Epidemiology of the Hepatitis E Virus in Reservoir Hosts

Inauguraldissertation

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften
(Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

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geboren am 13. April 1983

in Wien, Österreich

Greifswald, August 2013

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Tag der Promotion:	2. Dezember 2013

Es hat alles zwei Seiten.
Aber erst wenn man erkennt, daß es drei sind,
erfasst man die Sache.

Heimito von Doderer
Repertorium, 1969

Contents

Summary

Background

Hepatitis E virus (HEV) is the etiological agent of an acute self-limiting hepatitis in humans worldwide. The main route of infection is by ingestion of food or water contaminated with the virus. In Germany, several hundred human cases are reported each year, while preliminary studies suggest a high infestation rate of herds of domestic pig (*Sus scrofa domesticus*) and sounders of wild boar (*Sus scrofa*). Autochthonous cases are originating mainly from zoonotic transmission from domestic pig and wild boar, but other animals may also be involved. Recently, a novel strain of HEV (rat HEV) had been found in Norway rats (*Rattus norvegicus*) in Germany, that could contribute to human epidemiology. Therefore, the aim of this study was to assess the seroprevalence of both HEV and the novel rat HEV in human, domestic pig and rat.

For each of the three mammal species, an indirect immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was established, that based on an *Escherichia coli*-expressed carboxy-terminal segment (GT 3-Ctr, amino acid (aa) 326 – 608) of the capsid protein of the autochthonous genotype 3 (GT 3), derived from a wild boar from Germany. In parallel, a segment from rat HEV homologous to GT 3-Ctr was also expressed in *E. coli* (rat HEV-Ctr, aa 315 – 599) and was used in the ELISA. Hence, the established tests detect antibodies directed against HEV GT 3 when using GT 3-Ctr as antigen and rat HEV when using rat HEV-Ctr.

Results

The GT 3-based in-house human IgG test was validated using a commercial assay and showed high specificity and sensitivity. The average human popu-

lation (represented by a panel of blood donors from Berlin and Brandenburg) reached a seroprevalence of 12.3 % (37 / 301) with the in-house ELISA. A panel of forestry workers from Brandenburg had an even higher seroprevalence of 21.4 % (119 / 555). Furthermore, rat HEV-specific antibodies could be detected in several sera of forestry workers.
The novel rat HEV-based rat IgG ELISA could not be compared to similar tests, however, parallel testing with GT 3-Ctr and statistical inference allowed conclusion of a seroprevalence. Rats trapped from several sites in Germany had an overall seroprevalence of 24.5 % (36 / 147). The sera were reactive exclusively with rat HEV-Ctr.
As with the in-house ELISA for human sera, the porcine IgG test was validated using a commercial assay, yielding high specificity and sensitivity. A panel of domestic pigs from ten federal states of Germany showed a seroprevalence of 42.7 % (383 / 898) when tested with the in-house ELISA. Reactivity with rat HEV was present, but seemed to be caused mostly by cross-reactivity to GT 3-Ctr.

Conclusion

The HEV seroprevalence observed for human sera of the average population of Germany is among the highest in Europe and has been confirmed recently by other authors. The high seroprevalence found in forestry workers suggests that they should be counted as a risk group for HEV infection.
Populations of rats have been shown to be infested heavily with rat HEV, as rats from all trapping sites situated within cities had a high prevalence for rat HEV exclusively and no serum reacted exclusively with GT 3-Ctr.
Seroprevalence in domestic pigs was demonstrated to be distributed evenly across federal states and districts. However, a vast difference of infestation could be detected in different herds, suggesting either differences in husbandry conditions, or an external source of infection that acts locally only.
The rare but exclusive reactivity of human sera with rat HEV as well as the high cross-reactivity of swine sera with rat HEV suggests that viral strains other than the ones already known may contribute to cases of hepatitis E.

Zusammenfassung

Grundlage

Das weltweit vorkommende Hepatitis E virus (HEV) ist die Ursache für eine akut auftretende, selbstlimitierende Hepatitis beim Menschen. Hauptsächlich erfolgt die Infektion durch die orale Aufnahme von mit Virus kontaminierten Nahrungsmitteln und Wasser. In Deutschland werden jährlich mehrere hundert Fälle von Hepatitis E im Menschen gemeldet, auch von einer hohen Durchseuchung der Haus- und Wildschweinpopulationen (*Sus scrofa domesticus* und *Sus scrofa*) wird berichtet. Autochthone Erkrankungen in Deutschland werden primär durch zoonotische Übertragung von Haus- und Wildschweinen verursacht, doch andere Übertäger könnten ebenfalls beteiligt sein. Ein 2010 beschriebener HEV-Stamm aus der Wanderratte (*Rattus norvegicus*), Ratten-HEV, könnte für die Epidemiologie der Hepatitis E ebenfalls von Bedeutung sein. Daher war das Ziel dieser Studie die Abschätzung der Seroprävalenz von HEV (sowohl Ratten-HEV als auch humanpathogenes HEV) in Mensch, Hausschwein und Wanderratte.

Für jede der drei Säugetierspezies wurden indirekte Immunoglobulin G (IgG) Enzymimmunoassays (ELISAs) etabliert, die auf einem *Escherichia coli*-exprimierten carboxy-terminalen Segment des Kapsidproteins basieren (GT 3-Ctr, Aminosäureposition (aa) 326 – 608). Der dafür verwendete Stamm gehört dem in Deutschland autochthonen Genotyp 3 (GT 3) an und entstammt einem Wildschwein aus Deutschland. Parallel dazu wurde für den ELISA auch ein zu GT 3-Ctr homologes und in *E. coli* exprimiertes Segment des Ratten-HEV verwendet (Ratten-HEV-Ctr, aa 315 – 599). Infolgedessen erfassen die etablierten serologischen Tests Antikörper gegen GT 3, wenn sie GT 3-Ctr als Antigen ver-

wenden, und Antikörper gegen Ratten-HEV beim Einsatz von Ratten-HEV-Ctr als Antigen.

Ergebnisse

Der auf GT 3 basierende in-house Human-IgG-Test wurde mithilfe eines kommerziell erhältlichen Tests validiert und erwies sich als hoch spezifisch und sensitiv. Die Durchschnittsbevölkerung (repräsentiert durch eine Gruppe von Blutspendern aus Berlin und Brandenburg) zeigte mit dem in-house ELISA eine Seroprävalenz von 12,3 % (37 / 301). Eine Gruppe Waldarbeiter aus Brandenburg zeigte eine Seroprävalenz von 21,4 % (119 / 555). Desweiteren wurden Ratten-HEV-spezifische Antikörper in einigen Waldarbeitern gefunden.
Der neuartige, auf Ratten-HEV basierende Ratten-IgG-ELISA konnte nicht mit ähnlichen Tests verglichen werden. Durch parallele Testung mit GT 3-Ctr-Antigen konnte jedoch auf eine Seroprävalenz geschlossen werden. Ratten von verschiedenen Fangorten innerhalb Deutschlands wiesen eine durchschnittliche Seroprävalenz von 24,5 % (36 / 147) auf. Die Seren zeigten ausschließlich Reaktivität mit Ratten-HEV-Ctr.
Wie beim Human-IgG-ELISA wurde auch der Schweine-IgG-ELISA mittels eines kommerziellen Tests validiert und erreichte eine hohe Sensitivität und Spezifität. Ein Serumpanel von Hausschweinen aus insgesamt zehn deutschen Bundesländern erreichte im in-house-ELISA eine Seroprävalenz von 42,7 % (383 / 898). Wenige Seren reagierten auch mit Ratten-HEV-Ctr, was aber größtenteils auf eine Kreuzreaktivität mit GT 3-Ctr zurückgeführt werden kann.

Schlussfolgerung

Die beobachtete Seroprävalenz der deutschen Durchschnittsbevölkerung gehört zu den bislang höchsten in Europa und konnte von anderen Autoren bestätigt werden. Die höchste Seroprävalenz wurde für Waldarbeiter bestimmt und lässt vermuten, daß es sich bei diesen um eine Risikogruppe für HEV-Infektionen handelt.
Deutsche Rattenpopulationen zeigten eine hohe Durchseuchung mit Ratten-HEV. Die Tiere von allen innerstädtischen Fangorten wiesen eine hohe Reakti-

vität ausschließlich mit Ratten-HEV-Ctr auf; kein Serum reagierte mit GT 3-Ctr allein.

Die Seroprävalenz in Hausschweinen ist gleichmäßig auf alle getesteten Bundesländer und Landkreise verteilt. Im Vergleich einzelner Betriebe zeigte sich jedoch ein starker Unterschied in der Durchseuchung. Die Ursachen hierfür könnten entweder Unterschiede in der Haltung, oder eine externe, lediglich lokal wirkende Infektionsquelle sein.

Aufgrund der ausschließlichen Reaktivität einiger Humanseren mit Ratten-HEV-Ctr und wegen der hohen Kreuzreaktivität weniger Hausschweinseren mit Ratten-HEV-Ctr liegt der Schluss nahe, daß weitere, bisher unbekannte HEV-Stämme bei humanen HEV-Infektionen eine Rolle spielen könnten.

Chapter 1

Introduction

1.1 General introduction

The hepatitis E virus (HEV) is the etiological agent of an acute self-limiting hepatitis and is a major cause of enterically transmitted hepatitis worldwide (Khuroo, 2011). The virus belongs to the genus *Hepevirus*, which itself is a member of the family *Hepeviridae* (Meng et al., 2011).

In humans, the symptoms are similar to those of hepatitis A, causing hepatomegaly, jaundice, fever, anorexia, nausea and abdominal pain. The case fatality rate of 0.07 – 0.6 % is not very high in otherwise healthy patients (Aggarwal and Krawczynski, 2000). However, for immunocompromised patients and pregnant women the mortality rates are especially high, the latter reaching 20 – 25 %, although that concerns mostly India and Pakistan (Lewis et al., 2010; Tsega et al., 1993; Hussaini et al., 1997). The virus is highly endemic in many developing countries of Asia and Africa and epidemics have occurred on the Indian subcontinent, China, Southeast and Central Asia, the Middle East, and northern and western parts of Africa (Aggarwal and Naik, 2009). It is believed that most of the water-borne outbreaks of hepatitis in these regions were caused by HEV (Khudyakov and Kamili, 2011). The World Health Organization (WHO) estimated that one third of the world's population has been infected with HEV, most of them living in developing countries (WHO, 2009).

In these countries, the primary transmission route of the virus is fecal-oral, mostly by contaminated drinking water. Low sanitary standards contribute

to the frequency of HEV spreading. In many industrialized countries such as the United States, Japan and most European countries, HEV is regarded as an emerging pathogen (Purcell and Emerson, 2008). There, a zoonotic transmission route has been recognized that presumably has its main reservoirs in domestic pig (*Sus scrofa domesticus*), wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) (Meng, 2010a; Tei et al., 2004).

1.2 Virion structure and genome organization

1.2.1 Structure

The hepatitis E virus consists of an RNA-genome enclosed in an icosahedral, non-enveloped virion with an outer diameter of approximately 27 – 34 nm (Balayan et al., 1983; Bradley et al., 1988; Tam et al., 1991). The native virion is believed to be composed of 180 capsomers, hence having a triangulation number of three ($T = 3$ icosahedral lattice) (Xing et al., 2010). The density of the virion is 1.4 g/cm^3 in caesium chloride (CsCl) (Balayan et al., 1983). However, virus-like particles (VLPs) expressed in insect cells and devoid of RNA possess a slightly lower density (1.28 g/cm^3 in CsCl) (Li et al., 1997b). They are usually smaller with an outer diameter of 24 nm as they are built of only 60 capsomers ($T = 1$). Recently, VLPs with an icosahedral lattice of $T = 3$ and an outer diameter of 41 nm were generated, which resemble the native capsid more adequately (Xing et al., 2010). The virion features characteristic conserved spikes that proved to be highly immunogenic (Guu et al., 2009; Li et al., 2009; Yamashita et al., 2009).

The genome of HEV is an unsegmented single stranded RNA of positive polarity. It resembles a eucaryotic mRNA as it is capped at the 5'-terminus with 7-methylguanosine (m7G) and is polyadenylated at the 3'-terminus (Kabrane-Lazizi et al., 1999b; Reyes et al., 1990). Both 5'- and 3'-termini feature short untranslated regions (UTR) (see Figure 1.1) (Tam et al., 1991). The genome is 7.2 – 7.4 kilobases (kb) in length (without its polyadenylated tail) and contains three open reading frames (ORFs) in the forward direction (Tam et al., 1991). The 5'-terminus of the ORF1 and the 3'-termini of both the ORF2 and ORF3 may

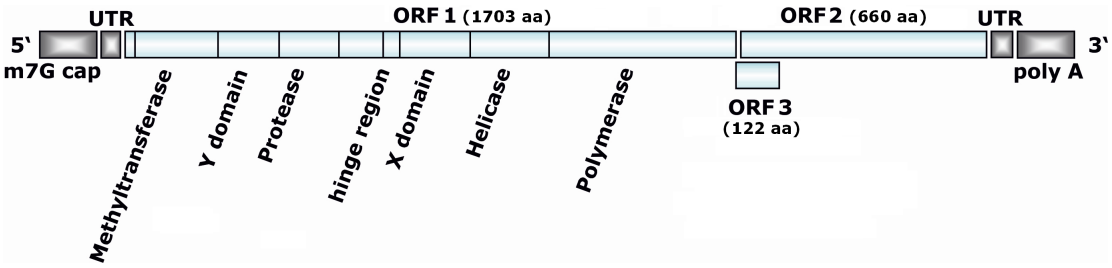

Figure 1.1: *Hepatitis E virus genome organization.* The lengths of the depicted genome are taken from subtype 3i (GenBank accession number FJ705359). The positive, single stranded RNA is 7.222 kb in length. It is capped with a 7-methylguanosine (m7G cap) at its 5’ terminus and has a poly-adenylated tail (poly A) at its 3’ terminus. Untranslated regions (UTRs) flank the three open reading frames (ORFs) on both ends. ORF1 encodes a polyprotein consisting of several nonstructural proteins and domains of unknown function. ORF2 encodes the capsid protein, ORF3 codes for a phosphoprotein.

overlap partly in a wide variety of constellations, depending on the genotype of the virus (see Figure 1.2). The length of the 5’ UTR may vary from 16 to 100 nucleotides (nts), while the length of the 3’ UTR may range from 39 to 127 nts.

1.2.2 Open reading frame 1

The open reading frame 1 (ORF1) covers the largest part of the genome (5,100 – 5,200 nts) and is believed to be translated directly from the genome, yielding all proteins necessary for viral RNA synthesis (Graff et al., 2006). The polyprotein resulting from the translation of ORF1-mRNA is then cleaved and hence forms four non-structural proteins. These proteins are a methyltransferase, a papain-like protease, a helicase and an RNA-dependent RNA polymerase (RdRp). Between the protein segments, the ORF1-encoded polyprotein contains several domains of unknown function (Y domain, proline-rich hinge domain, X domain) that are homologous to domains of other positive-strand RNA viruses (Koonin et al., 1992).

1.2.3 Open reading frame 2

The open reading frame 2 (ORF2) codes for the capsid protein (CP) with a length of 658 – 674 amino acids (aas) (Tam et al., 1991). It constitutes the outer structure of the virion and contains immunogenic epitopes, some of which elicit

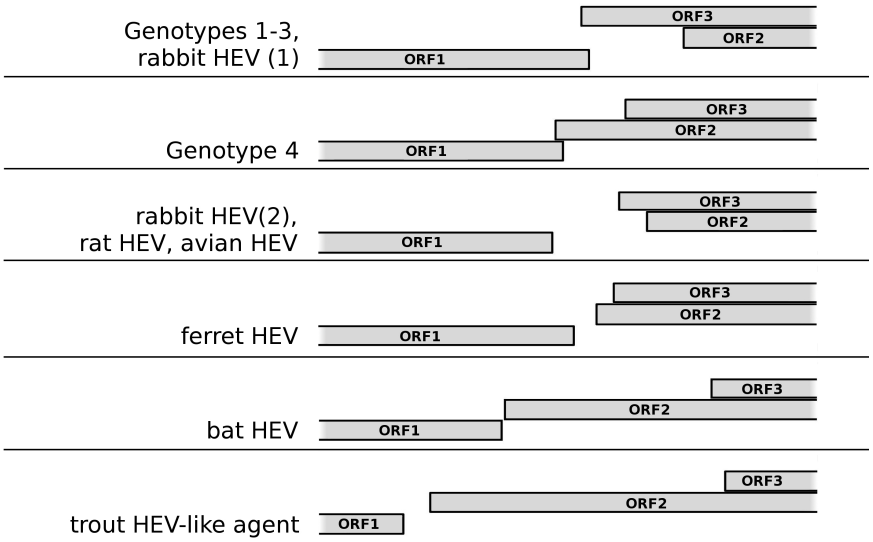

Figure 1.2: *Genome organization at the overlapping region of the three open reading frames (ORFs) from different HEV genotypes and strains.* The graphs represent a small, approximately 200 nt section of the genome, in which the overlap of the ORFs occur. Genomes investigated (GenBank accession numbers): Genotypes 1 – 3: AF076239, M80581, M74506, EU360977, AB189070, AF060668; rabbit HEV (1): JX565469, FJ906896, FJ906895; Genotype 4: AB220977, AJ272108, GU119961; rabbit HEV (2): GU937805; rat HEV: JN167537, JN167538, GU345042, GU345043, JX120573; avian HEV: GU954430, JN597006, AY535004; ferret HEV: JN998607, JN998606; bat HEV: JQ001749; trout HEV-like agent: NC015521

neutralizing antibodies, thus making it the immunodominant protein of the virus. Hence it is often used as a potent antigen in serological tests and poses as a promising target for vaccine development (Aggarwal and Jameel, 2008; Wu et al., 2012). The gene overlaps with ORF3 and is translated from a bicistronic subgenomic RNA that incorporates both ORF2 and ORF3 (Graff et al., 2006). The full-length protein is 72 kDa in size with some truncated recombinant versions capable of self-assembly into VLPs (Chandra et al., 2008b; Xing et al., 1999).

The protein contains an endoplasmic reticulum (ER) signal peptide at the N-terminus that is followed by an arginine-rich domain which binds the 5’-terminus of the HEV genome and may play a role in viral encapsidation (Guu et al., 2009). The arginine-rich domain is thought to neutralize the negatively charged RNA. The capsid protein exhibits three putative N-glycosylation sites at the aa residues 137, 310 and 562 (Zafrullah et al., 1999). Despite the considerable research, it still remains unclear whether they are glycosylated in the viral protein (Ahmad et al., 2011).

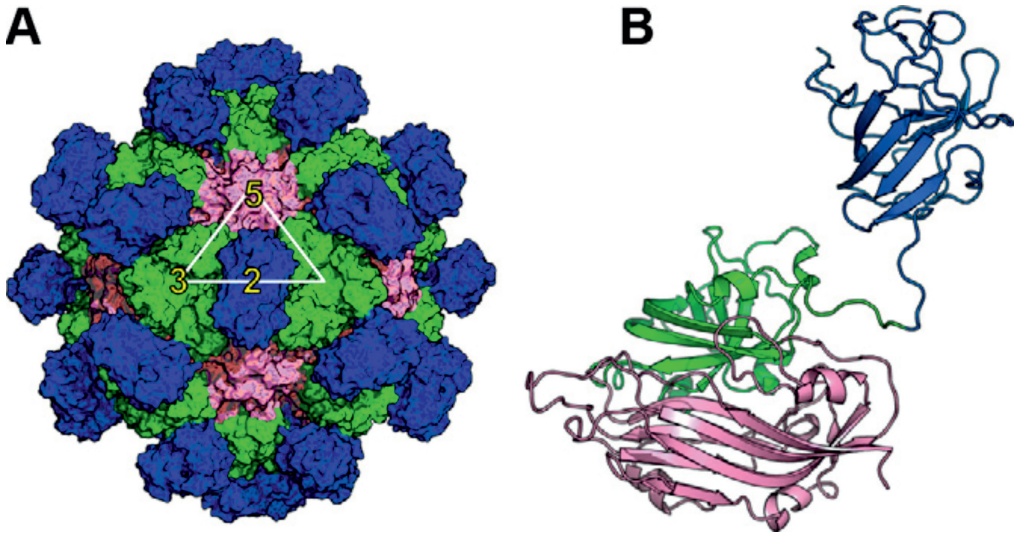

Figure 1.3: *Tertiary and quaternary structure of the ORF2-encoded capsid protein and the capsid.* (A) depicts the crystal structure of a virus-like particle with $T = 1$ symmetry, hence the particle consists of 60 monomers of the CP. The external diameter is approximately 27 nm, icosahedral 2-, 3- and 5-fold axes are presented. (B) presents the tertiary structure of pORF2. The S, M and P domains in both (A) and (B) are shown in pink, green and blue, respectively. Figure reprinted from Mori and Matsuura (2011)[1].

The protein encoded by ORF2 (pORF2) has been found to consist of the three domains S (shell, aa 129 – 319), M (middle, aa 320 – 455) and P (protruding, aa 456 – 606) by x-ray analysis of VLPs of HEV genotype (GT) 3 (see Figure 1.3) (Yamashita et al., 2009). Guu et al. (2009) made the same discovery by electron microscopy-analysis of VLPs of HEV GT 4, albeit naming the domains S, P1 and P2 with slightly deviating aa positions. Following the nomenclature of Yamashita et al. (2009), the domains P and M at the C-terminus of pORF2 form the outer surface of the virion and contain the most important epitopes for neutralizing antibodies (Meng et al., 2001; Purdy et al., 1993; Tang et al., 2011; Zhou et al., 2004).

1.2.4 Open reading frame 3

The open reading frame 3 (ORF3) encodes a regulatory protein with a length of 112 – 123 aa that is phosphorylated by a mitogen-activated protein kinase (MAPK) at Ser80 (Liu et al., 2011). The exact function of the protein is unclear, but it appears to be essential for viral infectivity *in vivo* (Huang et al., 2007).

[1] Reprinted from Virus Research, 161, Mori, Y., Matsuura, Y., Structure of hepatitis E viral particle, 59 – 64, Copyright 2011, with permission from Elsevier.

Notwithstanding, this is disputed, as replication, virion assembly and infection seem to be possible *in vitro* without the protein encoded by ORF3 (pORF3) (Emerson et al., 2006). The protein has been found attached to the cytoskeleton as well as on the surface of HEV particles in cell culture supernatant (Takahashi et al., 2008; Zafrullah et al., 1997). The pORF3 was shown experimentally to interact with the non-glycosylated form of pORF2 as well as numerous cellular proteins e. g. the α1-microglobulin/bikunin precursor, tumor suppressor gene 101 (pTSG101)-product and proteins with src homology 3 (SH3) domains (Tyagi et al., 2002; Surjit et al., 2006; Tyagi et al., 2004; Korkaya et al., 2001). Hence, numerous different functions have been hypothesized. There exist evidence that pORF3 may be involved in cell signaling, thereby promoting host cell survival (Chandra et al., 2008a). Furthermore, pORF3 is believed to be necessary for release of mature virions from the host cell (Kenney et al., 2012; Yamada et al., 2009).

1.3 Replication cycle

HEV is a hepatotropic virus and associated with the liver and bile (Lee et al., 2009). As no efficient *in vitro* culture system or small animal infection model was accessible for HEV, the replication cycle of HEV is not known in detail and only basic information is available. However, very recent developments in cell culture could provide the high yield necessary for more detailed future studies on viral replication (Okamoto, 2011; Nguyen et al., 2012; Shukla et al., 2012). The following model of replication has been proposed by Ahmad et al. (2011), Chandra et al. (2008b) and Jameel (1999), based on analogy to similar positive-strand RNA viruses.

For binding and entry of HEV, no specific receptor or route has been identified, but heparan sulfate proteoglycans might play a role. Also, a potential polysaccharide-binding site on both domains P1 and P2 of pORF2 may be involved (Guu et al., 2009). Viral trafficking involves proteins of the cytoskeleton and heat shock protein 90 (HSP90). However, the mechanisms involved in uncoating of RNA remain unclear. The release of the viral genome into the

cytosol results in the first translation of ORF1. This yields the non-structural proteins (methyltransferase, protease, helicase and RdRp). The cleavage processes of the ORF1-encoded polyprotein necessary depend presumably on cellular proteases, but may also involve the viral protease. The viral RdRp attaches to the ER using an ER transmembrane domain (Rehman et al., 2008) and replicates the HEV genome, yielding negative-sense RNA intermediates. This RNA is transcribed to viable positive-strand genomes as well as bicistronic subgenomic RNAs, which get translated to pORF2 and pORF3. The genome is then packaged by pORF2 that binds to it using an arginine-rich domain. The mechanism of maturation and secretion is also poorly understood. In the last years however, evidence suggested the involvement of pORF3 as well as lipids in packaging, release and secretion of the complete particle.

1.4 Human HEV infections

1.4.1 Human-pathogenic genotypes

There are four genotypes of the hepatitis E virus (genotypes 1 – 4) that are known to be human-pathogenic (Meng, 2006). These genotypes were originally defined by Burmese (GT 1), Mexican (GT 2), US (GT 3) and Chinese (GT 4) prototype strains of HEV (Lu et al., 2006). Schlauder and Mushahwar (2001) classified the virus into 9 different subtypes, using a 5’ region of ORF1. This genetic classification reaffirms the notion of 4 genotypes, but allocates several groups to the genotypes 3 and 4, accounting for their strong genetic divergence. Moreover, the classification into 24 subtypes by Lu et al. (2006) subdivides the genotypes even further. Hence, despite the agreement to the four genotypes, there exist several definitions and numbers of sub-genotypes depending on the genomic fragments analyzed (Arankalle et al., 1999).

To date, GT 1 consists of human strains from Asia and Africa, while GT 2 is represented by human strains from Mexico and Africa. GT 3 is formed by human strains from industrialized countries in Asia, Europe, Oceania, North and South America as well as swine strains from both developing and industrialized countries. GT 4 comprises human strains from Asia and swine strains from both

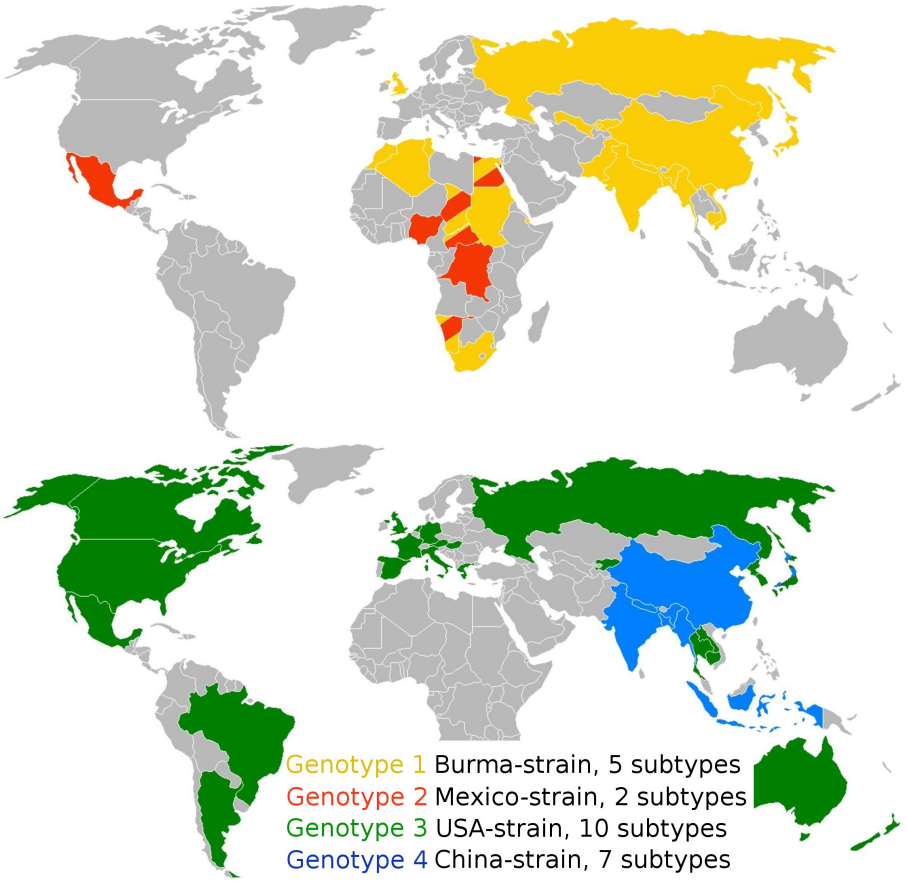

Figure 1.4: *Currently known geographic distribution of the human-pathogenic genotypes of HEV and their corresponding prototype strain*. The number of subtypes is according to Lu et al. (2006).

developing and industrialized countries (see Figure 1.4). GT 1 and GT 2 are believed to infect human only and to cause epidemics in developing countries, while GT 3 and GT 4 are zoonotic with domestic pig, wild boar and red deer as their main reservoirs (Teo, 2010). In swine, HEV infection can be found worldwide in both developing and industrialized countries.

1.4.2 Transmission in developing countries

Most of the water-borne outbreaks of hepatitis E are reported from the Indian subcontinent, Central and Southeast Asia, the Middle East and North Africa. These regions are described as the epidemic zone (Khuroo, 2011). There, the spread of HEV depends heavily on the water supply for fecal-oral transmission. Several studies demonstrated that in India transmission occurs

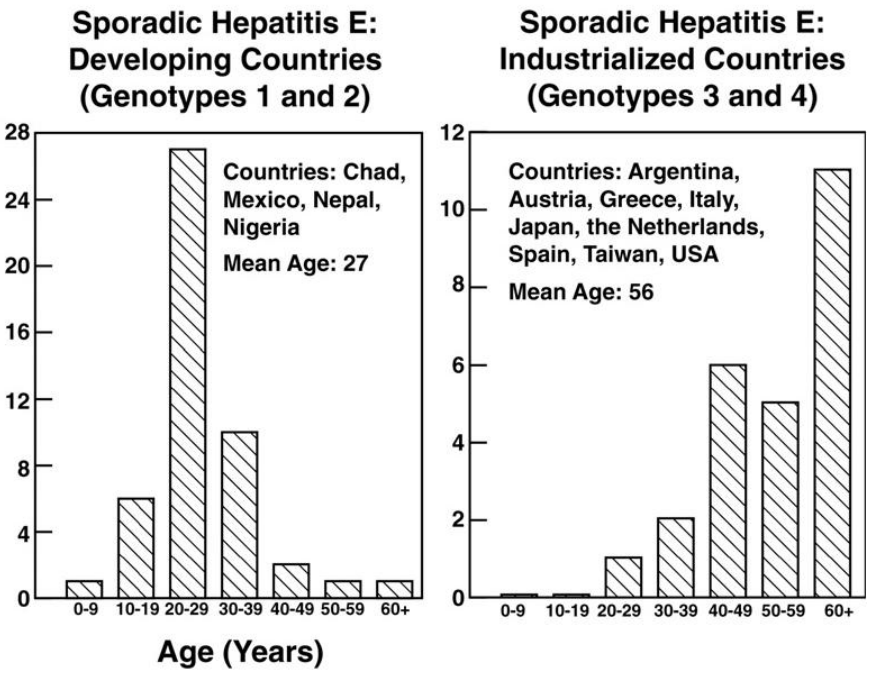

Figure 1.5: *Clinical attack rates of HEV by country of acquisition.* HEV affects different age groups depending on the genotype involved. In endemic developing countries, most clinical infections originate from the genotypes 1 and 2. For the most part, young adults are affected, with a mean age of 27 years. In industrialized countries, where only genotypes 3 and 4 occur autochthonously, the mean age of patients is higher (56 years). Figure reprinted from Purcell and Emerson (2008)[1].

mostly by water-borne infection while intrafamilial transmission is considered very rare, even during an epidemic (Aggarwal and Naik, 1994; Somani et al., 2003). This makes HEV especially a threat to public health in regions with poor sanitary conditions. Additionally, communities drawing their water from rivers, ponds or open ditches, expose their inhabitants to an even greater risk (Sedyaningsih-Mamahit et al., 2002; Toole et al., 2006). However, the primary route of infection may vary, as person-to-person transmission was described as the most likely cause of infection during an outbreak in Uganda (Teshale et al., 2010). Large outbreaks have occurred in Mexico (Velázquez et al., 1990), China (Aye et al., 1992; Bi et al., 1993), India (Naik et al., 1992; Vivek et al., 2010b), Vietnam (Corwin et al., 1996), Indonesia (Sedyaningsih-Mamahit et al., 2002), Namibia (Maila et al., 2004), Sudan (Guthmann et al., 2006), Uganda

[1]Reprinted from Journal of Hepatology, 48, Purcell, R. H., Emerson, S. U., Hepatitis E: An emerging awareness of an old disease, 494 – 503, Copyright 2008, with permission from Elsevier.

