Identification and molecular characterization of small mammal-associated hepeviruses for the development of novel animal models

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aa	amino acid
ADP	Adenosine diphosphate
Appr-1"-pase	Adenosine diphospate-ribose-1"-monophosphatase
BLAST	Basic Local Alignment Search Tool
cDNA	copy deoxynucleic acid
CP	capsid protein
CTV	cutthroat trout virus
cvHEV	common vole-associated hepatitis E virus
ELISA	Enzyme linked immunosorbent assay
GMP	Guanosine-monophosphate
GDP	Guanosine-diphosphate
GTP	Guanosine-triphosphate
Hel	Helicase
HEV	Hepatitis E virus
HTS	high-throughput sequencing
HVR	Hypervariable region
InDels	insertions/deletions
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kb	kilo base
kDa	kilo Dalton
Mt	methyltransferase
MDA5	melanoma differentiation associated gene 5
NCR	non-coding region

nm	nanometer
n. d.	not done
nt	nucleotide
NTPase	nucleoside triphosphatase
ORF	open reading frame
PCP	papain-like cysteine protease
PUUV	Puumala orthohantavirus
rabbitHEV	rabbit-associated hepatitis E virus
rabies virus	RABV
ratHEV	rat-associated hepatitis E virus
RdRP	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SL	stem-loop
SNP	single nucleotide polymorphism
SfHeV	Sogatella furcifera hepe-like virus
Tn5	*Trichoplusia ni*, BTL-Tn 5B1-4
VP	viroporin
VLP	virus-like particle

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1. Introduction

1.1 Hepatitis E Virus – Introducing the causative agent of hepatitis E

1.1.1 Classification and Taxonomy

Hepatitis E virus (HEV) belongs to the family *Hepeviridae,* including the two genera *Orthohepevirus* and *Piscihepevirus* (Purdy et al., 2017; Smith et al., 2014, see Figure 1). The genus *Piscihepevirus* only contains the species *Piscihepevirus A*, with strains detected in cutthroat trout (cutthroat trout virus; CTV) and related fish species (Batts et al., 2011). The genus *Orthohepevirus* contains four species, namely *Orthohepevirus A* – *Orthohepevirus D. Orthohepevirus A* contains currently seven genotypes (HEV-1 - HEV-7) and a proposed genotype HEV-8, with specific reservoir hosts (Smith et al., 2016; Woo et al., 2016).
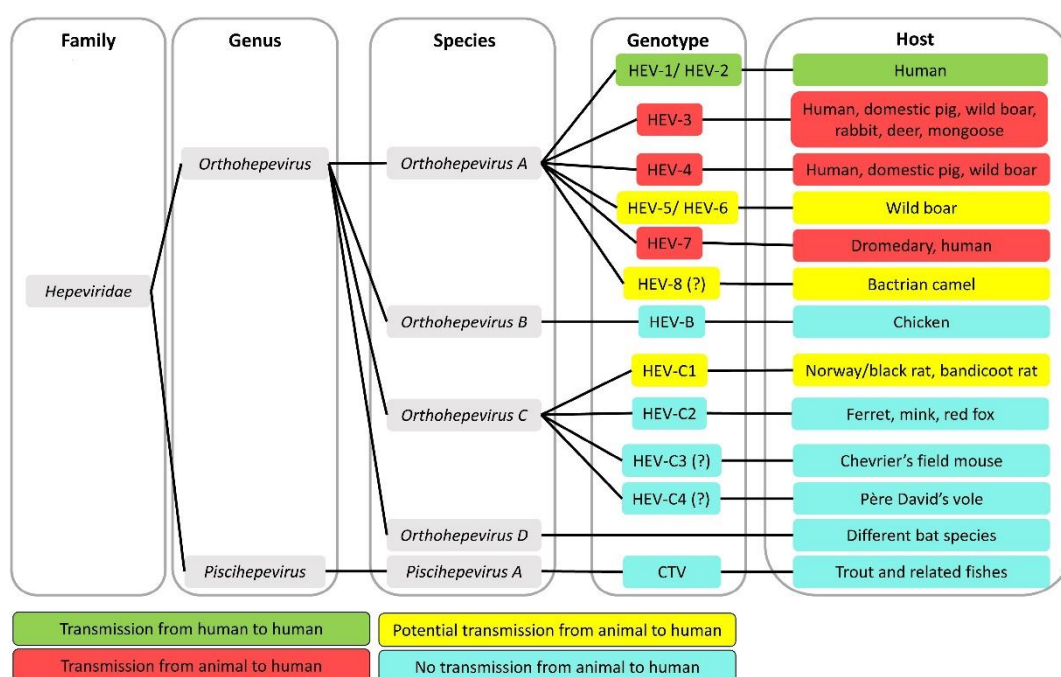
*Figure 1. **Taxonomy of the family** **Hepeviridae** **and host association of the genotypes.** The classification follows the taxonomy suggested by Smith et al., (2014 and 2016) and Batts et al., 2011, including proposed genotypes HEV-C3, HEV-C4 (Wang et al. (2018)) and HEV-8 (Woo et al. (2016)) labeled with a question mark. Possible transmission routes are indicated by different colors. For details see chapter 1.1.3 and 1.1.4.*

Orthohepevirus B genotypes were found in birds exclusively, namely chickens and wild bird species (Haqshenas et al., 2001; Huang et al., 2004; Reuter et al., 2016b). Small mammal-associated HEV strains found in rats, foxes, minks and ferrets were classified within the species *Orthohepevirus C*. Rat-associated HEV (ratHEV) belongs to the genotype HEV-C1, whereas carnivore-associated HEV strains are grouped together as

HEV-C2 (Smith et al., 2014). Two recently detected, rodent associated HEV strains, found in Chevrier's field mouse (*Apodemus chevrieri)* and Père David's vole (*Eothenomys melanogaster*), respectively, were proposed as genotypes HEV-C3 and HEV-C4 (Wang et al., 2018). Bat-associated HEV strains, members of the species *Orthohepevirus D*, were found in different bat species worldwide (Drexler et al., 2012). The described taxonomical classification is in line with the phylogenetic reconstruction of the protein encoded by the concatenated region of the open reading frame (ORF) 1 and ORF2 from representative members of the family *Hepeviridae* (see Figure 2).
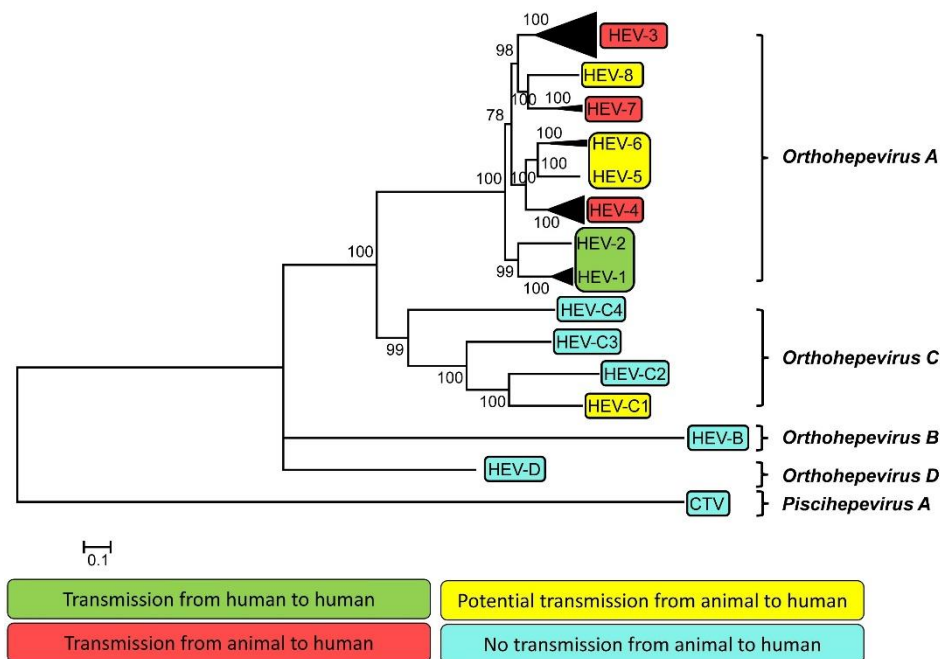
*Figure 2. **Phylogenetic relationship of genotypes and species within the family** **Hepeviridae.** The phylogenetic tree was generated by fusion of trees calculated by Maximum-likelihood (Substitution model: Jukes-Cantor with gamma distribution and 1000 bootstraps) and Bayesian analysis (15 million generations, burn-in phase of 25%). The amino acid sequences of the concatenated open reading frame (ORF) 1 and ORF2, used for the calculation, are reference sequences for hepatitis E virus (HEV), proposed by Smith et al., (2014 and 2016) and Batts et al., 2011. Additionally the proposed genotypes HEV-C3/HEV-C4 (Wang et al., 2018) and HEV-8 (Woo et al., 2016) were included as well. Maximum-likelihood and Bayesian calculations were done via CIPRES Online Portal (Miller et al., 2015). Support values above 70 are shown. The possible transmission routes are indicated by different colors. For details see chapters 1.1.3 and 1.1.4.*

Recent high-throughput sequencing (HTS) investigations revealed novel hepevirus-related pathogens in white-backed planthopper (*Sogatella furcifera*), an insect from southern China (N. Wu et al., 2018), and in agile frog (*Rana dalmatina*) from Hungary (Reuter et al., 2018). The frog-associated virus and the Sogatella furcifera hepe-like virus (SfHeV) are sharing the typical genomic attributes of other hepeviruses, with an unknown taxonomic position.

1.1.2 Structure and genome organization of hepeviruses

The hepevirus virion was initially described as a "non-enveloped", spherical particle with a diameter of about 27-34 nanometer (nm) (Mori and Matsuura, 2011). Recent investigations indicated its association with lipids and therefore it is described as "quasi-enveloped" (Yamada et al., 2009; Yin et al., 2016). Hepeviruses contain a single stranded ribonucleic acid (RNA) genome, having a positive polarity and a size ranging from 6.2 – 7.4 kilo bases (kb) (Tam et al., 1991). The single-stranded RNA genome contains three major ORFs, flanked by a 5' non-coding region (NCR), which is m^7G-capped, and a 3' NCR, with a polyadenylation. The NCRs differ in length among the different HEV-strains (Purdy et al., 2017). The ORFs are arranged by different overlapping patterns, even for members of the same HEV species. The major ORF1 encodes a nonstructural polyprotein of about 1700 aa (see Figure 3), that is translated from the genomic RNA. The potentially functional domains were predicted by computer-based search for homologues and conserved domains within the genome of HEV and other viruses (Koonin et al., 1992; Tam et al., 1991, see Figure 3). The cleavage of the ORF1-encoded polyprotein into smaller polypeptides, corresponding to the functional domains, was shown in a baculovirus expression system (Sehgal et al., 2006). Cell culture and/or animal experiments revealed the functionality of the predicted domains (see Figure 3):

- Methyl-/Guanyltransferase (Mt) is a 110 kilo Dalton (kDa) protein (P110) and catalyzes the transfer of a methyl group from S-adenosylmethionin to guanosine-triphosphate (GTP) and guanosine-diphosphate (GDP) to yield m^7GTP or m^7GDP and thus forms a covalent enzyme-m^7guanosine-monophosphate (GMP) complex needed for the capping of the viral RNA (Magden et al., 2001);

- The "Y-like domain" (Y) has a critical role in HEV replication cycle by affecting the gene regulation and/or membrane binding in intracellular replication complexes (Parvez, 2017);
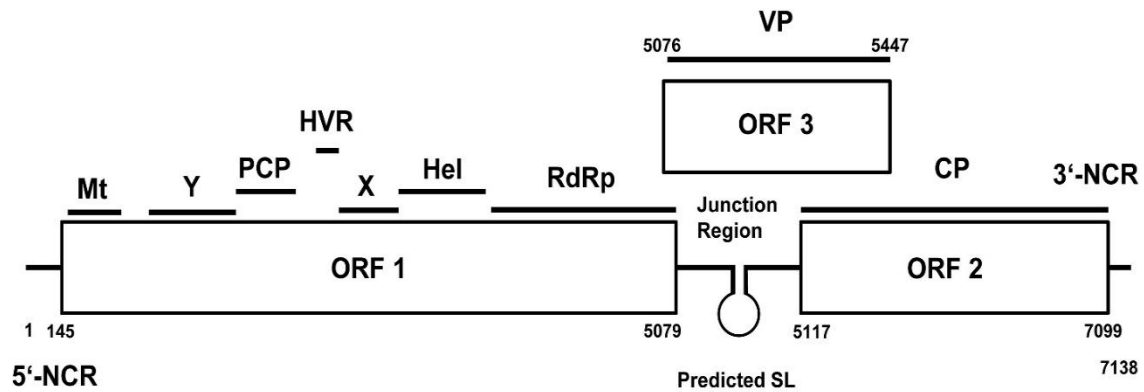
*Figure 3. **Genome organization of hepatitis E virus.** The location of the overlapping major open reading frames ORF1, ORF2, ORF3, and the 5'- and 3' non-coding regions (NCR) as well as the junction region with the predicted stem-loop (SL) structure are indicated based on HEV prototype strain SAR 55, accession number M80581 (Cao et al., 2010; Ding et al., 2017; Johne et al., 2014a; Pudupakam et al., 2009). Functional domains are: Mt, methyltransferase; Y, Y-like domain; PCP, papain-like cysteine protease; HVR, hypervariable region; X, X domain/ADP-ribose-binding site; Hel, Helicase; RdRP, RNA-dependent RNA polymerase; CP, capsid protein; VP, viroporin.*

- The papain-like cysteine protease (PCP) is involved in the proteolytic processing of the ORF1-encoded polyprotein as demonstrated by characterization of variant molecules with aa exchanges within its catalytic active center (Parvez, 2013). Additionally, it was shown that PCP downregulates melanoma differentiation associated gene 5 (MDA5)-mediated activation of interferon β induction, suggesting a role of PCP as an interferon I antagonist (Kim and Myoung, 2018);

- Homologues of the HEV cellular X domain/ADP-ribose-binding site activities were identified in rubella virus and different corona- and alphaviruses. The role of this domain for HEV replication was shown in the human hepatoma cell line HuH7/S10-3 using X-domain aa exchanges (Parvez, 2015);

- In addition to the PCP and X-domain, a proline rich/ hypervariable region (HVR) of different length was identified, but seems to have no influence on HEV infectivity (Pudupakam et al., 2009; Tsarev et al., 1992).

- The HEV Helicase (Hel) is a nucleoside triphosphatase (NTPase) that mediates the unwinding of the RNA duplex in 5' to 3' direction and the first steps of 5' cap synthesis (Karpe and Lole, 2010a, b);

- RNA-dependent RNA polymerase (RdRP) is needed for HEV replication and transcription. Electrophoretic mobility shift assays (EMSA) demonstrated a high affinity of recombinant RdRP for the 3'-end of the HEV genome, including the polyadenylated

region, secondary stem-loop (SL) structures and putative subgenomic (SG) promotor regions (Agrawal et al., 2001; Mahilkar et al., 2016).

The ORF2 and ORF3 are located within a bicistronic subgenomic RNA of HEV (Graff et al., 2006). For the translation of this subgenomic RNA a secondary SL structure was identified within the junction region between ORF1 and ORF2/ORF3 (Cao et al., 2010, see Figure 3). The ORF2 encodes the capsid protein (CP; see Figure 3), which is subsequently processed into multiple monomeric forms with sizes ranging from 74 to 88 kDa (Jameel et al., 1996). Expression of a N-terminally truncated ORF2 (amino acid, aa, residues 112 to 600) by a recombinant baculovirus in insect cell line *Trichoplusia ni*, BTL-Tn 5B1-4 (Tn5) cells, revealed two forms of the CP, a native one (58 kDa) and a slightly smaller one (50 kDa). As evidenced by electron microscopy, the smaller one was found to self-assemble into empty virus-like particles (VLPs) (Li et al., 1997). Analyses of different N-terminally and C-terminally truncated CPs in the baculovirus-mediated expression system resulted in the identification of aa residues 125 and 601 as essential elements for initiation of VLP assembly (Li et al., 2005b). In a recent study, three different forms of the CP were identified (ORF2 i, g/c) and the existence of two production pathways of the CP were proposed: ORF2 i is produced by delivering cytosolic ORF2 proteins to the virion assembly sites and thus is associated with infectious virus particles. The ORF2 g/c variants are generated in a nonproductive way and are absent in infectious particles. ORF2 proteins are pushed into the secretion route, get glycosylated/cleaved and secreted out of the cells. These ORF2 g/c forms were proposed to function as immunological baits in chronically HEV infected patients (Montpellier et al., 2018). The ORF3 encodes a multifunctional protein that is supposed to function as a viroporin (VP) and seems to be involved in virus release (Ding et al., 2017; Tyagi et al., 2002; Tyagi et al., 2004; Tyagi et al., 2005 and see Figure 3). Additionally, the ORF3-encoded protein interacts with the CP (Tyagi et al., 2002) and thus, could play a role in regulating the virion assembly.

A putative ORF4 exclusively for members of the species *Orthohepevirus C* was predicted. Three genotypes, HEV-C1, HEV-C2 and the proposed HEV-C3, share the putative ORF4, whereas it is absent in members of the proposed genotype HEV-C4 (Johne et al., 2014a; Wang et al., 2018). The ORF4 is overlapping with the ORF1 at its 5' end, has a size of about 552 nucleotides (nt) and encodes a putative protein of 183 aa residues (Johne et al., 2014a). Transfection of the human hepatoma carcinoma cell line PLC/PRF/5 cells with infectious ORF4-defective mutant copy deoxynucleic acid (cDNA) clones of ratHEV

demonstrated no difference in the replication to the corresponding wild type ORF4 cDNA clone. Therefore, the function of the putative ORF4 encoded protein remains still unclear (Tanggis et al., 2018).

