were stored in liquid nitrogen. Each aliquot contained a number of cells necessary to be confluent in one to two days in a 10 cm dish. All cell lines plates (10cm, 6 or 24-well plates) used were under quality control management system.

3.2.10.2 Passage and maintenance

In order to estimate the viability cells were evaluated macro- and microscopically. Cell layer was detached using EDTA-trypsin solution and diluted to the desired proportion. The dilution factor was determined according to previous information of cell growing from each cell line as well as the level of confluence.

Cells were maintained using medium with 10% FCS with penicillin, streptomycin and amphotericin and passaged twice a week. Old medium was removed and cell layers washed with the equal amount of EDTA-trypsin solution. Afterwards 1 ml of EDTA-trypsin solution was added and plates were incubated until the cells were completely detached from the plate. Finally 9 ml maintenance medium was added and cells were placed in the new plates into the desired dilution.

3.2.10.3 Determination of cell concentration

Cell suspensions were used in order to determine the number of viable cells. This was performed by the use of the trypan blue exclusion test. The principle of this test is based on trypan blue characteristics: As soon as the cell membrane is undamaged the trypan does not enter in the cell; however, when the cell is dead the membrane can be traversed giving a strong blue coloration. For this 20 µl of cell suspension were diluted in 180 µl of trypan blue solution. Live cells (without blue color) were observed in an inverted microscopy and counted in four big squares of a Fuchs-Rosenthal chamber (diagonale). The determination of the cell number was obtained by the following formula:

$$\frac{n \times 4 \times V \times 1000}{3.2} = \text{n of cells/ml}$$

(n= total of cells in the four squares, V= dilution factors)

3.2.11 Infection of A549 cell line

A549 cells were passaged and counted into a 24 well plate. The concentration of cells was around 1.2×10^6 cells/ml => 50µl/well = 6×10^3. A fragment of infected liver was cut and triturated with sterile sand and medium without FBS. The suspension was

centrifuged at 2700 g (3500 – 4000 rpm) for 10 minutes at 4°C. Supernatants were collected and sterile filtered (0.22 µl). Medium (containing 10% FBS) was removed and 200 µl of medium without FBS was added. Cell layers were infected with 4 µl, 20 µl and 100 µl of the suspension and incubated for 1h. Cell layers were washed with 1 ml of medium without FBS. Medium with 1%, 2%, 5% FBS and serum free medium with trypsin were added. Cells and supernatants were collected on days 0 (only supernatant), 2, 4, 6, 8, 10, 12, 14.

4 Results

4.1 HEV in domestic swine and wild boar

4.1.1 Detection of HEV in domestic swine

Hepatitis E is an emerging infectious disease distributed worldwide which occurs in both humans and animals such as domestic swine and wild boar. In contrast to the situation in humans, no clinical disease has been associated with HEV in animals so far, although sequences from human and animal HEVs are closely related and zoonotic transmission is known to take place. To elucidate the HEV prevalence in different animal populations of domestic swine and wild boar samples from Germany, The Netherlands and Morocco were tested for the presence of HEV RNA.

A panel of fecal samples from 105 domestic swine was available which had been used in another study. A fragment of 241 nucleotides from the capsid gene region of the HEV genome was amplified in one out of 105 fecal samples (0.95 %). The animal, a four month old female Pietrain breed, originated from Giessen (Hesse state) and was clinically healthy. The negative animals originated from different regions of Germany and one Dutch farm. Information about age and sex, if available, is shown in Figure 7.

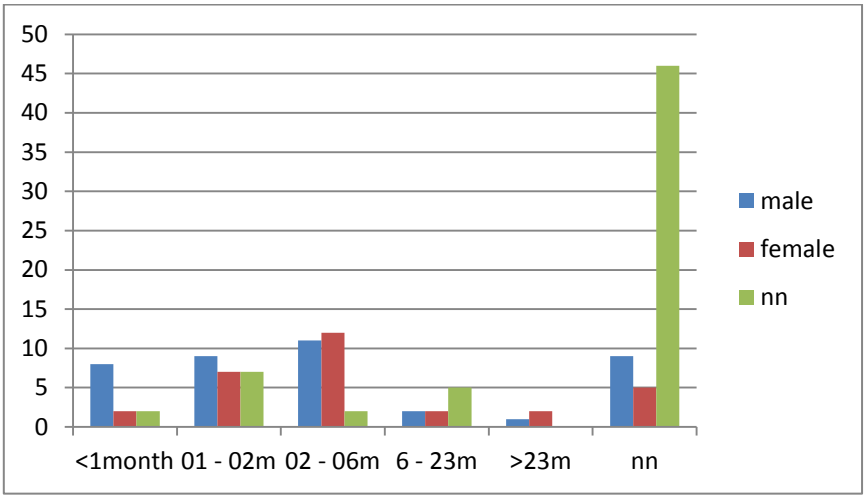

Figure 7: Domestic swine sampling: age and sex distribution. Green: no information on sex available. X-axis: age, nn: no age given.

4.1.2 Detection of HEV in wild boar

Wild boar sera were provided by “Landesbetrieb Hessisches Landeslabor” and originated from the classical swine fever surveillance program. HEV could be detected in 18 out of the 124 sera, corresponding to a detection rate of 14.5 %. The positive animals

were distributed in the sampling area (Fig. 8 and Table 26). No information about sex and age of the animals was available.

Until now only few HEV strains have been detected in Africa. To elucidate the situation there, 160 wild boar sera were obtained from a farm in Morocco. The animals were kept semi-intensive fenced. None of these samples was positive by RT-PCR. All animals were apparently healthy. Age and sex distribution are listed in figure 9.

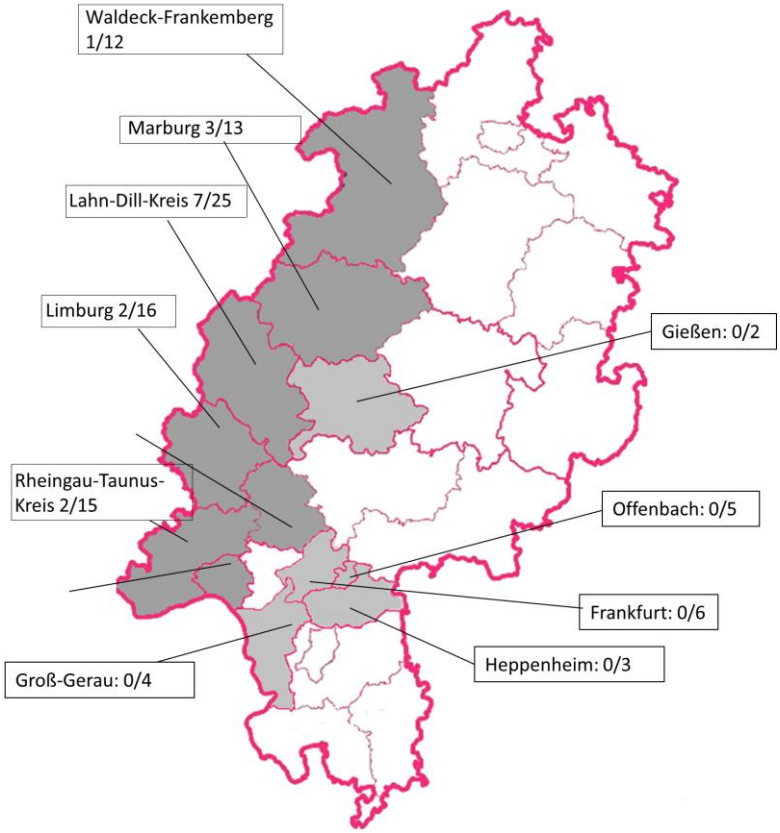

Figure 8: Geographical distribution of wild boar samples tested (positive samples / total samples). Dark grey indicates were positive samples were found, light grey shows regions where samples were collected but no positive have been found.

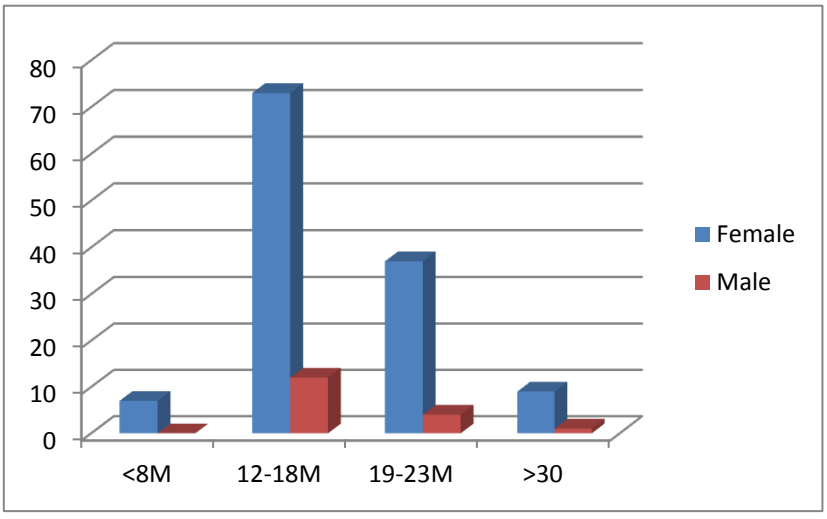

Figure 9: Age and sex distribution of wild boar in Morocco.

4.1.3 Phylogenetic analysis

PCR fragments of 241 nt (capsid position: from nt 449 to 691) obtained from samples of domestic swine and wild boar were cloned, sequenced and phylogenetically analyzed. The level of divergence between the obtained sequences is given in table 27. The nucleotide differences (pairwise corrected distances) between the sequences from domestic pigs and wild boar ranged from 11.57 to 21.45 %. Within the wild boar sequences differences from 0 to 21.61 % were observed.

Table 26: Regions where positive wild boar samples were found; subtyping according to 241 b and 2.1 kb; accession numbers

Region	N. pos	Sample Identification	Subtype		Accession numbers
			241b	2.1 kb	
Rheingau-Taunus-Kreis	2	WB 1,	3i, 3h	3i	**KF303501**
		WB 121	3a,3b, 3j		**KF303496**
Wiesbaden	2	WB 22	3i		**KF303486**
		WB 24	3i, 3h		**KF303485**
Lahn-Dill-Kreis/Wetzlar	7	WB 25	3i		**KF303494**
		WB 75	3i		**KF303489**
		WB 76	3i		**KF303490**
		WB 104	3i		**KF303493**
		WB 119	3i		**KF303491**
		WB 117	3a,3b, 3j		**KF303484**
		WB 124	3a,3b, 3j		**KF303498**
Marburg-Biedenkopf/Lahn	3	WB 34	3i, 3h		**KF303487**
		WB 91	3i		**KF303488**
		WB 122	3a	3a	**KF303499**
Waldeck-Frankenberg	1	WB 52	3i		**KF303492**
Hochtaunuskreis	1	WB 69	3a,3b, 3j	3a	**KF303500**
Limburg	2	WB 118	3i		**KF303495**
		WB 120	3a,3b, 3j		**KF303497**
Total	**18**				

The obtained sequences clustered in different branches within genotype 3 of HEV (Fig. 10). Bootstrap values were generally low (under 750), which denote a low reliability of the analysis. The wild boar isolates (WB122) and one sample from domestic swine (GiSw) grouped together with sequences from subtype 3a (AB074918 and AB089824). A second group comprising five isolates (WB69, WB117, WB120, WB121 and WB124) showed the closest relationship to sequences previously classified as subtypes 3a, 3j and 3b. The third group with the majority of our isolates clustered in subtype 3i together with German isolates (FJ998008 and FJ705359) and subtype 3h (AB290312) comprising a Mongolian swine isolate (Fig. 10 and Table 26).

Table 27: Phylogenetic analysis of 241 b of the capsid region of the HEV genome. Phylogenetic distances were corrected using the Kimura-2 parameter method. Numbers show nucleotide divergence (%) within wild boar and domestic swine positive samples.

	WB69	WB124	WB120	WB117	WB121	GiSw	WB122	WB22	WB118	WB52	WB1	WB25	WB104	WB76	WB119	WB75	WB91	WB24	WB34
WB69	WB69	0	0.42	0.42	5.18	12.63	11.52	19.54	20.66	17.78	17.2	17.72	17.21	17.21	15.54	16.65	17.2	16.53	16.48
WB124		WB124	0.42	0.42	5.18	12.63	11.52	19.54	20.66	17.78	17.2	17.72	17.21	17.21	15.54	16.65	17.2	16.53	16.48
WB120			WB120	0.83	4.74	13.1	12	19.02	20.14	17.28	16.7	17.21	16.71	16.71	15.04	16.15	17.3	16.03	15.98
WB117				WB117	5.63	13.16	12.04	20.14	21.27	18.36	17.8	18.29	17.78	17.78	16.09	17.21	17.8	17.09	17.04
WB121					WB121	16.21	14.5	15.22	16.28	13.58	13.1	13.53	10.96	10.96	9.46	10.46	13.6	12.42	17.85
GiSw						GiSw	11.57	18.43	19.54	17.85	17.3	17.78	20.83	20.83	21.45	21.45	17.3	18.87	17.09
WB122							WB122	20.74	19.62	22.61	22	22.51	21.98	21.98	22.61	21.36	16.1	18.81	15.93
WB22								WB22	2.54	5.71	5.24	5.68	7.12	7.12	8.58	8.58	7.6	14.61	20.31
WB118									WB118	6.62	6.15	6.59	8.05	8.05	9.53	9.53	8.54	15.1	20.22
WB52										WB52	0.42	0.83	4.79	4.79	6.18	6.18	7.12	9.92	19.7
WB1											WB1	0.42	4.33	4.33	5.71	5.71	6.65	9.42	19.1
WB25												WB25	4.77	4.77	6.15	6.15	7.09	9.89	19.62
WB104													WB104	0	1.26	1.26	7.6	12.47	19.1
WB76														WB76	1.26	1.26	7.6	12.47	19.1
WB119															WB119	0.84	9.07	13	19.7
WB75																WB75	8.09	14.07	20.92
WB91																	WB91	14.61	21.54
WB24																		WB24	8.05
WB34																			WB34

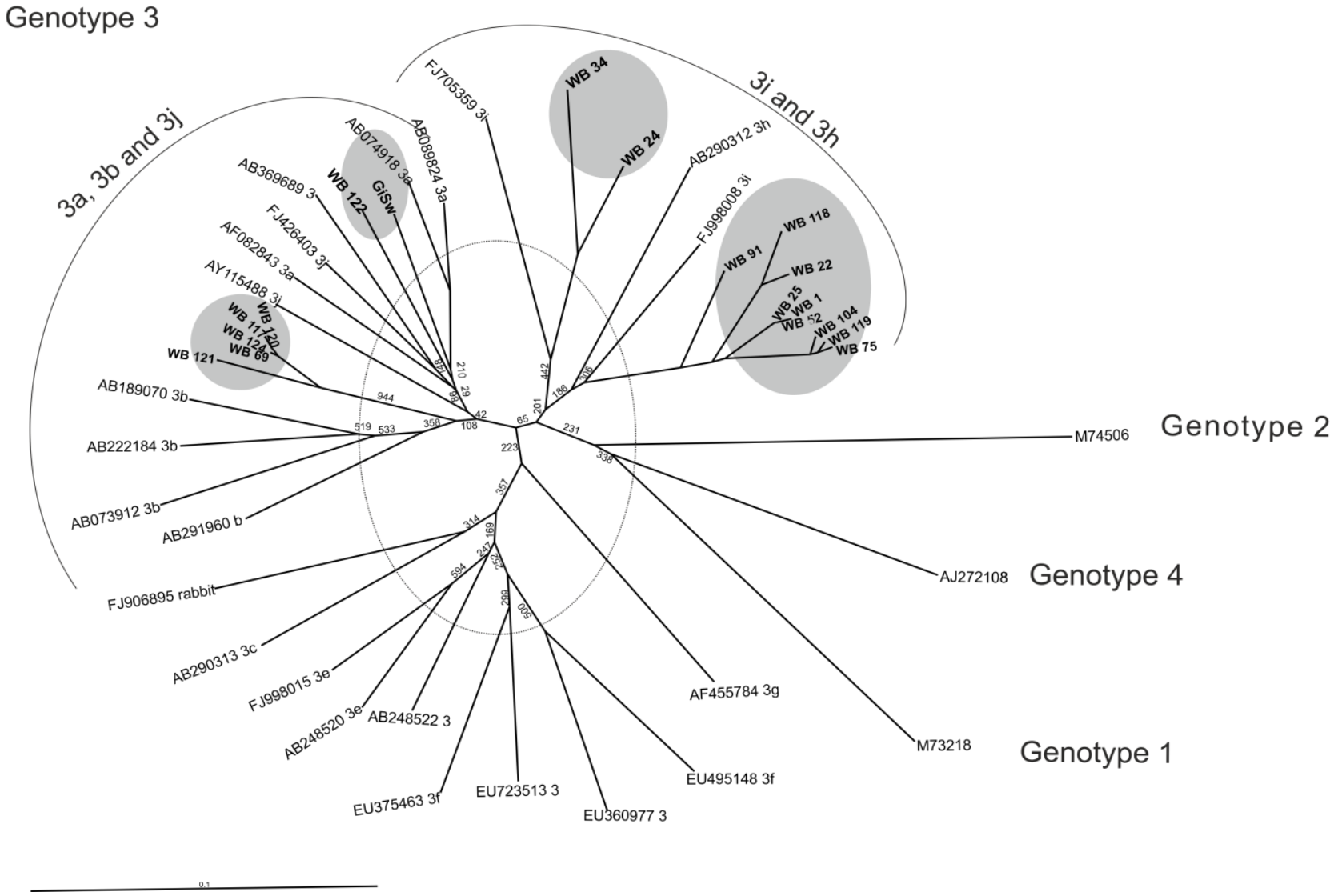

Figure 10: Phylogenetic analysis based on 241b of the capsid protein gene. Phylogenetic distances were calculated using the Kimura-2 parameter method. The phylogenetic tree was calculated using the neighbor-joining method. Branch lengths are proportional to the genetic distances. A bootstrap analysis was included (1000 replicates), numbers within the circle indicate bootstrap values. Additional sequences were obtained from GenBank (accession numbers indicated).