(Teshale et al., 2010) and Egypt (Shata et al., 2012). This concerns mostly the age group of 11 – 40 years, with males and females affected equally (see Figure 1.5) (Purcell and Emerson, 2008; Khuroo, 2011). Pregnant women have an even higher mortality (Hussaini et al., 1997).
GT 1 and GT 2, which are found in humans exclusively, are the most prevailing genotypes in developing countries (Purcell and Emerson, 2008). Although GT 3 and GT 4 could also be found, it is believed that these play only a minor role in these regions (Shukla et al., 2007). The prevalence of anti-HEV antibodies varies widely in developing countries from 22.5 % in rural Bangladesh (Labrique et al., 2009) to more than 60 % in rural Egypt (Fix et al., 2000) (see Table 1.1).

1.4.3 Transmission in developed countries

In industrialized areas, such as Europe, Japan and the United States, hepatitis E occurs in lower incidences and without epidemic outbreaks. Fecal-oral transmission is greatly hindered by good sanitary conditions and safe water supplies. Previously, hepatitis E was believed to be a disease associated with travel to endemic areas, but HEV is recognized recently as autochthonous virus that is acquired mostly locally (Drobeniuc et al., 2001; Mansuy et al., 2004; Preiss et al., 2006). In developed countries, no epidemics of GT 1 and GT 2 have been reported. Instead, the world-wide occurring GT 3 and the wide-spread GT 4 are autochthonous there. These genotypes elicit zoonoses and are thought to have their reservoirs mainly in domestic pig, wild boar and red deer. As transmission of GT 3 and GT 4 does not occur directly between humans, it appears that human do not play a role as a reservoir (Purcell and Emerson, 2008). There is evidence for zoonotic transmissions following the consumption of under-cooked meat or offal originating from these animals infected with HEV (Masuda et al., 2005; Matsuda et al., 2003; Takahashi et al., 2004; Tei et al., 2003; Yazaki et al., 2003). On these rare occasions, infection could be linked directly to the consumption of meat of an infected animal. RNA of HEV extracted from leftover deer meat could be sequenced and showed very high identities (99.7 – 100 %) to the HEV found in the patients (Tei et al., 2003). Similarly, in Xinjiang, China, HEV found in the local swine population had been shown to be virtually identical (up to 100 % nucleotide identity) with samples from the human population (Fu et al., 2010).

Table 1.1: *Reported seroprevalences in average human populations worldwide.*

country	number of investigated samples	number of positive samples	positive (%)	reference
Austria	1,094	25	2.3	Hofmann and Holzmann, 1995
Bangladesh	1,134	255	22.5	Labrique et al., 2009
Brazil	200	4	2	Parana et al., 1997
Burkina Faso	178	34	19.1	Traoré et al., 2012
Burundi	129	18	14	Aubry et al., 1997
Chile	1,360	109	8	Ibarra et al., 1997
China	546	124	22.7	Cheng et al., 2012
	44,816	14,608	32.6	Guo et al., 2010
	173	69	39.9	Li et al., 2011b
	456	37	8.1	Shenyang et al., 2011
	450	129	28.7	Chiu et al., 2013
Cuba	469	47	10	Villalba et al., 2010
Denmark	456	94	20.6	Christensen et al., 2008
Egypt	140	79	56.4	Darwish et al., 1996
	10,026	n/a	67.7	Fix et al., 2000
	95	43	45.2	Hady et al., 1998
France	1,998	64	3.2	Boutrouille et al., 2007
	529	88	16.6	Mansuy et al., 2008
	512	268	52.5	Mansuy et al., 2011
French Guiana	996	64	6.4	Talarmin et al., 1997
Germany	4,422	n/a	16.8	Faber et al., 2012
	1,019	69	6.8	Juhl et al., 2013
	116	18	15.5	Krumbholz et al., 2012
Ghana	239	11	4.6	Meldal et al., 2013
India	578	26	4.5	Khuroo and Khuroo, 2010
	2,279	330	14.5	Vivek et al., 2010a
Iran	551	51	9.3	Mohebbi et al., 2012
Italy	361	6	1.7	Pavia et al., 1998
	3,511	101	2.9	Vulcano et al., 2007
Japan	22,027	1,167	5.3	Takahashi et al., 2010
Moldova	255	63	24.7	Drobeniuc et al., 2001
Morocco	81	5	6.1	Benjelloun et al., 1997
the Netherlands	644	n/a	2	Bouwknegt et al., 2008a
	7,072	134	1.9	Verhoef et al., 2012
	1,275	5	0.4	Zaaijer et al., 1993
San Marino	2,233	33	1.5	Rapicetta et al., 1999
South Africa	767	n/a	10.7	Tucker et al., 1996
South Korea	361	43	11.9	Ahn et al., 2005
	96	17	17.7	Choi et al., 2003
	147	21	14.3	Park et al., 2012
	147	34	23.1	Park et al., 2012
Spain	1,280	96	7.3	Buti et al., 2006
	97	4	4.1	Galiana et al., 2008
	863	25	2.8	Mateos et al., 1998
	2,305	25	1.1	Fogeda et al., 2012
Russia	185	33	17.8	Obriadina et al., 2002
Sweden	108	10	9.3	Olsen et al., 2006
Switzerland	550	27	4.9	Kaufmann et al., 2011
Thailand	342	79	23.1	Hinjoy et al., 2012
Tunisia	687	n/a	5.4	Houcine et al., 2012
Turkey	1,350	80	5.9	Thomas et al., 1993
UK	500	80	16	Dalton et al., 2008b
	2,731	n/a	13.3	Ijaz et al., 2009
USA	400	66	16.5	Meng et al., 2002
	367	54	14.7	Obriadina et al., 2002

n/a, not available

Porcine livers available on the retail market as well as their products were found to be RNA-positive in Japan (Yazaki et al., 2003), the Netherlands (Bouwknegt et al., 2007), the United States (Feagins et al., 2007), India (Kulkarni and Arankalle, 2008), France (Colson et al., 2010) and Germany (Wenzel et al., 2011). Furthermore, these studies showed either the infective potential of the commercial meat by successful experimental infection of domestic pigs, or high similarities of the isolated sequences to HEV-sequences of local patients (Bouwknegt et al., 2007; Colson et al., 2010; Feagins et al., 2007; Wenzel et al., 2011; Yazaki et al., 2003).

Tei et al. (2004) as well as Wichmann et al. (2008) describe the consumption of uncooked meat of deer and wild boar as a significant epidemiological risk factor for HEV infection. Even ingestion of infected shellfish is a possible route of infection (Cacopardo et al., 1997; Koizumi et al., 2004). Moreover, in the United States, the third National Health and Nutrition Examination Survey (NHANES) showed a higher seroprevalence in persons with an elevated uptake of organ meats (Kuniholm et al., 2009).

A route of infection other than consumption of infected meat is the direct contact with infected animals or with their habitats, reflected in increased seroprevalences in veterinarians and slaughterhouse personnel (Kuniholm et al., 2009; Meng, 2003). Blood transfusion may also pose a risk, as cases of transmission by blood transfusion were reported from Japan (Matsubayashi et al., 2004; Mitsui et al., 2004), the United Kingdom (Boxall et al., 2006) and France (Haïm-Boukobza et al., 2012). This route of infection of genotypes 3 and 4 demonstrates the capability of a human-to-human transmission of these genotypes (Purdy and Khudyakov, 2011).

The incidences of hepatitis E are reported to be low in several industrialized countries (Mateos et al., 1998; Takahashi et al., 2010), while the seroprevalence of HEV may be higher than expected (see Table 1.1). This could be caused by the dose-dependency of the HEV-infection, where lower doses lead to a prolonged incubation period, less severe clinical symptoms and absence of biochemical evidence, but may still elicit an immune reaction (Aggarwal et al., 2001; Tsarev et al., 1994b).

In Germany, hepatitis E is a notifiable disease since the Federal Protection against Infection Act in 2001 and is monitored by the Robert Koch-Institut (RKI). Since 2001, the number of reported cases increased (31 cases in 2001; 388 cases in 2012) (RKI: SurvStat, 2013). Although the reason is not fully understood, it would be plausible to attribute this to a raised awareness by physicians, which could result in more diagnosed cases.

1.4.4 Clinical features of hepatitis E

Detailed descriptions of several major outbreaks as well as case-control studies in industrialized countries show patients with a wide variety of symptoms and severity of disease (Aggarwal et al., 2000; Khuroo, 1980; Sainokami et al., 2004; Wichmann et al., 2008). Additionally, the course of illness of experimentally infected male volunteers is described in detail (Balayan et al., 1983; Chauhan et al., 1993). The mildest form is an asymptomatic infection, while severe forms of hepatitis E lead to fulminant hepatic failure (Aggarwal, 2011).
The incubation period varies greatly and ranges from two weeks to two months with an average of 40 days (Pavio et al., 2010). The onset of the illness is characterized by a prodromic phase which leads to the icteric phase. During the prodromic phase, which usually lasts several days up to one month, the patient may show unspecific symptoms such as fever, anorexia, and abdominal pain of varying intensity (Aggarwal, 2011; Balayan et al., 1983; Chauhan et al., 1993). During this phase, the viremia and fecal shedding of the virus commences, the latter normally declining after one or two weeks (Aggarwal et al., 2000; Pavio et al., 2010). In individual cases however, prolonged viral shedding was reported to last more than 100 days (Nanda et al., 1995). During the icteric phase, which normally has a duration of approximately two weeks, the viremia and shedding cease and jaundice, hepatomegaly and elevated levels of serum alanine aminotransferase (ALT) and bilirubin may be observed (see Figure 1.6) (Aggarwal, 2011; Chauhan et al., 1993). During this stage, fulminant disease could be monitored in some patients. There is a broad range of secondary illness (e. g. acute pancreatitis, thrombocytopenia or nerve palsies) reported to be elicited by HEV (Aggarwal, 2011).

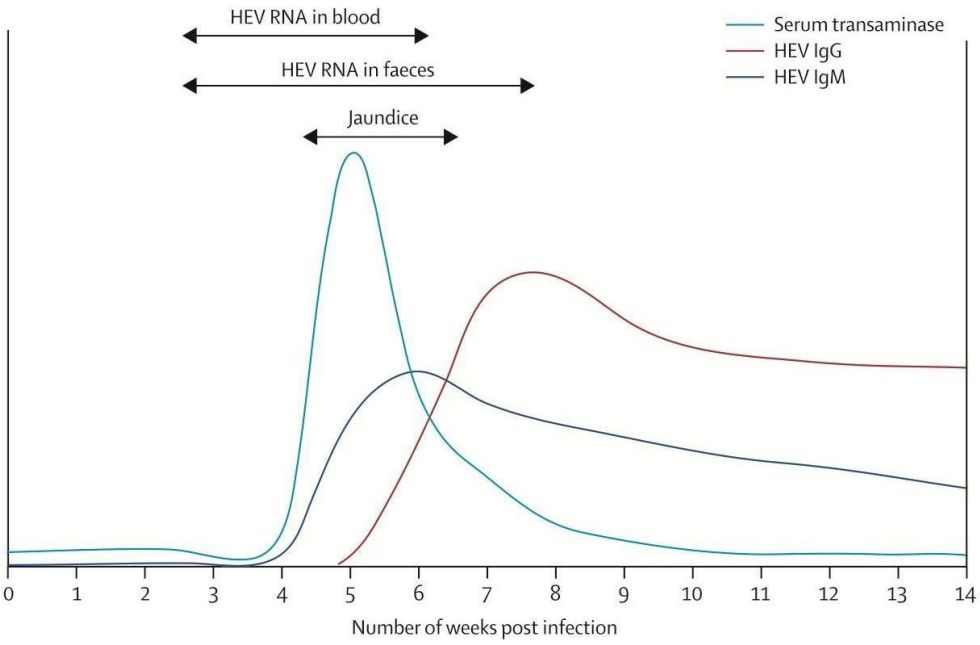

Figure 1.6: *Typical course of human HEV infection.* Figure reprinted from Dalton et al. (2008a)[1].

Clinical symptoms may become more severe in the presence of comorbidities like diabetes mellitus, compromised immune status, hypertension, obesity, arthritis, ischaemic heart disease or previous hepatitis A virus infection (Lewis et al., 2010; Pfefferle et al., 2012). The usually self-limiting disease may lead to prolonged viremia and fecal shedding and even become chronic in immunocompromised patients such as organ transplant recipients or HIV infected patients, leading to liver fibrosis and liver cirrhosis (Haagsma et al., 2008; Kaba et al., 2011).

The case fatality rate during outbreaks in developing countries is estimated to be 0.5 – 4 % of the hospitalized population and 0.07 – 0.6 % of the unhospitalized population (Aggarwal and Krawczynski, 2000). The case fatality rates for pregnant woman in the third trimester vary widely, depending on the region (Tsega et al., 1993). They are reported to be highest on the Indian subcontinent, reaching more than 20 % (Hussaini et al., 1997). Additionally, the incidence rate of hepatitis E is elevated in pregnant women of these countries, reaching 18 % in the third trimester (Khuroo et al., 1981). The reasons for this extraordinary

[1] Reprinted from The Lancet Infectious Diseases, 8, Dalton, H. R., Bendall, R., Ijaz, S., Banks, M., Hepatitis E: an emerging infection in developed countries, 698 – 709, Copyright 2008, with permission from Elsevier.

high mortality of pregnant women is thought to be associated to the hormonal changes during pregnancy, however the details are still unclear (Jilani et al., 2007; Navaneethan et al., 2008; Pal et al., 2005).
It is suggested that in developing regions, only 25 – 50 % of all infections are symptomatic, while the other 50 – 75 % are subclinical (Clayson et al., 1997). GT 3 and GT 4 are thought to be less virulent than GT 1 and GT 2 and lead to fewer clinical cases of hepatitis E (Purcell and Emerson, 2008). The reason for this could be differences in virulence and stability of viral strains. Nevertheless, hepatitis E caused by GT 3 may have a fulminant and even fatal outcome, as reported in Europe (Ijaz et al., 2005) and Japan (Suzuki et al., 2002). There are no reports on fulminant hepatitis E cases during pregnancy associated with GT 3 (Pavio and Mansuy, 2010). In industrialized countries, older patients (40 years of age and older) appear to be at an elevated risk to acquire HEV infection, which is in contrast to developing countries, were mostly young adults are affected (see Figure 1.5) (Ijaz et al., 2005; Purcell and Emerson, 2008; Suzuki et al., 2002). Males appear to have a higher risk for overt disease, as predominantly males suffer from autochthonous, clinically manifested infections, which is reflected rarely by the seroepidemiological studies (Lewis et al., 2010; Sainokami et al., 2004; Wichmann et al., 2008).

1.5 HEV in animal hosts

1.5.1 Species prone to infection

Domestic pig as well as wild boar are considered the main reservoirs for HEV GT 3 and GT 4 (de Deus et al., 2008b; Meng et al., 1997; Sonoda et al., 2004). Another reservoir host of GT 3 and GT 4 is deer, although its significance in human epidemiology is unclear (Matsuura et al., 2007; Takahashi et al., 2004; Tei et al., 2003). RNA of strains closely related to GT 3 were found in mongoose (*Herpestes javanicus*) (Nakamura et al., 2006) and rabbit (Zhao et al., 2009). However, in several animals, strains of HEV have been found that are only distantly related to the genotypes 1 – 4. The causing agent of big liver and spleen disease (BLS) in chicken (*Gallus gallus domesticus*) was identified as

avian HEV, which is not considered human-pathogenic (Huang et al., 2004; Meng et al., 2011; Payne et al., 1999). In the last decade, several other strains could be detected in rat (*Rattus norvegicus*) (Johne et al., 2010b), bat (Drexler et al., 2012) and ferret (*Mustela putorius*) (Raj et al., 2012) (see Figure 1.7). Additionally, a distantly related member of the family *Hepeviridae* could be found in trout (*Oncorhynchus clarkii*) (Batts et al., 2011).

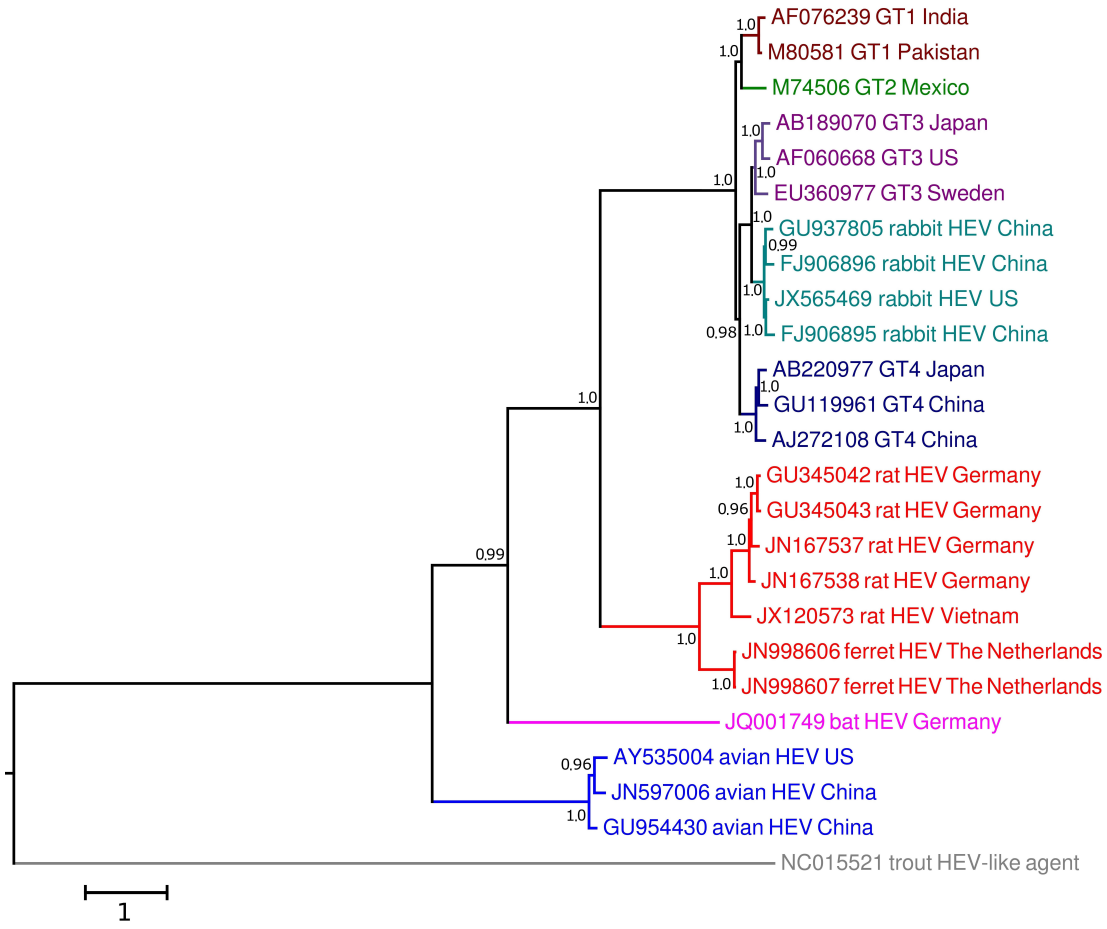

Figure 1.7: *Bayesian phylogenetic tree of representatives of the family* Hepeviridae. Posterior probabilities for Bayesian analysis are given at the branches. The scale bar indicates phylogenetic distances in nucleotide substitutions per site. The tree was built with full genome sequences of HEV with Mr. Bayes 3.1.2 on the CIPRES Science Gateway with 2,000,000 generations, which were sampled every 1,000 steps (Miller et al., 2010). The substitution model used was the two-parameter model of Hasegawa et al. (1985) with a gamma distribution across sites and a proportion of invariant sites (HKY + I + G).

1.5.2 HEV in domestic pig

In 1997, it was discovered that HEV could not only be found in human but also in domestic pigs of farms in the United States (Meng et al., 1997). Infection with GT 3 or GT 4 is common and widespread in swine farms and occurs worldwide in both developing and industrialized countries (Meng, 2003). In domestic pigs, only sequences of GT 3 and GT 4 could be detected and attempts to experimentally infect pigs with GT 1 or GT 2 proved unsuccessful (Cooper et al., 2005; Meng, 2003). However, several studies demonstrated successfully the experimental infection of pigs with GT 3 and GT 4, using strains extracted from swine as well as strains from human patients (Barnaud et al., 2012; Feagins et al., 2008b; Halbur et al., 2001). As in human, the transmission route in pig is believed to be fecal-oral. When used in studies to experimentally infect pigs with single doses of HEV, this route appears to be somewhat inefficient (Kasorndorkbua et al., 2004). Single doses given by the intravenous route were demonstrated to cause HEV infection in pigs more reliably (Barnaud et al., 2012; Kasorndorkbua et al., 2002). The oral route of infection is effective only when HEV is given in multiple doses, what implies a greater efficiency of transmission by a repeated ingestion of inoculum (Kasorndorkbua et al., 2004). This leads to the assumption that the most natural way of infection appears to be the repeated exposure to saliva, nasal secretions, urine, and feces from pigs in the same pen (Kasorndorkbua et al., 2004; Meng et al., 1998a,b). When pigs are infected with HEV either naturally or experimentally, the course of infection is asymptomatic or subclinical. Pathological lesions could be observed in hepatic and mesenteric lymph nodes as well as hepatocellular necrosis (Halbur et al., 2001; Meng et al., 1997). No deviations in the levels of liver enzymes could be detected in the serum of experimentally infected animals (Halbur et al., 2001; Meng et al., 1998a,b).

In commercial pig herds infested with HEV, piglets are infected with HEV at a very young age. When newly born to anti-HEV-positive sows, they are protected by parenterally acquired anti-HEV Immunoglobulin (Ig) G antibodies. Within 4 – 9 weeks, their immunity to HEV decreases, thus rendering them prone to HEV infection. This normally leads to IgG-seroconversion between an age of

14 – 21 weeks in an infested herd (Meng et al., 1997). RNA of HEV can be detected in feces between week 9 – 15 and has a very similar time window as viremia, which peaks at approximately week 15 (de Deus et al., 2008a; Leblanc et al., 2007; Nakai et al., 2006; Takahashi et al., 2005). Fecal shedding of the virus lasts 3 – 7 weeks (Meng et al., 1998a,b). When at slaughter age, pigs may still be positive for HEV-RNA in fecal matter as well as serum, although the majority of pigs are negative for HEV-RNA (Leblanc et al., 2007).
In commercial pig farms, studies assessed the basic reproduction number R_0 for HEV at 4.0 – 8.8 (Bouwknegt et al., 2008b; Satou and Nishiura, 2007). The parameter R_0 indicates the average number of secondary cases caused by a primary case in a fully susceptible population. This signifies that in the environment of a pig farm, one single animal with an HEV infection can infect 4 – 9 more animals. In despite of contaminated liver being found in Japanese grocery stores, more than 95 % of all farmed pigs in Japan were estimated to not contain virus anymore by the time being slaughtered. Thereby it is demonstrated that a slightly lower force of infection or a younger slaughter age may be sufficient to elevate greatly the amount of contaminated pork available on the retail market (Satou and Nishiura, 2007).

1.5.3 HEV in wild boar

In 1999, the first study found anti-HEV antibodies in 17 % of investigated free-living pigs (Chandler et al., 1999). It is believed that HEV is common in wild boar and that it could act as a reservoir host for the virus. Subsequently, genomic RNA of HEV was found in wild boars in several other countries (de Deus et al., 2008b; Reuter et al., 2009; Takahashi et al., 2004). Free-living wild boars can be found in most parts of the world and thereby form a vast, uncontrolled reservoir (Mitchell-Jones et al., 1999). In 2011, a novel genotype has been detected in Japan (Sato et al., 2011; Takahashi et al., 2011). Anti-HEV antibodies have been detected in wild boar populations from a number of countries including Spain (42.7 % positive; de Deus et al., 2008b), Japan (25.5 % positive; Michitaka et al., 2007) and the Netherlands (12 % positive; Rutjes et al., 2010) (see Table 1.2). In Germany, HEV-RNA was reported in sera, bile and liver of wild boars (Adlhoch et al., 2009b; Kaci et al., 2008; Schielke et al., 2009).

Table 1.2: *Reported prevalences in wild boar populations worldwide.*

country	number of investigated samples	positive (abs)	positive (%)	method of detection	references
France	285	7	2.5	RT-PCR	Kaba et al., 2010
	421	59	14	serological	Carpentier et al., 2012
Germany	189	10	5.3	RT-PCR	Kaci et al., 2008
	107	15	14	serological	Adlhoch et al., 2009b
	132	90	68.2	RT-PCR	
	148	22	14.9	RT-PCR	Schielke et al., 2009
Hungary	74	9	12.2	RT-PCR	Reuter et al., 2009
Italy	88	22	25	RT-PCR	Martelli et al., 2008
Japan	35	3	9	serological	Sonoda et al., 2004
	41	1	2.4	RT-PCR	
	392	100	25.5	serological	Michitaka et al., 2007
	392	12	3.1	RT-PCR	
	89	4	4.5	serological	Sakano et al., 2009
	89	1	1.1	RT-PCR	
	507	41	8.1	serological	Sato et al., 2011
	578	19	3.3	RT-PCR	
	450	16	3.6	RT-PCR	Takahashi et al., 2011
the Netherlands	1,029	165	12	serological	Rutjes et al., 2010
	106	8	8	RT-PCR	
Spain	150	64	42.7	serological	de Deus et al., 2008b
	138	27	19.6	RT-PCR	
	942	n/a	26.3	serological	Boadella et al., 2012
Sweden	159	13	8.2	serological	Widén et al., 2011

abs, absolute number; RT-PCR, reverse transcription-polymerase chain reaction; n/a, not available

1.5.4 HEV in rodents

The first indications of HEV-infections in rodents were found in Russia by immuno-electron microscopy (Karetnyi et al., 1993). This finding is supported by detection of HEV-reactive antibodies in numerous rodent species throughout the world. Antibodies were found in Norway rats and black rats (*Rattus rattus*) in Japan (Hirano et al., 2003). Furthermore antibodies were found in *Nectomys* spp. in Brazil (Vitral et al., 2005), in *Neotoma* spp., *Oryzomys* spp., *Peromyscus* spp., *Rattus* spp. and *Sigmodon* spp. in the United States (Favorov et al., 2000; Kabrane-Lazizi et al., 1999a) and in *Rattus* spp. and *Bandicota* spp. in India (Arankalle et al., 2001). All surveys detected IgG antibodies against parts of the capsid protein, with Favorov et al. (2000) detecting antibodies against both pORF2 and pORF3. Although the seroprevalence rates were found to be extremely high in some regions (91 % in parts of the United States), genomic RNA of HEV could not be detected for several years. Hence, antibody induction in rodents might be caused either by spillover infection (and clearance of the virus) or infection by an unknown, but cross-reactive novel virus that is only

distantly related to the human viral strains. Several studies demonstrated that BALB/c nude mice and Wistar rats could be successfully infected with known human genotypes (Huang et al., 2009; Maneerat et al., 1996), whereas infection of C57BL/6 mice was not possible (Li et al., 2008). Until 2010, no genomic RNA of HEV could be detected in naturally infected wild rodents.
By using broad-spectrum, nested reverse transcription-polymerase chain reaction (RT-PCR), Johne et al. (2010b) described HEV-like sequences in fecal samples of Norway rats in Hamburg, Germany. This newly detected virus has a high nucleotide sequence divergence to all the hitherto known genotypes 1 – 4 and avian HEV of 60 % and 43 %, respectively. Despite this divergence, the virus has a similar genomic organization and morphologic structure. Additionally, the virus is believed to have a hepatotropism, as suggested by immunohistochemistry and real-time RT-PCR (Johne et al., 2010a). The strain is thought to represent a novel genotype and could be found in America as well as Asia (Li et al., 2013a; Mulyanto et al., 2013; Purcell et al., 2011). Henceforth the strain will be denominated as rat HEV, as phylogenetic analyses and sequence comparisons of fragments as well as the whole genome show it to be separated consistently from all other HEV strains (see Figure 1.7) (Johne et al., 2010a,b). In 2012, HEV GT 3 was detected in wild rats in Japan as well as the United States, indicating that rats are susceptible to HEV GT 3 and therefore may contribute to a source of infection for domestic pig, wild boar and deer as well as human (Kanai et al., 2012; Lack et al., 2012). However, Wistar rats could not be infected experimentally with the genotypes 1, 3 and 4, but with rat HEV (Li et al., 2013b).

1.5.5 Avian HEV

Avian HEV belongs to another genus than the HEV genotypes 1 – 4 (Meng et al., 2011). It is the causative agent of BLS and has been detected in chicken in Australia (Payne et al., 1999), the United States (Huang et al., 2002b; Sun et al., 2004a), Italy (Massi et al., 2005), Hungary (Bilic et al., 2009; Morrow et al., 2008), Spain (Peralta et al., 2009a), China (Zhao et al., 2010), Austria, Canada, the Czech Republic, Germany, Israel, Poland and the United Kingdom

(Marek et al., 2010). In the United States and Canada, the disease is known as Hepatitis-Splenomegaly (HS) syndrome. The BLS was believed to be elicited by a separate virus, the BLS-virus (BLSV). However, it was demonstrated that the genomes of BLSV and avian HEV share 80 % nucleotide sequence similarity, showing that they are related strains of the same virus (Meng, 2010b). The virus is remotely related to the genotypes 1 – 4, having a nucleotide sequence identity over the full genome of 50 % (see Figure 1.7) (Haqshenas et al., 2001; Huang et al., 2004). Its genome is about 600 nucleotides shorter than the genotypes 1 – 4, equaling 6.6 kb in length (Huang et al., 2004). Avian HEV has antigenic epitopes in common with the genotypes 1 – 4, however several epitopes are unique to either avian HEV or the genotypes 1 – 4 (Guo et al., 2006). The identical epitopes make avian HEV a member of the same serotype as the mammalian human-pathogenic genotypes (Guo et al., 2006).

Avian HEV affects commercial broiler breeder hens as well as laying hens at 30 – 72 weeks of age (Peralta et al., 2009a; Meng, 2010a). The course of avian HEV infection is mostly subclinical. The avian HEV may cause a decrease in egg production that in some cases reached up to 20 %. The weekly case fatality rate may increase to 0.3 % for several weeks, sometimes exceeding 1 %, however, prior to death, clinical signs generally are not recognized (Meng, 2010a).

A serological survey in the United States based on the detection of anti-pORF2 IgG found 71 % of all chicken flocks and 30 % of all chickens positive (Huang et al., 2002b). However, these antibodies could also be found in healthy chickens (Sun et al., 2004a). Turkeys could be infected experimentally (Sun et al., 2004b). However it was not possible to infect rhesus monkeys, showing neither seroconversion nor fecal virus shedding, suggesting that avian HEV may be irrelevant for HEV epidemiology in mammals (Huang et al., 2004).

1.5.6 Novel HEV strains and hosts

Recently, novel strains of HEV, that are only distantly related to the strains of HEV already described, could be found. A new HEV-like virus that may represent a new genotype was identified in cutthroat trout (Batts et al., 2011). To date, this is the most distant strain of HEV (see Figure 1.7). Another most recent

finding is a putative novel genotype of HEV found in bats in Africa, Central America and Europe (Drexler et al., 2012). Furthermore, a virus closely related to rat HEV has been found in ferret (Raj et al., 2012). Unfortunately, these novel HEV strains are not classified by the International Committee on Taxonomy of Viruses (ICTV) yet.

Additionally, there exist several other reservoir hosts for strains closely related to GT 3 (Takahashi et al., 2004). Sika deer (*Cervus nippon*) and Yezo-deer (*Cervus nippon yesoensis*) positive for anti-HEV IgG were found in Japan (Matsuura et al., 2007; Sonoda et al., 2004; Tomiyama et al., 2009), while HEV-RNA could be found in roe deer (*Capreolus capreolus*), red deer and wild boar of Hungary (Forgách et al., 2010; Reuter et al., 2009). Both HEV-specific antibodies and RNA could be found in wild boar and red deer of the Netherlands (Rutjes et al., 2010) and in red deer of Spain (Boadella et al., 2010).

