Investigation of animal reservoirs of the Hepatitis E virus in Germany

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Zusammenfassung

Hepatitis E ist eine in Deutschland meldepflichtige Krankheit, die durch Hepatitis E-Viren (HEV) ausgelöst wird. Im Jahr 2010 wurden 220 Hepatitis E-Fälle an das Robert Koch-Institut (RKI) übermittelt, wovon 165 Fälle in Deutschland erworben wurden. Die Ansteckungsquelle dieser Fälle ist zurzeit unbekannt, es wird aber eine zoonotische Übertragung diskutiert. Wildschweine und Hausschweine gelten weltweit als die wichtigsten Tierreservoire für HEV; in mehreren Studien wurde von Hepatitis E-Fällen berichtet, die auf den Verzehr von HEV-kontaminiertem Wildschwein- oder Schweinefleisch zurückzuführen waren. Bei anderen Tieren, speziell bei Ratten, konnten bislang nur HEV-spezifische Antikörper nachgewiesen werden. Bis 2008 waren keine Daten zur HEV-Prävalenz in deutschen Haus- und Wildtieren verfügbar. Im Rahmen dieser Dissertation sollte eine erste Einschätzung der HEV-Prävalenz in Deutschland bei verschiedenen Tierarten, die als HEV-Reservoir dienen können, erfolgen, um mögliche Übertragungswege aufzudecken. In verschiedenen Studien wurden hierzu Nachweissysteme entwickelt und nachfolgend Wildschweine, Hausschweine sowie Ratten aus unterschiedlichen Regionen in Deutschland auf HEV oder HEV-spezifische Antikörper untersucht.

Mittels Real-time RT-PCR konnte in 14,9 % (22/ 148) der untersuchten Wildschwein-Leberproben HEV RNA detektiert werden. Die Nachweisrate lag in den ländlichen Regionen in Brandenburg und Thüringen (25,9 % bzw. 23,8 %) dabei signifikant höher als in den Städten Berlin und Potsdam (4,1 %). Die detektierten Genotypen 3a, 3c, 3h und 3i zeigten starke Sequenzhomologien zu humanen HEV-Stämmen von autochthonen Hepatitis E-Fällen. Das Genom des HEV-Stammes wbGER27 wurde komplett sequenziert und stellt somit die erste vollständige Nukleotidsequenz des Genotyps 3i dar. In einer zweiten Studie wurde die HEV-Seroprävalenz in deutschen Hausschweinen untersucht. Die Ergebnisse von drei verschiedenen Immunoassays wurden dabei miteinander verglichen. Die Seroprävalenzen waren generell hoch, schwankten jedoch zwischen 21,7 % und 64,8 % abhängig vom verwendeten Assay. Durch die Entwicklung einer Breitspektrum-nested RT-PCR für HEV konnte zum ersten Mal ein Hepatitis E-ähnliches Virus in Kotproben von Wanderratten in Deutschland nachgewiesen werden. Elektronenmikroskopische Aufnahmen zeigen ein HEV-ähnliches Virus von 32-34 nm Größe. Dieses Virus wird vorläufig als rat

HEV bezeichnet und zeigt etwa 50-60 % Sequenzhomologien zu den anderen HEV-Genotypen. Die Untersuchung von Organproben weiterer Wanderratten aus derselben Region führte zu der Detektion von zwei weiteren rat HEV-Stämmen, deren Genom komplett sequenziert werden konnte. Phylogenetische Analysen zeigten, dass rat HEV einen separaten Zweig zwischen anderen Säuger- und aviären HEV-Stämmen bildet. Durch eine spezifische Real-time RT-PCR für rat HEV und immunhistologische Untersuchungen konnte ein Hepatotropismus des Virus festgestellt werden.

Zusammenfassend konnte sowohl in Wildschweinen als auch in Hausschweinen aus Deutschland HEV direkt oder indirekt nachgewiesen werden; in Ratten wurde darüber hinaus ein HEV-ähnliches Virus entdeckt. Eine zoonotische Übertragung von HEV von den Tierreservoiren auf den Menschen scheint also in Deutschland möglich zu sein. Weitere Studien sind nötig, um zu ermitteln, inwieweit infektiöse Viren im Fleisch der Wildschweine oder Hausschweine enthalten sind und ob rat HEV auf den Menschen übertragbar ist. Rat HEV könnte zur Etablierung eines Nagermodells für die humane Hepatitis E verwendet werden. Mit einem solchen Modell wäre es unter anderem auch möglich, die Effizienz der verschiedenen Übertragungswege des HEV zu ermitteln. Mit Hilfe der neu entwickelten Nachweissysteme sollten andere Tierarten ebenfalls auf HEV-ähnliche Viren untersucht werden.

Summary

Hepatitis E is a notifiable disease in Germany, which is caused by the hepatitis E virus (HEV). In 2010, 220 hepatitis E cases have been recorded; 165 cases of these have been acquired in Germany. The reason for these cases has still to be elucidated but a zoonotic transmission seems to be likely. Wild boars and domestic pigs are worldwide regarded as the main animal reservoirs of HEV and several studies report food-borne hepatitis E cases after the consumption of undercooked or raw meat of wild boars or pigs. Other animal species, especially rats, have been discussed as HEV reservoir but so far only HEV-specific antibodies have been detected in these animals. By 2008, no data about the HEV prevalence in Germany in wild as well as domestic animals are available. Hence, the aim of the studies was to assess the HEV prevalence in different animal species in Germany, which are considered as HEV animal reservoirs, and to reveal possible zoonotic transmission routes. After the development and establishment of suitable detection methods, wild boars, domestic pigs and rats from different regions in Germany have been investigated for the presence of HEV or HEV-specific antibodies.

In average, 14.9% (22/148) of the investigated wild boar liver samples have been tested positive for HEV RNA using real time RT-PCR. However, in the rural regions of Brandenburg and Thuringia a significantly higher prevalence rate (25.9% & 23.8%, respectively) have been found compared to the cities Berlin and Potsdam (4.1%). From the HEV positive wild boars the HEV genotypes 3a, 3c, 3h and 3i have been detected, which show a high sequence identity to human HEV strains from autochthonous hepatitis E cases. The genome of the strain wbGER27 has been sequenced completely and represents the first full-length sequence of HEV genotype 3i. In a second study, the HEV seroprevalence has been determined in domestic pigs using three different immunoassays, which results have been compared to each other. In general, the HEV seroprevalences determined ranged between 21.7% and 64.8% depending on the used immunoassay. By the development of a novel HEV broad-spectrum RT-PCR it was possible to detect for the first time an HEV-like virus in faecal samples of wild Norway rats from Germany showing about 50 to 60% sequence identity to other HEV genotypes. Using electron microscopy an HEV-like virus of 32-34 nm in diameter was demonstrated tentatively designated as rat HEV.

Screening of organ samples of further wild Norway rats trapped at the same location resulted in the detection of two full-length genomic sequences of rat HEV. Phylogenetic analyses showed that rat HEV builds a separated branch between mammalian and avian HEV genotypes, probably representing a novel HEV genotype. Using a specific real time RT-PCR for rat HEV and immunohistochemical methods a hepatotropism of rat HEV could be revealed.

In summary, in wild boars and domestic pigs HEV or HEV-specific antibodies have been detected; in rats an HEV-like virus has been discovered. Thus, a zoonotic HEV transmission from animal reservoirs to humans might be possible in Germany. Further studies are needed, investigating the presence of infectious HEV in meat of wild boars and domestic pigs and assessing the transmissibility of rat HEV from rats to humans. In addition, rat HEV might be used for the establishment of a rodent model for human hepatitis E. Using such a rodent model, the efficiency of different transmission routes may be assessed. Finally, other animal species should be screened for the presence of HEV-like viruses using the novel detection methods.

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List of Abbreviation

A

aa	amino acid
Ab	antibody
(A)n	polyA-tail
ALP	alkaline phosphatase
ALT	alanine transaminase
AST	aspartate aminotransferase

B

BLS	big liver and spleen disease
BLSV	big liver and spleen disease virus
bp	base pairs

C

cm^3	cubic centimetre
CsCl	caesium chloride

E

e.g.	for example (exempli gratia)
ELISA	enzyme-linked immunosorbent assay
ET-NANBH	enterically transmitted-non-A/-non-B hepatitis
ER	endoplasmic reticulum

G

g	gram
GT	genotype

H

H	RNA helicase
HAV	hepatitis A virus
HBV	hepatitis B virus
HEV	hepatitis E virus
HIV	human immunodeficiency virus
HS	hepatitis-splenomegaly

I

ICTV	International Committee on Taxonomy of Viruses
IEM	immune electron microscopy
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M

K

kb	kilobase
kDa	kilodalton

M

M	middle domain ***or*** methyltransferase
m^7G	7-methylguanosine

N

NANBH	non-A/-non-B hepatitis
NCR	non-coding region
nm	nanometre
nt	nucleotide

O

ORF	open reading frame

P

P	protruding domain ***or*** papain-like protease
P1	three-fold protrusion
P2	two-fold spike
PCR	polymerase chain reaction

R

R ***or*** RdRp	RNA dependent RNA polymerase
R_0	basic reproduction ratio
RKI	Robert Koch-Institut
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction

S

S	shell domain ***or*** continuous capsid

T

T	triangulation number
TiHo	Tierärztliche Hochschule Hannover

U

USA	United States of America

V

V	proline-rich hinge domain
VLP	virus-like particle

X

X	X domain

Y

Y	Y domain

1 General Introduction

The hepatitis E virus (HEV) is the aetiological agent of an acute viral hepatitis in humans with symptoms similar to hepatitis A and a case fatality rate of 1 to 4% (Purcell & Emerson, 2008). For pregnant women, however, mortality rates up to 25% are reported (Aggarwal & Krawczynski, 2000). The virus is endemic in many developing countries. In addition, HEV is regarded as an emerging pathogen in industrialized countries and the number of cases is increasing over the last years (Purcell & Emerson, 2008; RKI, 2010). The main transmission route of the virus is faecal-oral via contaminated drinking water or food but also a zoonotic pathway has been identified with pigs and wild boars as the main reservoirs of this virus. The detection of HEV RNA as well as HEV-specific antibodies in several other animal species suggests that additional animals beside pigs and wild boars may act as virus reservoir (Meng, 2010a). In Germany, 220 cases of hepatitis E have been notified in 2010. Most of these cases are autochthonous but the distinct source of infection has still to be elucidated (M. Faber, personal communication; RKI, 2010).

1.1 Discovery of HEV

The history of the discovery of HEV is exemplary for the necessity of sensitive and specific diagnostic tools. During the seventies and eighties of the 19th century the existence of other viruses causing hepatitis in humans beside the formerly known hepatitis A virus (HAV) and hepatitis B virus (HBV) was first recognized because novel diagnostic tools for the detection of HAV and HBV failed to determine the causative agents of several hepatitis cases (Khuroo, 1980; Wong et al., 1980) (Figure 1). For the sake of simplicity these unidentified viruses causing hepatitis in humans were called non-A/-non-B hepatitis (NANBH) viruses

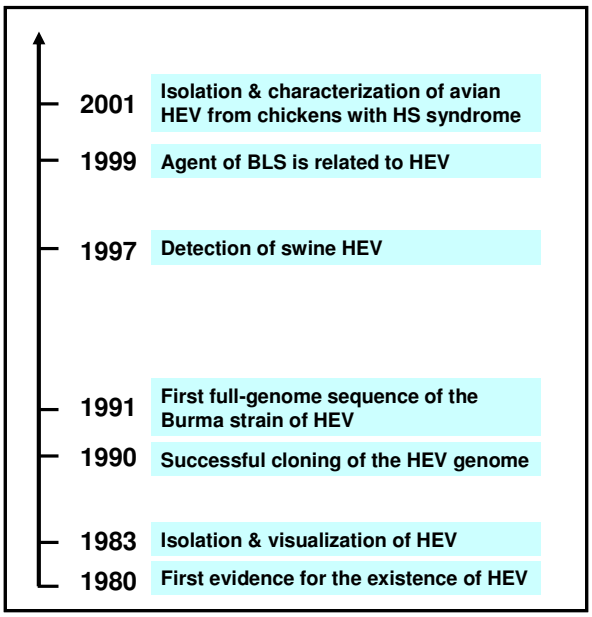

Figure 1: Timeline of important discoveries about HEV; BLS = big liver and spleen disease; HS = hepatitis-splenomegaly

(Reyes et al., 1990). Soon, it was suggested that more than one agent must be responsible for these cases of NANBH because many different transmission routes were reported: person-to-person, blood transfusion-associated, coagulation-factor and faecal-oral transmission (Khuroo, 1980; Shorey, 1985; Tabor, 1985; Villarejos et al., 1975).

In 1980, two independent studies were published within a few months, which strengthened the hypothesis of the existence of a NANBH virus transmitted by the faecal-oral route (Khuroo, 1980; Wong et al., 1980). The first article described an outbreak investigation study on 16,620 inhabitants of the Kashmir valley in India (Khuroo, 1980). The epidemiological analysis strongly indicated a water stream used as drinking water as the source of infection. Blood and stool samples were thereupon screened for the presence of HAV and HBV antigens and antibodies but neither of the two viruses could be identified as the aetiological agent of this epidemic. This led to the assumption that an additional virus causing hepatitis in humans might exist (Khuroo, 1980). In the other study, sera originating from different hepatitis epidemics as well as sporadic cases of hepatitis in India (the Delhi epidemic of 1955-1956, the Ahmedabad epidemic of 1975-1976, and sporadic cases in Pune from 1978 through 1979) were tested for markers of HAV and HBV. The epidemics of Delhi and Ahmedabad could be epidemiologically associated with faecal contamination of the drinking water. However, for most cases of hepatitis an aetiological agent could not be identified and there was growing evidence that a virus not yet known but similar to HAV might exist and might be responsible for a large proportion of hepatitis cases in India. Thereafter, other reports about epidemics or sporadic cases of hepatitis caused by this enterically transmitted-NANBH (ET-NANBH) virus have been published (Balayan et al., 1983; Chakraborty et al., 1982; Kane et al., 1984).

In 1983, Balayan et al. were able to purify the agent of a waterborne hepatitis outbreak after an experimental infection of a human volunteer (Balayan et al., 1983). Thus, it was possible to visualize virus-like particles (VLPs) with diameters between 27 to 30 nm in the stool of the volunteer using immune electron microscopy (IEM) (Figure 2). The term “Hepatitis E virus” was first proposed by Purcell and Ticehurst in 1988 and was then picked up in later publications (Purcell & Ticehurst, 1988; Reyes et al., 1990; Tam et al., 1991).

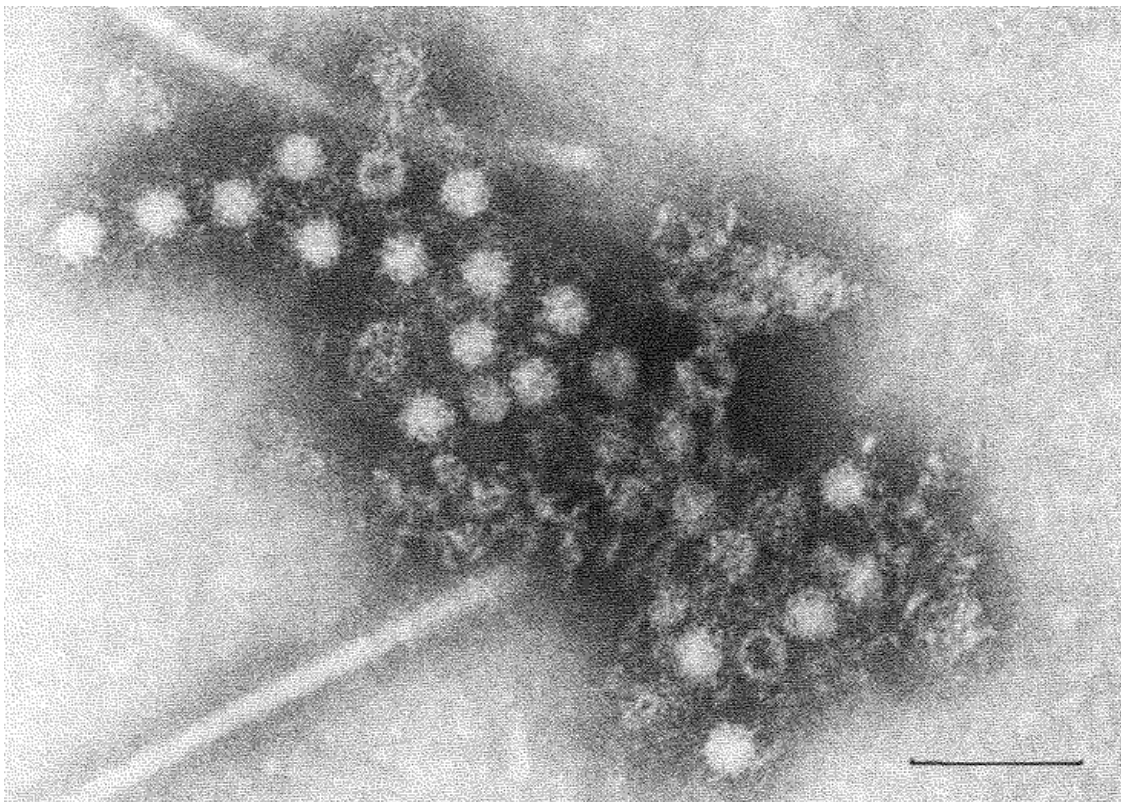

Figure 2: Immune electron microscopic picture of HEV (Balayan et al., 1983)

The successful cloning of the genome paved then the way for the molecular characterization of HEV (Reyes et al., 1990; Tam et al., 1991).

The possibility of a zoonotic transmission of HEV was first claimed by Balayan and his colleagues in 1990 when he succeeded in the experimental infection of a pig with a human HEV strain (Balayan et al., 1990). The detection of swine HEV as the first animal strain in domestic pigs in the United States in the year 1997 shed a different light on the transmission of HEV, especially in developed countries, where water treatment processes are well established (Meng et al., 1997). Since swine HEV is closely related to human HEV, a zoonotic way of transmission was now more evident (Erker et al., 1999; Meng et al., 1997). The aetiological agent of the big liver and spleen disease (BLS) in chickens was also found to be genetically related to HEV (Payne et al., 1999) and in 2001 avian HEV was detected in chickens with hepatitis-splenomegaly (HS) syndrome in the USA (Haqshenas et al., 2001). Later, anti-HEV antibodies as well as HEV RNA were found in several animal species in different regions all over the world (Meng, 2000).

1.2 Taxonomy & phylogeny

Due to structural and genomic similarities to caliciviruses, HEV was first classified into the family *Caliciviridae* (Bradley & Balayan, 1988; Okamoto, 2007). Later, in the 8th report of the International Committee on Taxonomy of Viruses (ICTV) the genus *Hepevirus* appears not assigned to any virus family with the type species *Hepatitis E virus* (Emerson et al., 2005a). According to this report, the genus *Hepevirus* comprises two species: the *Hepatitis E virus*, namely the mammalian HEV isolates, and the tentative species called *Avian hepatitis E virus*. Soon after publication of this latest report a new taxonomic proposal (2008.005-009V) was initiated to create the new family *Hepeviridae* with the genus *Hepevirus* and the type species *Hepatitis E virus* (Mayo & Ball, 2006), which was ratified by the ICTV in July 2009 (Carstens, 2010). The ICTV 2009 Master Species List (Version 5, August 2009) includes the family *Hepeviridae* unassigned to any order, the genus *Hepevirus* with the one type species *Hepatitis E virus* and the species *Avian hepatitis E virus* not assigned to any genus but to the same family. A new proposal posted in June 2010 (2010.002a-iV) suggests the creation of two new genera within the family *Hepeviridae* called *Orthohepevirus* and *Avihepevirus* and the removal of the sole genus *Hepevirus* (ICTV, 2011).

Although only one serotype is described by now (Anderson et al., 1999; Guo et al., 2006), a vast genomic heterogeneity is found among the hepatitis E viruses. Based on sequence alignments the mammalian HEVs are subdivided into four genotypes (GT), which can be further divided in several subtypes: five subtypes of GT1 (1 a-e), two subtypes of GT2 (2 a+b), ten subtypes of GT3 (3 a-j) and seven subtypes of GT4 (4 a-g) (Lu et al., 2006). The avian HEV isolates can be subdivided into three genotypes, also correlating with their geographical distribution (Bilic et al., 2009). Until now, there are no valid criteria defined for the assignment of a new isolate to a certain species or genotype within the genus *Hepevirus*.

1.3 Morphology & physical properties

HEV is a small non-enveloped icosahedral sphere of about 27 to 34 nm in diameter (Tam et al., 1991). The morphology of HEV resembles that of caliciviruses. The buoyant density in caesium chloride (CsCl) is between 1.35 and 1.40 g/cm³ (Balayan

et al., 1983). The sedimentation coefficient has been determined to be 183S (Bradley et al., 1988).

Native HEV particles are composed of 180 capsomers, which corresponds to a triangulation number of three (T=3). VLPs are smaller and are composed of 60 capsomers resulting in a triangulation number of one (T=1) (Xing et al., 1999). Recently, the crystal structure of HEV has been further characterized. Each capsid protein is built up by three major domains. These domains are indicated as the shell (S) domain (amino acids [aa] 129-319), the middle (M) domain (aa 320-455) and the protruding (P) domain (aa 456-606) according to Yamashita et al. (2009). The outer surface of the particle, which is supposed to be the target for antibodies, is mainly formed by the M and P domains (Khudyakov et al., 1994). According to Guu et al. (2009) the three domains are designated as continuous capsid (S) (aa 118-313), three-fold protrusions (P1) (aa 314-453) and two-fold spikes (P2) (aa 454-608) (see Figure 3).

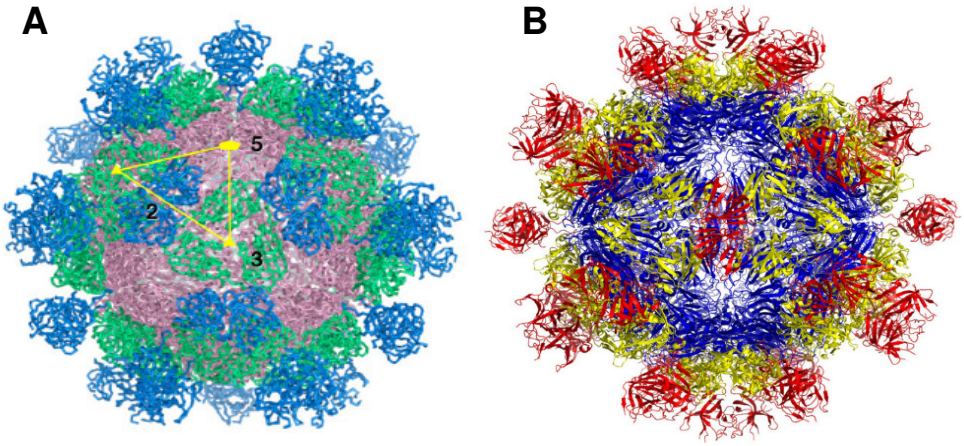

Figure 3: Crystal structure of HEV VLP; S-domain = pink, M-domain = green, P-domain = blue (Yamashita et al., 2009) **(A)**; S-domain = blue, P1 domain = yellow, P2 domain = red (Guu et al., 2009) **(B)**

1.4 Genomic organization

The genome of HEV consists of a linear single-stranded positive-sense RNA of about 7.3 kb in length, which contains three open reading frames (ORFs) (see Figure 4). The genome is capped at the 5´-end with 7-methylguanosine (m^7G) (Kabrane-Lazizi et al., 1999) and polyadenylated at the 3´-end (Reyes et al., 1990). At both ends of

the genome short non-coding regions (NCR) are found (Tam et al., 1991). The NCRs, the polyA-tail as well as the cap structure of the RNA are suggested to play crucial roles in viral replication (Chandra et al., 2008; Pavio et al., 2010). ORF1 is directly translated from the genome while ORF2 and ORF3 are translated from a bicistronic subgenomic RNA (Graff et al., 2006). The genome of avian HEV isolates is shorter with about 6.6 kb and shows only 50% nucleotide sequence identity compared to the genome of mammalian HEV. However, ORF1 to ORF3 are also present at the same positions (Haqshenas et al., 2001).

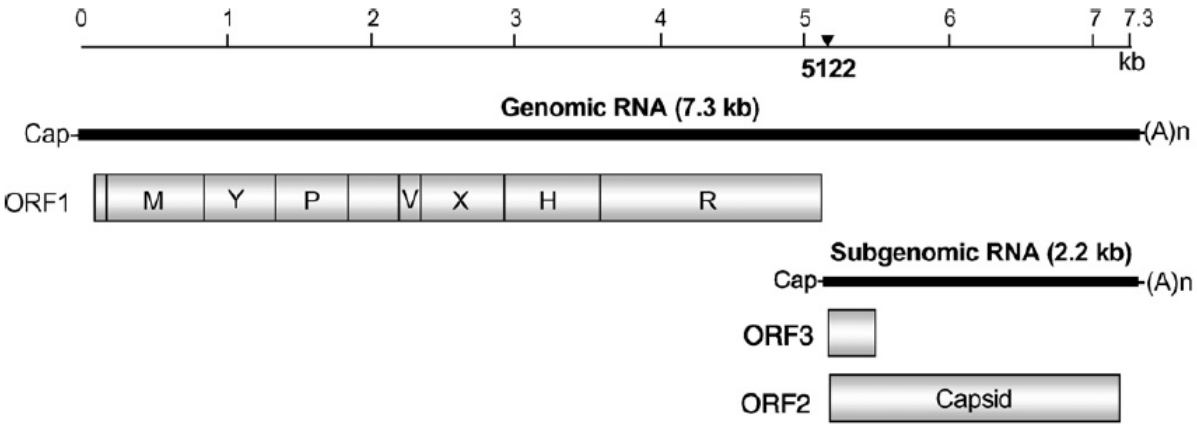

Figure 4: Genome organization of HEV; nucleotide positions according to strain Sar-55 (GenBank accession number AF444003); methyltransferase (M), Y domain (Y), papain-like protease (P), proline-rich hinge domain (V), X domain (X), RNA helicase (H), RNA dependent RNA polymerase (R), polyA-tail ((A)n) (Okamoto, 2007).