4.1.4 Sequencing of the complete capsid gene and phylogenetic analysis of HEV from domestic swine and wild boar samples

In order to broader the knowledge about the phylogenetic relationship of the obtained isolates the capsid protein was sequenced. For sequencing of the complete capsid gene (1983 nucleotides) of HEV in the samples from domestic swine and wild boar ORF 2 was divided into four regions named R1, R2, R3 and R4 (Fig. 11). R1 region corresponded to the 5' end of the capsid protein encoding sequence together with 205 nucleotides from the 3' end of ORF1 (Fig. 11). It was intended to sequence the entire capsid region from several HEV positive samples. However due to limited amounts of samples, additional sequences were obtained only from three wild boar samples (WB1, WB69 and WB122) and the positive domestic swine (GiSw).

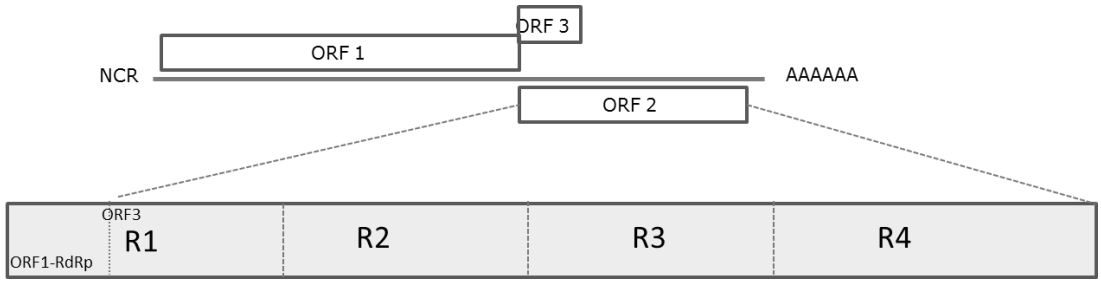

Figure 11: Regions for sequencing of the entire capsid gene. ORF 2 was divided into four regions named R1 to R4.

Based on complete capsid sequences higher bootstrap values and a more reliable separation of subtypes could be achieved (Fig. 12). Accordingly GiSw, WB122 and WB69 were placed into subtype 3a. The WB1 sequence clustered in one branch together with two previously reported viruses from wild boar in Germany classified as subtype 3i (FJ705359 and FJ998008) (Adlhoch et al., 2009b; Schielke et al., 2009). Pairwise comparison of the latter three sequences showed a remarkably high heterogeneity of 10.6 and 13.5 substitutions per 100 nucleotides, respectively. The heterogeneity within subtype 3i was thus much higher when compared with other subtypes; for instance within 3a, 3d and 3f (Table 28).

Table 28: Phylogenetic analysis of the entire capsid gene region of the HEV genome. Phylogenetic distances were corrected using the Kimura-2 parameter method. Numbers show nucleotide divergence (%) within wild boar and domestic swine samples. In bold: domestic swine and wild boar samples from our study. Boxes highlighted show the nucleotide divergence within subtypes 3a, 3d, 3e, 3f and 3i.

		G2		3f		3d		3e		3c	3g	3b				3a			3j		3i		3h	G4
		m74506	m73218	eu495148	eu375463	ab481226	ab248521	ab248520	fj998015	ab290313	af455784	ab073912	ab222184	ab089824	ab074918	wb122	wb69	GiSw	ay115488	wb1	fj705359	fj998008	ab290312	aj272108
G2	m74506		22.02	25.68	25.00	25.41	25.87	26.02	25.17	25.15	25.40	26.80	26.49	24.08	24.07	**26.18**	**25.32**	**25.53**	25.58	**26.77**	25.51	26.01	24.80	25.16
1a	m73218			23.55	22.90	23.07	23.22	23.46	23.67	22.28	23.11	22.43	23.73	23.89	23.71	**24.86**	**22.77**	**23.67**	22.80	**24.40**	24.84	22.97	22.34	24.25
3f	eu495148				7.89	14.30	13.92	14.13	12.92	12.00	15.16	18.25	18.54	17.56	17.60	**16.79**	**16.69**	**16.58**	16.27	**17.74**	17.45	17.46	18.53	22.52
	eu375463					14.75	14.56	14.05	13.74	13.46	16.00	17.68	18.03	15.86	16.84	**16.91**	**17.09**	**16.63**	16.26	**18.55**	17.77	17.50	18.03	21.64
3d	ab481226						1.59	9.63	9.62	12.65	13.91	17.89	16.97	16.57	17.29	**17.42**	**17.13**	**17.49**	17.30	**17.33**	17.65	17.54	18.48	22.17
	ab248521							9.32	9.38	13.23	13.92	17.89	17.25	16.71	17.36	**17.63**	**17.61**	**17.55**	16.89	**18.02**	17.80	17.89	18.63	22.11
3e	ab248520								9.70	13.00	15.32	18.88	18.56	17.95	18.27	**17.50**	**17.33**	**17.71**	17.65	**18.08**	19.11	17.97	18.68	23.85
	fj998015									11.80	14.33	17.36	19.06	17.35	18.01	**17.04**	**17.56**	**17.17**	17.45	**18.86**	17.94	17.51	18.02	22.18
3c	ab290313										15.30	19.46	19.02	18.10	17.93	**16.64**	**18.53**	**17.59**	18.08	**18.49**	18.27	17.28	17.90	22.32
3g	af455784											17.65	17.75	16.45	16.44	**17.25**	**17.34**	**16.23**	17.25	**18.69**	18.17	17.35	18.35	22.89
3b	ab073912												10.95	11.98	12.54	**11.71**	**11.82**	**13.03**	12.15	**16.21**	15.74	14.78	16.54	22.39
	ab089824													13.08	12.55	**12.25**	**13.17**	**13.10**	13.32	**16.35**	16.60	14.88	15.99	23.80
3a	ab089824														4.14	**7.94**	**8.69**	**9.05**	10.72	**15.38**	16.07	13.18	15.89	22.71
	ab074918															**8.22**	**9.03**	**9.21**	11.88	**15.37**	15.79	13.56	16.67	22.26
	wb122																**9.45**	**9.75**	**11.59**	**15.11**	**15.93**	**13.88**	**15.42**	**23.15**
	wb69																	**10.60**	**12.05**	**15.88**	**16.30**	**14.51**	**15.74**	**22.48**
	GiSw																		**11.62**	**16.14**	**15.89**	**15.72**	**16.24**	**23.48**
3j	ay115488																			**16.37**	15.26	14.78	16.03	23.50
3i	wb1																				**10.66**	**13.54**	**15.66**	**24.94**
	fj705359																					12.09	14.10	24.59
	fj998008																						13.07	23.43
3h	ab290312																							23.45
G4	aj272108																							

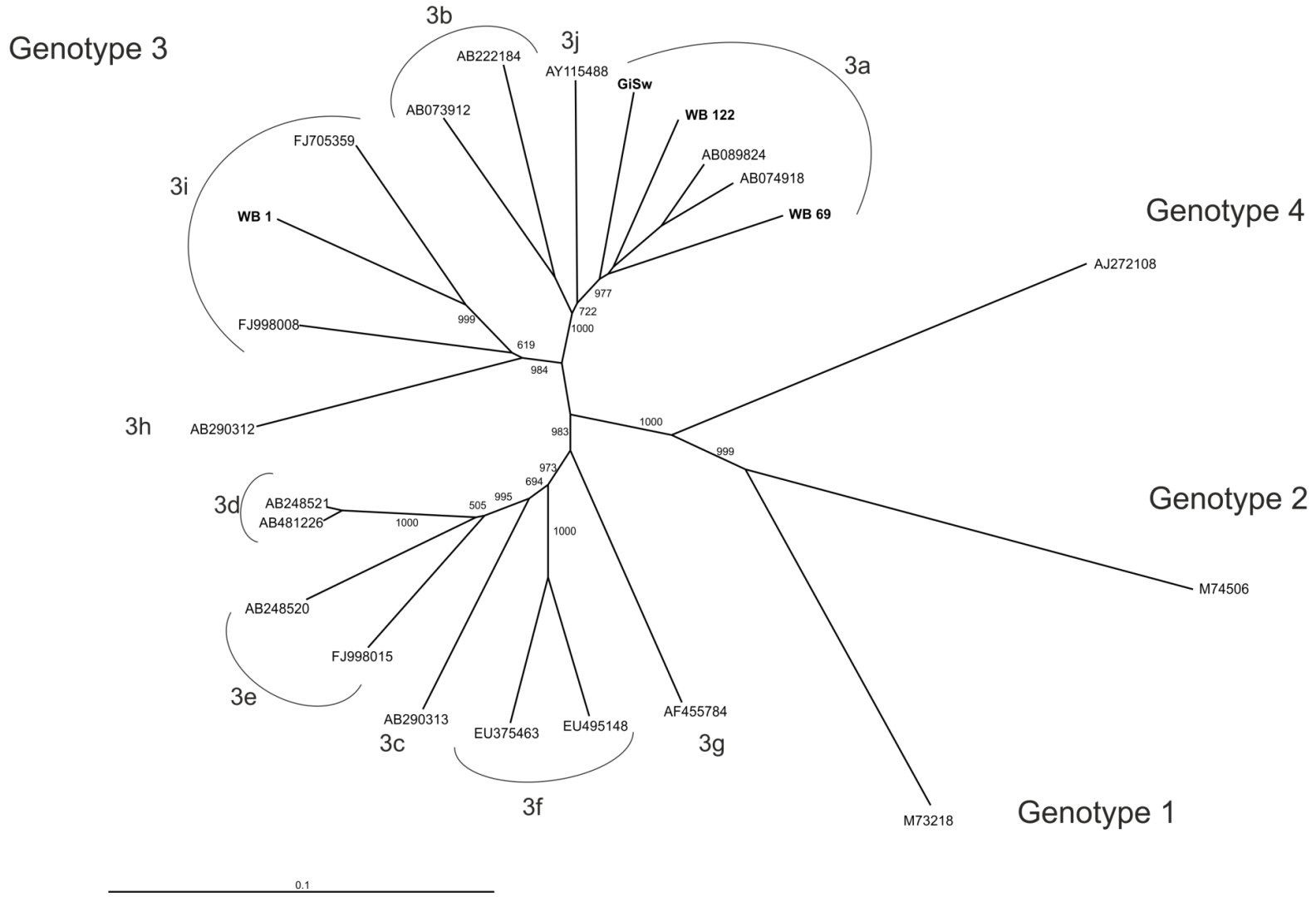

Figure 12: Phylogenetic analysis based on complete capsid gene sequences. Phylogenetic distances were calculated using the Kimura-2 parameter method. Tree was calculated by the neighbor-joining method. The branch lengths are proportional to the genetic distances. A bootstrap analysis of 1000 replicates was included; numbers indicate bootstrap values. Additional sequences were obtained from GenBank together with accession numbers indicated.

4.1.5 Search for recombinants

Recombination among closely related RNA viruses is a common event. The high heterogeneity observed among wild boar sequences and the difficulties to classify subtypes unambiguously made the search for recombination promising. Different approaches can be used in order to detect recombination events and recombination sites. The split decomposition method which allows to show conflicting phylogenetic signals was applied first. This method can be used to show alternative positions of these sequences in a given phylogenetic tree by plotting parallel edges between them forming an interconnected network. Such a network means a conflict in the phylogenetic analysis may be due to recombination. In the second approach we used the recombination detection package 3 (RDP 3); this software combines a number of different recombination detection methods (Martin et al., 2010).

The tree based on a 241 b fragment of the HEV capsid region was plotted using the split decomposition method. Conflicting phylogenetic signals on wild boar isolates were found (Fig. 13). The presence of a network instead of bifurcation connecting the isolates may indicate viral recombination.

Analyses using the RDP 3 suggested recombination events with regard to six sequences. The first recombination event concerned isolate WB24 with isolate WB25 as potential major parent and WB34 as potential minor parent. The recombination breakpoints began at position 21 and ended at position 154. The second event concerned isolate WB121 with WB75 and WB117 as potential major and minor parents. The recombination breakpoint started at position 4 and ended on position 163.

The graphical representation based on the PhylPro method is shown in Figure 14A. The trees were constructed using different regions of the 241 b fragment. On the left side the region with possible recombination breakpoint was used (from 4 to 163) (Fig. 14 B); and on the right side the tree is based on the region where no recombination was detected (from 163 to 4) (Fig. 14 C). It is possible to observe some isolates shifting position (even subtype) on the different position-based phylogenetic trees (Fig. 14B and 14C). Unfortunately it was not possible to obtain larger sequences from these isolates. It is unclear whether the recombination detected was a bias due to the fragment size or region or indeed due to the occurrence of recombination.