In Japan, 8 – 21 % of the tested mongoose were found positive for HEV-specific antibodies and viral sequences had been recovered (Li et al., 2006; Nakamura et al., 2006). Phylogenetic studies of HEV-sequences isolated from mongoose showed close similarity to GT 3 (Nakamura et al., 2006; Nidaira et al., 2012). Furthermore, HEV GT 3-specific nucleic acid could be detected in horse and rabbit in China (Zhang et al., 2008; Zhao et al., 2009), as well as in oyster in Korea (Song et al., 2010).

Furthermore, several studies reported serological evidence of HEV infection in numerous other animal species including dog (Arankalle et al., 2001; Liu et al., 2009; Zhang et al., 2008), cat (Song et al., 2010), goat (Sanford et al., 2012a; Zhang et al., 2008), cattle, buffalo, sheep, duck and pigeon (Shukla et al., 2007; Zhang et al., 2008). However, the significance of these exclusive serological findings is unclear.

1.6 Detection of HEV

For diagnosis of HEV in human or animal samples, either detection of viral RNA or detection of HEV-specific antibodies is feasible. Detection of RNA is accomplished by RT-PCR methods, amplifying genomic fragments in either of

the three ORFs (Adlhoch et al., 2009a; Huang et al., 2002a; Johne et al., 2010b; Jothikumar et al., 2006; Mansuy et al., 2004). Viral nucleic acid is present in the blood and stool of patients during the late prodromic phase and stays detectable in the feces for about 2 weeks (Aggarwal et al., 2000; Pavio et al., 2010). If the PCR-product is sequenced, it further allows determination of the HEV strain. Aside from blood and fecal matter, in experimental infections of swine, viral RNA could be detected in hepatocytes, Kupffer cells, bile epithelial cells and interstitial lymphocytes (Lee et al., 2009). Negative sense HEV-RNA could be detected in the small intestine, lymph node, colon and liver of swine, which is an indicator of active viral replication in these organs (Williams et al., 2001).

Serological methods are mostly based on detection of anti-HEV antibodies specific for pORF2 or pORF3. HEV elicits antibody responses of the immunoglobulin isotypes IgM, IgA and IgG in human and swine. HEV-specific IgM has an early onset and stays detectable for several weeks to months, which makes it ideal for clinical investigations and screening for recent or ongoing infections (Favorov et al., 1996). IgA has a similar onset but although being detectable in serum, it is not screened for in most studies (Chau et al., 1993; Osterman et al., 2013). IgG has its onset approximately two weeks later than IgM but stays detectable considerably longer, having been detected in human on some occasions 14 years after infection (Bryan et al., 2002; Khuroo et al., 1993; Myint et al., 2006). Laboratory diagnosis uses serum samples to detect anti-HEV antibodies with indirect enzyme-linked immunosorbent assays (ELISAs), western blot assays or line assays. For serological tests in domestic pig, meat juice may be a feasible alternative to serum (Wacheck et al., 2012b).

The proteins that most antibodies are directed against are pORF2 and pORF3, so these are utilized as antigens in most tests. The tests themselves base on a variety of genotypes and antigens: The pORF3 is small enough to be used in its entirety, while pORF2 is either used almost in its entirety or in small segments of the C-terminus, where several neutralizing epitopes are located (see Table 6.1) (Worm et al., 2002; Zhang et al., 2002). There are tests using short polypeptides of pORF2 or assembled VLPs (Baechlein et al., 2010; Li et al.,

2000). The antigen itself may be either synthesized chemically, expressed heterologously in *Escherichia coli* or in insect cells with the use of recombinant baculovirus. HEV constitutes of only one serotype and HEV-specific antibodies appear to be detectable with antigen of either of the four genotypes without significant loss of sensitivity. Nevertheless, all four genotypes as well as rat HEV are used as source of antigen in different tests (Arankalle et al., 2007; Herremans et al., 2007a; Li et al., 2011a; Obriadina et al., 2002).

Two of the most widespread commercial tests are the MP Diagnostics HEV ELISA kit (MP Biomedicals, Santa Ana, CA, USA; formerly Genelabs Diagnostics, Singapore), utilizing short C-termini of pORF2 and pORF3 of GT 1 and GT 2 as well as the Abbott HEV EIA (Abbott Diagnostics, Lake Forest, IL, USA), using the complete pORF3 and a significant portion of pORF2 of GT 1. Among the tests often used in European surveys is the HEV Ab-ELISA kit (Axiom, Bürstadt, Germany), which is a double-antigen sandwich ELISA basing on a significant portion of pORF2 of GT 1. The *recom*Line HEV (Mikrogen, Neuried, Germany) is another prominent test in European studies, a line assay using pORF3 and three segments of pORF2 of GT 1 and GT 3. The PrioCHECK HEV Ab porcine assay (Prionics, Schlieren-Zürich, Switzerland) is an indirect ELISA for IgG-detection in domestic pig and is based on pORF2 and pORF3 from both GT 1 and GT 3.

1.7 Prevention and therapy

Prevention of HEV infections is decisive as there exist experimental antiviral therapies only. The improvement of sanitation is the most effective method especially in developing countries, where general standards of hygiene are low and the morbidity and mortality of HEV and other water-related pathogens are elevated (Purcell and Emerson, 2008).

Developed countries need other methods of prevention as the autochthonous GT 3 and GT 4 are spread by a zoonotic transmission route. In these countries, efforts should be focused on protection of risk groups, such as veterinarians and slaughter house personnel (Meng et al., 2002).

As HEV is ingested with food, inactivation of the virus by sufficient heating is

also recommended. However, there exist several conflicting studies concerning the temperature and heating time necessary to inactivate HEV successfully. It was demonstrated that inactivation of the virus occurred when a contaminated porcine liver was boiled or stir-fried (191° C) for 5 min (Feagins et al., 2008a). Barnaud et al. (2012) reported that an internal temperature of 71° C for 20 min is necessary when using a similar setup. The opinions diverge even further when lower temperatures are considered, that are more common in household cooking. Some studies indicated that an incubation temperature of 56 – 60° C for 30 – 60 min may be sufficient (Emerson et al., 2005; Schielke et al., 2011), while others report remaining infectivity of virus incubated at 56° C (Feagins et al., 2008a; Tanaka et al., 2007). These contrary results could be caused by a diverse heat stability of the different viral strains that were used in these assays, as well as different matrices the virions were embedded in.

There are experimental antiviral treatments known, administering either ribavirin (Kamar et al., 2010b; Mallet et al., 2010) or pegylated interferon over a period of up to one year (Haagsma et al., 2010; Kamar et al., 2010a). As no large controlled and blinded trials have been done so far, the effect is not studied well enough to adopt or dismiss these potential treatments.

In 1994, an epidemiological study in Pakistan suggested that persons previously infected may be protected against re-infections by HEV (Bryan et al., 1994). It appears that pORF2 is the only protein eliciting protective antibodies (Ghabrah et al., 1998). The first tests aiming for a vaccine begun with re-challenge, as well as active and passive immunization in macaques (Arankalle et al., 1993; Tsarev et al., 1994a). Zhu et al. (2010) reported the results of a Phase III trial of the vaccine HEV 239 in China. 48,693 subjects received three doses of the vaccine, which prevented hepatitis E in the following 12 months with 100 % efficacy. This led to the approval of HEV 239 as the first vaccine against HEV by China's State Food and Drug Administration. It was developed by Xiamen Innovax Biotech and is sold exclusively in China under the brand name Hecolin (Wu et al., 2012). The vaccine is a bacterially expressed recombinant protein and consists of aa 368 – 606 of pORF2 of a GT 1 strain of Chinese origin. The protein forms VLPs of 27 nm in diameter when in neutral solution and contains at least two T-cell epitopes (Wu et al., 2007).

Chapter 2

Aim of the thesis

In recent years, HEV has been found in a number of animal reservoirs around the world. Domestic pig has been reliably identified as a reservoir. Autochthonous human cases in Germany and other developed countries are likely to occur by zoonotic transmission. It is widely believed that in most cases, the oral uptake of swine meat is the cause of infection, as several studies showed that the consumption of swine offal is a risk factor in numerous countries, including Germany. However, the prevalence and the risk of infection for the human population as well as livestock is still largely unknown. Furthermore, a variety of different methods of testing complicate the comparison of different epidemiological data.

Additionally, the novel genotype of rat HEV that was discovered recently in Norway rats, needs further attention, as its host, occurrence and route of infection are not investigated yet. Also, the prevalence and occurrence of HEV in rats has not been assessed sufficiently in Germany. Neither the significance of rat HEV as infectious agent, nor the significance of rats as carriers of HEV have been examined for their contribution to the HEV-epidemiology in livestock and human.

Therefore, the major objective of this study was to investigate the seroprevalence of HEV in human, swine and rat in Germany on the basis of the autochthonous genotypes GT 3 and rat HEV.

This objective comprises the following tasks:

- the establishment and validation of an indirect ELISA capable of detecting IgG antibodies against HEV GT3 in serum, utilizing a segment of pORF2 originating from GT 3.
- the establishment and validation of an indirect ELISA capable of detecting IgG antibodies against rat HEV in serum, utilizing the equivalent segment of pORF2 originating from rat HEV.
- the generation of hyperimmune sera of rat and rabbit for cross-reactivity studies and as positive controls, using both a segment of pORF2 originating from HEV GT 3 and rat HEV.
- the serological testing of an average human population and a risk group, both originating from Germany.
- the serological testing of commercial swine populations throughout Germany.
- the serological testing of urban rat populations from Germany.

Chapter 3

Publication I

Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs

Key information of the publication

- Establishment of a novel GT 3-based in-house ELISA.
- High seroprevalence in the German average human population (12 %).
- Identification of forestry workers as a risk group for HEV infection (21 % seroprevalence).
- First indication that rat HEV may be involved in human epidemiology.

Reprinted from Medical Microbiology and Immunology, 201, 2012, 189 – 200, Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs. Dremsek, P., Wenzel, J. J., Johne, R., Ziller, M., Hofmann, J., Groschup, M. H., Werdermann, S., Mohn, U., Dorn, S., Motz, M., Mertens, M., Jilg, W., Ulrich, R. G., Copyright 2012, with kind permission of Springer Science & Business Media.

Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs

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Keywords:
Hepatitis E virus, genotype 3, ratHEV, capsid protein, ELISA, forestry workers, blood donors, Germany

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Abstract

Hepatitis E virus (HEV) is the causative agent of an acute self-limiting hepatitis in humans. In industrialized countries, autochthonous cases are linked to zoonotic transmission from domestic pigs, wild boar and red deer. The main route of human infection presumably is consumption of contaminated meat. Farmers, slaughterers and veterinarians are expected to be risk groups as they work close to potentially infected animals.

In this study we tested four *Escherichia coli*-expressed segments of the capsid protein (CP) of a German wild boar-derived HEV genotype 3 strain for their diagnostic value in an indirect immunoglobulin G (IgG) ELISA. In an initial validation experiment a carboxy-terminal CP segment spanning amino acid (aa) residues 326- 608 outperformed the other segments harbouring aa residues 112–608, 326–660 and 112–335. Based on this segment an indirect ELISA for detection of anti-HEV IgG antibodies in human sera was established and validated using a commercial line immunoassay as reference assay. A total of 563 sera from forestry workers of all forestry offices of Brandenburg, eastern Germany and 301 sera of blood donors from eastern Germany were surveyed using these assays. The commercial test revealed seroprevalence rates of 11% for blood donors and 18% for forestry workers. These rates are in line with data obtained by the in-house test (12% and 21%). Hence, the in-house test performed strikingly similar to the commercial test (sensitivity 0.9318, specificity 0.9542). An initial screening of forestry worker and blood donor sera with a corresponding CP segment of the recently discovered Norway rat-associated HEV revealed several strong positive sera exclusively in the forestry worker panel. Future investigations have to prove the performance of this novel IgG ELISA in large scale seroepidemiological studies. In addition, the observed elevated seroprevalence in a forestry worker group has to be confirmed by studies on groups of forestry workers from other regions. The epidemiological role of ratHEV in human disease should be assessed in a large-scale study of risk and non-risk groups.

Introduction

Hepatitis E virus (HEV) is the causative agent of an acute, usually self-limiting hepatitis. The primary route of faecal-oral transmission is via contaminated drinking water. Hepatitis E is a major public health concern to people in developing countries in Asia and Africa that are usually affected by large outbreaks [1]. Nevertheless, there are increasing numbers of reports of sporadic autochthonous HEV infections in developed countries including the USA, Japan and different European countries [2]. The typical course of the disease is characterized by hepatomegaly, jaundice, fever, anorexia, nausea and abdominal pain. Usually the viremia is transient and occurs mainly during the prodromic phase, followed by faecal excretion of the virus [3]. However, in developed countries most cases of hepatitis E are believed to have a subclinical course [4]. In general, the case fatality rate of hepatitis E is low with 1 to 4% but higher than that of hepatitis A [5].

HEV is the only member of the genus *Hepevirus* of the family *Hepeviridae* [6,7]. The spherical non-enveloped virion has a diameter of approximately 27-34 nm. Using heterologously expressed virus-like particles the capsid was demonstrated to have an icosahedral (T=3) symmetry [8]. The viral genome is a single-stranded RNA of positive polarity and about 7.2 kb length (genotype 3i strain wbGER27 7,222 nucleotides (nt); [9]; GenBank accession number FJ705359.1). The genome is flanked by a 5´ untranslated region (UTR), capped at the 5´-end with 7- methylguanosine (m7G) and a 3´ UTR followed by a polyadenylated tail. It contains three partially overlapping open reading frames (ORF1, ORF2 and ORF3). ORF1 is the largest open reading frame of 5,112 nt encoding a polyprotein of 1,703 amino acids (aa) (wbGER27) with various enzymatic functions [10]. ORF2 of 1,983 nt encodes a capsid protein of 660 aa (wbGER27) representing the immunodominant protein mainly used for serodiagnostics [11]. The ORF3 of 369 nt encodes a small cytoskeleton associated phosphoprotein of 122 aa (wbGER27). It represents a viral accessory protein which is likely to affect the host response to infection [10].

In general, HEV can be subdivided phylogenetically into four genotypes and several subgenotypes: Genotypes 1 and 2 are believed to be present only in humans with genotype 1 found in Asia and Africa and genotype 2 in Mexico and Africa. Genotype 3 seems to have a world-wide distribution with current detection in Asia, Europe, Oceania, North and South America, whereas the occurence of genotype 4 seems to be limited to Asian countries [12]. Interestingly, in contrast to genotypes 1 and 2, genotypes 3 and 4 represent zoonotic pathogens. Wild boar and domestic pigs are thought to be the major reservoirs [13]. There are several lines of evidence in support of this assumption. Firstly, sequence similarities of HEV strains from human patients and from swine suggested an epidemiological association [13]. Experimental infection studies revealed the susceptibility of domestic pigs for human strains as well as of primates for swine strains [13]. Further, direct molecular epidemiological evidence for food-borne HEV transmission to human confirmed the zoonotic nature of this agent and swine and sika deer as a reservoir [14, 15]. Moreover, HEV was molecularly detected in other mammals, including mongoose, cattle and sheep, and serologically in several further mammals [16]. Besides genotypes 1–4, other HEV strains with limited nt sequence similarity have been found in wild boar [17, 18], rabbits [19], rats [20] and chicken [21-23]. Avian HEV strains largely differ from the mammalian strains, suggesting that they might represent a separate genus [23]. The determination of the complete genomic sequence of ratHEV suggested that these strains represent a novel genotype, however, the zoonotic potential of this virus remains unclear [24]. The recent identification of a HEV-related agent in different fish species raised major questions on the evolution and host adaptation of HEV [25].

Due to the short-termed viremia, laboratory diagnosis of HEV infections in humans is mainly based on serological assays. Immunoglobulin (Ig) class M antibodies can be detected a few days after the onset of clinical symptoms, but usually disappear within 4–5 months. IgG-class antibodies appear several days later but remain

detectable for up to 14 years [26]. Serological assays have been developed using recombinant ORF2- and ORF3-derived proteins expressed in *E. coli*, yeast, baculovirus-infected insect cells and mammalian cells. Alternatively, synthetic peptides or chimeric constructs harbouring multiple epitopes have been applied. These assays comprise ELISA and line immunoassay formats with several commercial tests available [11].

In Germany, a total of 910 human hepatitis E cases have been notified since the introduction of the Federal Protection against Infection Act in 2001 (Robert Koch- Institut, SurvStat, www.rki.de; data as of September 14, 2011). Several autochthonous human cases have been reported since 2006 [27-29]. A phylogenetic and case-control study confirmed the presence of autochthonous human infections with HEV genotype 3 [30]. Recent studies indicated a risk of chronic HEV infection during immunosuppression after solid organ transplantation or acute lymphoblastic leukaemia [31-33]. An initial molecular study in archived wild boar samples demonstrated the presence of HEV for at least ten years in Germany [34]. Further molecular biological investigations on wild boar tissue samples confirmed a broad geographical distribution of HEV and resulted in the identification of different circulating genotype 3 subtypes [9, 34, 35]. Also, the consumption of undercooked wild boar meat has been identified as a risk factor for autochthonous HEV infections [30]. The recently detected sequence similarity of HEV sequences found in porcine livers from retail markets and in patients from the same geographical region suggested the importance of undercooked pig products in food as a source of zoonotic HEV infection for humans in Germany [36].

Here we describe the development and validation of a novel indirect HEV genotype 3-based IgG ELISA and its application for a seroepidemiological study in forestry workers and blood donors from eastern Germany. In addition, these serum panels were investigated in parallel in an IgG ELISA with a corresponding ratHEV-derived recombinant antigen.

Materials and Methods

Human serum samples

In 2008, a panel of 563 serum samples was collected from 499 male and 64 female forestry workers from ten different forestry districts in the federal state of Brandenburg, eastern Germany (Fig. 1; [37]). All participants provided informed consent. The control group comprises 301 serum samples of healthy blood donors from the blood donation unit of the Charité University Hospital Berlin. The donors are residents of the federal states Berlin and Brandenburg.

Cloning of HEV capsid protein-encoding sequences, expression and purification of the recombinant HEV capsid protein derivatives

For expression in *E. coli* a modified pET-19b vector was generated by substituting the original BamHI/NcoI fragment by an oligonucleotide duplex harboring the translation initiation codon, 10 histidine codons and a unique SpeI restriction site (Fig. 2A). The entire ORF2 and 5´ and 3´ truncated segments thereof, taken from a German wild boar-derived HEV genotype 3i [9], were amplified by RT-PCR using specific primers harbouring an XbaI restriction site overhang and inserted into the vector pCR-TOPO2.1 (Invitrogen, Darmstadt, Germany) (Fig. 2B). The inserts with the correct sequences were then subcloned into the SpeI-linearized modified pET- 19b vector (Fig. 2B). In parallel a pET-19b construct encoding aa residues 315-599 of the capsid protein of ratHEV strain R4 was generated ([20]; Johne, Dremsek et al., submitted). The entire coding sequence of the non-structural protein 1 (NS1) of West Nile virus (WNV) was amplified from cell culture supernatant of the New York flamingo isolate of 1999 (accession number AF196835). The amplification product obtained by RT-PCR using primers adding a 5' NdeI restriction site and a 3' BamHI restriction site was inserted into a non-modified NdeI

and BamHI cleaved pET-19b vector (Johne, Dremsek et al., submitted). The different expression plasmids were retransformed for heterologous expression into *E. coli* strain BL21 (DE3) (Novagen Merck KGaA, Darmstadt, Germany). The synthesis of the recombinant proteins was induced by addition of 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For determination of an expression kinetics, 50 ml cultures of the recombinant bacteria were induced at an OD600 value of 0.5. Samples of 1 ml each were drawn at timepoints 0 min,15 min, 30 min, 60 min, 90 min, 120 min, 150 min, 3 h, 4 h and after overnight incubation. The bacteria were pelleted and resuspended in 50 µl buffer containing 0.5 g/l SDS and 0.5% Triton X-100. The samples were mixed with 50 µl loading buffer, boiled and 5 µl were applied on an SDS-PAGE. The purification of the His-tagged capsid protein derivatives was performed with the Ni-NTA chromatography system under denaturing conditions according to the protocol of the manufacturer (Qiagen, Hilden, Germany).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The synthesis and the purity of the recombinant CP derivatives was analysed by SDS-PAGE using 12.5 or 13% gels stained by Coomassie brilliant blue. For Western blot analysis the proteins separated by SDS-PAGE were blotted onto a PVDF membrane. The membrane was blocked in 5% skim milk overnight at 4 °C and incubated for 1 h at room temperature (RT) in anti-His monoclonal antibody (mAb) (Novagen, Merck KGaA, Darmstadt, Germany) diluted 1:2,500 in PBS-Tween 20. The antigen-antibody reaction was detected by adding horse-radish peroxidase (HRP) labeled anti-mouse Ig (Dako Cytomation, Glostrup, Denmark), diluted 1:2,000, visualized by adding ECL reagent and hydrogen superoxide (Amersham, GE Healthcare, UK) and documented using a VersaDoc Model MP 5000 Imaging-System (Bio-Rad, Munich, Germany).

Commercial serological assay

To validate the in-house ELISA, a commercial *recom*Line HEV IgG test (Mikrogen, Neuried, Germany) was used. This test is based on HEV genotype 1 and genotype 3 antigens. The assay was performed and read according to the manual of the manufacturer. In brief, 20 µl of serum was added to each test strip immersed in 2 ml dilution buffer and incubated for 1 h at RT. The test strips were washed three times. Incubation with HRP conjugate solution was carried out for 45 min at RT. After additional washing the kit substrate solution (1.5 ml) was added. The reaction was stopped as soon as the cut-off control band was clearly visible (6–10 min). Interpretation of test results was performed densitometrically by means of the recomScan software. The final test result (neg., borderline, pos.) was reported based on the sum of predefined "points" assigned to each positive test strip band.

In-house indirect IgG ELISA

In an initial setting four different recombinant HEV GT-3 CP derivatives were tested according to a standard protocol previously established for investigations of human sera with recombinant hantavirus antigen [38]. This standard protocol was adopted for detection of anti-HEV IgG antibodies using a serum pool of five forestry workers found positive and a second serum pool of five forestry workers found negative in the reference assay. During this optimization process for all constructs, different types of microtiter plates and different concentrations of the capsid antigen as well as of the serum pools were assayed. Thereafter further optimization was done with WB-Ctr (see Fig. 2B) alone, altering the concentrations of the secondary antibody, different incubation times for the secondary antibody as well as different bovine serum albumin (BSA) concentrations in blocking. The estimation of the best protocol was accomplished by judging the ratio of the results for the anti-HEV positive and negative serum pools. The final protocol is based on a 1 h coating at 37 °C with 100 µl of 1 µg/ml protein in 0.05

M carbonate buffer (pH 9.8) per well, 1 h blocking at RT with 200 µl of PBS containing 3% BSA and 0.05% Tween 20, 1 h incubation at 37 °C with 100 µl serum diluted 1:400 in PBS with 1% BSA and 0.05% Tween 20, and a final 1 h incubation at 37 °C with 100 µl of HRP-conjugated anti-human IgG (rabbit polyclonal, P0214, Dako, Glostrup, Denmark) diluted 1:6,000 in PBS with 1% BSA and 0.05% Tween 20. The enzyme reaction was performed using 100 µl 3,3',5,5'- Tetramethylbenzidine (Peroxidase EIA Substrate Kit, Bio-Rad, Hercules, California, USA) as substrate, stopped by adding 100 µl 1M H_2SO_4 and visualized at 450 nm. The same protocol was used for the ratHEV-derived antigen and the negative control antigen.

Determination of the cut-off value for the in-house indirect IgG ELISA

For the evaluation of the ELISA we used the commercial line assay as a reference method. To find a variable, plate-dependent cut-off value based on the positive and negative control serum pools, a deviation of the receiver operating characteristic (ROC) curve was evaluated by several methods. To this end, cut-off values being defined as percentage of the positive control, of the negative control with or without the blank value substracted, a summand added to the negative control with or without the blank value substracted and the cut-off of the index ((reading-neg)/(posneg)) were tested. The optimal method of cut-off calculation was determined by the area under curve (AUC) value. The optimal cut-off value was selected based on the minimum ROC-distance, i.e. the distance between the curve and the upper left corner of the graph.

Statistical analysis

The statistical significance of the difference of the antigenic efficacy as ELISA coat as well as the significance regarding sex and risk-group/control-group affiliation were examined using a Wilcoxon rank sum test with continuity correction. The analysis of the ELISA results from the different forestry districts were done with a two-sided Fisher's Exact Test. All calculations were performed using R, version 2.13.0 (2011- 04-13) [39].

Results

Expression, purification and characterization of HEV capsid protein derivatives

An initial approach to express the entire ORF2 of HEV genotype 3i strain wbGER27 failed. The expression of the entire ORF2 was neither detected in crude lysates in the stained SDS-PAGE nor by Western blot analysis using the His-tag-specific mAb independently of the induction time. In addition, a nickel chelate affinity purification approach also failed to enrich the entire capsid protein (data not shown). Therefore, four truncated, partially overlapping segments of the ORF2-encoding region were generated (Fig. 2B). The analysis of total lysates from *E. coli* transformed with the four recombinant plasmids revealed the synthesis of the CP derivatives WB-tr, WBNtr, WB-C and WB-Ctr (see Fig. 2B) corresponding to their respective molecular weights. A subsequent Western blot analysis of these four crude lysates with a Histag- specific mAb confirmed the authenticity of these recombinant proteins with an amino-terminal His-tag (data not shown). The main peak of protein synthesis was detected by an expression kinetics approach after a 3 h induction with IPTG for all four proteins (data not shown). These recombinant proteins were found to be insoluble in inclusion bodies, but were solubilised in 8 M urea prior to purification. Separating the inclusion bodies from the soluble proteins by centrifugation followed by a single step of nickel chelate affinity chromatography and elution in a low pH buffer and imidazole resulted in highly purified proteins of the expected molecular weights (Fig. 3).

ELISA development and validation

To identify the most useful recombinant antigen for serodiagnostics, all four purified antigens and a negative control antigen, i.e. WNV NS1 protein, were tested for their reactivity with an anti-HEV positive and a negative serum pool. As expected, the ratio of the OD values for the positive and negative serum pool was close to 1 for the negative control antigen (Fig. 4). The truncated amino-terminal CP derivative behaved similarly, i.e. it was not able to discriminate between the positive and negative pools. The almost entire CP (WB-tr) and the carboxy-terminal derivatives (WB-C and WB-Ctr) performed better with significant differences to the negative control antigen and to each other (Fig. 4). The WB-Ctr antigen demonstrated an average ratio of 10.6 (range 8.1-14.1) of the OD values for the positive and the negative serum pool and was therefore selected for subsequent investigations.

To determine the performance of the novel in-house IgG ELISA, 555 serum samples from forestry workers and 298 serum samples from blood donors were screened in parallel by this assay and the commercial *recom*Line HEV IgG assay serving as reference test. For the majority of anti-HEV-positive and -negative forestry worker (Table 1) and blood donor sera (Table 2) the results were concordant. A modified ROC curve analysis using seven different approaches resulted in AUC values of 0.9774 to 0.9499 and corresponding sensitivities of 0.9167 to 0.9318 and specificities of 0.9667 to 0.9376 (Fig. 5). Based on the method yielding the highest AUC value the variable cut-off value was defined at 5.425% of the difference of the positive control and the negative control resulting in a sensitivity of 0.9318 and a specificity of 0.9542 (Fig. 5A).

HEV seroprevalence of the forestry worker and blood donor panels

The average seroprevalence for the forestry workers reached 17.8% and 21.4% when using the commercial and the in-house test, respectively. A more precise analysis demonstrated that in all ten forestry districts anti-HEV positive individuals were detected, independently of the test used (Table 3). The prevalences for the forestry districts ranged from 5.6% (Doberlug-Kirchhain, *recom*Line) and 9.1% (Peitz, in-house test) to 25% (Alt Ruppin, both tests), 26.8% and 28% (Belzig and Wünsdorf, in-house test).

When compared to the forestry worker panel, for the blood donor panel lower seroprevalences of 11.1% (*recom*Line) and 12.3% (in-house test) were determined. The seroprevalences for male blood donors were almost identical for both assays (12.1% versus 12.9%), whereas the values for female blood donors varied between 7.5% for the *recom*Line assay and 10.3% for the in-house ELISA. Independently from the test used, the seroprevalence for male subjects was slightly higher than for female subjects, but not of statistical significance in both tests and with all populations (blood donors, forestry workers or both). The analysis of age and anti - HEV prevalence revealed no significant association of these two factors, regardless of the test and of the population viewed (blood donors, forestry workers or both) (Fig. 6).

Reactivity of the forestry worker and blood donor serum panels with a recombinant ratHEV antigen

In addition to the IgG ELISA analysis of the serum panels with the genotype 3i HEV antigen, the sera were screened in parallel with the corresponding ratHEV-derived antigen. This antigen was found to be expressed in *E. coli* at high level and could be readily purified in the same manner as the genotype 3i antigen (Johne, Dremsek et al., submitted). Interestingly, in the forestry worker panel several sera with an almost exclusive and relatively strong reactivity with the ratHEV CP derivative were observed (Fig. 7A). On the other hand, only a very few of the blood donor sera were found to be very weakly reactive with the ratHEV antigen (Fig. 7B).

Discussion

Here we report on the diagnostic value of different segments of the CP of HEV genotype 3i. Due to failure in expression of the entire ORF2 in the *E. coli* expression system, carboxy- and amino-terminal segments of the CP were generated in *E. coli*. In line with previous investigations [40-42], the carboxy-terminal segments spanning aa residues 326–608 and 326–660 were found to be highly reactive with a pool of anti-HEV positive human sera. Additionally, immunodominant antigenic regions were previously found in this region using peptide-based assays [43, 44]. The observation of the strong antigenicity of the carboxy-terminal region is also in line with the localisation of the protruding domain in the CP structure between aa 456 and aa 606 [45] and the localization of the immunodominant virus-neutralizing epitope inside this part of CP [46]. Peptide scanning demonstrated a large number of linear epitopes in the carboxy-terminal region, but linear epitopes were found to be scattered throughout the entire protein [47]. However, the amino-terminal segment harbouring aa 112–335 was not able to discriminate between the seropositive and seronegative pools.

Therefore an indirect IgG ELISA was developed based on the most reactive carboxy-terminal segment. When testing two different serum panels, a panel of forestry workers and a panel of blood donors, the performance of the novel in-house assay was similar to that of the commercial reference test used. Based on the ROC-mediated definition of a cut-off value the sensitivity and specificity of the novel assay was determined to be 0.9318 and 0.9542, respectively. Thereby, this novel genotype 3-based IgG ELISA performs comparably to other serological assays developed using *E. coli*-expressed [48] or baculovirus-expressed ORF2 antigens [49, 50].

The seroprevalences of 11.1% and 12.3% observed in the blood donor panel were surprisingly high, but seem to be in the same range as recent studies found in Germany [51, 52, 66]. The similar values obtained for this panel using two different assays might clearly argue against a large number of false-positive sera, as discussed recently [11]. Similarly high seroprevalences in the average population were previously reported for other non-epidemic regions in Europe, i.e. in Denmark with 20.6% [53], and the UK with 16% [54]. However, for some non-epidemic countries, i.e. The Netherlands (1.1%; [55]), Italy (1.0%; [56]), Switzerland (3.2%; [57]), Spain (2.8%; [58]), USA (1.1%; [59]) and Japan (5.3%; [60]) rather low prevalences were reported. These conflicting findings underscore the need for developing standardized serological assays for comparative analyses of HEV seroprevalences. The rather high seroprevalences found in this study on one hand and the low number of notified hepatitis E cases in Germany on the other might indicate a high number of infections with mild or unspecific symptoms that are therefore not recorded. In addition, this finding raised important questions on the transmission route of HEV genotype 3. Therefore, additional epidemiological studies have to prove the role of transmission routes other than oral uptake of contaminated food, blood transfusion and organ transplantation.