The largest part of the genome with about 1,400 codons is **ORF1** positioned at the 5´-end of the genomic map and encoding one polyprotein, which is processed into several non-structural proteins. Upon computer-based sequence analyses of the ORF1 polyprotein the following conserved functional motifs and domains are predicted: methyltransferase, Y domain, papain-like protease, proline-rich hinge domain, X domain, helicase and RNA dependent RNA polymerase (RdRp) (Koonin et al., 1992).

ORF2 encodes the capsid protein, which consists of 660 aa resulting in a protein of about 88 kDa in size, and is positioned at the 3´-end of the genome. The capsid proteins as well as truncated capsid proteins are capable of self assembly into VLPs (Chandra et al., 2008). Immunogenic epitopes are mainly located at the surface of

this protein (Meng, 2010a). The capsid protein contains an endoplasmic reticulum (ER) signal peptide and three putative N-glycosylation sites at the aa residues 137, 310 and 562. The capsid protein is shown to interact with the 5´-end of the viral RNA and is thus suspected to play a role in viral RNA encapsidation (Pavio et al., 2010).

The third in-frame start codon of **ORF3** is supposed to be the initiation site for the translation of a small phosphoprotein of 123 aa, which is connected to the cytoskeleton (Chandra et al., 2008). This protein seems to be necessary for viral infectivity *in vivo* (Graff et al., 2005; Huang et al., 2007) but not *in vitro* (Emerson et al., 2006). Takahashi et al. (2008) found the protein on the surface of HEV virions, which were newly released from infected cells. Various different putative functions of the ORF3 protein are discussed, which can be summarized as promotion of host cell survival, modulation of acute phase response and immunosuppression of the host (Chandra et al., 2008; Dalton et al., 2008; Meng, 2010a).

1.5 Infection & viral replication

As typical for hepatotropic viruses HEV is primarily found in the liver, especially in hepatocytes and Kupffer cells (Lee et al., 2009). Since no efficient cell culture system is known so far, only limited information about the replication cycle of HEV is available. However, according to other positive strand RNA viruses the following model for the replication of HEV has been proposed (Chandra et al., 2008; Jameel, 1999; Reyes et al., 1993): After the adsorption of the virus to a certain cell receptor the virus enters the cell and the viral RNA is uncoated. First the translation of ORF1 is initiated. The resulting polyprotein is cleaved by cellular proteases; possibly the viral papain-like protease may be involved in the cleavage. Expression of the viral RdRp in HepG2 cells revealed that the replication complex of HEV associates with the ER using an ER transmembrane domain found in the RdRp, which also interacts with the 3´-end of HEV RNA (Agrawal et al., 2001; Rehman et al., 2008). Thereafter, the positive strand is transcribed into a negative strand, which serves as template for the genomic positive strand and for the subgenomic positive strand. The structural proteins encoded by ORF2 and ORF3 are then translated from the bicistronic subgenomic RNA (Graff et al., 2006). After virus assembly progeny virions are able to exit the cell by an unknown mechanism (Chandra et al., 2008) (see Figure 5).

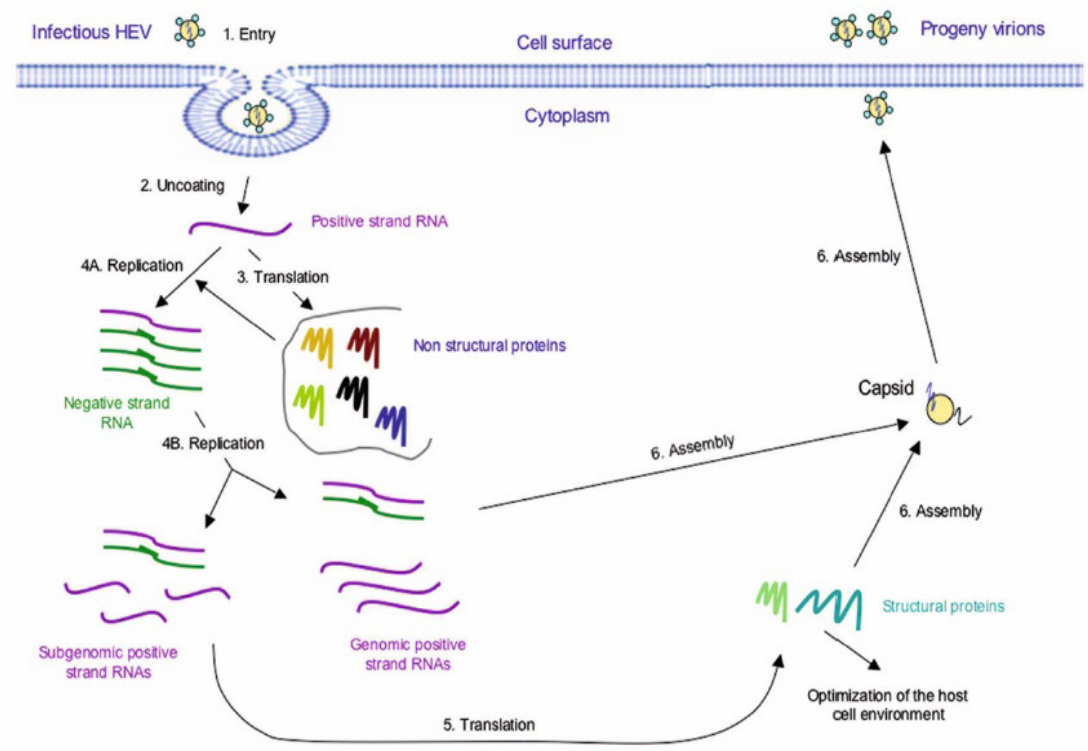

Figure 5: Replication cycle of HEV (Chandra et al., 2008)

1.6 The disease hepatitis E

HEV is the aetiological agent of an acute viral hepatitis called hepatitis E. In developed countries subclinical hepatitis E infections are supposed to be predominant (Dalton et al., 2008). In general, the case-fatality rate of hepatitis E is low with 1 to 4% (Purcell & Emerson, 2008). Nevertheless, in developing countries clinical and even fulminant cases of hepatitis E resulting in acute liver failure are continuously reported (Pavio et al., 2010; Smith, 2001). For pregnant women mortality rates up to 25% have been observed, which has been mainly assessed for endemic regions such as India and Pakistan (Hussaini et al., 1997; Khuroo et al., 1995; Patra et al., 2007). The reasons for the high mortality of pregnant women have still to be elucidated (Jilani et al., 2007; Khuroo et al., 1981; Pal et al., 2005; Patra et al., 2007; Prusty et al., 2007). In contrast, other studies did not find a correlation between pregnancy and an increased risk for an acute liver failure (Bhatia et al., 2008; Kasorndorkbua et al., 2003; Tsarev et al., 1995). An underlying prior infection may also lead to increased mortality (Hamid et al., 2002).

Typically, the disease occurs after an incubation time of about 2 to 10 weeks and is characterized by a self-limiting jaundice, which is hard to distinguish from hepatitis due to other viral infections and is often accompanied by unspecific symptoms like fever, arthralgia, malaise, anorexia, nausea and pain of the upper abdomen. Hepatomegaly may be thereupon palpated (Pavio et al., 2010; Pischke et al., 2010). The increased levels of the liver enzymes in the serum as bilirubin, alanine transaminases (ALT) and γ-glutamyltransferases are indicators for an affected liver often accompanied by a decolouration of stool and dark urine (Pischke et al., 2010). Hepatitis E is assumed to be an immune-mediated disease since the viruses themselves do not cause a cytopathic effect in liver cells and hepatocate cytolysis may be induced by the host immune response itself (Pavio et al., 2010). Viremia normally occurs during the prodomal stage while faecal excretion of the virus may start a few days prior to jaundice and may continue until 2 to 3 weeks after the onset of jaundice (Pavio et al., 2010). Antibodies of the immunoglobulin (Ig) class M are detected early at about 3 weeks post-infection but their concentration declines within a few months while IgG occurs later but may persist several years (Aggarwal & Krawczynski, 2000; Pelosi & Clarke, 2008). Symptoms as well as increased liver enzyme values usually resolve within 6 weeks (Pelosi & Clarke, 2008; Pischke et al., 2010) (see Figure 6).

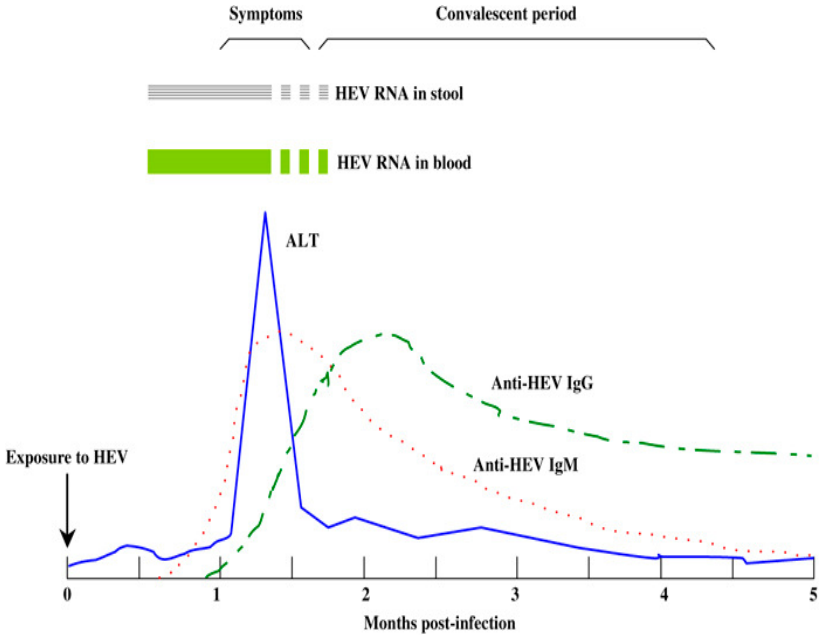

Figure 6: Time course of HEV infection in humans (Pelosi & Clarke, 2008)

Recently, also cases of chronic or persistent hepatitis E have been reported in immunocompromised solid-organ transplant patients and in patients with immunosuppressive therapy or HIV infection (Dalton et al., 2009; Gerolami et al., 2008; Haagsma et al., 2008; Kamar et al., 2008; Pischke et al., 2010; Tamura et al., 2007). One case of HEV reactivation after complete recovery from acute hepatitis E has been described (le Coutre et al., 2009).

Non-human primates and pigs are susceptible to HEV infection but beside a moderate increase of liver enzymes or minor histological lesions no clinical symptoms are visible (Aggarwal et al., 2001; Halbur et al., 2001; Meng et al., 1997). In contrast, the avian HEV strains may cause the big liver and spleen disease or the hepatitis-splenomegaly syndrome in chickens (Payne et al., 1999).

1.7 Geographical distribution & epidemiology

HEV is known to be endemic in Asia, Africa and Latin America (Chandra et al., 2008; Okamoto, 2007; Purcell and Emerson, 2001). In developing countries, large outbreaks with thousands of cases of hepatitis E were reported, e.g., in China, Sudan, Chad and recently in Uganda (Aye et al., 1992; Nicand et al., 2005; Okamoto, 2007; Teshale et al., 2010; Zhuang et al., 1991). In these regions, HEV is claimed to be responsible for more than 50% of the cases of acute viral hepatitis (Aggarwal et al., 1997; Dalton et al., 2008; Yarbough, 1999). The target population seems to be young to middle aged male adults (15 to 40 years) (Chandra et al., 2008). Epidemic as well as sporadic cases of hepatitis E are reported to be mostly associated with conditions of low hygiene and faecal contamination of the drinking water (Aggarwal & Naik, 2009). In contrast, person-to-person transmission is uncommon (Aggarwal & Naik, 1994; Somani et al., 2003). However, in industrialized countries, HEV is recently recognized as a pathogen of emerging concern with increasing number of cases reported every year. In Germany, the disease hepatitis E is notifiable since 2001. Since then, an increase of the reported hepatitis E cases is apparent (see Figure 7). Especially, in the year 2010 significant more cases (220 cases) are reported compared to the years before (SurvStat, 2011). The reasons for this increase of reported cases are not known by now. One possible explanation could be raised public attention, which may result in more diagnosed cases (Christensen et al., 2008; Pavio et al., 2010). In the past, it was assumed that most of the cases in

developed countries were associated to travel to endemic areas but further examination revealed that the majority of the cases in Germany are acquired without any history of travel. The source of infection for these autochthonous cases of hepatitis E has still to be elucidated but a zoonotic transmission is assumed (RKI, 2010).

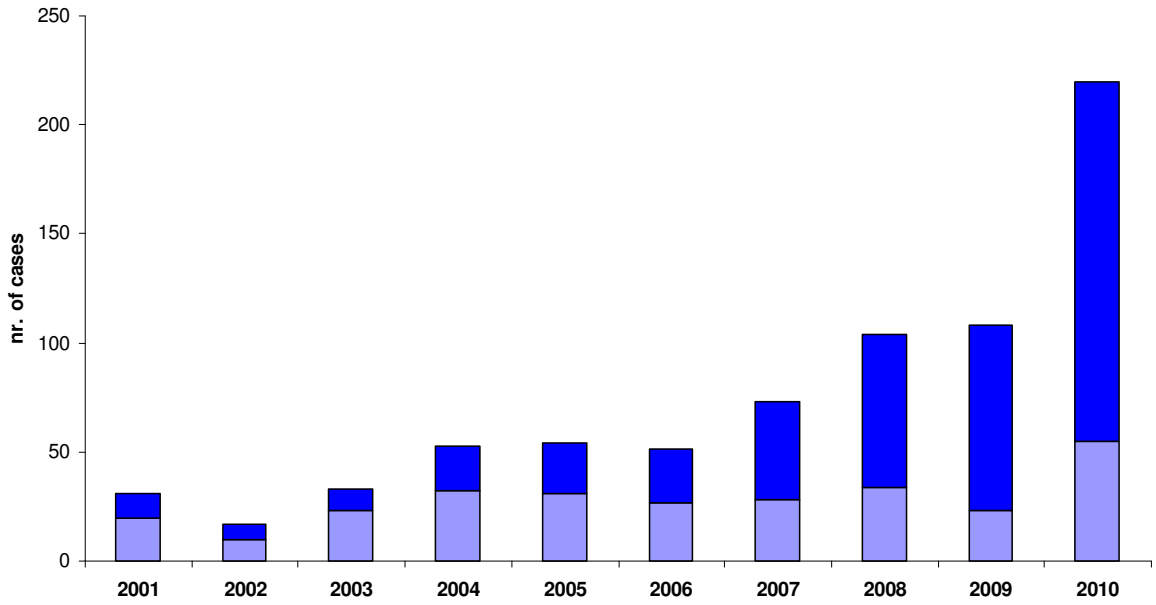

Figure 7: Number of HEV cases in Germany (RKI, 2010; M. Faber, personal communication); travel-related, autochthonous HEV infections.

After molecular characterization of various HEV isolates all over the world, it became evident that in different geographical regions different HEV genotypes are predominant. Viruses belonging to GT1 are detected in Asia, Africa and South America, GT2 is found in Mexico and in parts of Africa, whereas GT3 can be detected worldwide in humans and several animals. By now, GT4 is only recorded from humans and pigs in Asia (Chandra et al., 2008; Dalton et al., 2008; Kim et al., 2010; Purcell & Emerson, 2008) (see Figure 8).

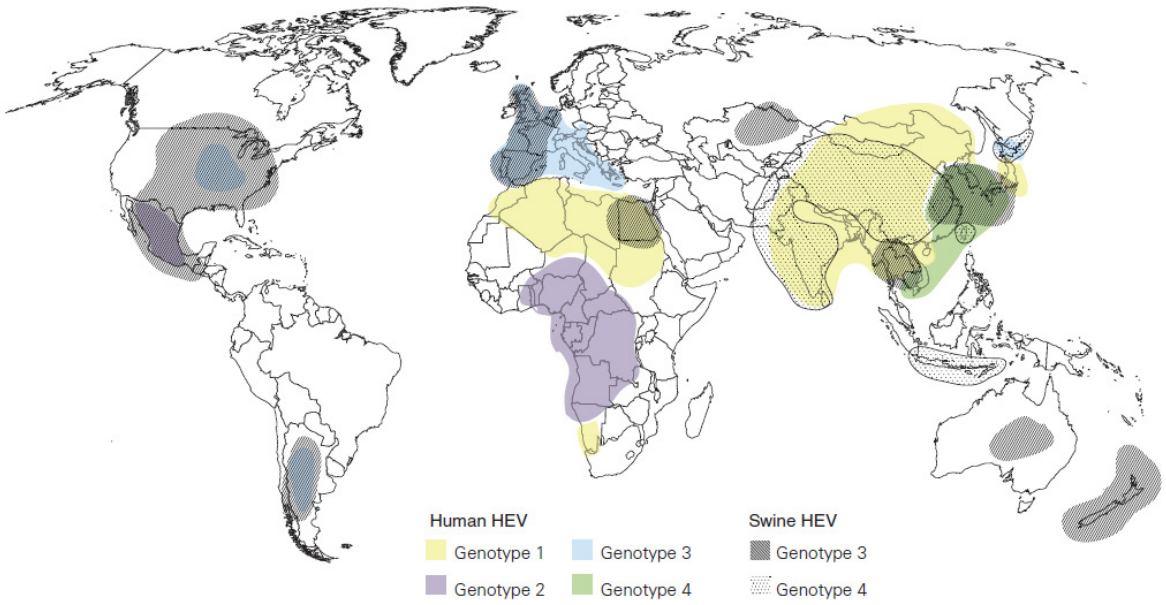

Figure 8: Worldwide distribution of HEV genotypes (Aggarwal & Naik, 2009).

There is some evidence that genotypes 3 and 4 may be less pathogenic than genotypes 1 and 2 since in regions predominant for GT3 or GT4 mainly subclinical cases are suggested (Aggarwal & Naik, 2009; Halbur et al., 2001; Pavio et al., 2010; Purcell & Emerson, 2008). In addition, no fulminant hepatitis E case during pregnancy associated with GT3 has been reported by now (Pavio et al., 2010).

According to the high number of cases in developing countries also high **seroprevalences** (15% to 60%) are reported in these regions (Dalton et al., 2008). A seroprevalence of up to 70% was detected in Egypt (Stoszek et al., 2006). In contrast, the seroprevalence in non-endemic regions generally varies from 0.4% to 7.4% (Chandra et al., 2008) and increases with age (Arankalle et al., 1995). In Germany, a seroprevalence between 1 to 3% has been reported (Dawson et al., 1992). Nevertheless, some studies in non-endemic countries revealed also high seroprevalence rates. In the south of France a seroprevalence of up to 16% has been found (Mansuy et al., 2008) and in a study in Denmark even 50.4% of farmers and 20.6% of blood donors were positive for anti-HEV antibodies (Christensen et al., 2008). A study of normal blood donors in the USA showed that about 17% were positive for antibodies against HEV (Meng et al., 2002). Additionally, people like swine farmers or veterinarians, who have contact to pigs or other animals that may

serve as reservoir for HEV, show significantly higher seroprevalence rates than the comparison groups (Drobeniuc et al., 2001; Karetnyi et al., 1999; Meng et al., 2002; Withers et al., 2002). However, reported seroprevalence rates have to be discussed carefully due to the use of different serological assays, which might explain some variation of the data.

1.8 Transmission routes of HEV

In developing countries with poor sanitation and low standards of hygiene, HEV is mainly transmitted via the **faecal-oral route** by contaminated drinking water or food (Chandra et al., 2008; Dalton et al., 2008). Many outbreaks can be linked to a contamination of the water source (Clayson et al., 1998; Ippagunta et al., 2007; Khuroo, 1980; Wong et al., 1980).

First, it was suggested that the hepatitis E cases in developed countries could be explained by travelling to endemic regions but several reports about hepatitis E cases in patients from the USA, New Zealand, Japan or Europe without a history of travel to HEV endemic regions refuted this hypothesis (Chapman et al., 1993; Dalton et al., 2007; Heath et al., 1995; Ijaz et al.; 2005; Mansuy et al., 2004; Pina et al.; 2000; Sainokami et al., 2004; Tsang et al., 2000; Widdowson et al., 2003). Additionally, the anti-HEV prevalence in healthy people in developed countries is relatively high (Chandra et al., 2008), which might hint to an indigenous source of infection. Since person-to-person transmission of HEV is rare, other routes of transmission have been discussed (Aggarwal & Naik, 1994; Somani et al., 2003). The detection of HEV in animals, which show high genomic similarities to human strains, indicated a **zoonotic transmission** for HEV (Erker et al., 1999; Meng et al., 1997). Animal experiments finally revealed that HEV GT3 and GT4 are able to cross species barriers (Bouwknegt et al., 2007; Chandra et al., 2008; Feagins et al., 2007; Meng et al., 1998; Yazaki et al., 2003). In contrast, genotypes 1 or 2 are only found in humans and are consequently mostly involved in travel-associated cases (Lewis et al., 2010).

Nowadays, zoonotic transmission is suspected to be responsible for the increasing number of autochthonous hepatitis E cases in industrializes countries, with wild boars and pigs regarded as the main virus reservoirs (Bouwknegt et al., 2009). The high seroprevalence in certain risk groups such as swine farmers or veterinarians with

contact to pigs or other animals that may serve as reservoirs for HEV also indicates a zoonotic transmission (Drobeniuc et al., 2001; Karetnyi et al., 1999; Meng, 2010a; Meng et al., 2002; Withers et al., 2002). However, most evidence for a zoonotic HEV transmission is derived from reports investigating a food-borne transmission route. Especially in Japan, several cases could be directly linked via sequence comparisons to the consumption of deer or wild boar meat originating from HEV-infected animals (Masuda et al., 2005; Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003). A small outbreak of hepatitis E was also recognized in France after the consumption of a raw pig liver sausage. Sausages from the supermarket, which sold these sausages during the outbreak, thereupon screened for HEV RNA were also positive (Colson et al., 2010). HEV of GT3 or GT4 were detected in porcine livers sold in grocery stores in the USA, Japan, the Netherlands, Korea and India (Bouwknegt et al., 2007; Feagins et al., 2007; Jung et al., 2007; Kulkarni & Arankalle, 2008; Yazaki et al., 2003). In Germany, the consumption of wild boar meat and offal has also been identified as risk factors for autochthonous HEV infections (Wichmann et al., 2008).

In contrast, in India the swine HEV is genetically different to the human isolates (Arankalle et al., 2002; Chandra et al., 2008; Shukla et al., 2007), which indicates that zoonotic transmission might play a minor role than the faecal-oral route in developing countries (Arankalle et al., 2002; Shukla et al., 2007). However, the faecal-oral route via contaminated seafood may also be possible for HEV GT3 as shown by an outbreak investigation on a cruise ship, which identified contaminated shellfish as possible source of infection (Said et al., 2009). Avian HEV has not been detected in humans by now (Dalton et al., 2008).

Other transmission routes like the vertical transmission or transfusion of infected blood products have been described but their relative importance has still to be elucidated (Aggarwal & Naik, 2009; Borgen et al., 2008; Boxall et al., 2006; Colson et al., 2007; Khuroo et al., 2009; Matsubayashi et al., 2008).

1.9 Animal reservoirs of HEV

Domestic pigs and wild boars are regarded as the main virus reservoirs for HEV GT3 and 4 (Meng et al., 2009; Pavio et al., 2010). Deer is also known to be a reservoir of HEV (Tei et al., 2003). Additionally, HEV RNA as well as HEV-specific antibodies have been detected in other animal species (Meng, 2000).

After the discovery of the first HEV animal strain in **pigs** in the USA in 1997 (Meng et al., 1997) several studies determined the prevalence of anti-HEV antibodies or HEV RNA in the sera, faeces, slurry or livers of pigs in many different countries demonstrating a wide distribution of HEV in pigs worldwide. HEV seems therefore to be enzootic in pigs (Lewis et al., 2010; Pavio et al., 2010). However, the seroprevalence strongly varies among different studies between 46% and 100% in the examined pig herds (Blacksell et al., 2007; Meng et al., 1997). The highest seroprevalence rates are reported from the USA, New Zealand, Mexico and Spain (Casas et al., 2009a; Cooper et al., 2005; Garkavenko et al., 2001; Meng et al., 1997; Seminati et al., 2008). In Europe, a seroprevalence of 80% can be determined in average ranging from 40% in France to 98% in Spain (Casas et al., 2009a; Kaba et al., 2009; Seminati et al., 2008). Sequence analyses revealed only the detection of GT3 or 4 in pigs (Arankalle et al., 2002; Cooper et al., 2005). The sequences of swine HEV are closely related to human HEV strains or are sometimes even identical (Banks et al., 2004; de Deus et al., 2008b; Herremans et al., 2007; van der Poel et al., 2001). The experimental infection of pigs with GT1 or 2 failed (Meng, 2003). For Germany, no data about HEV prevalence was available until this study.

Piglets normally show an HEV infection between 9 to 18 weeks of age (Meng, 2010a). The dynamics of HEV infection in pigs follows that of other viral infections in these animals: first, passive immunity is acquired with a progressive decline of the passive antibodies after 4 to 8 weeks. The concentration of anti-HEV of the IgM class increases beginning with 9 weeks of age. After the 22nd week of age nearly all pigs in an infected herd are IgG positive. Faecal shedding of HEV is reported between 4 to 18 weeks with a peak of high virus concentration in faeces at weeks 15 to18 (Blacksell et al., 2007; de Deus et al., 2008b; Fernandez-Barredo et al., 2006; Leblanc et al., 2007; Nakai et al., 2006; Pavio et al., 2010). Viremia also peaks around week 15 (de Deus et al., 2008b). Beside minor lesions or moderately elevated liver enzymes in serum no additional symptoms are visible in pigs (Aggarwal et al., 2001; Halbur et al., 2001; Meng et al., 1997). However, pigs may still be positive for HEV RNA at the time of slaughter (de Deus et al., 2007; Di Bartolo et al., 2008; Fernandez-Barredo et al., 2007) (see Figure 9).