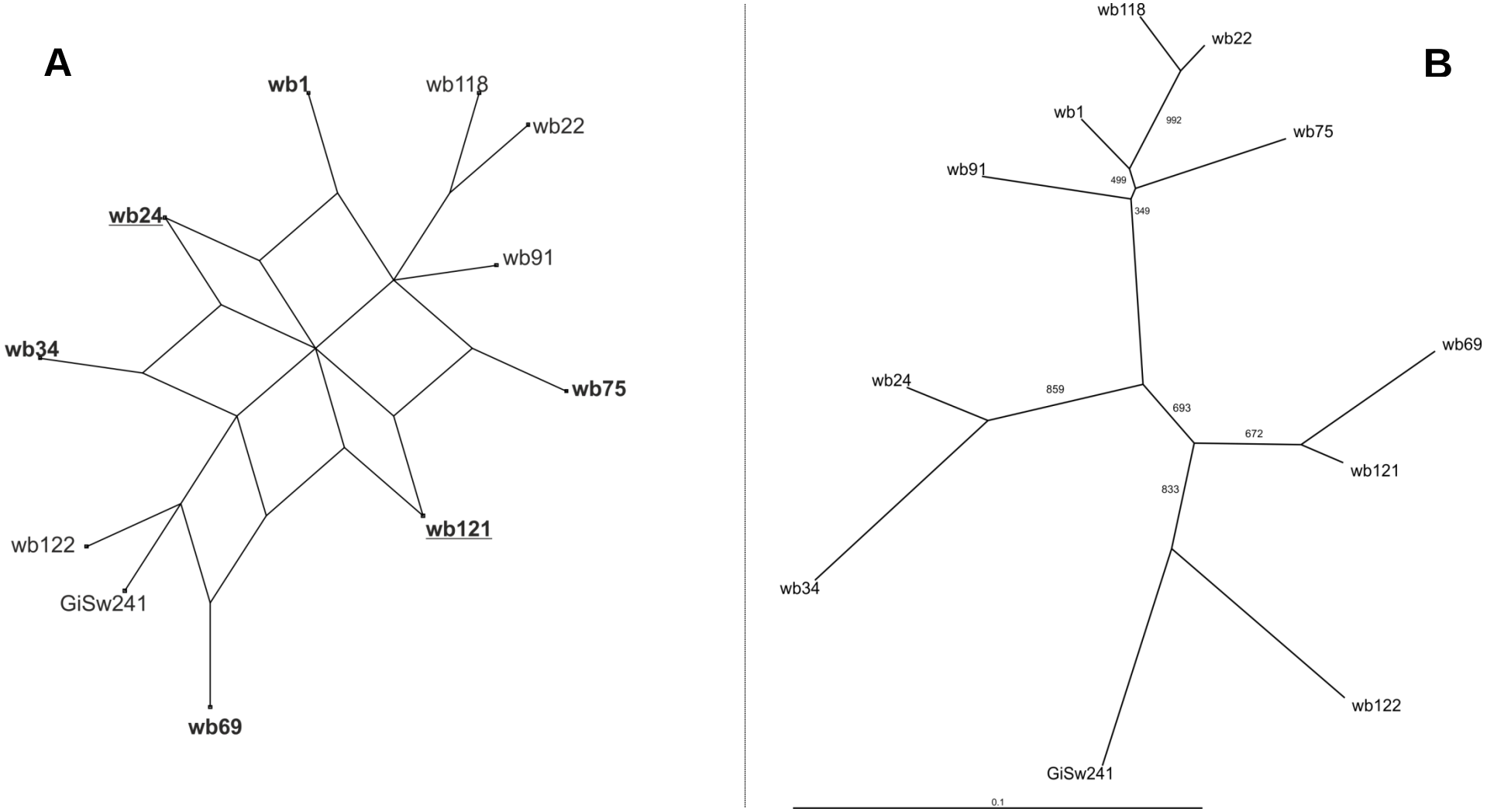

Figure 13: The plotted trees show the networks generated by split decomposition suggesting alternative positions (A) comparison with the tree calculated by the neighbor-joining method. The Branch lengths are proportional to the genetic distances and bootstraps values are indicated.

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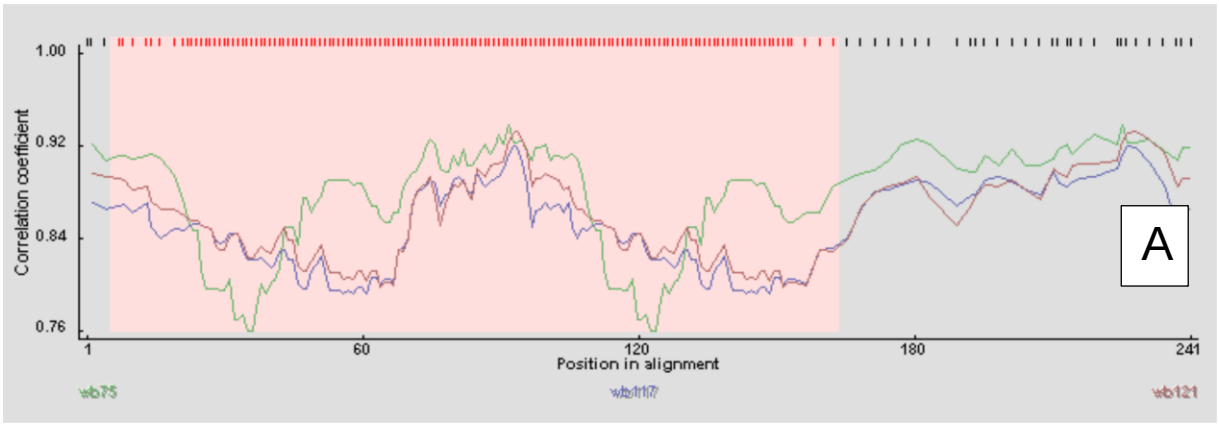


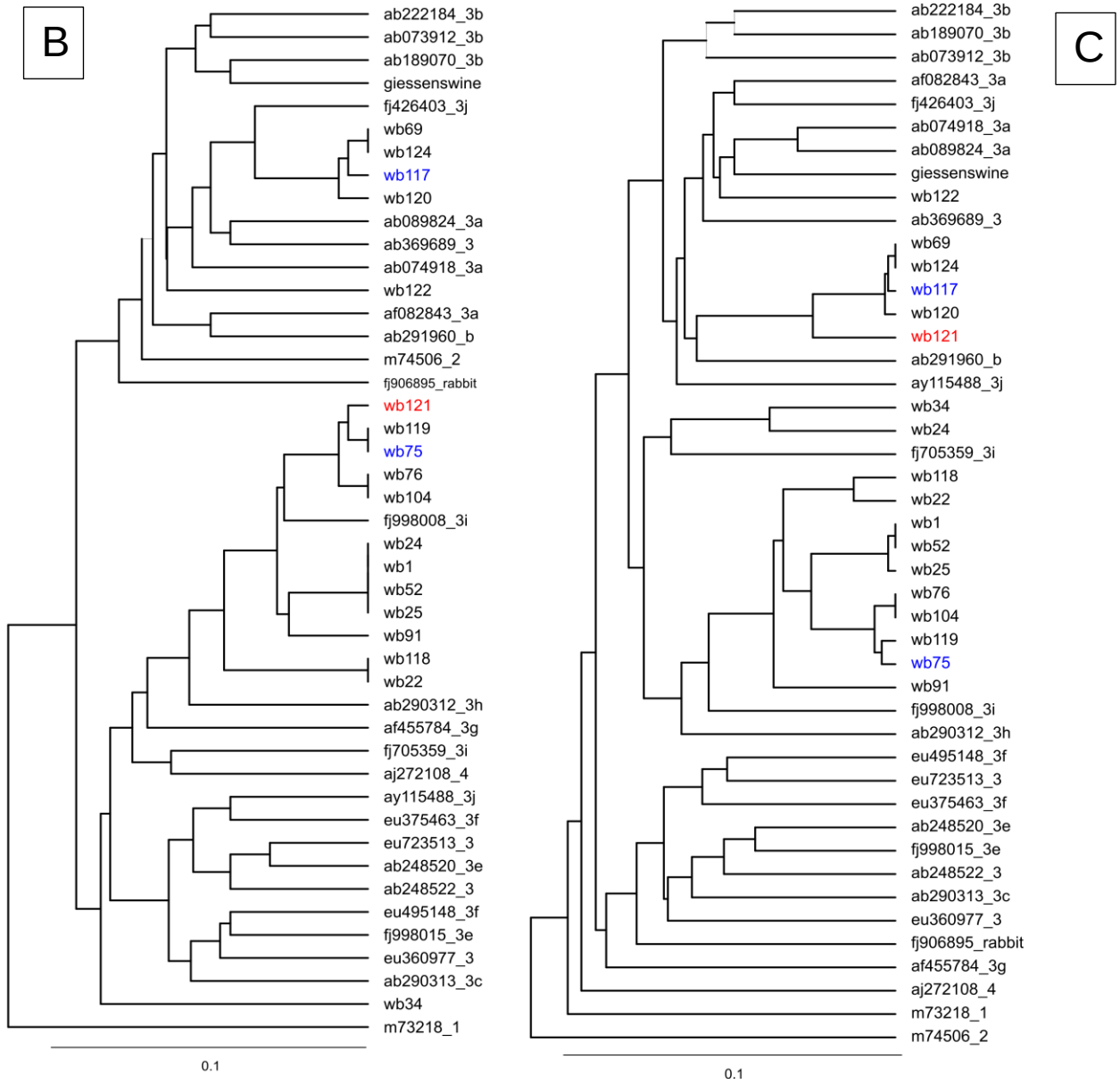


Figure 14: Search for viral recombination using PhylPro method (A) which shows where recombination may have occurred in the alignment. Phylogenetic trees show different positions of isolates WB75, WB117 and WB121 with the area where recombination possibly occurred 4 – 163 (C) and from 164 to 4 (B). Phylogenetic trees are calculated by UPGMA method.

4.1.6 Complete sequence of HEV isolate from domestic swine

The complete genome of the GiSw HEV was amplified using a different set of primers. Subsequently, each fragment was cloned and sequenced. The ORF1 was divided into six overlapping regions, and primers were designed for each region (Table 29). In order to obtain the complete genomic sequence, ORF1 was assembled using the overlapping fragments and placed together with the ORF2/3 sequence (described previously) (Fig. 15).

The genome of GiSw HEV consisted of three ORFs with a size of 5122 nt (ORF1), 1983 nt (ORF2) and 369 nt (ORF3) flanked by 5' and 3' non-coding regions. The non-coding regions (NCR) from both 3' and 5' were not included in the sequencing. The encoding sequence was compared to genomic sequences obtained from GenBank, as shown in Figures 16 (genotype 3) and 17 (genotypes 1–5).

Like deduced from other HEV complete sequences GiSw ORF1 encoded a polyprotein containing putative conserved domains: methyltransferase (MT) 34-355 aa, papain-like cystein protease (Pr) 432 – 592 aa, helicase (H) 980 – 1199 aa and RNA-dependent RNA polymerase (RdRp) 1412 – 1594 aa.

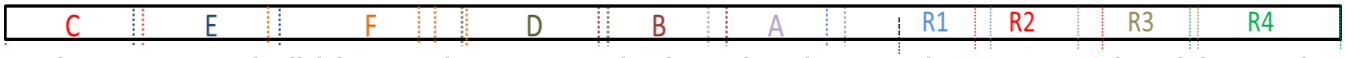


Figure 15: Genomic divisions used to sequence the domestic swine HEV GiSw. For sequening of the complete HEV genome, the genome was divided into overlapping regions. A – F: ORF1, R1 – R4: ORF2.

Table 29: Primers for amplification of ORF1.

Region	Name	Amplicon Size
ORF1 A	orf1HEV-1F	871
	orf1HEV-1R	
ORF1 B	ORF1-2F	831
	ORF1-2R	
ORF1 C	ORF1-3F	974
	ORF1-3R	
ORF1 D	ORF1-4F	919
	ORF1-4R	
ORF1 E	ORF1-5F	842
	ORF1-5R	
ORF1 F	ORF1-6F	938
	ORF1-6R	
ORF1 F2	HEV ORF1f2-R	820
ORF1 F3	ORF1_2340F	157
	ORF1_2496R	

Phylogenetic analysis demonstrated a close relationship between the GiSw and genotype 3 sequences. Pairwise genetic distances ranged from 9.88 to 26.35 % as can be seen in Table 30. GiSw clustered in a branch together with FJ426404 and FJ426403 found in Korean pigs, AF060669 and AF060668 from human patients from the US, AF082843 found in domestic swine also from US and AB591734 in Mongoose in Japan (Fig. 16). Regarding to members of other HEV genotypes the number of substitution per 100 nucleotides in the pairwise distance matrix was 32.7 from GT1, 32.1 from GT2, 31.0 from GT4 and 32.3 from GT 5 (Table 30).

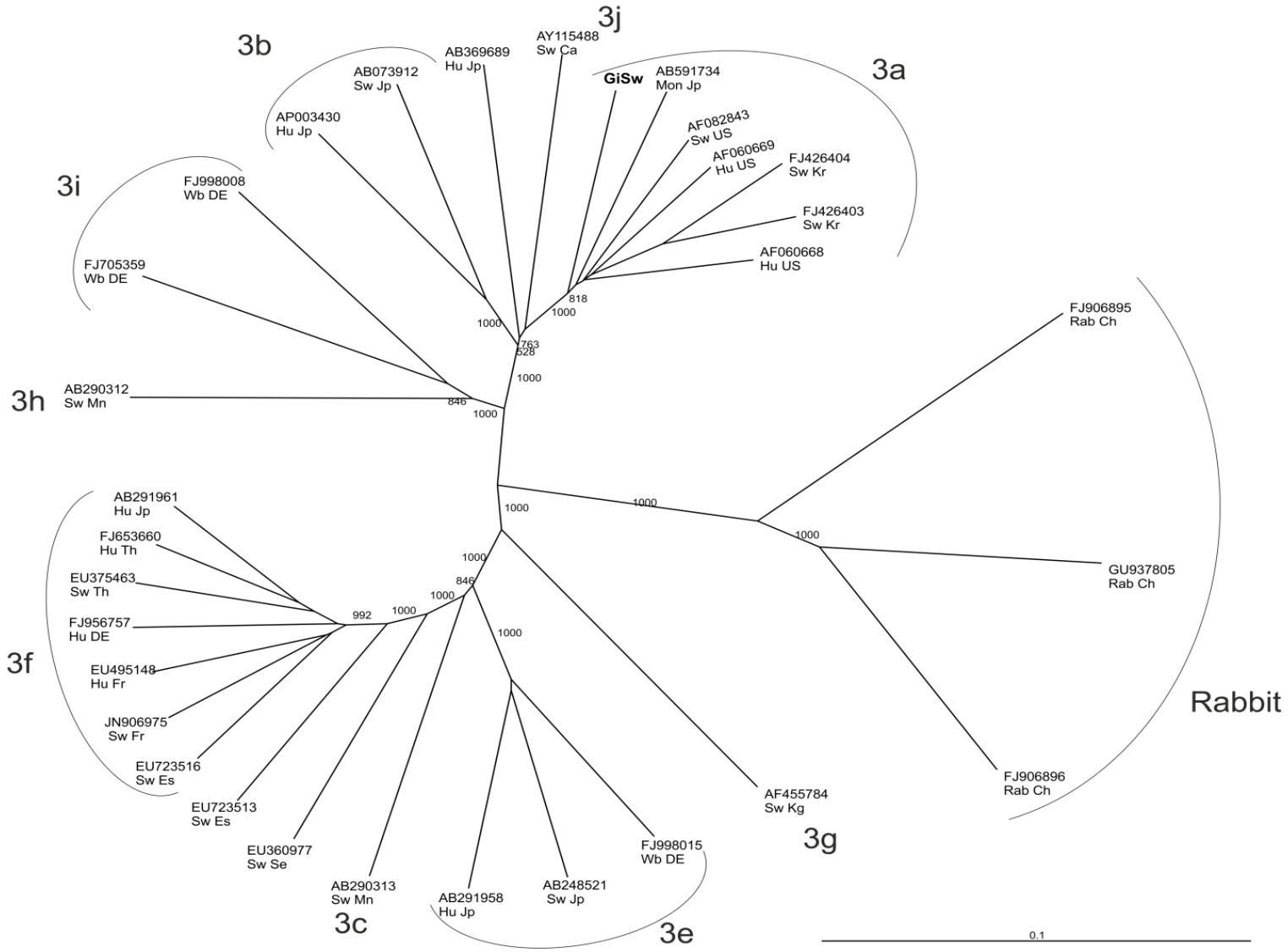


Figure 16: Phylogenetic analysis of HEV genotype 3 based on complete sequences (excluding NTRs). Phylogenetic distances were calculated using the Kimura-2 parameter method. Tree was calculated by the neighbor-joining method. The branch lengths are proportional to the genetic distances. A bootstraps analysis of 1000 replicates was included; numbers indicate bootstrap values. Additional sequences were obtained from GenBank with (accession numbers indicated). Host and country where HEV was found is given (Sw: Swine, Hu: Human, Wb: wild boar, Mon: mongoose, Rab: Rabbit).

Table 30: Pairwise comparison based on complete genomic sequences (excluding NTRs) including the GiSw sequence.