The seroprevalence in the forestry worker group (18% and 21%) was found higher than that of the blood donor control group (11% and 12%). This difference was found to be significant ($p<0.01$), but both groups are not sufficiently matching in sex, age and residence (see Fig. 6). Nevertheless, these findings still confirm a HEV seroprevalence in the investigated part of Germany of more than 10%. In the past, forestry workers have not been considered as a typical risk group for HEV infection. However, hunting activities are usually included in the professional work of forestry workers, including the group investigated in this study. This risk might be especially taken into account if the HEV prevalence in the wild boar population at site is high, as previously reported for Brandenburg [35, 9]. These findings may also underline other transmission routes for HEV such as direct contact to wild boar blood or feces or their aerosols. Another recent study in Germany has reported an increased HEV seroprevalence in slaughterers [52]. Studies in other European countries and the USA have also demonstrated increased HEV seroprevalences in farmers and veterinarians exposed to swine (for references see [53, 61, 62]) .

On the other hand, other investigations did not find differences in seroprevalence between risk and non risk

groups [63, 64] emphasizing the above mentioned need for test harmonization. Recently, a scoping study which used systematic review/meta-analysis methodology confirmed a significant association between occupational exposure to swine and human HEV IgG seropositivity in 10 out of 13 cross-sectional studies [65]. Interestingly, a recent study demonstrated a HEV seroprevalence of 30% in psychiatric patients that was ascribed to patients of addiction therapy, including alcohol, benzodiazepine and intravenous drug addicts [66].

In our study we did not find a statistically significant association of seropositivity with age and sex in neither group and independently of the test used. Until now there are controversial findings on the influence of sex and age on the level of the seroprevalence [2]. Thus, a recent study found in a group older than 40 years a significantly higher prevalence than in a group younger than 14 years, but no significant differences when analysing age groups step by step [66]. Interestingly, 62.5% of the notified hepatitis E cases in Germany were found in men (Robert Koch- Institut, SurvStat, www.rki.de; data as of September 14, 2011). Future studies should prove if sex-hormone-mediated factors or alcohol abuse may influence the outcome of HEV infections.

A parallel analysis of forestry worker and blood donor sera in the genotype 3i- and a similar ratHEV-IgG ELISA suggested for the first time the occurrence of human infections by ratHEV or a related agent in a few cases. The two recombinant antigens used for this serological investigation shared a total aa sequence identity of 54% with an identity of 45% in the protruding domain [45] indicating antigenic differences in the CP of genotype 3 and ratHEV. Recent investigations of hyperimmune sera from rats demonstrated clear antigenic differences between both genotypes, at least for the antigen segment used (Johne, Dremsek et al., submitted). Cross-protection studies will help to determine if ratHEV belongs to the same single serotype with the four major genotypes of HEV. Future studies in risk groups, such as pest management or sewage workers, and control groups have to further elaborate the potential of ratHEV to cause human infections and disease. In addition, this finding again raises the question of the transmission route of ratHEV or a related virus to humans.

In conclusion, a novel genotype 3-based ELISA was developed and showed similar sensitivity and specificity as compared to a commercial reference test. Application of the novel assay and the reference test resulted in the detection of HEV-specific antibodies in blood donors and forestry workers from eastern Germany with an unexpected high frequency. An initial analysis of both groups with a similar ratHEV-based ELISA demonstrated for the first time the occurrence of human infections. Future larger seroepidemiological studies in non-risk and risk groups should further investigate the epidemiological importance of ratHEV.

Acknowledgements

We would like to thank Alexandrina Schmidt, Ute Wessels, Thomas Büchner, Maya Gussmann, Monika Ehrl and Bianka Ehrlich for excellent technical assistance. The purified recombinant WNV NS1 protein was kindly provided by Katja Schmidt. This work was funded in part by the German Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) through the Federal Institute for Agriculture and Nutrition (BLE), grant numbers 07HS026 and 07HS0271 (contract no.: 506122) and the Grimminger-Stiftung. PD acknowledges support by the Förderverein of the Friedrich-Loeffler-Institut.

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Figure legends

Fig. 1 Schematic map of Germany (A) and location of the 10 forestry districts of the federal state of Brandenburg, eastern Germany (B).

Fig. 2 Schematic presentation of the modified expression vector pET-19b (A) and the encoding sequences of the hepatitis E virus capsid protein along the genome of genotype 3i strain wbGER27 (B).
The pET-19b vector carries a phage T7-derived promoter (PT7) and transcription terminator (TT7) upstream and downstream of the insertion site, respectively. The original pET-19b vector was modified by insertion of an oligonucleotide duplex harboring a translation initiation codon, ten histidine codons (His10) and a unique SpeI restriction site into the BamHI/NcoI digested vector. Met, methionine; His, histidine; UTR, untranslated region.

Fig. 3 Analysis of the purified *E. coli*-expressed capsid protein derivatives, the truncated almost complete capsid protein (WB-tr), the truncated amino-terminal part of capsid protein (WB-Ntr), and the carboxy-terminal part (WB-C) and truncated carboxy-terminal part (WB-Ctr) of the capsid protein of genotype 3i strain wbGER27 in a Coomassie blue-stained 13% SDS-polyacrylamide gel. The West Nile virus (WNV), strain New York, non-structural protein 1 (NS1) expressed in the same heterologous system and purified in the same way was used as a control antigen.
M, molecular weight marker (PageRuler unstained SM0661, Fermentas). The molecular weights given above the recombinant protein bands were predicted using a web-based calculator (http://www.bioinformatics.org/sms/prot_mw.html).

Fig. 4 Comparison of the reactivity of the purified capsid protein derivatives with anti-HEV positive and negative human serum pools in ELISA.
Five sera each found to be negative or positive in the reference assay were pooled and used for analysis in the indirect ELISA using the truncated almost complete capsid protein (WB-tr), the truncated amino-terminal part of capsid protein (WB-Ntr), and the carboxy-terminal part (WB-C) and truncated carboxy-terminal part (WB-Ctr) of the capsid protein of genotype 3i strain wbGER27. For determination of the antigen with the strongest specific reactivity the ratio of the corrected OD values of the positive pool and the negative pool was calculated. The columns show the mean value and standard error of the mean for five replicates. Statistically significant correlations are marked with an asterisk ($p<0.01$).

Fig. 5 Determination of the optimal variable cut-off value for the genotype 3i wbGER27 capsid protein derivative based indirect ELISA by a modified receiver operating characteristic (ROC) analysis using three different approaches. The graphs show the performance of the novel in-house test (and its sensitivities and specificities at the cut-off of the minimal ROC distance) when the cut-off value is defined as percentage of the difference of the positive control and the negative control (A), as the percentage of the positive control (B) and by finding a factor of the negative control (C). The selection of the optimal cut-off value for the further investigations (as given in A) was based on the highest area under curve (AUC) value.
pos, positive; neg, negative; fact, factor.

Fig. 6 Ratios of anti-HEV-positive and -negative male forestry workers (A,E) and female forestry workers (B, F) and anti-HEV-positive and -negative male blood donors (C,G) and anti-HEV-positive and -negative female blood donors (D,H) according to age based on the analyses with the *recom*Line reference assay (A-D) and the novel

in-house assay (E-H). White segments of the columns represent seronegative subjects, black segments represent seropositive subjects.

Fig. 7 Reactivity of forestry worker (A) and blood donor sera (B) with corresponding recombinant capsid protein derivatives of genotype 3i (WB-Ctr, Y-axis) and ratHEV (R-Ctr, X-axis) in the ELISA. For each serum sample a parallel analysis in both ELISAs was performed. The values depicted represent percentage-values of the corrected OD values in relation to the positive anti-HEV-GT3i and anti-ratHEV control set to 100%. The black vertical line denotes the percentage (26.0%) of the blooddonor sample with the strongest reactivity with ratHEV antigen.

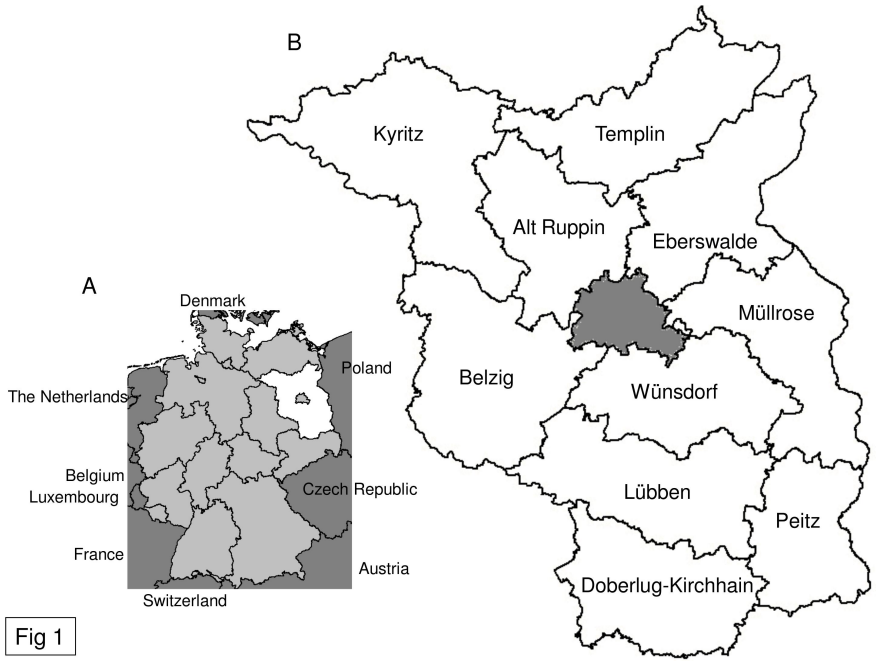

Fig 1

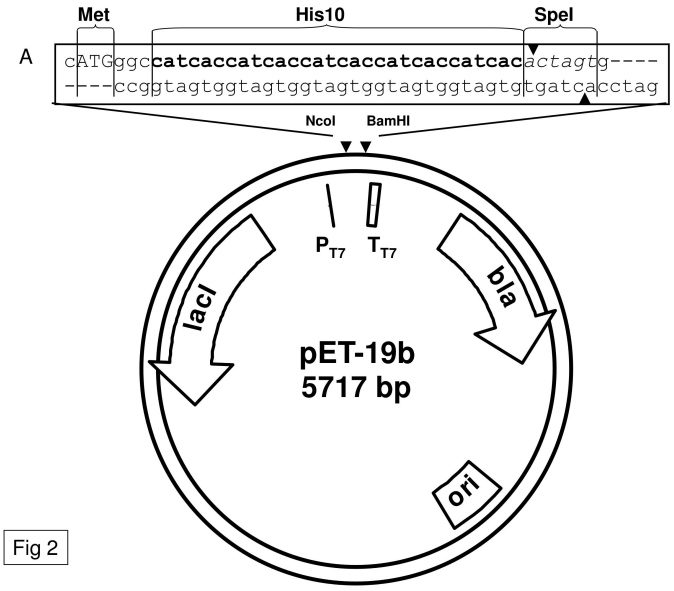

Fig 2

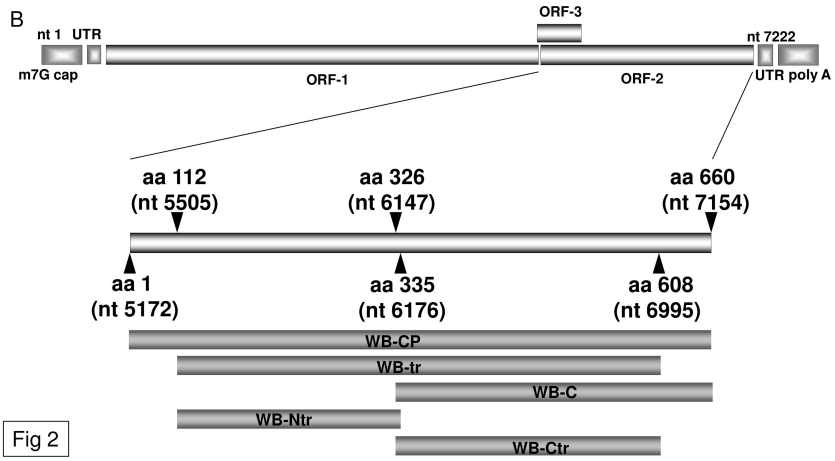

Fig 2

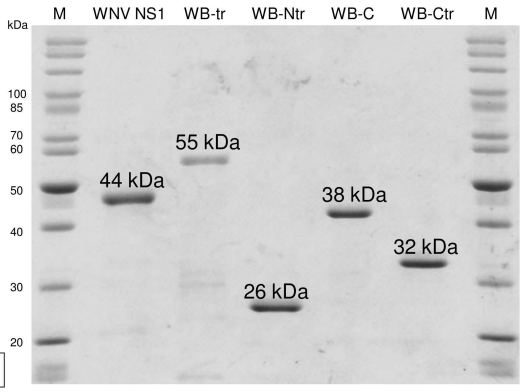

Fig 3

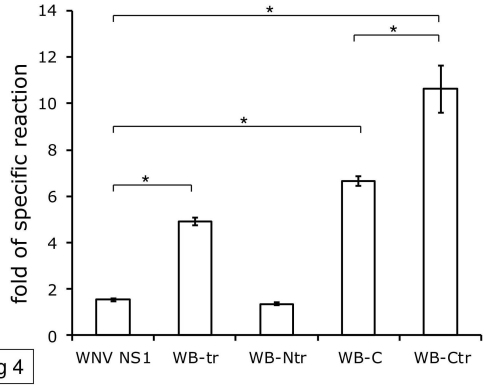

Fig 4

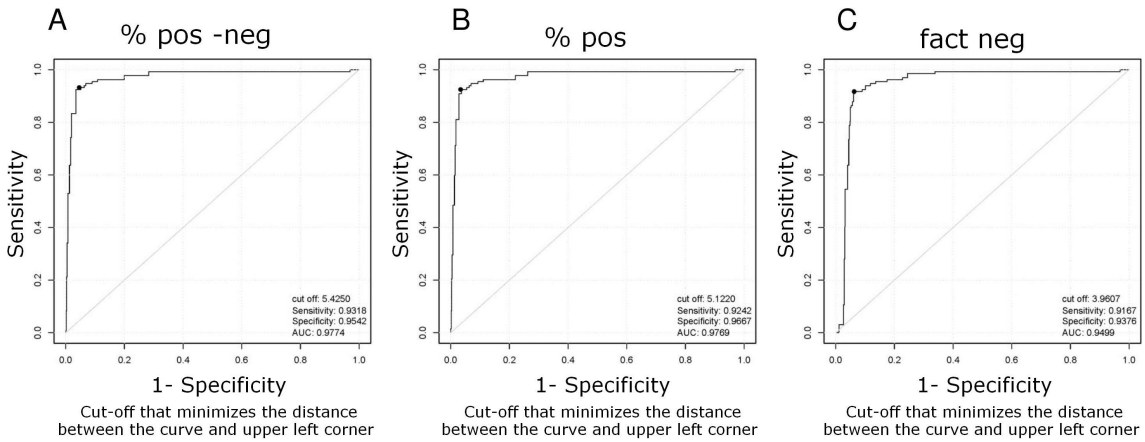

Fig 5

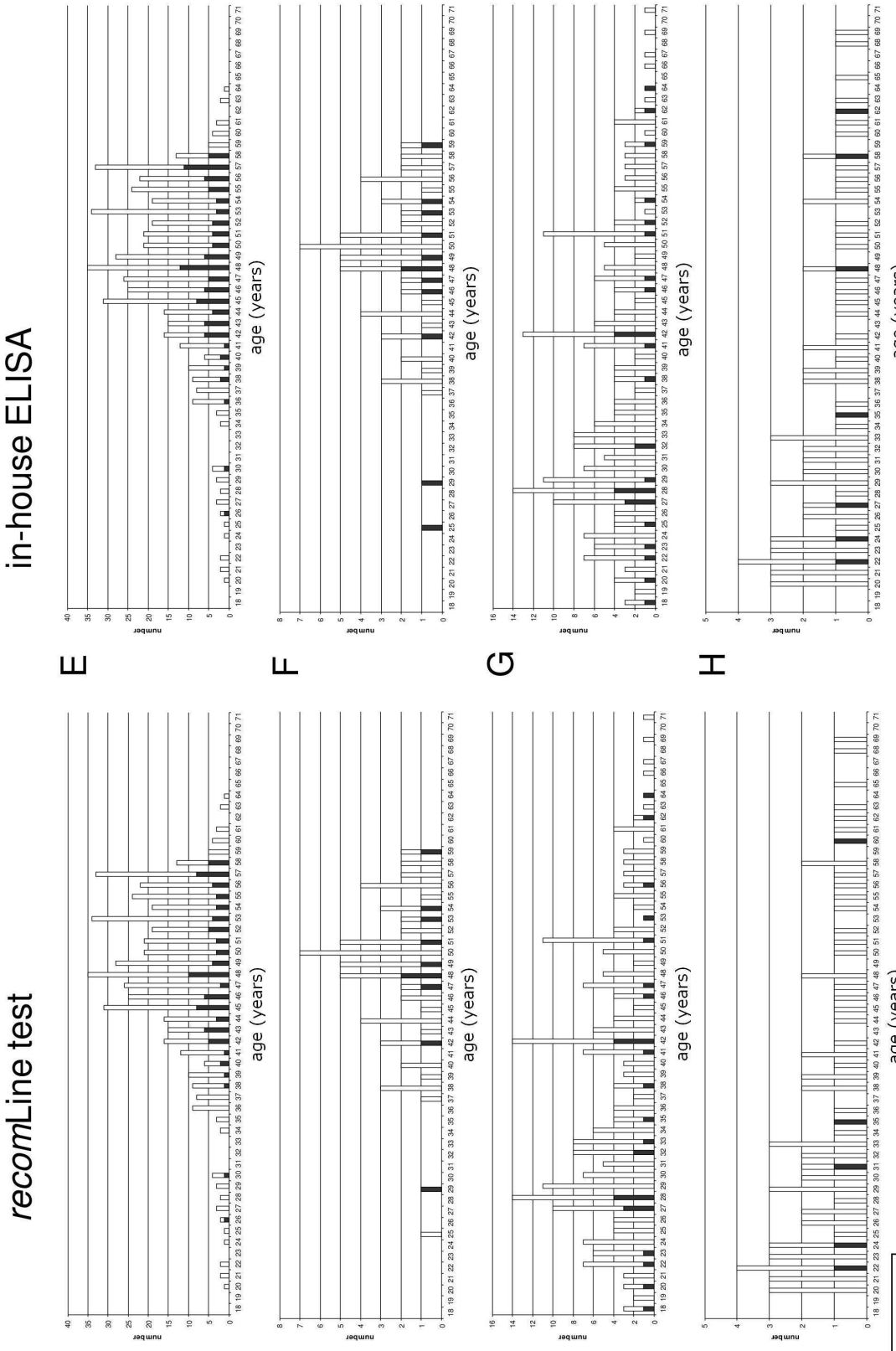

Fig 6

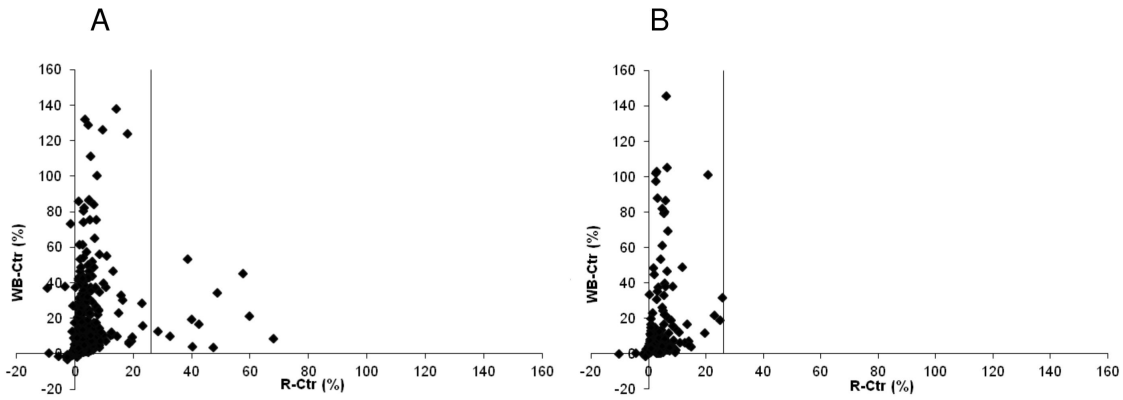

Fig 7

TABLE 1. Comparison of the reactivity of the forestry worker sera with the novel in-house IgG ELISA and the commercial *recom*Line assay.

		Results of novel in-house IgG ELISA		
		positive	negative	total
Results of commercial IgG line immunoassay	positive	**96**	3	99
	negative	23	**433**	456
	total	119	436	555*

*8 additional sera showed a borderline reactivity in the reference assay and were therefore not included in further investigations.

TABLE 2. Comparison of the reactivity of the blood donor sera with the novel in-house IgG ELISA and the commercial *recom*Line assay.

		Results of novel in-house IgG ELISA		
		positive	negative	total
Results of commercial IgG line immunoassay	positive	**27**	6	33
	negative	10	**255**	265
	total	37	261	298*

*3 additional sera showed a borderline reactivity in the reference assay and were therefore not included in further investigations.

TABLE 3. Number of seroreactive samples from female and male forestry workers from the 10 different forestry districts in Brandenburg, eastern Germany.

	Male forestry workers: Number of seropositive samples/total number of investigated samples		**Female forestry workers: Number of seropositive samples/total number of investigated samples**		**Total forestry workers: Number of seropositive samples/total number of investigated samples**	
Forestry District	***recom*Line assay**	**novel in-house ELISA**	***recom*Line assay**	**novel in-house ELISA**	***recom*Line assay**	**novel in-house ELISA**
Alt Ruppin	8 / 28	8 / 28	0 / 4	0 / 4	8 / 32 25.0 %	8 / 32 25.0 %
Doberlug-Kirchhain	1 / 31	3 / 31	1 / 5	1 / 5	2 / 36 5.6 %	4 / 36 11.1 %
Belzig	15 / 62	16 / 62	2 / 9	3 / 9	17 / 71 23.9 %	19 / 71 26.8 %
Wünsdorf	19 / 82	24 / 82	4 / 18	4 / 18	23 / 100 23.0 %	28 / 100 28.0 %
Lübben	10 / 45	11 / 45	0 / 0	0 / 0	10 / 45 22.2 %	11 / 45 24.4 %
Kyritz	8 / 41	9 / 41	0 / 4	0 / 4	8 / 45 17.8 %	9 / 45 20.0 %
Peitz	4 / 50	5 / 50	0 / 5	0 / 5	4 / 55 7.3 %	5 / 55 9.1 %
Eberswalde	13 / 71	17 / 71	1 / 5	1 / 5	14 / 76 18.4 %	18 / 76 23.7 %
Templin	5 / 45	6 / 45	1 / 6	2 / 6	6 / 51 11.8 %	8 / 51 15.7 %
Müllrose	6 / 38	8 / 38	1 / 6	1 / 6	7 / 44 15.9 %	9 / 44 20.5 %
Total	**89 / 493 18.1 %**	**107 / 493 21.7 %**	**10 / 62 16.1 %**	**12 / 62 19.4 %**	**99 / 555 17.8 %**	**119 / 555 21.4 %**

Chapter 4

Publication II

Rat hepatitis E virus: Geographical clustering within Germany and serological detection in wild Norway rats (*Rattus norvegicus*)

Key information of the publication

- Establishment of a rat HEV-antigen based in-house ELISA.
- Low cross-reactivity of the capsid proteins of rat HEV and HEV GT 3.
- Detection of genomic RNA of rat HEV and of anti-rat HEV antibodies in rats from several cities of Germany.
- Clustering of rat HEV sequences on a small geographical scale.

Reprinted from Infection, Genetics and Evolution, 12, Johne, R., Dremsek, P., Kindler, E., Schielke, A., Plenge-Bönig, A., Gregersen, H., Wessels, U., Schmidt, K., Rietschel, W., Groschup, M. H., Guenther, S., Heckel, G., Ulrich, R. G., Rat hepatitis E virus: Geographical clustering within Germany and serological detection in wild Norway rats (*Rattus norvegicus*), 947 – 956, Copyright 2012, with permission from Elsevier.

Infection, Genetics and Evolution 12 (2012) 947–956

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Rat hepatitis E virus: Geographical clustering within Germany and serological detection in wild Norway rats (*Rattus norvegicus*)

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ARTICLE INFO

Article history:
Received 28 September 2011
Received in revised form 10 February 2012
Accepted 12 February 2012
Available online 28 March 2012

Keywords:
Rattus norvegicus
Hepatitis E virus
RT-PCR
Serology
Phylogeny

ABSTRACT

Zoonotic hepatitis E virus (HEV) infection in industrialised countries is thought to be caused by transmission from wild boar, domestic pig and deer as reservoir hosts. The detection of HEV-specific antibodies in rats and other rodents has suggested that these animals may represent an additional source for HEV transmission to human. Recently, a novel HEV (ratHEV) was detected in Norway rats from Hamburg, Germany, showing the typical genome organisation but a high nucleotide and amino acid sequence divergence to other mammalian and to avian HEV strains. Here we describe the multiple detection of ratHEV RNA and HEV-specific antibodies in Norway rats from additional cities in north-east and south-west Germany. The complete genome analysis of two novel strains from Berlin and Stuttgart confirmed the association of ratHEV to Norway rats. The present data indicated a continuing existence of this virus in the rat populations from Berlin and Hamburg. The phylogenetic analysis of a short segment of the open reading frame 1 confirmed a geographical clustering of the corresponding sequences. Serological investigations using recombinant ratHEV and genotype 3 capsid protein derivatives demonstrated antigenic differences which might be caused by the high amino acid sequence divergence in the immunodominant region. The high amount of animals showing exclusively ratHEV RNA or anti-ratHEV antibodies suggested a non-persistent infection in the Norway rat. Future studies have to prove the transmission routes of the virus in rat populations and its zoonotic potential. The recombinant ratHEV antigen generated here will allow future seroepidemiological studies to differentiate ratHEV and genotype 3 infections in humans and animals.

1. Introduction

The hepatitis E virus (HEV) was identified as the causative agent of an acute hepatitis in humans (for Ref. see Khuroo, 2011). Although the majority of clinical infections is characterised by a self-limited course, in rare cases a lethal outcome or chronic infections have been reported. Previously reported case fatality rates of 0.5–4% during outbreaks may represent an overestimation, as in population surveys the rates ranged from 0.07–0.6% (Aggarwal, 2011). Recently, chronic infections have been mainly documented for immunosuppressed solid organ transplant recipients, exclusively for genotype 3 (for Ref. see Schlosser et al., in press). In outbreak regions the disease is typically characterised by symptoms like jaundice, malaise, anorexia, abdominal pain, hepatomegaly, nausea and vomiting, fever and pruritus (Aggarwal, 2011). In Europe jaundice was found to be the most common symptom. Asthenia, fever, joint and muscle pains as well as abdominal pain are other common symptoms (Pavio and Mansuy, 2010).

The small non-enveloped virion of HEV has a spherical shape similar to that of other small round structured viruses. Sucrose gradient centrifugation purified virions were found to have a diameter of 32–34 nm (Bradley et al., 1988). Recent studies indicated the

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http://dx.doi.org/10.1016/j.meegid.2012.02.021

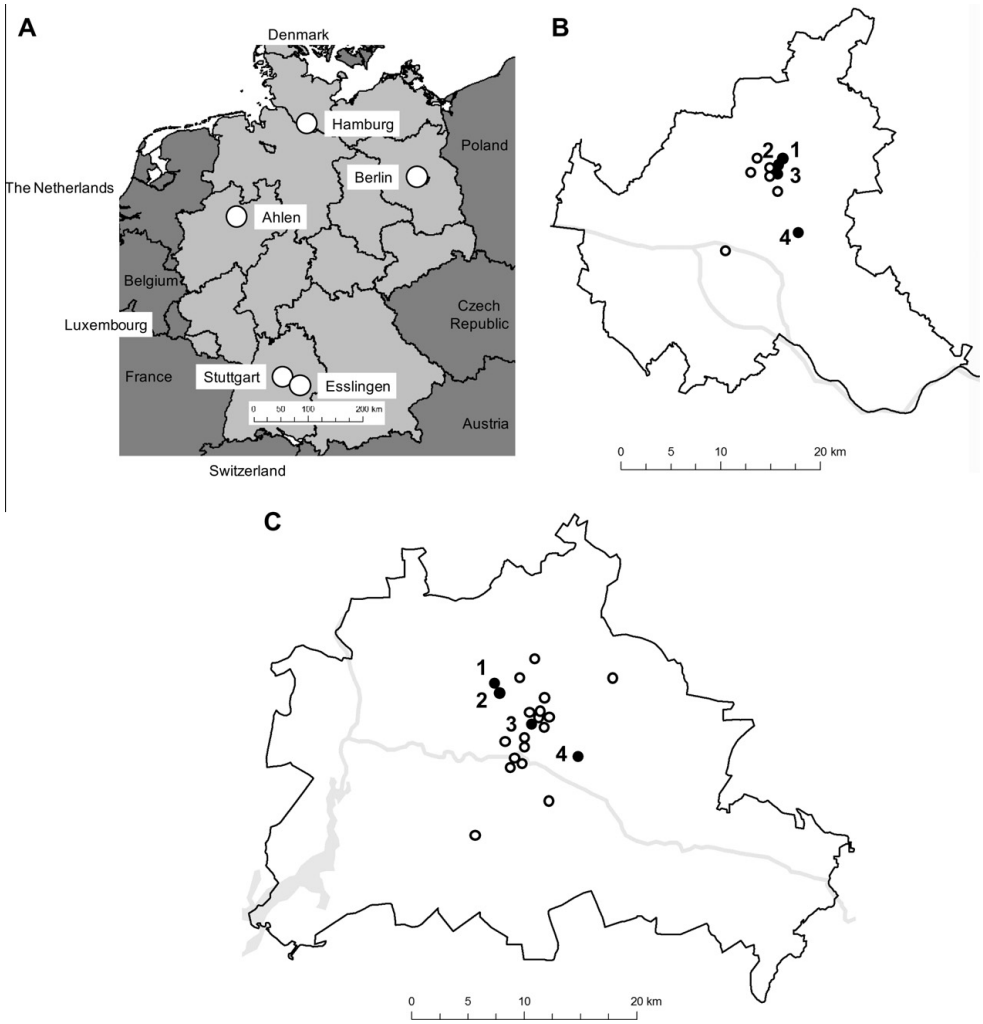

Fig. 1. Location of the trapping sites of Norway rats (*Rattus norvegicus*) in Germany (A) and more precise localisation of the trapping sites in the cities of Hamburg (B) and Berlin (C). Empty circles in (B) and (C), trapping sites where only RT-PCR-negative samples were collected; full circles, trapping sites with RT-PCR-positive samples (numbers correspond to those in Fig. 5 and Table 2).

association of lipids with the surface of the virions (Takahashi et al., 2010). The capsid of the virion comprises the capsid protein (CP) of about 660 amino acid (aa) residues. This protein is encoded by the open reading frame (ORF) 2 and target for the main immune response. The gene product of ORF3 of 123 aa was also found to be associated with the surface of virions, but additionally represents a phosphoprotein associated with the cytoskeleton. The 5′ part of the genome contains the large ORF1 which encodes a polyprotein with different enzymatic functions. The genome of the virus is completed by a 5′ untranslated region with a cap modification and a 3′ untranslated region preceding the poly(A) tail (for review see Ahmad et al., 2011).