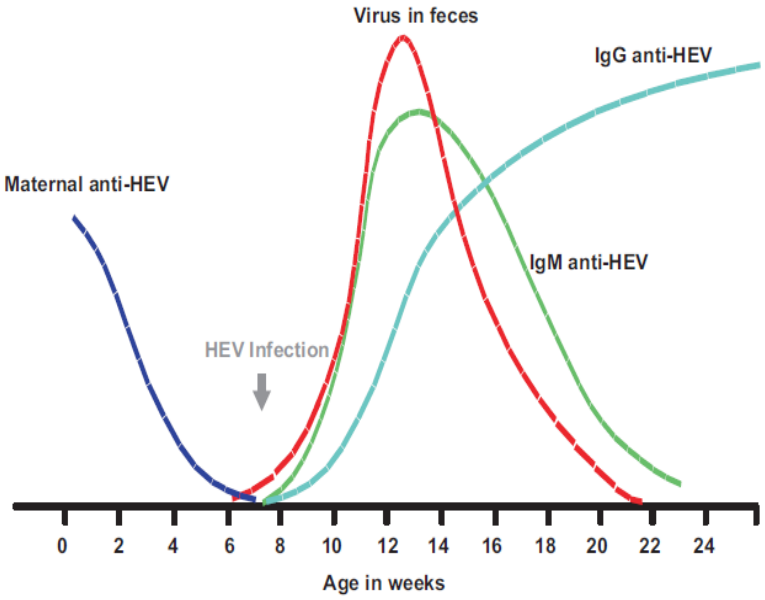

Figure 9: Natural time course of HEV infection in pigs (Pavio et al., 2010)

Wild boars are regarded as the second most important reservoir of HEV. In 1999, serological examinations of wild boars indicated for the first time that wild boars might act as reservoir of HEV additional to domestic pigs (Chandler et al., 1999). Several subsequent studies in different countries supported this hypothesis by the detection of anti-HEV specific antibodies or HEV RNA in wild boar sera, bile, faeces or liver (Meng, 2010a). The first wild boar HEV was detected in Japan (Sonoda et al., 2004) but HEV is also found in the wild boar population in Europe (de Deus et al., 2008a; Kaba et al., 2010; Kaci et al., 2008; Martelli et al., 2008; Reuter et al., 2009; Rutjes et al., 2010; Rutjes et al., 2009). All HEV isolates derived from wild boars belong to GT3 and 4 (Kim et al., 2010; Michitaka et al., 2007; Nishizawa et al., 2005; Sonoda et al., 2004). Between the HEV strains from wild boars and human strains a high genetic relationship is observed (Reuter et al., 2009; Takahashi et al., 2004) and the acquirement of an hepatitis E infection after the consumption of undercooked wild boar meat has been repeatedly described (Li et al., 2005; Masuda et al., 2005; Matsuda et al., 2003). The seroprevalence of HEV-specific antibodies in wild boars varies between 4 to 71% and the prevalence of HEV genome ranges from 1 to 68% (Adlhoch et al., 2009; de Deus et al., 2008a; Kaba et al., 2010; Martelli et al., 2008; Michitaka et al., 2007; Reuter et al., 2009; Rutjes et al., 2009; Sonoda et al., 2004). In Germany, the first proof of wild boars as HEV reservoir was accomplished by Kaci

et al. when in 5.3% of wild boar sera collected in 1995/1996 HEV GT3 RNA could be detected (Kaci et al., 2008). Adlhoch et al. found 68.2% of German wild boars to be positive for HEV of the genotypes 3i, 3h, 3f and 3e (Adlhoch et al., 2009).

A small outbreak of hepatitis E was reported from Japan after the consumption of uncooked **deer** meat, which indicated that deer might act as an additional reservoir of HEV (Tei et al., 2003). Subsequent studies were conducted to determine the prevalence of HEV in the deer population. The seroprevalence in Japan varies from 2 to 34.8% (Matsuura et al., 2007; Sonoda et al., 2004; Tomiyama et al., 2009). In Hungary, 11% of the examined roe-deer were tested positive for HEV RNA (Reuter et al., 2009). However, Matsuura and colleagues suggest that deer may only play a minor role as HEV reservoir (Matsuura et al., 2007).

The big liver and spleen disease virus (BLSV) is responsible for decreased egg production and a slightly increased mortality of **chickens** in Australia. Sequence comparison revealed that BLSV is related to HEV, however with only 62% nucleotide sequence identity to human HEV strains (Payne et al., 1999). In the USA and Canada, the hepatitis-splenomegaly syndrome emerges, which is caused by avian HEV sharing about 50% nucleotide sequence identity to human HEV strains (Haqshenas et al., 2001). However, the BLSV and the avian HEV share about 80% sequence identity indicating that both are distinct strains of the same virus (Meng, 2010b). Avian HEV seems to be enzootic in chicken flocks in the USA and also in Spain 89.7% of chicken flocks were positive for antibodies against avian HEV (Huang et al., 2002b; Peralta et al., 2009). Antibodies specific for avian HEV are also prevalent in healthy chickens (Sun et al., 2004). Sequence analyses have shown that avian HEV strains are genetically very heterogenic. Nevertheless, avian HEV strains share certain antigenic epitopes with human and swine HEV strains but exhibit also unique epitopes that do not show any antigenic cross-reactivity (Guo et al., 2008; Guo et al., 2006; Haqshenas et al., 2002). Although the virus could be transmitted to young turkeys, an attempt to infect rhesus monkeys with avian HEV failed and it was therefore concluded that avian HEV is not capable to infect humans (Huang et al., 2004; Sun et al., 2004).

The first indication of an involvement of **rodents** in the transmission of hepatitis E has been published in 1993 when Karetnyi et al. detected HEV in the sera of rodents

caught next to a Russian village, where an outbreak of hepatitis E occurred (Karetnyi et al., 1993). The experimental infection of laboratory rats or mice has been investigated, but with contradictory results: some studies show successful infection, whereas others do not (Karetnyi et al., 1993; Li et al., 2008; Maneerat et al., 1996). Experiments with Balb/c nude mice infected by swine HEV resulted in the detection of HEV antigens and HEV RNA in several organs, histopathological changes in the liver and the spleen of the mice and increased levels of aspartate aminotransferase (AST), alkaline phosphatase (ALP) and HEV-specific antibodies (Huang et al., 2009). Several studies report the detection of HEV-specific antibodies in rodents from different countries like India, Vietnam, Brazil, Japan and the USA (Arankalle et al., 2001; Favorov et al., 2000; Hirano et al., 2003; Kabrane-Lazizi et al., 1999; Meng et al., 2002; Vitral et al., 2005). In the USA, prevalence rates of HEV-specific antibodies as high as 90% have been reported for some wild rodent populations (Favorov et al., 2000; Kabrane-Lazizi et al., 1999). However, HEV RNA has not been convincingly detected in wild rodents by now. The only article describing the detection of HEV GT1 RNA in rodents has been retracted due to a laboratory contamination (He et al., 2002; He et al., 2006).

Mongooses are also suspected as virus reservoir since HEV GT3 was demonstrated in these animals in Japan (Li et al., 2006; Nakamura et al., 2006). HEV RNA was detected in 4% of work **horses** in Egypt (Saad et al., 2007). In Japan, 16.8% of the examined horses were positive for anti-HEV IgG and in one sample the genome of HEV GT3 could be detected by reverse transcription polymerase chain reaction (RT-PCR) (Zhang et al., 2008). Interestingly, in Denmark the contact to horses was also identified as one risk factor for an HEV infection (Christensen et al., 2008). Recently, HEV genome was also detected in **rabbits** and **cows** belonging to GT3 and GT4, respectively (Hu & Ma, 2010; Zhao et al., 2009).

Antibodies against HEV are also found in buffaloes, goats, sheep, ducks, pigeons, camels, cats and dogs, which are therefore regarded as additional reservoirs and sources of infection of hepatitis E (Arankalle et al., 2001; Kuno et al., 2003; Meng, 2000; Shukla et al., 2007; Yu et al., 2009; Zhang et al., 2008).

1.10 Animal experiments

The experimental infection of animals is a possibility to assess infectivity, excretion, host range, organ tropism and pathogenesis of viruses. Small animal models may be applicatory, whereas pigs and non-human primates can be used as model organisms for humans.

The experimental infection of cynomolgus macaques, chimpanzees and rhesus monkeys with human HEV strains is possible (Aggarwal et al., 2001; Balayan et al., 1983; Huang et al., 2008; Yu et al., 2010). In addition, the experimental infection of rhesus monkeys and chimpanzees with swine HEV has been successfully conducted (Meng et al., 1998). Conversely, also human HEV strains of different genotypes could be used to infect pigs revealing that HEV is able to cross species barriers (Feagins et al., 2008; Halbur et al., 2001; Lu et al., 2004). Beside these animals, marmosets, lambs, Wistar rats and Balb/c mice have been successfully inoculated with mammalian HEV, although the susceptibility of these species could not be reproduced in all of these cases (Huang et al., 2009; Kane et al., 1984; Maneerat et al., 1996; Meng, 2010b; Tabor, 1985; Usmanov et al., 1994). An animal model for avian HEV in specific-pathogen-free chickens has been established (Billam et al., 2005). In contrast, the experimental infection of rhesus monkeys with avian HEV failed showing that avian HEV may not be transmissible to humans (Huang et al., 2004). In almost all studies, animals are infected intravenously with HEV, although this kind of inoculation does not reflect the natural route of transmission. The minimal infectious dose is not known by now (Pavio et al., 2010). However, pigs infected orally with HEV need large amounts of virus; generally, oral infection is often not successful (Bouwknegt et al., 2009; Bouwknegt et al., 2008b; Casas et al., 2009b; Kasorndorkbua et al., 2004). The absence of an efficient small animal model or an efficient cell culture system still hampers investigations on HEV infection, replication, protein processing and pathogenesis (Pavio et al., 2010).

1.11 Diagnostic tools for the detection of HEV

Since no efficient cell culture system exists so far, the detection of HEV is mainly dependent on molecular methods, which involve **RT-PCR** in conventional or real time mode and immunoassays (Chandra et al., 2008). Many different protocols for RT-PCR assays exist, most of them target the conserved regions within ORF1 (Preiss et

al., 2006; Zhao et al., 2007), within ORF2 or the overlapping region of ORF2/ORF3 (Adlhoch et al., 2009; Ahn et al., 2006; Enouf et al., 2006; Gyarmati et al., 2007; Herremans et al., 2007; Huang et al., 2002a; Jothikumar et al., 2006; Mansuy et al., 2004; Orru et al., 2004; Preiss et al., 2006). Due to the genetic heterogeneity of HEV, it was useful to develop PCR assays that are able to detect all four genotypes in one reaction (Grimm & Fout, 2002; Gyarmati et al., 2007). For the detection of avian HEV specific RT-PCR protocols have been developed (Huang et al., 2002b; Peralta et al., 2009; Sun et al., 2004). Real time RT-PCR or nested RT-PCR assays are necessary to increase sensitivity and specificity of the assays (Enouf et al., 2006; Huang et al., 2002a; Jothikumar et al., 2006; Meng et al., 1997; Zhao et al., 2010).

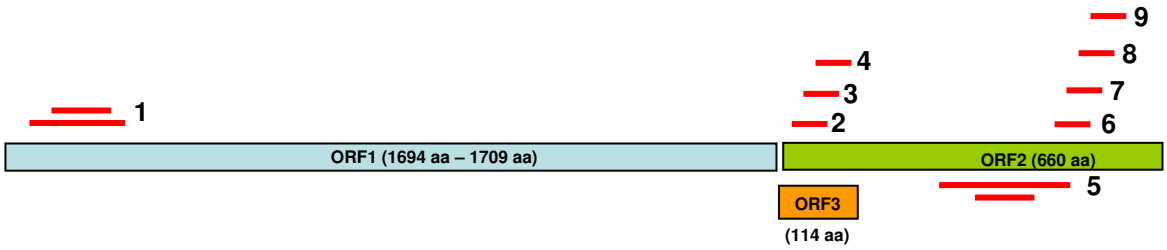

Figure 10: Targets of different published RT-PCR assays; 1 Preiss et al., 2006; 2 Enouf et al., 2006; 3 Orru et al., 2004; 4 Jothikumar et al., 2006; 5 Huang et al., 2002 ; 6 Ahn et al., 2006 ; 7 Gyarmati et al., 2007 ; 8 Adlhoch et al., 2009 ; 9 Mansuy et al., 2004 (Dremsek et al., 2010).

For the detection of HEV antigens or HEV-specific antibodies several immunoassays, mostly enzyme-linked immunosorbent assays (ELISA) or western blots, are available (Huang et al., 2002b; Legrand-Abravanel et al., 2009; Mast et al., 1998; Peralta et al., 2009; Rose et al., 2010). However, there is no immunoassay, which serves as “gold standard” for the detection of HEV-specific antibodies. The use of different antigenic peptides and different HEV genotypes as antigens further complicates comparison of results (Bouwknegt et al., 2008a; Ghabrah et al., 1998; Mast et al., 1998). For serological testing, recombinant ORF2 or ORF3 proteins, truncated versions of the proteins or peptides are used, which may be produced in insect cells, *Escherichia coli*, yeast or baculovirus-mediated expression systems (Dremsek et al., 2010; Rose et al., 2010). Due to the existence of only one serotype, the used genotype seems to play only a minor role in contrast to the size and region of the recombinant proteins used for detection of anti-HEV antibodies (Ma et al., 2009).

No efficient **cell culture** system is available for propagation of HEV so far (Chandra et al., 2008). However, HEV has been shown to infect certain carcinoma cell lines: A549, HepG2/C3A and PLC/PRF/5 (Emerson et al., 2005b; Huang et al., 1999; Tanaka et al., 2007; Yunoki et al., 2008). HEV may even produce infectious progeny viruses in A549 and PLC/PRF/5 in the absence of any cytopathic effects (Tanaka et al., 2007; Yunoki et al., 2008). A combination of tissue culture infection and immunofluorescence can be used to demonstrate the presence of infectious HEV (Emerson et al., 2005b). Nevertheless, for an efficient amplification of the virus a very high titre of infectious HEV is needed (Tanaka et al., 2007). Infectious cDNA clones transfected into cells are also used to analyse the replication cycle and to further characterize viral proteins (Graff et al., 2008).

Using **electron microscopy** HEV strongly resembles *caliciviruses* and is hard to distinguish from other small round viruses (Bradley & Balayan, 1988).

1.12 Prevention & control of HEV

Prevention and control of HEV is crucial, especially in developing countries, where morbidity and mortality are relatively high. Sanitation and access to save drinking water is the most effective way to control the emergence of HEV and other water-related diseases in developing countries (Pavio et al., 2010).

In developed countries, where high hygienic standards are common, other measures have to be taken into consideration, since a zoonotic transmission is suspected to play the major role in HEV epidemiology. Information and surveillance especially of populations at higher risk of HEV infection such as veterinarians, swine handlers and hunters might be useful, e.g., communicating hygienic measures when handling animals or animal products (Pavio et al., 2010), e.g., HEV is reported to be sensitive to low temperatures and iodinated disinfectants (Meng, 2010a).

A sufficient heating of meat or meat products may also prevent an HEV infection. However, only limited information is available about the heat stability of HEV. Some studies report that temperatures above 56°C applied for 30 to 60 minutes resulted in the inactivation of most of the viruses (Emerson et al., 2005b; Huang et al., 1999) although infectious viruses were still detectable under these conditions (Emerson et al., 2005b; Feagins et al., 2008; Tanaka et al., 2007). Temperatures above 70°C are

more efficient in HEV inactivation (Feagins et al., 2008; Tanaka et al., 2007). However, Yunoki et al. showed that proteins or magnesium may act as stabilizers, which can increase heat stability of HEV (Yunoki et al., 2008).

Furthermore, vaccination of the population would be reasonable, especially in developing countries. At the moment no vaccine is available but a successful phase II clinical trial of a VLP-based HEV vaccine candidate was conducted in Nepal (Purcell & Emerson, 2008; Shresta et al., 2007).

2 Aims of the studies

The number of autochthonous HEV infections is increasing in Germany over the last years. The route of virus transmission for these cases is not clear but a zoonotic transmission seems to be likely. This is supported by several cases of food-borne acquired hepatitis E as reported from other countries and by HEV sequences from animals, especially from pigs and wild boars, which have high sequence identities with human HEV isolates. Additionally, an epidemiological study revealed the consumption of offal and wild boar meat as risk factors for an autochthonous HEV infection in Germany.

Against this background, the aims of the studies were to investigate the presence and prevalence of HEV in German wild as well as domestic animals, which come into consideration as HEV reservoirs. Wild boars and domestic pigs are well-known HEV animal reservoirs and were investigated for the presence of HEV RNA and HEV-specific antibodies, respectively. Additionally, rats, which have been suspected as HEV animal reservoir for at least 17 years now, were screened for the presence of the HEV genome. To this end, serological assays for the detection of HEV-specific antibodies in pigs had to be compared and the development of a broad-reactive RT-PCR, which can be used to detect also so far unknown HEV-like viruses, was necessary. A genomic characterization of the animal HEV strains detected in Germany, detailed phylogenetic analyses as well as comparison of human HEV strains with endemic animal HEV strains should reveal zoonotic transmission routes. The results of the studies should help to assess the epidemiological role of HEV animal reservoirs in Germany and thus serve as the basis for decisions about required countermeasures in order to prevent human hepatitis E cases.

3 HEV in wild boars

3.1 Summary of Paper 1

Detection of hepatitis E virus in wild boars of rural and urban regions in Germany and whole genome characterization of an endemic strain

Anika Schielke; Katja Sachs; Michael Lierz; Bernd Appel; Andreas Jansen and Reimar Johne

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Wild boars are regarded as a main reservoir of HEV. In Japan, several cases of hepatitis E are reported, which can be directly linked to the consumption of rare or undercooked wild boar meat. Even in Germany, the consumption of wild boar meat has been identified as a risk factor to acquire an HEV infection. Since 2008, the existence of HEV in the German wild boar population is proven.

In this study, liver samples from wild boars originating from the federal states Brandenburg and Thuringia and the cities of Berlin and Potsdam were screened for HEV RNA using real time RT-PCR in order to determine the prevalence of HEV in German wild boars. Out of these 148 samples, 22 specimens were tested positive for HEV, which resulted in an average detection rate of 14.9%. HEV was found in all age groups and all geographical regions investigated. However, in the urban regions of the cities Berlin and Potsdam, the prevalence of HEV (4.1%) was significantly lower than in the rural regions of Brandenburg and Thuringia (25.9% and 23.8%, respectively). Genotyping was possible for 14 out of the 22 HEV positive samples and phylogenetic analyses showed the presence of the genotypes 3a, 3c, 3h and 3i. The HEV sequences clustered according to their geographical origin. Three sequences of wild boar HEV strains could be compared with human HEV strains from autochthonous cases in Germany. The wild boar HEV strain wbGER27 showed 97.9% nucleotide sequence identity compared with a human HEV strain in a 147 bp fragment of ORF2. The whole genome of the HEV strain wbGER27 was thereupon sequenced and showed the highest sequence identity with an HEV strain isolated from a pig in Mongolia. The HEV strain wbGER27 may be claimed as the first full-length sequence of HEV GT3i.

It can be concluded that wild boars serve as an important HEV reservoir in Germany and that human hepatitis E cases may be linked to contact to wild boars or consumption of wild boar meat.

3.2 Key Messages of Paper 1

- **14.9% of German wild boars are positive for HEV RNA**
- **HEV prevalence is significantly higher in rural compared to urban regions**
- **HEV sequences belong to GT3 & cluster according to their geographical origin**
- **High sequence identity between human and wild boar HEV strains**
- **First full-length genomic sequence of HEV GT3i**

3.3 Own contribution to Paper 1

For this study I performed RNA extraction and PCR analysis of the liver samples as well as sequence analyses. I also participated in the amplification and analysis of the whole genome sequence of isolate wbGER27. Additionally, I wrote the major part of the manuscript.

3.4 Paper 1

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Detection of hepatitis E virus in wild boars of rural and urban regions in Germany and whole genome characterization of an endemic strain

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Abstract

Background: Hepatitis E is an increasingly diagnosed human disease in Central Europe. Besides domestic pigs, in which hepatitis E virus (HEV) infection is highly prevalent, wild boars have been identified as a possible source of human infection. In order to assess the distribution of HEV in the wild boar population of Germany, we tested liver samples originating from different geographical regions for the presence of the HEV genome and compared the detected sequences to animal and human HEV strains.

Results: A total of 148 wild boar liver samples were tested using real-time RT-PCR resulting in an average HEV detection rate of 14.9% (95% CI 9.6–21.6). HEV was detected in all age classes and all geographical regions. However, the prevalence of HEV infection was significantly higher in rural as compared to urban regions ($p < 0.001$). Sequencing of the PCR products indicated a high degree of heterogenicity of the detected viruses within genotype 3 and a grouping according to their geographical origin. The whole genome sequence of an HEV isolate (wbGER27) detected in many wild boars in the federal state of Brandenburg was determined. It belongs to genotype 3i and shows 97.9% nucleotide sequence identity to a partial sequence derived from a human hepatitis E patient from Germany.

Conclusion: The results indicate that wild boars have to be considered as a reservoir for HEV in Germany and that a risk of HEV transmission to humans is present in rural as well as urban regions.

Background

Hepatitis E virus (HEV) causes a human disease with acute hepatitis as the major clinical symptom. Although the case-fatality rate of hepatitis E is low in the general population, rates up to 25% have been observed in pregnant women [1]. In developing countries, HEV infection is one of the most important causes of infectious hepatitis leading to epidemics associated with contaminated water resources [2]. The hepatitis E cases in North America and Central Europe could be either traced to imported infec-

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tions from endemic regions or to autochthonous HEV infections [3-5]. In Germany, an increasing number of non-travel related hepatitis E cases have been notified in the last years leading to an increase from 44% of 54 hepatitis E cases in 2005 to 63% of 73 hepatitis E cases in 2007 for the autochthonous infections [6].

HEV is a single-stranded RNA virus and the only member of the unassigned genus *Hepevirus* [7]. Until now, four genotypes and several subtypes have been defined [8]. Genotypes 1, 2 and 4 are found only in distinct geographical regions of the world whereas genotype 3 seems to have a worldwide distribution [8]. Among genotype 3 and 4, HEV strains closely related to human HEV have been detected in pigs, deer and wild boar indicating the possibility of a zoonotic transmission [2,9,10]. HEV strains isolated from pigs in the Netherlands have been shown to be closely related to HEV strains from human cases of hepatitis E of the same region indicating that autochthonous HEV infections may be acquired from pigs in Central Europe [4,11,12].

Wild boars (*Sus scrofa*) have shown a significant increase in the population density throughout Europe and the USA over the past decades. Subsequently, migration to urban areas and close contact between wild boars and humans has been observed [13]. In Berlin, the capital city of Germany, the estimated number of wild boars living in urban areas is 5.000 animals [13]. Reports on human hepatitis E cases after consumption of uncooked meat from wild boar strengthened the hypothesis of a zoonotic origin of human HEV infections [14-16]. In Japan, wild boars have been suggested to serve as a reservoir for HEV infections as a broad variety of strains including those closely related to human HEV strains has been detected in this animal species [9]. A high prevalence of HEV infection was demonstrated in a wild boar population of Italy [17]. In Germany, HEV sequences have been detected in archived sera of wild boar originally sampled in 1995/1996 demonstrating that the virus has been present in the wild animal population for a longer time [18,19]. Recently, consumption of wild boar meat has been identified as a risk factor for autochthonous HEV infections in Germany [6].

In order to determine the actual distribution of HEV in wild boars from Germany, liver samples were tested for the presence of HEV and subsequently genotyped. By comparing samples derived from different urban and rural regions, possible differences in the epidemiology of the infections were investigated. The availability of the generated HEV sequences may serve as a basis for comparing actual and future human isolates to identify transmission events between wild boar and humans.

Methods

Samples

Liver tissue samples were collected from wild boars hunted in the study area (federal states of Brandenburg and Thuringia, cities of Berlin/Potsdam) for population control between 2005 and 2008. Wild boars were categorized according to age (teeth method; shoats: <1 year, yearlings: 1–2 years, adults: >2 years), sex, and location of death for most samples. Wild boar samples were considered to originate from urban areas in case that they have been sampled in settled areas (as defined by administrative districts) of more than 10,000 people. The remainder samples were considered to originate from rural areas. All samples had been stored at -80°C until analysis.

RNA extraction and PCR analysis of samples

RNA was isolated from liver suspensions using the RNeasy Mini Kit (Qiagen, Hilden, Germany) along with QIAshredder collumns (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted RNA was tested by real-time RT-PCR according to Jothikumar et al. [20] in an ABI PRISM 7500 cycler using the Quantitect Probe RT-PCR Kit (Qiagen, Hilden, Germany). Positive samples were additionally tested by RT-PCR according to Schlauder et al. [21] and modified by Herremans et al. [4] amplifying a 197 bp product of open reading frame (ORF)-2 using the One-Step RT-PCR Kit (Qiagen, Hilden, Germany). For amplification of a 287 bp product of ORF-1, a nested RT-PCR was performed according to Preiss et al. [5] using the One-Step RT-PCR Kit (Qiagen, Hilden, Germany) for the first round of RT-PCR and the TaKaRa Ex Taq (Takara Bio Europe S.A.S., Saint-Germain-en-Laye, France) for the nested PCR. PCR products were separated on ethidium bromide-stained 1.5% agarose gels and visualized by UV light.