1.1.3 Transmission routes of hepatitis E virus

The routes of HEV transmission are very complex and not all of them are fully understood. Most likely pathways are via faecally contaminated water, blood products, food, environment and direct contact with animals as well as infected humans (Lewis et al., 2010). The possible route of transmission differs strongly among the different HEV genotypes. The genotypes HEV-1 and HEV-2 are restricted to humans and were responsible for large hepatitis E outbreaks in the past years. The genotypes HEV-3 and HEV-4 are zoonotic pathogens and can cause an infection in humans (Li et al., 2005a; Takahashi et al., 2002; Pavio et al., 2015; Smith et al., 2016). These pathogens can be found in different reservoir hosts like domestic pigs or wild boars, deer and rabbits. The most probable way of infection for humans is by the consumption of undercooked meat of infected animals, like from deer, rabbits, pigs or wild boars (Abravanel et al., 2017; Izopet et al., 2012; Li et al., 2005a; Sonoda et al., 2004; Yazaki et al., 2003). Furthermore, camelids are also reservoir hosts for a zoonotic HEV-strain, HEV-7, which was found in a dromedary species (Arabian camel; *Camelus dromedaries*) and infected a human, who was frequently consuming milk and meat from Arabian camel (Lee et al., 2016). Similarly, the proposed genotype HEV-8, which was found in a Bactrian camel (*Camelus bactrianus*) is most likely non-zoonotic (Takahashi et al., 2014; Woo et al., 2016). The genotypes HEV-5 and HEV-6 were found exclusively in wild boar (*Sus scrofa*) with no hints for a zoonotic potential (Takahashi et al., 2014). All members of the species *Orthohepevirus B*, *Orthohepevirus D* and *Piscihepevirus A* have no or an unknown zoonotic potential (Batts et al., 2011; Drexler et al., 2012; Huang et al., 2004).

1.1.4 Hepatitis E virus infection in humans

HEV is the main causative agent of an acute hepatitis in humans (Rein et al., 2012). After two to eight weeks of incubation flu-like symptoms arise, followed by emesis, fever, pain of the limbs or headache and epigastralgia before signs of acute hepatitis and liver failure occur. The fatality rates are very low in general and range between 0.2% and 4%, but case fatality rates of up to 25% can occur in immunocompromised persons, i.e. pregnant women or persons who received an organ transplantation (Kumar et al., 2013). These high rates of

mortality were observed during larger hepatitis E outbreaks in endemic regions of China, India, Somalia and Uganda (Kamar et al., 2012). Immunocompromised persons can also develop a chronic HEV infection, which is associated with liver failure and liver cirrhosis (Gerolami et al., 2008; Kamar et al., 2008). HEV infections are not only a problem of developing countries with known endemic regions, but in industrialized countries also HEV infections occur as sporadic autochthonous cases and in exposure risk groups (Clemente-Casares et al., 2003, 2016; King et al., 2018; Sayed et al., 2015). Serological investigations of blood donors and the general population revealed highly divergent seroprevalences (2.1 - 52.2%) in different European countries and even between parts of one country (Clemente-Casares et al., 2016). A serosurvey in blood donors from the United States of America (USA) showed an age-dependence and general decrease in anti-HEV seroprevalences of 21.8% (2006) to 16% (2012) (Sayed et al., 2015). Seroprevalences of 4.2% and 9.7% were detected in blood donors from New Zealand in the year 2007 and in the years 2014/2015, respectively (King et al., 2018). Additionally, HEV could be detected in sewage and human samples from different high human-density cities in Spain, Greece, France, Sweden and the USA, indicating a circulation of different HEV-strains even in one location/city (Clemente-Casares et al., 2003).

More than one third of the world-wide human population, approximately more than two billion humans, lives in highly endemic regions for the genotypes HEV-1 and HEV-2, like South-East Asia, the Middle East, India, Central Asia, Middle and South America (World Health Organization, 2017). The consumption of water, contaminated by human excreta, is the most probable transmission route for these two genotypes (Nelson et al., 2018). A vertical, transplacental transmission or intrauterine infection with HEV has been reported in India, with fatality rates of the mothers up to 100% and HEV-RNA detection rates of 50% in their children (Khuroo et al., 1995; Singh et al., 2003).

Hepatitis E is a notifiable disease in Germany since 2001 and cases have to be reported to the Robert Koch-Institute, if at least one of the following methods can detect HEV-RNA or anti-HEV antibodies (Robert Koch-Institute, 2015a):

- Direct detection of HEV by detecting HEV-RNA via reverse transcription-polymerase chain reaction (RT-PCR) from serum or stool;
- Indirect detection of HEV infection by serological assays; measuring of immunoglobulin M (IgM) antibodies (e.g. by enzyme-linked immunosorbent assay

(ELISA)) or by observing an increased titer of HEV-specific immunoglobulin G (IgG) antibodies (e.g. via ELISA).

The number of recorded HEV infections in Germany increased from less than 50 cases in the year 2001 to more than 3000 cases in 2018 (Figure 4). This increased number of reported HEV infections might be explained by better diagnostic assays for HEV and a greater awareness of the physicians (Robert Koch-Institute, 2015b).
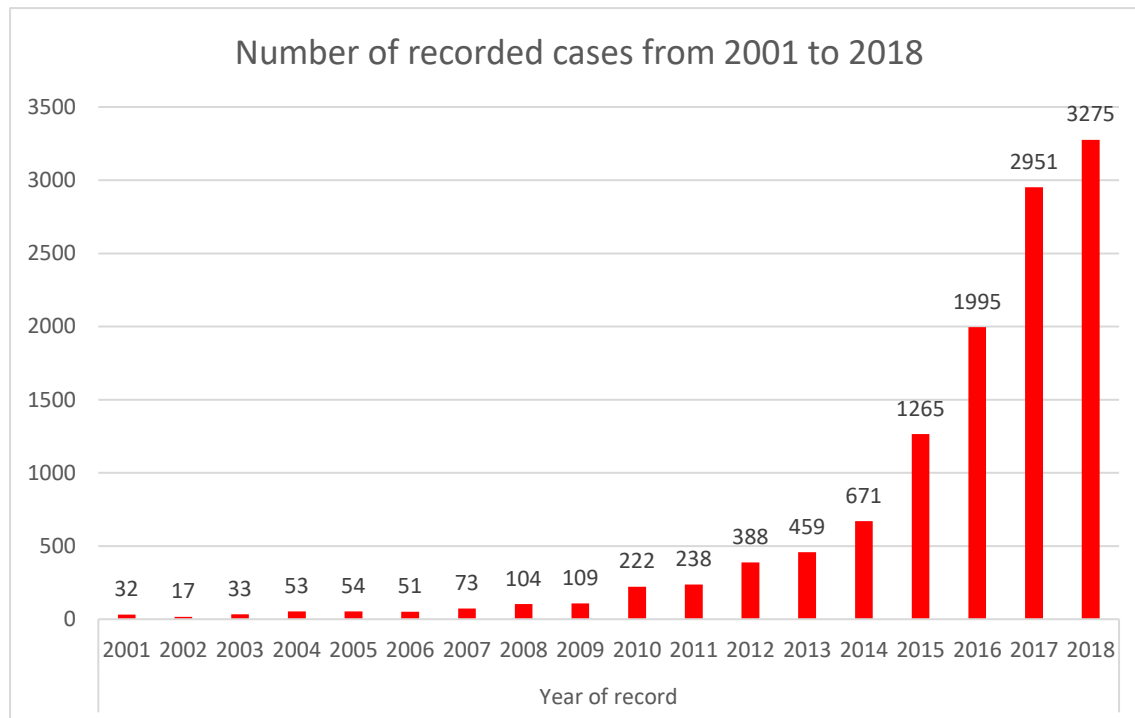
*Figure 4. **Recorded cases of human hepatitis E virus (HEV) infections in Germany.** Taken from the Robert Koch-Institute (Faensen and Krause, 2004, accessed 07.01.2019).*

The general seroprevalence for humans living in Germany was calculated to be 16.8% (samples taken between 2008 and 2011) (Faber et al., 2012). Another study in southeast Germany demonstrated a decrease of the anti-HEV antibody prevalence from 50.7% in 1996 to 34.4% in 2011 (Wenzel et al., 2014). Investigation of risk groups with an occupational exposure to pigs, like butchers, meat inspectors, pig farmers or veterinarians, demonstrated a higher seroprevalence (28.3%) than the corresponding control group (15.5%, Krumbholz et al., 2012). A large-scale study for the presence of anti-HEV antibodies in humans living in North Rhine-Westphalia and Lower Saxony, known federal states of Germany with a high domestic pig density, revealed a higher seroprevalence for the group with direct contact to pigs (13.2 - 32.8%) in comparison to the group without direct contact to pigs (7.7 - 21.7%). The investigation of forestry workers, another potentially risk group for HEV-infections, revealed an anti-HEV antibody prevalence of

21% (Dremsek et al., 2012). In addition to the exposure risk, the HEV seroprevalence increased with age as observed in studies in Germany and USA (Faber et al., 2012; Krumbholz et al., 2014a, b; Sayed et al., 2015).

Until today, there is only one vaccine (HEV 239 vaccine/Hecolin) available, which is only licensed in China and not in Germany or the European Union (Park, 2012). Another HEV-1 recombinant protein is also efficacious against HEV (Shrestha et al, 2007). Despite these two HEV-1 derived vaccines novel vaccines, including a HEV-4 derived peptide vaccine, are in different stages of development (Cao et al., 2017; Kulkarni et al., 2016; Wen et al., 2016; Xia et al., 2016). To date, there is no approved specific therapy for the treatment of acute or chronic HEV-3 or HEV-4 infection. The off-label use of ribavirin seems to be suitable for the elimination of chronic HEV infection (Kamar and Pischke, 2018; Todt et al., 2018).

1.2. Hepatitis E virus in small mammals

1.2.1 Hepatitis E virus-specific antibodies in rodents

Rodents represent the most diverse group of small mammals, according to the number of taxa and number of individuals (Wilson and Reeder, 2005). They are well known carriers of a wide number of zoonotic viral, bacterial and parasite agents (Meerburg et al., 2009). In addition, rodents carry rodent-specific pathogens that are most likely non-zoonotic, however, the current knowledge on these rodent-associated pathogens is still very limited (Drewes et al., 2017; Olival et al., 2017; Z. Wu et al., 2018).

Large serosurveys in rodents suggest the presence of additional hepeviruses (see Supplementary Table 1). During 1994 and 1998 more than 800 rodents, including 26 different species of 15 genera, were collected throughout the USA and investigated for the prevalence of anti-HEV antibodies. The majority of the positive animals belonged to the genus *Rattus*, with a prevalence of 59.7% (166/278). In addition, animals belonging to the genera *Neotoma*, *Peromyscus*, *Oryzomys*, *Sigmodon*, *Mus* and *Myodes* were tested positive for HEV-specific antibodies (Supplementary Table 1). Serological investigations of different rodent specimens (*Bandicota bengalensis*, *Rattus* spp. and *Mus* spp.) from India resulted in the detection of anti-HEV IgG antibodies in the rodent species *Rattus rattus rufescens*, *Bandicota bengalensis* and *Rattus rattus andamanensis* (Arankalle et al., 2001).

1.2.2 Discovery and characterization of rat-associated hepatitis E virus (ratHEV)

A novel rat-associated HEV (ratHEV) was discovered by investigating 30 faecal samples of wild Norway rats (*Rattus norvegicus*) trapped around the sewerage of Hamburg, Germany (Johne et al., 2010b). Two faecal samples were positive by HEV broad-spectrum RT-PCR and revealed partial genome sequences with 50% and 60% sequence similarity to avian and human HEV strains, respectively. In a follow-up study, additional nine animals were trapped around the same location like twelve months before and the complete genome of the novel ratHEV prototype strains R63 and R68 were determined and characterized. RatHEV shares the typical genomic attributes of hepeviruses, like the genomic organization or the size of the genome (Johne et al., 2010a). These initial findings were confirmed by the detection of additional ratHEV-positive rats in populations from other cities of Germany (Johne et al., 2012).

In 2012, Norway rats and Black rats (*Rattus rattus*) were found to be infected with a zoonotic HEV-3-strain. Some of the rats were trapped next to pig farms, whereas others were trapped at urban and rural sites (Kanai et al., 2012; Lack et al., 2012).

1.2.3 Novel hepeviruses in rodents and birds of prey

Nearly 300 specimens from seven wild small mammal species from Yunnan province, China, have been collected between 2014 and 2015 and tested for the presence of HEV-RNA by a broad-spectrum RT-PCR. HEV-RNA was found exclusively in two rodent species, Chevrier's field mouse and Père David's vole, with HEV-RNA detection rates of 29% and 7%, respectively. By determining the complete genomes of four representative strains, two of each specie, followed by a phylogenetic analysis and characterization, two novel genotypes within the species *Orthohepevirus C*, HEV-C3 and HEV-C4, were proposed (Wang et al., 2018). By a HTS approach of different rodents from Sao Paulo State, Brazil, two novel members of the species *Orthohepevirus C* were found, one in the hairy-tailed bolo mouse (*Necromys lasiurus*) and the second one in the delicate vesper mouse (*Calomys tener*). Thus, this study increased the knowledge about the broad host range of HEV and described HEV in additional members of the family Cricetidae (de Souza et al., 2018).

Attempting to find novel HEV-strains in birds of prey from Hungary, faecal samples of common kestrel (*Falco tinnunculus*) and red-footed falcon (*Falco vespertinus*) were

investigated by HTS and RT-quantitative real-time PCR (RT-qPCR). Thereby, HEV-RNA was detected in faecal samples of both species and one complete genome was generated. Interestingly, this kestrel-derived sequence shows the highest similarity towards members of the rodent and carnivore associated species *Orthohepevirus C*, instead towards the bird associated *Orthohepevirus B*. A dietary origin of infection could not be excluded as explanation of this interesting finding in a bird of prey (Reuter et al., 2016a). Broad spectrum RT-PCR investigation of liver samples of more than 330 different rodents of five species from Hungary resulted in the detection of HEV-RNA exclusively in eleven common voles (*Microtus arvalis*). Phylogenetic reconstruction revealed a close relationship towards the previously described kestrel-derived HEV-strain (Kurucz et al., 2018).

1.2.4 Rabbit-associated hepatitis E virus in rabbit breedings, wild life populations and pet animals

Rabbit-associated hepatitis E virus (rabbitHEV) was described for the first time in a Chinese breeding of farmed Rex rabbits, a breed of European rabbits (*Oryctolagus cuniculus*). In this initial study of rabbits from two farms from Gansu province, China, the seroprevalence was 57% and the HEV-RNA detection rate 7.5% (Zhao et al., 2009). Thereafter, rabbitHEV was found in different other breedings in China (J. Geng et al., 2011; Y. Geng et al., 2011), but also in breedings in USA (Cossaboom et al., 2011), Mongolia (Jirintai et al., 2012), the Netherlands (Burt et al., 2016) and Korea (Ahn et al., 2017). Additional investigations revealed the presence of rabbitHEV in pet rabbits from Italy and the Netherlands (Burt et al., 2016; Caruso et al., 2015; Di Bartolo et al., 2016) and in different wild life populations and archived serum samples of rabbits from Germany (Eiden et al., 2016; Hammerschmidt et al., 2017). No rabbitHEV-RNA was detected in hares so far, although HEV-reactive antibodies were detected (Hammerschmidt et al., 2017).

RabbitHEV shares the typical genome organization with other HEV strains: three ORFs with a genome length of about 7.2 kb (Zhao et al., 2009). In contrast to all other HEV strains, rabbitHEV has a unique and so far, rabbitHEV-specific 93nt- in-frame insertion within the X-domain (Zhao et al., 2009). Based on phylogenetic analysis of full-length sequences, rabbitHEV strains belong to the zoonotic HEV-3 clade of HEV, but forming a well-separated cluster, designated as HEV-3ra (Smith et al., 2016).

1.3 Objectives

To understand the zoonotic transmission and pathogenicity of HEV as well as to evaluate anti-HEV vaccines and antiviral drugs, the development of small mammal animal models is needed. Therefore, the objectives of this study were:

-to search for novel hepeviruses in other rodents

-to identify rat and rabbit populations with ratHEV and rabbitHEV infections

-to characterize the sequence variation of these hepeviruses within their reservoirs and evaluate their persistence within the populations

2. Publications

2.1 Paper I

Ryll, R., Heckel, G., Corman, V. M., Drexler, J. F., Ulrich, R. G., 2019. Genomic and spatial variability of a European common vole hepevirus. Archives of Virology, https://doi.org/10.1007/s00705-019-04347-1.

ORIGINAL ARTICLE

Genomic and spatial variability of a European common vole hepevirus

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Abstract

Rodents host different orthohepeviruses, namely orthohepevirus C genotype HEV-C1 (rat hepatitis E virus, HEV) and the additional putative genotypes HEV-C3 and HEV-C4. Here, we screened 2,961 rodents from Central Europe by reverse transcription polymerase chain reaction (RT-PCR) and identified HEV RNA in 13 common voles (*Microtus arvalis*) and one bank vole (*Myodes glareolus*) with detection rates of 2% (95% confidence interval [CI]: 1-3.4) and 0.08% (95% CI: 0.002-0.46), respectively. Sequencing of a 279-nucleotide RT-PCR amplicon corresponding to a region within open reading frame (ORF) 1 showed a high degree of similarity to recently described common vole-associated HEV (cvHEV) sequences from Hungary. Five novel complete cvHEV genome sequences from Central Europe showed the typical HEV genome organization with ORF1, ORF2 and ORF3 and RNA secondary structure. Uncommon features included a noncanonical start codon in ORF3, multiple insertions and deletions within ORF1 and ORF2/ORF3, and the absence of a putative ORF4. Phylogenetic analysis showed all of the novel cvHEV sequences to be monophyletic, clustering most closely with an unassigned bird-derived sequence and other sequences of the species *Orthohepevirus C*. The nucleotide and amino acid sequence divergence of the common vole-derived sequences was significantly correlated with the spatial distance between the trapping sites, indicating mostly local evolutionary processes. Detection of closely related HEV sequences in common voles in multiple localities over a distance of 800 kilometers suggested that common voles are infected by cvHEV across broad geographic distances. The common vole-associated HEV strain is clearly divergent from HEV sequences recently found in narrow-headed voles (*Microtus gregalis*) and other cricetid rodents.