The usual way of transmission in developing countries is the faecal-oral route. This transmission route has caused large outbreaks in countries with low sanitation standards and was found to be associated with genotypes 1 and 2. On the contrary, during the recent decennia autochthonous hepatitis E cases were increasingly identified in industrialised countries in Europe, Asia and America. Interestingly, these infections were found to be caused by genotypes 3 and 4. Molecular epidemiological evidence and sequence similarities of swine and human HEV sequences confirmed the food-borne transmission of these genotypes from reservoir hosts (Meng et al., 1998; Wang et al., 2002; Li et al., 2005; Tei et al., 2003, 2004; Takahashi et al., 2004). Additional evidence for a zoonotic transmission of these genotypes came from experimental transmission of "human" HEV strains to pig and of "pig" HEV strains to primates (Balayan et al., 1990; Meng et al., 1998; Arankalle et al., 2006). Recently, a novel genotype has been detected in wild boar in Japan (Sato et al., 2011; Takahashi et al., 2011). In addition to the mammalian HEV genotypes avian strains have been

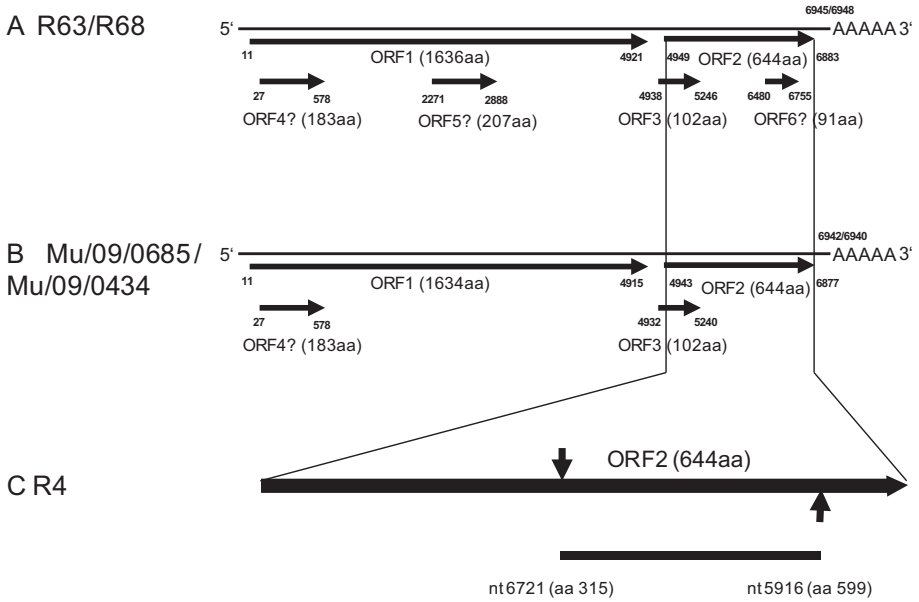

Fig. 2. Genome organisation of rat hepatitis E virus, strains R63 and R68 from Hamburg (A), strain Mu/09/0685 from Berlin and strain Mu/09/0434 from Stuttgart (B) and localisation of the coding sequence of the recombinant ratHEV capsid protein derivative from strain R4 used for the generation of the recombinant antigen (C).

identified as causative agents of the big liver and spleen disease and of the similar hepatitis-splenomegaly syndrome (Payne et al., 1999; Haqshenas et al., 2001). The avian strains of different geographical origin were suggested to represent a separate genus of HEV with at least three genotypes (Marek et al., 2010). Most recently a HEV-related agent was identified in different fish species (Batts et al., 2011).

Molecular studies demonstrated the presence of HEV RNA in tissues of wild boar (*Sus scrofa)*, domestic pig (*S. scrofa domesticus)* and deer species and therefore confirming them as reservoir for zoonotic transmission (Meng, 2010). The detection of HEV-reactive antibodies in other mammalian species, such as cattle, sheep, goat, horse and cat, may indicate the existence of further susceptible potential reservoir animals (Vitral et al., 2005; Sakano et al., 2009; Geng et al., 2010). Recently, a putative novel HEV genotype was identified in rabbits from China which is broadly distributed in rabbit farms in China (Zhao et al., 2009; Geng et al., 2011). However, its closer similarity to genotype 3 (Geng et al., 2011) might be explained by a spillover event of a genotype 1–4 derived strain in the past (Johne et al., 2010b).

For a long time serological investigations suggested spillover infections of human genotypes 1–4 to rodents or the presence of an additional partially cross-reactive HEV-like agent in rodents. HEV-reactive antibodies have been detected in different rat species, i.e. Norway or brown rats (*Rattus norvegicus*), black rats (*R. rattus*) and other *Rattus* species, family Muridae, subfamily Murinae, from India, Japan and USA (Arankalle et al., 2001; Favorov et al., 2000; Kabrane-Lazizi et al., 1999; Hirano et al., 2003). In addition, anti-HEV antibodies were detected in other species of the Murinae subfamily, i.e. Bandicota bengalensis (Arankalle et al., 2001), *Mus musculus*, and representatives of the Cricetidae subfamilies Arvicolinae, Sigmodintinae and Neotominae, i.e. *Myodes* (*Clethrionomys*) *gapperi*, *Oryzomys palustris* and *Sigmodon hispidus*, and different *Neotoma* and *Peromyscus* species, respectively (Favorov et al., 2000).

The development of a novel broad-spectrum RT-PCR resulted in the first detection of a novel HEV-like agent in faecal samples from two Norway rats from Hamburg, northern Germany (Johne et al., 2010a). The determination of the entire nucleotide (nt) sequence of two additional strains from Norway rats from the same trapping site demonstrated a genome organisation (see Fig. 2A) and electron microscopic structure similar to previously identified HEV strains (Johne et al., 2010b). In addition, real-time RT-PCR and immunohistochemical studies suggested a hepatotropism of this novel virus. However, phylogenetic analyses and sequence comparisons showed marked differences to all other mammalian HEV strains as well as to the avian HEV strains. These differences prompted us to state ratHEV as a novel genotype of HEV (Johne et al., 2010b, 2011).

Here we describe the detection of novel ratHEV strains in three additional cities in Germany. The studies suggest a continuous presence of this virus in different Norway rat populations in Germany. Phylogenetic and sequence analyses indicated a local evolution of ratHEV in the corresponding rat populations. Serological investigations using recombinant CP derivatives of ratHEV and genotype 3 HEV demonstrated strong antigenic differences and revealed ratHEV-specific antibodies in rats from different areas of Germany.

2. Materials and methods

2.1. RT-PCR and cloning

A 3′-terminal fragment of ORF2 of the ratHEV strain R4, isolated from faeces of a wild Norway rat from Hamburg, Germany (Johne et al., 2010a, GenBank accession number GQ504009), was RT-PCR amplified using primers 5′-GGA AGA GTC AAC CTC AGG GAT GT-3′ and 5′-CCG AAT TCC CGG GAT CC$(T)_{17}$V-3′ (complementary to the poly(A) tail of the genome). The PCR product was cloned into

vector pCR4-TOPO (Invitrogen, Carlsbad, California, USA). A 3′-terminal truncated sequence harbouring XbaI sites for subcloning and encoding aa residues 315 to 599 of the CP (Fig. 2C) was obtained from this template by PCR amplification using forward primer 5′-TAT ATC TAG AAC AAT GAC AGC CCC GCA TAA GAT CAA GCG GCT-3′ and reverse primer 5′-TAT ATC TAG ATT ACT GCT CAG TCG GGC TGG GGC CGA TA-3′. Thereafter, the CP-encoding sequence was subcloned into a modified pET19b vector with a SpeI cloning site at the 3′-end of a decahistidine codon stretch (Dremsek et al., 2011) obtaining plasmid pET19b-R3tr.

The cloning of a corresponding 3′-terminal segment of the CP-encoding region (aa residues 326 to 608) of the HEV genotype 3i strain wbGER27 (Schielke et al., 2009; GenBank accession number FJ705359) into the modified pET19b vector to obtain plasmid pET19b-WB3T was described previously (Dremsek et al., 2011).

The entire coding sequence for the non-structural protein 1 (NS1) of West Nile virus (WNV), New York flamingo isolate of 1999 (accession number AF196835), was RT-PCR amplified using the forward primer 5′-ACA GCA TAT GGA CAC TGG GTG TGC CAT AAA C-3′ and reverse primer 5′-ACA GGG ATC CAA GCA TTC ACT TGT GAC TGC AC-3′. The amplification product was cleaved by BamHI and NdeI and inserted into the original non-modified pET-19b vector cleaved by the same enzymes.

2.2. Recombinant protein expression in Escherichia coli and purification

For protein expression the recombinant plasmids were re-transformed into the *E. coli* strain BL21(DE3). Transformed and non-transformed *E. coli* cells were grown in Luria Bertani medium containing 100 μg/ml ampicillin until an optical density at 600 nm of 0.5 and foreign protein synthesis was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM for 3 h. The purification of the His-tagged CP derivatives and the WNV NS1 protein was performed by nickel chelate affinity chromatography following the protocol of the manufacturer under denaturing conditions (Qiagen, Hilden, Germany).

2.3. SDS polyacrylamide gel electrophoresis (SDS–PAGE), immunoblot analysis and enzyme-linked immunosorbent assay (ELISA)

The synthesis and the purity of the recombinant CP derivatives was analysed by SDS–PAGE using 13% gels stained by Coomassie brilliant blue. For immunoblot analysis the proteins separated by SDS–PAGE were blotted onto an Immobilon-P Transfer Membrane (Merck Millipore, Billerica, Massachusetts, USA). The membrane was blocked in 5% skim milk overnight at 4 °C and incubated for 1 h at RT in murine anti-His monoclonal antibody (Novagen, Merck KGaA, Darmstadt, Germany) diluted 1:2500 in PBS-Tween 20. The antigen–antibody reaction was detected by adding horse-radish peroxidase (HRP) labelled anti-mouse immunoglobulin (Dako Cytomation, Glostrup, Denmark), diluted 1:2000, visualised by adding ECL reagent and hydrogen superoxide (Amersham, GE Healthcare, UK) and documented using a VersaDoc Imaging-System Model 5000 (Bio-Rad, Munich, Germany).

For ELISA, 2 μg per well of the purified antigens were coated on a Multisorp microtiter plate (Nunc, Roskilde, Denmark). For end-point titration the sera of immunized rabbit and rats were serially diluted in PBS containing 0.5% BSA and 0.05% Tween 20 with an initial dilution of 1:1000. The chest cavity fluids from wild rats, diluted 1:15, and the diluted rabbit and rat sera were incubated for 2 h on the microtiter plate. After threefold washing with PBS-Tween 20 the HRP-labelled anti-rat immunoglobulin (Sigma–Aldrich, St. Louis, Missouri, USA), diluted 1:12500, or anti-rabbit immunoglobulin (Promega, Mannheim, Germany), diluted 1:2500, were incubated for 1 h. After additional washing, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (Bio-Rad, Munich, Germany) was added. The enzyme reaction was stopped by addition of 1 M H_2SO_4 and measured at 450 nm with an ELISA reader Tecan Sunrise (Tecan Group Ltd, Männedorf, Switzerland).

In parallel a commercial double-sandwich, genotype 1 antigen-based ELISA (Axiom HEV Ab, Axiom GmbH, Bürstadt, Germany) for detection of anti-HEV antibodies was performed according to the protocol of the manufacturer.

2.4. Rat and rabbit immunizations

Female Wistar rats were immunized intraperitoneally using 200 μg of the truncated carboxy-terminal segment of the CP of ratHEV. They were immunized three times at intervals of 4 weeks with incomplete Freund's adjuvant. For the immunization with the genotype 3 CP equivalent, 200 μg of antigen were given intramuscularly three times at intervals of 3–4 weeks with incomplete Freund's adjuvant. Blood samples were collected at 4 weeks p.i.

A male rabbit was immunized subcutaneously three times at intervals of 4 weeks with 200 μg of the truncated carboxy-terminal segment of the CP of ratHEV in incomplete Freund's adjuvant. Blood samples were collected at 4 weeks p.i..

2.5. Rat trapping and necropsy

Norway rats (*Rattus norvegicus*) were trapped in Hamburg, Berlin, Stuttgart and Esslingen using snap traps. At an additional site close to Ahlen, Westphalia, dead rats were collected at two farms with pig husbandries after a field trial with a rodenticide (Fig. 1). During necropsy liver tissue and chest cavity fluid samples were collected and stored at −20 °C.

2.6. RNA isolation, RT-PCR analysis and sequencing of PCR products

Liver tissue samples were homogenised using QIAshredder columns (Qiagen) and RNA was extracted with the RNeasy Mini kit (Qiagen). A nested broad-spectrum RT-PCR for detection of hepeviruses was performed as described previously (Johne et al., 2010a) and PCR products derived from two rat samples trapped in 2009 (sample numbers Mu/09/0434 and Mu/09/0685) were sequenced using the nested PCR primers. Based on these sequences and previously determined ratHEV sequences (Johne et al., 2010a,b), a real-time RT-PCR protocol for detection of ratHEV was established. Briefly, primers rHEV-F (5′-TAC CCG ATG CCG GGC AGT-3′), rHEV-R2 (5′-ATC YAC ATC WGG GAC AGG-3′) and probe rHEV-P2 (5′-6FAM-AAT GAC AGC ACA GGC ACC GGC GCC-BHQ-3′) were used together with the Quantitect Probe RT-PCR kit (Qiagen). Reverse transcription was conducted at 50 °C for 30 min, followed by an activation step at 95 °C for 15 min. A total of 45 PCR cycles each consisting of 10 s at 94 °C, 20 s at 55 °C and 1 min at 72 °C were performed. All available rat liver samples were re-tested by this real-time RT-PCR and positive samples were subjected to the hepevirus-specific nested broad-spectrum RT-PCR (Johne et al., 2010a). PCR products were subsequently sequenced and the determined sequences were deposited at GenBank with accession numbers JN167530–JN167536.

2.7. Sequencing of novel entire ratHEV genomes and sequence analysis

Four additional primer pairs for amplification of short partially overlapping fragments of the ratHEV genome were designed based on an alignment of available ratHEV genome sequences (Johne et al., 2010b) together with other non-rodent HEV strains (Supplementary Table S1). The PCR products generated by these primer pairs and by the nested broad-spectrum PCR mentioned above were sequenced using the respective primers. Based on the

determined sequences, strain-specific primer pairs were constructed for amplification of the remaining parts of the genomes using the LongRange 2Step RT-PCR kit (Qiagen). The sequences at the genome ends were determined using a strategy as described by Schielke et al. (2009). Briefly, for amplification of the 5′-ends of the genomes, the 5′ RACE System kit (Invitrogen GmbH, Karlsruhe, Germany) was used according to the supplier's protocol together with strain-specific primers. The 3′-ends of the genomes were amplified using primer pA1 [5′-CCG AAT TCC CGG GAT CC(T)17 V-3′, complementary to the poly(A) tail] in reverse transcription, followed by PCR with primer 5′-CCG AAT TCC CGG GAT CC-3′ (binding site on primer pA1) and a strain-specific primer. All amplification products were separated by electrophoresis on ethidium bromide-stained agarose gels. Bands of the expected length were excised, purified using the QIAquick Gel Extraction kit (Qiagen) and subsequently cloned using the TOPO TA Cloning kit for sequencing (Invitrogen). The inserts of the plasmids were sequenced with primers M13 Forward and M13 Reverse (Invitrogen), as well as strain-specific primers, in an ABI 3730 DNA Analyser (Applied Biosystems). The genome sequences were 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 numbers JN167537 and JN167538. Sequence alignments were performed using the MEGALIGN module of DNASTAR. The CLUSTAL W method was used with the IUB (nt) or Gonnet 250 (aa) residue-weight tables (Thompson et al., 1994) in alignments to calculate pairwise identities.

2.8. Phylogenetic analysis

Sequences were aligned at the aa level, reverse-translated to nt using the program Geneious (Drummond et al., 2011) and revised manually. Phylogenetic relationships were inferred by Bayesian algorithms (BI) implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and by Maximum Likelihood Algorithms (ML) using the online tool PhyML (Guindon et al., 2010) and the program MEGA 5 (Tamura et al., 2011). The optimal mutation model was selected based on the Bayesian Information Criterion (BIC) in jModeltest 0.1.1 (Posada, 2008). The TrNef model (Tamura and Nei, 1993) with a gamma-shaped distribution of rates across sites (Γ) was selected as the optimal nt substitution model for the alignment based on complete genome sequences, as well as for the alignment based on the short ORF1-derived sequences. The Bayesian analyses for the complete genome sequences were run twice for two million generations with every 1000th generation sampled, using one cold and three heated chains, implemented in the web-based cluster Cipres Portal (Miller et al., 2009). The BI for the partial ORF1 sequences were run four times for one million generations with every 1000th generation sampled. The first 25% of the samples were discarded as burn-in and convergence was determined by examining the log likelihood values and the split frequencies. The ML analyses were run with default values using PhyML, and twice in MEGA, using the Tamura-3-parameter model (Tamura, 1992) and 10000 bootstrap replicates. All trees were visualised in MEGA. GenBank accession numbers of all the sequences included in this study are directly shown in the corresponding phylogenetic trees.

3. Results

3.1. Antigenicity of the recombinant ratHEV CP and its cross-reactivity

The truncated carboxy-terminal region of the CP spanning aa residues 315 to 599 of ratHEV strain R4 was expressed at high level in *E. coli*. The initial analysis of crude lysates of *E. coli* cells analysed by SDS PAGE revealed a CP derivative of the expected molecular weight of 32.7 kDa. A control lysate of non-transformed cells lacked the corresponding protein band. Analysis of soluble and insoluble fractions upon ultrasound lysis revealed that the main portion of the CP derivative is insoluble under native conditions (data not shown).

Purification of the CP derivative by affinity chromatography under denaturing conditions resulted in a final amount of 4 mg protein per 150 ml culture. Analysis of the subsequent fractions, i.e. flow-through, washing and elution fractions, confirmed the majority of the recombinant protein to be bound to the affinity column and almost exclusive elution upon addition of the elution buffer at a pH of 5.9. The SDS–PAGE and immunoblot analysis of the elution fraction revealed a highly pure recombinant ratHEV protein of the expected size with an intact amino-terminal His-tag. In addition, the purified HEV genotype 3 CP derivative and the WNV NS1 negative control antigen were detected in the stained gel and in the immunoblot at the expected sizes (data not shown).

Immunization of laboratory Norway rats with the ratHEV-derived recombinant CP (R-Ctr) resulted in the induction of high-titered ratHEV-CP-specific antibodies (Fig. 3A). Similarly, immunization of a rat with the corresponding segment of the genotype 3 HEV strain (WB-Ctr) induced a high-titered homologous antibody response (Fig. 3B). An endpoint titration of the sera with the homologous and heterologous recombinant antigens revealed a titer difference of about 16-fold (Fig. 3A and B). Similar data were obtained for a rabbit immunized with the ratHEV antigen (data not shown). A parallel ELISA analysis using the purified *E. coli*-expressed WNV NS1 negative control antigen demonstrated a low background reaction indicating that nearly all detected antibodies in rats and the rabbit are directed to the recombinant HEV antigens (data not shown).

3.2. Serological detection of ratHEV infections in rats from four trapping sites in Germany

A total of 147 Norway rats were collected during 2007–2010 at five sites in the northern, central and south-western part of Germany (Fig. 1, Table 1). A parallel analysis of the rat blood samples with the ratHEV and the genotype 3 HEV antigen demonstrated sera with a strong reactivity with the ratHEV antigen and almost no reactivity with the genotype 3 antigen for animals from Hamburg, Berlin, Stuttgart and Esslingen (Fig. 4B-E). On the contrary, all investigated blood samples from Ahlen did not contain any HEV-reactive antibodies (Fig. 4A). Only a very few rat samples from Berlin demonstrated an equal reactivity to the heterologous genotype 3 antigen. Following a cut-off value definition of the average OD values of the analysed samples from Ahlen plus three times the standard deviation, the average seroprevalence of the rats from Hamburg, Berlin, Stuttgart and Esslingen was defined to be 41.2%, 34.4%, 14.7% and 21.4%, respectively, (Table 1). A parallel investigation of all samples with the commercial genotype 1 based ELISA showed only a very low reactivity of one sample originating from Berlin (data not shown).

3.3. RT-PCR detection of ratHEV and phylogenetic analysis of ratHEV sequences

Liver samples of wild Norway rats trapped at five sites in Germany were analysed for the presence of ratHEV genome using a novel ratHEV-specific real-time RT-PCR. No HEV RNA was detected in 21 rat liver samples from Ahlen (Table 1). In the liver samples of rats from Hamburg, Berlin, Stuttgart and Esslingen at least one per trapping year was found to be real-time RT-PCR positive (Table 1). The detection rates were calculated on average to be 35.3%, 11.5%, 2.9% and 7.1%, respectively.

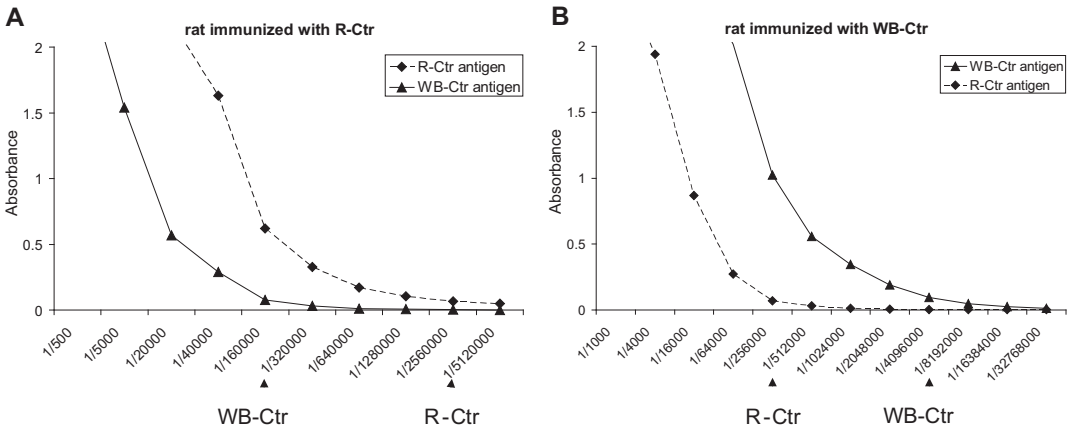

Fig. 3. Cross-reactivity of sera from rats immmunized with recombinant capsid protein derivatives of ratHEV (A) and genotype 3i HEV (B) with homologous and heterologous antigens. Triangles mark the endpoint titer of the sera with the respective antigen used in the test.

Table 1
Frequency of ratHEV real-time RT-PCR detection and serological reactivity of chest cavity fluids in ratHEV IgG ELISA in Norway rats from five trapping sites in Germany.

Geographical origin	Trapping year	Total number of samples	Real-time RT-PCR positive samples	%	IgG ELISA-positive samples	%
Ahlen	2007	7	0		0	
	2008	14	0		0	
	Sub-total	21	0	0	0	0
Hamburg	2009	2	1		0	
	2010	15	5		7	
	Sub-total	17	6	35.3	7	41.2
Stuttgart	2008	34	1	2.9	5	14.7
Esslingen	2009	14	1	7.1	3	21.4
Berlin	2009	25	1		6	
	2010	36	6		15	
	Sub-total	61	7	11.5	21	34.4
Total		147	15	10.2	36	24.5

Samples showing positive reaction in the real-time RT-PCR were subjected to a nested consensus RT-PCR (Johne et al., 2010a) in order to amplify genome fragments for sequencing. By direct sequencing of the nested RT-PCR products, a 216 nt fragment of ORF1 could be retrieved from two samples derived from Hamburg, five samples derived from Berlin and one sample each derived from Stuttgart and Esslingen (Table 2). A closer inspection of the PCR results showed that nested consensus RT-PCR products could only be sequenced from samples showing a low ct-value in the real-time RT-PCR (data not shown). Therefore, the discrepancy between the detection rates of both assays may be explained by a higher sensitivity of the real-time RT-PCR. All generated sequences were aligned with the corresponding published sequences of three ratHEV strains from Hamburg and HEV strains of other hosts. Between the ratHEV sequences, pairwise identities of 81.5%-98.6% at nt level and 91.7–100.0% at aa level were calculated.

Phylogenetic analysis based on BI and ML algorithms congruently revealed a geographical clustering of the short ORF1-derived sequences from rats sampled in Germany. The ratHEV sequences of animals from Hamburg, Esslingen/Stuttgart and Berlin were grouped into three distinct clades (Fig. 5A). Additionally, ratHEV sequences of rats from Berlin were differentiated into three clades with geographical clustering according to the trapping sites 1, 2 and 3/4 with a maximal distance of 7 km between site 1 and 4 (Fig. 5A, Fig. 1C). On the contrary, sequences from Hamburg originated from rats trapped at very close sites 1, 2 and 3 did not show any geographical clustering (Fig. 5A, Fig. 1B).

3.4. Complete genome sequence analysis of novel ratHEV strains

The complete genome sequences were determined for ratHEV strain Mu/09/0685 derived from Berlin and ratHEV strain Mu/09/0434 derived from Stuttgart. The lengths of the complete genomes (excluding the poly(A) tail) of Mu/09/0685 and Mu/09/0434 are 6942 nt and 6940 nt, respectively. The difference observed in genome lengths between both strains is due to a deletion/insertion polymorphism in the non-coding 3′-terminal region of the genomes. The three major open reading frames ORF1, ORF2 and ORF3 with homologies to the HEV polyprotein, the CP and the phosphoprotein, respectively, could be identified in both strains (Fig. 2B). Compared to the two published genome sequences of ratHEV strains R63 and R68 (Johne et al., 2010b), strains Mu/09/0685 and Mu/09/0434 have a deletion of 6 nt in the hypervariable region of ORF1 (nt positions 2537–2542 in R63 and R68). Pairwise nt sequence identities of the genomes of ratHEV strains R63, R68, Mu/09/0434 and Mu/09/0685 ranged between 86.8% and 95.3%. The aa sequence identities of the encoded proteins were 94.9–97.7% for ORF1, 96.6–98.8% for ORF2 and 77.7–93.2% for ORF3. Out of three additional ORFs postulated to be present in the ratHEV genome (Johne et al., 2010b), only ORF4 was found in both ratHEV strains at nt positions 27–578 overlapping the ORF1 in a +1 reading frame. The nucleotide sequence identity between the sequences of the four ratHEV strains was higher (92.9–96.9%) in the ORF1/ORF4 region (nt 29–578) than in other similar sized regions of ORF1 (nt 581–1130: 84.9–94.7% – containing 5–8 stop codons in the +1 and 10–11 stop codons in the +2 reading frame; nt 1133–1682: 84.7–95.8% - containing 5–7 stop codons in the +1 and 5–10 stop codons in the +2 reading frame). The aa sequence identities of the putative 183 aa-long protein are 82.6–94.0% between the four ratHEV strains, which is slightly higher than that for the protein encoded by ORF3. The putative ORF4-encoded protein contains a relatively high proportion of leucine (18–19%) and arginine residues (11–12%). A BLASTp homology search of aa sequences deposited in

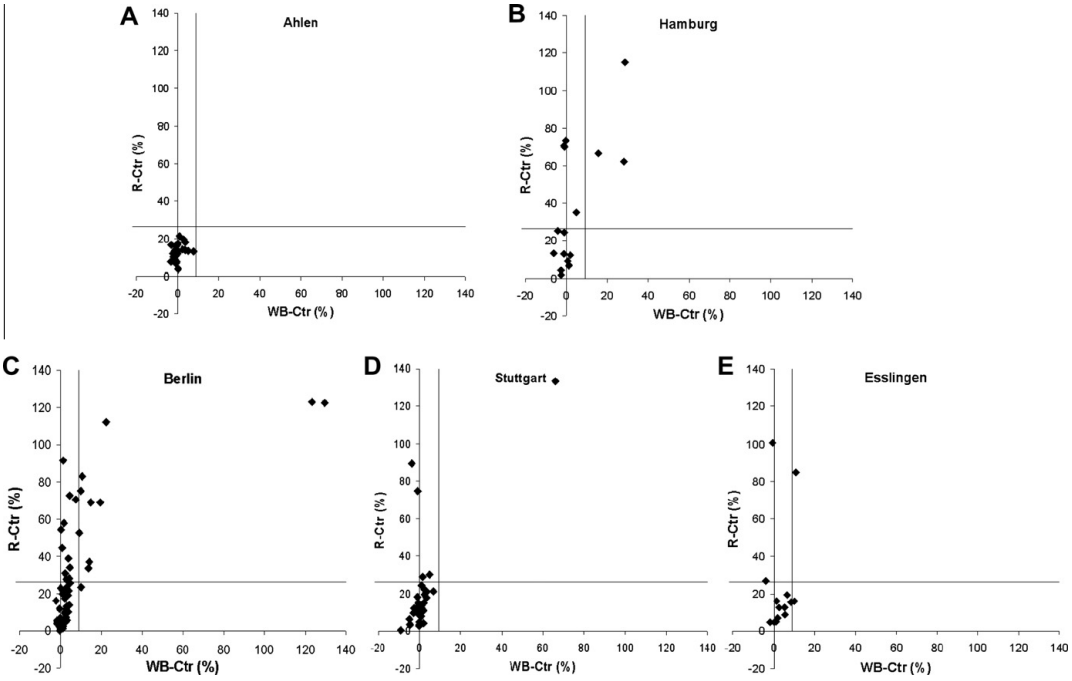

Fig. 4. Results of the serological screening of chest cavity fluid samples of rats from Ahlen (A), Hamburg (B), Berlin (C), Stuttgart (D) and Esslingen (E) with ratHEV and HEV genotype 3 recombinant capsid proteins. Based on the reactivity observed for the sera from Ahlen (A) cut-off values were defined as the average OD value plus three times their standard deviation for estimation of seropositive samples from the other trapping sites (B–E). These values were 26.3% of the positive control for R-Ctr and 9.2% of the positive control for WB-Ctr.

Table 2
Detection of ratHEV-reactive antibodies in real-time RT-PCR-positive Norway rats and accession numbers of ratHEV sequences.

Number	Geographical origin (site)[a]	Trapping year	Real-time RT-PCR	Accession number	ratHEV IgG-ELISA
Mu/10/2587	Hamburg (4)	2009	+	–[b]	–
Sm10/2575	Hamburg (1)	2010	+	JN167535	+
Mu/10/2578	Hamburg (2)	2010	+	–[b]	+
Mu/10/2579	Hamburg (2)	2010	+	–[b]	–
Mu/10/2582	Hamburg (3)	2010	+	–[b]	–
Sm10/2585	Hamburg (1)	2010	+	JN167536	+
Mu/09/0685	Berlin (3)	2009	+	JN167537	–
Mu/10/1772	Berlin (2)	2010	+	JN167530	–
Mu/10/1775	Berlin (2)	2010	+	JN167531	–
Mu/10/1778	Berlin (1)	2010	+	JN167532	+
Mu/10/1779	Berlin (4)	2010	+	JN167533	–
Mu/10/1788	Berlin (2)	2010	+	–[b]	+
Mu/10/1791	Berlin (2)	2010	+	–[b]	–
Mu/09/0434	Stuttgart	2008	+	JN167538	–
Mu/10/1542	Esslingen	2009	+	JN167534	–

+, positive; –, negative.
[a] For details see Fig. 1A–C.
[b] No specific sequence amplified by broad-spectrum RT-PCR.

GenBank revealed only very low sequence identities with known proteins.

A phylogenetic analysis of the entire ratHEV strain sequences from Berlin and Stuttgart demonstrated a clustering with sequences from the ratHEV strains from Hamburg, and confirmed a clear separation from other mammalian HEV sequences and the avian and fish HEV strains (Fig. 5B).

4. Discussion

Here we describe novel complete and partial ratHEV genome sequences from wild rats from Berlin, Stuttgart and Esslingen and additional novel partial sequences from Hamburg. The complete sequence analysis of both novel strains from Berlin and Stuttgart confirmed the recently described genome organisation of ratHEV with major ORFs (ORF1, ORF2 and ORF3) in a typical organisation. Interestingly, in the two new entire genome sequences the prediction of an additional putative reading frame ORF4 (Johne et al., 2010b) overlapping the ORF1 in a +1 reading frame was confirmed. In contrast, the previously predicted ORF5 and ORF6 were disrupted by a premature stop codon or did not contain a start codon at all. Additional investigations will have to prove the expression and functional role of the putative ORF4-encoded protein, which is predicted to have an unusual aa content.