Amplification of the whole genome sequence of isolate wbGER27

The genome of isolate wbGER27 was amplified by RT-PCR in seven parts and by application of RACE protocols. First, four PCR-products were generated using the primer sets 1, 3 and 5 previously described by Xia et al. [22]. Then, primers ORF2F (5'-ACG TCT AGA ATG TGC CCT AGG GCT KTT CTG-3', nt 5172–5192, nucleotide numbering according to wbGER27) and ORF2R (5'-ACG TCT AGA TTA AGA CTC CCG GGT TTT RCC YAA-3', nt 7154–7131) were used to amplify the complete ORF-2-encoding region (constructed on the basis of an alignment of 24 HEV full-length sequences, not shown). Based on the sequences determined for these PCR products, specific primer pairs were constructed (5'-CCC GGT CGA CAG AGG TGT ATG T-3' [nt 870–890] and 5'-CAT CAA AAA CAA GCA CCC TTG GG-3' [nt 1382–1360]; 5'-ATT CAT GCA GTG GCT CCT GAT T-3' [nt 2606–2627] and 5'-ATC

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ACG AAA TTC ATA GCA GTG TG-3' [nt 4681–4659]) for amplification of the remaining parts of the genome. For RACE amplification of the 3'-end of the wbGER27 genome, reverse transcription was performed using the primer pA1 (5'-CCG AAT TCC CGG GAT CCT_{17} V-3', complementary to poly A tail), followed by PCR with primers 5'-CCG AAT TCC CGG GAT CC-3' (binding site on primer pA1) and 5'-ATT CGG CTC TTG CAG TCC TTG A-3' (nt 6982–7003). For RACE amplification of the 5'-end of the wbGER27 genome, the 5' RACE System Kit (Invitrogen GmbH, Karlsruhe, Germany) was used according to the supplier protocol with the gene-specific primers 5'-CCA ACT GCC GGG GTT GCA TCA A-3' (nt 191–170) and 5'-GAA TCT CAG TTT GCA CAC GAG A-3' (nt 161–140). All RT-PCRs were performed using the QIAGEN LongRange 2Step RT-PCR Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out in a 20 µl reaction at 42°C for 90 min. PCR was subsequently performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA) using 5 µl of cDNA in 50 µl reactions and 93°C for 3 min, 35 cycles of 93°C for 30 sec, 56°C for 30 sec and 68°C for 5 min, and a final incubation at 68°C for 7 min.

Sequence analysis

RT-PCR products considered for sequence analysis were purified using the Qiaquick DNA purification kit (Qiagen, Hilden, Germany) and subsequently cloned into the vector pCR4-TOPO using the TOPO TA Cloning Kit for Sequencing (Invitrogen GmbH, Karlsruhe, Germany). The inserts of the plasmids were sequenced using M13 Forward and M13 Reverse primers (Invitrogen GmbH, Karlsruhe, Germany) as well as gene-specific primers in an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA). The sequence of the wbGER27 genome was assembled from the determined sequence pieces using the SeqBuilder module of the DNASTAR software package (Lasergene, Madison, USA) and submitted to the GenBank database with accession number FJ705359. The partial sequences determined here were deposited with GenBank accession numbers FJ748515 – FJ748531. Sequence similarity searches were performed using the BLAST 2.2.14 search facility [23] and the GenBank database. Phylogenetic trees were constructed on the basis of the nucleotide sequences using the MegAlign module of the DNASTAR software package (Lasergene, Madison, USA) with the CLUSTAL W method and a bootstrap analysis with 1000 trials and 111 random seeds.

Statistical analysis

For comparison of categorical variables between groups, we used the summary χ^2 test and Fisher's exact test. Calculations were done using Intercooled Stata 10 software (Stata Corporation, Texas, USA). A p-value of <0.05 was considered significant. The exact binomial method was used to calculate 95% confidence intervals of single proportions.

Results

Detection of HEV RNA in wild boar liver samples from Germany

A total of 148 liver samples from wild boar originating from different regions of Germany were analysed by real-time RT-PCR for the detection of the HEV genome. By this, 22 samples were tested positive resulting in an overall detection rate of 14.9% (95%CI 9.6–21.6). A detailed analysis showed that 14 out of 54 (25.9%; 95%CI: 14.9–39.7) and 5 out of 21 (23.8%; 95%CI 8.2–47.2) were tested positive in the rural areas of the federal states of Brandenburg and Thuringia, respectively. In the cities of Berlin/Potsdam, 3 out of 73 (4.1%; 95%CI 0.9–11.6) wild boars were tested positive. The difference of detection rates among wild boars originating from rural vs. urban areas was highly significant ($p < 0.001$). The distinct distribution of positively and negatively tested areas is shown in Figure 1. The detection rate was highest in

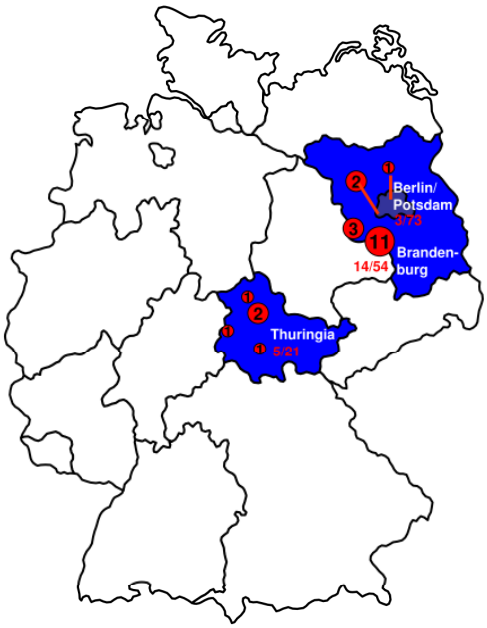

Figure 1
Geographical origin of wild boar samples. In the map of Germany, the federal states of Brandenburg and Thuringia are coloured in blue, the cities of Berlin/Potsdam are in dark blue. The areas, in which HEV positive wild boars have been detected, are marked by red circles containing the number of positive animals. The total number of positives out of all samples investigated from a federal state or from the cities is indicated by red numbers.

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shoats (19,7%) and adult animals (12,9%), while 5,9% of yearlings were tested positive for HEV. The detection rate was unrelated to sex (p = 0.1).

Genotyping of detected HEV strains

The positive samples were further analysed by RT-PCR amplifying a 197 bp fragment of ORF-2. Bands of the expected length could be detected in 14 out of the 22 samples and the DNA sequence could be determined. Phylogenetic analysis of the 148 bp sequence (excluding the primer sequences) indicated that all isolates belonged to genotype 3. Further subtyping was performed by comparison with prototype sequences of genotype 3 subtypes [8]. Although the resulting phylogenetic tree (Figure 2) generally shows low bootstrap values, which is most probably due to the short sequence used, a grouping according to the assigned subtypes is evident for the prototype strains. The sequences of wild boars clustered within different subgroups according to their geographical origin: the 9 sequences from Brandenburg clustered in genotype 3i, the two sequences from Thuringia clustered in genotype 3h, the two sequences from Potsdam clustered in genotype 3a and the isolate from Berlin branched between genotypes 3c and 3g.

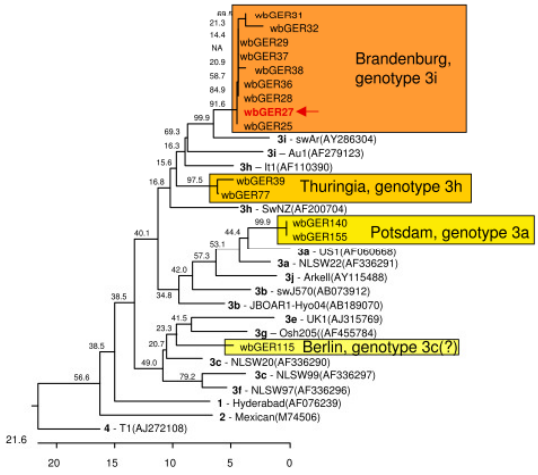

Figure 2
Genotyping of wild boar HEV strains. The phylogenetic tree was constructed based on a 148 base pair nucleotide sequence of ORF-2 using reference sequences. The genotypes according to Lu et al. [8] are indicated in bold face. The actual isolates from wild boars are marked in coloured boxes with respect to their geographical origin and deduced genotype. Isolate wbGER27, which was selected for whole genome sequencing, is shown in red and marked by a red arrow. The tree is scaled in nucleotide substitution units.

Comparison of HEV sequences to human HEV strains from Germany

To enable a comparison of the wild boar isolates with human HEV isolates derived from autochthonous infections acquired in Germany, sequences were retrieved from the GenBank database. As only partial sequences of ORF-1 were available, amplification of the corresponding region was tried by nested RT-PCR analysis of the positively tested wild boar samples. A PCR product with the expected length was only detected in three cases (isolates wbGER27, wbGER77 and wbGER155). As these samples also had shown the lowest ct values in real-time RT-PCR, the amount of HEV genome may be considered as the limiting factor for a positive ORF-1 PCR. The PCR products were compared to sequences of 15 genotype 3 isolates derived from recent human hepatitis E cases from Germany [6]. A very close relationship between the wild boar isolate wbGER27 and the human isolate V0706586 is evident from the phylogenetic tree (Figure 3), which reflects 97.9% nucleotide sequence identity between both strains within the 287 bp fragment analysed. With 92.1% nucleotide sequence identity, the human isolate V0609076 was most closely related to the wild boar isolate wbGER155. The human isolate V0703163 and the wild boar isolate wbGER77 showed 89.7% nucleotide sequence identity.

Determination and sequence analysis of the full-length genome of wbGER27

To get more information about the isolate wbGER27, which was closely related to the human isolate V0706586

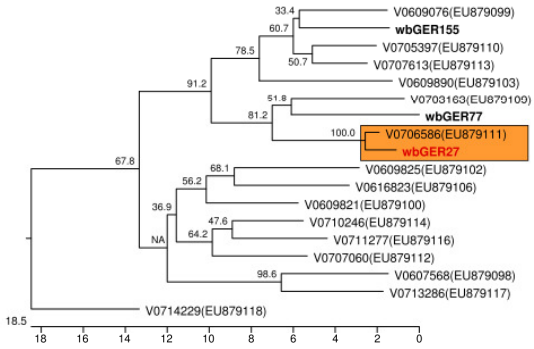

Figure 3
Phylogenetic relationship between genotype 3 HEV strains derived from wild boars and humans from Germany. The tree was constructed on the basis of a 287 bp sequence fragment of ORF-1. The actual isolates from wild boars are shown in bold face. The closely related isolates wbGER27 from wild boar and V0706586 from human are indicated with a coloured box. The tree is scaled in nucleotide substitution units.

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and which was nearly identical to the other 8 sequences detected in wild boars from Brandenburg, its whole genome sequence was determined. It consists of 7222 nucleotides (excluding the poly A tail). A BLAST search of the GenBank database using the full-length genome sequence of wbGER27 revealed the highest degree of identity with strain swMN06-A1288, which was originally detected in a pig from Mongolia. This close relationship is also reflected by a phylogenetic tree constructed on the basis of 20 HEV full-length sequences derived from the GenBank database (Figure 4). As no definitive subtype has been assigned to this Mongolian isolate, a grouping of wbGER27 is difficult. However, as it shows only up to 85.3% nucleotide sequence identity to the other isolates and as analysis of the ORF-2 fragment indicated grouping into genotype 3i, this isolate may be considered as the first full-length sequence of genotype 3i. Similar relationships were evident by analysing the deduced amino acid sequences of ORF-1, ORF-2 and ORF-3, with the highest identities of 96.2%, 97.6% and 90.2%, respectively, to those of isolate swMN06-A1288. An analysis of the non-coding regions revealed highly conserved sequences in the 5'-end as well as in the last 23 nucleotides directly adjacent to the poly A tail, but sequence variability in the residual 3' non-coding region.

Discussion

Our investigations show that HEV is highly prevalent in the German wild boar population with an average detection rate of 14.9% in liver samples. This proportion is higher than that demonstrated in a previous study showing that HEV could be detected in 5.3% of archived German wild boar sera [19]. The differences in detection rates

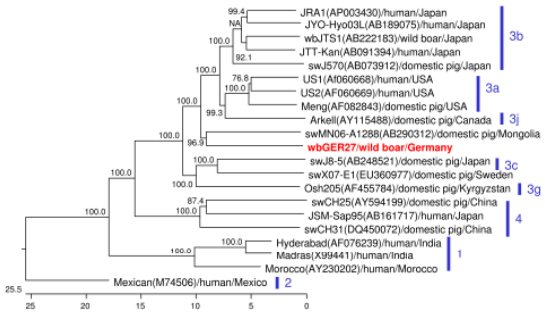

Figure 4
Comparison of the entire genome sequence of the wild boar isolate wbGER27 with 20 full-length sequences of HEV. Strain designations, accession numbers, host species and geographical origin of the isolates are indicated. Isolate wbGER27 is shown in red colour. Assigned genotypes are indicated with blue bars. The phylogenetic tree is scaled in nucleotide substitution units.

may be explained by the use of different sample material and different storage durations of the samples. A high prevalence of 25% has been also reported for wild boars from Italy, however, only a single population had been investigated in this study [17]. In Japan, several studies reported the detection of HEV or HEV-specific antibodies in the wild boar population leading to the assumption that these animals serve as a reservoir for human HEV infection [9,10,24].

Differences in the determined prevalences may also be caused by the different populations investigated. One of the most obvious finding of our study is the different detection rate in rural vs. urban regions, indicating that a more efficient virus spread among the wild boar population is possible in rural settings. The ecological and/or biological variations between rural vs. urban wild boar populations, which may explain these differences, remain elusive so far. Although with a low number, however, HEV was also detected in urban regions thus indicating that either direct or indirect transmission of HEV from wild boar to humans has to be taken into account in cities also. Notably, the shift from sylvatic to synanthropic occurrence of this game species might lead to a future increase of the infection pressure from HEV on the human population.

We detected a number of different subtypes in the wild boars which clustered due to their geographical origin. This finding argues against short-term epidemics of a certain strain and supports the assumption that several HEV subtypes are endemic in the wild boar population underlining the role of this animal species as a virus reservoir. Clustering of HEV strains according to their geographical distribution has been previously reported for domestic pigs and humans [3,4,11,12]. For domestic pigs in Germany, no data on the prevalence of HEV infection and on the distribution of specific genotypes are available so far. However, in analogy with other European countries [11,22,25,26], a high prevalence of infection with a variety of genotype 3 HEV strains could be expected. Therefore both, domestic pigs and wild boars, have to be considered as reservoirs for HEV in Germany, which may be important for the development of strategies for prevention of HEV infections. In case of wild animals, eradication of the virus infection is more difficult and other groups of the human population have to be considered to be exposed to the virus than in the case of domestic pigs.

Most important, significant homologies were detected between the HEV isolates of wild boars and those derived from autochthonous human cases of hepatitis E, which had been acquired in Germany. Unfortunately, no further information on the distinct geographical origin within Germany or on possible contacts to wild boars was avail-

able for these human cases. However, the exceptional high degree of nucleotide sequence homology between the wild boar isolate wbGER27 and the human isolate V0706586 suggests a direct connection between both by direct or indirect (food-borne or by surfaces, environment, or other carrier animals) transmission from wild boar to human. Alternatively, contact of wild boar and human to the same, so far unknown, virus source has to be taken into consideration. The full-length genome sequence of isolate wbGER27 may help to identify further transmission events as it can be compared to any genome fragment generated from a human HEV isolate. Until now, no other HEV full-length sequences derived from wild animals of Europe are available. The generation of more full-length sequences will be necessary due to the detected genetic heterogenicity of the isolates as shown for pigs in Europe [22,26].

Conclusion

In summary, the results indicate that wild boars may be an important reservoir for HEV in Germany possessing a significant risk for HEV infection of humans. This risk is obvious for hunters, which may be infected during dissection of wild boars. However, consumption of undercooked wild boar meat or contact with faecal contaminations of wild boars may also be taken into consideration. Moreover, HEV was detected with no significant differences in all age groups of wild boars which is in contrast to the situation in domestic pigs, where the age class of 10 to 15 weeks of age is predominantly infected [25,27], thus increasing the risk of virus transmission. The distinct reasons for these differences are not known so far. However, the more intensive contacts between domestic pigs in animal production may explain a more rapid virus spread as compared to the epidemiological situation with rarer contacts between wild boar herds. In Germany, up to 500,000 wild boars are hunted yearly [28], out of these more than 2,000 wild boars in the urban region of Berlin [29]. Further studies on the role of wild boars in the epidemiology of HEV infections are necessary to develop effective measurements for prevention of virus transmission to humans.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AS carried out the PCR analyses and participated in the sequence alignments. KS and ML participated in the design of the study and collected the samples. BA was included in drafting the manuscript and critical revision. AJ participated in the design of the study and performed the statistical analyses. RJ participated in the design of the study and sequence alignments, and drafted the manuscript.

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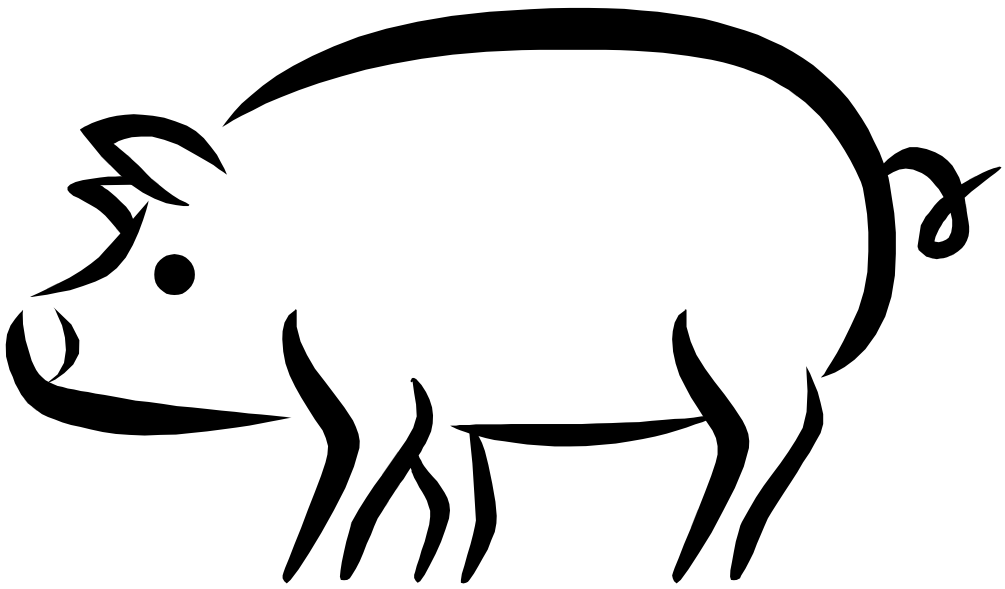
4 HEV in pigs



4.1 Summary of Paper 2

Prevalence of Hepatitis E virus-specific antibodies in sera of German domestic pigs estimated by using different assays

Christine Bächlein; Anika Schielke; Reimar Johne; Rainer G. Ulrich; Wolfgang Baumgärtner and Beatrice Grummer

Veterinary Microbiology 2010, 144(1-2):187-191

Since in 1997 the first HEV animal strain has been detected in a domestic pig in the USA, HEV has been found to be enzootic in pigs worldwide and pigs are regarded as the main animal reservoir of the virus. However, data concerning the prevalence of HEV in the German pig livestock are still lacking.

In this study, the seroprevalence of HEV-specific antibodies in pigs in Germany has been determined using three different immunoassays: a commercially available ELISA (HEV Ab-ELISA kit, [Axiom, Bürstadt, Germany]), an in-house ELISA developed at the University of Veterinary Medicine Hannover (TiHo-ELISA), and a commercially available immunoblot called recomBlot test (Mikrogen, Neuried, Germany). In total, 1072 sera from 142 farms collected between 2007 and 2008 originating from eleven different federal states were screened for the presence of anti-HEV antibodies, which would indicate a prior HEV infection if the pigs are tested positive. Using the TiHo-ELISA an average seroprevalence of 49.8% ranging from 15.6% in Mecklenburg-Western Pomerania to 70.7% in Bavaria was determined for the 1072 sera. Of these 1072 sera, 321 sera were randomly selected and retested using the commercially available Axiom-ELISA. For these sera the Axiom-ELISA determined a seroprevalence of 64.8% in contrast to the TiHo-ELISA, which determined a seroprevalence of 43.9% for the same samples. Concordant results were obtained for only 56.1% of these sera tested by the two ELISAs. A subset of 23 sera was additionally tested by using the immunoblot, which resulted in only 30.4% concordant results between the three test systems. This study shows that different prevalences result when different test systems are applied. All test systems have in common that they use antigens originating from HEV genotype 1 but the antigen used for the TiHo-ELISA is a composite of the C-terminal amino acids 1 to 30 of

ORF2 and amino acids 1 to 29 of ORF3, respectively, whereas the antigen of the Axiom-ELISA is a recombinant capsid protein derivative consisting of the C-terminal amino acids 394 to 606. For the immunoblot four recombinant denatured polypeptides covering the whole ORF2 and ORF3 are blotted. In addition, the double-antigen sandwich principle of the Axiom-ELISA enables the detection of all antibody classes independent of the host species, whereas the other assays only detect IgG.

It can be concluded that standardized test systems are needed for comparison of seroprevalences. However, all three assays used in this study confirm a high seroprevalence in the German pig population. Anti-HEV antibodies were detected in all age groups but piglets had a lower seroprevalence. These results are comparable to that reported for other European countries such as Spain and France.

4.2 Key Messages of Paper 2

- **High seroprevalence of HEV-specific antibodies in the German pig livestock**
- **Detection of anti-HEV antibodies in all age groups, but piglets show a lower seroprevalence**
- **Seroprevalence is highly dependent on used immunoassay, thus standardized methods are needed to enable comparisons**

4.3 Own contribution to Paper 2

I was involved in preparation and distribution of the samples between the project partners. Additionally, I performed the experiments using the Axiom-ELISA. I wrote the respective parts of the manuscript and was engaged in critical reading and revision of the whole manuscript.

4.4 Paper 2

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Short communication

Prevalence of Hepatitis E virus-specific antibodies in sera of German domestic pigs estimated by using different assays

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ABSTRACT

Hepatitis E virus is the causative agent of an acute hepatitis in humans. In industrialized countries, autochthonous hepatitis E cases in the past were mainly of undetermined origin, whereupon nowadays some cases may be linked to zoonotic transmission of HEV from pigs and wild boars. In contrast to several European countries the HEV status of German domestic pigs and a possible risk of transmission are unknown so far. Here, a novel peptide-based ELISA was used to detect HEV-specific antibodies in 1072 sera from German domestic pigs resulting in an average seroprevalence of 49.8% indicating widespread HEV infections in these animals. A comparative testing of 321 randomly selected sera revealed a seroprevalence of 64.8% when using a commercially available ELISA and 43.9% for the novel peptide-based ELISA but concordant results were obtained in both tests only for 56.1% of the sera. Additional re-testing of 23 randomly selected sera with a modified commercially available immunoblot revealed discordant results also. The use of different antigens and the measurement of different immunoglobulin classes are considered to be responsible for the observed variations of the results. Though the present study revealed a high seroprevalence of HEV in the German domestic pig population and a potential risk of transmission to humans, the differing results of the tests highlight the necessity of a standardization of serological assays for comparative seroprevalence and longitudinal studies.

1. Introduction

Hepatitis E virus (HEV) is a non-enveloped, single-stranded RNA virus mostly causing a mild to moderate self-limiting hepatitis in humans. The positively orientated RNA genome possesses three open reading frames (ORFs), which code for the non-structural proteins (ORF1), the capsid protein (ORF2) and a phosphoprotein that is associated with the cytoskeleton (ORF3) (Zafrullah et al., 1997).

In developing countries, where HEV is endemic, the virus is transmitted to humans via contaminated drinking water and mostly affects adults. In Europe, numerous symptomatic hepatitis E cases without a travelling history to HEV-endemic regions were reported in the previous years (Buti et al., 2004; Mansuy et al., 2004; Wichmann et al., 2008). The assumption of domestic pigs and wild boar representing HEV reservoirs in industrial countries was confirmed by the detection of HEV-specific RNA and antibodies in these animals (Rutjes et al., 2007; de Deus et al., 2008; Di Bartolo et al., 2008; McCreary et al., 2008).

HEV is representing a unique serotype (Anderson et al., 1999), but is subgrouped into at least four different

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genotypes. Genotypes 1 and 2 occur in humans only and can be found in Southeast Asia, Mexico and Central Africa, respectively (Schlauder and Mushahwar, 2001). HEV genotypes 3 and 4 can be found in humans and pigs (Purcell and Emerson, 2008).

Although HEV obviously circulates in the German wild boar population at least for the last 14 years (Kaci et al., 2008; Adlhoch et al., 2009; Schielke et al., 2009), studies on domestic pigs were still pending.

Here, we report on the application of a novel peptide-based ELISA for a seroprevalence study in German domestic pigs. The absolute values of the generally high HEV seroprevalences differed between the novel ELISA and two commercially available tests. We discuss the possible reasons for the variation of results and suggest a standardization of test systems for future seroepidemiological studies.

2. Materials and methods

2.1. Serum samples

A total of 1072 sera were randomly collected from 142 farms of 11 federal states of Germany during 2007/2008. For 264 sera data on the age of the pigs were available. Positive and negative reference field sera from domestic pigs from Spain had been previously characterized using an HEV genotype 1 ELISA (Martin et al., 2007; sera were kindly provided by M. Casas, CReSA, Barcelona, Spain).