Introduction

The family *Hepeviridae* comprises two genera, genus *Piscihepevirus*, with only one fish-associated virus [2], and genus *Orthohepevirus*, with four species. Species *Orthohepevirus A* comprises eight genotypes, of which HEV-1 to HEV-4 and HEV-7 have been found in humans. Genotypes HEV-1 and HEV-2 seem to be restricted to humans. The

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other genotypes occur in various animals, including pigs and wild boar (HEV-3 to HEV-6), rabbits (HEV-3), and camelids (HEV-7 and HEV-8) [44]. The species *Orthohepevirus B* and *Orthohepevirus D* include only non-zoonotic avian HEV and bat HEV strains, respectively. Members of the species *Orthohepevirus C* were first detected in Norway rats (*Rattus norvegicus*; genotype HEV-C1) and in different carnivore species (genotype HEV-C2) [20, 37].

Hepeviruses have a single-stranded RNA genome of positive polarity with a size of about 6.9–7.2 kilobases (kb), a short 5' untranslated region (UTR), three major open reading frames, and a 3'-UTR with a poly-A tail [23, 44]. Open reading frame (ORF) 1 is 4.6 to 5.2 kb in length and encodes a polyprotein including different non-structural proteins (Mt, methyltransferase; Y, Y-like domain; PCP, papain-like cysteine protease; X, X domain/ADP-ribose-binding module; Hel, helicase; RdRp, RNA-dependent RNA polymerase) [23]. ORF2 is about 2.0 kb in length and encodes the capsid protein [55]. ORF3 is approximately 342 nucleotides (nt) long and encodes a multifunctional protein that seems to function as an ion channel (viroporin) and is needed for the release of viral particles [9, 54]. In addition to these three ORFs, members of the species *Orthohepevirus C* share an additional putative fourth ORF [20].

Rodents represent the largest mammalian order, in terms of both the number of species and the number of individuals, and have various life history traits [50]. They are well-known carriers of a large number of zoonotic viral, bacterial and parasite agents [31], but although the number of pathogens identified in rodent species is steadily increasing, our current knowledge of rodent-borne agents is still very limited [34, 53].

Norway rat-associated HEV (rat HEV) was detected first in Germany [18, 19], but was found later in Norway rats and Black rats (*Rattus rattus*) from various other European countries, Asia and the USA [26, 27, 32, 33, 35, 36, 39, 51]. This virus has also been detected in Asian house shrews (*Suncus murinus*), which might have been due to spillover infections [13]. Similarly, rat HEV RNA was detected in a Syrian brown bear (*Ursus arctos syriacus*) in a zoo in Germany, probably due to a spillover infection from free-living rats in the same zoo [46]. Serological and experimental infection studies suggest that rat HEV could have zoonotic potential although this is controversial [7, 10, 20, 36, 42]. Recently, HEV-C1-related RNA was detected in an immunocompromised patient from Hong Kong, China, and a healthy, immunocompetent patient from Halifax, Canada, underlining the need for a critical assessment of the zoonotic potential of members of the genotype HEV-C1 [1, 47]. Additionally, HEV-3 strains of the species *Orthohepevirus A* were detected in rats, probably transmitted by spillover infection [22, 25, 39].

Recently, two novel rodent-associated hepeviruses were identified in a Chevrier's field mouse (*Apodemus chevrieri*) and a Père David's vole (*Eothenomys melanogaster*) from China, and proposed as genotypes HEV-C3 and HEV-C4 [49] within the species *Orthohepevirus C*. Additional novel, unassigned rodent-associated HEV-strains were detected in cricetid and murine rodents in Brazil and China, namely in a hairy-tailed bolo mouse (*Necromys lasiurus*), a delicate vesper mouse (*Calomys tener*), a striped field mouse (*Apodemus agrarius*), a Chinese striped hamster (*Cricetulus barabensis*), a grey-sided vole (*Myodes rufocanus*), a narrow-headed vole (*Microtus gregalis*), a grey dwarf hamster (*Cricetulus migratorius*), and a kolan vole (*Eothenomys inez*) [8, 53]. Furthermore, a HEV-strain originating from a kestrel (*Falco tinnunculus*) showed higher sequence similarity to members of the species *Orthohepevirus C* than to the avian-associated HEV-strains [38]. Investigation of more than 330 rodents from five different species in Hungary resulted in the detection of HEV RNA exclusively in eleven common voles (*Microtus arvalis*). Phylogenetic reconstruction of the short screening fragment within the coding region of the RdRp of HEV revealed a close relationship to the previously described kestrel-derived HEV-strain. The complete genome sequences of these novel HEV strains were not determined [24].

Here, we describe the screening of nearly 3,000 rodents originating from Central Europe by broad-spectrum, nested reverse transcription polymerase chain reaction (RT-PCR) for the presence of HEV RNA. We determined and analyzed the complete genome sequences of novel hepevirus strains associated with common voles.

Materials and methods

Animal collection

A total of 2,961 rodents were used for this study (for detailed information see Table 1 and Figure 1). This includes 2,662 animals collected previously and screened for novel rodent hepaciviruses [12] and 299 animals trapped along a transect at the border of Germany and the Czech Republic [3, 41].

RNA extraction

Viral RNA was extracted from serum pools or individual liver samples. Viral RNA was extracted from about 30 mg of liver tissue or 10-50 µL of serum. RNA was purified using a MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche, Penzberg, Germany) for tissue specimens and a DNA and Viral NA Small Volume Kit (Roche) for serum.

Molecular detection of HEV RNA

For the detection of HEV RNA, a nested RT-PCR targeting the RdRp-encoding domain of the ORF1 of HEV was used

Table 1 Results of RT-PCR screening of rodents collected in Germany and the Czech Republic

Country	Federal state/ region	Trapping site	Number of RT-PCR positive/total number of animals tested						
			Microtus arvalis	*Microtus agrestis*	*Apodemus flavicollis*	*Apodemus agrarius*	*Apodemus sylvaticus*	*Myodes glareolus*	Subtotal
Germany	Berlin	Berlin	-	-	0/10	0/40	-	-	0/50
	Brandenburg	Muckrow (Muc)[b]	1/16	-	-	-	0/2	-	1/18
		four other sites	0/14	-	0/3	0/3	0/14	0/1	0/35
	Baden-Wuert-temberg	Weissach (Wei)[b]	1/24	0/4	0/34	-	0/2	0/150	1/214
		Ditzingen (Dit)[a]	-	-	0/20	-	-	1/28	1/48
		ten other sites	0/25	0/2	0/118	-	0/7	0/213	0/365
	Bavaria	Falkenstein (Fal)[b]	2/32	-	-	-	-	-	2/32
		eleven other sites	0/73	-	0/1	-	0/4	0/1	0/79
	Hesse	seven sites	0/15	0/13	0/17	-	0/27	0/88	0/160
	Mecklenburg-Western Pomerania	nine sites	0/20	0/18	0/76	0/20	-	0/73	0/207
	North Rhine-Westphalia	twelve sites	0/11	-	0/80	-	0/35	0/177	0/303
	Lower Saxony	twelve sites	0/3	-	0/192	-	0/55	0/187	0/437
	Thuringia	Creuzburg (Cre)[a]	1/14	0/1	0/1	0/4	0/16	-	1/36
		eight other sites	0/211	0/113	0/145	0/19	0/13	0/288	0/789
Subtotal	**9**	**79**	**5/458**	**0/151**	**0/697**	**0/86**	**0/175**	**1/1206**	**6/2,773**
Czech Republic	Plzen Region	Hayek (Hay)[b]	5/39	-	-	-	-	-	5/39
		Zalesi (Zal)[b]	3/17	-	-	-	-	-	3/17
		four other sites	0/132	-	-	-	-	-	0/132
Subtotal	**1**	**6**	**8/188**	-	-	-	-	-	**8/188**
Total	**10**	**85**	**13/646**	**0/151**	**0/697**	**0/86**	**0/175**	**1/1206**	**14/2,961**

[a] Trapping site with at least one hepatitis E virus (HEV)-RNA-positive animal (see Fig. 1, filled squares or circles); [b] Trapping sites where additionally at least one complete genome sequence of the novel common-vole-associated HEV was obtained (see Fig. 1, trapping sites indicated by asterisks)

as described before [11]. The PCR product has an expected size of 279 nt, without the primers. PCR products were separated by agarose gel electrophoresis and visualized by staining with Midori Green Advance (Biozym, Hessisch Oldendorf, Germany).

Complete genome sequence determination

For complete genome sequence determination of five representative HEV strains of the common vole-associated HEV, a primer-walking approach was used, including a 5′ and 3′ rapid amplification of cDNA ends (5′/3′ RACE System, Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out under the following conditions: 50 °C for 15 min, 94 °C for 2 min, 40 PCR cycles at 94 °C for 15 s, 60 °C for 30 s, 72 °C for 1-2

min, and a final extension at 72 °C for 10 min. The primers used for determination of the complete genome sequences are listed in Supplementary Table 1. RT-PCR products were sequenced directly using a BigDye Terminator 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

Sequence and phylogenetic analysis

Sequence alignments were produced using BioEdit [14], and phylogenetic analysis was done using MEGA 6 [48]. The amino acid (aa)-sequence-based phylogenies were reconstructed by a maximum-likelihood analysis with the Jones-Taylor-Thornton (JTT) substitution model with invariant sites, a gamma distribution shape parameter of five, and 1,000 bootstrap replicates. The phylogenetic analysis

Fig. 1 Map of the trapping sites of common voles (*Microtus arvalis*) and bank voles (*Myodes glareolus*) in Germany and the Czech Republic. Trapping sites of hepatitis E virus (HEV)-RNA-positive common voles and bank voles are indicated by filled squares and circles, respectively. Asterisks indicate the origins of complete common vole-derived HEV sequences
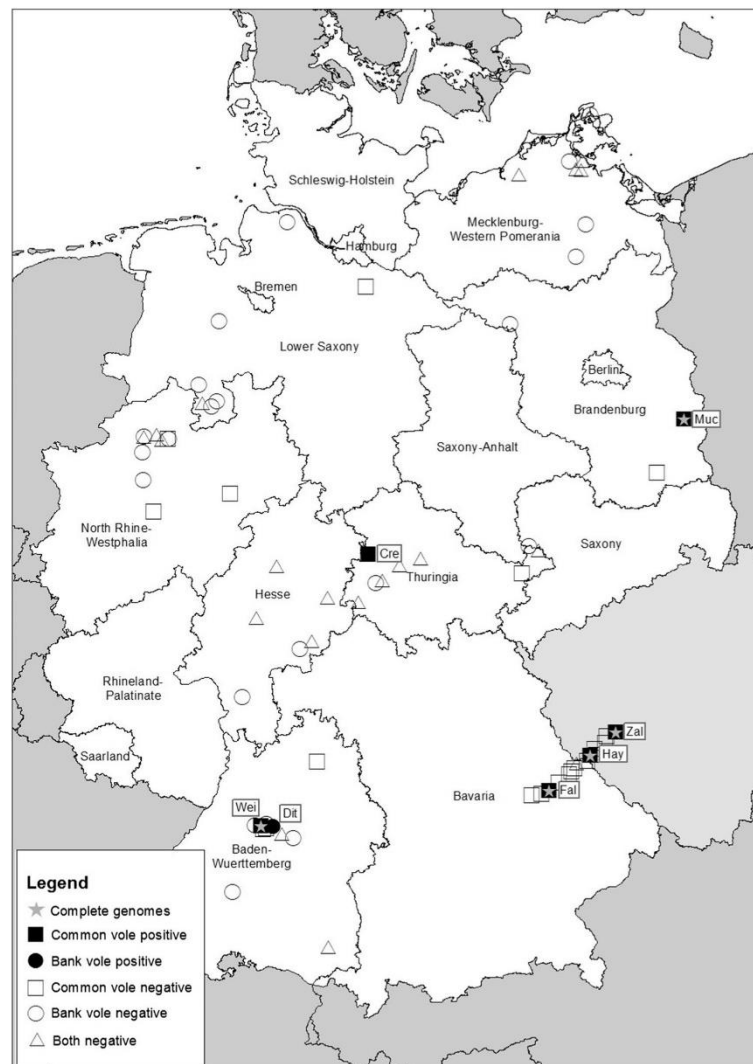
used the proposed reference sequences of HEV [44, 45], sequences for the proposed genotypes HEV-8, HEV-C3, and HEV-C4 [49, 52], two unassigned sequences [8, 38], five rodent-associated HEV-strains from China [53] and recently described HEV sequences from common voles [24]. If available, complete genome sequences were used. Otherwise, a fragment of the RdRp-encoding region was used for phylogenetic analysis. Pairwise distances were calculated at the nt and aa level with MEGA 6 [48]. Tests for potential recombination events were done using Bootscan

[29], implemented within RDP4 software [30], using the reference sequences listed above. For Simplot analysis with a window size of 100 nt and a step size of 25 nt, scripts were written in R [6]. The identification of conserved protein coding domains was done using CDD/SPARCLE, using the default settings [28]. For the prediction of RNA secondary structures within the intergenic region between ORF1 and ORF2, the program mfold [57] was used with default settings. We tested for largely local transmission of cvHEV resulting in "isolation-by-distance" patterns by computing

pairwise genetic and geographic distances between sampling locations [40]. Pairwise genetic distances were calculated using MEGA 6 [48] for all common vole-associated HEV sequences, including the kestrel-derived HEV sequence (see Fig. 2A, clades I-III), and geographic distances were calculated using the "dist"-function, implemented in R [6], by using the coordinates of the individual trapping sites. We tested for statistical significance between half matrices at the nt and aa level using Mantel tests, implemented in the package "ade4" of R. The 95% confidence interval (CI) was calculated in R by using the binom.test() function [5, 6].

Results

Screening of rodents by nested RT-PCR

RT-PCR screening of 432 serum pools with 2,961 individual samples (Table 1) resulted in the detection of 14 positive pools with a band of the expected size in agarose gel electrophoresis. Subsequent analysis of individual samples from the positive pools showed 14 animals, including 13 common voles and one bank vole (*Myodes glareolus*), to be RT-PCR positive. HEV-RNA-positive animals originated from seven trapping sites (Fig. 1). The detection rate of HEV RNA was 2% (13/646; 95% CI: 1-3.4) for all common voles tested and 0.08% (1/1206; 95% CI: 0.002-0.46) for all bank voles tested using broad-spectrum nested RT-PCR (Table 1). For single trapping sites, the detection rate in common voles reached 4.2% (1/24, site Wei; 95% CI: 0.1-21.1), 7.1% (1/14, site Cre; 95% CI: 0.18-33.86), 6.2% (2/32, site Fal; 95% CI: 0.76-20.8 and 1/16, site Muc; 95% CI: 0.1-30.2), 12.8% (5/39, site Hay; 95% CI: 4.2-27.4), 17.6% (3/17, site Zal; 95% CI: 3.7-43.4) and in bank voles 3.5% (1/28, site Dit; 95% CI: 0.01-18.34). For the locations of the trapping sites, see Fig. 1.

Phylogenetic analysis and spatial relationships

Phylogenetic analysis based on predicted aa sequences of a portion of ORF 1 (GenBank accession numbers MK192405 to MK192409 and MK192412 to MK192420) revealed a monophyletic group of three major clades, I–III, including the previously reported common vole-associated HEV sequences and a kestrel-derived HEV strain from Hungary (Fig. 2A, indicated by an arrow) within the species *Ortho-hepevirus C*. Clade I includes sequences from common voles trapped at different sites at the border between Germany (GER) and the Czech Republic (CZE; Fig. 1). The second clade, clade II, includes only sequences derived from four common voles trapped at Hayek (Hay), CZE. Clade III includes sequences from voles from four trapping sites in GER, including three sequences from common voles and

one sequence from a bank vole, and several common vole derived HEV sequences and a kestrel-derived sequence from Hungary [24].

Pairwise sequence identity values for the sequences within clade I ranged from 91.7 to 100% at the nt level and were 100% at the aa level. The identity values for nt and aa sequences within clades II and III were similar (clade II: 99.6 to 100% and 100%, respectively; clade III: 86.6 to 99.6% and 98.9 to 100%, respectively). Comparing the sequences from clades I, II and III with the kestrel-derived sequence, the sequence identity ranged from 83.7 to 92.4% and 94.6 to 98.9% at the nt and aa level, respectively (Supplementary Table 2).

The nt and aa sequence divergence of all common vole-derived HEV sequences and the spatial distances of the corresponding trapping sites showed significant correlations ($r = 0.7$; $p < 0.00001$ and $r = 0.6$; $p < 0.00001$, respectively; see Fig. 3).

The phylogenetic tree revealed that the common vole (and kestrel-) associated HEV sequences clustered together with a narrow headed vole-derived sequence from China (HEV-RtMg/XJ2016, indicated by a dotted arrow in Fig. 2A). Members of the species *Orthohepevirus C* not associated with cricetid rodents were only found in a separate clade (rat-associated HEV-C1, carnivore-associated HEV-C2, and *Apodemus chevrieri*-associated putative genotype HEV-C3).

Generation of complete genome sequences from novel common vole-associated hepatitis E virus

Complete genome sequences of this novel common vole-associated HEV (cvHEV) were obtained by a primer-walking approach for five common vole-derived strains (GenBank accession numbers MK192405 to MK192409; Fig. 1). The genomes of these novel strains all have the same organization but with slight variation in length, ranging from 7020 to 7077 nt (Fig. 4A and B). The 5' UTR has a length of 33 nt, and ORF1 has a length of 4914-4971 nt and encodes a polyprotein of 1638-1657 aa. ORF2 had a length of 1992 nt and encodes a protein of 664 aa residues. The 3' UTR has a length of 55 nt, excluding the poly-A tail. The different lengths of ORF1 are caused by short in-frame insertions/deletions (indels) of different lengths within the Y-domain and PCP- and RdRp-encoding regions of ORF1 (Fig. 4C).