The more precise analysis of the ORF2-encoded CP revealed a high level of aa sequence identity between the four ratHEV strains of 96.9–98.8%, but a low sequence identity to the CP aa sequences of genotypes 1–4 (56.0–57.9%), avian HEV strains (46.1–46.8%) and fish-HEV like agent (18.7–19.0%). Cross-reactivity studies with sera of Norway rats immunized with *E. coli*-expressed CP either of ratHEV or genotype 3 HEV clearly indicated antigenic differences although slight cross-reactions were observed. This is in line with recently published results using ratHEV-derived virus-like particles expressed in insect cells as antigens (Li et al., 2011). Interestingly, the aa sequence identity in the recombinant proteins of ratHEV and genotype 3 HEV used in this study is 54%, whereas it is even only 45% for the protruding domain part of this

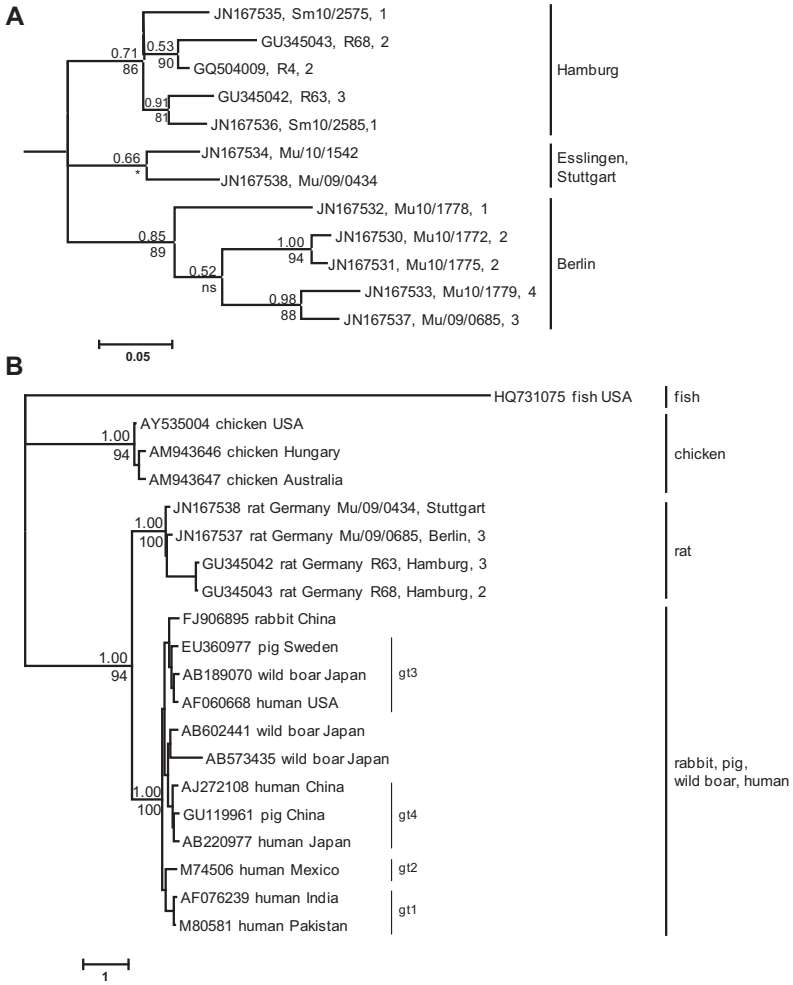

Fig. 5. Phylogenetic trees of 216 nt-long ORF1-derived nucleotide sequences of novel ratHEV strains from Hamburg, Esslingen, Stuttgart and Berlin (A) and complete genome sequences of novel ratHEV strains from Stuttgart and Berlin with published sequences from ratHEV strains R63, R68 and R4 from Hamburg (Johne et al., 2010a,b) as well as mammalian, avian and fish HEV strains (B). The GenBank accession numbers, strain designations, hosts and geographical origins of the strains are indicated. Numbers after strain designation in ratHEV strains from Hamburg and Berlin refer to the trapping sites as indicated in Fig. 1B and C, respectively. The scale bar indicates phylogenetic distances in nucleotide substitutions per site. Posterior probabilities for Bayesian analyses are given above the branches, support values for Maximum Likelihood (ML) analyses below the branches, respectively. * indicates a different topology based on ML algorithms, ns refers to posterior probabilities < 0.50 and support-values < 50%.

immunodominant region. The low level of sequence identity may also explain the failure of detection of HEV-specific antibodies in rats using a commercial genotype 1-based ELISA (Johne et al., 2010b; this paper). In addition, these findings may raise the question whether ratHEV represents not only a separate genotype, but also a separate serotype.

The detection of new ratHEV sequences in wild Norway rats from Berlin, north-east Germany, and Stuttgart/Esslingen, south-west Germany, together with the confirmation of the virus in rats from Hamburg may indicate a broad geographical distribution of ratHEV in Germany. Recently, ratHEV has also been detected in wild rats from the USA and Vietnam indicating that this virus infection is not restricted to Germany (Purcell et al., 2011; Li et al., 2011). The detection of ratHEV in rats from the same sites of the German cities in different years may additionally suggest a continuing presence of the virus in the local rat populations. When analysing all investigated 126 rats from Hamburg, Stuttgart, Esslingen and Berlin, the majority ($n = 39$) demonstrated only ratHEV-specific antibodies ($n = 30$) or ratHEV specific RNA ($n = 9$). In contrast, in six animals a parallel presence of viral RNA and of anti-ratHEV-CP antibodies was observed. The lacking association of RT-PCR and serological ratHEV detection may indicate that the ratHEV infections are mostly not persistent in Norway rats. This observation is in line with the results from an experimental infection study in rats published recently (Purcell et al., 2011). The here observed absence of the RT-PCR and serological detection of ratHEV in Norway rats from the Ahlen area may indicate absence of this virus from local rat populations; however, this conclusion

needs additional studies at this site because of the low number of animals tested.

The phylogenetic analysis of the novel entire and partial ratHEV sequences clearly demonstrated a geographical clustering. Phylogenetic investigations based on the ORF1-derived nt sequence revealed a small-scale geographical clustering of ratHEV sequences of Norway rats from Berlin. The location of the trapping sites and the phylogenetic affinities of the ratHEV sequences, however, did not suggest a simple linear distance/sequence identity relationship. This might be in line with the findings in Hamburg where all ratHEV sequences in this and the previous studies (Johne et al., 2010a,b) originated from closely neighbouring trapping sites of a maximal distance of approximately 3 km. The phylogenetic clustering of the sequences from Hamburg cannot be explained by the geographical origin of the sequences. Future phylogeographical and population genetics studies should further investigate the transmission routes of ratHEV.

In conclusion, our study demonstrated the presence of ratHEV infections in Norway rats from additional regions in Germany. Phylogenetic analysis of the ratHEV sequences indicated a geographical clustering. Serological investigations using a carboxy-terminal region of the ratHEV CP revealed an antigenicity in immunized rats which clearly differed from that of the corresponding CP segment of a genotype 3 HEV strain. This serological difference may also allow serological screening for ratHEV-specific antibodies in other animal species and humans. Indeed, a pilot study in forestry workers from eastern Germany indicated the presence of ratHEV-specific antibodies in some of the investigated sera (Dremsek et al., 2011). Future investigations should prove the transmission routes of ratHEV and its epidemiological role for human HEV infections.

Acknowledgements

We would like to thank Mathias Schlegel, Dörte Kaufmann, Konrad Wanka, Ulrike M. Rosenfeld, Sabrina Schmidt, Theres Wollny, Josephine Schlosser, Bärbel Hammerschmidt, Franziska Thomas, Hanan Sheikh Ali, Daniel Windolph, Stephan Drewes and Thomas Büchner for excellent technical assistance, Matthias Stange, Mario Heising, the public pest control officers and the sewer company of Hamburg, and the animal keeping staff of the Wilhelma, Stuttgart and the Nymphaea, Esslingen, for their support, and Markus Keller, Bernd Köllner, Nicole Klemann, Stefan Endepols, Mario Ziller, Christoph Staubach and Elke Lange for their support und helpful discussions. This work was funded in part by the German Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) through the Federal Institute for Agriculture and Nutrition (BLE), grant numbers 07HS026 and 07HS027, and the Grimminger-Stiftung. Rat trapping in Ahlen was supported by Rodenticide Resistance Action Committee of CropLife International.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.02.021.

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Chapter 5

Publication III

Hepatitis E virus seroprevalence of domestic pigs in Germany determined by a novel in-house and two reference ELISAs

Key information of the publication

- Establishment of a novel in-house ELISA for detection of HEV-specific IgG antibodies in domestic pigs.
- High seroprevalences of domestic pig in German herds (average 43 %).
- Uniform seroprevalence in the federal states (18.8 –50.3 %).
- Highly diverse seroprevalence in the herds (0 –100 %).

Reprinted from Journal of Virological Methods, 190, Dremsek, P., Joel, S., Baechlein, C., Pavio, N., Schielke, A., Ziller, M., Dürrwald, R., Renner, C., Groschup, M. H., Johne, R., Krumbholz, A., Ulrich, R. G., Hepatitis E virus seroprevalence of domestic pigs in Germany determined by a novel in-house and two reference ELISAs, 11 – 16, Copyright 2013, with permission from Elsevier.

Journal of Virological Methods 190 (2013) 11–16

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Hepatitis E virus seroprevalence of domestic pigs in Germany determined by a novel in-house and two reference ELISAs

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Article history:
Received 20 November 2012
Received in revised form 16 January 2013
Accepted 11 March 2013
Available online xxx

Keywords:
Hepatitis E virus
Recombinant antigen
ELISA
Seroprevalence
Domestic pigs
Germany

A B S T R A C T

Autochthonous hepatitis E virus (HEV) infections by zoonotic transmission of genotype 3 (GT3) have been reported increasingly from industrialized countries. In this paper the development and validation of an IgG ELISA for the detection of HEV-specific antibodies in domestic pigs is described. Comparison of the diagnostic value of *Escherichia coli*-expressed HEV-GT3 capsid protein (CP) derivatives revealed a carboxy-terminal derivative as most suitable. Validation of the in-house assay using a commercially available IgG ELISA revealed a high diagnostic specificity and sensitivity. The average HEV seroprevalence of domestic pigs from Germany and the federal state Baden–Wuerttemberg determined by the in-house test was 42.7% and 50.3%, respectively. The seroprevalence in different districts of Baden–Wuerttemberg ranged from 34.9% to 60%, but from 0% to 100% between different herds. These data were compared to those achieved by two commercially available ELISA kits and an in-house ratHEV-based ELISA. In conclusion, the CP-based in-house test proved sensitive and specific, indicating that the ORF3-encoded protein might be dispensable for diagnostics. The novel assay also allowed a parallel analysis by a homologous ratHEV-derived antigen. Thus, the novel IgG ELISA represents a useful tool for future standardized seroprevalence studies in domestic pigs from Germany and other regions of Europe.

1. Introduction

The hepatitis E virus (HEV) was discovered during an epidemic of acute hepatitis in India in 1978 (Khuroo, 1980). It belongs to the genus *Hepevirus* within the family *Hepeviridae* which is not assigned to any order (Emerson et al., 2004; Meng et al., 2012). The majority of human infections and large outbreaks in countries with lower sanitary conditions are caused by infections with the genotype (GT) 1 and GT2 (Mushahwar, 2008). These infections are transmitted by the fecal-oral route, mainly by contaminated drinking water (Aggarwal and Jameel, 2011). Sporadic hepatitis E cases in industrialized countries were found frequently to be caused by autochthonous HEV infections with GT3 and GT4 (Meng, 2011). These human infections are mediated by zoonotic transmission through meat and meat products from wild boar, domestic pig and deer (Colson et al., 2010; Yazaki et al., 2003; Matsuda et al., 2003; Tei et al., 2003). In addition to the zoonotic transmission, organ transplantation and blood transfusion may represent alternative routes of transmission (Bajpai and Gupta, 2011; Schlosser et al., 2012). Moreover, the observed increased seroprevalences in veterinarians, pig farmers, slaughter house personnel and swine traders also suggested a direct contact to HEV-infected animals as potential transmission route (Vivek and Kang, 2011; Krumbholz et al., 2012). The average seroprevalence in the human population

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http://dx.doi.org/10.1016/j.jviromet.2013.03.010

of several industrialized countries was found to be surprisingly high (Dalton et al., 2008; Guo et al., 2010; Faber et al., 2012).

Recent molecular investigations demonstrated additional mammal-associated hepeviruses. Thus, in Norway rats *Rattus norvegicus* from Germany a novel genotype (ratHEV) was identified (Johne et al., 2010a, 2010b, 2012) which was also detected in Vietnam and the US (Li et al., 2011; Purcell et al., 2011). Further, novel, highly divergent hepevirus strains were detected in bats from different continents and in ferrets in the Netherlands (Drexler et al., 2012; Raj et al., 2012). In addition to mammalian hepeviruses, different strains of avian HEV were found to be responsible for the hepatitis-splenomegaly syndrome (Huang et al., 2002). The most divergent HEV-like virus was found in seven trout species (Batts et al., 2011).

Hepeviruses exhibit a single stranded RNA genome of positive polarity with the typical features of a eukaryotic mRNA (Meng et al., 2012). The genome size without poly-A tail has been found to be 6945 nucleotides (ratHEV, GU345043.1) to 7415 nucleotides (genotype 3, HQ389544.1) for mammalian hepeviruses and 6654 nucleotides for avian hepeviruses (AY535004.1). The general genome organization is very similar throughout all members of the virus family with a large open reading frame (ORF) 1 at the 5′ end and the overlapping ORF2 and ORF3 at the 3′ region (Tam et al., 1991; Meng et al., 2012). ORF1 encodes a non-structural polyprotein (Pudupakam et al., 2009), ORF2 codes for the capsid protein (CP) encompassing 660 amino acid (aa) residues and ORF3 encodes a phosphoprotein that appears to have a variety of functions (Chandra et al., 2011; Liu et al., 2011).

The number of human hepatitis E cases recorded in Germany seems to have increased in previous years, reaching a total number of 1344 since 2001 when hepatitis E became a notifiable disease (Robert Koch-Institut, www.rki.de, SurvStat, data as of 2/1/2013). A recent study reported that the proportion of autochthonous cases increased from 30–40% to 78% of all recorded cases in Germany (Faber et al., 2012). In line with this, the presence of HEV-GT3 was confirmed molecularly in wild boars from Germany (Adlhoch et al., 2009; Schielke et al., 2009). In addition, blood and meat juice samples from domestic pigs were found to contain HEV-specific antibodies (Wacheck et al., 2012). A further serosurvey in domestic pigs demonstrated a broad variation in results when using different assays, thus underlining the necessity of standardization of serological assays used for seroprevalence studies (Baechlein et al., 2010; Pavio et al., 2010; Khudyakov and Kamili, 2011).

In the current paper the establishment and validation of a novel HEV-GT3 IgG ELISA for domestic pigs and its use for a seroprevalence study in Germany is described. The performance of the novel assay was compared to those of a commercial IgG ELISA and a commercial double-antigen sandwich ELISA. In addition, all pig sera were investigated in parallel by in-house IgG ELISA using the corresponding antigen of ratHEV.

2. Material and methods

2.1. Serum panels of domestic pigs

For the validation of the test, a total of 898 field sera from domestic pigs were included. Previously, 332 sera of domestic pigs were collected from pig herds in the federal states of Brandenburg ($n = 59$), Hesse ($n = 19$), Mecklenburg-Western Pomerania ($n = 43$), Saxony ($n = 50$), Saxony-Anhalt ($n = 53$), Schleswig-Holstein ($n = 58$) and Thuringia ($n = 50$; see Fig. 1A) and tested by the commercial double-antigen sandwich ELISA (Baechlein et al., 2010). The serum panel was completed by samples from Baden–Wuerttemberg ($n = 461$), Lower Saxony ($n = 50$), Rhineland-Palatinate ($n = 50$) and a few additional sera from Mecklenburg-Western Pomerania

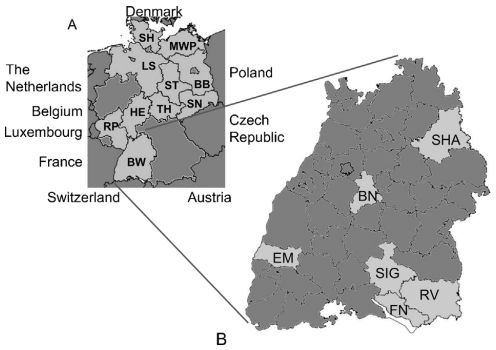

Fig. 1. Origin of the domestic pig field serum samples from the federal states of Schleswig-Holstein (SH), Mecklenburg-Western Pomerania (MWP), Lower Saxony (LS), Saxony-Anhalt (ST), Brandenburg (BB), Rhineland-Palatinate (RP), Hesse (HE), Thuringia (TH), Saxony (SN) and Baden–Wuerttemberg (BW) of Germany (A), and the districts of Böblingen (BN), Emmendingen (EM), Friedrichshafen (FN), Ravensburg (RV), Schwäbisch-Hall (SHA) and Sigmaringen (SIG) within the federal state of Baden–Wuerttemberg (B).

($n = 5$) (see Fig. 1A). The samples from Baden–Wuerttemberg were obtained from six districts (Fig. 1B) and 24 herds (with 9–48 animals per herd). In addition, 16 serum samples from one non-infected and two experimentally GT 3e (accession no EF494700.1) infected domestic pigs *Sus scrofa domesticus* from a specific pathogen, i.e. HEV, free (SPF) facility were used for the test validation (Barnaud et al., 2012).

2.2. Recombinant antigens

The GT3i strain wbGER27 (accession no FJ705359.1)-derived CP derivatives containing aa residues 112–608 (GT3-tr), 112–335 (GT3-Ntr), 326–608 (GT3-Ctr) and 326–660 (GT3-C) and ratHEV (accession no GQ504009.1) CP derivative spanning aa residues 315–599 (ratHEV-Ctr) were expressed as His-tagged proteins in *Escherichia coli* and purified as described recently (Dremsek et al., 2012; Johne et al., 2012). As a negative control, a His-tagged non-structural protein 1 (NS1) derivative of West Nile virus (WNV) was used, since it was expressed and purified under the same conditions (Johne et al., 2012).

2.3. Reference assays

A double-antigen sandwich ELISA (HEV EIA; Axiom, Bürstadt, Germany) for the detection of HEV-specific antibodies of all isotypes and the PrioCHECK® HEV Ab porcine assay (Prionics, Schlieren-Zurich, Switzerland) for the detection of IgG antibodies were performed according to the manufacturers' protocols.

2.4. Indirect in-house IgG ELISA

The in-house test was based on a protocol developed for an indirect IgG ELISA to detect anti-HEV antibodies in human sera using a carboxy-terminal segment of HEV-GT3 CP (GT3-Ctr; Dremsek et al., 2012). This protocol was modified based on the results obtained for two anti-HEV positive and two negative control sera from domestic pigs identified by the commercial double-antigen sandwich ELISA. Therefore, various test parameters were optimized to obtain the highest ratio of the corrected optical density (OD) values for the anti-HEV positive and negative sera. In the protocol, coating was done overnight at 4 °C with 0.2 μg/well GT3-Ctr protein in carbonate buffer on medisorp plates (Nalge

Nunc International, Penfield, NY, USA). Blocking at room temperature (RT) for 1 h with 200 μl PBS containing 1% BSA and 0.05% Tween 20 was followed by 2 h incubation at 37 °C with 100 μl serum diluted 1/200 in PBS with 0.5% BSA and 0.05% Tween 20 (DB), threefold washing with 0.1% PBS Tween 20, and 1 h incubation at 37 °C with 100 μl polyclonal horse-radish peroxidase (HRP)-conjugated goat anti-swine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:45,000 in DB. Following an additional threefold washing, the HRP-mediated reaction was initiated by 3,3′,5,5′-Tetramethylbenzidine (Peroxidase EIA Substrate Kit, Bio-Rad, Hercules, CA, USA) and stopped by adding H_2SO_4. The OD values of each serum were measured in antigen-coated and antigen-free wells at 450 nm with 620 nm as the reference. The corrected OD values were calculated by subtracting the OD values obtained for the non-coated wells from the corresponding OD values obtained for the coated wells. The same standard protocol was used for testing the performance of other GT3 CP segments and a corresponding CP segment of ratHEV.

2.5. Determination of the cut-off value for the indirect in-house IgG ELISA

To find the most appropriate cut-off and to describe the test thoroughly, receiver-operated characteristic (ROC) analyses were performed following classical and modified ROC protocols implementing plate-dependent variables (Dremsek et al., 2012). The optimal method for calculating the cut-off was selected by the highest area under curve (AUC) value and the resulting sensitivity and specificity values, following which the optimal cut-off value of the chosen method was determined by the minimum ROC distance.

2.6. Seroprevalence studies

The GT3-Ctr antigen-based IgG ELISA was used for the determination of HEV seroprevalence in domestic pigs from Germany. The pig sera were investigated in parallel by two commercially available HEV ELISAs and an in-house ratHEV antigen-based ELISA.

2.7. Statistical analysis

The diagnostic values of the different recombinant antigens were compared using a Wilcoxon rank sum test with continuity correction. The concordance indications were calculated as Cohen's kappa. The prevalences in the pig populations were calculated with a 95% confidence interval. All calculations and ROC-graphics were performed using R, version 2.13.0 (2011-04-13, R Development Core Team, 2011).

3. Results

3.1. Establishment and validation of a novel IgG ELISA for the detection of HEV-specific antibodies in domestic pigs

Initially, the four different *E. coli*-expressed and purified GT3-derived CP derivatives were evaluated for their reactivity with selected positive and negative control sera, following the in-house ELISA standard protocol (see Section 2). The CP derivative GT3-Ctr harbouring aa residues 326–608 demonstrated reproducibly the highest sensitivity in this initial setting, whereas GT3-C harbouring aa residues 326–660 revealed a significantly lower reactivity. The reaction of the other two CP derivatives was as low as the WNV-NS1 negative control protein (data not shown).

To validate the novel GT3-Ctr antigen-based IgG ELISA, a total of 914 sera including 898 field sera from Germany as well as 16 sera from two experimentally infected pigs and a non-infected control animal were investigated in parallel by the novel in-house and

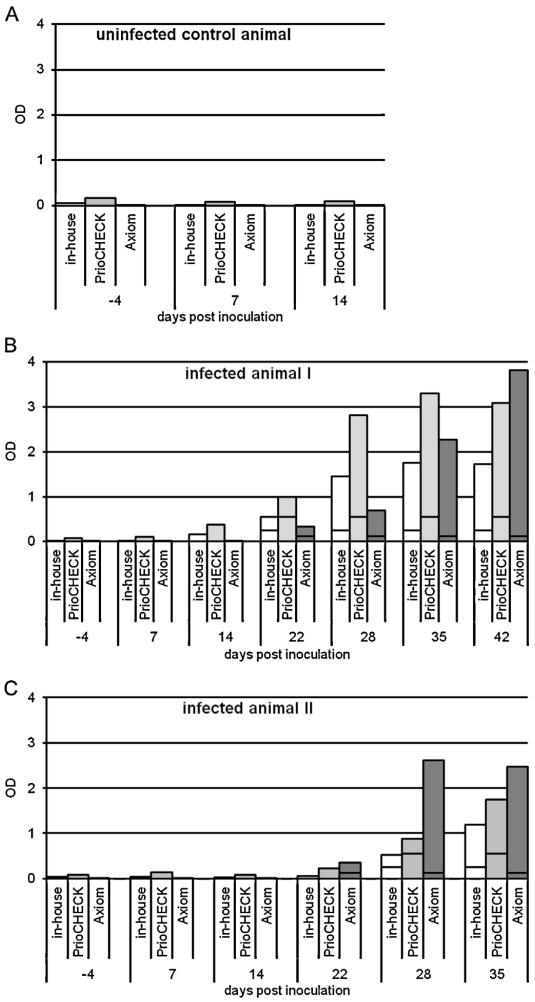

Fig. 2. Comparison of the reactivity of the in-house IgG ELISA (in-house), the commercial IgG ELISA (PrioCHECK) and the double-antigen sandwich ELISA (Axiom) with follow-up sera of one uninfected domestic pig (A) and experimentally HEV-genotype 3 infected domestic pigs I (B) and II (C). The columns of the commercial tests show the OD, the columns of the in-house ELISA show the corrected OD. Each column representing a positive value displays the cut-off as a black horizontal line.

the commercial IgG ELISAs. Definition of the cut-off using both global and modified ROC analysis yielded high and very similar AUCs (data not shown). Based on the modified ROC analysis the cut-off at 2.1457% of the positive control minus the negative control of each plate was selected. The selection of this cut-off resulted in a diagnostic sensitivity of 0.8550 and of a diagnostic specificity of 0.9190. The concordance of the results of the reference IgG ELISA and the novel in-house assay reached 0.7776.

To prove the performance of the novel in-house IgG ELISA, the reactivity of domestic pig follow-up sera from the infection experiment was compared using the novel assay, the commercial IgG ELISA and the commercial double-antigen sandwich ELISA (Fig. 2A–C). The sera from the negative control animal did not show any significant reactivity with any of the three assays (Fig. 2A). In

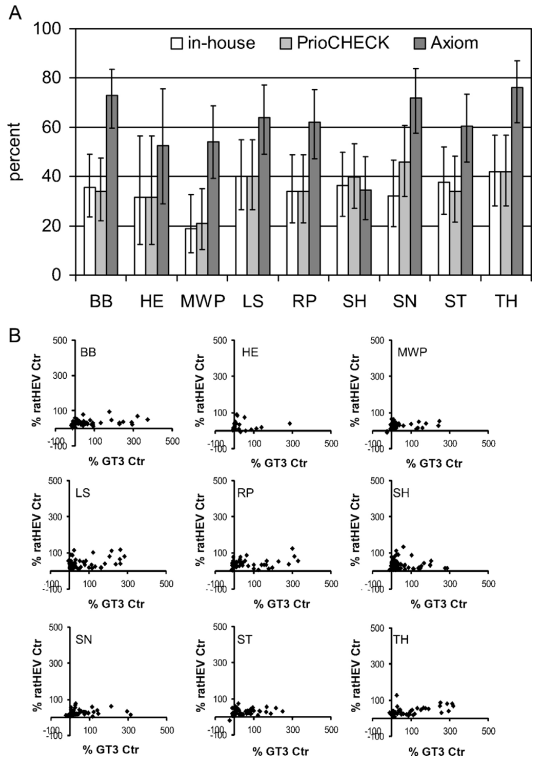

Fig. 3. Results of the investigations of serum panels from the federal states of Brandenburg (BB), Hesse (HE), Mecklenburg-Western Pomerania (MWP), Lower Saxony (LS), Rhineland-Palatinate (RP), Schleswig-Holstein (SH), Saxony (SN), Saxony-Anhalt (ST), Thuringia (TH) with the in-house and commercial IgG ELISAs and the commercial double-antigen sandwich ELISA (A) and using the in-house ELISA with GT3 and ratHEV CP antigens in a dot plot (B). The x-axis of the dot plots shows reactivity in the GT3-based ELISA, the y-axis shows reactivity in the ratHEV-based ELISA. Both axes are scaled to percent of reactivity of the respective positive controls. Error bars denote a 95% confidence interval.

animal I, first seroreactivity was observed by all three assays at 22 days post infection (Fig. 2B). In the sera from the other experimentally infected animal II the double-antigen sandwich test (Axiom) yielded a positive result on day 22, while the IgG tests detected antibodies slightly later on day 28 (Fig. 2C). The specificity of the reactivity of the in-house IgG ELISA was confirmed by the exclusive reactivity of the sera with the GT3 antigen in the absence of reactivity with the corresponding ratHEV antigen (data not shown).

3.2. Seroepidemiological studies

3.2.1. Seroepidemiological study in the German domestic pig population in general

The results of both assays for the 437 sera from the 9 federal states investigated were very similar (Fig. 3A), and the concordance of the reference IgG ELISA and the novel in-house assay reached 0.8148 (data not shown). A comparison of the results of these two IgG ELISAs to those obtained by the double-antigen sandwich ELISA demonstrated higher discrepancies; the average prevalences in this serum panel were 34.5% and 36.1% for the in-house and reference IgG ELISAs respectively, but 61.3% for the double-antigen sandwich ELISA. The seroprevalences in the different federal states ranged from 18.8–20.8% (Mecklenburg-Western Pomerania) to 32–46% (Saxony) when using the in-house and reference IgG assays, but from 34.5% (Schleswig-Holstein) to 76% (Thuringia) when using the double-antigen sandwich ELISA (Fig. 3A). The majority of the GT3-reactive sera demonstrated no or a lower level of reactivity to the ratHEV antigen in the corresponding in-house IgG ELISA (Fig. 3B).

3.2.2. Regionally detailed seroepidemiological study in south–west Germany

In order to investigate local seroprevalences in detail, the novel in-house ELISA was used for a seroprevalence study on a panel of 461 sera from six districts in Baden–Wuerttemberg, south–west Germany (Fig. 1B). The average seroprevalence was demonstrated to be 50.3% by the in-house assay, whereas the reference IgG ELISA revealed a seroprevalence of 52.7%. The Cohen's kappa value of both assays was 0.7266 (data not shown). Evaluation of the same serum panel with the double-antigen sandwich ELISA showed a prevalence of 75.3%. A comparison of the results on the district level showed an even distribution in seroprevalences when using the IgG ELISAs, ranging from 34.9–40.7% (Emmendingen) to 60–80% (Friedrichshafen) with the in-house test and the reference IgG ELISA. In contrast, the seroprevalences in the double-antigen sandwich ELISA ranged from 69.5% (Sigmaringen) to 100% (Friedrichshafen) (Fig. 4A). A comparison of the reactivity of the serum panel with the in-house GT3- and ratHEV-based ELISAs confirmed a stronger reactivity to the GT3 antigen and a lower cross-reactivity with the corresponding ratHEV antigen for most pig sera (Fig. 4B).

The 24 different herds showed very similar ratios of positive animals with the in-house test and the reference IgG ELISA ranging from 0% to 100% with one herd each having a prevalence of 100% (Fig. 4C). The double-antigen sandwich ELISA almost always yielded ratios equally high or higher with 7 herds showing a prevalence of 100% (Fig. 4C).

4. Discussion

The serological detection of a HEV infection is usually based on recombinant ORF2- and ORF3-derived antigens (Khudyakov and Kamili, 2011). Previous investigations demonstrated the C-terminal region of the CP as immunodominant and the antigen of choice for serological diagnostics (Purdy et al., 1993; Meng et al., 2001; Tang et al., 2011; Dremsek et al., 2012). This region was found to be exposed on the surface of the capsid and to harbour neutralizing epitopes, whereas the N-terminal region was shown to be buried within the particle (Mori and Matsuura, 2011; Tang et al., 2011; Shata et al., 2012). Moreover, homologous C-terminal CP derivatives of GT3 and ratHEV were found to allow a differentiation of antibodies specific for the homologous and heterologous antigens (Johne et al., 2012). The current study confirms the diagnostic value of the C-terminal region of GT3-HEV for the detection of GT3-specific antibodies in domestic pigs. In line with a previous study in human sera (Dremsek et al., 2012), the N-terminal region seems of negligible value for serodiagnostics. The in-house IgG ELISA demonstrated a very similar performance compared to the commercial IgG ELISA (PrioCHECK) used as reference assay with an average concordance of 0.7776. Thus, the use of an additional ORF3-derived antigen and the combination of antigens of GT3 and GT1 in the PrioCHECK test did not influence the overall performance of the IgG ELISA, at least in regions with circulation of GT3 alone. The double-antigen sandwich ELISA (Axiom) demonstrated a much lower concordance to the IgG tests with kappa values of 0.3883 (in-house/Axiom) and 0.4385 (PrioCHECK/Axiom). One obvious reason might be the consequence of the Axiom test format including the detection of IgA and IgM antibodies, which may allow for an earlier detection of an HEV infection (Chau et al., 1993). In addition, the

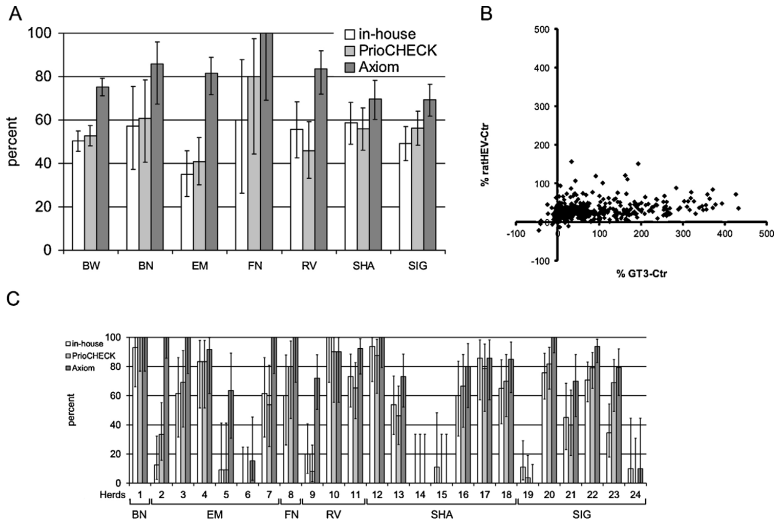

Fig. 4. HEV seroprevalences determined for the districts (A) and the herds (C) of the federal state Baden–Wuerttemberg as determined by the in-house and commercial IgG ELISAs and commercial double-antigen sandwich ELISA. Böblingen (BN), Emmendingen (EM), Friedrichshafen (FN), Ravensburg (RV), Schwäbisch-Hall (SHA) and Sigmaringen (SIG). (B) Comparison of the results of the in-house test with GT3 and ratHEV antigen. The dot plot illustrates the reactivity of samples in GT3-HEV and ratHEV in-house ELISA. The *x*-axis shows the reactivity in the GT3-based ELISA, the *y*-axis shows the reactivity in the ratHEV-based ELISA. Both axes are scaled to percent of reactivity of the respective positive controls. Error bars denote a 95% confidence interval.

sensitivity and specificity might also differ because of the exclusive use of GT1-derived antigen(s) in the double-antigen test.