2.2. Novel peptide-based ELISA (TiHo-ELISA)

The newly developed ELISA (TiHo-ELISA) is based on a synthetic peptide representing a composite of carboxy-terminal 30 amino acid (aa) residues of ORF2 protein and carboxy-terminal 29 aa residues of ORF3 protein, both originating from the Burmese HEV genotype 1 strain (Tam et al., 1991). Ninety-six well plates were coated with these HEV ORF2/ORF3 peptides purchased from Acris Antibodies GmbH (Herford, Germany). Sera were applied in a dilution of 1:250 in phosphate buffered saline (PBS) with 1% horse serum. After a stringent wash with 3 M urea, polyclonal rabbit anti-pig IgG conjugated with horseradish peroxidase (Sigma–Aldrich, Saint Louis, USA) was used in a dilution of 1:10,000. As substrate, tetramethylbenzidine (TMB) was added for 10 min followed by stopping of the enzymatic reaction with 1 M hydrochloric acid. The optical density (OD) was automatically scored in an ELISA reader (Tecan Sunrise, Tecan, Crailsheim, Germany).

2.3. Commercial recombinant protein-based ELISA (Axiom-ELISA)

The commercially available HEV Ab-ELISA kit (Axiom, Bürstadt, Germany) is a double-antigen sandwich ELISA based on a recombinant Burmese HEV genotype 1 capsid protein derivative covering the carboxy-terminal aa residues 394–606. Due to its test principle, it can detect HEV-specific antibodies independently of the host species and immunoglobulin class. The assay was performed by strictly following the manufacturer's instructions including the recommended thresholds for definition of a positive serum.

2.4. Commercial immunoblot test

The recomBlot test (Mikrogen, Neuried, Germany), primarily developed for the detection of anti-HEV antibodies in human serum, based on four recombinant proteins of HEV genotype 1: three overlapping ORF2 derived polypeptides that completely cover the capsid protein and the entire ORF3 protein. This test was performed following the recommendations of the manufacturer with the following modifications: sera at a dilution of 1:200 were incubated with the blot strips for 2 h. After washing, peroxidase-conjugated polyclonal rabbit anti-pig IgG (Sigma–Aldrich, Saint Louis, USA) was used to detect specific antibodies. TMB was used as substrate for up to 15 min. The results were defined as positive (strong bands visible), equivocal (weak bands visible) or negative (no band visible).

3. Results

3.1. Testing of domestic pig sera with the TiHo-ELISA

For the new ELISA, a cut-off value was defined using four negative control sera as defined by Martin et al. (2007). These sera also reacted negative in the two commercial tests used in our study. The cut-off value resulted from the average ODs of up to 27 testings of these negative controls plus a threefold standard deviation. Two positive reference sera, which have also been confirmed by the immunoblot test and the Axiom-ELISA to contain anti-HEV antibodies, showed OD values well above the calculated cut-off value in the TiHo-ELISA in multiple investigations. For all subsequent investigations one of the negative and both positive controls were applied on each plate (Fig. 1). To reduce background reactions and to identify antibodies with high avidity an additional incubation step with 3 M urea was applied.

In total, 1072 porcine serum samples have been screened and about half of the sera (534 = 49.8%) were tested positive in the TiHo-ELISA. The seroprevalence in the different federal states ranged from 15.6% in Mecklenburg-Western Pomerania to 70.7% in Bavaria. In 111 of 142 investigated farms (78.2%) at least one anti-HEV-antibody positive animal was identified with all farms in Bavaria and Baden-Wuerttemberg being affected (Table 1). Additional information about gender or age of the pigs was available for 264 samples. The prevalence in the adult females and males was 50% or close to 50%, respectively, but lower in the younger animals, whereas the average prevalence in the whole investigated panel was about 30% (Table 2).

3.2. Comparison of the TiHo-ELISA with the Axiom-ELISA

To verify the results of the TiHo-ELISA, 321 randomly chosen field sera, representing the minimum sample number for comparison as calculated for a 95% confidence level and a 5% confidence interval (http://www.survey-system.com/sscalc.htm), were analysed in parallel with

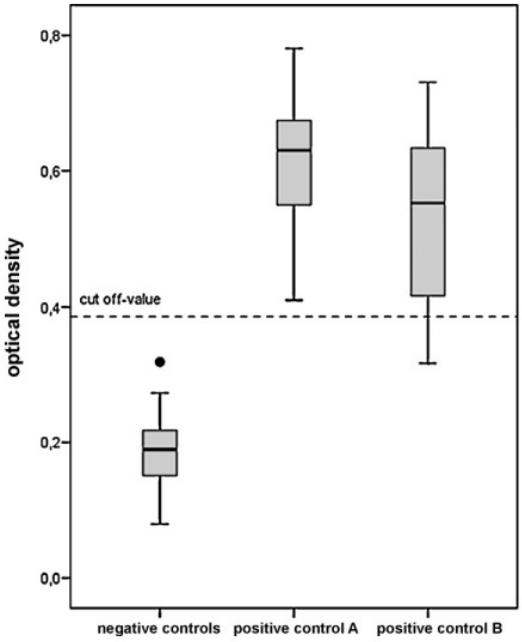

Fig. 1. Distribution of optical density (OD) values for the negative and positive controls as determined by the TiHo-ELISA and definition of the cut-off value. Given are the cumulated values of the four negative controls and the values of positive control A and positive control B which have been tested up to 27 times in duplicate; the dot represents an outlier; dotted line: cut-off value 0.386.

the Axiom-ELISA. This test classified 208 (64.8%) of the samples as positive. Using the same set of samples, the TiHo-ELISA rated 141 (43.9%) of the samples as positive (Table 3). A closer analysis of the results revealed that only 180 (56.1%) of the analysed sera showed identical results in both tests. The kappa-value was calculated as 0.182 showing only slight concordance between both ELISA tests.

3.3. Comparison of ELISA results with immunoblot data

Further investigations of 23 randomly selected sera were done with the modified recomBlot test. The sera were chosen because of their reactivity in the TiHo-ELISA: 12 anti-HEV positive and 11 anti-HEV negative sera of differing origin were included. In this test, five sera reacted positive, six sera were classified as equivocal and 12 sera did not show any reactivity with one of the recombinant proteins. In conclusion, seven (30.4%) of the sera revealed identical results in all three tests (Table 4). Unfortunately, we could not test a larger number of sera, because the manufacturer discontinued the production of the test.

4. Discussion

There is an increasing evidence that hepatitis E is a zoonosis. Pigs, either domestic or feral, can be infected with HEV and phylogenetic analyses revealed a geographical clustering of porcine and human HEV strains indicating a molecular epidemiological evidence for pig

Table 1
Overview of reactivity of the pig sera in the TiHo-ELISA from the different federal states of Germany. Results comprise the total number of positive sera as well as the total number of infected farms (n. a., not available).

	Federal state											
	Schleswig-Holstein	Mecklenburg-Western Pomerania	Lower Saxony	Brandenburg	Saxony-Anhalt	North Rhine-Westphalia	Thuringia	Saxony	Rhineland-Palatinate	Bavaria	Baden-Wuerttemberg	Total
Total number of investigated sera	151	45	159	59	53	174	50	50	88	123	120	**1072**
Number of anti-HEV-antibody positive sera	106	7	25	24	30	74	26	28	49	87	78	**534**
%	70.2	15.6	15.7	40.7	56.6	42.5	52.0	56.0	55.7	70.7	65.0	**49.8**
Total number of investigated farms	18	11	10	n. a.	11	17	10	21	21	10	13	**142**
Number of farms with anti-HEV-antibody positive pigs	13	3	6	n. a.	10	13	7	18	18	10	13	**111**
%	72.2	27.3	60.0	n. a.	90.9	76.5	70.0	85.7	85.7	100.0	100.0	**78.2**

Table 2
Overview of the different age groups of animals tested positively for HEV-specific antibodies in the TiHo-ELISA.

	North Rhine-Westphalia	Number of anti-HEV positive animals	%	Brandenburg	Number of anti-HEV positive animals	%	Lower Saxony	Number of anti-HEV positive animals	%	Total number of investigated sera	Total number of anti-HEV positive animals	%
Piglets	27	13	48.1	–	–		74	4	5.4	101	17	16.8
Fatteners	27	15	55.6	15	5	33.3	54	10	18.5	96	30	31.3
Sows	–	–		15	12	80.0	31	11	35.5	46	23	50.0
Boars	6	4	66.7	15	6	40.0	–	–		21	10	47.6
Total	60	32	53.3	45	23	51.1	159	25	15.7	264	80	30.3

Table 3
Comparison of results for 321 randomly selected pig sera from Germany obtained by two different antibody ELISA tests.

	TiHo-ELISA		
	Positive	Negative	Total
Axiom-ELISA			
Positive	104	104	208
Negative	37	76	113
Total	141	180	

to human HEV transmission. In this study, we show that HEV is widespread in the German domestic pig population as also previously reported for HEV in wild boars (Kaci et al., 2008; Adlhoch et al., 2009; Schielke et al., 2009). These data are in line with high seroprevalences observed in domestic pigs from Spain and France (Seminati et al., 2008; Casas et al., 2009; Kaba et al., 2009). In Europe, Germany plays a major role in the pig meat production, and it is known that sporadic cases or limited outbreaks of hepatitis E can be related to the consumption of undercooked HEV containing wild boar meat (Matsuda et al., 2003). Besides demonstrating a high HEV seroprevalence, the TiHo-ELISA also revealed that HEV-specific antibodies are common in all age groups of pigs but with only a small portion of piglets exhibiting anti-HEV antibodies. Most of the serum samples from piglets originated from Lower Saxony where the general seroprevalence is relatively low. Therefore further analyses in different federal states seem to be necessary. Additionally, the farming structure may contribute to the recording of differing seroprevalences between the federal states. In Baden-Wuerttemberg and Bavaria, where the highest anti-HEV prevalences were recorded, the piggeries are generally smaller with less structuring of pig production, which is probably also related to inefficient disinfection of stables or to potential contact with wild boars.

Analyses with another ELISA and with a commercial immunoblot confirmed the high seroprevalence of HEV in German pigs; however a closer examination of the test results revealed that both ELISAs and the immunoblot test disaccorded strongly. Several conceivable explanations are possible for this observation. All three tests based on polypeptides of HEV genotype 1, but different regions of the immunogenic proteins were presented as antigens. The TiHo-ELISA and the Axiom-ELISA use antigens

Table 4
Comparison of results for 23 randomly selected sera from German pigs obtained by three different HEV-antibody assays.

No. of serum	Origin	TiHo-ELISA	Axiom-ELISA	recomBlot
1	Schleswig-Holstein	Positive	Positive	Positive
2	Rhineland-Palatinate	Positive	Positive	Positive
3	Rhineland-Palatinate	Positive	Positive	Positive
4	Baden-Wuerttemberg	Positive	Positive	Equivocal
5	North Rhine-Westphalia	Positive	Positive	Equivocal
6	Rhineland-Palatinate	Positive	Positive	Equivocal
7	Brandenburg	Positive	Negative	Negative
8	Saxony-Anhalt	Positive	Negative	Negative
9	Rhineland-Palatinate	Positive	Negative	Positive
10	Rhineland-Palatinate	Positive	Negative	Equivocal
11	Bavaria	Positive	Negative	Equivocal
12	Rhineland-Palatinate	Positive	Negative	Equivocal
13	Rhineland-Palatinate	Negative	Negative	Negative
14	Rhineland-Palatinate	Negative	Negative	Negative
15	Rhineland-Palatinate	Negative	Negative	Negative
16	Rhineland-Palatinate	Negative	Negative	Negative
17	Saxony	Negative	Positive	Positive
18	Saxony	Negative	Positive	Negative
19	Thuringia	Negative	Positive	Negative
20	Brandenburg	Negative	Positive	Negative
21	Saxony-Anhalt	Negative	Positive	Negative
22	Rhineland-Palatinate	Negative	Positive	Negative
23	Rhineland-Palatinate	Negative	Positive	Negative

corresponding to the carboxy-terminal region of the capsid protein but of different size. The immunoblot test uses polypeptides completely covering ORF2 and ORF3 proteins, but in a denatured form. The presence of antibodies with different binding specificities to the HEV polypeptides may therefore influence the test result. Furthermore, the Axiom-ELISA was developed for the simultaneous detection of antibodies of all classes. In contrast, the TiHo-ELISA and the modified immunoblot test detect porcine IgG antibodies only. The presence of IgM in sera from acutely infected pigs and IgG in sera from reconvalescent pigs may therefore also explain contradictory results using the different test systems.

Besides, the inclusion of an incubation step with 3 M urea in the TiHo-ELISA protocol dropped background reactions remarkably resulting in the detection of antibodies exclusively with high avidity to the antigen. This phenomenon was revealed by Allmang et al. (2001), who showed that in horses naturally infected with Borna disease virus, IgG with high avidity to the viral nucleoprotein could be selected by treatment with urea. Varying results using different assays to determine the HEV seroprevalence in pigs have also been reported previously (Peralta et al., 2009) showing that there is an urgent need for standardized serological assays for the detection of HEV-specific antibodies in pigs in general. Comparability of assays may for example be achieved by the use of homologous porcine sequences of HEV genotype 3 as it has been proposed by others (Jimenez de Oya et al., 2009; Peralta et al., 2009). Such assays will be needed to enable comparison of results of surveillance studies conducted by independent research groups in different countries and also for reliable testing results in order to define a distinct pig farm as free from HEV.

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5 HEV in rats

5.1 Summary of Paper 3

Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR

Reimar Johne; Anita Plenge-Bönig; Michael Hess; Rainer G. Ulrich; Jochen Reetz and Anika Schielke

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Several studies report the detection of HEV-specific antibodies in rodents indicating a potential role of these animals in the transmission of HEV. However, HEV RNA has not been verified in rodents by now.

In this study, we found for the first time an HEV-like virus in rats, which was tentatively called rat HEV. In total, 30 faecal samples of wild Norway rats (*Rattus norvegicus*) originating from Hamburg, Germany, were screened for the presence of HEV RNA using a novel nested broad-spectrum RT-PCR targeting a highly conserved region within ORF1. This assay was newly developed here and was shown to detect HEV strains from wild boars and chickens. Two faecal specimens of rats were positive using this RT-PCR, namely the samples R4 and R8. Genome fragments of 4019 nt of R4 and 1545 nt of R8 could be sequenced showing a sequence identity based on nucleotide level of 59.9% to human and 49.9% to avian HEV strains. The deduced amino acid sequences of R4 and R8 revealed 56.2% and 42.9% sequence identity to human and avian HEV strains, respectively. Phylogenetic analyses showed a clustering of the two rat HEV strains representing a clearly separated branch between mammalian and avian HEV isolates. A further characterization of the viral genome fragment demonstrated the presence of a typical hydrophobic region in the signal sequence of ORF2, an accumulation of arginine residues in the arginine-rich domain of ORF2 as well as some highly conserved regions in the S and M domain of ORF2 compared to avian and mammalian HEV strains. Using negative-staining electron microscopy empty as well as filled viral particles with diameters between 32 to 34 nm reminiscent of HEV could be demonstrated in the faecal sample of R4. Solid-phase immune electron microscopy using a human serum positive for HEV-specific antibodies resulted in a considerable

increase of the number of HEV-like viruses that could be detected. Unfortunately, the isolation of infectious viruses using three different rat liver cell lines failed. Several reasons may be responsible as for example a too low viral titre or degradation of the viruses due to long storage conditions. Additionally, the cell lines may not be susceptible to rat HEV.

In summary, the first description of an HEV-like sequence in rats explains the detection of HEV-specific antibodies in rodents. Further experiments are necessary for a more detailed characterization of rat HEV.

5.2 Key Messages of Paper 3

- **Development of a novel nested broad-spectrum RT-PCR for HEV-like viruses**
- **First detection of an HEV-like virus in rats, tentatively designated as rat HEV**
- **Preliminary genomic characterization of rat HEV revealed only limited sequence identities of rat HEV with mammalian and avian HEV strains**
- **Visualization of HEV-like viruses in one faecal sample using electron microscopy**
- **Isolation of infectious rat HEV using different cell lines failed**

5.3 Own contribution to Paper 3

I was responsible for the RNA extraction from the faecal samples and performed the nested broad-spectrum RT-PCR. I participated in the cloning and sequencing of the positive samples and conducted phylogentic analyses. Additionally, I wrote major parts of the manuscript.

5.4 Paper 3

≪paper no. vir016584 charlesworth ref: vir016584≫

Animal RNA

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Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR

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Hepatitis E is a rare human disease in developed countries. It is caused by hepatitis E virus (HEV), which is probably transmitted zoonotically to humans from domestic pigs and wild boars. Multiple reports on the detection of HEV-specific antibodies in rats have suggested the presence of an HEV-related agent; however, infectious virus or a viral genome has not been demonstrated so far. Here, a nested broad-spectrum RT-PCR protocol was developed capable of detecting different HEV types including those derived from wild boar and chicken. Screening of 30 faecal samples from wild Norway rats (*Rattus norvegicus*) from Hamburg (Germany) resulted in the detection of two sequences with similarities to human, mammalian and avian HEV. Virus particles with a morphology reminiscent of HEV were demonstrated by immunoelectron microscopy in one of these samples and the virus was tentatively designated rat HEV. Genome fragments with sizes of 4019 and 1545 nt were amplified from two samples. Sequence comparison with human and avian strains revealed only 59.9 and 49.9 % sequence identity, respectively. Similarly, the deduced amino acid sequence for the complete capsid protein had 56.2 and 42.9 % identity with human and avian strains, respectively. Inoculation of the samples onto three different permanent rat liver cell lines did not result in detectable virus replication as assayed by RT-PCR with cells of the fifth virus passage. Further investigations are necessary to clarify the zoonotic potential of rat HEV and to assess its suitability to serve in a laboratory rat animal model for human hepatitis E.

INTRODUCTION

Hepatitis E, caused by hepatitis E virus (HEV), is a worldwide human disease that is endemic in many developing countries. In industrialized countries, sporadic cases are increasingly reported, which can be traced either to imported infections from endemic regions or to autochthonous HEV infections (Clemente-Casares *et al.*, 2003; Dalton *et al.*, 2008; Gyarmati *et al.*, 2007; Purcell & Emerson, 2008). Hepatitis E is characterized by a self-limiting jaundice of varying severity, which is hard to distinguish from a hepatitis of other viral origin, and is often accompanied by non-specific symptoms such as fever, headache and pain in the upper abdomen. Although the case fatality rate of hepatitis E is low in the general population (0.5–3 %), rates of up to 20 % have been observed for pregnant women (Shrestha *et al.*, 2007; Smith, 2001; Wichmann *et al.*, 2008).

HEV is classified as the only member of the genus *Hepevirus*. This genus is subdivided into four distinct genotypes and the avian HEV strains (Bilic *et al.*, 2009), which are not included in any of the other genotypes. All mammalian HEV isolates described to date comprise the same serotype (Lu *et al.*, 2006; Takahashi *et al.*, 2005). The HEV virion appears as a non-enveloped icosahedral sphere of approximately 27–34 nm in diameter. The crystal structure of HEV-like particles has recently been solved (Yamashita *et al.*, 2009). The particles are composed of a single capsid protein, which folds into three major

The GenBank/EMBL/DDBJ accession numbers for the sequences of rat hepatitis E virus determined in this study are GQ504009 and GQ504010.

Supplementary data are available with the online version of this paper.

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domains: the shell (S) domain, the middle (M) domain and the protruding (P) domain. The outer surface of the particle, which is a target for antibodies, is mainly formed by the M and P domains. The genome of HEV is a single-stranded, positive-sense RNA of about 7.2 kb and contains three open reading frames (ORFs) (Aggarwal & Krawczynski, 2000; Purcell & Emerson, 2008). These three ORFs encode a non-structural polyprotein (ORF1), the capsid protein (ORF2) and a phosphoprotein (ORF3) associated with signal transduction. The genome is capped at the 5′ end and polyadenylated at the 3′ end (Smith, 2001; Xia *et al.*, 2008).

HEV is transmitted mainly via the faecal–oral route by contaminated drinking water, but zoonotic transmission via undercooked meat has also been reported (Li *et al.*, 2005; Matsuda *et al.*, 2003; Tei *et al.*, 2003). HEV-specific antibodies as well as viral RNA have been detected in several animal species (Smith, 2001; Meng, 2009). The first HEV animal strain was detected in a domestic pig from the USA in 1997 (Meng *et al.*, 1997). Other hosts of the virus include wild boar and deer (Martelli *et al.*, 2008; Schielke *et al.*, 2009; Tei *et al.*, 2003). In 2001, avian HEV was detected in chickens with hepatitis–splenomegaly syndrome in the USA (Haqshenas *et al.*, 2001). Recently, HEV has been found in farmed rabbits in China, possibly representing a novel genotype (Zhao *et al.*, 2009). Viruses isolated from swine or wild boar show the closest genetic relationship to human HEV strains (Schielke *et al.*, 2009; van der Poel *et al.*, 2001; Ward *et al.*, 2009).

An initial indication for a potential role of rodents in the transmission of hepatitis E was found by the detection HEV antigen in the sera of rodents caught next to a Russian village where an outbreak of hepatitis E was reported (Karetnyï *et al.*, 1993). Trials to infect laboratory rats or mice experimentally with human HEV led to contradictory results, as some studies reported successful infection whilst others did not (Karetnyï *et al.*, 1993; Li *et al.*, 2008; Maneerat *et al.*, 1996). Several studies have shown the detection of HEV-specific antibodies in rodents from different countries including India, Vietnam, Brazil, Japan and the USA, including commensal rodents such as Norway (*Rattus norvegicus*) and black (*Rattus rattus*) rats, but also wild rodents such as deer mouse (*Peromyscus maniculatus*) and cotton rat (*Sigmodon hispidus*) (Arankalle *et al.*, 2001; Favorov *et al.*, 2000; Hirano *et al.*, 2003; Kabrane-Lazizi *et al.*, 1999; Meng *et al.*, 2002; Vitral *et al.*, 2005). In the USA, prevalence rates of HEV-specific antibodies as high as 90 % have been reported for some rat populations (Favorov *et al.*, 2000; Kabrane-Lazizi *et al.*, 1999). However, HEV RNA has not been convincingly detected in commensal or wild rodents to date.

The extent of genomic heterogeneity of HEV complicates the detection of novel strains (Gyarmati *et al.*, 2007). Therefore, there is a need for a nested broad-spectrum RT-PCR for the simultaneous detection of the known HEV genotypes, including avian HEV strains, to be developed and tested for its suitability to detect novel HEV strains. In this study, 30 faecal samples from wild rats collected in Hamburg, Germany, were tested using such an RT-PCR for the existence of HEV-like viruses. Two HEV-like sequences derived from these samples were further characterized by phylogenetic investigations. The zoonotic potential of the detected HEV-like strain and its possible future application in an animal model for human hepatitis E is discussed.

RESULTS

Development of a nested RT-PCR for the detection of hepeviruses

By alignment of 22 full-length genome sequences of HEV derived from human, pig, wild boar and chicken (see Supplementary Table S1, available in JGV Online), a conserved region was identified within ORF1. Two degenerated primer pairs were constructed (Table 1) with binding sites within this region and a nested RT-PCR protocol was developed. Application of the optimized protocol to the genotype 3 HEV strain wbGER27 and to the avian HEV strain 05-2294, which are only distantly related to each other, is shown in Fig. 1(a). After RT-PCR with primers HEV-cs and HEV-cas, bands at the expected position were visible in both cases; however, the avian HEV product had a lower intensity. Using the nested PCR primers HEV-csn and HEV-casn in an RT-PCR protocol, products of the expected length were detected for both templates. After nested PCR (RT-PCR with primers HEV-cs and HEV-cas followed by nested PCR with primers HEV-csn and HEV-casn), broad bands of the expected

Table 1. Oligonucleotides used in the hepevirus broad-spectrum PCR

Step	Primer designation	Sequence (5′→3′)*	Product length (bp)
RT-PCR	HEV-cs	TCGCGCATCACMTTYTTCCARAA	469–472
	HEV-cas	GCCATGTTCCAGACDGTRTTCCA	
Nested PCR	HEV-csn	TGTGCTCTGTTTGGCCCNTGGTTYC†G	331–334
	HEV-casn	CCAGGCTCACCRGARTGYTTCTTCCA	

*D=A, G or T; M=A or C; N=A, C, G or T; R=A or G; Y=C or T.
†According to a mismatch found in the described rat HEV sequences, C may be changed to M at this position.

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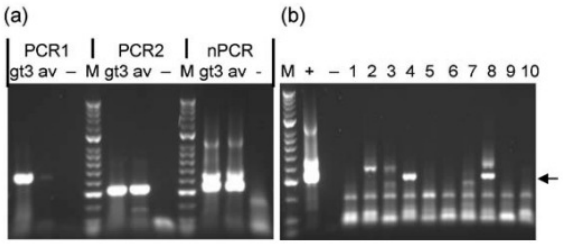

Fig. 1. Nested broad-spectrum RT-PCR for the detection of hepeviruses. RT-PCR products were visualized after electrophoresis on ethidium bromide-stained agarose gels. M, DNA size markers (HyperLadder II; Bioline). (a) RNA isolated from liver samples containing HEV genotype 3 (gt3) strain wbGER27 or avian HEV strain 05-2294 (av), or a negative control containing water (–) was analysed by RT-PCR with primers HEV-cs and HEV-cas (PCR1), by RT-PCR with primers HEV-csn and HEV-casn (PCR2) or by nested RT-PCR using primers HEV-cs and HEV-cas followed by primers HEV-csn and HEV-casn (nPCR). (b) Nested RT-PCR analysis of rat faecal samples R1 to R10 (lanes 1–10), HEV strain wbGER27 (+) and water (–). The arrow indicates the position of the specific nested PCR product.

length were amplified for both samples with additional slower-migrating bands of lower intensity, which most likely represented the remaining products of the initial RT-PCR.