ORF3 has a length of 363 nt and encodes a protein of 121 aa, overlapping with ORF2 and starting within the non-coding region between ORF1 and ORF2 (Fig. 4A-C). It contains an alternative start codon (UUG encoding leucine) at the 5' end, as it was described previously for the kestrel-derived HEV genome [16, 38]. In addition, all five novel genome sequences contain an in-frame indel within the ORF2/ORF3 overlapping region in comparison to the

Fig. 2 Phylogenetic reconstruction of amino-acid (aa)-sequence-based trees for novel rodent-associated hepatitis E virus (HEV) sequences. The phylogenetic relationships of the novel common vole-associated HEV were calculated by maximum-likelihood analysis using the Jones-Taylor-Thornton (JTT) substitution model with invariant sites, a gamma-distributed shape parameter, and 1,000 bootstrap replicates. Support values below 70 are not shown. The phylogenetic analysis of HEV was based on proposed reference sequences [44, 45], sequences for the proposed genotypes HEV-8, HEV-C3 and HEV-C4 [49, 52], two unassigned sequences [8, 38], five rodent-associated HEV-strains from China [53], and a recently discovered HEV from common voles in Hungary [24]. Next to the individual identification code for each positive animal, the abbreviation of the species (Mgla, *Myodes glareolus*; Marv, *Microtus arvalis:* Cb, *Cricetulus barabensis*; Mr, *Myodes rufocanus*; Cl, *Eothenomys melanogaster*; Ei, *Eothenomys inez*; Mg, *Microtus gregalis*), the trapping site, and country are shown. An unassigned kestrel-derived HEV sequence is indicated by an arrow. (A) Relationships based on the protein encoded by the 92-aa screening fragment encoded within ORF1. (B) Relationships based on the concatenated ORF1/ORF2 encoded sequence, with a length of 2139-2384 aa
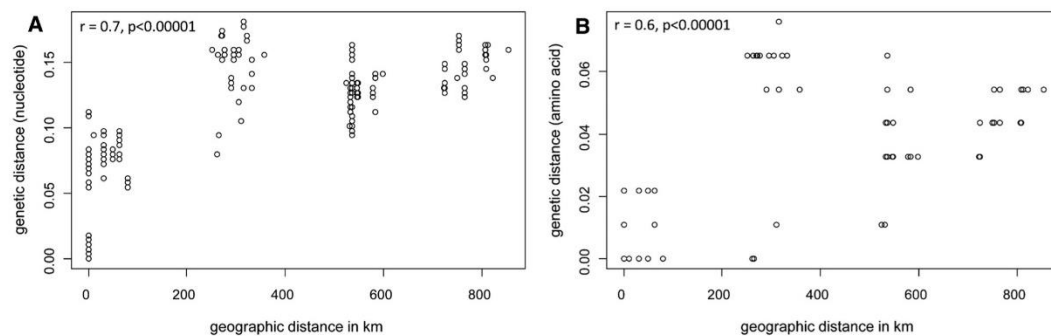

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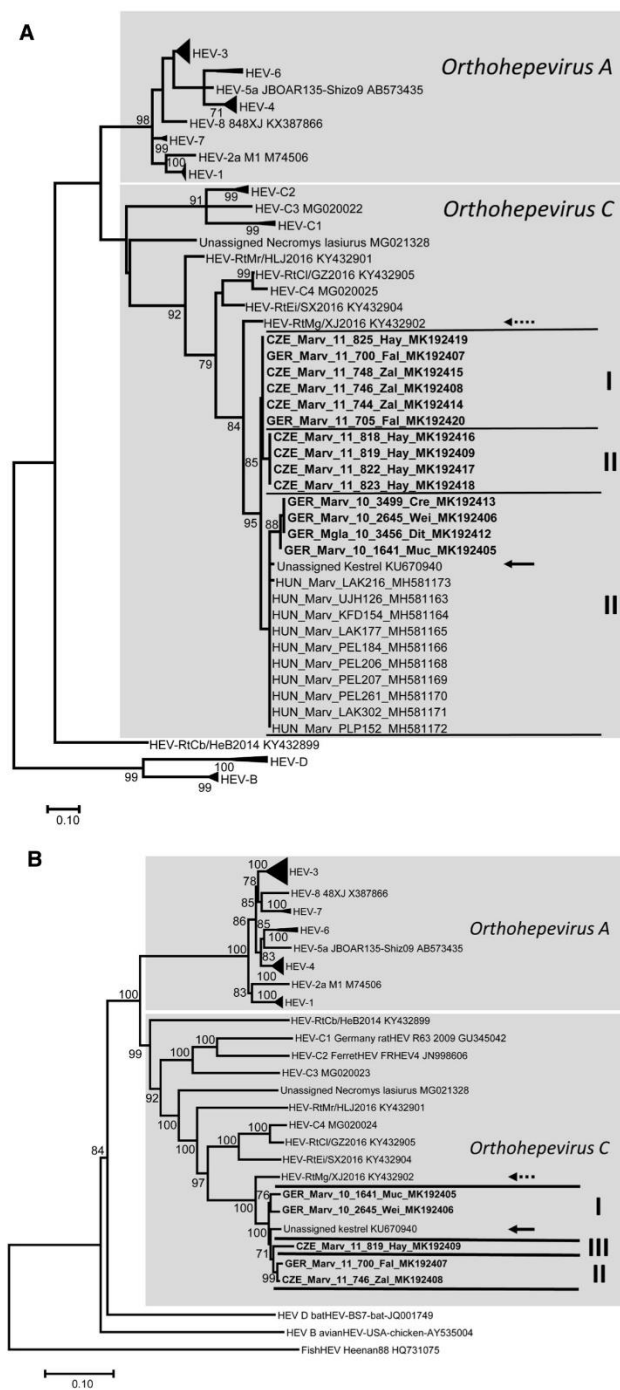
Fig. 3 Relationships between geographical distance and genetic divergence based on a portion of the RdRp coding region of rodent-associated hepatitis E virus (HEV) sequences at the nt level (A) and the corresponding amino acid sequence level (B). Sequences of all common vole-associated HEV strains (see Fig. 2A) were included.

Pairwise geographic distances were calculated by using the coordinates (longitude and latitude) of each trapping site, the "dist" function implemented in R [6], and the genetic distance (p-distance), which was calculated using MEGA 6 [48]. Pairwise distance plots were generated using the "plot" function implemented in R [6]

kestrel-derived sequence (Fig. 4B and C). In contrast to other members of the species *Orthohepevirus C*, an additional putative ORF4 overlapping with the ORF1 was not found at the 5' end (Fig. 4A) [20].

An RNA secondary structure prediction revealed a similar stem-loop structure in all five novel common vole-derived genomes and the kestrel-derived sequence at the same position within the junction region between ORF1 and ORF2 (Fig. 4A and Supplementary Fig. 1) as described for members of the species *Orthohepevirus A* [4].

Comparison of the complete common vole-associated HEV genome sequence with those of other members of the family *Hepeviridae*

Phylogenetic analysis and pairwise sequence comparison of the concatenated ORF1 and ORF2 nt and aa sequences showed almost the same patterns as the analysis of the screening fragment (Fig. 2B and Supplementary Tables 2 and 3). The cvHEV sequences again had the highest similarity to the narrow headed vole-derived sequence from China (HEV-RtMg/XJ2016). Together with other cricetid-rodent-associated HEV strains, they formed a sister clade to murid-rodent- and carnivore-associated HEV strains. The Chinese striped hamster-associated sequence formed a separate clade (Fig. 2B).

In a comparison to the kestrel-derived HEV sequence, the sequence identity values ranged from 83.7 to 86.2% and 94.6 to 96.7% for the clades I, II and III at the nt and aa level, respectively. The similarity of the concatenated ORF1 and ORF2 sequence to other members of the species *Orthohepevirus C* ranged from 59.6 to 67.8% and 61.1 to 76.6% at the nt and aa level, respectively (Supplementary Table 3).

Simplot analysis of the novel cvHEV revealed no obvious differences compared to other members of the species *Orthohepevirus C*. Additionally, no recombination events were detected by Bootscan analysis (data not shown).

Five conserved protein coding domains were identified in all five complete genome sequences of the novel cvHEV, the reference sequence originating from a kestrel (GenBank accession number KU670940), and the narrow-headed vole-derived sequence (GenBank accession number KY432902): viral methyltransferase, Appr-1"-p processing enzyme, RNA helicase, RNA-dependent RNA polymerase, structural protein 2 (Supplementary Table 4).

Discussion

By screening nearly 3,000 rodents from Central Europe, we identified a novel HEV strain in 14 samples and determined the complete genome sequences of five isolates of this strain from common voles collected at different sites. The multiple detection of this strain in common voles from different trapping sites (this study and [24]) and its complete absence in other rodent species from the same region suggests that this novel virus is specific for common voles. The detection of cvHEV in one bank vole might be explained by a spillover infection. These findings again underline the necessity of multiple detections of a pathogen in a single species from different geographical regions in order to make a reliable conclusion about a reservoir-virus association, as discussed previously for hantaviruses [15].

The average infection rate observed here (2%; 95% CI: 1-3.4) was low; however, when calculating the detection rate for single trapping sites it ranged between 4.2% (95% CI: 1-21.1) and 17.6% (95% CI: 3.7-43.4). A previous study in

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A

Mt | Y | PCP | X | Hel | RdRp | Junction Region | VP | ORF 3 | CP

ORF 1 — ratHEV (HEV-C1)
ORF 4 / Predicted SL / ORF 3 / ORF2

ORF 1 — ferretHEV (HEV-C2)
ORF 4 / ORF 3 / ORF2

ORF 1 — Putative HEV-C3
ORF 4 / ORF 3 / ORF2

ORF 1 — Putative HEV-C4
ORF 3 / ORF2

ORF 1 — Kestrel-derived HEV
ORF 3 / ORF2

ORF 1 — cvHEV
ORF2

B

Mt | Y | PCP | X | Hel | RdRp | VP | ORF 3 | CP

ORF1 — ORF2 — 1641
ORF 3

ORF1 — ORF2 — 2654
ORF 3

ORF1 — ORF2 — 700
ORF 3

ORF1 — ORF2 — 746
ORF 3

ORF1 — ORF2 — 819
ORF 3

ORF1 — ORF2 — Kestrel-derived HEV

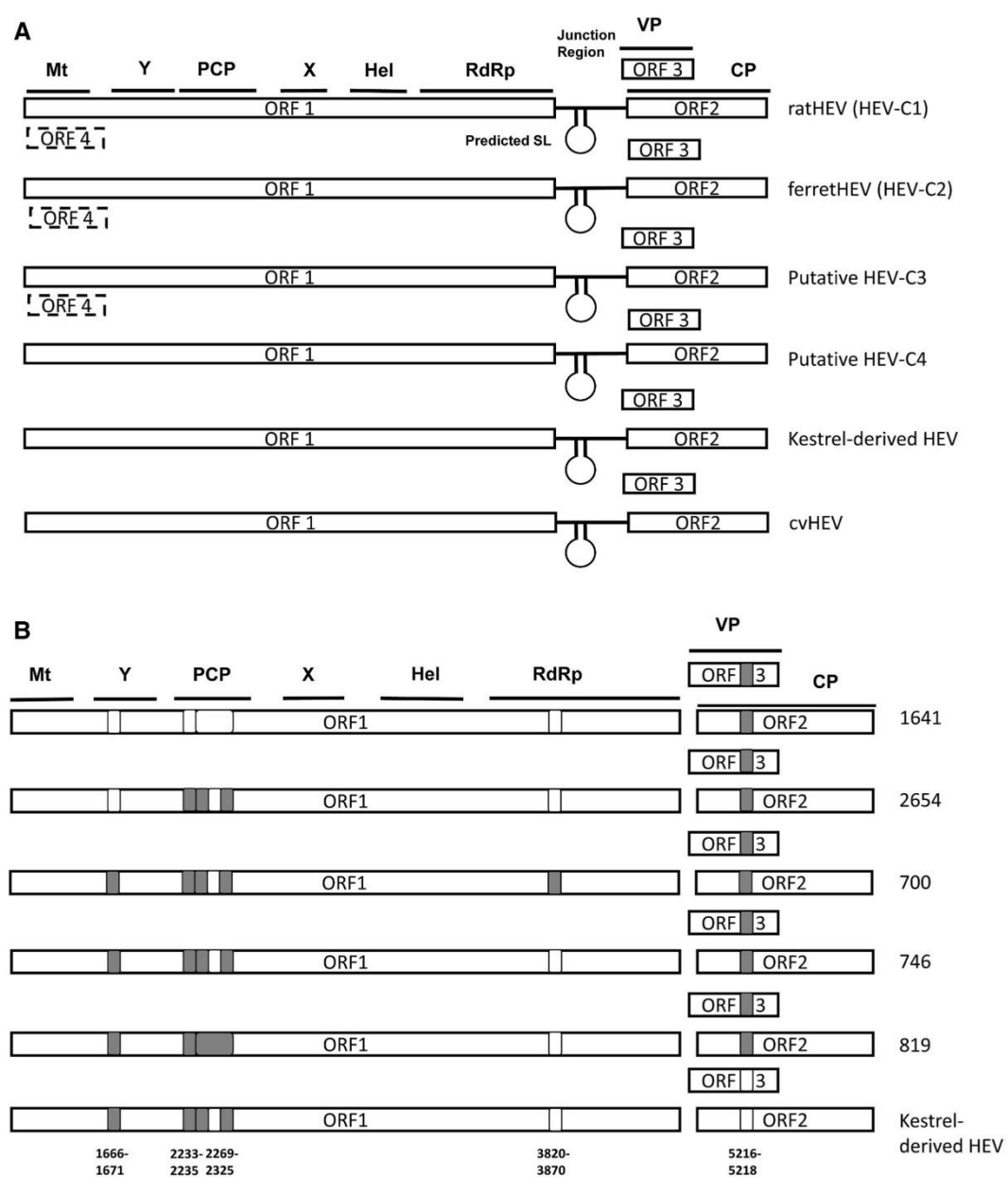1666-1671 | 2233-2235 | 2269-2325 | 3820-3870 | 5216-5218

◄**Fig. 4** Comparison of the genomic organization of members of the species *Orthohepevirus C* (genotypes HEV-C1 and HEV-C2), including the putative genotypes HEV-C3/HEV-C4, the kestrel-derived HEV and the novel common vole-associated HEV. (A) A schematic representation showing the length and position of the open reading frames (ORFs) 1, 2 and 3, including the putative ORF4 (dotted lines). The functional domains encoded by ORF1 (Mt, methyltransferase; Y, Y-like domain; PCP, papain-like cysteine protease; X, X domain/ADP-ribose-binding module; Hel, helicase; RdRp, RNA-dependent RNA polymerase [23]), the ORF2-encoded capsid protein (CP), and the ORF3-encoded viroporin (VP) are located at the corresponding positions, and the junction region with a stem-loop (SL) secondary structure between the ORF1 and the overlapping ORF2/ORF3 of rat HEV (HEV-C1, GU345042), ferret HEV (HEV-C2, JN998606), putative HEV-C3 (MG020023), putative HEV-C4 (MG020024), kestrel-derived HEV (KU670940), and common-vole associated HEV (cvHEV, strain MK192496) are shown. (B) Insertions or deletions (indels) of the five cvHEV strains 1641, 2645, 700, 746 and 819 (GenBank accession numbers MK192405-MK192409) compared to the kestrel-derived HEV-strain (KU670940) are indicated by empty or filled squares. Indels are located at the nucleotide positions 1666-1671, 2233-2235, 2269-2325, 3820-3870 and 5216-5218. Nucleotide sequences of indels are highlighted by a grey colored box. (C) Nucleotide sequences of the indels within ORF1 and ORF2/ORF3

Hungary indicated a detection rate of 10.2% at the single site investigated [24]. Future investigations would have to apply a cvHEV-specific RT-PCR assay for potentially improved sensitivity.

The detection of this virus in common voles from Germany, the Czech Republic (this study) and Hungary [24] suggests a broad geographical distribution and individual strains of the novel cvHEV in Europe. This conclusion was confirmed by an "isolation-by-distance" pattern that showed a strong, positive correlation between the geographic and genetic distances of the novel cvHEV strains. This suggests that the spread and evolution of cvHEV occur mostly at a local or regional spatial scale, probably tightly associated with common voles [40]. Further investigations are needed to evaluate if the presence of various cvHEV strains can be explained by their association with different lineages of common voles in Europe [41].

The high sequence similarity and phylogenetic relationship of the cvHEV sequences, and in particular those from Hungary, may indicate a dietary origin of the kestrel-derived HEV strain [38]. Supporting this assumption, the kestrel-derived HEV strain and the cvHEV strains share attributes that are unique among the members of the family *Hepeviridae*: an alternative noncanonical start codon for ORF3 and the absence of a putative ORF4, previously found in members of genotypes HEV-C1 and HEV-C2 and the putative genotype HEV-C3 ([16], Fig. 4A).