	AJ272108	AB602441	AF455784	JN906974	AF082843	GiSw	AY115488	AP003430	FJ906895	GU937805	M73218	M74506
GT4 (AJ272108)		28.02	30.48	30.31	30.43	30.99	32.09	30.27	32.79	31.98	31.25	32.95
GT5 (AB602441)			31.17	32.02	32.77	32.31	33.01	32.18	33.72	32.76	32.35	33.97
GT3.2 (AF455784)				19.55	21.13	20.81	21.33	21.46	26.40	24.61	31.32	32.27
GT3.2 (JN906974)					21.29	21.67	22.51	21.49	26.20	25.42	31.76	32.41
GT3.1 (AF082843)						10.72	13.87	14.18	26.23	25.12	32.14	31.44
GiSw							14.24	14.39	26.19	25.46	32.67	32.15
GT3.1 (AY115488)								15.19	26.92	25.20	32.31	32.85
GT3.1 (AP003430)									25.26	25.10	31.71	32.67
GT3.3 (FJ906895)										18.04	33.24	35.12
GT 3.3 (GU937805)											32.42	34.08
GT2 M73218												28.96
GT1 M74506												

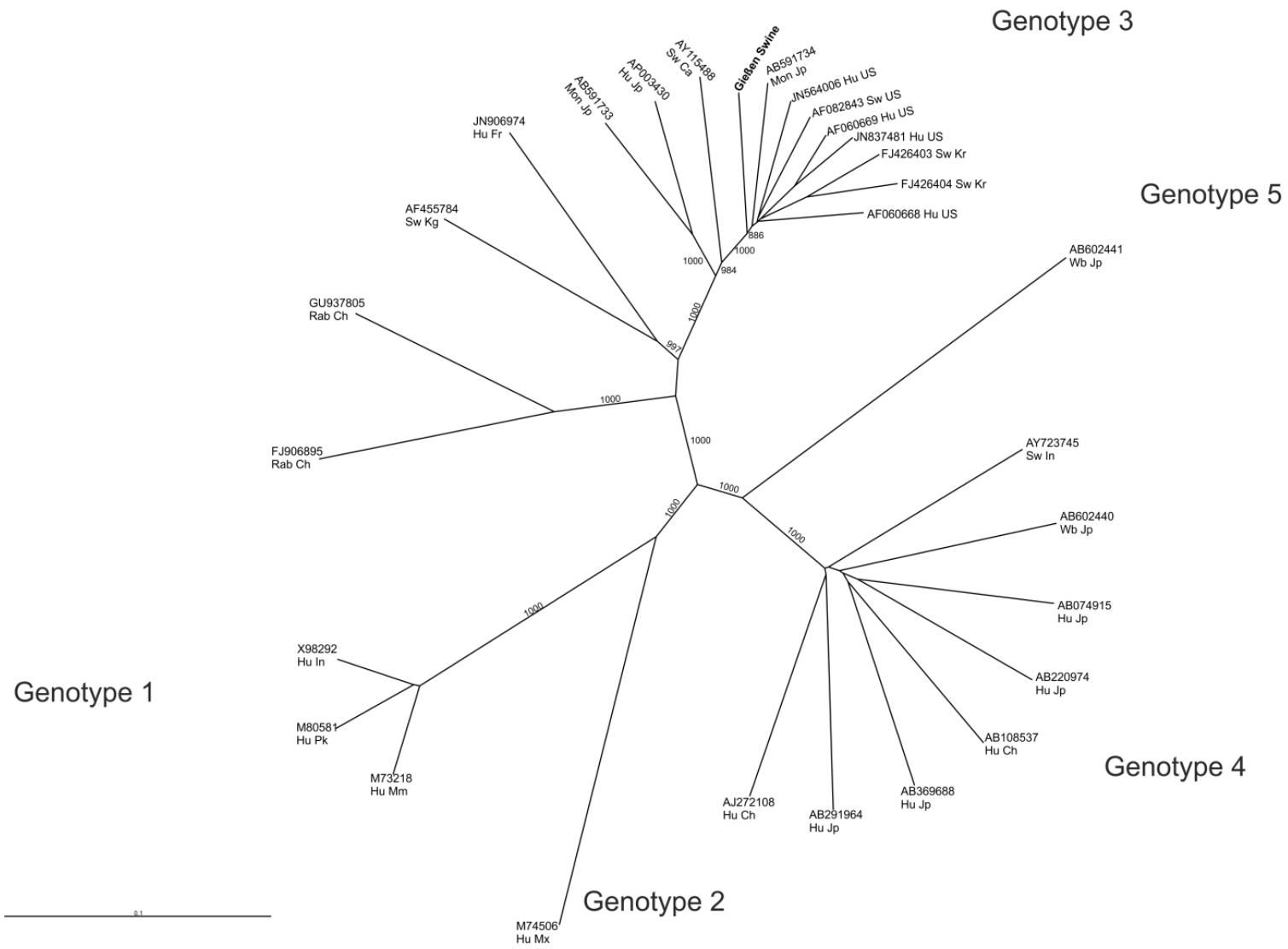


Figure 17: Phylogenetic analysis of HEV based on complete sequences (excluding NTRs) of HEV isolates (GT1 – 5). Phylogenetic distances were calculated using the Kimura-2 parameter method. Tree was calculated by the neighbor-joining method. The branch lengths are proportional to the genetic distances. A bootstraps analysis of 1000 replicates was included; numbers indicate bootstrap values. Additional sequences were obtained from GenBank with (accession numbers indicated). Host species and country of origin are indicated (Sw: Swine, Hu: Human, Wb: wild boar, Mon: mongoose, Rab: Rabbit).

4.2 Genetic variability in HEV isolates [1]

The International Committee on Taxonomy of Viruses (ICTV) does usually not consider classification below the specie level. The ICTV has defined four HEV genotypes and there is no official classification system for subtyping. Some research groups only use genotypes for classification (Sonoda et al., 2004; Takahashi et al., 2003; Tei et al., 2003; Wibawa et al., 2004), while others use one of the proposed subtyping systems. Arankalle and colleagues (Arankalle et al., 1999) suggested to divide genotype (GT) 1 into four sub-genotypes (a, b, c and d), while Tsarev and coworkers (Tsarev et al., 1999) proposed one extra group (I2) in GT 1. Wang et al. (Wang et al., 1999) proposed to divide GT 1 in five groups and GT 3 into 2 groups; Schlauder and Mushahwar (Schlauder and Mushahwar, 2001) divided GT1-4 into 11 independent subtypes. These systems were used to classify human strains at a time when few sequences from animal isolates were available. The most widely accepted system for subtyping of HEV sequences was published by Lu et al. (2006). This system placed the HEV sequences available at that time into 24 subtypes: GT 1(a-e), GT 2 (a,b), GT 3 (a-j) and GT 4 (a-g). Due to limited availability of sequence information the subtyping was based on both complete genomic and partial sequences from five different genomic regions. Complete genomic sequences were available for only few subtypes of genotype 3 (3a, 3b, 3g and 3j) and 4 (4c, 4d and 4g). In the meantime the number of HEV sequences has increased considerably from less than 10 complete sequences in the year 1991 up to more than 90 in July 2012 (Fig. 18).

4.2.1 Evaluation of the current system

In our study three regions used by Lu et al. (2006) were reanalyzed and novel sequence information was added: ORF1 (first 287 nt 5' end), ORF2 (301 nt of 5' end and 148 nt: 6390-6537). The use of these different genomic regions resulted in variable grouping of HEV isolates and did not allow a clear differentiation between certain subtypes. Furthermore, the respective phylogenetic trees were based on extremely low bootstrap values and did not allow a clear designation of subtypes (Fig. 19 A, B and C). For instance, using the region from ORF1 it was not possible to differentiate between subtypes 3i, 3h and 3c as proposed by Lu et al. (2006); instead, all three were placed within a single branch. In

[1] Adapted from Oliveira-Filho et al., 2013

addition the sequences classified as subtypes 3e and 3f by Lu et al. (2006) were placed into separate branches, but with very low bootstrap values (Fig. 19 A). Using one region of ORF2 (148 nt) it was not possible to differentiate between subtypes 3i, 3b, 3h as well as between 3e, 3f, 3g. Isolates representing these subtypes were mixed in two branches and a large number of potential new subtypes could be formed (Fig. 19 B). According to our data sequences previously classified as subtypes 4a and 4f by Lu et al. (2006) belong actually to one subtype within genotype 4 (Fig. 19 and 21).

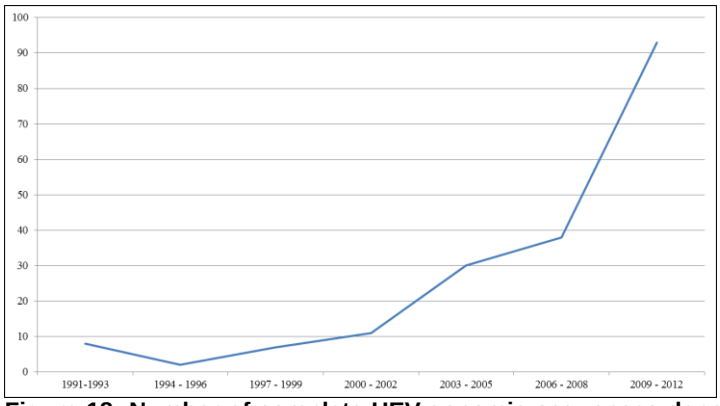
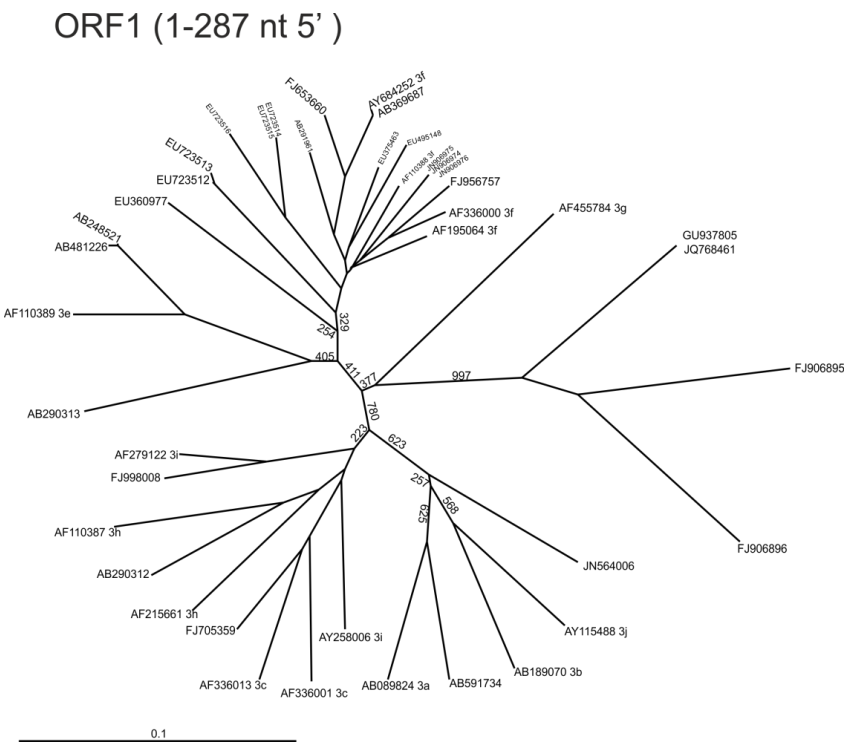


Figure 18: Number of complete HEV genomic sequences deposited in GenBank from 1991 to July 2012 (adopted from Oliveira-Filho et al., 2013).



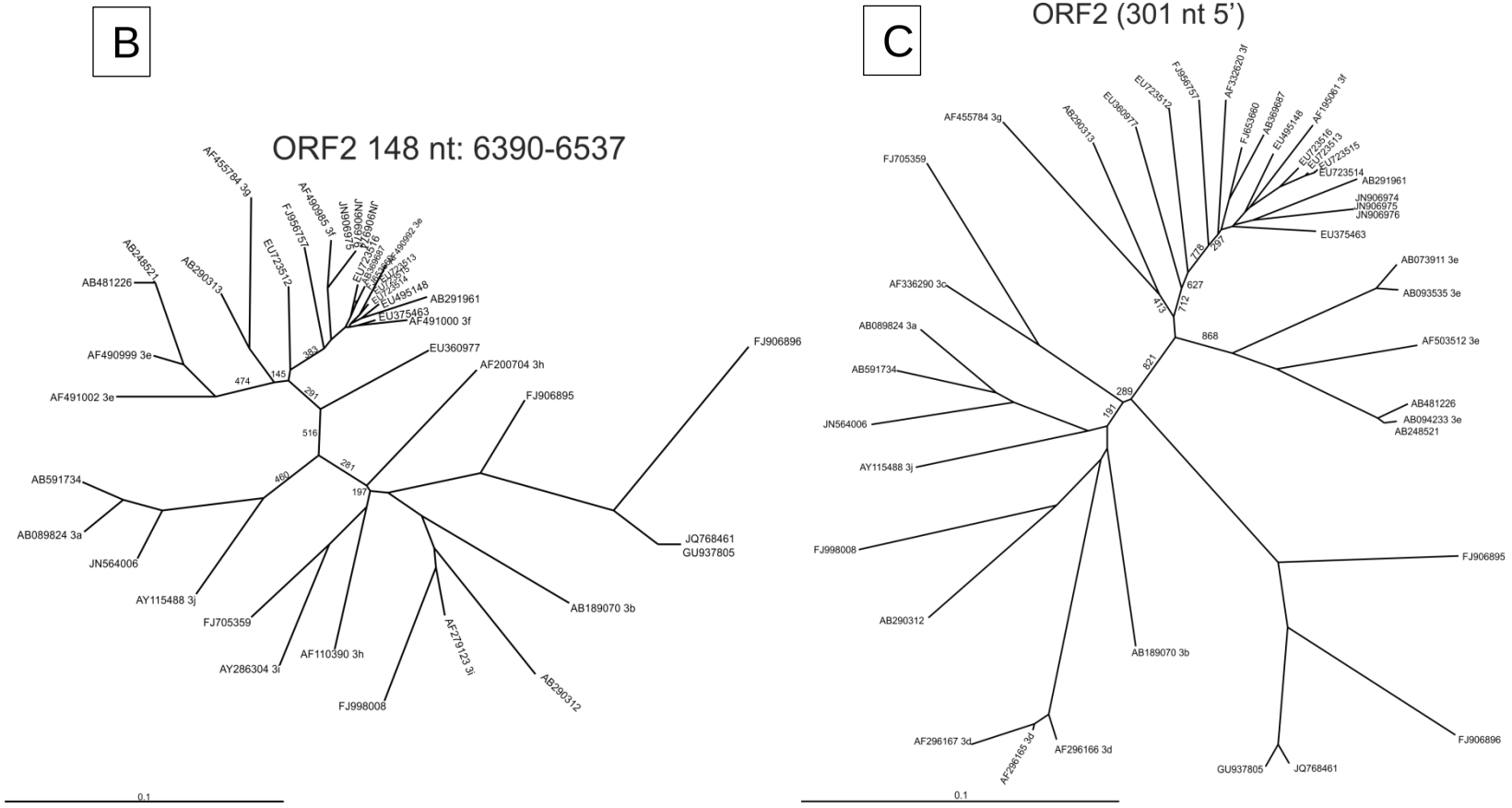


Figure 19: Phylogenetic trees based on different genomic regions of the HEV genome: ORF1: 5' 287 nt (A), ORF2: 148 nt 6690 - 6537 (B) and ORF2 5' 301 nt (C). Phylogenetic distances were calculated using the Kimura-2 parameter method. Tree was calculated by the neighbor-joining method. The branch lengths are proportional to the genetic distances. A bootstraps analysis of 1000 replicates was included; numbers indicate bootstrap values.

4.2.2 Grouping of HEV based on complete genomic sequences

As the next step HEV complete genomic sequences were used for phylogenetic analyses instead of partial fragments. It was expected that this approach would lead to a higher reliability of the analyses.

Based on analyses performed here, the current HEV genomic sequences cluster into seven major branches, including established genotypes (GT) 1, GT 2, GT 4, three branches for GT 3 and a new branch formed by two wild boar isolates from Japan provisionally termed “GT 5” (Fig. 20 and 21). The maximum nucleotide differences observed within the established genotypes are 13.16 % (GT 1), 27.10 % (GT 3) and 19.96 % (GT 4); only one and two complete sequences are available for GT 2 and GT 5, respectively. Genotype 3 showed a particularly high heterogeneity and could be separated into three subgroups, based on tree topology, nucleotide divergence and epidemiological features. Subgroup 3.1 contains human and animal sequences from Asia (Japan, China, Korea, Mongolia), North America (USA, Canada) and Germany. Subgroup 3.2 comprises mainly sequences recently obtained from Europe, Japan, Thailand and one distantly related sequence from Kyrgyzstan. Subgroup 3.3 contains HEV sequences from rabbits farmed in China. Subdivision of GT 3 into three subgroups reduced the nucleotide divergence within the subgroups: 19.22 % (subgroup 3.1), 20.11 % (subgroup 3.2) and 18.01 % (subgroup 3.3). These values are comparable to the distances observed within genotypes 1 and 4 (Fig. 20). Our phylogenetic analyses support the idea that newly identified wild boar isolates from Japan form a novel separate genotype (“GT 5”).