Screening of rat faecal samples for hepeviruses

A total of 30 faecal samples of wild rats collected in the city of Hamburg, Germany, was screened using the nested broad-spectrum RT-PCR for the presence of hepevirus RNA sequences. In the case of samples R4 and R8, products of the expected length were detected (Fig. 1b); all other samples showed only non-specific products. The PCR products of samples R4 and R8 were cloned and sequenced. A sequence similarity search of GenBank using the BLAST search facility revealed the highest sequence identity of R4 and R8 sequences with those of HEV strains Arkell (pig HEV genotype 3 strain from Canada) and CN9802 (human HEV genotype 4 strain from China), respectively. The nucleotide sequence identity between the R4 and R8 sequences in this fragment spanning nt 4285–4616 (numbering according to genotype 3 strain Meng) was found to be 85.4 %. The identified virus represented by these two novel strains was tentatively designated rat HEV.

Identification of virus particles by transmission electron microscopy

The faecal samples R4 and R8 were investigated by negative-staining electron microscopy. In sample R4, but not in R8, a few filled and empty virus particles with a diameter of 32–34 nm and a shape reminiscent of hepeviruses were detected (Fig. 2a, b). Using solid-phase immunoelectron microscopy with a human serum positive for HEV-specific antibodies and sample R4, the number of detectable HEV-like particles increased considerably (Fig. 2c). No HEV-like particles were found in this test when the serum or the faecal sample was omitted (not shown).

Cultivation trials of rat HEV in tissue culture

Efforts were made to propagate rat HEV by inoculation of samples R4 and R8 onto three different rat liver cell lines. In one series of experiments, trypsin was added to the cultures, as it is known for rotaviruses and influenza viruses that activation with trypsin is necessary for efficient virus propagation. The supernatants were separately passaged five times onto the same cell line. A cytopathic effect was evident beginning with the second passage in the clone 9 cell line inoculated with R4 without trypsin. However, the isolated agent had a shape and diameter typical of a picornavirus, as demonstrated by electron microscopy (not

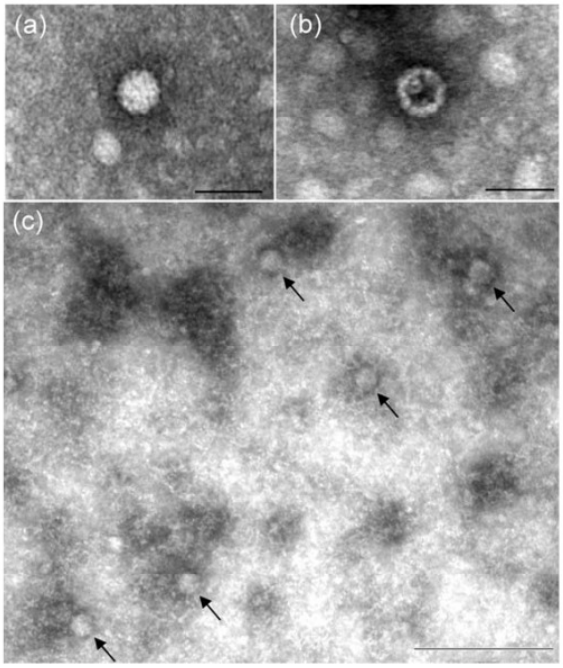

Fig. 2. Demonstration of HEV-like particles in the rat faecal sample R4. The transmission electron micrographs show (a) a filled particle with a diameter of 32 nm and (b) an empty particle with a diameter of 34 nm. (c) Solid phase immunoelectron microscopy using a human serum positively tested for HEV-specific antibodies and sample R4. The arrows indicate adsorbed HEV-like particles. Negative staining was performed with uranyl acetate. Bars, 50 nm (a, b); 200 nm (c).

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shown). This culture supernatant and those from the fifth passages of all inoculated cell lines were tested using the nested broad-spectrum RT-PCR for detection of the hepevirus genome, as well as with an RT-PCR with the rat HEV-specific primers ORF2-s and ORF2-as (see Supplementary Table S2, available in JGV Online). However, a specific product could not be demonstrated (not shown).

Genome sequence analysis of rat HEV

Additional parts of the rat HEV genome were amplified from the faecal samples by RT-PCR using primers with sequences delineated from an alignment of available HEV genome sequences and from the novel rat HEV sequences described here (see Supplementary Table S2). Although many additional primers were tested (not shown), only a 4019 nt segment of strain R4 and a 1545 nt segment of strain R8 could be amplified, corresponding to nt 3146–7200 and 4240–5836 (nucleotide numbering according to HEV genotype 3 strain Meng) for isolates R4 and R8, respectively. Unexpectedly, closer examination of the determined nucleotide sequences of both rat HEV strains indicated a mismatch in the binding site of primer HEV-csn (an exchange of C to A at nt 25 of the primer sequence; see Table 1).

The 4019 nt sequence of rat HEV strain R4 was most closely related to human genotypes 1 and 3 strains, but with only 58.4 % nucleotide sequence identity. Comparison of the nucleotide sequences of the 1545 nt fragment of both rat HEV strains with the corresponding region of HEV strains from humans and different animal species revealed identities ranging from 49.9 (comparison of rat HEV R4 with avian HEV) to 59.9 % (rat HEV R8 with human HEV genotype 1). The nucleotide sequence identity of the rat HEV strains R4 and R8 in this fragment was 90.4 % with each other. A phylogenetic tree constructed on the basis of this sequence fragment showed that both rat HEV strains clustered together and represented a branch clearly separated from that represented by HEV genotypes 1–4 and the rabbit HEV isolate and that represented by avian HEV (Fig. 3). In this comparison, the rabbit HEV isolate turned out to be closely related to HEV genotype 3 with 82.1 % nucleotide sequence identity to the pig isolate swX07-E1.

Analysis of deduced amino acid sequences

Due to the close relationship of strains R4 and R8 and the availability of a longer sequence, strain R4 was chosen for further analysis of the rat HEV genome sequence. The obtained sequence of R4 represented the 658 3′-terminal codons of ORF1 and the entire ORF2 (644 codons) and ORF3 (102 codons) sequences. The deduced amino acid sequences were compared with those of genotypes 1–4 and avian HEV, resulting in sequence identities ranging from 52.1 to 58.7 % for the partial ORF1, 42.9 to 56.2 % for ORF2 and 24.8 to 32.8 % for ORF3. The corresponding phylogenetic trees in all cases showed a branching of rat HEV between avian HEV and the HEV genotypes 1–4 (Fig. 4).

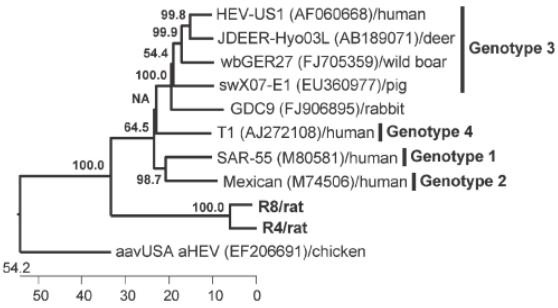

Fig. 3. Phylogenetic relationship of rat HEV strains R4 and R8 with nine HEV strains derived from human and different animal species. The tree was constructed using a 1545 nt fragment of the rat HEV genome together with the corresponding region of the other isolates. The strain designations, GenBank accession numbers and the corresponding hosts are indicated. The respective genotypes are given on the right. The tree is scaled in nucleotide substitution units and was constructed using MEGALIGN software (CLUSTAL W, IUB residue weight table, 1000 trials and 111 random seeds in bootstrap analysis).

A detailed alignment of the deduced amino acid sequences of the capsid protein (ORF2) identified conserved as well as highly variable regions among rat HEV and HEV genotypes 1–4 (Fig. 5). Most of the conserved amino acid positions were located in the S domain, which is known to have important functions in capsid assembly, and in the M domain, which has been shown to be tightly associated with the S domain and to form parts of the outer capsid surface. The P domain, which forms the protruding parts of the outer capsid surface, was more variable among the different viruses. However, two stretches of the rat HEV sequence in this region (aa 528–556 and 572–584) were nearly identical to the corresponding sequences of the other mammalian HEVs. The sequence identities in the remaining regions were low; however, a typical hydrophobic region in the signal sequence and an accumulation of arginine residues in the arginine-rich domain were evident. Tyrosine at position 288 (numbering according to genotype 3), which has been shown to be crucial for capsid formation in HEV genotype 3 (Yamashita *et al.*, 2009), was mutated to phenylalanine in rat HEV. However, it was shown in the same study that phenylalanine can functionally replace tyrosine at this position.

DISCUSSION

HEV is suspected to be a zoonotic virus, which could be transmitted from pigs, wild boars or Sika deer to humans

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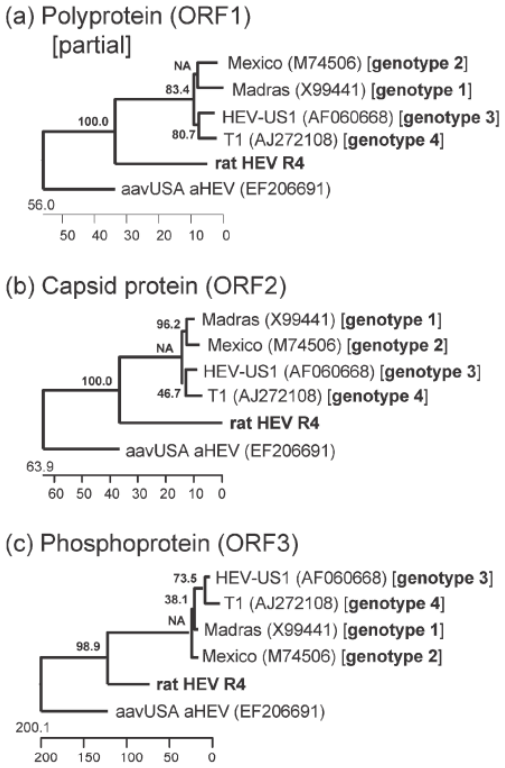

Fig. 4. Phylogenetic analysis of the deduced amino acid sequences of rat HEV and genotypes 1–4 as well as avian HEV based on a 658 aa fragment of ORF-encoded polyprotein (a), the complete ORF2-encoded capsid protein (b) and the complete ORF3-encoded phosphoprotein (c). The branches are labelled with the strain designations, GenBank accession numbers and the respective genotypes. The trees are scaled in amino acid substitution units and were constructed using MEGALIGN software (CLUSTAL W, PAM250 residue weight table, 1000 trials and 111 random seeds in bootstrap analysis).

(Schielke *et al.*, 2009; Tei *et al.*, 2003; van der Poel *et al.*, 2001). In addition, HEV-like viruses have been identified in chickens (Bilic *et al.*, 2009; Haqshenas *et al.*, 2001) and rabbits (Zhao *et al.*, 2009). Our investigation resulted in the detection of a novel virus in the faeces of wild rats from a large city in Germany, which demonstrated only limited sequence identity to HEV. This finding may suggest the presence of additional unknown HEV-like viruses in other animal species.

For sensitive detection of such HEV-like viruses, a nested broad-spectrum RT-PCR was developed here. The binding sites of the primers were chosen within a highly conserved region of ORF1. This region shows strong similarities to conserved domains of RNA-dependent RNA polymerases and contains putative functionally important regions for nucleotide and template binding (Koonin *et al.*, 1992). These essential functions may ensure the presence of such highly conserved sequences in many HEV-like viruses. A nested RT-PCR protocol was chosen due to its higher sensitivity compared with a single-step RT-PCR, as reported previously for other viruses (Johne *et al.*, 2005). The general applicability of this novel RT-PCR approach was verified by detection of two only distantly related HEV strains from wild boar and chicken whose sequences were not included in the initial alignment for primer design. In addition, the nested broad-spectrum RT-PCR could readily identify the novel rat HEV and may therefore also be suitable for detection of other, so far unknown hepeviruses in other animal species. However, as suggested above, a further adjustment of primer sequences based on the rat HEV sequences described here might improve the performance of the assay, i.e. by the inclusion of a wobble site (M instead of C) at nt 25 of primer HEV-csn (see Table 1).

Two closely related rat HEV sequences were amplified from two different rat faecal samples. However, virus particles were identified in only one of the samples, and infectious virus could not be isolated from any of the samples. The results of the RT-PCR and electron microscopy suggested that only a small amount of virus was present in both samples. For sample R8, amplification of a larger genome fragment was successful only after application of a nested RT-PCR protocol, indicating that this sample contained an even lower level of virus compared with sample R4. Isolation of HEV in tissue culture is generally difficult and is dependent on the amount of virus used for inoculation (Graff *et al.*, 2005; Tanaka *et al.*, 2007, 2009). The low rat HEV concentration in the faecal samples may therefore explain the failure of virus isolation. Alternatively, the cell lines used may not be susceptible to rat HEV, or the virus may have been degraded due to a long (unknown) retention time in the samples.

HEV-specific antibodies have repeatedly been detected in different rat species such as *R. norvegicus*, *R. rattus* and *Rattus exulans* and other rodent species (Arankalle *et al.*, 2001; Favorov *et al.*, 2000; Hirano *et al.*, 2003; Kabrane-Lazizi *et al.*, 1999; Meng *et al.*, 2002; Vitral *et al.*, 2005); however, no HEV-specific genome sequences have been convincingly demonstrated in rodent species to date. The high sequence divergence of rat HEV identified here compared with all other previously known HEV strains may be responsible for these findings. The rat HEV sequence determined here shows that RT-PCR systems usually used for HEV genome amplification (Jothikumar *et al.*, 2006; Kaci *et al.*, 2008; Schlauder *et al.*, 1999; van der Poel *et al.*, 2001; Wichmann *et al.*, 2008) cannot be used for the detection of this novel virus due to multiple nucleotide substitutions in the primer-binding regions. However, the presence of regions with highly conserved amino acid sequences within the capsid protein may explain the previously observed serological reactions of rodent samples

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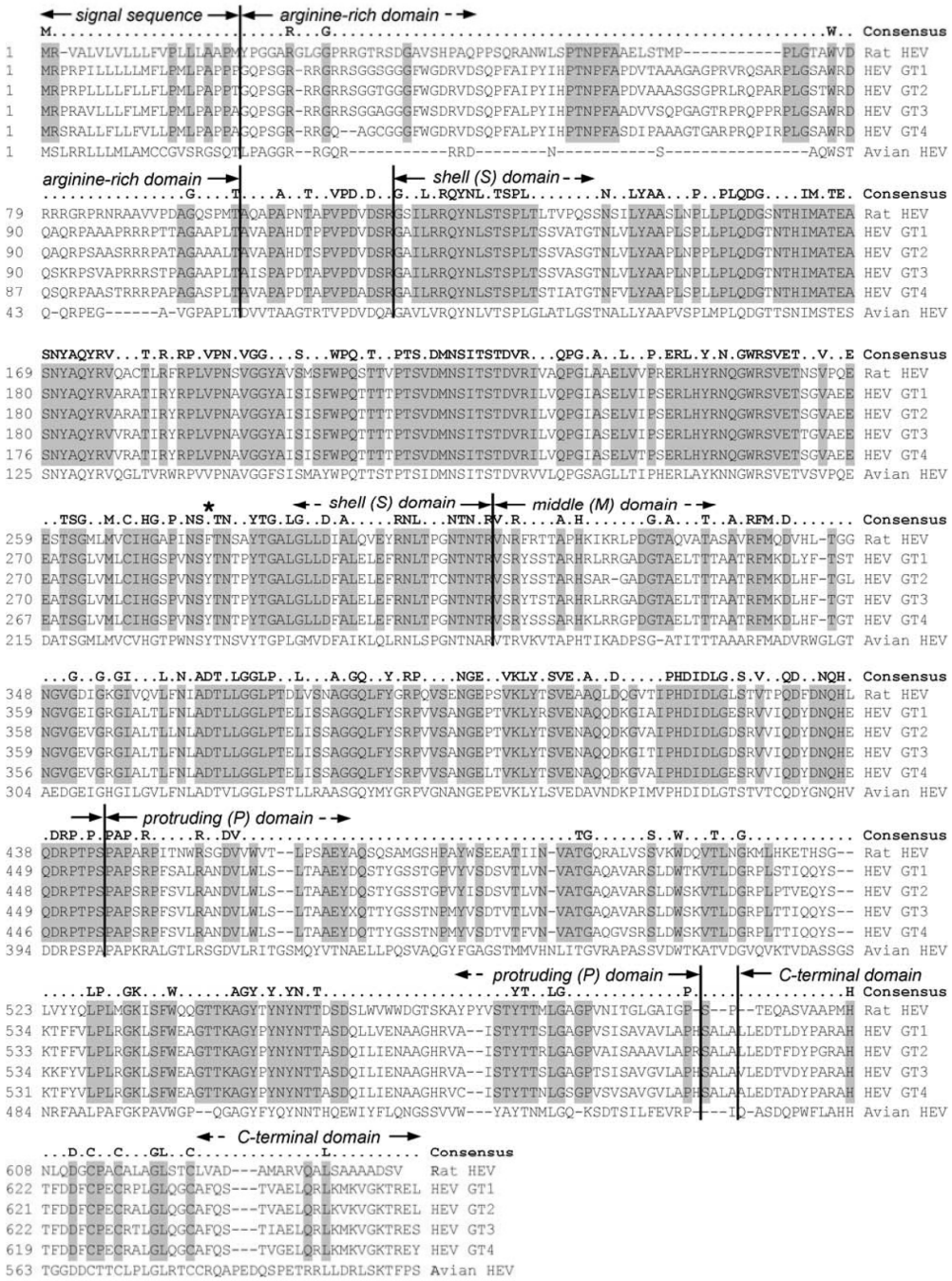

Fig. 5. Alignment of the deduced amino acid sequences of the ORF2-encoded capsid protein of rat HEV strain R4 and representatives of genotypes 1–4 (GenBank accession nos X99441, M74506, AF060668 and AJ272108), as well as avian HEV (GenBank accession no. EF206691). Residues that are conserved among all HEV isolates are shown as a consensus above the sequences; residues that are conserved in all mammalian isolates are shaded grey. The structural domains of the HEV capsid protein according to Yamashita *et al.* (2009) are indicated in italics. Amino acid position 288 in genotype 3, which has been shown previously to exert an important function in capsid assembly, is marked with an asterisk.

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with non-rodent HEV strains due to the presence of cross-reacting antibodies.

A final taxonomic classification of viruses within the genus *Hepevirus* has not been established so far (Emerson *et al.*, 2005; Meng, 2009). Our sequence analyses indicate that three distinct phylogenetic groups are formed by avian HEV, rat HEV and the remaining HEVs isolated from humans and different animal species, which may correspond to three different virus species. The third group contains the well-known HEV genotypes 1–4, as well as the newly described rabbit HEV. The latter virus appears to be relatively closely related to genotype 3 in our analysis, which is in contrast to the analysis of Zhao *et al.* (2009) classifying it as a novel HEV genotype. Reliable thresholds for the definition of HEV species and genotypes will be needed to clarify the taxonomic relationships within the genus *Hepevirus*.

Some of the HEV genotypes have been suggested to be transmitted zoonotically between animals and humans (Meng, 2009; Purcell & Emerson, 2008). The zoonotic potential of rat HEV is not known so far. The low nucleotide sequence identities between rat HEV and human HEV may indicate an independent evolution of both viruses with no transmission between the two hosts. However, it cannot be excluded that transmission of rat HEV to humans has already occurred but has remained undetected due to the use of inappropriate RT-PCR systems. It can be speculated that the relatively high seroprevalence of HEV-specific antibodies in the human population of developed countries (Christensen *et al.*, 2008; Mansuy *et al.*, 2008), which does not correlate with the low PCR detection rates for the virus, may be caused – at least in part – by so far unknown HEV types including rat HEV. Moreover, this spillover infection might cause a milder course of the infection in humans, explaining the discrepancy between the high seroprevalence and the low number of clinical cases in humans.

Laboratory rats (*R. norvegicus*) are well established for use in various human disease models. For human hepatitis E, HEV infections of monkeys, pigs and chicken have been tested as animal models (Billam *et al.*, 2005; Huang *et al.*, 2007; Pudupakam *et al.*, 2009; Vitral *et al.*, 1998). Recently, HEV infection of BALB/c nude mice carrying a severe T-cell defect was described (Huang *et al.*, 2009). However, there is still a need for a reliable rodent model for studies of HEV pathogenesis, immunology and vaccine development. Further studies should focus on rat HEV infection in laboratory rats to clarify whether this virus infection could serve as a model for human hepatitis E.

METHODS

Samples. In total, of 30 faecal samples of wild Norway rats (*R. norvegicus*) were collected at different places above ground and underground (in the sewage system) in the city of Hamburg, Germany, in the spring of 2007 and 2008. To confirm the host origin, all of the samples were tested by real-time PCR for the presence of DNA of the cytochrome *b* gene of Norway rats. Primers for this real-time PCR were constructed on the basis of an alignment of rodent cytochrome *b* genes, and the specificity of the PCR was demonstrated previously using DNA of different rodent species (data not shown). The original faecal samples were stored at −20 °C for up to 24 months. For further analysis, a 1 : 10 faecal suspension was prepared using PBS and stored at −80 °C. A liver suspension from a wild boar containing HEV genotype 3 strain wbGER27 (Schielke *et al.*, 2009) and a liver suspension from a chicken containing avian HEV strain 05-2294 (Bilic *et al.*, 2009) were centrifuged at 4190 ***g*** for 5 min and the supernatants were stored at −80 °C.

Negative-stain and solid-phase immunoelectron microscopy. The supernatants of the faecal samples were applied to carbon-coated, polioform, 400-mesh copper grids (Plano) for 10 min, fixed with 2.5 % aqueous glutaraldehyde solution for 1 min, stained with 2 % aqueous uranyl acetate solution for 1 min and examined by transmission electron microscopy using a JEM-1010 microscope (JOEL) at 80 kV accelerated voltage.

For immunoelectron microscopy, grids were incubated for 30 min in a solution containing 20 µg protein A (Sigma) ml^{-1}. Thereafter, a human serum, which had tested positive for HEV-specific antibodies using a *recom*Blot HEV IgG immunoblot assay (Mikrogen), was added at a 1 : 50 dilution to the grid for 30 min. The faecal suspension was added for 30 min and then negatively stained and examined as above.

Tissue culture. Three rat cell lines of liver origin were cultivated according to the instructions of the supplier. The suspension cell line N1-S1 (ATCC CRL-1604), originally isolated from a Novikoff hepatoma, was maintained in Iscove's modified Dulbecco's medium supplemented with 4 mM L-glutamine, 1.5 g sodium bicarbonate l^{-1}, 10 % fetal calf serum (FCS) and 0.5 % gentamicin. The adherent cell line clone 9 (ATCC CRL-1439), originally isolated from a normal liver, was cultivated in Ham's F12K medium supplemented with 10 % FCS and 0.5 % gentamicin. The adherent cell line MH1C1 (ATCC CCL-144), originally isolated from a chemically induced hepatoma, was maintained in Ham's F12K medium with 2.0 mM L-glutamine, 1.5 g sodium bicarbonate l^{-1}, 15 % horse serum, 2.5 % FCS and 0.5 % gentamicin. For infection trials, the faecal suspensions were clarified by centrifugation at 4190 ***g*** for 15 min and the supernatants were sequentially filtered through syringe filters (Whatman) with pore sizes of 1.2, 0.45 and 0.2 µm. A hundred microlitres of the purified suspension was used for inoculation onto the cell cultures grown in 24-well plates (Falcon 3047; Becton Dickinson). After 1 h incubation at 37 °C, the suspension was removed from the cells and replaced by growth medium. In some experiments, 0.5 % trypsin/0.2 % EDTA (PAN Biotech) was added to the medium diluted to a final trypsin concentration of 1, 0.125 or 0.125 µg ml^{-1} for N1S1, clone 9 and MH1C1 cells, respectively. These trypsin concentrations corresponded to the highest concentration tolerated by the cells, as determined in previous experiments. Serum was not included in the medium when trypsin was added. Inoculated cells were incubated for 6 days at 37 °C. Thereafter, the cultures were frozen at −20 °C, thawed and the cellular debris removed by low-speed centrifugation. A 100 µl of the supernatant was used for inoculation of fresh cell cultures as above. Further passages of the supernatant were carried out accordingly.

Nested broad-spectrum RT-PCR. Primers for the nested broad-spectrum RT-PCR for the detection of hepeviruses (Table 1) were selected on the basis of an alignment of 22 full-length HEV sequences of genotypes 1–4 derived from humans, pigs and wild boars and one full-length sequence of an avian HEV isolate (GenBank accession numbers are listed in Supplementary Table S1). RNA was isolated

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from the faecal suspensions using a QIAamp Viral RNA Mini kit (Qiagen). For homogenization of the liver samples, QIAshredder columns (Qiagen) were applied prior to RNA extraction using an RNeasy Mini kit (Qiagen). A first RT-PCR was performed using a One-Step RT-PCR kit (Qiagen) with primers HEV-cs and HEV-cas in a 2720 thermal cycler (Applied Biosystems). The thermal profile comprised 42 °C for 60 min and 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 74 °C for 45 s, with a final incubation at 74 °C for 5 min. An aliquot of the RT-PCR product (5 µl) was used in a nested PCR with a TaKaRa ExTaq kit (TaKaRa Bio) and the primers HEV-csn and HEV-casn. The thermal profile consisted of 95 °C for 5 min and 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, with a final incubation at 72 °C for 5 min. Nested PCR products were separated by electrophoresis on ethidium bromide-stained agarose gels.