The cvHEV strains show a high degree of sequence similarity to other members of the species *Orthohepevirus C* and form a large cluster with HEV strains from different cricetid rodents, including the putative HEV-C4 genotype [49]. A coevolution scenario might explain the phylogenetic relationships of the rodent-associated hepeviruses, at least in part. The common vole-associated HEV is closely related to the narrow-headed vole-associated HEV strain from China, and both reservoir species belong to the genus *Microtus*. The next most closely related HEV strains were identified
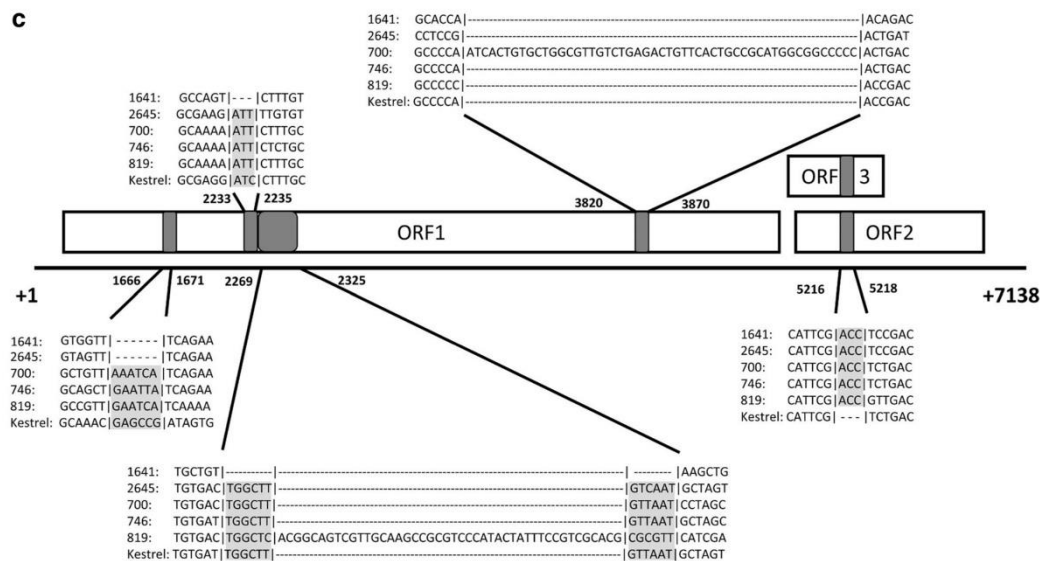

Fig. 4 (continued)

in other arvicoline rodents, followed by a strain from a sigmodontine rodent from South America. Members of the previously proposed genotype HEV-C3 are most closely related to rat HEV (genotype HEV-C1) and have also been discovered in the murine species *Apodemus chevrieri*, which belongs to the same subfamily as rats, which are reservoirs of rat HEV. The most divergent HEV strain within the species *Orthohepevirus C* was found in Chinese striped hamster (HEV-RtCb/HeB2014 KY432899).

Interestingly, multiple insertions/deletions were identified in the genomes of common vole-associated HEV strains; all indels were in-frame, and none of them interrupted the ORF1 coding sequence. The positions of these indels within ORF1 of common vole-associated HEV differed from those of indels previously detected in HEV-3 strains from chronically infected patients or in rabbit HEV strains [17, 21, 43, 56]. The occurrence of these indels suggests genomic plasticity in the ORF1 region, and this might be used in the future as a marker for molecular epidemiological studies.

In conclusion, this study confirms that members of the species *Orthohepevirus C* are associated with rodents and refutes an evolutionary origin of these viruses in avian hosts. The overrepresentation of cricetid-rodent-associated hepeviruses needs to be confirmed by large-scale studies of other rodent families.

Compliance with ethical standards

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Supplementary Figure 1 Predicted RNA secondary structures of the junction region between the ORF1 and ORF2 for the five cvHEV genomes 1641, 2645, 700, 746 and 819 and the kestrel-derived HEV genome used as reference. For the prediction of RNA secondary structure within the intergenic region between ORF1 and ORF2 the negative strand RNA region spanning nt positions 4981 to 5047 was analysed by the program mfold [1], with its default settings. The conserved AGA triplet motifs in the subgenome (SG) promotor region are boxed with solid lines. Additionally, the HEV bicistronic SG start site is indicated, as well as the start sites of the ORF2 and ORF3, by arrows. Dotted lines label the stop codon of the ORF1. The "energy" is given below of each prediction.
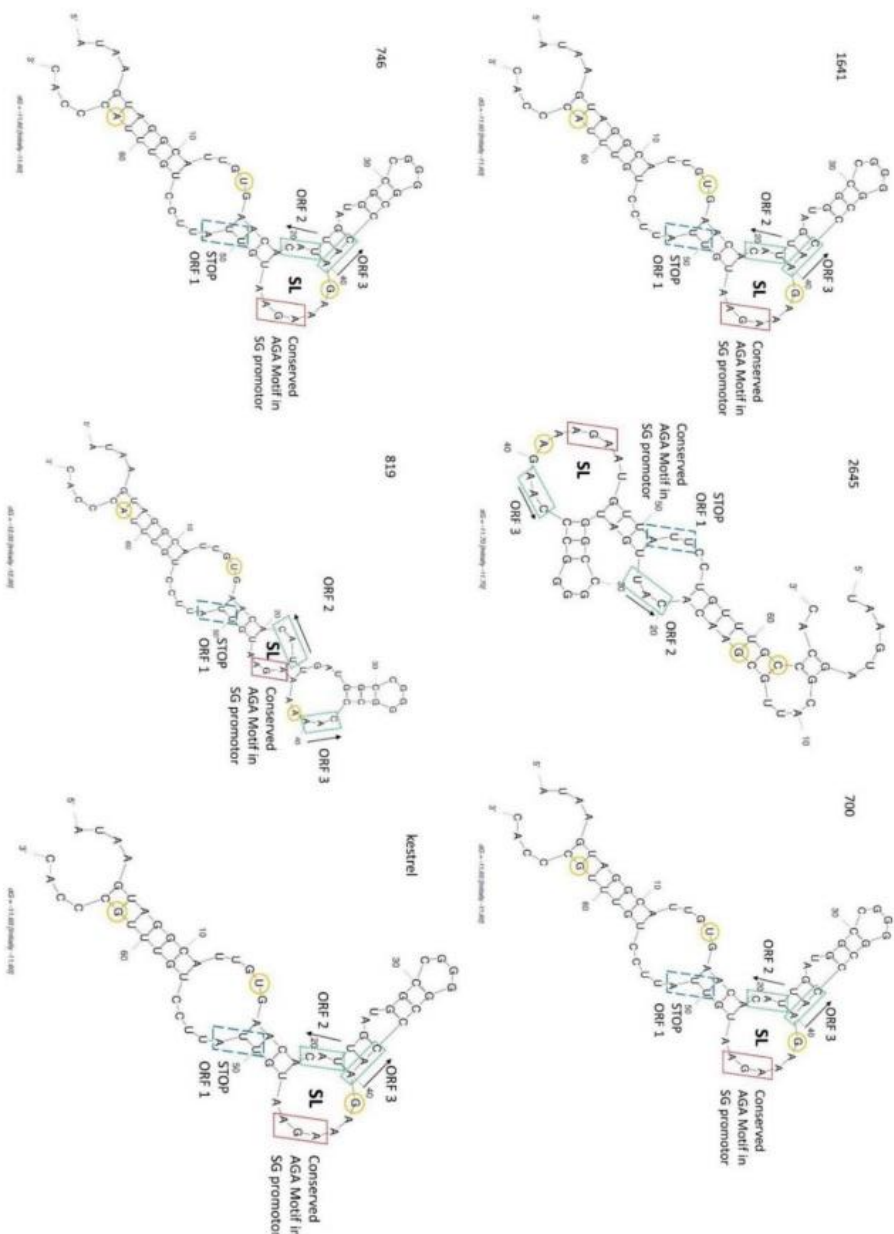
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Supplementary Table 1 Primers used for primer walking approach to determine complete genomes of novel common vole-associated hepatitis E virus.

Primer[a]	Sequence (5'->3')
104 as	AAAGCATTAGCCAACGAGGCGGTG
234 as	TGTGCACCCACTTCGAGGCA
360 as	TGGCGACATTTTTCAAATCCGGA
3 s	GGATCCTGTGGTATTGATCCC
816 as	CGATGTCYARCARRAARTGACARC
55 s	TCCGTGCYSCMGGGGGTTAC
1352 as	TCAAARTARCAVCCDGCDSWVACCCAATC
1123 s	CCACYGATGARTAYGCVCTCA
2353 as	GGCTGCTCAAAACTCGTTGG
2265 s	TGTGAYTGGCTBGTBAAYGCWAGYAAYCC
2955 as	GGKGGCATDGMHGGSGCYTCATCAAC
2913 s	CAGCATGCKGCCATHCGYMAWGT
3789 as	GCATABCKVCCRACAAGRGTRGTRAGKAC
3565 s	GATGAAGCGGACATGATCCCA
4354 as	TAATCATCCACACCGGCATCC
4239 s	TTTCCGCCGGGTTTTTGAGA
4979 as	CCGGGGCCCAAGAAAGAATG
4715 s	GARAAGAAYTGGGGSCCHGRT
5618 as	GTRGGVGCDGTRGADGAYTGVGGCCAAAAHGABAT
4929 s	CGGGCCTCYTTYACYGARATT
6335 as	ATDGGRATRCCCTGGKCTARYT
5912 s	GAGTATCGCAACCTCACCCC
6307 as	GAGACACCCAGGTCAAGGTC
6173 s	GGGCAGTTGTTTTATGGCCG
7016 as	TTTTTTTTTGTYKTGCGRAMBGCAGAA

[a] Positions according to kestrel-derived hepatitis E virus strain (accession number KU670940)

Supplementary Table 2 Pairwise sequence comparison of the screening fragment within the open reading frame 1 of the novel common vole-associated hepatitis E virus to related hepatitis E virus sequences on nucleotide and amino acid level. Sequences used for the calculation of the pairwise sequence identities were the unassigned hepatitis E virus (HEV) sequence from a kestrel [2] and the common vole-associated HEV strains, described here and those recently identified in Hungary [1]. Additionally, the screening fragment of one novel rodent-associated HEV-strain (HEV-RtMg/XJ2016 KY432902) from China was included, too [7]. Pairwise distance calculation was done with MEGA [5], above/below the diagonal the pairwise amino acid/nucleotide sequence identities are shown. The corresponding clades (see Figure 2 A) are condensed (I-III) and novel complete genomes (see Figure 2 B) are written in bold.

	Kestrel-derived HEV	HEV-RtMg	I (**700**, 705, 744, **746**, 748, 825)	II (818, **819**, 822, 823)	III (3456, 3499, **2645**, **1641**, UJH126, KFD154, LAK177, PEL184, PEL206, PEL207, PEL261, LAK302, PLP152, LAK216)
Kestrel-derived HEV	Id	89.1	94.6 – 94.6	96.7 – 96.7	94.6 – 98.9
HEV-RtMg	76.8	Id	90.2	88	87.0 – 90.2
I (**700**, 705, 744, **746**, 748, 825)	85.9 – 88.8	77.9 – 80.1	91.7 – 100/ 100	97.8 – 100	93.5 – 96.7
II (818, **819**, 822, 823)	85.9 – 86.2	75.7 – 76.1	90.2 – 93.8	99.6 – 100/ 100	92.4 – 96.7
III (3456, 3499, **2645**, **1641**, UJH126, KFD154, LAK177, PEL184, PEL206, PEL207, PEL261, LAK302, PLP152, LAK216)	83.7 – 92.4	73.6 – 78.6	83.0 – 90.6	81.9 – 87.3	86.6 – 99.6/ 98.9 – 100

Supplementary Table 3 Pairwise sequence comparison of the open reading frame 1 (ORF1), ORF2 and concatenated ORF1/ORF2 nucleotide (nt) and corresponding amino acid (aa) sequences of the five novel common vole-associated hepatitis E virus (HEV) strains towards representative HEV-strains of the genera *Piscihepevirus* and *Orthohepevirus*. Sequences used for the calculation of the pairwise sequence similarity were the five complete genomes of the novel common vole-associated HEV, described here, and the reference sequences for the genera *Piscihepevirus* and *Orthohepevirus* [3, 4], including the putative genotypes HEV-C3 and HEV-C4 [6], kestrel-derived HEV-strains [2] and recently identified rodent-associated HEV-strains (HEV-RtCb/HeB2014, HEV-RtMr/HLJ2016, HEV-RtMg/XJ2016, HEV-RtEi/SX2016 and HEV-RtCl/GZ2016) from China [7]. Pairwise distance calculation was done with MEGA [5] and the group specific mean identities (in percentage) are shown.

Genotype	Similarity of the cvHEV sequences to sequences of other genotypes in %							
	ORF1		ORF2		ORF3		ORF1/ORF2	
	nt	aa	nt	aa	nt	aa	nt	aa
PisciHEV	37.4	24.3	26.6	5.7	30.2	14.0	33.4	17.43
HEV-B	48.4	44.1	48.1	44.5	29.9	3.3	48.3	44.2
HEV-C1	58.4	59.2	65.5	70.4	30.8	11.7	61.0	63.4
HEV-C2	57.2	57.9	63.7	66.6	31.7	8.6	59.6	61.1
HEV-C3	57.0	58.5	64.5	69.2	37.8	13.3	59.8	62.5
HEV-C4	67.4	76.0	68.6	77.6	48.0	28.4	67.8	76.6
HEV-D	50.8	44.7	54.5	54.0	25.6	9.8	52.2	48.1
HEV-1	54.7	53.0	63.0	65.5	44.0	22.5	57.8	57.6
HEV-2	54.2	52.6	62.1	64.6	43.9	25.1	57.1	57.0
HEV-3	53.9	53.1	62.7	64.7	42.9	23.9	57.1	57.4
HEV-4	54.0	52.9	61.8	64.0	36.1	17.3	56.9	57.0
HEV-5	54.3	52.7	61.3	64.6	35.2	19.9	56.9	57.1
HEV-6	53.9	53.2	62.0	63.9	37.9	19.4	56.9	57.2
HEV-7	54.6	53.5	62.2	65.0	38.9	18.9	57.4	57.8
HEV-8	54.9	53.1	62.5	63.9	42.1	22.8	57.7	57.1
Kestrel	83.4	96.0	85.9	98.2	92.2	81.8	84.4	96.8
HEV-RtCb	54.7	54.6	64.1	68.2	67.7	85.6	58.2	59.6
HEV-RtMr	64.2	70.5	69.3	77.8	51.9	72.9	66.1	73.2
HEV-RtMg	75.8	91.6	79.5	94.8	38.8	56.6	77.1	92.8
HEV-RtEi	65.8	74.6	71.1	81.7	52.2	68.8	67.8	77.2
HEV-RtCl	67.3	76.5	71.3	80.8	52.6	71.3	68.8	78.1

Supplementary Table 4 Prediction of different conserved domains among the genome of the novel common vole-associated hepatitis E virus strains, in comparison to a kestrel-derived hepatitis E virus genome and a novel rodent-associated hepatitis E virus (HEV-RtMg/X/2016) from China.

Name	Accession	Description	Interval	1641	2645	700	746	819	Kestrel	HEV-RtMg	Number of samples per domain
Vmethyltransf	pfam01660	Viral methyltransferase	148-1095	+	+	+	+	+	+	+	7
PRK07003	PRK07003	DNA polymerase III subunits gamma and tau; Validated	1662-2234						+		1
PRK12323	PRK12323	DNA polymerase III subunits gamma and tau; Provisional	1627-2403				+				1
Macro_Afl521_BAL_like	cd02907	Macro domain, Afl521- and BAL-like family	2242-2559			+	+				2
YmdB	COG2110	O-acetyl-ADP-ribose deacetylase (regulator of RNase III)	2242-2559			+	+			+	3
Macro	pfam01661	Macro domain; This domain is an ADP-ribose binding module	2284-2541		+	+	+	+	+	+	6
Alpp	smart00506	Appr-1"-p processing enzyme;	2272-2544	+	+	+	+	+	+	+	7
Viral_helicase1	pfam01443	Viral (Superfamily 1) RNA helicase	2770-3411	+	+	+	+	+	+	+	7
RecD	COG0507	ATP-dependent exoDNase (exonuclease V)	3265-3408	+	+	+	+	+			5
RdRP_2	pfam00978	RNA dependent RNA polymerase	3814-4857	+	+	+	+	+	+	+	7
RNA_dep_RNAP	cd01699	RNA_dep_RNAP; RNA-dependent RNA polymerase (RdRP)	4102-4530	+	+	+	+	+	+	+	7
SP2	pfam03014	Structural protein 2	5103-6911	+	+	+	+	+	+	+	7
Number of domains per sample				8	8	10	11	7	8	8	

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2.2 Paper II

Ryll, R., Bernstein, S., Heuser, E., Schlegel, M., Dremsek, P., Zumpe, M., Wolf, S., Pepin, M., Bajomi, D., Muller, G., Heiberg, A.C., Spahr, C., Lang, J., Groschup, M.H., Ansorge, H., Freise, J., Guenther, S., Baert, K., Ruiz-Fons, F., Pikula, J., Knap, N., Tsakmakidis, I., Dovas, C., Zanet, S., Imholt, C., Heckel, G., Johne, R., Ulrich, R.G., 2017. Detection of rat hepatitis E virus in wild Norway rats (*Rattus norvegicus*) and Black rats (*Rattus rattus*) from 11 European countries. Veterinary microbiology 208, 58-68.

Detection of rat hepatitis E virus in wild Norway rats (*Rattus norvegicus*) and Black rats (*Rattus rattus*) from 11 European countries

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ABSTRACT

Rat hepatitis E virus (HEV) is genetically only distantly related to hepeviruses found in other mammalian reservoirs and in humans. It was initially detected in Norway rats (*Rattus norvegicus*) from Germany, and subsequently in rats from Vietnam, the USA, Indonesia, China, Denmark and France.

Here, we report on a molecular survey of Norway rats and Black rats (*Rattus rattus*) from 12 European countries for ratHEV and human pathogenic hepeviruses. RatHEV-specific real-time and conventional RT-PCR investigations revealed the presence of ratHEV in 63 of 508 (12.4%) rats at the majority of sites in 11 of 12 countries. In contrast, a real-time RT-PCR specific for human pathogenic HEV genotypes 1–4 and a nested broad-spectrum (NBS) RT-PCR with subsequent sequence determination did not detect any infections with these

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genotypes. Only in a single Norway rat from Belgium a rabbit HEV-like genotype 3 sequence was detected. Phylogenetic analysis indicated a clustering of all other novel Norway and Black rat-derived sequences with ratHEV sequences from Europe, the USA and a Black rat-derived sequence from Indonesia within the proposed ratHEV genotype 1. No difference in infection status was detected related to age, sex, rat species or density of human settlements and zoological gardens.