In comparison to GT 1 and 3, GT 4 showed a different pattern when the nucleotide divergence was compared. The spectrum of nucleotide distances between genomic sequences placed in GT 4 was narrow when compared to the other genotypes; this can be seen by the difference between the first (15.34 %) and the third (18.55 %) quartile (Fig. 20). Apparently most GT 4 sequences are equally distant to each other, pointing to a separation into sub-groups.

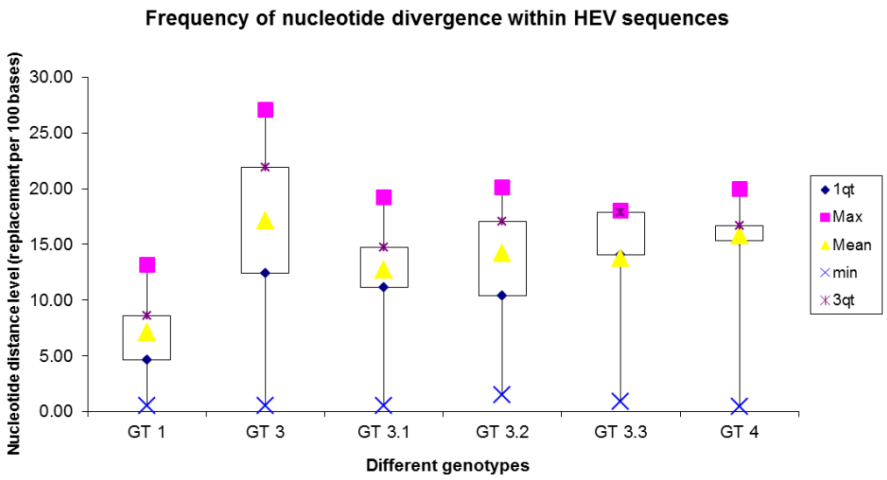


Figure 20: Box-and-whisker plots of nucleotide divergences within genotypes 1, 3 and 4. Note the decreasing level of heterogeneity when GT 3 is split into the subgroups 3.1, 3.2 and 3.3 (adopted from Oliveira-Filho et al., 2013).

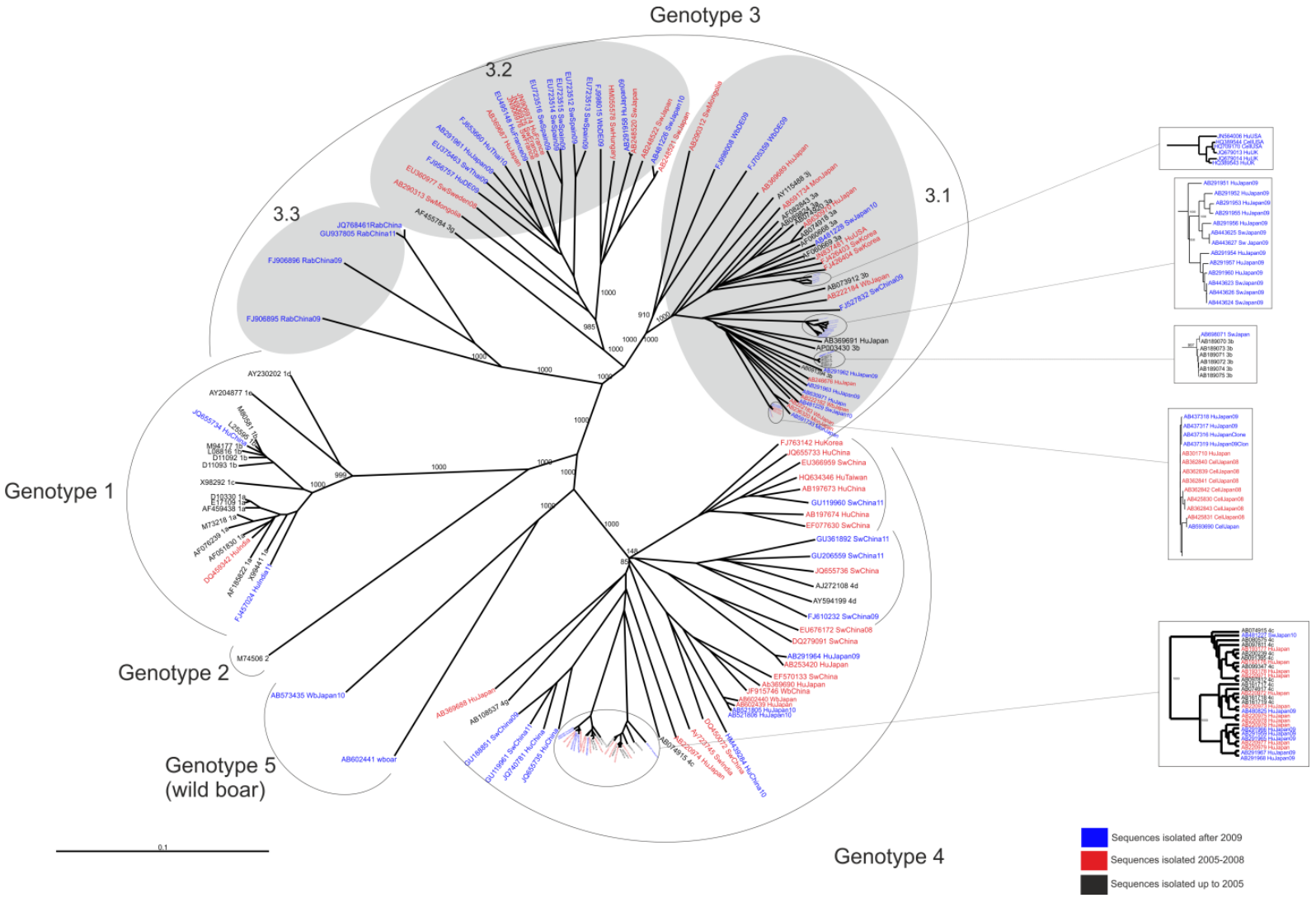


Figure 21: Phylogenetic tree based on 186 complete genomic sequences of HEV isolates. The tree was calculated by the neighbor-joining method. The branch lengths are proportional to the genetic distances. Bootstrap values of 1000 replicates are indicated (adopted from Oliveira-Filho et al., 2013).

4.2.3 HEV-like viruses

Recently, HEV-like viruses have been identified in rats, bats and ferrets (Drexler et al., 2012; Johne et al., 2010b; Raj et al., 2012). The genomic sequences of these viruses showed a high degree of divergence (64.03 – 81.21 %) when compared to the new Japanese wild boar isolates. When these sequences were compared to the isolates from the established HEV genotypes (1-4) on nucleotide basis, distances ranged from 27.11 to 34.05 %. Our phylogenetic analysis together with sequences from HEV-like viruses clearly place Japanese wild boar virus within HEV as a new genotype (Fig. 22).

Nucleotide distances ranged from 63.23 % to 81.60 % between viral sequences from rats, ferrets and bats compared to HEV GT1-4 (Table 31). Based on the phylogenetic analyses recently discovered HEV-like viruses as well as avian HEV can be considered as new genera within the family *Hepeviridae* (Fig. 22).

A virus found recently in cutthroat trout was claimed to be a member of the *Hepeviridae* family. This assumption was based in sequence analysis conserved motifs (helicase) (Batts et al., 2011). However, in our analysis using complete genomic sequences, the cutthroat trout virus (CTV) did not show a measurable degree of relatedness to HEV or the other members of the *Hepeviridae* (Table 31).

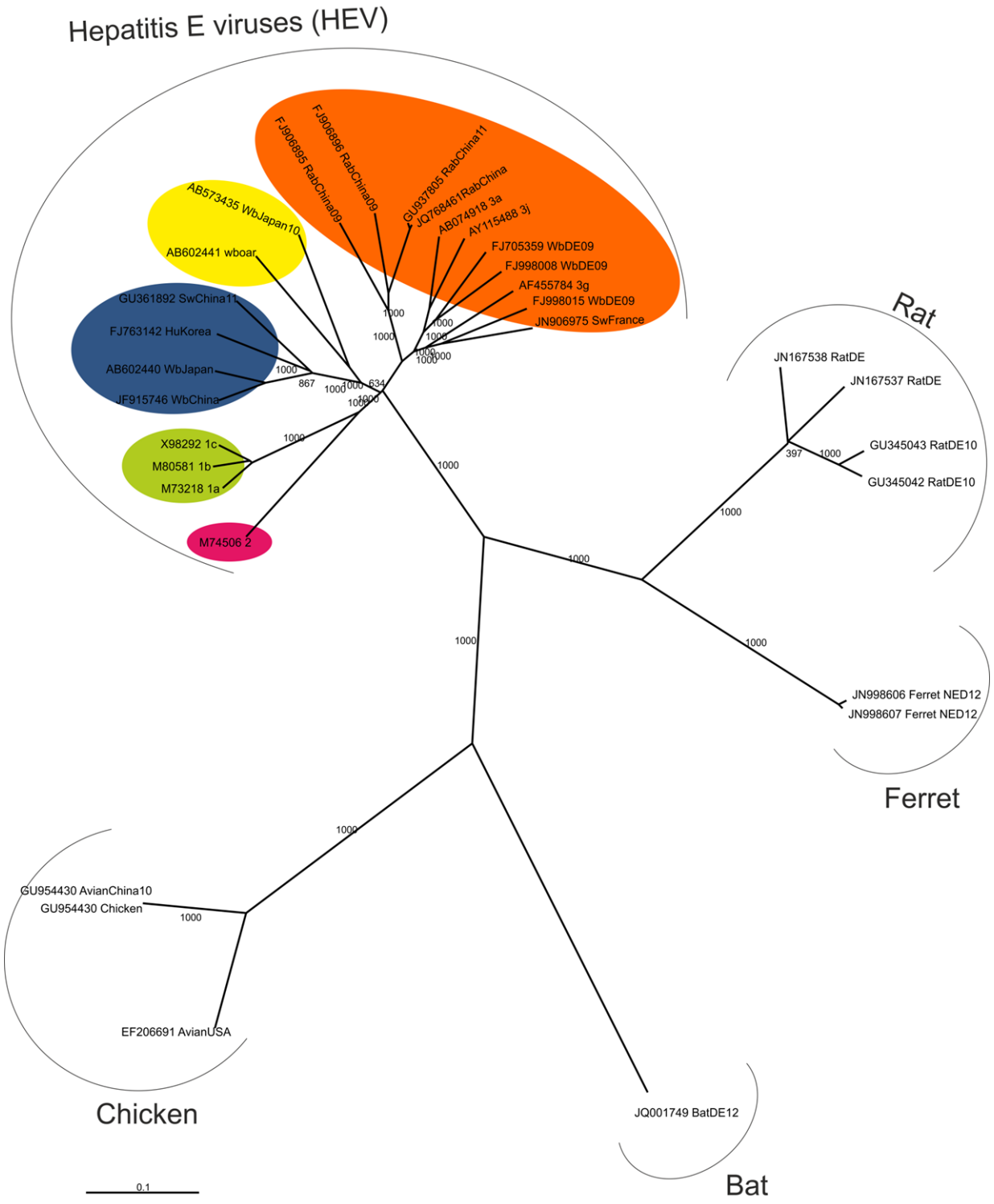


Figure 22: Phylogenetic tree based on complete genomic sequences of HEV and HEV-like isolates. Colors indicate HEV genotypes: GT 1 green, GT 2 pink, GT 3 orange and GT 4 blue and GT 5 yellow. The tree was calculated by the neighbor-joining method. The branch lengths are proportional to the genetic distances. Bootstrap values of 1000 replicates are indicated.

Table 31: Comparison between wild boar (GT 5), HEV-like viruses (found in rat, ferrets and Bats) and Cutthroat trout virus (CTV) to HEV (GT 1-4). Distances matrix based on complete genomic sequences. Values indicate the number of substitutions per 100 bases corrected by Kimura-2 parameter method.

	GT 5	Rat	Ferrets	Chicken	Bats	Fish
Min	27.11	66.49	63.23	78.15	78.48	>100
Mean	31.50	69.02	65.36	80.07	79.72	>100
Max	34.05	71.05	67.63	82.71	81.60	>100

4.2.4 Subtyping of genotypes 3 and 4

The use of the complete capsid gene sequences instead of genomic sequences provided reliable phylogenetic trees and is considered adequate to classify available sequences into genotypes. The phylogenetic trees calculated either by the neighbor-joining or the maximum-likelihood methods led to similar topology (Fig. 23).

Based on tree topology and pairwise nucleotide differences, GT 3.1 could be divided into three subtypes, GT 3.2 into three subtypes and GT 3.3 into two subtypes. However, the sequences within the groups showed high nucleotide divergence levels (up to 15.45 %between 3.1, 3.2 and 3.3).

In GT 4 several major branches were observed using both (Maximum-Likelihood and Neighbor-Joining) tree construction methods. However, acceptable bootstraps values were obtained only for three out of eight groups. Thus it was not possible to establish a reliable subtype classification comprising the majority of GT 4 isolates.

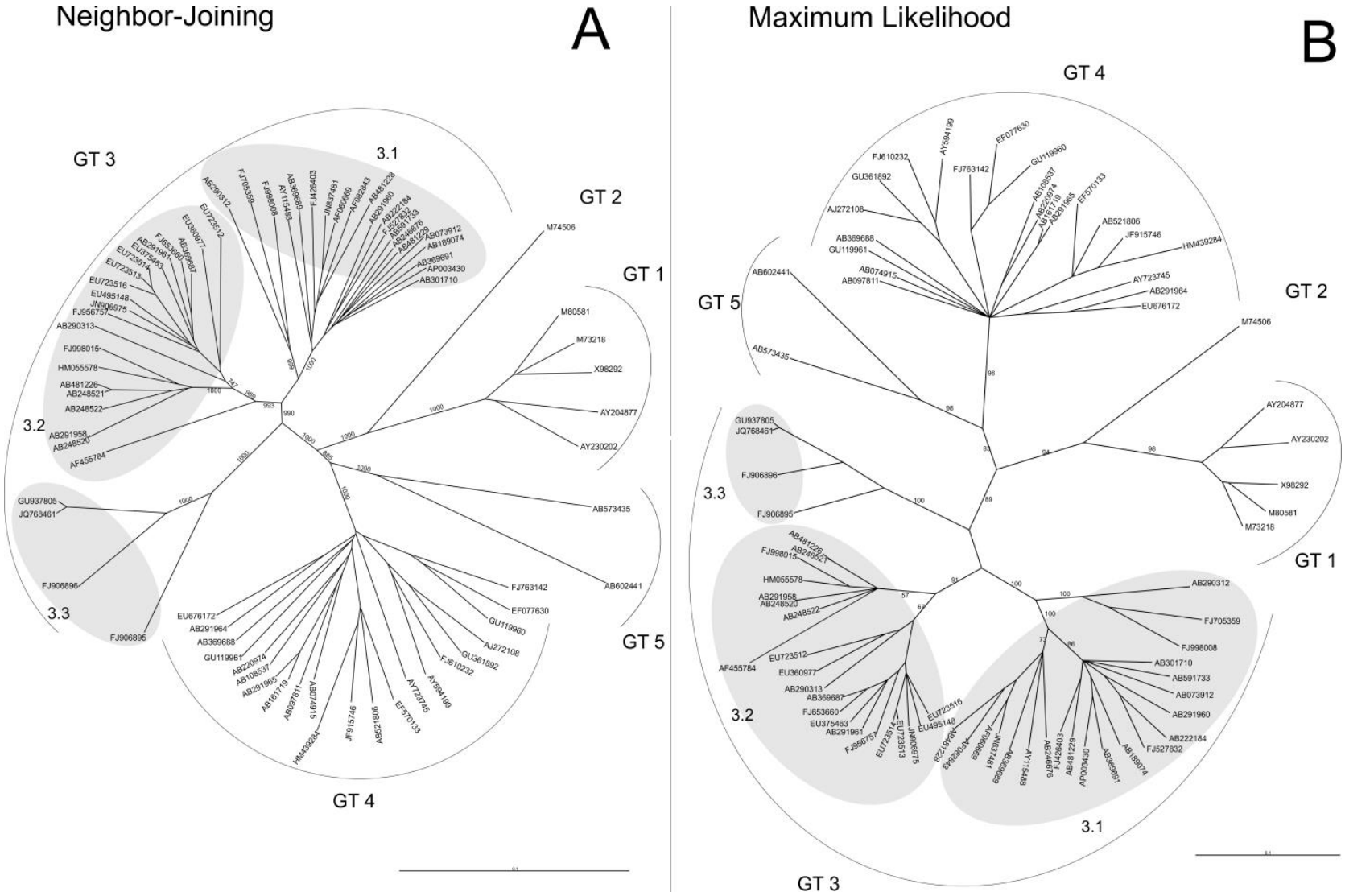


Figure 23: Phylogenetic trees based on complete capsid gene sequences of HEV. Trees were calculated by neighbor-joining (A) and maximum likehood (B) methods. Bootstrap values of 1000 (A) and 100 (B) replicates are indicated.