Amplification and sequencing of rat HEV genome. Primers used for amplification of parts of the rat HEV genome were generated using the sequence alignment mentioned above, and primers were selected with binding sites on the rat HEV sequences obtained in this study. Primer sequences that successfully amplified parts of the rat HEV genome are listed in Supplementary Table S2. RNA isolated from the faecal suspension as described above was used together with a LongRange 2Step RT-PCR kit (Qiagen). Reverse transcription was performed in a 20 µl reaction at 42 °C for 90 min using two primers. PCR was subsequently performed with 5 µl cDNA, applying different thermal profiles depending on the primer sequence and the expected product lengths. For rapid amplification of cDNA ends (RACE) of the 3′ end of the rat HEV genome, reverse transcription was performed using the primer pA1 [5′-CCGAATTCCCGGGATCC$(T)_{17}$V-3′, complementary to the poly(A) tail], followed by PCR with primers 5′-CCGAATTCCCGGGATCC-3′ (binding site on primer pA1) and primer ratORF2-s. PCR products were separated by electrophoresis on ethidium bromide-stained agarose gels. Bands of the expected length were excised, purified using a QIAquick Gel Extraction kit (Qiagen) and subsequently cloned into the vector pCR4-TOPO using a TOPO TA Cloning kit for Sequencing (Invitrogen). The inserts of the plasmids were sequenced using M13 Forward and M13 Reverse primers (Invitrogen), as well as gene-specific primers, in an ABI 3730 DNA Analyser (Applied Biosystems).

Sequence analysis. The sequences of the genome segments were assembled from the determined sequences using the SeqBuilder module of the DNASTAR software package (Lasergene). ORFs were identified and amino acid sequences were deduced from the nucleotide sequences using the same module. Sequence alignments and construction of phylogenetic trees were performed using the MEGALIGN module of the abovementioned software package. The accession numbers of HEV sequences included in the analyses are shown in Supplementary Table S1. The CLUSTAL W method was used with the IUB (nucleotide) or PAM250 (amino acids) residue weight tables (Thompson *et al.*, 1994) in alignments, and bootstrap analysis of phylogenetic trees was performed with 1000 trials and 111 random seeds.

ACKNOWLEDGEMENTS

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Supplementary Table S1. GenBank accession numbers of the nucleotide sequences used in this study

Strain designation	Host origin	Genotype	GenBank accession no.
Hydcrabad	Human	1	AF076239
Madras	Human	1	X99441
Morocco	Human	1	AY230202
SAR-55	Human	1	M80581
Mexico	Human	2	M74506
HE-JA10	Human	3	AB089824
HEV-US1	Human	3	AF060668
HEV-US2	Human	3	AF060669
JDEER-Hyo03L	Wild deer	3	AB189071
JJT-Kan	Human	3	AB091394
JKK-Sap	Human	3	AB074917
JRA1	Human	3	AP003430
JYO-Hyo03L	Human	3	AB189075
Meng	Pig	3	AF082843
swMN06-A1288	Pig	3	AB290312
swMN06-C1056	Pig	3	AB290313
swJ570	Pig	3	AB073912
swX07-E1	Pig	3	EU360977
wbJTS1	Wild boar	3	AB222183
swCH25	Pig	4	AY594199
swCH31	Pig	4	DQ450072
T1	Human	4	AJ272108
GDC9	Rabbit	–	FJ906895
aavUSA aHEV	Avian	–	EF206691

Johne, R., Plenge-Bönig, A., Hess, M., Ulrich, R. G., Reetz, J. and Schielke, A. (2010). Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. *J Gen Virol* **91**, 750–758.

Supplementary Table S2. Primers successfully used for amplification of genome fragments of rat HEV in samples R4 and R8

Primer	Sequence (5′→3′)*	Binding site within the fragment (specificity for sample)
ratHEV-inv-s	GGGGCRCCYGAGTGGATGTGGA	1358–1379 (R4), 302–323 (R8), 2327–2349 (R4)†, 2827–2805 (R4)†
ratORF2as	CTGCCGGCGTAGAATGGAGCCAC	2377–2355 (R4)
ratORF2s	GGAAGAGTCAACCTCAGGGATGT	2776–2798 (R4)
ORF2-s	CCCTTACTGCCTYTKCAGGAYGG	2456–2478 (R4), 1400–1422 (R8)
ORF2-as	GTGGAAGTGATGGAATTCATRTC	2661–2639 (R4), 1605–1583 (R8)
ORF1m-s	CGCCGGGTTGTGATKGAYGAGGC	1–23 (R4)
8-4400-s	GTGGAAGCTGTATCACCTGCTCC	319–341 (R8)
8-5650-as	GGCCTGCACTCGGTACTGGGCA	2539–2518 (R4), 1483–1462 (R8)
8-4450-s‡	TGCTGCAGGCTCCCCAAGAGAG	354–375 (R8)
8-5600-as‡	GCTTCAGTCGCCATGATATGCGTG	2508–2485 (R4), 1452–1429 (R8)

*K=G or T, R=A or G, Y=C or T.

†Primer binding due to mis-priming.

‡Used as nested PCR primer.

Johne, R., Plenge-Bönig, A., Hess, M., Ulrich, R. G., Reetz, J. and Schielke, A. (2010). Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. *J Gen Virol* **91**, 750–758.

5.5 Summary of Paper 4

Novel Hepatitis E Virus Genotype in Norway Rats, Germany

Reimar Johne; Gerald Heckel; Anita Plenge-Bönig; Eveline Kindler; Christina Maresch; Jochen Reetz, Anika Schielke and Rainer G. Ulrich

Emerging Infectious Diseases 2010, 16:1452-1455

The detection of a novel HEV-like virus in the faeces of wild Norway rats from Hamburg reported in the previous paper was motivating to screen six Norway rats trapped in manholes of the sewer system in Hamburg, Germany, at the same location, where rat HEV had been detected before. Initial necropsy and serology with an HEV GT1-based ELISA indicated no morphological abnormalities or HEV-reactive antibodies. Nevertheless, screening of the liver samples using the previously developed nested broad-spectrum RT-PCR revealed two samples as positive for HEV-like viruses (no. 63 and no. 68). For these two samples the whole genome sequences were obtained by RT-PCR with degenerated primers, which enabled a detailed characterization of the genomes and reliable phylogenetic analyses. The whole genome sequences consist of 6945 nt and 6948 nt, respectively. The genomic organization of rat HEV is typical for HEV but three additional putative ORFs of 280 to 600 nt were predicted. However, further experimental proof of these additional ORFs is needed. Phylogenetic trees based on the alignment of different parts of the genome or the whole genome sequence revealed all a clear separation of rat HEV from the mammalian and avian HEV strains. In addition, a real time RT-PCR specific for the ORF2 of rat HEV was developed and used to determine the viral load in different organs and the blood of the positive rats. The results of the real time RT-PCR as well as immunohistochemical analysis revealed a hepatotropism of rat HEV, which is comparable to mammalian and avian HEV. It can be concluded that rat HEV represents a novel HEV genotype within the genus *Hepevirus*.

5.6 Key Messages of Paper 4

- **Detection of further HEV-like viruses in rats**
- **Two whole genome sequences of rat HEV**
- **Rat HEV probably represents a novel HEV genotype**
- **Hepatotropism of rat HEV**

5.7 Own contribution to Paper 4

I performed RNA extraction from different rat organs and blood samples. I developed the real time RT-PCR for rat HEV and applied it to the samples. I participated in cloning and sequencing of the whole genome sequences. Additionally, I wrote parts of the article and helped by critical reading of the whole manuscript.

5.8 Paper 4

DISPATCHES

Novel Hepatitis E Virus Genotype in Norway Rats, Germany

Reimar Johne, Gerald Heckel, Anita Plenge-Bönig, Eveline Kindler, Christina Maresch, Jochen Reetz, Anika Schielke, and Rainer G. Ulrich

Human hepatitis E virus infections may be caused by zoonotic transmission of virus genotypes 3 and 4. To determine whether rodents are a reservoir, we analyzed the complete nucleotide sequence of a hepatitis E–like virus from 2 Norway rats in Germany. The sequence suggests a separate genotype for this hepatotropic virus.

Hepatitis E virus (HEV) is a nonenveloped virus, diameter 30–34 nm, that belongs to the genus *Hepevirus*. Its single-stranded, positive-polarity RNA genome of 6.6–7.3 kb harbors 3 major open reading frames (ORFs) flanked by a capped 5′ end and a poly A at the 3′ end. ORF1 at the 5′ end of the genome codes for several nonstructural proteins, ORF2 encodes the immunodominant capsid protein, and the partially overlapping ORF3 codes for a cytoskeleton-associated phosphoprotein with multiple functions (*1*).

Hepatitis E, an acute self-limiting disease, occurs worldwide; large outbreaks have occurred in developing countries, as was recently reported from Uganda (*2*). Initially, hepatitis E was believed to be endemic only to developing countries in Asia, Africa, and Central America, but recent studies have demonstrated autochthonous infections in industrialized countries (Europe, Japan) (*3*). In contrast to the fecal–oral transmission of HEV that occurs in developing countries, it is suspected that these human infections result from zoonotic transmission of HEV genotypes 3 and 4; domestic pigs, wild boars, and deer represent major reservoir hosts (*1*,*4*). However, rodents, especially commensal rodents, may represent an additional HEV reservoir and may play a role in the epidemiology of hepatitis E. HEV-reactive antibodies have been detected in several rat species (*Rattus norvegicus*, *R. rattus, R. exulans)* but also in some noncommensal wild rodent species (*5*–*8*). By using broad-spectrum, nested, reverse transcription–PCR (RT-PCR), we recently detected HEV-like sequences in fecal samples of Norway rats (*R. norvegicus*) trapped as part of the Rodent-borne Pathogens network (which coordinates activities with regard to rodent trapping during outbreaks) (*9*,*10*). These sequence fragments had high nucleotide sequence divergence to genotypes 1–4 and to avian HEV strains.

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The Study

During July 8–16, 2009, a total of 6 Norway rats, 3 male and 3 female, 65–432 g, were trapped in manholes of the sewer system of Hamburg, northern Germany, at the same locations where ≈12 months before HEV RNA had been detected in rat feces (*10*). Standardized necropsy (*9*) found no morphologic abnormalities. Initial serologic screening with a commercial genotype 1–based ELISA (Axiom, Bürstadt, Germany) detected no reactive antibod-

Table. Nucleotide and deduced amino acid sequence identities between human, rabbit, and avian HEV strains compared with HEV isolated from 2 Norway rats, Germany, July 2009*

Strain, GenBank accession no.	Rat no., GenBank accession no.							
	63, GU345042				68, GU345043			
	Genome, nt	ORF1, aa	ORF2, aa	ORF3, aa	Genome, nt	ORF1, aa	ORF2, aa	ORF3, aa
Genotype 1, F076239	55.9	47.6	56.2	27.5	55.7	47.4	56.4	30.4
Genotype 2, M74506	55.3	48.7	55.4	28.4	55.2	48.6	55.5	29.4
Genotype 3, F060668	55.7	48.0	57.2	24.8	55.7	47.7	57.3	26.7
Genotype 4, J272108	55.5	48.2	55.9	27.5	55.3	47.8	56.1	26.5
Rabbit HEV, J906895	55.1	48.7	56.7	23.5	55.1	48.6	56.8	25.5
Avian HEV/Hungary, AM943646	50.2	46.5	45.9	26.9	49.9	46.4	46.3	26.9
Avian HEV/Australia, AM943647	49.9	46.6	46.1	26.9	49.3	46.5	46.5	26.9
Avian HEV/USA, AY535004	49.5	46.7	46.1	26.9	49.8	46.7	46.5	26.9

*HEV, hepatitis E virus; ORF, open reading frame.

ies in transudates of any of the 6 rats. Liver RNA from 1 female (no. 68, 311 g) and 1 male (no. 63, 313 g) rat yielded an amplification product of the expected size (331–334 nt) and a sequence identity of 83.8%–94.6 % with the HEV sequences recently obtained from rat feces (data not shown). Using a strategy according to Schielke et al. (*4*), we determined the entire rat HEV genome sequences from each sample to be 6,945 nt and 6,948 nt; the sequences differed by an insertion–deletion polymorphism in the 3′ noncoding region. The sequence identity between each complete sequence was 95.3% and reached 55.1%–55.9% to HEV genotypes 1–4 and 49.3%–50.2% to avian HEV strains (Table). Using prediction software, we identified the major ORFs 1, 2, and 3 in the new genomes in an organization typical for HEV (Figure 1, panel A). In contrast to HEV genotypes 1–3, rat HEV ORFs 1 and 3 do not overlap. Three additional putative ORFs of 280–600 nt that overlap with ORFs 1 or 2 were predicted for each rat HEV genome (Figure 1, panel A). However, before the meaning of these findings can be verified, sequence information from additional rat HEV strains and experimental proof are needed. Phylogenetic analyses of a 1,576-nt segment available for all published rat HEV sequences demonstrated clear separation from mammalian genotypes 1–4 and avian strains (Figure 1, panel B). The same 3 phylogenetic clusters were obtained when the complete genomes were analyzed (Figure 1, panel C) and when the nucleotide and deduced amino acid sequences of ORF1, ORF2, and ORF3 were investigated separately (data not shown).

To compare viral load in different tissues of the 2 HEV–positive rats, we developed a real-time RT-PCR selective for a region in the ORF2 of rat HEV. Parallel analysis of RNA isolated from 10 mg of each tissue or 10 µL of blood reproducibly showed the highest viral load to be in the liver; cycle threshold values for liver were 20.5 and 21.6 for each animal and lower for all other tissues (online Appendix Figure, www.cdc.gov/EID/content/16/9/1452-appF.htm). Further, immunohistochemical analysis, using anti-HEV serum, detected viral antigen in the cytoplasm of a few hepatocytes from each HEV-positive rat. Antigen

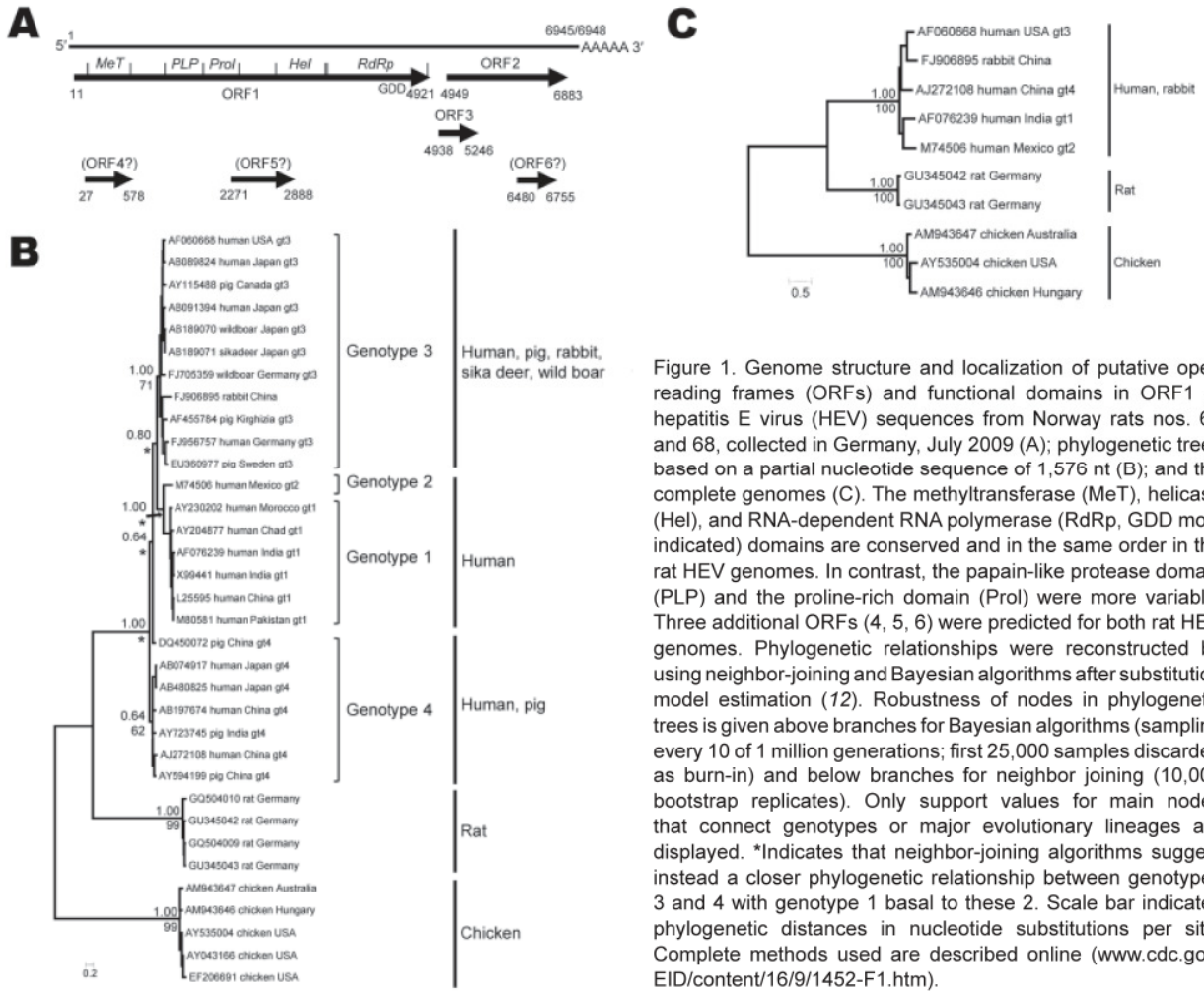

Figure 1. Genome structure and localization of putative open reading frames (ORFs) and functional domains in ORF1 of hepatitis E virus (HEV) sequences from Norway rats nos. 63 and 68, collected in Germany, July 2009 (A); phylogenetic trees based on a partial nucleotide sequence of 1,576 nt (B); and the complete genomes (C). The methyltransferase (MeT), helicase (Hel), and RNA-dependent RNA polymerase (RdRp, GDD motif indicated) domains are conserved and in the same order in the rat HEV genomes. In contrast, the papain-like protease domain (PLP) and the proline-rich domain (Prol) were more variable. Three additional ORFs (4, 5, 6) were predicted for both rat HEV genomes. Phylogenetic relationships were reconstructed by using neighbor-joining and Bayesian algorithms after substitution model estimation (*12*). Robustness of nodes in phylogenetic trees is given above branches for Bayesian algorithms (sampling every 10 of 1 million generations; first 25,000 samples discarded as burn-in) and below branches for neighbor joining (10,000 bootstrap replicates). Only support values for main nodes that connect genotypes or major evolutionary lineages are displayed. *Indicates that neighbor-joining algorithms suggest instead a closer phylogenetic relationship between genotypes 3 and 4 with genotype 1 basal to these 2. Scale bar indicates phylogenetic distances in nucleotide substitutions per site. Complete methods used are described online (www.cdc.gov/EID/content/16/9/1452-F1.htm).

was also observed in some activated hepatic stellate cells (Figure 2). Hematoxylin-eosin staining showed a marginally increased number of monocytes and granulocytes in sinusoids as well as a moderately increased number of lymphocytes and plasma cells in some Glisson triads of the livers (data not shown).

Conclusions

Phylogenetic analyses and nucleotide and amino acid sequence comparisons demonstrated that the complete rat HEV genome sequences were consistently well separated from those of mammalian genotypes 1–4 and the tentative avian genotype. This finding suggests that these sequences represent an additional genotype (Figure 1, Table). In our analyses, the recently described HEV strain found in domestic rabbits, proposed to represent a separate genotype (*13*), clustered with human HEV genotypes irrespective of the genome part, nucleotide, or deduced amino acid sequences analyzed (Figure 1, panels B, C, and data not shown). Therefore, this strain may represent the consequence of recent spillover rather than the result of long-term virus–host coevolution. In contrast, the nonzoonotic avian HEV strains strongly differ from the mammalian HEV genotypes 1–4 (Figure 1, panels B, C). Although in the genus *Hepevirus* no species demarcation criteria have been defined, the marked sequence diversities suggest that the rat HEV represents an additional virus species other than HEV-1, HEV-2, HEV-3, HEV-4, and the tentative species avian HEV, which are currently classified in this genus (*14*).

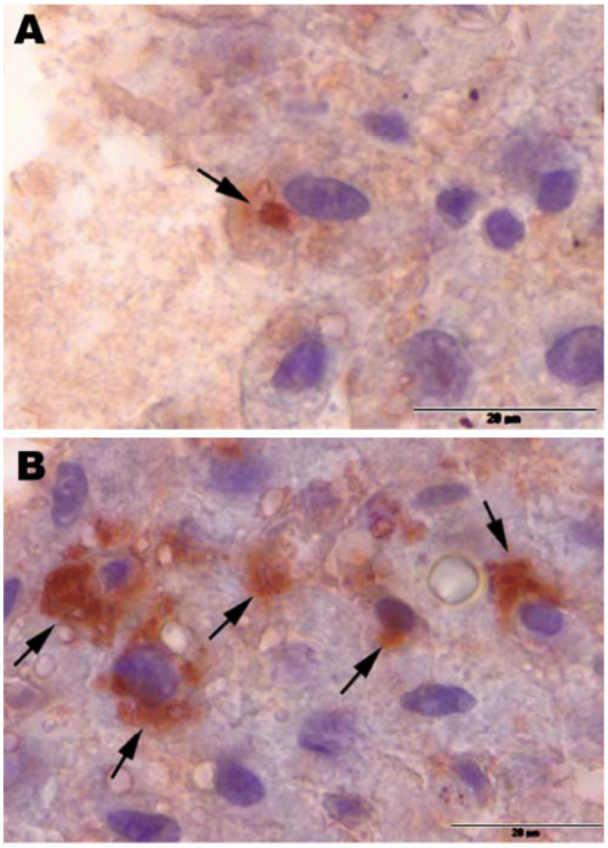

Figure 2. Immunohistochemical staining (peroxidase-antiperoxidase (PAP) technique) of liver samples from 2 rat-hepatitis E virus (HEV)–positive Norway rats from Germany, July 2009. Arrows indicate immunohistochemical positive reactions in the cytoplasm of single hepatocytes (A) and in a few foci in hepatocytes and stellate cells (B). For PAP staining, deparaffinized slides of liver samples were incubated with anti-HEV–positive human serum, which had been previously used to detect rat HEV by using solid phase immunoelectron microscopy (*10*), for 1 h at 37°C with protein A (Sigma-Aldrich, Steinheim, Germany) at a dilution of 1:100 for 45 min at 37°C and finally with PAP complexes from rabbits (Sigma, St. Louis, MO, USA) at a dilution 1:200 for 45 min at 37°C. AEC (3-amino-9-ethylcarbazol; Sigma Chemie GmbH, Deisenhofen, Germany) was used as the substrate chromogene. The slides were counterstained with hematoxylin and subsequently analyzed by light microscopy. Scale bars = 20 µm.

Detection of rat HEV RNA and antigen in the liver cells of the infected Norway rats may indicate hepatotropism of this virus. Therefore, regarding its organ and cell-type tropism, this virus seems to be similar to the human and pig HEV genotypes (*15*). Because the virus was also detected in the intestine and, in the previous study, in feces (*10*), fecal–oral transmission as for genotypes 1–4 is plausible. The common properties of this virus and the human HEV genotypes suggest the usefulness of developing an HEV model in laboratory rats. In addition, the detection of rat HEV in animals from an urban region in Germany raises questions about the putative epidemiologic role of rat HEV for hepatitis E in humans.

Acknowledgments

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Dr Johne is a senior scientist at the Federal Institute for Risk Assessment, Berlin. His research interests are the epidemiology, molecular evolution, and pathogenesis of food-borne viruses.

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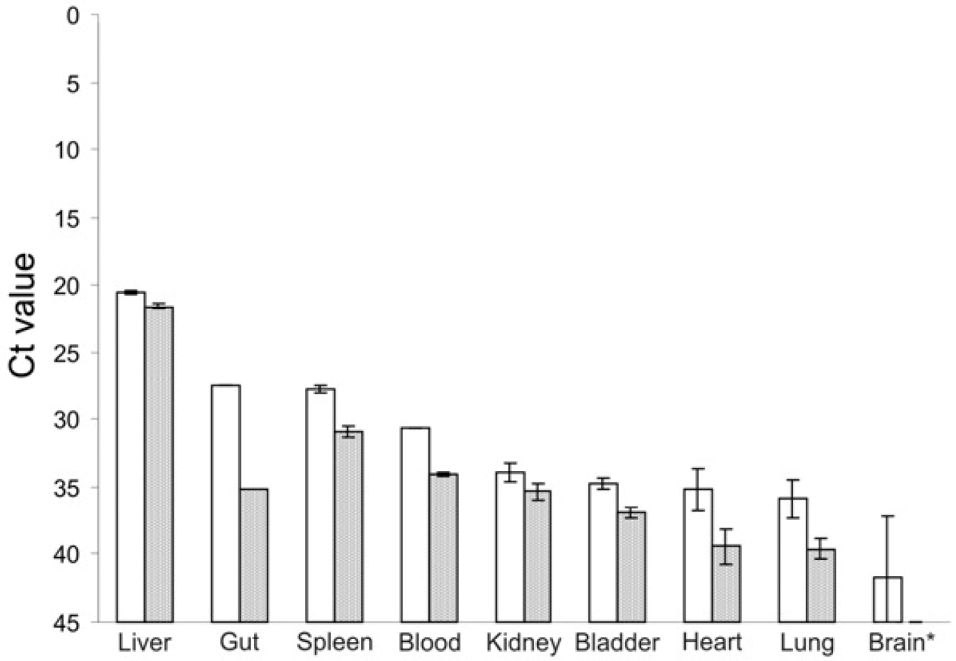

Appendix Figure Results of real-time reverse transcription–PCR showing cycle threshold (Ct) values of viral load in different tissues of Norway rats nos. 63 (white bars) and 68 (gray bars). For RNA isolation, 10 mg of tissue or 10 µL of blood were homogenized and used. The PCR was selective for a region in the open reading frame 2 of rat hepatitis E virus (rHEV) and was based on primers rHEV-forward (5′-TACCCGATGCCGGGCAGT-3′) and rHEV-reverse (5′-ATCCACATCTGGGACAGG-3′) and probe (5′-6FAM-AATGACAGCACAGGCACC-BBQ-3′). Error bars indicate SD. *Brain sample from rat no. 63 contained blood.