In conclusion, our investigation shows a broad geographical distribution of ratHEV in Norway and Black rats from Europe and its presence in all settlement types investigated.

1. Introduction

The family *Hepeviridae* comprises an increasing number of viruses in mammals, birds and fish (Johne et al., 2014; Pérez-Gracia et al., 2015). Initially, hepatitis E virus (HEV) was the only member of this virus family, which was divided into four genotypes. The genotypes 1 and 2 are supposed to exclusively infect humans, whereas genotypes 3 and 4 are zoonotic with wild boar, domestic pig and deer representing animal reservoirs (Meng, 2013). In chicken, additional divergent genotypes were discovered and designated as avian HEV, which can be associated with the diseases Big Liver and Spleen Disease and Hepatitis-Spleno-megaly Syndrome (Handlinger and Williams 1988; Ritchie and Riddell 1991; Gerber et al., 2015). The International Committee on Taxonomy of Viruses (ICTV) currently classifies the human pathogenic HEV genotypes 1–4 into species *Orthohepevirus A*, avian HEV into *Orthohepevirus B*, bat HEV into *Orthohepevirus D* and the carnivore and rat HEV into *Orthohepevirus C* (Smith et al., 2014; http://ictvonline.org/

virusTaxonomy.asp, Accessed 07 April 2017).

The hepevirus genome is a positive stranded RNA of approximately 6.7–7.3 kilobases (kb) (Meng et al., 2012). The genome contains the typical sequence elements of an eukaryotic mRNA with a cap structure at its 5′-end and a polyadenylation at its 3′-end (Tam et al., 1991). For all hepeviruses, three major open reading frames (ORF) were identified with almost the same organization, but differences in the junction or overlapping region of ORF1 and ORF2/ORF3 (Johne et al., 2014). The ORF1 of 4.6 to 5.2 kb is located at the 5′-end of the genome and codes for a polyprotein comprising several nonstructural proteins including regions with similarity to methyltransferases, papain-like proteases, helicases and RNA-dependent RNA polymerases (Koonin et al., 1992). The capsid protein of 600–675 amino acid residues is encoded by ORF2 and contains three domains with the carboxyterminal domain being exposed on the surface of the virion (Yamashita et al., 2009). The overlapping ORF3 codes for a small phosphoprotein of strongly varying length in avian, mammalian and fish hepeviruses (Zafrullah et al.,
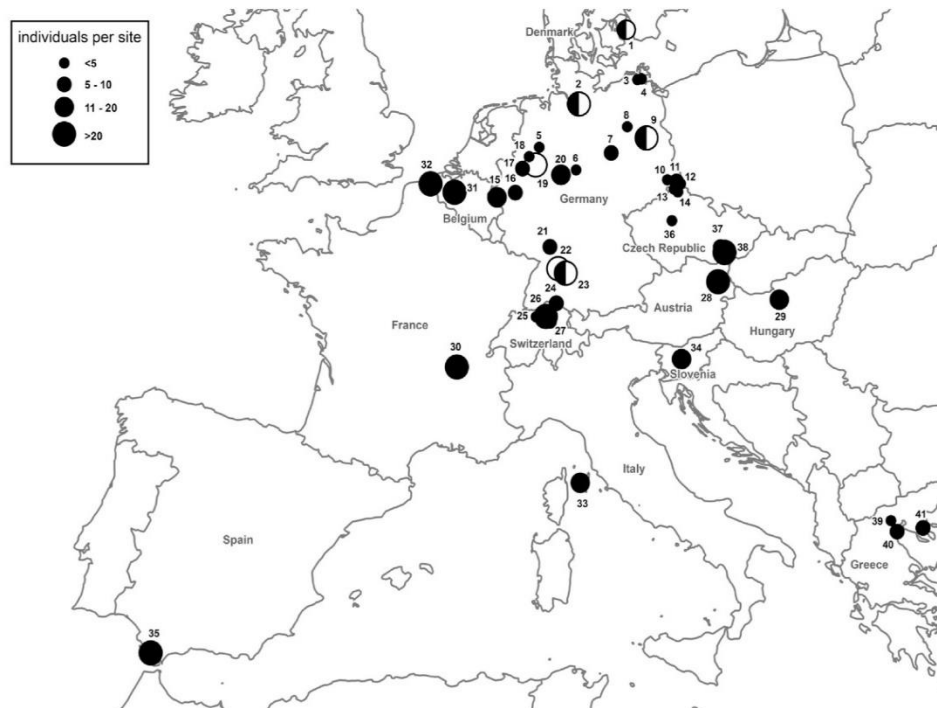
Fig. 1. Geographical map representing the rat collection sites in Denmark (1, Copenhagen), Germany (2, Hamburg; 3, Elmenhorst; 4, Stahlbrode; 5, Osnabrück; 6, Wolbrechtshausen; 7, Magdeburg; 8, Kampehl; 9, Berlin; 10, Neschwitz; 11, Königshain; 12, Görlitz; 13, Niederoderwitz; 14, Zittau; 15, Aachen; 16, Köln; 17, Oer-Erkenschwick; 18, Münster; 19, Ahlen; 20, Warburg; 21, Heidelberg; 22, Stuttgart; 23, Esslingen; 24, Möggingen), Switzerland (25, Gränichen, 26, Dübendorf, 27, Zurich), Austria (28, Vienna), Hungary (29, Budapest), France (30, five sites close to Lyon), Belgium (31 Dender, 32 Ijzer), Italy (33 Pianosa Island), Slovenia (34 close to Ljubljana), Spain (35 Cadiz), Czech Republic (36 Prague, 37 Brno, 38 Northern Moravia), Greece (39 Thessaloniki, 40 Kilkis, 41 Chalkidiki). All or some of the rats from sites 1, 2, 9, 19, 22 and 23 were investigated for ratHEV previously (Johne et al., 2010a,b, 2012; Wolf et al., 2013; indicated by empty or half-filled circles, respectively) and were included here for analysis of demographic, reservoir and human settlement type association of ratHEV infections (see Heuser et al., 2017).

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1997; Holla et al., 2013; Johne et al., 2014). This protein is essential for virus egress and found to be associated with lipid membranes (Okamoto, 2013). Interestingly, ratHEV as well as ferretHEV contain an additional putative open reading frame (ORF4), overlapping ORF1 at its 5′-end, of still unknown function (Johne et al., 2010a; Raj et al., 2012).

Using a broad-spectrum RT-PCR assay, a novel, only distantly-related hepevirus was identified in 2010 in Norway rats (*Rattus norvegicus)* from Hamburg, Germany (Johne et al., 2010a, 2010b). This initial finding was confirmed by detection of closely related sequences in Norway rats from other cities in Germany (Johne et al., 2012). Detection of related sequences in rats from the USA, Vietnam, Denmark, France, China and Indonesia suggests a host specificity of ratHEV for rats of the genus *Rattus* and indicated its broad geographical distribution (Li et al., 2013b,d; Mulyanto et al., 2013, 2014; Purcell et al., 2011; Widen et al., 2014; Wolf et al., 2013). The host specificity of this virus was also demonstrated by infection experiments using laboratory rats and other mammals (Cossaboom et al., 2012; Li et al., 2013c). However, recent studies in China suggested a broader host range of the virus or frequent spillover infections of bandicoot rats and even shrews (Guan et al., 2013; Li et al., 2013d). The genotypes G1, G2 and G3 of ratHEV were previously defined on the basis of a complete genome sequence comparison; a further comparison of 31 ORF 2-derived sequences of 281-bp length revealed two additional sequences of a non-designated clade (ND), which clustered with G1 (Mulyanto et al., 2014). All G1 ratHEV sequences in previous studies originated from *R. norvegicus* or *Black rats (Rattus rattus)*, whereas ratHEV sequences of G3 originated exclusively from *R. rattus*. In contrast, genotype G2 was detected in *R. rattus, Rattus tanezumi, Rattus rattoides losea* and the Asian house shrew *Suncus murinus* (Li et al., 2013b, 2013d; Mulyanto et al., 2013).

The zoonotic potential of ratHEV is currently controversially discussed. Serological studies in forestry workers from Germany showed a few seropositive individuals (Dremsek et al., 2012). In addition, febrile patients from China showed a stronger reactivity with ratHEV antigen than with genotype 1 and 3 antigens (Shimizu et al., 2016). Furthermore, ratHEV was shown to replicate in a human-derived cell line (Jirintai et al., 2014; Li et al., 2015). In contrast, experimental infection of monkeys and domestic pigs with ratHEV failed (Cossaboom et al., 2012; Purcell et al., 2011). Reproducible experimental infections of nude rats and Wistar rats with ratHEV (Li et al., 2013c; Purcell et al., 2011) and the availability of a recently developed reverse genetics system for ratHEV (Li et al., 2015) led to the suggestion to use ratHEV-infected laboratory rats as an infection model for hepeviruses. On the other hand, Norway rats were found to be infected with human pathogenic genotype 3 associated strains, suggesting a potential role for zoonotic transmission (Lack et al., 2012; Kanai et al., 2012).

Here, we describe a molecular survey of Norway and Black rats from 12 European countries for ratHEV and human pathogenic HEV genotypes, and evaluated influences of sex, age, rat species and human settlement type on ratHEV prevalence.

2. Material and methods

2.1. Rat collection, dissection and sample collection

The collection of Norway rats in Copenhagen and Berlin has been already described previously (Sachsenroder et al., 2014; Wolf et al., 2013). Additional Norway rats were collected in Germany, Denmark, Austria, Switzerland, Czech Republic, Belgium, France, Slovenia and Greece; Black rats were collected in Italy, Slovenia, Greece and Spain (Fig. 1).

The dissection and collection of tissue samples followed standard protocols. For the evaluation of the influence of sex, age, reservoir species and human settlement type on ratHEV prevalence, previously published results for rats from Hamburg, Berlin, Stuttgart, Esslingen and Copenhagen (Johne et al., 2012, 2010a, 2010b; Wolf et al., 2013) were also included.

2.2. RNA isolation, real-time and conventional RT-PCR and sequencing

After homogenizing rat liver tissue using a TissueLyser (Qiagen, Hilden, Germany), RNA was extracted with the RNeasy Mini Kit (Qiagen). A ratHEV-specific real-time RT-PCR (Johne et al., 2012, RTD, see Fig. 2) and a real-time RT-PCR specific for HEV genotypes 1–4 (Jothikumar et al., 2006) were performed as previously published. The QuantiTect Probe RT-PCR Kit (Qiagen) was used in a 7500 Real Time PCR System (Applied Biosystems Life Technologies, Darmstadt, Germany) and the data were evaluated using 7500 Software v2.0.1 (Applied Biosystems Life Technologies, Darmstadt, Germany).

A one-step RT-PCR (designated SW-RT-PCR; see Fig. 2) was then performed using a SuperScriptIII One-Step RT-PCR with PlatinumTaq Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) in a C1000 Thermal Cycler (Bio-Rad Laboratories, Munich, Germany). Reverse transcription was conducted at 42 °C for 50 min, followed by a denaturation step at 94 °C for 2 min. A total of 45 PCR cycles each consisting of 30 s at 94 °C, 30 s at the primer-specific annealing temperature (Table 1), 1 min at 68 °C and a final incubation at 68 °C for 10 min were performed.

Additionally, a slightly modified nested broad-spectrum (NBS) RT-PCR was performed to test the samples for all possible HEV strains, including ratHEV and human pathogenic genotypes as described (Johne et al., 2010b; see Fig. 2). A first RT-PCR was performed using a One-Step RT-PCR kit (Qiagen) with primers HEV-cs and HEV-cas in a 2720 thermal cycler (Applied Biosystems). The thermal profile comprised 42 °C for 60 min and 95 °C for 15 min, followed by 40 cycles of 94 °C for
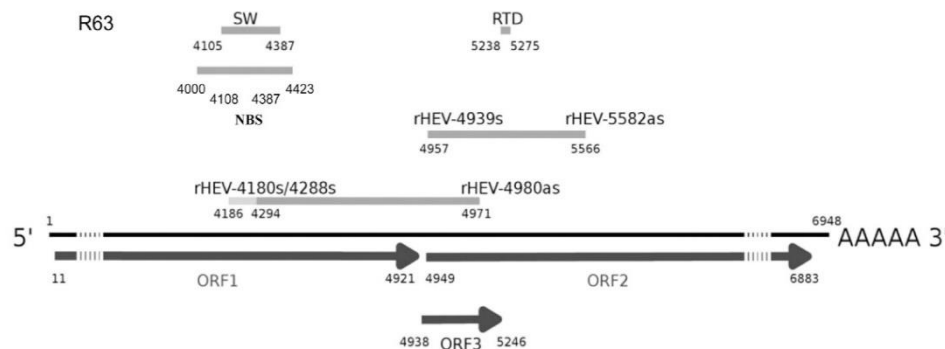

Fig. 2. Genome organization of ratHEV, prototype strain R63 (accession number GU345042), and location of primer binding sites for real-time (RTD) and conventional screening SW-/NBS-RT-PCR and primer-walking RT-PCRs as well as the corresponding amplification products.

Table 1
Oligonucleotides used in the RT-PCR and corresponding annealing temperatures.

RT-PCR assay	Primer pair	Primer sequence	Annealing temperature	Reference
SW-RT-PCR	rHEV-SW-for and	5′-GCNCTGTTYGGCCCNTGGTT	56 °C	Wolf et al. (2013)
	rHEV-SW-rev	5′-GGYTCACCRGARTGYTTYTTCCA		
NBS-RT-PCR	HEV-cs	5′-TCGCGCATCACMTTYTTCCARAA	50 °C	Johne et al. (2010b), modified
	HEV-cas	5′-GCCATGTTCCAGACDGTRTTCCA		
	HEV-csn	5′-TGTGCTCTGTTTGGCCCNTGGTTYCG	50 °C	
	HEV-casn	5′-CCAGGCTCACCRGARGYTTCTTCCA		
Primer-walking RT-PCR I	rHEV-4180s/	5′-AGGAGAARTTGGAGGCCGC	58 °C	This paper
	rHEV-4288 s and	5′-GAGTGCYTRYTGATGAAGGAGGC		
	rHEV-4980as	5′-CAGCAGCGGCACGAACAGC		
Primer-walking RT-PCR II	rHEV-4939s and	5′-GTAGCATGTGTGCGGAATGCGTGT	58 °C	This paper
	rHEV-5230as/	5′-GTCATTGGCGACTGCCCGGCATC		
	rHEV-5582as	5′-GTGATGGAATTCATRTCCACCGACGT		

NBS, nested broad-spectrum.

30 s, 50 °C for 30 s and 74 °C for 45 s, with a final incubation at 74 °C for 5 min. An aliquot of the RT-PCR product (5 μl) was used in a nested PCR with a GoTaq kit (Promega) and the primers HEV-csn and HEV-casn. The thermal profile consisted of 95 °C for 5 min and 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, with a final incubation at 72 °C for 5 min.

To generate a longer sequence stretch, overlapping the SW-/NBS-RT-PCR products and including the 3′-end of ORF 1, the 5′-region of ORF 2 and a partial or complete ORF 3, selected samples were analyzed by a primer walking-based attempt using two different primer pairs and following the protocols of the SW-RT-PCR (see Fig. 2 and Table 1; Primer-walking RT-PCR-I/II).

RT-PCR products were purified using a MiniElute PCR Purification Kit (Qiagen) or a NucleoSpin Gel and PCR Clean-up Kit (Machery-Nagel, Düren, Germany), separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

For sequencing, the purified RT-PCR product was amplified by PCR using the same primers and the following temperature profile: 96 °C for 1 min, followed by 30 cycles of 96 °C for 15 s, 50 °C for 15 s and 60 °C for 90 s. Amplicons were purified using a Sigma Spin Post-Reaction Clean-up Column Kit (Sigma-Aldrich, Hamburg, Germany) and sequenced on an ABI 3100 Avant DNA-Sequencer (Applied-Biosystems, Darmstadt, Germany). Sequences were assembled and aligned using BioEdit 7.2.0 (Hall, 1999) and MEGA 7 (Kumar et al., 2016), respectively. The novel HEV sequences were deposited at GenBank (for accession numbers see Fig. 3 B-D).

2.3. Phylogenetic analysis

The General Time Reversible + discrete Gamma distribution (GTR + G) model was the best suited substitution model determined by MEGA 7 for both regions spanning nucleotides (nt) 4105–4387 (numbering based on strain R63, acc. no. GU345042) and nt 4105–5226. The phylogenetic analyses were performed by Bayesian algorithms via MrBayes v.3.2.2 and CIPRES online portal (Ronquist et al., 2012) and by Maximum likelihood algorithm performed via MEGA7 (Kumar et al., 2016).

2.4. Evaluation of demographic, rat species and human settlement type influence

The statistical evaluation of demographic, rat species and human settlement type influences on individual ratHEV infection status was performed similarly to the previously described methodology for other infectious agents on a sub-sample (Heuser et al., 2017). Briefly, generalized linear modelling (GLM) with a binomial error distribution was applied using individual infection status as the response variable, with sex and age classes (< 200 g (juvenile) and > 200 g (adult)) (Webster et al., 1995)) as demographic predictors as well as the association of ratHEV with a particular *Rattus* species (*R. norvegicus* vs. *R. rattus*) and human settlement type, based on human population density (urban (> 1500 inhabitants/km²), small town (300–1500 inhabitants/km²), rural (< 300 inhabitants/km²)) (database: Geostat initiative, 2012). Rats collected in zoological gardens were put in a separate category.

Table 2
Results of real-time (rt) RT-PCR screening and conventional/nested RT-PCR (SW-RT-PCR) analysis of rats.