4.2.5 Geographic distribution

The 187 complete HEV sequences analyzed in this study originated from 21 countries in 4 continents. The majority of isolates (78.07 %) was from Asia with 92 (49.2 %) from Japan, 32 (17.11 %) from China and 7 (3.74 %) from India. More detailed information about the number and origin of samples is provided in table 32. GT 1 has only been found in Africa (Morocco) and Asia (Myanmar, China, Pakistan, India, Nepal), GT 2 in Mexico, GT 3.1 in Asia (Japan, Korea and Mogolia), Europe (Germany) and North America (Canada and USA), 3.2 in Asia (Japan, Thailand, Kyrgyzstan and Mogolia) and Europe (France, Germany, Spain and Sweden), GT 3.3 (rabbit) only in China. Complete HEV genomic sequences of GT 4 is available only from Asia (Japan, China, India and Korea), however partial HEV sequences have been reported recently in Europe (Colson et al., 2012; Hakze-van der Honing et al., 2011). We did not obtain any epidemiological evidence like geographical or host distribution to support further dissection of HEV into subtypes.

Table 32: Geographic origin of the HEV complete genomic sequences.

Continent	Country	Number	(%)	Genotype
Asia	Japan	92	49.20%	1,3,4,5
	China	32	17.11%	1,3,4
	India	7	3.74%	1,4
	Korea	3	1.60%	3
	Mongolia	2	1.07%	3
	Thailand	3	1.60%	3
	Kyrgyzstan	1	0.53%	1
	Pakistan	2	1.07%	1
	Nepal	1	0.53%	1
	Myanmar	2	1.07%	1
	Taiwan	1	0.53%	4
Africa	Chad	1	0.53%	1
America	USA	6	3.21%	3,4
	Mexico	1	0.53%	2
	Canada	1	0.53%	3
Europe	Germany	8	4.28%	3
	Spain	5	2.67%	3
	France	4	2.14%	3
	Sweden	1	0.53%	3

	Hungary	1	0.53%	3
	UK	2	1.07%	3
Cells	-	11	5.88%	
Total		187		

4.3 Expression of HEV capsid protein

HEV capsid protein of the Gießen swine isolate (GiSw) was sequenced and its amino acid sequence was deduced. ORF2 with 1983 nucleotides encodes a capsid protein with 661 aa and a predicted molecular weight of about 71 kDa. Using different continuous linear B cell epitope prediction methods several immunogenic regions could be detected (Fig. 24). The HEV capsid protein contains several epitopes and immunodominant regions and thus is highly immunogenic. For instance a strong antigenic reactivity has been reported for aa 450 – 460, and an immunodominant region for aa 546 – 580 (Khudyakov et al., 1994). The region between aa 607 – 659 was the shortest fragment to be recognized by two neutralizing mAbs (Zhou et al., 2004).

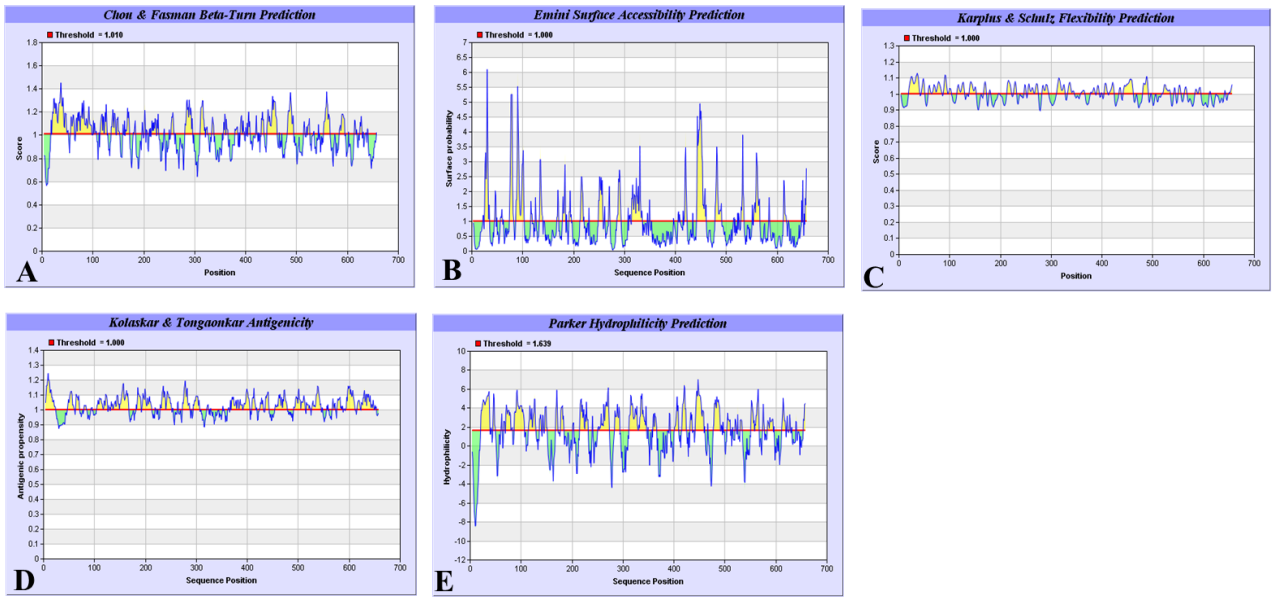


Figure 24: Prediction of epitopes for the GiSw capsid protein based on Chou & Fasman Beta-Turn (A), Emini Surface Accessibility (B), Karplus Schulz Flexibility (C), Kolaskar & Tongaonker Antigenicity (D), Parker Hydrophilicity (E) prediction methods. The red line shows the threshold, yellow and green colors have different meaning according to the method employed: A and C: score of antigenicity, B: score of surface probability,D:levels of antigenic propensity,E: hydrophilicity.

4.3.1 Construction of recombinant capsid protein (ORF 2) in pET vector

pET 26 (+) vector is a commercial vector from Novagen™ used to express proteins in the *E. Coli* system under control of the T7 promoter. The pET 26 plasmid was modified by insertion of ubiquitin to the encoding sequence in front of the multiple cloning site. Accordingly, foreign proteins are expressed as fusion proteins with ubiquitin at the N-terminus and a poly histidine tag at the C-terminus. It was planned to express two different regions of the capsid protein namely aa position 1 – 278 in the N-terminal and a aa 543 – 617 located in the C-terminal region (Fig. 25 and 26).

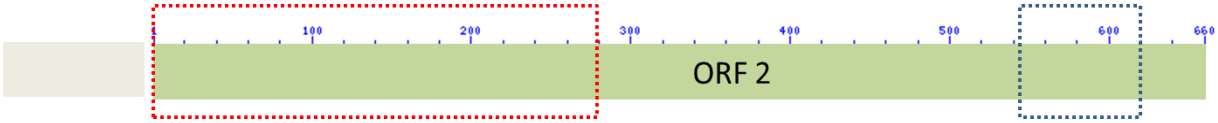


Figure 25: Regions of HEV ORF2 expressed in *E. coli*.

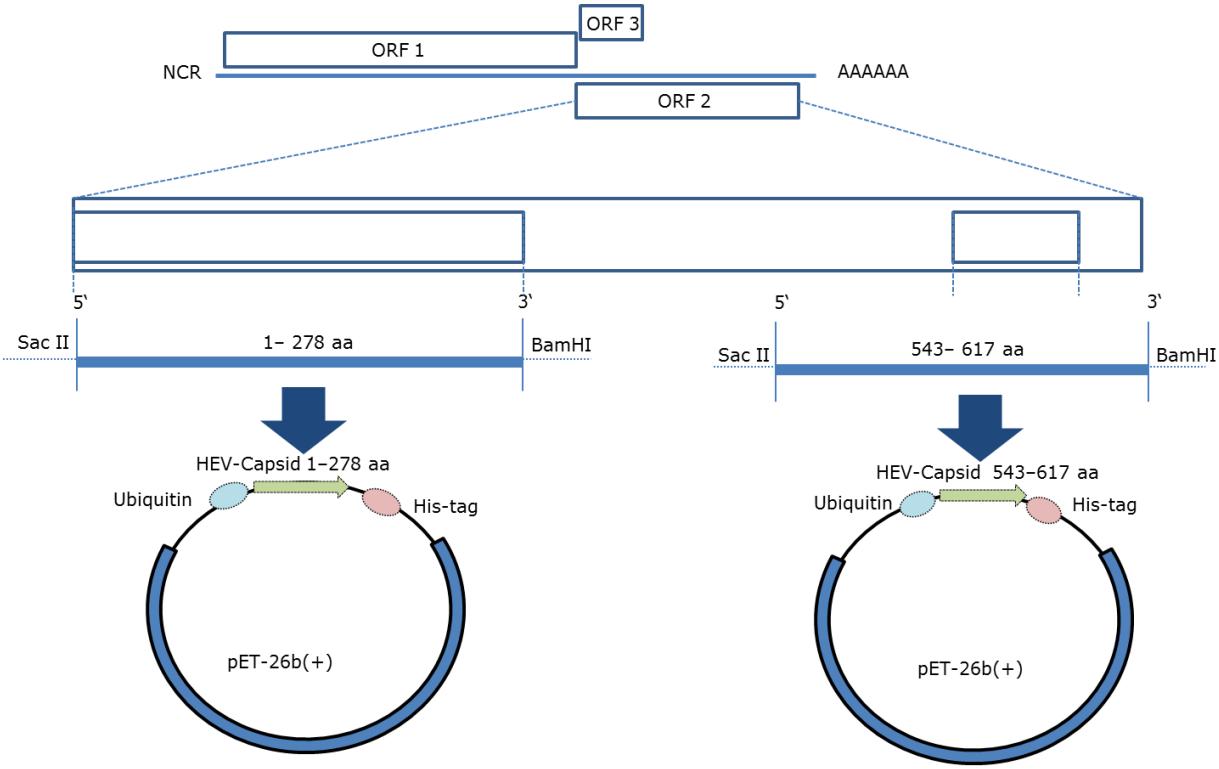


Figure 26: Strategy for construction of HEV Cap 2.1 and R4b recombinant vectors.

For the amplification PCR was performed with the plasmid which contains the R4 and HEV 2.1 regions. Primers were designed including SacII and BamHI restriction sites. The two different regions of the capsid protein in the N- and C-terminus were expressed using the pET vector, as can be seen in Figures 25 and 26.

4.3.2 Expression of the two capsid protein regions

Western blot analysis of extracts from the bacterial cell pellets using anti–ubiquitin and anti–His-tag monoclonal antibodies (Mab) (Fig. 27) demonstrated the presence of the recombinant proteins detected in the expected sizes (Fig. 27).The fusion proteins showed estimated molecular weights of 17.2 kDa (Ubi-R4bHEVcap-His) or 38 kDa (Ubi-HEV 2.1-His) (Fig. 27 and 28).

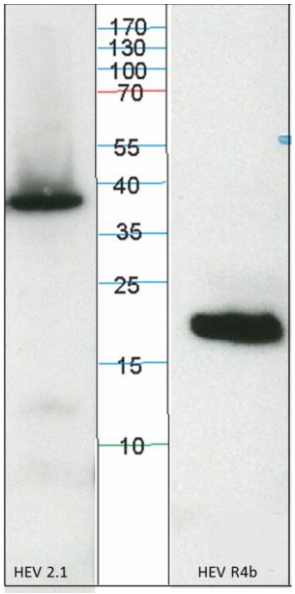


Figure 27: Western blot of fusion proteins HEVcap2.1 (left) and R4b (right) using anti-ubiquitin monoclonal antibodies showing bands in the expected sizes.

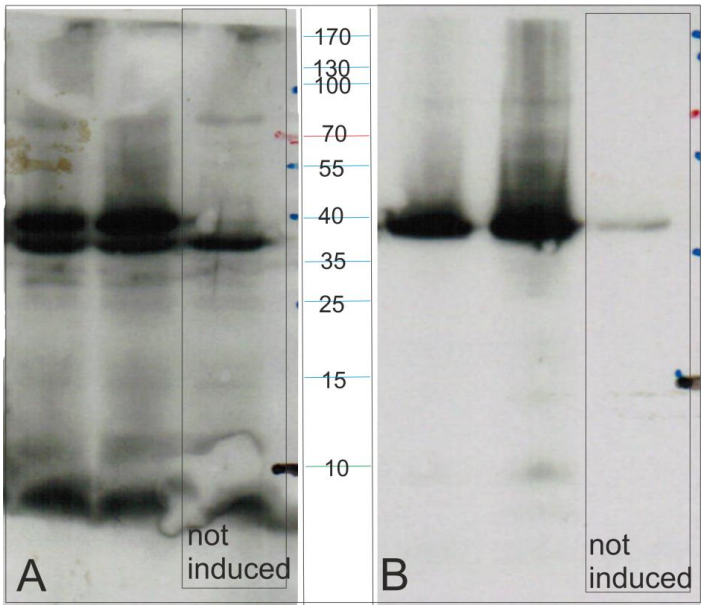


Figure 28: Western blot of fusion proteins R4b and HEVcap2.1. Reactivity with anti-His tag (A) and anti-ubiquitin (B) monoclonal antibodies. Note the presence of a double band in A and the presence of bands without IPTG induction.

4.3.3 Reactivity of fusion proteins

Both proteins were successfully expressed and reacted with both the anti-ubiquitin and anti-His-tag monoclonal antibodies (Fig. 27 and 28). Expressed products were purified by ultracentrifugation and ion exchange chromatography using Ni-NTA Columns (Qiagen). Proteins were eluted using different concentrations of Imidazol as shown in Figures 29 A and B.