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*HEV, hepatitis E virus; ORF, open reading frame.

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6 General Discussion

6.1 Background

In Germany, hepatitis E is a notifiable disease since 2001. Since then, an increase of the reported hepatitis E cases is apparent (see Introduction, Figure 7). In the beginning, most cases were registered as travel-associated. However, in 2007 about 50% of hepatitis E cases were supposed to be autochthonous and nowadays more than two-thirds of the notified cases have been acquired in Germany (RKI, 2010). Although the exact route of transmission for these autochthonous cases has still to be elucidated, a zoonotic transmission seems to be likely. Several HEV animal reservoirs are known (Meng, 2010a). For pigs, wild boars and deer a zoonotic food-borne transmission from animals to humans has been repeatedly reported (Colson et al., 2010; Masuda et al., 2005; Matsuda et al., 2003; Tei et al., 2003). However, HEV RNA and HEV-specific antibodies have also been detected in many other animal species, such as chickens, rodents and others (for details see Introduction). In Germany, HEV is present in the wild boar population since at least 1995 (Kaci et al., 2008). In addition, an epidemiological study in Germany revealed the consumption of offal and wild boar meat as risk factors for an autochthonous HEV infection (Wichmann et al., 2008). However, detailed data about the presence of HEV in wild or domestic animals in Germany were not available at the beginning of these studies.

These facts gave reason to investigate the presence and prevalence of HEV in German wild as well as domestic animals, which are considered as HEV reservoirs. Thus, wild boars, domestic pigs and wild rats were investigated for the presence of HEV RNA or HEV-specific antibodies. A genomic characterization of the animal HEV strains detected in Germany, detailed phylogenetic analyses as well as comparison of human HEV strains with endemic animal HEV strains should reveal zoonotic transmission routes. These data may help to assess the epidemiological role of the HEV animal reservoirs in Germany and thus represent a basis for decisions on required counter measures.

6.2 Animal reservoirs of HEV in Germany

6.2.1 Prevalence of HEV in German wild boars

Wild boars are known as reservoir for HEV and several human hepatitis E cases can be linked to the consumption of undercooked or raw wild boar meat (Li et al., 2005; Masuda et al., 2005; Matsuda et al., 2003). In Germany, an epidemiological study also identified the consumption of wild boar meat as one risk factor for hepatitis E (Wichmann et al., 2008). The existence of HEV in the German wild boar population was first shown in 2008 when Kaci et al. (2008) detected HEV in 5.3% of wild boar sera collected in Mecklenburg-Western Pomerania in 1995/1996 using RT-PCR. Due to the long storage time of these sera and the lack of testing of tissue samples, the HEV prevalence may be underestimated in this study. These facts gave reason for a more detailed investigation concerning the HEV prevalence in the German wild boar population.

Therefore, liver samples from wild boars originating from the federal states Brandenburg and Thuringia and the cities of Berlin and Potsdam collected between 2005 and 2006 were screened for HEV RNA using real time RT-PCR. The livers were chosen as sampling material due to the known hepatotropism of HEV (Lee et al., 2009). Out of these 148 samples, 22 specimens were detected as positive for HEV, which resulted in an average detection rate of 14.9%. However, in the urban regions of the cities Berlin and Potsdam, the prevalence of HEV was significantly ($p < 0.001$) lower (4.1%) than in the rural regions of Brandenburg and Thuringia (25.9% and 23.8%, respectively).

In another study from Germany, which has also been published in 2009, an average HEV prevalence of 68.2% in wild boars is reported (Adlhoch et al., 2009). Liver, bile as well as serum samples originated from wild boars from Baden-Württemberg, Brandenburg, Brandenburg/Saxony and Rhineland Palatine in this study. The HEV prevalence ranged from 22% in Baden-Württemberg to 100% in Brandenburg.

In other European countries, HEV prevalence rates determined in wild boars range from 2.5% in France to 25% in Italy (de Deus et al., 2008a; Kaba et al., 2010; Martelli

et al, 2008; Reuter et al., 2009; Rutjes et al., 2010; Rutjes et al., 2009). Seroprevalence rates of HEV-specific antibodies in wild boars were reported from the Netherlands with 12% and Spain with 42.7% (de Deus et al., 2008a; Rutjes et al., 2010). In Germany, an average seroprevalence of 26.2% to 29.9% was determined depending on the immunoassay (Adlhoch et al., 2009).

In Japan, where several food-borne hepatitis E cases though the consumption of undercooked wild boar meat have been reported, the HEV prevalence ranges from 1.1% to 42.9% (Michitaka et al., 2007; Nishizawa et al., 2005; Sakano et al., 2009; Takahashi et al., 2004) and the HEV seroprevalence is found between 4.5% and 71.4% (Michitaka et al., 2007; Sakano et al., 2009).

Comparing these prevalence data it becomes obvious that there are large differences in the prevalence rates not only between different countries but also within the same country. The reasons for these differences may be diverse and are not always clear. Definitely, the use of different test systems, like different PCR assays or immunoassays, has a great influence on the results (see sections 6.3.1 & 6.3.2). The storage of the sampling material but also the sample material itself may play a crucial role in the resulting prevalence rate. For instance, Adlhoch et al. (2009) reported that some animals are tested positive for HEV RNA in serum and bile but no virus was detectable in the liver of the same animals. In contrast, in Brandenburg only one animal was found to be positive for HEV when screening the serum samples but 100% of the same animals were HEV positive when examining their livers, which were sampled at the same time (Adlhoch et al., 2009). Furthermore, the selection of animals may cause differences in prevalence rates. All of the wild boars from Brandenburg tested by Adlhoch et al. (2009) were derived from a single hunting event (C. Adlhoch, personal communication), thus only one time point in a very restricted area has been investigated. In our study, the tested animals from Brandenburg originated from different locations and time points.

Another possible explanation for varying prevalence rates could be differences between distinct geographical regions. In our study, we show that in rural regions in Germany significantly more wild boars are infected with HEV compared to urban regions. However, the reasons why the regional conditions might influence the

prevalence rate are currently not understood. One possible explanation might be a change in the social behaviour of the wild boars in urban areas with fewer contacts among each other resulting in a reduced chance to infect the whole sounder. This is the first study, which differentiates between HEV prevalence rates detected in wild boars from urban or rural areas. However, studies in Korea and Malaysia revealed that HEV is mainly found in human populations from rural areas (Ahn et al., 2005; Seow et al., 1999). Thus, a detailed description of the sampling location might be necessary in future studies and may explain some deviation in prevalence rates. Comparison of animal and human data with respect to a distinct area may also help to identify transmission events.

In both studies from Germany, HEV could be detected in all age groups of the wild boars and no age-dependency was evident (Adlhoch et al., 2009). The fact that all age groups are affected equally implies an increased risk for human infection as all shot animals have to be considered to contain HEV with the same frequency. In contrast, domestic pigs are mainly infected early in life resulting in an increasing HEV antibody prevalence with age of the pigs (Pavio et al., 2010). Therefore, at the time of slaughter, most pigs have already cleared the infection and do no longer contain infectious viruses. The reasons for these differences between wild boars and domestic pigs are not known so far, but it could be speculated that the keeping of large herds of domestic pigs with the same age may contribute to an early HEV infection.

The prevalence rate of 14.9% determined in this study demonstrates that Germany's situation is compareable to other countries in Europe and worldwide concerning the prevalence of HEV in wild boars (Reuter et al., 2009; Rutjes et al., 2010; Rutjes et al., 2009; Sakano et al., 2009). Since HEV was found in all age groups and all geographical regions investigated, the virus seems to be enzootic in the German wild boar population. It can be concluded that wild boars are a main reservoir for HEV in Germany. As a consequence, hunters and other people with contact to wild boars represent a definite risk group and protective measures may be necessary when handling raw wild boar meat. To which extent cooked wild boar meat may contain infectious HEV is dependent on virus concentration and the thermal stability of HEV. Some publications report that HEV is nearly completely inactivated at temperatures

between 56°C and 60°C applied for one hour (Emerson et al., 2005b; Huang et al., 1999), whereas other reports mention that temperatures of 70°C to 95°C are necessary for complete inactivation (Feagins et al., 2008; Tanaka et al., 2007). Thus, further experiments concerning the heat stability of HEV are needed.

6.2.2 Prevalence of HEV in domestic pigs in Germany

HEV has been suggested to be enzootic in domestic pigs worldwide (Lewis et al., 2010). However, for Germany no data about the prevalence of HEV in the domestic pig population were available. Therefore, an investigation of the German pig livestock was initiated. In contrast to the investigation of the German wild boars, in which the presence of the HEV genome was tested, the sera of the domestic pigs were tested for the presence of HEV-specific antibodies enabling a more indirect estimation of the HEV distribution. In addition, different serological assays were subsequently compared with each other. The conducted study shows that HEV is widespread in the German pig population. The investigation of 1072 sera using the TiHo-ELISA resulted in a seroprevalence of 49.8%. HEV-specific antibodies were found in all age groups but piglets showed the lowest seroprevalence. However, if subsets of serum samples were retested, the seroprevalences ranged from 21.7% to 64.8% depending on the used immunoassay. The influence of the used immunoassay on seroprevalence rates is discussed in section 6.3.1.

Once HEV is present in a herd it usually easily spreads from pig to pig, which can be measured by the basic reproduction ratio (R_0) (Bouwknegt et al., 2008b). In a recent study from the Netherlands, R_0 has been estimated to be 8.8 for contact-exposure, which means that one HEV infected pig may infect about 9 other pigs in the same stable (Bouwknegt et al., 2008b). Thus, the seroprevalence is also dependent on the number of samples collected at the same farm. The lower prevalence of HEV-specific antibodies in the sera of the piglets may be explained by a decline of maternal antibodies after 4 to 8 weeks before new antibodies are rising due to HEV infection. Piglets normally show an HEV infection between 8 to 9 weeks of age and the IgG response evolves later (Pavio et al., 2010).

The prevalence rates determined in this study are comparable to observations in other European countries and worldwide (Lewis et al., 2010). In general, the HEV prevalence in pigs is high, both in endemic as well as in non-endemic regions worldwide (Banks et al., 2004; Blacksell et al., 2007; Jiang et al., 2008; Seminati et al., 2008; Shukla et al., 2007; Yu et al., 2009). In Europe, an HEV prevalence as high as 98% have been reported for pig herds in Spain (Casas et al., 2009a; Seminati et al., 2008). In other European countries such as the United Kingdom, Sweden, France, the Netherlands and Italy prevalence rates are also high and range from 22% in the Netherlands to 85.5% in the United Kingdom (Banks et al., 2004; de Deus et al., 2008b; Martelli et al., 2010; Rutjes et al., 2007). Interestingly, from developing countries also high HEV prevalence rates in the pig populations have been reported, although the faecal-oral route seems to play the major role as transmission route as revealed by the predominant detection of HEV GT1 in infected humans in these countries (Shukla et al., 2007; Tian et al., 2009; Yan et al., 2007; Yu et al., 2009; Zhang et al., 2008).

This study reveals the importance of domestic pigs as HEV reservoir in Germany. However, further studies are needed as basis for a reliable risk assessment concerning pig meat as transmission route of HEV in Germany. This should include PCR testing of pigs and control measures before the meat is distributed.

6.2.3 Prevalence of HEV in rats in Germany

The existence of HEV-like viruses in rodents was suggested for a long time but could not be proven by the detection of the HEV genome in rodents. In our study, using a nested broad-spectrum RT-PCR, HEV-like viruses designated as rat HEV could be detected for the first time in rodents. In total, 36 samples (faeces and liver) of wild Norway rats (*Rattus norvegicus*) originating from Hamburg, Germany, were screened for the presence of HEV RNA using the novel assay, which resulted in an average detection rate of 11.1%. When investigating the faecal samples a prevalence rate of 6.7% (2/30) has been found. In contrast, a prevalence rate of 33.3% (2/6) has been determined when the livers were used for RNA extraction. The time the faecal samples were exposed to the environment is not known. Thus, some of the HEV RNA may have already been degraded before processing these samples. A

screening of further rats for the presence of rat HEV is necessary to obtain a reliable prevalence rate of this virus in wild rats. As no other prevalence rates of HEV genomes are currently available, comparison to rodent data of other countries are difficult. Nevertheless, in Russia 5 of 23 rodents (21.7%) have been found to be positive for HEV antigens using immune electron microscopy (Karetnyi et al., 1993). Different seroprevalences are reported for rodents from several other countries. Especially in the USA, very high seroprevalences are detected ranging from 33% to 90% (Easterbrook et al., 2007; Favorov et al., 2000; Kabrane-Lazizi et al., 1999) and also for Vietnam, India, Japan and Brazil different HEV seroprevalences from 2.1% to 50% are reported (Arankalle et al., 2001; Meng et al., 2002; Hirano et al., 2003; Vitral et al., 2005). As all of these seroprevalences have been determined using HEV GT1 as antigen, it is not clear whether only rat HEV-specific antibodies have been measured. Further studies should be performed using rat HEV antigens. Additionally, serological cross-reactivity of rat HEV and human HEV should be investigated to enable interpretation of HEV seroprevalence in rodents.

Due to this study, the existence of rat HEV has been proven in Germany. However, a further screening of rodents using the broad-reactive nested RT-PCR or rat HEV-specific tests is necessary to attain reliable results concerning the prevalence rate for Germany and other countries. Testing of other areas in Germany and other rodents than rats should help to assess distribution and variability of HEV-like viruses in rodents. The obtained data may also help to estimate the role of rat HEV for the epidemiology of human hepatitis E.

6.3 Development of diagnostic tools

6.3.1 Comparison of serological assays for pigs

Only one serotype has been described for HEV by now (Anderson et al., 1999; Guo et al., 2006). Although the avian HEVs show some distinct epitopes, all *Hepeviruses* share also common epitopes (Guo et al., 2008; Guo et al., 2006; Haqshenas et al., 2002). However, there is no immunoassay, which serves as “gold standard” for the detection of HEV-specific antibodies. Since a standardized test system does not exist, it is difficult to constitute specimens as truly positive or truly negative for the

presence of HEV-specific antibodies. In addition, most commercially available test kits are specified for the detection of human HEV-specific antibodies and have not been tested for pig sera. The use of different antigenic peptides and different HEV genotypes as antigens further complicates comparison of results.

The primary aim of the present study was to estimate the HEV seroprevalence in domestic pigs in Germany. However, in order to obtain reliable results, three different immunoassays had to be used for the detection of HEV-specific antibodies and the resulting seroprevalence data were subsequently compared to each other. The used test systems included two ELISAs, a commercial (AXIOM ELISA) and an assay developed at the TiHo Hannover (TiHo ELISA) and one commercial immunoblot (recomBlot). Although all three assays revealed a high HEV seroprevalence in German domestic pigs (see section 6.2.2) a high inter-assay discordance between the results has been observed.

Discordance between different immunoassays has been reported previously, which may be traced back to several factors (Bouwknegt et al., 2008a; Ghabrah et al., 1998; Mast et al., 1998). First, the class of immunoglobulin, which can be detected by the assay, has great impact on the results. The AXIOM ELISA is capable to detect antibodies of all classes in contrast to both other used immunoassays, which are only capable to detect IgG. During infection, IgM could be usually detected early and transiently, whereas IgG is rising later but is long-lasting (Pavio et al., 2010). This fact may explain that the highest seroprevalence determined in this study was derived using the AXIOM ELISA detecting IgG as well as IgM and IgA, which is also determined to be a suitable marker for HEV viremia (de Deus et al., 2008b; Takahashi et al., 2005).

Second, the kind of antigen used may influence the result. All three immunoassays are based on a human HEV GT1 antigen. Since a high seroprevalence has been determined using these antigens and only one serotype is described by now, the choice of the genotype seems to play only a minor role (Engle et al., 2002; Meng et al., 2001). However, the immunoassays use antigens of different size. The TiHo ELISA is based upon a small ORF2-derived fusion protein of 59 aa, the AXIOM ELISA is based upon an ORF2 protein derivative of 212 aa and the recomBlot is

based on four overlapping polyproteins covering the whole ORF2 (660 aa) and the entire ORF3 protein (123 aa). In general, larger antigens are found to be more sensitive for detecting their respective antibodies (Ghabrah et al., 1998; Mast et al., 1998). Full-length recombinant proteins expressed from ORF2 may be less suitable as antigens due to their diminished solubility and thus truncated versions of the capsid protein may even be more sensitive in detecting HEV-specific antibodies (McAtee et al., 1996). The use of the ORF3 protein as antigen is questionable since tests based upon antigens derived from ORF3 are found to be less sensitive than assays based upon expressed ORF2 (Ghabrah et al., 1998). Possible reasons for the reduced seroreactivity may be a less vigorous immune response to this small protein or a shorter half-life of antibodies specific for ORF3 protein (Ghabrah et al., 1998). Additionally, different expression systems are discussed to influence the seroreactivity as well (Mast et al., 1998).
Third, both ELISAs use the C-terminus of the capsid in its native form, whereas the immunoblot is based on its denatured form as antigen, which might reduce some antibody-antigen interaction.

As discussed in section 6.2.2, the HEV seroprevalence varies greatly in Europe, which might be also explained by the use of different immunoassays (Banks et al., 2004; Casas et al., 2009a; Rutjes et al., 2007). Hence, for comparison of seroprevalences in different populations, regions or countries a standardized test system is of great importance. Validation of such a test system would also require the availability of standardized sera, which may be produced by experimental infection of pigs under controlled conditions.

6.3.2 Development of a broad-reactive PCR for detection of HEV-related agents

Since no efficient cell culture system exists so far, the detection of HEV is mainly dependent on molecular methods, such as PCR-based detection methods. However, the extent of genomic heterogeneity of HEV complicates the detection of unknown strains due to the use of specific primer sequences (Gyarmati et al., 2007). Therefore, a nested broad-spectrum RT-PCR for the simultaneous detection of all known HEV genotypes including avian HEV was developed in the present study. Degenerated primer pairs were selected on the basis of an alignment of 22 full-length

genome sequences of HEV originating from humans, pigs, wild boars and chickens, which revealed a highly conserved region within the gene coding for the RNA-dependent RNA polymerase within ORF1. The application of a nested RT-PCR protocol is useful for increased sensitivity and specificity of the assay (Enouf et al., 2006; Huang et al., 2002a; Jothikumar et al., 2006; Meng et al., 1997; Zhao et al., 2010). Sequencing of the PCR product thereafter allows the detailed analysis of phylogeny and epidemiology of the virus. As the primer sequences are highly degenerated, non-specific PCR products have to be expected. Therefore, sequencing is absolutely necessary to confirm HEV detection, which makes the application of the method relatively labour-intensive.

Finally, this broad-reactive PCR assay succeeded in the detection of a new genotype tentatively called rat HEV, which can be clearly separated from mammalian and avian HEV strains. Sequence alignments reveal only limited sequence identity of rat HEV to the other known genotypes, thus other HEV-specific PCR assays could not detect this virus. This might be the reason for its delayed discovery although the presence of an HEV-like virus in rodents has already been suggested since the 1990s (Karetnyi et al., 1993).

The presence of additional currently unknown HEV-like viruses in other animal species seems to be likely since antibodies against HEV have also been detected in other animals (see Introduction). The application of the broad-spectrum RT-PCR assay may help to identify these viruses. However, it has to be taken into consideration that not all HEV-like viruses might be detected by this assay as even single nucleotide exchanges can inhibit a PCR. Assays targeting other conserved regions of the HEV genome should be developed in order to increase the chance of a broad HEV detection.

6.4 Characterization of HEV strains of animal reservoirs in Germany

6.4.1 Genomic characterization of wild boar HEV strains from Germany

In the conducted study, HEV has been detected in 14.9% of German wild boars. Genotyping was possible for 14 out of the 22 HEV positive liver samples and phylogenetic analyses revealed the presence of the genotypes 3a, 3c, 3h and 3i. The HEV sequences clustered according to their geographical origin, which has also been shown for swine HEV strains before (Huang et al., 2002a). The genotypes 3a and 3c have been detected in Berlin, genotype 3i in Brandenburg and genotype 3h in Thuringia. Adlhoch et al. found genotypes 3e and 3f at the border of Brandenburg and Saxony, whereas genotype 3h has been found in Baden-Württemberg and 3i in Brandenburg and Rhineland Palatine (Adlhoch et al., 2009). The detection of different HEV subtypes in different areas supports the hypothesis that a long-term evolution of HEV has taken place in separated regions. The finding of genotype 3i in samples from Brandenburg from different time points in two independent studies further supports this hypothesis and confirms the suitability of the genotyping method.

The whole genome of the HEV strain wbGER27 was therefore sequenced. The HEV strain wbGER27 may be claimed as the first full-length sequence of HEV GT3i showing the highest sequence identity with an HEV strain from a pig in Mongolia. This surprising result has to be discussed carefully as only a few GT3 full-length sequences are available so far. However, intensive contacts including pig trade cultivated between Eastern Germany and Mongolia in former times may be an explanation. For comparison of human and wild boar HEV strains from Germany three sequences of wild boar HEV were compared with human HEV strains from autochthonous cases in Germany. The GT3i wild boar HEV strain wbGER27 showed 97.9% sequence identity on nucleotide level compared to a human HEV strain.

Interestingly, in a patient from Berlin suffering from an autochthonous HEV infection genotype 3c has been revealed, the same genotype, which has been detected in wild boars from Berlin in this study (le Coutre et al., 2009). Thus, these findings

strengthen the hypothesis of a zoonotic HEV transmission from wild boars to humans in Germany.

6.4.2 Genomic characterization of rat HEV

Due to the application of the broad-range nested RT-PCR (section 6.3.2), it was possible to gain sequences of an HEV-like virus from rats for the first time. This novel HEV-like virus is tentatively designated as rat HEV. All rat HEV strains sequenced so far cluster together and represent a clearly separated branch between the mammalian HEV genotypes 1 to 4 and the avian HEVs. Sequence identities reach 55.1% to 55.9% to HEV genotypes 1 to 4 and 49.3% to 50.2% to avian HEV strains. Thus, it could be concluded that rat HEV may represent a novel HEV genotype.

The predicted genome organization of rat HEV is typical for HEV (Meng, 2010a). Three additional ORFs, which have been predicted in this study for all rat HEVs analysed so far, need further experimental proof. An alignment of ORF2 of rat HEV revealed conserved as well as highly variable regions with most of the conserved regions in the S domain and M domain, whereas the P domain is more variable but the aa 528 to 556 and aa 572 to 584 are nearly identical to sequences of genotypes 1 to 4. The M and P domains are supposed to be the main targets for antibodies (Khudyakov et al., 1994). Several studies have detected HEV-specific antibodies in rodents (Arankalle et al., 2001; Favorov et al., 2000; Hirano et al., 2003; Kabrane-Lazizi et al., 1999; Meng et al., 2002; Vitral et al., 2005). As HEV GT1 has been used in most of these assays as antigens it could be speculated that cross-reacting antibodies against rat HEV have detected epitopes of the conserved regions within the M domain and the two conserved regions within the P domain.

So far, the transmissibility of rat HEV to humans is not known. Based on the data of genome sequence analyses, rat HEV is only distantly related to the known human HEV strains. Therefore, it may be speculated that rat HEV is adapted to rats and does not infect humans. However, virus transmission cannot be ruled out without laboratory testing. As the usually applied PCR assays are not capable of detecting rat HEV, such studies have to be performed using rat HEV-specific assays. In addition, experimental infection studies with monkeys as performed with HEV GT1,

GT3, GT4 and avian HEV (Aggarwal et al., 2001; Huang et al., 2008; Huang et al., 2004; Meng et al., 1998) may be necessary in order to assess the zoonotic potential of rat HEV.

6.5 Future prospects

These studies reveal the importance of animal reservoirs for HEV in Germany and the possibility of a zoonotic virus transmission. The wild boar population is increasing in Western Europe and the USA over the past decades (Schley & Roper, 2003). About 500,000 wild boars are hunted in Germany per year. Thereof about 2,000 synanthropic wild boars are hunted in the urban region of the city Berlin (Jansen et al., 2007). Thus, contact between humans and wild boars is possible and especially hunters are at risk to attain an HEV infection. HEV is also highly prevalent in the German domestic pig livestock. However, further experiments are necessary to assess the distinct transmission routes between wild boars/pigs and humans. Pig meat or wild boar meat on the market should therefore be analysed for the presence of HEV to assess its role for autochthonous HEV infections. Studies on heat stability of HEV may be necessary to assess the risk of a food-borne zoonotic transmission from wild boar or pig meat to humans and to give recommendations for adequate heating temperatures. So far, conducted experiments on the thermal stability of HEV are not sufficient for a reliable risk assessment.

To assess whether rat HEV is transmissible to humans, animal experiments with non-human primates may be necessary. Especially if humans are not susceptible to rat HEV, the virus may be a promising candidate as surrogate for the other HEV genotypes. Infection of rats with rat HEV could serve as a small animal model for further studies on stability, pathogenicity, replication and transmission of human HEV.

Additionally, the expression of new recombinant proteins may promote the development of further specific immunoassays and vaccines, which might support more detailed prevalence studies and protection of certain populations at risk.

Screening of other animals for the presence of additional so far unknown HEV-like viruses may be useful to discover new transmission routes. Especially food producing animals for example cattle, which have been already tested positive for HEV-specific antibodies and HEV RNA (Geng et al., 2010; Hu & Ma, 2010), should be screened for HEV-like viruses using broad-reactive PCR assays.

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I List of Publications

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II Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich diese Arbeit selbst verfasst habe sowie keine anderen als die angegebenen Quellen und Hilfsmittel in Anspruch genommen habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form keiner anderen Prüfungsbehörde vorgelegt wurde.

Anika Schielke Berlin, Januar 2011

III Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Due to protection of data privacy the curriculum vitae is not included.