The average seroprevalence in domestic pigs from 10 federal states reached 42.7–44.7% (in-house and PrioCHECK), whereas the Axiom test found 68.5% of all German sera to be positive. The seroprevalence of pigs coming from different districts of Baden–Wuerttemberg differed markedly, independently from the test used. This variation of prevalence was even more obvious when comparing the results for the 24 herds from Baden–Wuerttemberg. A similar strong variation of the seroprevalences of pig herds has been reported previously (Baechlein et al., 2010). This variation is also in line with earlier observations where wild boars were found to deviate strongly in HEV-seroprevalence, even in neighboring regions of Germany (Adlhoch et al., 2009).

Previous investigations of forestry workers from Brandenburg, eastern Germany, have suggested the presence of antibodies specific for ratHEV or a closely related other hepevirus (Dremsek et al., 2012). In the current investigation the majority of domestic pig sera were found to be stronger or exclusively reactive with the GT3 antigen, but a few animals demonstrated a stronger reactivity to the ratHEV antigen. Future molecular and serological studies of rats from pig herds have to prove the presence of ratHEV and if GT3-HEV may also infect Norway rats in pig herds as previously reported (Lack et al., 2012).

In conclusion, the in-house ELISA developed demonstrated a high sensitivity and specificity comparable to that of an already licensed commercial IgG ELISA. Using both tests a comparable high seroprevalence was demonstrated in domestic pigs from different federal states of Germany. The parallel investigation of pig sera with in-house GT3- and ratHEV-based ELISAs may allow a diagnostic differentiation between GT3- and ratHEV-specific antibodies.

Acknowledgements

The authors would like to thank Elodie Barnaud for providing sera from experimentally HEV infected domestic pigs, Katja Schmidt for providing the WNV NS1 control antigen, Ina Römer, Dörte Kaufmann, Bärbel Hammerschmidt, Kathrin Baumann for excellent technical assistance, and Elke Lange for her support and helpful discussions. In addition, the authors are very grateful to the veterinary offices of Baden–Wuerttemberg that were involved in the acquisition of the samples, the pig health service of the Tierseuchenkasse Baden–Wuerttemberg and the laboratories for processing and transport of the samples. The authors would also like to express their gratitude to Malcolm Banks (Weybridge, UK) for proofreading and editing of the manuscript.

This work was funded in part by the German Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) through the Federal Institute for Agriculture and Nutrition (BLE), grant numbers 07HS026 and 07HS027, and the Grimminger-Stiftung.

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Chapter 6

Results and discussion

6.1 Serological diagnostics

6.1.1 General overview

Indirect serological tests are able to detect subjects within the late phase of an acute HEV infection as well as subjects with a previous infection. These tests are based on the detection of antibodies directed against an antigenic segment of a viral protein. All three pORFs of HEV incorporate immunogenic sequences, i. e. the Y, V and X-domains of the polyprotein encoded by ORF1 (pORF1), the entire pORF3 as well as its C-domain, and several segments of pORF2 (see Figure 6.1) (Osterman et al., 2012). In despite of the widespread occurrence of antigenic epitopes throughout the proteins of HEV, the most immunogenic epitopes are encoded near the C-terminus of pORF2 and in pORF3. Furthermore, antibodies specific for certain epitopes may have a shorter persistence than others. The titers of anti-pORF3 antibodies usually decline within several months and generally faster than antibodies against pORF2, thus limiting the application of pORF3 as diagnostic antigen and rendering pORF2 the most promising antigen (Li et al., 1994). Furthermore, neutralizing antibodies are formed only against the protruding domain of pORF2 (aa 456 – 606), a region which is accessible to antibodies in the complete virion (Guu et al., 2009; Yamashita et al., 2009). Hence, pORF2 seems to be the best diagnostic antigen, as antibody-production against it appears to be mandatory for clearing of HEV. However, segments of pORF1 and pORF3 may still be of importance in

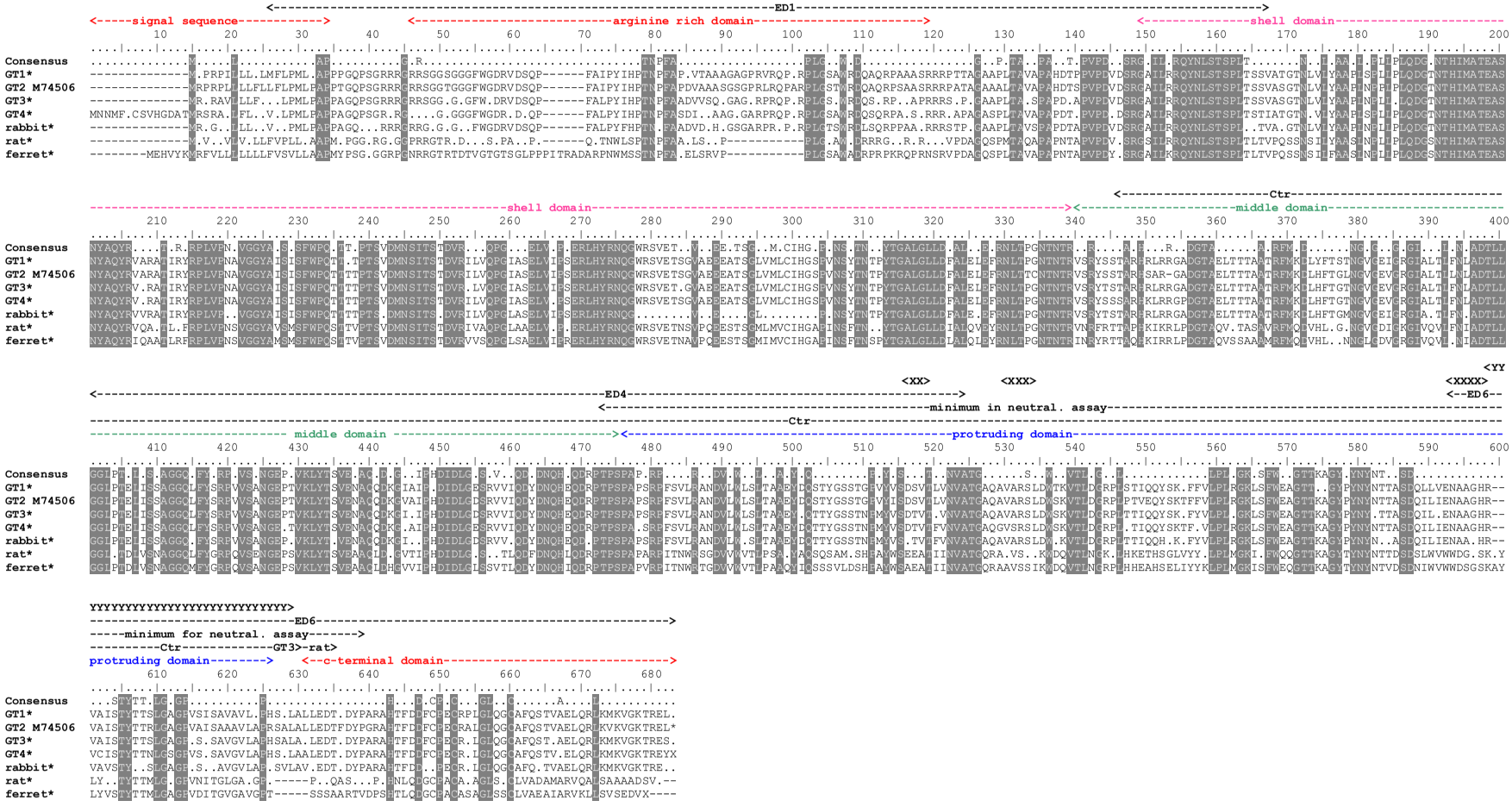

Figure 6.1: *Amino acid sequence of pORF2, with prominent features.* Assignment and denomination of functional domains from Mori and Matsuura (2011). Epitope domains ED1, ED4, ED6 (Khudyakov et al., 1999). Epitopes that bind neutralizing antibodies are marked with an "X" for antibody 8C11 (Tang et al., 2011) and with a "Y" for antibodies HEV#4 and HEV#31 (Schofield et al., 2000). Minimum neutralization epitope from Meng et al. (2001). GT1*, consensus sequence of AF076239 and M80581; GT3*, consensus sequence of EU360977, AB189070 and AF060668; GT4*, consensus sequence of AB220977, AJ272108 and GU119961; rabbit*, consensus sequence of JX565469, GU937805, FJ906896 and FJ906895; rat*, consensus sequence of JN167537, JN167538, GU345042, GU345043 and JX120573; ferret*, consensus sequence of JN998606 and JN998607; -, gap; ., non-consensus site; residues at shaded sites have a frequency of 0.95 or higher.

serological tests and have been used as additional antigens to confirm positive samples (Osterman et al., 2012).

Indirect ELISA, as described in Publications I – III, is a widespread method of serological testing. It relies on detection of the antibody isotopes IgG, IgA or IgM. IgG is the most abundant isotype in serum. It is an excellent marker and often used in seroepidemiological surveys, as its persistence may be of several years (Khuroo et al., 1993). IgA is not screened for in commercial tests, however the duration of IgA in serum is longer than IgM and it seems to be a reliable marker (Chau et al., 1993; Osterman et al., 2013; Tian et al., 2006). Nonetheless it may be ineffective in cases of parenteral infection (Herremans et al., 2007b). This could mean that infections by blood transfusion or solid organ transplantation are not reflected by an elevated titer of anti-HEV IgA. Titers of IgM wane typically within several months. However, IgM is a reliable marker to detect recent or current infection. As indirect IgM ELISAs may be prone to unintentional, additional detection of abundant IgG, μ-capture assays are considered more accurate (Aggarwal, 2013). Thereby, the solid phase is coated with an anti-μ-antibody, capturing IgM antibodies of the sera, which then bind to conjugated antigen of HEV.

An alternative method is the double-antigen sandwich ELISA. Thereby, the solid phase is coated with antigen, which can be bound by specific antibodies of the serum. A secondary antigen, conjugated to an enzyme, may be bound by the now immobilized antibodies. This method is able to detect antibodies regardless of their isotype and host species.

The CP has been employed widely for serological testing, hereby using different segments (see Table 6.1). To find the most promising segments, the protein has been screened for epitopes with synthetic peptides, the most relevant are located at the termini of the protein in epitope domains 1, 4 and 6 (see Figure 6.1) (Khudyakov et al., 1999). It is believed that many antigenic epitopes are complex and conformation-dependent, as segments of several hundred amino acids perform better than short peptides (Khudyakov and Kamili, 2011). Additionally, using longer segments of the CP may be advantageous, as short epitopes may vary from one strain to another. Thus, antigens incorporating multiple epitopes exhibit a broader range of cross-reactivity. However, exceeding

Table 6.1: *Serological assays based on segments of pORF2 as antigen.*

GT	expression system	fusion protein	length (aa) from	length (aa) to	diagnostic system	references
1	*E. coli*	GST	394	660	EIA	Anderson et al., 1999
1	*E. coli*	GST	394	660	IB	Li et al., 1994
1	*E. coli*	GST	99	660	IB	Li et al., 1994
1	*E. coli*	GST	394	660	IB	Li et al., 1997a
1	*E. coli*	GST	452	617	EIA	Obriadina et al., 2002
1	*E. coli*	—	394	660	ICT	Chen et al., 2005
1	*E. coli*	—	394	660	EIA	Hu et al., 2008
1	*E. coli*	—	458	607	EIA	Zhou et al., 2004
1	BV	—	1	660	EIA	Arankalle et al., 2007
1	BV	—	1	660	EIA, IB	Haqshenas et al., 2002
1	BV	—	112	660	EIA	Li et al., 2000
1	BV	—	112	607	EIA, IB	Peralta et al., 2009b
1	BV	—	1	660	EIA	Tsarev et al., 1993
1	BV-S	—	112	660	EIA	Mizuo et al., 2002
1, 2	*E. coli*	GST	31	660*	EIA	Obriadina et al., 2002
3	BV	—	1	660	EIA, IB	Haqshenas et al., 2002
3	BV	—	1	660	EIA	Meng et al., 2002
3	BV	—	112	607	EIA, IB	Peralta et al., 2009b
4	BV	—	1	660	EIA	Arankalle et al., 2007
rat	BV	—	101	644	EIA	Li et al., 2011a
avian	*E. coli*	—	338	606	EIA, IB	Haqshenas et al., 2002
avian	*E. coli*	—	338	606	EIA	Peralta et al., 2009a

aa, amino acid; BV, baculovirus-mediated expression in insect cells; BV-S, baculovirus-mediated expression in silkworm pupae; GST, glutathione S-transferase; *, mosaic protein, formed by segments from pORF2 and pORF3; EIA, Enzyme immunoassay; IB, immunoblot; ICT, immunochromatographic test

the optimal length of the antigenic segment is likely to hinder proper antibody binding, probably due to a different presentation of conformation-dependent epitopes (Li et al., 1994, 1997a).

6.1.2 Development of the GT 3 in-house ELISAs

6.1.2.1 Selection of antigens

For the detection of anti-HEV-antibodies in human, swine and rat in Germany, the CP of an HEV GT 3 strain was chosen to supply the antigen needed for the development of an indirect ELISA. This strain (HEV GT 3i, wbGER27, GenBank accession number FJ705359) was found in a German wild boar and is autochthonous in Germany (Schielke et al., 2009).

Several segments of the CP were considered as antigens, including GT 3-Ntr, GT 3-tr, GT 3-C and GT 3-Ctr (see Figure 2 in Publication I). The most favorable

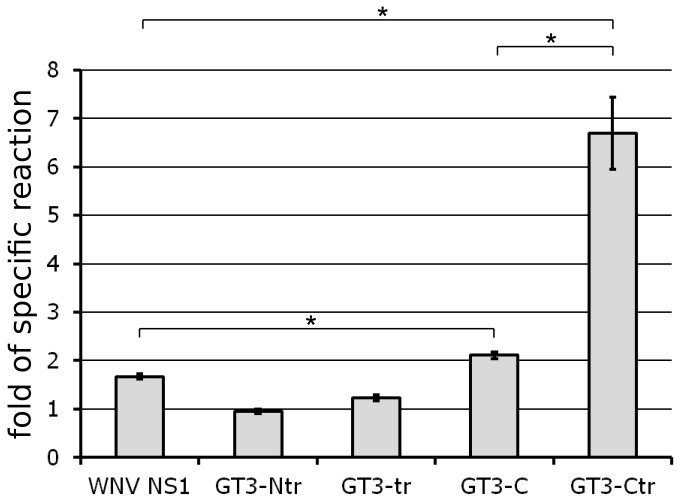

Figure 6.2: *Reactivity of the four segments of the capsid protein of HEV with control sera of domestic pig.* The graph depicts the ratio of the reactivity (corrected OD) of the positive control and the negative control as arithmetic mean value of five independent measurements. West Nile Virus (WNV) nonstructural protein 1 (NS1) is used as a negative control (see Publication I). The interval shown is the standard error of the mean. Significances were calculated only for values connected with a horizontal line. Correlations marked with an asterisk are statistically significant ($P<0.01$).

segment for detection of antibodies in humans and domestic pigs was GT 3-Ctr, spanning from aa 326 to aa 608 and thereby incorporating both the middle and protruding domain, both of which have been proven accessible in VLPs (Yamashita et al., 2009) (see Figure 4 in Publication I and Figure 6.2). Consequently, the indirect IgG ELISA for detection of HEV in human and swine was established basing on the GT 3-Ctr segment as antigen. This segment is at approximately the same position as most antigens given in Table 6.1. Moreover, it harbors the protruding domain, which contains several epitopes of neutralizing antibodies (Khudyakov et al., 1999; Mori and Matsuura, 2011; Tang et al., 2011).

The feasibility of GT 3-Ctr is in line with previous studies that show this region as a powerful antigen in serological tests (see Table 6.1) and in immunization experiments (Riddell et al., 2000). A study involving synthetic peptides and recombinant proteins to immunize mice showed that the minimum length of a segment to elicit neutralizing antibodies is aa 452 to aa 617, which is in the same range as GT 3-Ctr and the protruding domain (see Figure 6.1) (Meng et al., 2001; Yamashita et al., 2009).

GT 3-C also performed very well, albeit its reactivity was not as strong as GT 3-Ctr (see Figure 4 in Publication I and Figure 6.2). This may be explained by the increased length of the segment, that could hinder proper display of epitopes. The longer fragment is likely to hinder proper antibody binding, an effect described previously by Li et al. (1994).
To validate the novel in-house ELISAs, widely used commercial tests were employed. These tests were indirect IgG tests basing on GT 3, hence their performances are directly comparable to the performances of their corresponding in-house tests. For validating the in-house ELISA of human sera, the *recom*Line HEV IgG test (Mikrogen, Neuried, Germany) was used as a reference assay. For the validation of the in-house test for porcine sera, PrioCHECK HEV Ab porcine assay (Prionics, Schlieren-Zürich, Switzerland) was selected as a reference assay.

6.1.2.2 Validation of the novel assays

To validate the in-house ELISAs, a modified receiver operating characteristic (ROC) analysis was used (Dremsek et al., unpublished data). ROC-analysis has been a well-established standard framework for evaluating diagnostic tests and for defining suitable cutoffs for almost three decades (Hanley and McNeil, 1982). The ROC-analysis depends on diagnostic procedures that provide directly comparable values (e. g. OD-values). From these values and a given pre-classification of the samples by a reference assay, an optimal global cutoff can be estimated.
As results may fluctuate, owed to differences of individual microtiter-plates, it may not be feasible to rely on the OD-values of the test. Instead, to minimize deviations of results, the "global" cutoff was abandoned and replaced by a variable cutoff. The variable cutoff is based on the values of the positive and negative control of a single microtiter-plate and used for classification of values measured on this plate only.
There are several methods known to define, but not to optimize variable cutoffs. However, as the ROC-analysis cannot handle values of different internal standards, six methods were adapted to be evaluable in ROC-analysis and to

yield optimized cutoffs. The pre-classification of the samples was done by commercial reference assays that were designated as gold standard, one (*recom*Line HEV IgG assay) for validating the in-house human IgG ELISA and one (PrioCHECK ELISA) for validating the in-house porcine IgG ELISA.
Using the in-house ELISAs, each serum was tested in wells with an HEV antigen, yielding the uncorrected OD-value. In addition, each serum was tested in wells without antigen coating to account for the background noise. When subtracted from the uncorrected OD-value, this results in the corrected OD-value. Given the measurements:

"value" — corrected OD-value for the sample
"pos" — corrected OD-value for positive control
"neg" — corrected OD-value for negative control
"raw_neg" — uncorrected OD-value for negative control

the following parameterized terms were considered as cutoff-definitions.

a. proportion of "pos" : cutoff := pos : param
b. multiple of "neg" : cutoff := neg · param
c. multiple of "raw_neg" : cutoff := raw_neg · param
d. proportion of "pos"−"neg" : cutoff := (pos−neg) : param
e. offset to "neg" : cutoff := neg + param
f. offset to "raw_neg" : cutoff := raw_neg + param

where "param" is the parameter to be optimized.
All calculations were performed using R, Version 2.13.07 (R Development Core Team, 2011). The optimization of the parameter of each cutoff-term was performed using a standard minimizer which was applied to the error-function defined dependent on the corresponding ROC-curve (Brent, 1973).
In addition, a relative index

g. result := (value−neg) / (pos−neg)

was calculated as the diagnostic result, and the cutoff determined with the usual methods.
For the human HEV IgG ELISA, the area under the ROC-curve (AUC) was very high for all methods used (a: 0.9769, b: 0.9499, c: 0.9752, d: 0.9774, e: 0.9752, f: 0.9767, g: 0.9752; see Figure 5 in Publication II). For the porcine IgG

ELISA, the AUC-values were marginally lower (a: 0.9636, b: 0.8403, c: 0.9588, d: 0.9628, e: 0.9604, f: 0.9593, g: 0.9624), but have the same outcome. The best results for both tests could be achieved for *a: proportion of* "*pos*" and *d: proportion of* "*pos*"−"*neg*". As the ELISAs themselves performed very well, all AUC-values were in the same range. For both tests, *d: proportion of* "*pos*"−"*neg*" was chosen to determine the cutoff, with a factor of 5.425 and 2.381 for the human IgG test and porcine IgG test, respectively. When compared to its respective reference test (*recom*Line HEV IgG assay), the in-house human IgG ELISA reached a high sensitivity (0.9318) and specificity (0.9542). The similarity of the in-house swine IgG ELISA and the PrioCHECK HEV Ab porcine assay was slightly lower but still yielded a sensitivity of 0.8550 and a specificity of 0.9191.
The "global" ROC-analyses yielded AUC-values similar to the variable ROC-analyses (0.9694 for the human IgG ELISA and 0.9592 for the porcine IgG ELISA), however still lower than most values based on the variable calculation methods. This indicates that even well-performing diagnostic tests may profit from the variable ROC-analysis.

6.1.3 Development of the rat HEV in-house ELISA

To detect anti-rat HEV antibodies, a segment (rat HEV-Ctr, aa 315 – 599) of pORF2 from rat HEV strain R4 (GenBank accession number GQ504009) was expressed and purified by the same method as the corresponding segment GT 3-Ctr. An indirect IgG ELISA was established to detect rat HEV in rats using hyperimmune rat sera as controls. A global cutoff was set at the mean value of minimally reactive rat samples plus three times its standard deviation, using the samples collected in Ahlen.
The ELISA using rat HEV was further coupled with the human and porcine IgG ELISAs based on GT 3. Thereby, the antigens of rat HEV and HEV GT 3 were used to coat different wells, what leads to the potential of discerning a GT 3-positive from a rat HEV-positive serum. The data obtained with GT 3-Ctr may be collated directly to the data obtained with rat HEV-Ctr, as the antigens have homologous sequences. This collated data is depicted by dot-plots (see Figure 7

in Publication I, Figure 4 in Publication II and Figures 3B and 4B in Publication III). Accordingly, the ELISAs for detection of anti-HEV GT 3 in human and swine could also detect anti-rat HEV antibodies when both antigens, rat HEV-Ctr and GT 3-Ctr were used for coating.

6.1.4 Cross-reactivity and differentiation of antibodies specific for GT 3 and rat HEV

The neutralizing antigenic epitopes of HEV genotypes 1 – 4 are believed to be conserved, as significant cross-reactivity was found on multiple occasions (Meng et al., 2001; Sanford et al., 2012b). This conservation of neutralizing epitopes offers cross-protection from other HEV strains (Khudyakov and Kamili, 2011). Additionally, broad serological reactivity throughout the genotypes 1 – 4 was found (Arankalle et al., 2007; Herremans et al., 2007b). This conservation is reflected in the aa sequence identity of 86 % or higher, that can be found in the highly immunogenic P domains of the genotypes 1 – 4 (data not shown). In despite of this broad cross-reactivity, there exist differences in sensitivity of detection of different genotypes, depending on the antigen. Hence, it was suggested to discern different genotypes by serotyping with the use of antigens from different strains (Osterman et al., 2012).

The genotypes 1 – 4 are only distantly related to rat HEV (see Figure 1.7). When comparing the P domains of GT 3 and rat HEV, the low aa sequence identity of 45 % suggests only a weak cross-reactivity. To assess the cross-reactivity of the antigens used in the in-house test, immunization of rats and rabbits were performed with GT 3-Ctr as well as rat HEV-Ctr. The Ctr segments are slightly longer than the P domain, resulting in an aa sequence identity of 54 % (see Table 6.2). Nonetheless is the Ctr-segment highly conserved in the genotypes 1 – 4 (aa identity of 0.893 or higher, colored cyan in Table 6.2) as well as in rat HEV (aa identity of 0.802 or higher, colored orange in Table 6.2). The generated hyperimmune sera were tested with in-house ELISAs by endpoint titration and demonstrated a titer difference of 16 to 32-fold (see Figure 3 in Publication II and Figure 6.3). The cross-reactivity appeared to be symmetric, showing similar values in animals immunized with GT 3-Ctr and in

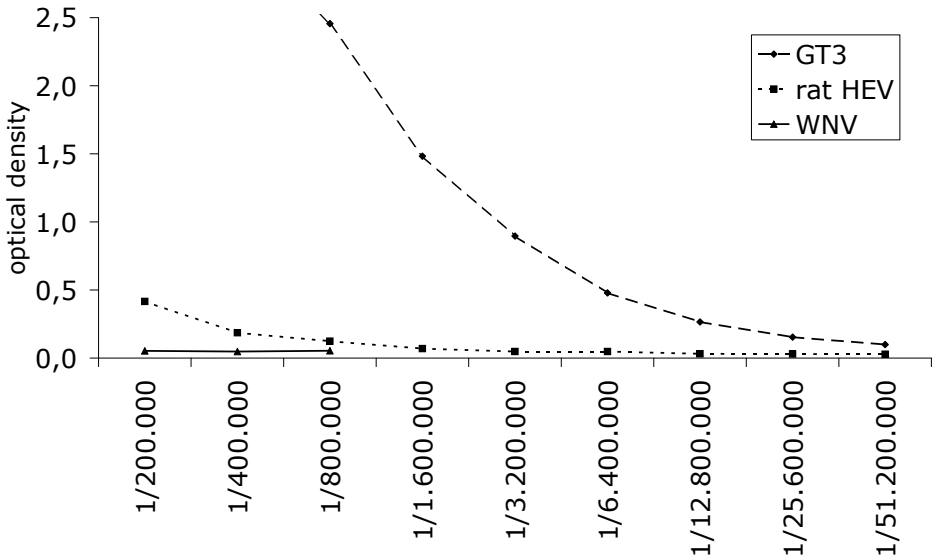

Figure 6.3: *Titration of sera from a rabbit immunized with GT 3-Ctr by in-house ELISA using GT 3-Ctr and rat HEV-Ctr as antigens to assess cross-reactivity.* The titer is 1/25,600,000 with the ELISA based on GT 3-Ctr and 1/800,000 with the ELISA based on rat HEV-Ctr. The antigen WNV NS1 was used as a negative control.

animals immunized with rat HEV-Ctr. A recent publication reported a similar cross-reactivity in rats immunized with VLPs derived from baculovirus infected insect cells (Li et al., 2011a). In despite of the use of VLPs for immunization and detection, the results are strikingly similar to the data presented here, suggesting that the length of the Ctr-segments was sufficient and leading to representative results.

These initial findings of cross-reactivity in hyperimmune sera indicated that it might be possible to identify the most likely causative virus by means of serotyping alone.

6.2 HEV seroprevalence in human from Germany

The novel Ctr-based ELISA detecting HEV in human was used to assess the IgG seroprevalence in the German population in Berlin / Brandenburg and to find demographic characteristics. Furthermore it was used to test if forestry workers form a risk group and carry HEV-specific antibodies more often than the sample population of Berlin / Brandenburg, that acts as a control group. The ELISA was done in parallel with antigen of both HEV GT 3 and rat HEV to detect possible

Table 6.2: *Amino acid identity matrix of the Ctr-segment of pORF2.*

	GT 1 AF076239	GT 1 M80581	GT 2 M74506	GT 3 EU360977	GT 3 AB189070	GT 3 AF060668	GT 4 AB220977	GT 4 AJ272108	ferret JN998607	ferret JN998606	rat HEV JN167537	rat HEV JN167538	rat HEV GU345042	rat HEV GU345043
GT 1 AF076239	ID													
GT 1 M80581	0.978	ID												
GT 2 M74506	0.908	0.922	ID											
GT 3 EU360977	0.925	0.939	0.922	ID										
GT 3 AB189070	0.922	0.936	0.911	0.982	ID									
GT 3 AF060668	0.915	0.925	0.908	0.975	0.975	ID								
GT 4 AB220977	0.904	0.918	0.901	0.954	0.943	0.932	ID							
GT 4 AJ272108	0.893	0.908	0,897	0.961	0.943	0.936	0.978	ID						
ferret JN998607	0.571	0.575	0.578	0.585	0.585	0.582	0.578	0.582	ID					
ferret JN998606	0.564	0.568	0.575	0.575	0.575	0.571	0.568	0.571	0.989	ID				
rat HEV JN167537	0.534	0.541	0.534	0.538	0.541	0.538	0.531	0.534	0.812	0.802	ID			
rat HEV JN167538	0.541	0.548	0.541	0.545	0.548	0.545	0.538	0.541	0.815	0.805	0.982	ID		
rat HEV GU345042	0.541	0.548	0.541	0.545	0.548	0.545	0.538	0.541	0.815	0.805	0.975	0.978	ID	
rat HEV GU345043	0.541	0.548	0.541	0.545	0.548	0.545	0.538	0.541	0.815	0.805	0.982	0.985	0.992	ID

antibodies not only against the known human-pathogenic HEV genotypes but also against the novel rat HEV.

In the survey described in Publication I, 555 serum samples from forestry workers of Brandenburg and 301 serum samples from blood donors of Berlin were tested for IgG-antibodies specific for HEV GT 3 and for IgG-antibodies specific for rat HEV. The commercial *recom*Line HEV IgG assay was used as reference test to evaluate the novel in-house ELISA. The test was validated with a modified ROC-analysis described in Chapter 6.1.2.2. The *recom*Line incorporates N-terminal, central and C-terminal pORF2 segments as well as the pORF3 of both GT 1 and GT 3. In the serum panel used, this multitude of antigens did not seem to pose an advantage in sensitivity or specificity when compared to the in-house ELISA that based on the C-terminal pORF2 segment only. This suggests that for epidemiological studies in Germany, a C-terminal portion of pORF2 of GT 3 may suffice.

The prevalence obtained from the average population of Berlin and Brandenburg is 12.3 % with the in-house ELISA. It was very high and comparable to prevalences reported in Germany (Faber et al., 2012; Krumbholz et al., 2012), although other surveys showed lower seroprevalences (Dawson et al., 1992; Juhl et al., 2013). However, it can be misleading to compare seroprevalences that base on different tests. Krumbholz et al. (2013) tested two human serum panels with and without professional contact to domestic pigs using several tests, including the *recom*Well HEV IgG ELISA (Mikrogen, Neuried, Germany), the in-house ELISA (see Publication I), the Axiom test and the MP Diagnostics HEV ELISA kit (MP Biomedicals, Santa Ana, CA, USA) (see Figure 6.4). The results demonstrated that different serological tests may lead to grossly opposing seroprevalence data, even if the tests base on the same principle. Similarly, a high divergence in interassay agreement, sensitivity and specificity was found in IgM tests (Drobeniuc et al., 2013). This interassay discordance hinders greatly the attempt to compare the results of seroprevalence studies based on different tests. The reason for the discordance is not known, but there are several possibilities, as the use of different protein segments as antigens. Additionally, the genotype of the antigen may pose a significant role, as different

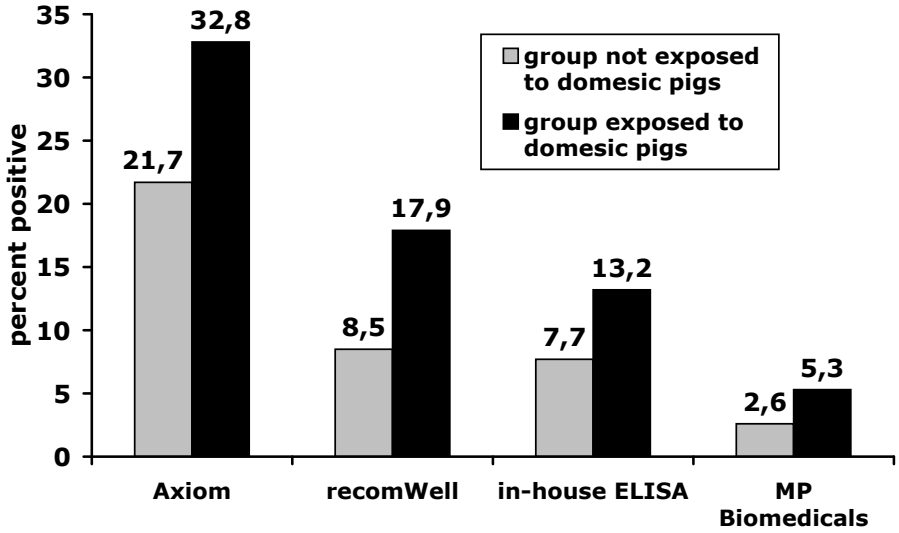

Figure 6.4: *Seroprevalence data of human subjects with and without professional contact to domestic pigs.* Data kindly provided by Sebastian Joel; Krumbholz et al. (submitted).

genotypes may elicit reactivities of different intensities (Osterman et al., 2012). It has been discussed that this problem may be avoided if more emphasis is laid on the validation of serological tests, incorporating greater panels of sera from patients infected with a wide range of genotypes by different modes of transmission (Aggarwal, 2013; Khudyakov and Kamili, 2011).