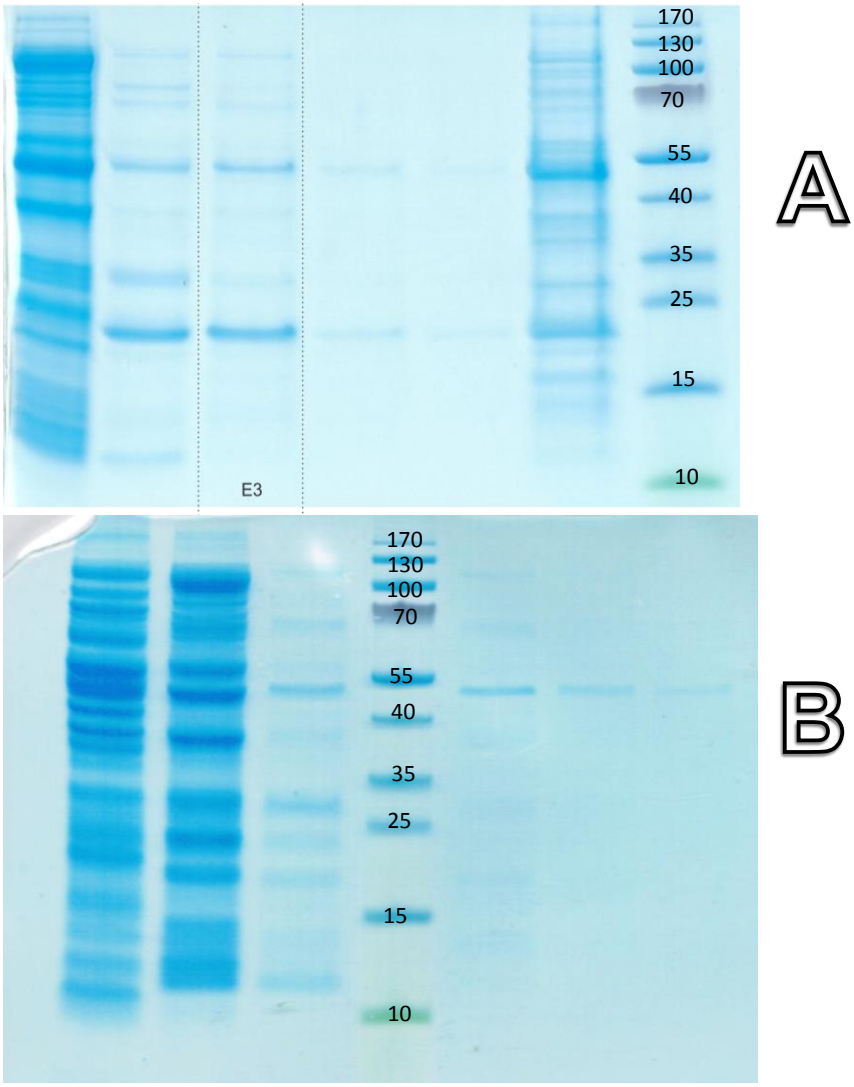


Figure 29: Coomassie blue stained polyacrylamide gel. Elution with different concentrations of imidazol. Protein R4b (A) with expected size of 17.2 kDA and HEV2.1 (B) with expected size of 38 kDA. E: Elution steps. Selected fraction used in further experiments with R4b: elution 3 (E3).

Purified protein R4b was tested in an immunoblot with one human and four swine anti-HEV positive sera known to be positive by ELISA. First a positive human serum was used. For swine, the four sera tested by a commercial ELISA (MP Diagnostic) were

positive, weak positive, strong positive, and negative. The sera were incubated overnight in a dilution of 1:50 (Fig. 30). In western blot the human serum showed a very discrete band which might indicate that it is reacting with our protein (Fig. 30). All swine sera were negative (data not shown).

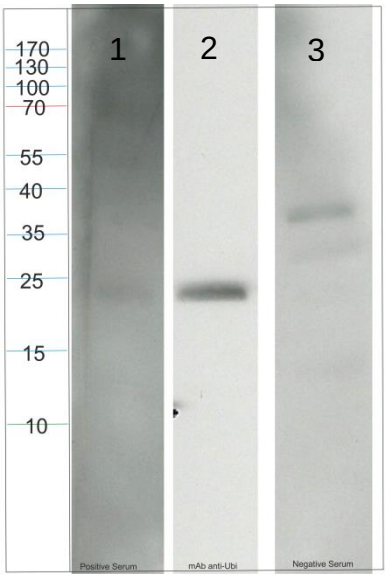


Figure 30: Immunoblot with R4b protein; human positive serum (1) and human negative serum (3) mAb anti-ubiquitin (2).

4.4 Cultivation of HEV

So far there is no reliable cell culture system for propagation of HEV. Some cell lines such as human adenocarcinomic alveolar basal epithelial cells (A549) and human hepatoblastoma cells (PLC/PFR/5) have been reported to be permissive for HEV. However, no cytopathic effect could be associated with HEV propragation (Okamoto, 2011a; Tanaka et al., 2007). Thus, the growth of HEV in cells is measured by quantification of HEV RNA in the cells. A quantitative RT-PCR (qRT-PCR) assay was developed for this purpose.

The RNA integrity, viral infectivity as well as the amount of viruses present in the domestic swine and wild boar samples (tested positive by RT-PCR in the first part of this thesis) was unknown. Therefore, for infection of cells a liver fragment from pigs experimentally infected with a genotype 3 HEV Dutch strain (DQ996399) was used (Bouwknegt et al., 2008); kindly provided by Prof Dr. Wim van der Poel.

4.4.1 Realtime RT-PCR for HEV detection

New primers and a probe were designed based on the Dutch strain DQ996399 (Bouwknegt et al., 2008). The primers might amplify other HEV genotype 3 strains but, due to the high specificity, it is unlikely that the probe will bind with another viral strain. A quantitative PCR assay with the respective primers and probe was successfully validated for the detection and quantification of HEV in liver and in cell culture (Fig. 31 A and B). Cells were tested together with a housekeeping gene 18s. Accordingly, the Taqman assay was efficient to amplify the standard positive samples as evidenced by the fluorescence curve demonstrated in figure 31 A. The standard curve was generated using 10-serial fold dilutions. It is important to note the quantification does not represent the amount of viral RNA as there has been no reverse transcription control in the test.

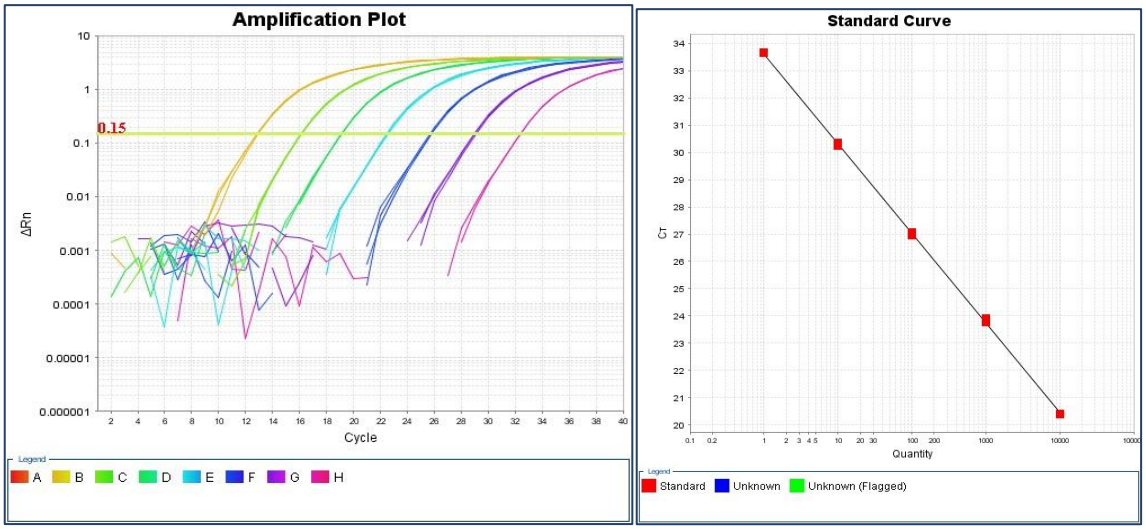


Figure 31: qRT-PCR validation based on serial diluted plasmid: (A) amplification of the standard positive and (B) standard curve. Slope =-3.316, intercept = 36.873, r = 0.996, E=100 %.

4.4.2 Infection experiments

A549 cells were infected with liver suspension from an experimentally infected pig (DQ996399). Infection was performed in the presence of different amounts of FCS in the medium (1, 2, 5 and 10 %). HEV RNA could be detected and quantified in cell supernatant samples and also in the cellular fraction. The amount of viral RNA in cell culture supernatants first decreased and then increased progressively from day 5 until day 15 in plates where 1 % FCS (Fig. 32) was used; however the amount of viruses detected was too low (2.79×10^2). No cytopathic effect (CPE) was detected and after the second passage

(after 28 days) it was not possible to detect HEV RNA either in cells or in supernatants. Using media with 2 % FCS higher and almost constant ct values were founded (Table 33). In the presence of concentration of 5% FCS HEV was detected only on days 1 and 2 after infection and the amount of virus estimate was low (higher ct values).

Another infection experiment has been performed with shrew hepatocytes (kindly provided by Dr. Dieter Glebe, Institute of Medical Virology, JLU Giessen) and two distinct viruses: swine liver suspension (as was performed for the A549 cell line infection) and human positive serum (kindly provided by Dr. Christian Schüttler, Institute of Medical Virology JLU Giessen). Hepatocytes were kept for seven days. Culture supernatant was collected on days 1, 4 and 7 and at the end hepatocytes were harvested and analyzed by electron microscopy (EM). No HEV RNA was detected on days 1, 4 and 7 and no viral particles were observed by EM in hepatocytes on day 7 P.I. (data not shown).

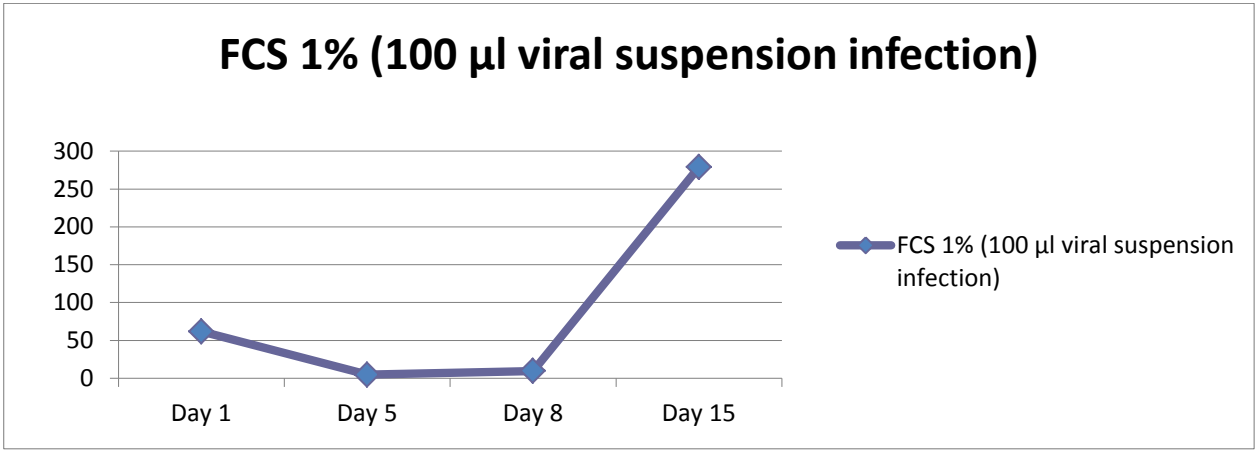


Figure 32: Increase of viral copies in the supernatant after infection with 100µl of liver suspension according to the day collected in the preliminary experiment.

Table 33: Ct values of the infection with A549 cell line using different FCS concentrations.

	FCS 1% 100 µl	FCS 2 % 100µl
Day 1	32.16	32.15
Day 5	36.13	34.25
Day 8	35.11	33.83
Day 15	28.82	34.12

5 Discussion

5.1. Prevalence of hepatitis E virus (HEV) in swine and wild boar

Domestic swine and wild boar have been reported as the major animal reservoir of HEV. Some retrospective studies suggest that HEV circulated in both domestic swine and wild boar for decades (Casas et al., 2009; Kaci et al., 2008).

Within this study, HEV could be detected in 18 out of the 124 sera from wild boar, corresponding to a detection rate of 14.5 %. This implies that HEV is endemic in the wild boar population of western Hesse / Germany. The presence of HEV in wild boar has been shown in many countries including Germany (Kaci et al., 2008; Martelli et al., 2008). The high prevalence rate observed in the current study is comparable to those previously found in other parts of Germany (Adlhoch et al., 2009a; Schielke et al., 2009) and other European countries such as Spain (de Deus et al., 2008b) and Italy (Martelli et al., 2008). Lower prevalence rates were reported in France, the Netherlands and in a retrospective study for Germany (Kaba et al., 2009; Kaci et al., 2008; Rutjes et al., 2009).

In contrast to the widespread occurrence in wild boar HEV was detected in only one out of 105 domestic swine analyzed. This rate is considerably lower than in other countries e.g. Brazil 9.3 % (dos Santos et al., 2009), Canada 34.4 % (Ward et al., 2008), China 47.9 % (Geng et al., 2011), India 12.3 % (Arankalle et al., 2003), Japan 14.5 % (Tanaka et al., 2004), United States 35.4 % (Huang et al., 2002) and also other European countries such as France 31.2 % (Kaba et al., 2009), Italy 29.9 % (Martelli et al., 2010), Spain 37.7 % (de Deus et al., 2007) and the Netherlands 22.0 % (van der Poel et al., 2001). Prevalence rates comparable to the current showed were reported China 0.8 % (Geng et al., 2010), Japan 1.8 % (Sakano et al., 2009), Taiwan 1.3 % (Wu et al., 2000), India 2 % (Vivek and Kang, 2011), Korea 1.9 % (Lee et al., 2009a), Indonesia 1 % (Utsumi et al., 2011), Bali 1% (Wibawa et al., 2004) and Congo 2.5 % (Kaba et al., 2010a).

According to the data in this thesis HEV is apparently more widespread in the wild boar population than in domestic swine. There are however a number of technical factors that might have influenced the results. The difference in prevalence rates may be influenced by the kind of samples tested, like serum for wild boar and feces for domestic

swine. Experimental studies showed that HEV could be detected more frequently and for longer periods in feces in comparison to blood samples (Bouwknegt et al., 2009). Another factor is the presence of inhibitors of RT-PCR; we did neither test for inhibitors nor quantify the amount of HEV RNA. According to both field and experimental studies detection rates are correlated with age of animals. HEV RNA appears to be more easily detectable in domestic pigs up to six months of age in comparison to older animals (dos Santos et al., 2009; Huang et al., 2002; Lee et al., 2009b). For animals in our study we have limited information about age.

Social, behavioral and environmental differences between domestic swine and wild boar may play a role in viral transmission. Wild boar, as free-living opportunistic omnivores, may be exposed to constant re-infection. For domestic swine good hygiene conditions and the restriction of animal interaction probably influences the detection rates. It is remarkable that wild boar sera samples from Morocco were all negative for HEV RNA. A commercial ELISA gave no indication for antibodies against HEV (data not shown). Several outbreaks of HE in humans have been reported in different African countries such as Chad, Egypt, Kenya, Morocco, Somalia, Sudan and Uganda; the majority of HE viruses belonged to genotypes 1 or 2 not detected in animals up to now; infection was linked to contaminated food and water (Benjelloun et al., 1997; Teshale et al., 2010; Tsarev et al., 1999; van Cuyck et al., 2003). On the basis of our results one might assume that the wild boar population in Morocco is unlikely to be reservoir of HEV. Animals tested however came from an isolated population and we cannot infer whether the results are statistically significant for the general population.

5.1.1. Phylogenetic analyses

HEV is currently divided into four genotypes, GT 1 – GT 4. The subtype classification so far is not consensual. The currently most accepted classification of 24 HEV subtypes has been proposed by Lu and colleagues (Lu et al., 2006). They divided GT 3 into 10 subtypes (a-j). The authors concluded that the observed variability may be due to the extended host range found for GT 3 (Lu et al., 2006). Isolates found in this study clustered in two distinctly branches of GT 3 demonstrating the heterogeneity of HEV within the wild boar population.

Phylogenetic analyses based on a 241 b fragment of the capsid gene did neither provide reliable trees nor separation into subtypes. Phylogenetic analyses based on complete capsid gene sequences led to a higher reliability as shown by high bootstraps values and allowed a convincing separation in subtypes. Trying to fit our data into the existing system of subtyping we have faced problems: Analyses based on different regions of the genome used by Lu et al placed our isolates in different subtypes. We therefor concluded that the subtyping classification in proposed was inconsistent. The data in this study are limited since it was not possible to obtain complete capsid gene sequence for all isolates.

The divergence observed within HEV sequences in the German wild boar population is remarkable. According to the analysis using the entire capsid gene, the two German isolates (FJ705359 and FJ998008) and the WB 1 isolate (KF303501) differ by 10.66 – 13.54 %. This is a high divergence when compared to other subtypes. Even higher nucleotide divergence was found by comparison of WB1 (Rheingau-Taunus-Kreis) and WB 69 (Hochtaunuskreis) (Figure 8), which were collected in neighbouring regions and showed a nucleotide divergence of 15.88 %. High heterogeneity plus distribution pattern indicates constant reinfection or immune evasion in the population. Constant re-infection with different strains together with the occurrence of viral recombination may explain such heterogeneity.