Country	Site number[1]	Total number of rats sampled	ratHEV rt RT-PCR[2]		GT 1–4 rt RT-PCR[2]		SW-RT-PCR		NBS-RT-PCR		ratHEV (total)[3]	
Germany	2–5, 7–24	156	5/145	3.4%	0/145	0.0%	17/154	11.0%	0/2	0.0%	17/156	10.8%
Hungary	29	18	1/18	5.5%	0/18	0.0%	2/18	11.1%	n.d.	–	2/18	11.1%
Denmark	1	11[4]	2/11	18.1%	0/11	0.0%	3/11[4]	27.2%[4]	n.d.	–	3/11[4]	27.2%[4]
Austria	28	43	0/43	0.0%	0/43	0.0%	7/43	16.2%	n.d.	–	7/43	16.2%
Switzerland	25–27[5]	29	4/29	13.7%	0/29	0.0%	4/29	13.7%	n.d.	–	4/29	13.7%
France	30	28	3/28	10.7%	0/28	0.0%	5/28	17.8%	n.d.	–	5/28	17.8%
Italy	33	17	n.d.	–	0/17	0.0%	0/17	0.0%	1/17	5.8%	1/17	5.8%
Spain	35	50	n.d.	–	0/50	0.0%	2/50	4.0%	2/50	4.0%	2/50	4.0%
Greece	39–41	20	n.d.	–	0/18	0.0%	0/20	0.0%	2/18	11.1%	2/20	10.0%
Slovenia	34	18	n.d.	–	0/18	0.0%	0/18	0.0%	0/18	0.0%	0/18	0.0%
Belgium	31,32	60	n.d.	–	0/60	0.0%	6/60	10.0%	5/60	8.3%	8/60	13.3%
Czech Republic	36–38	58	n.d.	–	0/58	0.0%	9/58	15.5%	10/58	17.2%	12/58	20.6%
Total		508[4]	15/274	5.4%	0/494	0.0%	55/506[4]	10.8%[4]	20/225	8.8%	63/508[4]	12.4%[4]

NBS, nested broad-spectrum; n.d., not determined.

[1] numbers according to trapping sites, for detailed information see Fig. 1.
[2] All ct values of positive samples in ratHEV rt RT-PCR were lower than 35; all ct values of GT1-GT4 rt RT-PCR were higher than 35 and set to be negative.
[3] samples positive in real-time RT-PCR and/or SW/nested-RT-PCR.
[4] includes a published ratHEV positive specimen (Wolf et al., 2013).
[5] includes 6 samples from the vicinity of Zurich.

Model selection was performed using the *drop1* function. Goodness of fit of all performed regression models was assessed using the Le Cessie-van Houwelingen test statistic implemented in the *rms*-package. All analyses were performed in R (R Core Team, 2015).

3. Results

3.1. Collection of rats and initial real-time RT-PCR screening of rats

From 2005 to 2016 a total of 508 rats were collected in 12 European countries (Fig. 1). This sample contained 420 Norway rats from trapping sites in Germany (23 sites, 156 rats), Denmark (1 site, 11 rats), Austria (1 site, 43 rats), Switzerland (3 sites, 29 rats), Czech Republic (3 sites, 58 rats), Belgium (2 sites, 60 rats), France (1 site, 28 rats), Slovenia (1 site, 1 animal) and Greece (3 sites, 16 rats) and 88 Black rats from trapping sites in Italy (1 site, 17 rats), Slovenia (1 site, 17 rats), Greece (2 sites, 4 rats) and Spain (1 site, 50 rats). Initially, liver-derived RNA preparations of a Norway rat sample subset were tested in parallel by real-time RT-PCR assays either targeting ratHEV or HEV genotypes 1 to 4. The ratHEV-specific real-time RT-PCR (RTD) resulted in the detection of 5 out of 145 (3.4%) samples from Germany (Table 2). Norway rat samples from Hungary, Denmark, Switzerland and France were also positive for ratHEV-RNA by ratHEV-specific real-time RT-PCR with a detection range of 5.5% (1/18)–18.1% (2/11; see Table 2). The Ct values of positive samples ranged between 20 and 34. In the real-time RT-PCR targeting the human pathogenic genotypes 1–4 none of the Norway rat samples showed a Ct value < 35, used as cut-off (Table 2).

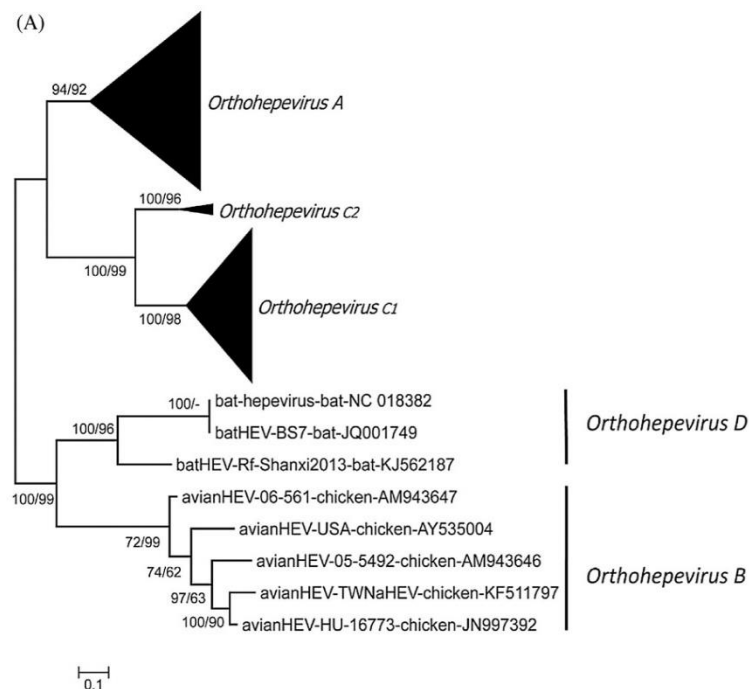### 3.2. Conventional SW-RT-PCR and NBS-RT-PCR analysis

A conventional RT-PCR approach using ORF1-specific SW-RT-PCR (nt positions 4105–4387, prototype strain R63, accession number GU345042, see Fig. 2) and NBS RT-PCR (nt positions 4000–4423, see Fig. 2) resulted in the detection of HEV-specific RNA in 17 of 156

(10.8%) samples from Germany (Table 2). The prevalence for samples from the sites in the other ten countries reached from 4% (2/50) to 27.2% (3/11; Table 2). The prevalences in Norway rats and Black rats were 10%-27.2% (2/20 and 3/11) and 4%-5.8% (2/50 and 1/17, respectively. None of the single Norway rat and 17 Black rats from Slovenia was HEV-RNA positive (Table 2).

Using a primer-walking based approach for thirteen samples from nine sites in Germany, France, Spain, Belgium, Austria and Denmark, a 1122/1125-base pair (bp) long region including parts of ORF1, ORF2 and partial or entire ORF3 (see Fig. 2) was RT-PCR amplified and sequenced. The different lengths of the sequences B1 and B4 from France were caused by a triplet indel, i.e., insertion/deletion of three nucleotides (data not shown).

3.3. Sequence comparison and phylogenetic analysis

Phylogenetic analysis of the 280 nt fusion-product of the SW-/NBS-RT-PCR assays showed that almost all novel sequences, independently whether from Norway or Black rats, clustered together with ratHEV sequences, species *Orthohepevirus C*, genotype HEV-C1, well separated from sequences of species *Orthohepevirus C*, genotype HEV-C2 (Fig. 3 A and B). In one Norway rat sample from Belgium (KS16/825) a sequence with 88.8% sequence similarity to genotype 3 HEV sequences was found (see below); in no other sample human pathogenic genotype-related sequences were found. This HEV genotype 3 − like sequence from the single Norway rat sample from Belgium clustered in the phylogenetic tree with three rabbit HEV strains from China and a human rabbit HEV sequence from France within species *Orthohepevirus A* (Fig. 3C); attempts to generate a longer sequence failed. The phylogenetic analysis of the concatenated 1122/1125 nt product of the coding sequences revealed clustering of all novel sequences within the ratHEV genotype G1 defined by Mulyanto et al. (2014), in sister clade relationship with ratHEV genotypes G2 and G3 (Fig. 3D). Genotype G1 contains the prototype sequence R63 from a Norway rat from Hamburg,

(A)

Fig. 3. Phylogenetic reconstructions of all species within the genus *Orthohepevirus* based on a 280 nt section of ORF1 (A-C) or 1122 nt concatenated sequence from ORF1 and ORF2/ORF3 (D). (A) shows the overall topology for the major groups in *Orthohepevirus*; the detailed phylogenetic relationships for the condensed groups (black triangles) are displayed in (B) and (C). Consensus phylogenetic trees were based on Bayesian analyses with 8,000,000 (A) or 6,000,000 (D) generations and a burn-in of 25%. Maximum-Likelihood analyses were run with 1000 bootstraps and 50% cut-off. Posterior probability/bootstrap values of > 50 are given at the supported nodes. Novel sequences are given in bold and labeled by a star. Identical sequences were omitted from the analysis and only different sequence types are presented (Berlin KS11/573 = KS11/576,/578,/580,/587, Esslingen Mu10/1564 = Mu10/1567,/1568,/1571, Warburg Mu10/697 = Mu10/698, Zurich KS12/1361 = KS12/1363, Czech Republic KS14/73 = KS14/75,/76,/99, and KS14/70 = KS14/80,/98).

Reference sequences for all species within genus *Orthohepevirus* and for genotypes within species *Orthohepevirus A* were taken from Smith et al., 2014, 2016, respectively.

(B) The two sequences of ratHEV clade ND (not designated) clustered separately from the *Orthohepevirus C1* genotypes G1, G2 and G3, while partial ORF2 data joined them with G1 (Mulyanto et al., 2014).

Rnor, *Rattus norvegicus*; Rrat, *Rattus rattus*; Bind, *Bandicota indica*; Smur, *Suncus murinus*; Rratto, *Rattus rattoides losea*; Rtan, *Rattus tanezumi*.
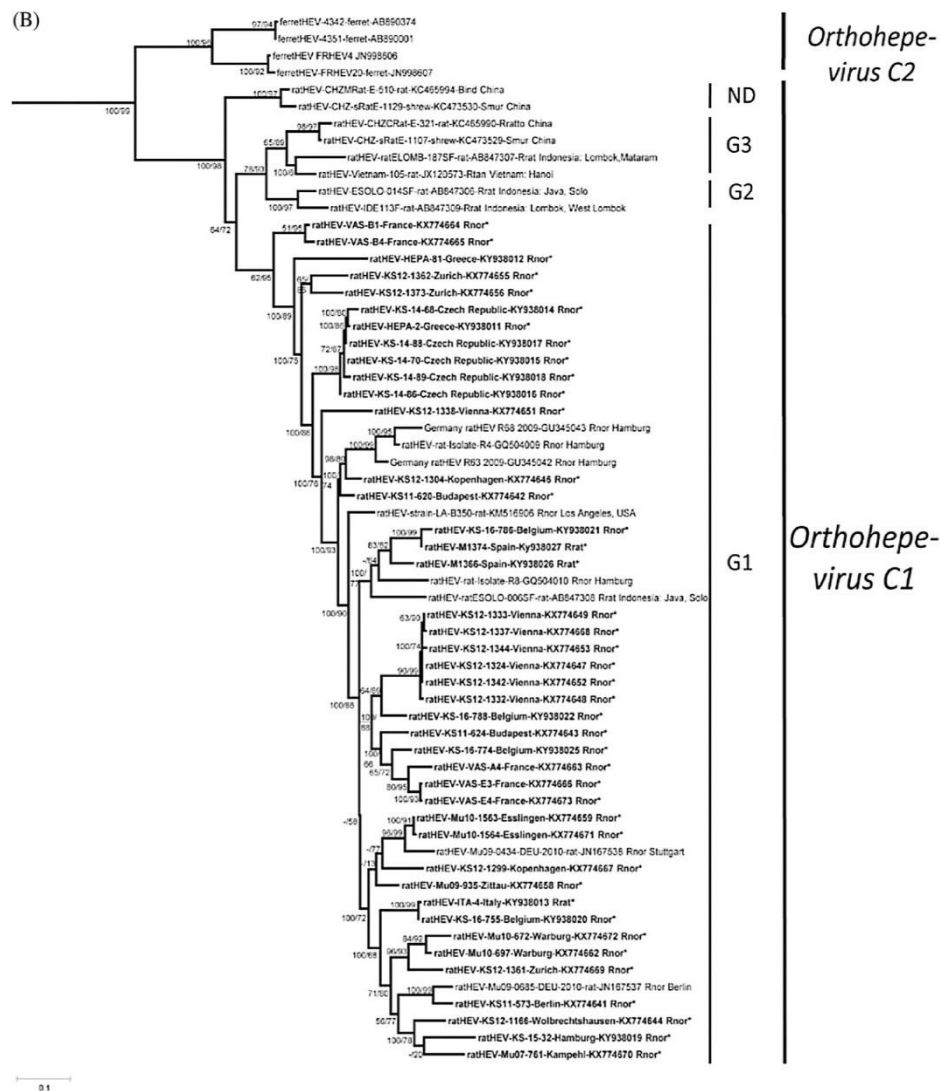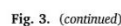
2. Publications

Fig. 3. *(continued)*

Norway rat-derived sequences from different European countries and the USA, Black rat-derived sequences from Spain and Italy and one sequence originating from a Black rat collected in Solo, Indonesia (Fig. 3B and D).

A novel sequence from rats in Berlin, detected in five animals, clustered with a previously determined sequence from Berlin and two novel sequences from rats in Esslingen, with one found in four animals, clustered with a sequence detected previously in Stuttgart, a site close to Esslingen (Johne et al., 2012; see Fig. 3B, and legend to Fig. 3). Similarly, two sequences from Warburg formed a well-separated subclade and all sequences from Czech Republic were highly related (Fig. 3B). Most novel ratHEV sequences from Vienna formed a well-supported cluster, but one sequence (KS12/1338) was highly divergent. Both sequences from Spain are closely related, independently if the 280 nt or 1222 nt products were analyzed (Fig. 3B and D). Interestingly, ratHEV

sequences from three trapping sites close to Lyon (B and E/A) formed two well-separated subclades and sequences from Zurich belonged also to two subclades (Fig. 3B). Sequences from Norway rats from Belgium were found at highly divergent positions within the tree (Fig. 3B).

Comparison of ORF1-derived sequences from the fusion product of SW-/NBS-RT-PCR from the same site resulted in an intra-cluster sequence similarity of 79.6% to 100% for the nucleotide and 86.8% to 100% for the corresponding amino acid sequences (Table 3). When analyzing the nucleotide sequence similarity within partial ORF1 or the overlapping ORF1/ORF2/ORF3 regions between different sites, the values reached similar levels of 81.0% to 96.1% and 87.2% to 91.5%, respectively (Supplementary Table and Table 4). The corresponding aa sequence similarities of ORF1-encoded protein and concatenated ORF1- and ORF2-encoded proteins ranged between 93.4% and 100% and 95.9% and 98.6%, respectively (Supplementary Table and Table 4).

(C)

Fig. 3. (continued)

3.4. Association of ratHEV infections with age, sex, rat species and human settlement density

For a total of 668 rats, including those of this study (n = 508) and those investigated previously (n = 160; Johne et al., 2012, 2010a; Wolf et al., 2013), no association with age, sex or the *Rattus* species and the individual ratHEV infection status could be detected (Table 5). In addition, ratHEV was detected in rats from all four settlement types investigated. Human population density did not seem to have an effect on ratHEV occurrence, as prevalences in small towns and rural sites did not differ significantly from high density urban areas. The prevalence in zoological gardens was lower compared to urban areas, though not formally significant (Table 5). For all models goodness of fit analysis did not provide any evidence of a lack of fit.

4. Discussion

In this study, we investigated Norway and Black rat samples from 12 European countries for the presence of ratHEV and other hepeviruses using ratHEV-specific real-time RT-PCR (Johne et al., 2010a) and human HEV genotype 1–4-specific real-time RT-PCR (Jothikumar et al., 2006) as well as conventional RT-PCR assays (SW-RT-PCR (Wolf et al., 2013) and NBS RT-PCR (Johne et al., 2010b)). Using these four methods, almost exclusively ratHEV was detected in Norway and Black
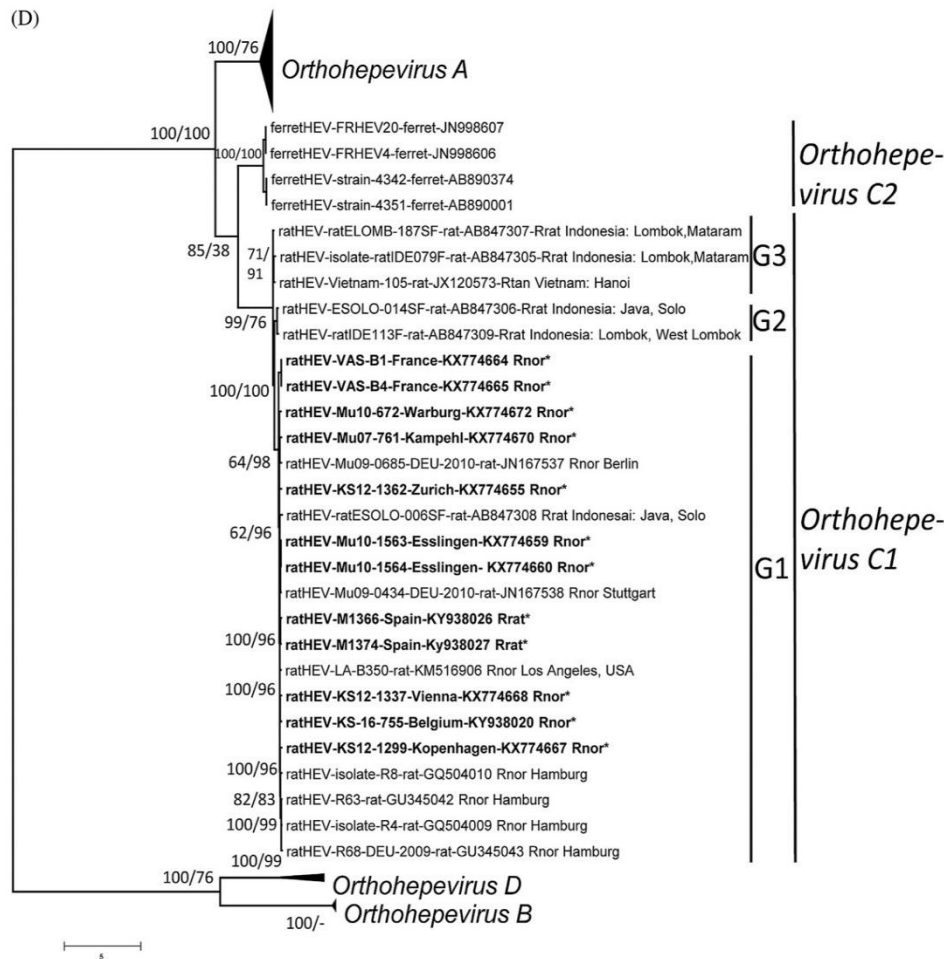
(D)

Fig. 3. (continued)

rats from 11 of 12 countries. This finding is in line with the previously demonstrated inability in experimentally infecting Norway rats with human pathogenic genotypes (Li et al., 2013a, 2013c; Purcell et al., 2011) and results from earlier field studies in Norway rats (Johne et al., 2012, 2010a). Similar to previous studies reporting the human pathogenic HEV genotype 3 in Norway rats (Lack et al., 2012; Kanai et al., 2012), in one Norway rat from Belgium a short rabbit HEV-like genotype 3 sequence was detected. This might be explained by a spillover infection of this strain from a rabbit reservoir. Rabbits and rats may share their habitats in this region of Belgium, either in wildlife habitats or when wild (pest) rats search for food close to private rabbit husbandry.