Several risk groups of infection with HEV have been proposed and identified in industrialized countries. Elevated seroprevalence was found in farmers, slaughterers, meat inspectors, veterinarians (Krumbholz et al., 2012; Withers et al., 2002) (see Table 6.3), inmates (Christensen et al., 2002) and psychiatric patients (Reinheimer et al., 2012). Forestry workers have been somewhat neglected and not been considered as a risk group for HEV infection. Still, forestry workers often have their working areas in the habitat of wild boar and red deer. Additionally, their hunting activities are higher when compared to the average population. Consequently, they are more likely to come in contact with fecal matter, raw blood and offal of wild boar and red deer and to consume their meat. Wild boar has been identified as a reservoir host for HEV in Berlin, Brandenburg and neighboring Mecklenburg-Western Pomerania (Kaci et al., 2008; Schielke et al., 2009; Adlhoch et al., 2009b). Therefore, forestry workers

Table 6.3: *Seroprevalences in human populations with an elevated occupational risk of infection.*

country	profession	number of investigated samples	positive (abs)	positive (%)	references
Chile	health care worker	72	9	12.5	Ibarra et al., 1997
Cuba	swine worker	69	28	40.5	de la Caridad Montalvo Villalba et al., 2013
Denmark	farmer	283	144	50.3	Christensen et al., 2008
Egypt	sewage worker	78	40	51.3	El-Esnawy et al., 1998
	sewage worker	43	22	51	Albatanony and El-Shafie, 2011
France	forestry worker	593	185	31.2	Carpentier et al., 2012
Germany	health care worker	511	20	3.9	Nübling et al., 2002
	work with pig meat	106	30	28.3	Krumbholz et al., 2012
Ghana	pig handler	105	n/a	38.1	Adjei et al., 2009
India	sewage worker	147	83	56.5	Vaidya et al., 2003
Madagascar	slaughter house worker	427	60	14.1	Temmam et al., 2013
Moldova	pig handler	264	135	51.1	Drobeniuc et al., 2001
Thailand	animal worker	270	72	26.7	Pourpongporn et al., 2009
the Netherlands	swine veterinarian	49	n/a	11	Bouwknegt et al., 2008a
Spain	swine worker	101	19	18.8	Galiana et al., 2008
Sweden	pig farmer	115	15	13	Olsen et al., 2006
UK	swine farmer	413	10	2.4	Meader et al., 2010
USA	swine veterinarian	295	68	23.1	Meng et al., 2002
	swine worker	165	18	10.9	Withers et al., 2002

abs, absolute number

occupied in these regions were presumed to have an elevated risk of infection. The seroprevalence for the forestry workers of Brandenburg obtained with the in-house ELISA was 21.4 % and ranged from 9.1 % to 28 % in the different forestries. A seroprevalence this high suggests a high risk of infection. Unfortunately, it was not possible to match the sex and age of the average population to the forestry workers, thereby making all statistical comparisons of forestry workers and blood donors inviable. Statistical significance of this finding is ineligible to calculate. However, the elevated risk for forestry workers was confirmed recently by a French study that reported 31 % seroprevalence in forestry workers and came to similar conclusions regarding infection by the wild boar population (Carpentier et al., 2012).

The pseudonymized serum samples were allocated to data on the sex and age of their donors, enabling the investigation for basic demographic characteristics of HEV seroprevalence. A very similar seroprevalence in male and female test subjects could be found. Several seroepidemiological studies have come to the same result, although males might have a higher risk for overt disease (Lewis et al., 2010; Wichmann et al., 2008). This is also the case in Germany, where no elevated seroreactivity for males could be found (Faber et al., 2012). Nonetheless, 62 % of all hepatitis E cases were notified in male patients (RKI: SurvStat, 2013; data as of March 06, 2013). The reason is unknown to date but may lie in a higher rate of comorbidities in males, which could cause an HEV-infection to become clinical. Similar data can also be observed in other viral diseases: e. g. hantavirus and WNV both have equal seroprevalences in both sexes in human. However, the *Hemorrhagic Fever with Renal Syndrome*, which is associated with infections with hantavirus, and the *WNV neuroinvasive disease* are reported more often in males than in females (Carson et al., 2012; Mertens et al., 2011).

No elevation of seroprevalence with increased age could be found in this study. However, several studies indicate the contrary (Carpentier et al., 2012; Christensen et al., 2008; Dalton et al., 2008b; Lewis et al., 2010). In Publication I, the failure to detect an increase of seroreactivity with age is likely to be caused by the serum panel used. The serum samples are not distributed

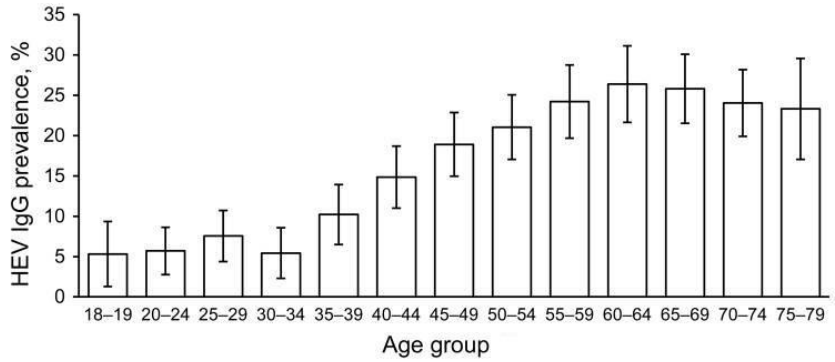

Figure 6.5: *Estimated prevalence of anti-hepatitis E virus IgG, by age group, Germany, 2008–2011.* Error bars indicate a confidence interval of 95 % (reprinted from Faber et al. (2012), published by *Emerging infectious diseases* in the public domain).

equally across all age-groups, but instead peak at age 49 and age 28 for forest workers and blood donors, respectively. A much larger seroepidemiological study, involving 4,422 participants, showed a linear increase of seroreactivity with age commencing at age 30 and leveling off at age 60 (Faber et al., 2012). This indicates that most infections occur after the third decade of life, in combination with a significant rise of clinical symptoms (see Figures 6.5 and 1.5). The leveling off does not mean a halt of new infections, as infections with GT 3 are generally transient and the antibody titers wane within several years in most patients.

For the population of the blood donors as well as the forestry workers, dot-plots were drawn, depicting the reactivity of each serum with the GT 3-Ctr and the rat HEV-Ctr antigen (see Figure 7 in Publication I). As expected, most sera do not significantly react with the antigen of rat HEV. However, several sera of forestry workers do exhibit an almost exclusive reactivity with rat HEV. This leads to the assumption that they could have been infected with rat HEV or a closely related strain, which does not necessarily originate from rat. Although domestic pigs and rhesus monkeys could not be infected intravenously with rat HEV (Cossaboom et al., 2012; Purcell et al., 2011), it is still unclear if rat HEV is capable of infecting human (Meng, 2013).

The divergence of high seroprevalences and relatively low numbers of reported cases of HEV can be found in many parts of the world, including Germany (Purdy and Khudyakov, 2011). One explanation might be that most infections

of HEV are devoid of or with only mild or unspecific symptoms. This would lead to numerous subjects with anti-HEV titer but with few cases reported. The often-stressed dose-dependency of HEV could also be heavily involved. Another explanation bases on the assumption that HEV strains other than genotypes 1 – 4 contribute to human epidemiology. These may be of low human pathogenicity but reactive in serological tests. Most recently, rat HEV and several other novel HEV strains could be found in hitherto unknown hosts (Johne et al., 2010b; Nakamura et al., 2006; Raj et al., 2012; Zhao et al., 2009). Thus, several reservoir hosts and transmission routes other than the ones mentioned before may be worthwhile to consider.

6.3 HEV seroprevalence in domestic pig in Germany

In numerous studies, effort was taken to assess the prevalences in herds and farms handling domestic pigs. The prevalences recorded tend to be very high in several countries, e.g. 87 % in Italy (Bartolo et al., 2011), 71 % in Madagascar (Temmam et al., 2013) and 60 % in Switzerland (Wacheck et al., 2012a), while the seroprevalences reported from Spain (20 %; de Oya et al., 2011) and France (31 %; Rose et al., 2011) were considerably lower. Recently, a prevalence of 69 % has been reported from pigs at Bavarian slaughterhouses by using meat juice as an alternative sample material (Wacheck et al., 2012b). Although this data is of high significance as Bavaria is a major producer of pork (as are Lower Saxony and North Rhine-Westphalia), this finding does not necessarily allow inference on other federal states of Germany (Statistisches Bundesamt, Wiesbaden, 2012).

To obtain an initial impression of the seroprevalence across Germany, approximately 50 sera from each of ten federal states were collected. To also get seroprevalence data in a higher geographical resolution, in Baden-Wuerttemberg a total of 461 sera from 6 districts and 24 herds was collected. This panel of swine sera was tested using the in-house ELISA. In parallel, the PrioCHECK ELISA was run as a reference assay to validate the new ELISA with a modified ROC-analysis (see Chapter 6.1.2.2). This data demonstrates significant concur-

rence of the reference test and the in-house ELISA. The PrioCHECK ELISA uses not only a segment of pORF2 but also pORF3 of both GT 1 and GT 3, additional antigens that did not seem to have a significant impact on the results of our serum panel. The test results of this commercial test could be approximated well by the in-house ELISA without the use of pORF3 or GT 1-derived antigens. This resembles the situation described in Chapter 6.2, leading to the impression that for epidemiological studies in Germany, the C-terminal pORF2 of GT 3 may be sufficient.

With the in-house test, the prevalence reached a total of 43 % in Germany. The highest and lowest prevalences were 19 % and 50 % in Mecklenburg-Western Pomerania and Baden-Wuerttemberg, respectively, while the prevalences of most states ranged from 32 % to 42 %. Likewise, Krumbholz et al. (submitted) found seroprevalences distributed evenly throughout the states and in the same range. The highest value, measured in Baden-Wuerttemberg, approximates the data obtained in its neighboring state Bavaria by Wacheck et al. (2012b). Noteworthy, seroprevalences in different herds deviated strongly and significantly from one another. Several herds seem completely devoid of HEV while other herds had 100 % of their tested animals found seropositive (see Figure 4C in Publication III). These inconsistencies have been found in other studies as well (Baechlein et al., 2010, Krumbholz et al., submitted).

As in Publication I, dot-plots were drawn, depicting the reactivity of each serum with the GT 3-Ctr and the rat HEV-Ctr antigen (see Figures 3B and 4B in Publication III). Most sera react poorly with rat HEV-Ctr, which suggests primarily infection with HEV GT 3, as anticipated. However, there is a fraction of sera reacting with rat HEV-Ctr. This may be due to infection with rat HEV or another related strain. An inaccuracy of the in-house test seems unlikely, as specific pathogen free (SPF) animals that were experimentally infected with GT 3 do not show any reactivity with rat HEV-Ctr (see Figure 6.6).

Overall, the strong differences in infestation of pig herds may indicate that HEV-free pork-production is an achievable goal. By examining both the HEV-free and the highly infested husbandries, the reasons for a stationary infestation could be investigated. However, the causes could be manifold. One obvious

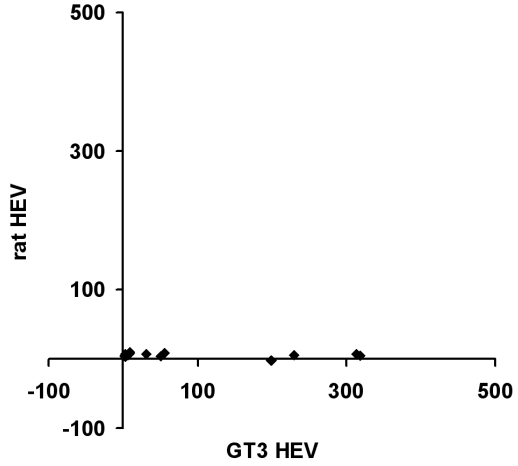

Figure 6.6: *Reactivity of domestic pigs experimentally infected with HEV GT 3.* The x-axis of the dot plot shows reactivity in the GT 3-based ELISA, the y-axis shows reactivity in the rat HEV-based ELISA. Both axes are scaled to percent of reactivity of the respective positive controls. The samples of the infected animals were kindly provided by Dr. Nicole Pavio. The experimental infection is described in Barnaud et al. (2012).

cause is that a once-infested breeding facility appears to stay in this state merely due to the high reproduction number R_0 of HEV and in despite of the transient nature of infection (Bouwknegt et al., 2008b; Satou and Nishiura, 2007), while fattening facilities could be infested by the breeding facilities where their livestock is obtained. Another cause of infection may be contaminated pig feed or drugs. Additionally, infection of pigs by a virus-shedding human cannot be excluded. Furthermore, there is a possibility that pigs may be infected from the surrounding wildlife. Recently, it has been reported that rats trapped in the vicinity of pig farms were found positive for HEV GT 3 in Japan (Kanai et al., 2012). Rodents are common in the environment of pig farms and could facilitate a bidirectional exchange of pathogens of farms with the surrounding wildlife, as deer and wild boar. This would lead to the assumption that facilities engaging in free-range pork production would be at a higher risk of HEV-infestation.

6.4 Infection of rats with HEV in Germany

The objective of this study was to detect anti-HEV antibodies in rat directed against either the novel rat HEV genotype or GT 3. For this reason the chest cavity fluid of 147 Norway rats trapped in 4 cities (Hamburg, Berlin, Stuttgart, Esslingen) and in the vicinity of one city (Ahlen) of Germany was screened with the in-house ELISA using both antigens GT 3 HEV and rat HEV (see Figure 1A in Publication II). The results are displayed in a dot-plot as done with the human and porcine sera (see Figure 4 in Publication II). A high rate of the samples reacted strongly with the antigen of rat HEV. In contrast, almost none of the samples reacted with the GT 3 antigen. An additional test (AXIOM HEV EIA) detected no antibodies reactive with GT 1. The low cross-reactivity of antigens from HEV GT 3 and rat HEV seems to hinder the detection of rat HEV with tests basing on GT 1 (Axiom test) and GT 3 (in-house ELISA). The almost complete absence of reactivity with these tests indicates that the rats tested had specific antibodies only against rat HEV or a closely related virus. Hence, HEV of the genotypes 1 – 4 do not appear to be of importance in the animals tested.

There is no validated test for seroreactivity of anti-rat HEV to use as a reference assay. Hence no sensitivity or specificity of the in-house test could be calculated by means of a gold standard, as it was done with the in-house ELISAs for human and porcine IgG. To classify the results of this ELISA, a cutoff was set, based on the results of the samples trapped in the vicinity of Ahlen. Samples from the surroundings of this town did react only marginally with both antigens. Designating these results as negative, a cutoff was chosen, set at the arithmetic mean of all samples from Ahlen plus three times its standard deviation, as nearly all (99.73 %) negative values should lie within three standard deviations of the mean. This method has been employed before in a similar fashion by Li et al. (2011a) in their serological survey of rat HEV. The seroprevalence in Germany calculated on that basis was somewhat high (24.5 % in total), reaching its maximum in Hamburg with 41.2 %.

A real-time RT-PCR with its primers designed exclusively for amplification of rat HEV was developed. This real-time RT-PCR was used to detect genomic RNA of HEV in homogenized liver samples of the rats. If positive, a template for

sequencing was generated by a nested broad-spectrum RT-PCR (Johne et al., 2010b). Several animals were found to be positive for rat HEV. Only a low number of samples were RT-PCR-positive as well as seropositive, which suggests that rat HEV results in an acute, non-persistent infection, as do the other mammalian genotypes. In line, Purcell et al. (2011) concludes that infections with rat HEV are not robust with a very short period of viremia and fecal shedding. In the course of infection no clinical indication of infection could be observed and no alteration of ALT levels (Li et al., 2013b).

Detection of anti-HEV antibodies in rodents in Indonesia (Mulyanto et al., 2013), Japan (Hirano et al., 2003; Kanai et al., 2012), and the United States (Easterbrook et al., 2007; Purcell et al., 2011) is reported frequently. All report detection based on an antigen originating from the genotypes 1 – 4, which is in contrast to the finding of Publication II, that GT 3 is not adequate to detect HEV in rats. It could be possible that the prevalences and titers measured in the mentioned publications would have been even higher if a test based on antigen of rat HEV had been used, as has been suggested by Mulyanto et al. (2013). To date, only one other group tested rats with a serological assay based on rat HEV, detecting positive animals in Vietnam (Li et al., 2011a). There exist also a report that almost no seropositive rats could be found with tests based on the classic genotypes in the United States, which could suggest a seroprevalence low enough to be undetectable without antigen of rat HEV (Dong et al., 2011). Although most groups tested seroreactivity only, some also isolated successfully viral RNA. Notably, most publications detected exclusively sequences clustering with rat HEV (Li et al., 2011a, 2013a; Mulyanto et al., 2013; Purcell et al., 2011). Only one survey reports several sequences grouping with HEV GT 3 and a single sequence of rat HEV (Lack et al., 2012). Another one describes sequences clustering with GT 3 only, which were obtained from rats in the surroundings of a pig farm (Kanai et al., 2012). Aside from these findings, there are successful infection experiments with HEV originating from swine and human in Wistar rats and BALB/c nude mice (Huang et al., 2009; Maneerat et al., 1996).

There is uncertainty regarding the aptitude of rat HEV to infect other animals

than rodents. To date, neither rhesus monkeys nor domestic pigs could be infected successfully with rat HEV, despite the successful infection of rats (Cossaboom et al., 2012; Li et al., 2013b; Purcell et al., 2011).

6.5 Taxonomy

The taxonomic classification of HEV has changed in the previous years. Initially, HEV was believed to belong to the family of *Caliciviridae* due to its morphological similarities and related biologic properties. However, phylogenetic analyses excluded HEV from the calicivirus cluster and it was assigned to the family *Hepeviridae* (Berke and Matson, 2000; Emerson et al., 2004). In the previous three years, a multitude of hitherto unknown HEV-like agents have been detected in various vertebrate species (see Chapters 1.5.4 and 1.5.6) (Batts et al., 2011; Drexler et al., 2012; Johne et al., 2010b; Raj et al., 2012; Takahashi et al., 2011; Zhao et al., 2009). This makes it necessary to reorganize the "new" strains and the "old" genotypes into a taxonomic order. A system based on the host was proposed, that introduces 4 genera: *Orthohepevirus*, which includes genotypes 1 – 4 as well as genotype 5 (rat/ferret HEV) and 6 (novel isolates from wild boar, see Takahashi et al., 2011); *Avihepevirus*; *Piscihepevirus*; *Chiropteranhepevirus* (Meng, 2013). Another proposal suggested classification by utilizing p-values of segments of the HEV-genome (Smith et al., 2013). Hereby, human-pathogenic HEV strains and closely related strains form one of four species: genotypes 1 – 4 in their current formation as well as two additional genotypes from the novel wild boar strains. The other species would be formed by avian HEV, bat HEV and rat/ferret HEV, while HEV from trout would constitute a different genus. A third suggestion uses distance-based classification criteria of aa sequences of a segment of RdRp (Drexler et al., 2012). Following this scheme, the following entities would be formed: human HEV, rat/ferret HEV, bat HEV and avian HEV. Human HEV would comprise the genotypes 1 – 4 in their current formation and a fifth genotype consisting of the novel wild boar strains. In all three proposals, rabbit HEV is assigned to GT 3, to which it is closely related, while rat HEV is depicted as only distantly related to the genotypes 1 – 4.

In addition to the taxonomic questions, it is currently unclear if all the recently discovered members of the family *Hepeviridae* belong to a single serotype. There have been reports on cross-reactivities between distantly related strains, as avian HEV and GT 1 / GT 3 or rat HEV and GT 3 (Haqshenas et al., 2002, Publication II). Nevertheless, antigenic differences of rat HEV and HEV GT 3 CP allowed the differentiation of antibodies raised against these viruses (Publication I, II and III). Unfortunately, there is only few data available on cross-protection. Initial immunization experiments with avian HEV and rat HEV have shown only limited cross-protective characteristics in infections with GT 3 (Sanford et al., 2012b).

6.6 Challenges and future prospects of HEV research

The genome organization of rat HEV is very similar to that of the human-pathogenic genotypes 1 – 4, but contains an additional 552 nts-long putative ORF (ORF4) of unknown function. This ORF4 could be found in all rat HEV strains from different sites in Germany and from Vietnam (GenBank accession number JX120573), in HEV strains from ferret (GenBank accession numbers: JN998607, JN998607) and in bat HEV (Drexler et al., 2012; Johne et al., 2010a; Raj et al., 2012, Publication II). The position of the putative ORF is at the 5'-terminus of the viral genome, entirely overlapping with ORF1 and with a +2 frame relative to ORF1. In rat/ferret HEV, the aa sequence identity of the putative ORF4-encoded protein is considerable (0.612), but lower than the aa sequence identity of the overlapping ORF1-encoded portion of the same region (0.802). The ORF4 found in bat HEV is 108 nts longer than in rat/ferret HEV strains and diverges greatly from them (Drexler et al., 2012). Future studies have to prove if this ORF is conserved in rat HEV strains from other geographic regions and to validate experimentally a possible function of this ORF. For this purpose the rat HEV infection model could be helpful, that has been reported recently (Li et al., 2013b). In addition, the recently described cell culture systems based on HEV strains from chronically infected hepatitis E patients may help to establish

similar systems for rat HEV. With reverse genetics studies it could be possible to characterize the functions of ORF4 and the other gene products of rat HEV in the replication cycle.

To date, it is unclear what animals rat HEV is able to infect, beside its reservoir. It has been suggested that ferret is susceptible to a rat HEV-related strain and antibodies reactive with pORF2 of rat HEV have been found in human (Raj et al., 2012, Publication I). The finding of rat HEV-reactive antibodies in forestry workers may indicate the presence of additional rat HEV-related hepeviruses in wild rodents. Therefore, an effort should be made to search for HEV-like agents in other small mammals, such as wild rodents, but also house mouse (*Mus musculus*). Similarly, the course of infection of rat HEV and its pathogenic potential has to be investigated, as it remains unclear if rat HEV is able to elicit chronic infections and prolonged viral shedding. Also, the establishment of animal models for transmission of zoonotic genotypes of HEV in a natural population could be helpful to assess their replication and possible hosts.

The results described here demonstrate the public health impact of HEV in Germany and the potential role of animal reservoirs. The seroprevalences detected in this study are not easily comparable to the results of other studies, as many serological tests have low concordances (Drobeniuc et al., 2013; Khudyakov and Kamili, 2011; Krumbholz et al., 2013). Thus, comparison of different assays is only possible when they were proven to have a high concordance as well as similar sensitivity and specificity. This problem might be caused by the use of different antigens, i. e. different genotypes, portions of the antigens (truncated proteins, peptides, entire proteins), expression systems, antigen structure (VLPs, denatured proteins) or the test format used (ELISAs of different formats, immunoblot). Recently developed efficient cell-culture systems may allow to produce virions in high quantities. Therefore, future investigations should focus on testing antigenic differences and consequently assess the diagnostic value of authentic viral protein and different recombinant antigens. In particular, the use of highly cross-reactive antigens or the application of antigens from different genotypes within a single test may improve the sensitivity of future diagnostic tests. Finally, future studies will have to validate

tests with generally higher numbers of samples and involving samples positive for several genotypes. This may result in novel serological assays with high diagnostic sensitivity and specificity that can be used for seroepidemiological studies to obtain comparable data.

Alternatively, tests reacting specifically with single strains may be useful for serotyping, as was attempted here with antigens originating from HEV GT 3 and rat HEV. Identification of non-cross-reactive epitopes within immunodominant proteins may help in the future to differentiate HEV infections serologically, at least for more divergent HEV strains, e. g. rat HEV and HEV GT 3. The differentiation might be improved by generation of further truncated antigens that harbor even more non-cross-reactive epitopes. In addition, the generation of rat HEV- and HEV GT 3-specific monoclonal antibodies may allow the development of competition assays for serotyping. The serotyping is especially important in geographical regions with a co-occurrence of these strains. Thus, these investigations may help to prove if rat and domestic pig represent reservoirs for rat HEV as well as GT 3 HEV as reported previously (Meng et al., 1997). Additionally, they may help to identify possible sources of infection after the relatively short-lived viremia in human and pig. In addition, larger seroepidemiological studies based on tests including rat HEV antigen could help clarifying the frequency of antibodies against rat HEV in matched cohorts of average human population and risk groups, such as forestry workers and sewage workers. This may lead to the finding of new risk groups primarily prone to infection with rat HEV. Thereby, these novel assays will allow new insights into the current controversy on the potential of rat HEV to cause infections and disease in human. Finally, these future investigations will help to assess the HEV strains occurring in reservoir populations and thereby to develop strategies for prevention of HEV infections and disease.

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Own contribution to publications

Publication I: Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs, printed in *Medical Microbiology and Immunology*

For this study, I generated recombinant constructs of HEV, and expressed and purified the encoded proteins. I tested all protein segments for their efficacy in detecting anti-HEV antibodies in a serological assay and established as well as validated an in-house ELISA. Furthermore, I tested all human sera in the in-house ELISA. I analyzed the data and designed a variant of the ROC-analysis, which found final adjustment and approval by Dr. Mario Ziller. I wrote the sections "Material and Methods" and "Results" of the manuscript and was significantly involved in writing the introductory and summarizing sections.

Publication II: Rat hepatitis E virus: Geographical clustering within Germany and serological detection in wild Norway rats (*Rattus norvegicus*), printed in *Infection, Genetics and Evolution*

All proteins that were used in this study were introduced in Publication I. The protein segments originating from the capsid protein of HEV were all expressed and purified by myself. Based on the protein segments, I established an indirect in-house ELISA. Furthermore, I tested all rat sera in the in-house ELISA and analyzed the data statistically. Moreover, I coordinated and evaluated the described immunization experiments in rat and rabbit. I wrote the respective

parts of the manuscript and I wrote the introductory and summarizing sections together with Dr. Ulrich.

Publication III: Hepatitis E virus seroprevalence of domestic pigs in Germany determined by a novel in-house and two reference ELISAs, printed in *Journal of Virological Methods*

As in Publication II, all proteins that were used in this study were introduced in Publication I. I expressed and purified the ORF2-encoded protein segments of HEV GT 3 and rat HEV. I tested them for their efficacy as antigens in an indirect ELISA. Thereafter I established and validated an indirect in-house ELISA. Furthermore, I tested all swine sera in the in-house ELISA and several sera in two commercial assays, the AXIOM EIA and PrioCHECK ELISA. I analyzed the data statistically, with Dr. Mario Ziller having the final oversight as designated statistician. Additionally, I wrote the sections "Material and Methods" and "Results" of the manuscript, while the introductory and summarizing sections were written by Dr. Ulrich and me.

In agreement:

. . . . . .

Signature PD Dr. rer. nat. Rainer G. Ulrich

List of publications

Publications part of this thesis

Publication I

Dremsek, P., Joel, S., Baechlein, C., Pavio, N., Schielke, A., Ziller, M., Dürrwald, R., Renner, C., Groschup, M. H., Johne, R., Krumbholz, A., Ulrich, R. G. 2013. Hepatitis E virus seroprevalence of domestic pigs in Germany determined by a novel in-house and two reference ELISAs. *J Virol Methods.* 190(1-2):11 – 6.

Publication II

Johne, R.[1], **Dremsek, P.**[1]**,** Kindler, E., Schielke, A., Plenge-Bönig, A., Gregersen, H., Wessels, U., Schmidt, K., Rietschel, W., Groschup, M. H., Guenther, S., Heckel, G., Ulrich, R. G. 2012. Rat hepatitis E virus: Geographical clustering within Germany and serological detection in wild Norway rats (*Rattus norvegicus*). *Infect Genet Evol.* 12(5):947 – 956.

Publication III

Dremsek, P., Wenzel, J. J., Johne, R., Ziller, M., Hofmann, J., Groschup, M. H., Werdermann, S., Mohn, U., Dorn, S., Motz, M., Mertens, M., Jilg, W., Ulrich, R. G. 2012. Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs. *Med Microbiol Immunol.* 201(2):189 – 200.

Additional publications

Papac-Miličević, N., Breuss, J. M., Zaujec, J., Ryban, L., Plyushch, T., Wagner, G. A., Fenzl, S., **Dremsek, P.,** Cabaravic, M., Steiner, M., Glass, C. K., Binder, C. J., Uhrin, P., Binder, B. R. 2012. The Interferon Stimulated Gene 12 Inactivates Vasculoprotective Functions of NR4A Nuclear Receptors. *Circ Res.* 110(8): e50 – 63.

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Dremsek, P. Neues Hepatitis E-Virus bei Wanderratten. *Arbeitsgruppe "Mäuse".* 29.–30.11.2011, Schwerin, Germany. Oral presentation.

Dremsek, P., Wenzel, J. J., Johne, R., Ziller, M., Hofmann, J., Groschup, M. H., Werdermann, S., Mohn, U., Dorn, S., Motz, M., Mertens, M., Jilg, W., Ulrich, R. G. Seroprevalence study in forestry workers from eastern Germany using a novel genotype 3 hepatitis E virus-specific immunoglobulin G ELISA. *Medical Biodefense Conference.* 25.–28.10.2011, Munich, Germany. Poster.

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Acknowledgements

I wish to thank Prof. Dr. rer. nat. habil. Dr. med. vet. h.c. Thomas C. Mettenleiter and Prof. Dr. med. vet. Martin H. Groschup for giving me the opportunity to research at the Friedrich-Loeffler-Institut.

I owe my deepest gratitude to my thesis advisor PD Dr. rer. nat. Rainer G. Ulrich. He provided me with excellent, dedicated supervision and granted assistance whenever necessary. With his vast scientific experience and invaluable inputs, he guided me throughout the thesis outstandingly.

Moreover, I want to thank our partners at the Bundesinstitut für Risikobewertung (PD Dr. med. vet. Reimar Johne and Dr. rer. nat. Anika Schielke) and the Christian-Albrechts-Universität zu Kiel (PD Dr. med. Andi Krumbholz and Sebastian Joel) for our fruitful and close collaboration and their valuable discussions and inputs.

My thanks go to Eveline Kindler for her kindness, patience and skill in introducing me to the merits of building phylogenetic trees.

Likewise, I thank Dr. rer. nat. Mario Ziller for his help in implementation of the variable ROC analysis and his statistical support.

Naturally, I am indebted to the denizens of the laboratory of Dr. Ulrich: Stephan Drewes, Dörte Kaufmann, Nastasja Kratzmann, Christian Kretzschmar, Ulrike M. Rosenfeld, Ina Römer, Mathias Schlegel, Sabrina Schmidt, Hanan Sheikh Ali, Franziska Thomas, Konrad Wanka, Ute Wessels and Theres Wollny all contributed to maintaining the very best atmosphere that one could wish for, inside as well as outside the laboratory. This cannot be said without highlight-

ing Kathrin Baumann for her immense dedication to the group, while dancing her maneuvers remarkably. It took her mere weeks to become the central hub.

Furthermore, I am thankful to all the other colleagues at the Friedrich-Loeffler-Institut for their support.

I thankfully acknowledge the financial support of the *Förderverein des Friedrich-Loeffler-Instituts* and the *Grimmingerstiftung*.

I want to take this opportunity to express my gratitude to my Michaela. She acted as my pillar, anchoring me in the realms of reality while lifting my spirit up high and to its operative levels. She sustained patiently the vast spatial distance between us.

Eventually I want to thank my parents Peter and Sabine who once again granted surpassing, unlimited and unparalleled support, supply and replenishment. They encouraged me and stood by me even from a thousand miles afar.