Zoonotic transmission of HEV has been reported to be associated with the consumption of deer, swine and wild boar meat products (Colson et al., 2010; Tamada et al., 2004; Tei et al., 2003). The HEV genotype 3 strains detected in humans (AB074918, AB089824) and GiSw, WB 69 and WB 122 cluster together and cannot be genetically distinguished. The presence of similar HEV strains both in animals and humans suggests that HEV circulate between domestic animals, free living animals and humans. This highlights the zoonotic potential of HEV as indicated by an earlier epidemiological study with hepatitis E patients in Germany (Wichmann et al., 2008).

5.1.2. Recombination of HEV

Recombination has been reported for several viruses including influenza viruses, herpesviruses and vaccinia viruses (Burnet and Lind, 1951; Fenner and Comben, 1958;

Wildy, 1955). It is a common in positive sense RNA viruses like Corona- and Flavivirus (Bull et al., 2007; Coyne et al., 2006). Recombination is thought to drive viral evolution (Worobey and Holmes, 1999). Two mechanisms of RNA recombination have been proposed: Replicative template-switching and non-replicative breakage and rejoining (Becher and Tautz, 2011).

Intra-genotype recombination within HEV genotype 1 has already been demonstrated with “China D” and “Nepal 15” isolates (van Cuyck et al., 2005). In addition inter-genotype recombination has been reported between members of genotypes 3 and 4 (Fan, 2009). It has also been suggested that the single Mexican genotype 2 sequence is a product of inter-genotype recombination (Fan, 2009). Recombination may occur in a host infected with different HEV strains (van Cuyck et al., 2005). It is not clear whether recombination plays an important role in HEV virulence as shown for other positive strand RNA viruses (Mathijs et al., 2010). According to the results within this thesis recombination may have occurred and helps to explain the heterogeneity found in our samples, e.g. the subtype change of WB69 as well as difficulties in assigning other isolates to subtypes (Figures 10 and 12). However, it is important to note that the results presented here are limited as we were not able to sequence larger fragments of the viruses.

5.2. Classification of HEV

Viruses are classified in a universal taxonomic scheme developed and updated officially by the International Committee on Taxonomy of Viruses (ICTV). Currently the ICTV classifies viruses in orders, families, subfamilies, genera and species (King et al., 2011). Accordingly there is no general official definition for genotypes, genogroups, subgroups and subtypes and the classification criteria vary for each virus family.

A proper classification of HEV and HEV-like viruses is important to understand the epidemiology of hepatitis E. It has been suggested that the clinical impact, including severe hepatic disease resulting in fulminant hepatic failure, might be related to the HEV genotype and subtype involved (Lewis et al., 2010). The lack of an unambiguous subtype classification scheme hinders a more detailed mapping of the molecular epidemiology of HEV. Moreover, the continuous increase of available sequence information makes it necessary to establish a generally accepted system for subtype classification.

In the first part of this thesis (detection of HEV in wild boar and domestic swine) it was not possible to obtain a clear definition of subtypes from all sequences found in domestic swine and wild boar. We therefore decided to perform a comprehensive analysis with all HEV complete sequences available.

The phylogenetic analyses of HEV performed here led to inconsistencies at the subtype level and challenged the current system proposed by Lu and co-workers (2006). Subtypes had been established using different regions of the genome. However, this did not result in a statistically significant assortment of viruses in phylogenetic analyses, which was reflected by low bootstrap values. Bootstrap values of 95 % or greater are statistically significant and do support a clade. Values of at least 70 % may only be taken as an indication while values below 50 % should be rejected (Soltis and Soltis, 2003). In the main branches of the phylogenetic trees based on small fragments (Fig. 19 A, B and C) compiled in this work the bootstrap values for partial sequences were below 50 %. This explains the inconsistences we found in the subtype classification proposed by Lu et al. (2006). The latter shows low accuracy in defining subtypes, and parts of it could not be reproduced. Accordingly the currently most accepted subtype classification system (Lu et al., 2006) is not very precise and may not be suitable for clinical and epidemiological studies.

In contrast, phylogenetic analyses based on complete HEV genomic sequences led to a consistent separation of established genotypes (GTs) and recently discovered isolates from rabbits, ferrets, rats and wild boar (Oliveira-Filho et al., 2013). High bootstrap values at the lower bifurcations demonstrated the robustness of the phylogenetic analysis. Topology of the tree and the high nucleotide distances observed between these HEV-like viruses and the established HEV genotypes suggest that the former should be placed in separate genera (Figures 21 und Table 30).

Remarkable differences were observed with regard to heterogeneity within established HEV genotypes. The divergence within GT 1 is lower than within genotypes 3 and 4. So far, GT 1 has only been found in humans. In contrast, viruses grouped in GT 3 and 4 have been reported in humans and different animal species. The restricted host range may be connected to the lower divergence found within GT 1. On the other hand, a

limited amount of complete genomic sequences available for GT 1 in comparison to GT 3 and GT 4 may have biased the analysis.

Our approach significantly improved the robustness of the analyses as demonstrated by high bootstrap values (Fig. 21). The separation of GT 3 into three subgroups (3.1, 3.2 and 3.3) is supported by the topology of phylogenetic trees based on both complete genomic and capsid gene sequences and the respective calculated nucleotide distances (Fig. 20, 21 and 22). The level of heterogeneity within GT 3 decreased (to around 20 % as observed for GT 1 and GT 4) when GT 3.1, 3.2 and 3.3 were considered as separate subgroups (Figure 20). These three GT 3 subgroups can be further divided into several subtypes at a statistically significant level. However available epidemiological data do currently not support further subdivisions. The genotype 4 isolates clustered in several highly heterogeneous branches which precluded a further separation, however only few groups could be at statically significant levels. It is questionable whether the degree of divergence alone should serve for separation into subtypes. In our opinion a constant addition of new subtypes is not helpful as the separation is not supported by epidemiological data. Subtyping should be useful in analysis of sequences when serve as suitable variables for epidemiological and clinical studies or help to understand pathogenesis. Separation of genotypes 3 and 4 into subtypes using currently available data sets does not improve the understanding of HEV epidemiology and pathogenesis.

The cutthroat trout virus (CTV) has been suggested to represent a member of the *Hepeviridae* family based on phylogenetic analyses (Batts et al., 2011). According to the deduced amino acid sequence of ORF1, CTV is 73-74 % distant from HEV and 84 – 86 % from Caliciviruses, Togaviruses and Picornaviruses. The genome organization of CTV differs from HEV, avian HEV (aHEV) and rodent HEV-like viruses with regard to the position of ORF3 (Batts et al., 2011). According to our analysis it is not clear whether CTV actually belongs to the *Hepeviridae*. Our approach was suitable for comparison of HEV with aHEV, ferret, rodent and bat HEV-like sequences, which exhibit a considerable degree of heterogeneity. This approach failed in the case of CTV.

5.3. Development of diagnostic tools

The HEV capsid protein has been expressed using different systems like baculoviruses (Li et al., 1997), *E. coli* (Hu et al., 2008; Zhang et al., 2001a) and vaccinia virus (Carl et al., 1994; Jiménez de Oya et al., 2012). Protein expression using bacterial-based systems has several advantages when compared to eukaryotic systems; it is relatively easy to handle, provides a rapid establishment of the expression system and allows the production of large amounts of protein (Cabrita et al., 2006; Stevens, 2000).

The proteins HEV R4b (ORF 2: from aa 543 to 617) and HEV 2.1 (from aa 1 to 278) were expressed as fusion proteins in the pET 26b+ vector as shown by Western blots (WB) with antibodies against the his-tag and ubiquitin. For HEV 2.1 two specific bands occurred (Figure 28); the reason for this is not clear. The protein may form dimers (approx. 40 kDa). Oligomerization of HEV capsid protein fragments has been reported after expression in *E. coli* systems using as vectors pGEX20 (Zhang et al., 2001a) and pMD 18-T(Li et al., 2009a). In addition it has been reported that the peptides were more immunogenic in WB in their dimeric than in monomeric form (Zhang et al., 2001a).

Tests using the HEV R4b peptide have shown a discrete band with a human serum from patient tested HEV positive by ELISA (figure 30). Negative human sera shown non-specific bands on western blot; the reason for that still has to be elucidated. Cross-reactive antibodies against the tag sequences of expressed proteins i.e. ubiquitin and his-tags may have been responsible. Anti-ubiquitin antibodies have been detected in human patients suffering from systemic lupus erythematous (Muller et al., 1988). No band on the expected size was observed when negative control sera from SPF swine were used.

The polypeptides produced within this study may be the starting point for improvement of tools and diagnostic tests. Additional analysis of these polypeptides, different expression strategy (e.g. without tags or different vectors) as well as further studies with both human and swine sera should be carried out.

5.4. Cultivation of HEV

Replication of viruses in tissue culture cells represent a routine approach and has been used for decades in order to diagnose, identify and characterize viruses (Bryden et

al., 1977; Covalciuc et al., 1999; Dulbecco and Vogt, 1953; Eagle, 1955). Infection of cell lines is the most common method for viral propagation (Flint et al., 2009). The growth and propagation of viruses in cells can be monitored microscopically by occurrence of cytopathic effects, electron microscopy, immunological assays and detection of viral genomes (Flint et al., 2009). An efficient cell culture system for HEV would make it possible to study viral replication and to generate large amounts of virus for further studies.

Several approaches have been followed in order to cultivate HEV, unfortunately without clear success. In two recent publications human adenocarcinomic alveolar basal epithelial cells (A549) and human hepatoblastoma cells (PLC/PFR/5) have been reported to be permissive for HEV (Takahashi et al., 2012; Tanaka et al., 2007). For this, 21 different cell lines were infected with a fecal suspension from a patient positive for HEV genotype 3; viral genome was followed by quantitative RT-PCR (Tanaka et al., 2007). Latter on the same system was successful for growth of HEV from feces and blood samples (Okamoto, 2011a) and HEV from swine and wild boar commercial liver (Takahashi et al., 2012). Recently a three dimensional cell culture system based on PLC/PFR/5 cell line has been reported; viral propagation was measured by quantitative RT-PCR from the culture suspension and viral particles could be demonstrated within the cells by electron microscopy (Berto et al., 2013).

In this work A549 cells were infected using different amounts of HEV and different concentrations of FCS. A slight increase in the HEV RNA levels measured by qRT-PCR in the supernatants was the only hint of a possible viral replication. The amount of viral RNA in cell culture supernatant however, was still lower than the amount used for the initial infection. In addition, no cytopathic effect (CPE) has been detected and after the second passage it has not been possible to detect HEV RNA in either cells or supernatants. Concerning the success and applicability of the cultivation system using A549 cell line our results are not in agreement with what has been demonstrated in previous studies (Okamoto, 2011b; Takahashi et al., 2012; Tanaka et al., 2007). The reason for that remains unclear. Different experimental conditions and different viral strain used in our experiment may have played a role.

Our data are preliminary with just a few experiments performed with one cell line. Accordingly the model using the A549 cell line is not suitable for use in further experiments, even though the A549 cell line seems to be somehow permissive to HEV infection.

5.4.1. Infection of primary hepatocytes

Infection of tree shrew (*Tupaia belangeri*) hepatocytes with Hepatitis B virus (HBV) has been reported as a good *in vitro* model (Glebe et al., 2003). According to our knowledge no HEV propagation systems has been developed using tree shrew hepatocytes. Hence, we wanted to determine whether the shrew hepatocytes are suitable for HEV infection. For the established HEV cultivation systems using cell lines, 12 days post infection in the 2D system and 24 days after infection in the 3D system were necessary to evidence infection (Berto et al., 2013; Tanaka et al., 2007).

In our study no signs of replication as judged by EM and qRT-PCR have been found. The cells could not be kept for more than seven days either due to inadequate maintenance conditions or due to natural limitations. Infection for longer periods has been required in order to demonstrate the HEV infection in both 2D and 3D systems. Thus, we cannot draw any conclusions regarding to permissibility of tree shrew hepatocytes to HEV infection. Further studies should be carried out with shrew hepatocytes using better maintenance conditions.

6 Summary

Hepatitis E is an emerging zoonotic disease distributed worldwide. The causative agent Hepatitis E virus (HEV) is also present in animals such as swine, wild boar, deer, rabbits and rodents, however no clinical disease has been associated with HEV in animals. The limitations concerning diagnosis and the lack of clinical and epidemiological information about HEV in different animal populations make it difficult to assess the risk for the human population. Due to the lack of an efficient cell culture system, little knowledge is currently available about replication mechanisms, pathogenesis and biology of HEV. Thus, the aims of this study were to detect HEV in different animal populations, to study the genetic variability of HEV, to express the capsid protein for use in diagnostic test and to cultivate HEV in primary cells and cell lines.

This study indicates that HEV is present in both wild boar and domestic swine populations in Germany. A high genetic heterogeneity has been found among the wild boar viruses. All HEV isolates from animals described in this study are closely related to human isolates indicating a potential zoonotic risk regarding the consumption of meat products especially from wild boar.

Extensive phylogenetic analyses were performed in order to study the genetic variability of HEV and to evaluate the classification at subtype and genotype level. Phylogenetic analyses on the basis of complete genomic as well as whole capsid sequences were shown to be adequate for defining HEV genotypes. The results of the phylogenetic analyses suggest modification in the current taxonomy of genotype 3 and to refine the established system for typing of HEV. In addition a classification for hepeviruses recently isolated from bats, ferrets, rats and wild boar is suggested.

Parts of the HEV capsid protein (ORF 2: aa 1 to 278 and aa 543 to 617) were expressed as fusion proteins which can be used to develop test systems. Furthermore, a qRT-PCR assay was developed. Numerous approaches were performed to cultivate HEV in cell lines and shrew hepatocytes; however, virus propagation could not be shown.

7 Zusammenfassung

Hepatitis E ist eine zoonotische Erkrankung mit weltweiter Verbreitung und zunehmender Bedeutung. Der Erreger, das Hepatitis E Virus (HEV), kommt auch bei Tieren wie Hausschweinen, Wildschweinen, Hirschen, Hasen und Nagetieren vor. Bisher wurden bei Tieren keine Erkrankungen durch HEV beschrieben. Die Einschränkungen bezüglich Diagnostik sowie das Fehlen von klinischen und epidemiologischen Daten über HEV bei verschiedenen Tierarten erlaubt es nicht, die Bedrohung für den Menschen abschließend zu beurteilen.

Infolge des Fehlens eines effizienten Zellkultursystems ist nur wenig über die Replikation, die Pathogenese und die Biologie von HEV bekannt. Ziele dieser Arbeit waren, HEV in verschiedenen Tierpopulationen zu detektieren, die genetische Variabilität von HEV zu untersuchen, das Kapsidprotein für den Einsatz in Testsystemen zu exprimieren sowie HEV in primären Zellen bzw. in Zelllinien zu vermehren.

Die vorliegende Studie zeigt, dass HEV in Wildschweinen und Hausschweinen vorkommt. Eine hohe genetische Heterogenität wurde bei den Viren aus Wildschweinen gefunden. Alle HEV Isolate von Tieren, die hier beschrieben werden, sind nahe mit humanen Isolaten verwandt, was auf die Gefahr einer zoonotischen Übertragung durch den Verzehr von Fleischprodukten insbesondere von Wildschweinen hinweist.

Umfangreiche phylogenetische Analysen wurden durchgeführt, um die genetische Variabilität von HEV zu untersuchen und die bestehende Klassifizierung auf Subtyp- und Genotyp-Ebene zu evaluieren. Phylogenetische Analysen auf der Basis des kompletten Genoms und des gesamten Kapsidproteingens waren geeignet, um HEV Genotypen zu definieren. Die Ergebnisse der phylogenetischen Analysen legen nahe, dass die gegenwärtige Taxonomie von HEV modifiziert und das etablierte Einstufungssystem verfeinert werden sollten. Zusätzlich wird eine Klassifizierung von Hepeviren, die vor kurzem aus Fledermäusen, Frettchen und Wildschweinen isoliert wurden, angeregt.

Teile des Kapsidproteins von HEV (ORF 2: AA 1 bis 278 und AA 543 bis 617) wurden als Fusionsproteine exprimiert und können zur Entwicklung weitergehender Testsysteme verwendet werden. Darüberhinaus wurde ein qRT-PCR Test für HEV entwickelt. Zahlreiche Ansätze zur Kultivierung von HEV in der Zelllinie A549 sowie in Hepatozyten von Spitzmäusen wurden durchgeführt; Virusvermehrung konnte jedoch nicht nachgewiesen werden.

8 References

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