This study demonstrates the occurrence of ratHEV not only in Norway rats, as previously reported for Germany, France and Denmark, but for the first time in Europe also in Black rats, namely from Italy and Spain. This finding is in line with studies in Asia, where ratHEV has been demonstrated in different *Rattus* species and in Greater bandicoot rats (*Bandicota indica*) (Guan et al., 2013; Li et al., 2013d).

In addition, in our study ratHEV was not only detected in rats from urban areas, but also in rats from small towns and rural areas. The detection of ratHEV in rural areas complements our previous finding of

a local absence of ratHEV in a rural area close to Ahlen (Johne et al., 2012), which may suggest site-specific differences and a heterogeneous distribution of ratHEV not primarily driven by human settlement. In addition, ratHEV was identified in pest rats from zoological gardens raising questions on the potential transmission of this virus to zoo animals. In fact, serological investigations have detected HEV-specific antibodies in captive macaques and HEV-RNA in different mammalian and avian species in a wildlife rescue center in China (Korzaia et al., 2007; Zhang et al., 2008). The recently developed in-house ELISA technology based on ratHEV- and HEV genotype 3-derived recombinant capsid protein derivatives (Dremsek et al., 2012; Johne et al., 2012) may be used in the future for differentiation of antibodies raised against these viruses in zoo animals.

The phylogenetic analysis of the novel ratHEV sequences showed for almost all a high similarity to ratHEV genotype G1 defined recently (Mulyanto et al., 2014), independently whether the sequences originated from Norway or Black rats. In line with a previous investigation (Purdy and Sue, 2017), the resolution of the phylogenetic analysis using the short-sized ORF1 region was lower than the resolution for the larger segment of ORF1/ORF2/ORF3. The observed phylogenetic clustering of many sequences from the same or neighbouring sites may indicate the

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Table 3
Intracluster nucleotide (nt) and amino acid (aa) sequence identity for the region spanning nt positions 4108–4387 (SW-/NBS-RT-PCR product) or aa positions 1369–1462, based on strain R63, acc.no. GU345042.

Site (number)	Number of Sequences[a]	Nt identity (in %)	Aa identity (in %)
Berlin, B, DE (9)	5	100	100
Warburg, NW, DE (20)	3	95.3–100	100
Esslingen, BW, DE (23)	5	99.6–100	100
Copenhagen, DK (1)	2	88.2	98.7
Vienna, AUT (28)	7	84.2–100	94.7–100
Zurich, CH (27)	4	81.0–100	96.1–100
Budapest, HU (29)	2	89.6	97.4
Lyon, FR (30)	5	84.2–99.6	96.1–100
Cadiz, ES (35)	2	91.4	100
Northern Moravia, CZ (38)	5	90–99.6	86.8–100
Dender, BE (31)	5	79.6–89.2	92.3–98.9

DE, Germany; DK, Denmark; AUT, Austria; CH, Switzerland; HU, Hungary; FR, France; ES, Spain; CZ, Czech Republic; BE, Belgium.

[a] For the trapping sites Pianosa island (Italy), Thessaloniki (Greece), Chalkidiki (Greece) and three trapping sites in Germany (Kampehl, Wolbrechtshausen and Zittau), only single sequences were obtained and therefore they were not included in this analysis.

persistence of ratHEV strains within the local populations. The separate clustering of sequences from the same geographical origin might be caused by an incursion (and perhaps establishment) of additional, highly divergent ratHEV strains by invading rats. In line with this assumption, sequences from the USA (strain LA-8350) and Indonesia (strain SOLO-006SF) cluster also within genotype G1 of ratHEV (Fig. 3B and D).

The previous finding of the majority of rats being only HEV RNA or anti-ratHEV antibody positive suggested non-persistent infections in individual rats (Johne et al., 2012). In line with this assumption, we did not find here a significantly higher RNA prevalence in adult rats compared to juvenile animals. These findings of non-persistent infections of rats are also in line with results of experimental infection studies in Norway rats (Purcell et al., 2011). At this time we cannot exclude age-dependent differences in susceptibility and mortality of rats for ratHEV infection, possibly associated with co-infections with other pathogens or genetic or environmental factors.

5. Conclusion

The detection of ratHEV in Norway and Black rats from 11 European countries indicates a broad geographical distribution of ratHEV suggesting an (almost) continent-wide occurrence and no specific association with human population density. Phylogenetic investigations indicated clustering of all European ratHEV sequences within ratHEV genotype G1. Well-separated subclades of sequences from the same or neighbouring sites might indicate the incursion of novel ratHEV strains into local Norway rat populations with a parallel persistence of a local ratHEV strain. This necessitates future studies on the population structure and potential invasion of individuals into existing rat populations and their association with ratHEV incursion. In addition, the finding of ratHEV infections in zoological gardens may allow future studies on the zoonotic potential of ratHEV based on the investigation of putative natural ratHEV transmission to non-human primates. Finally, the finding of a rabbit HEV-like sequence in a single Norway rat necessitates further studies, especially in habitats with sympatric occurrence of rabbits or pigs and rats, to evaluate potential spillover infections of human pathogenic genotype(s) and their potential public health impact.

Authors contributions

Designed the study: RGU, RJ, GH. Performed the experiments: SB,

Table 4
Intercluster nucleotide (nt, above diagonal) and amino acid (aa, below diagonal) sequence identity in percentage, for the concatenated region spanning nt positions 4105–4921 and 4949–5566 or concatenated aa sequence of positions 1369–1640 of ORF1-encoded protein and positions 1–207 of ORF2 encoded capsid protein, based on R63, acc.no. GU345042.

aa/nt	Kampehl,BB, DE (1[b])	Warburg, NW, DE (1[b])	Esslingen, BW, DE (2[b])	Copenhagen,DK (1[b])	Vienna,AUT (1[b])	Zurich, CH (1[b])	Lyon, FR (3[b])	Dender,BE (1[b])	Cadiz, ES (2[b])	Reference (2[b])
Kampehl, BB, DE (1[b])	ID	87.8	87.8	87.0	87.3	87.1	83.9–87.6	86.7	85.3–86.4	86.2–86.5
Warburg, NW, DE (1[b])	95.5	ID	86.9–87.0	87.6	87.8	87.8	83.5–88.6	87.3	86.2–86.7	87.1
Esslingen, BW, DE (2[b])	95.7	96.3–96.9	ID	87.8–87.9	89.9–90.2	87.6–87.9	83.7–89.9	87.9–88.0	87.3–88.4	87.5–87.8
Copenhagen, DK (1[b])	95.5	95.7	96.0	ID	87.6	87.2	84.0 − 87.6	85.8	87.5–87.7	88.4–89.0
Vienna, AUT (1[b])	96.0	97.4	97.4–98.0	96.6	ID	87.3	83.9–91.5	91.3	86.7–88.2	87.8–87.9
Zurich, CH (1[b])	94.9	95.5	96.3	95.5	96.9	ID	83.7–87.9	86.2	87.3–88.0	86.6–86.7
Lyon, FR (3[b])	92.9–97.1	93.2–97.4	92.9–97.7	95.7–96.9	93.5–98.0	93.5–96.6	ID	85.0–91.8	84.2–88.1	82.6–87.6
Dender, BE (1[b])	94.9	95.5	96.3	95.2	96.6	95.7	93.2–97.4	ID	87.8–88.2	86.3–86.8
Cadiz, ES (2[b])	94.1–94.3	95.5–95.7	95.5–96.3	95.5–96.3	96.6–96.9	96.0	94.1–96.9	95.7–96.0	ID	87.4–88.0
Reference (2)	95.2–97.1	95.5–95.7	95.7–96.6	94.3–94.9	96.0–96.6	94.6–95.5	92.4–97.1	96.0–96.3	94.3–95.2	ID

Reference: ratHEV sequences of R63 and R68 prototype strains from rats collected in Hamburg (Johne et al., 2010a).
DE, Germany; BB, Brandenburg; NW, North Rhine-Westphalia; BW, Baden-Wuerttemberg; DK, Denmark; AUT, Austria; CH, Switzerland; FR, France; BE, Belgium; ES, Spain.
[b] Number of sequences per site.

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Table 5
Results of the binomial generalized linear model for factors associated with ratHEV infection. Reference categories are juvenile/subadult individuals in high human density urban areas. There were no effects of sex and species (*Rattus norvegicus* vs. *Rattus rattus*) and therefore these factors were removed during model selection.

Source of Variation	ratHEV				
	Coef.	Std.Err	z-value	p-value	OR (+/−CI)
Intercept	−1.601	0.214	−7.490	< 0.001	0.20 (0.13–0.30)
Weight [juvenile]	−0.332	0.257	−1.293	0.196	0.72 (0.43–1.18)
Pop [town]	0.035	0.325	0.107	0.915	1.04 (0.54–1.93)
Pop [rural]	−0.271	0.309	−0.876	0.381	0.76 (0.41–1.38)
Pop [zoo]	−0.868	0.435	−1.998	0.05	0.42 (0.17–0.93)

OR = odds ratio; CI = Confidence interval.

RR, EH, MS, PD, MZ, SW. Analyzed the data: SB, RR, CI, RJ, GH, RGU. Contributed materials: MP, DB, GM, ACH, JL, MHG, HA, JF, SG, KB, FRF, JP, NK, JT, CD, SZ. Wrote the manuscript: RR, SB, CI, GH, RJ, RGU. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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.vetmic.2017.07.001.

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Supplementary Table

Intercluster of nucleotide (nt, above diagonal) and amino acid (aa, below diagonal) sequence identity, in percentage, for region spanning nt positions 4105–4387 or aa positions 1369–1462, based on strain R63, acc.no. GU345042.

aa/nt	Berlin, DE (5)*	Kampehl, BB, DE (1)	Wolbrechtsh ausen, LS, DE (1)	Warburg, NW, DE (3)	Zittau, SA, DE (1)	Esslingen, BW, DE (5)	Copenhagen, DK (2)	Vienna, AUT (7)	Zurich, CH (4)	Budapest, HU (2)	Lyon, FR (5)	Cadiz, ESP (2)	Brno, Prag, Northern Moravia, CZ (5)	Pianosa Island, ITA (1)	Thessaloniki, Chalkidiki, GR (2)	Dender, Ijzer, BE (6)	Reference (2)
Berlin, DE (5)*	ID	86.0	86.4-88.2	86.7-87.1	87.8	85.7-86.0	84.6-85.3	83.2-86.7	82.1-86.0	84.2-86.0	82.8-86.0	85.3-87.8	75.3-84.2	85.3	79.2-83.2	78.5-83.2	83.9
Kampehl, BB, DE (1)	86.0	ID	87.5	86.4-86.7	96.1	85.7-86.0	86.0-87.8	84.6-86.4	83.5-85.3	85.3-87.5	81.0-84.6	83.2-84.9	80.3-86.0	86.0	82.1-84.9	76.0-86.0	86.0
Wolbrechtshausen, LS, DE (1)	98.7	96.1	ID	86.4-86.0	96.1	85.7-86.0	84.6-86.0	82.8-88.5	83.5-88.2	85.7-88.9	81.4-88.2	85.7-87.5	78.1-87.1	85.7	82.8-86.0	79.9-86.4	88.5
Warburg, NW, DE (3)	97.4	97.4	97.4	ID	96.1	85.7-87.8	85.3-88.9	82.4-87.5	82.1-91.0	85.7-86.4	82.8-90.0	84.2-84.6	77.8-86.0	87.5	84.9	88.5	85.3
Zittau, SA, DE (1)	98.7	96.1	100	97.4	ID	88.2-89.6	88.5-89.6	82.8-88.5	83.5-88.2	88.5-88.9	81.4-88.2	85.7-87.5	78.5-87.5	88.9	81.7-88.5	80.6-89.6	87.5
Esslingen, BW, DE (5)	96.1	97.4	97.4	97.4	97.4	ID	86.4-88.5	83.2-88.2	83.5-88.2	87.8-89.6	83.9-90.0	86.7-88.2	76.3-84.9	87.5	80.6-87.8	78.1-88.2	86.0
Copenhagen, DK (2)	97.4	96.1-97.4	97.4-98.7	98.7	97.4-98.7	86.4-88.5	ID	85.7-89.2	84.9-86.4	88.2-94.3	82.4-88.2	86.4-88.9	80.3-88.2	86.4	82.8-87.5	88.5	88.5
Vienna, AUT (7)	96.1-100	94.7	96.1-98.7	96.1-97.4	96.1-98.7	94.7-96.1	96.1-98.7	ID	81.0-86.7	84.6-90.7	81.4-88.2	83.5-87.1	78.9-88.2	87.8	77.0-88.5	77.0-91.4	85.7
Zurich, CHE (4)	96.1-98.7	94.7-96.1	96.1-100	96.1-98.7	96.1-100	96.1-97.4	97.4-98.7	81.0-86.7	ID	83.2-88.5	82.8-88.9	83.2-88.2	76.7-87.8	87.5	82.4-87.5	74.6-87.8	86.4
Budapest, HU (2)	97.4	94.7-97.4	98.7	97.4	98.7	96.1-98.7	96.1-100	94.7-97.4	97.4-98.7	ID	81.4-89.6	87.1-89.6	78.5-89.6	84.9	81.0-88.5	82.1-91.0	85.7
Lyon, FR (5)	96.1	94.7-96.1	96.1-97.4	97.4	96.1-97.4	96.1-97.4	97.4-98.7	94.7-97.4	97.4-98.7	94.7-98.7	ID	83.9-87.8	76.0-89.6	90.7	81.7-88.5	81.0-90.7	81.0
Cadiz, ESP (2)	96.7	95.6	96.7	97.8	96.7	96.7	97.8-98.9	95.6-100	96.7-100	95.6-97.8	96.7-97.8	ID	87.9-100	96.7	86.7-87.1	88.2	88.2
Brno, Prag, Northern Moravia, CZ (5)	84.6-96.7	84.6-95.6	84.6-96.7	85.7-97.8	84.6-96.7	84.6-96.7	85.7-98.9	84.6-100	84.6-100	83.5-97.6	84.6-97.8	87.9-100	ID	83.5	82.8-86.7	79.6-87.5	84.9
Pianosa Island, ITA (1)	98.9	96.7	100	97.8	100	97.8	97.8	96.7-98.9	96.7-100	98.9	96.7-97.8	96.7	84.6-99.3	ID	82.8-83.5	79.6-99.6	84.9
Thessaloniki, Chalkidiki, GR (2)	95.6	94.5	95.6	96.7	95.6	95.6	96.7-97.8	94.5-98.9	95.6-98.9	94.5-96.7	95.6-96.7	95.6-97.8	83.5-93.4	83.5	ID	86.4	85.3
Dender, Ijzer, BE (6)	94.3-98.9	92.3-96.7	93.4-100	94.5-97.8	93.4-100	93.4-97.8	94.5-98.9	92.3-98.9	93.4-100	92.3-98.9	93.4-97.8	95.6-97.8	80.2-97.8	92.3-100	90.1-96.7	ID	88.5
Reference (2)	94.7	96.1	96.1	96.1	96.1	98.7	96.1-97.4	93.4-94.7	94.7-96.1	94.7-97.4	94.7-96.1	91.2-97.8	97.8	98.9	96.7	98.9	ID

Reference: ratHEV sequences of prototype strains R63 and R68 from rats collected in Hamburg (Johne et al., 2010)

DE, Germany; BB, Brandenburg; LS, Lower Saxony; NW, North Rhine-Westphalia; SA, Saxony-Anhalt; BW, Baden-Wuerttemberg; DK, Denmark; AUT, Austria; CH, Switzerland; FR, France

*Number of ratHEV sequences per site.

2.3 Paper III

Spahr, C., **Ryll, R.**, Knauf-Witzens, T., Vahlenkamp, T. W., Ulrich, R. G., & Johne, R., 2017. Serological evidence of hepatitis E virus infection in zoo animals and identification of a rodent-borne strain in a Syrian brown bear. Veterinary microbiology, 212, 87-92.
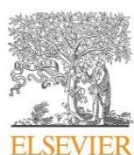
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Short communication

Serological evidence of hepatitis E virus infection in zoo animals and identification of a rodent-borne strain in a Syrian brown bear

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ABSTRACT

Hepatitis E virus (HEV) is the causative agent of hepatitis E, an emerging infectious disease of humans. HEV infections have also been described in various animal species. Whereas domestic pigs and wild boars are well-known animal reservoirs for HEV, the knowledge on natural HEV infection in zoo animals is scarce so far. Here, we analysed 244 sera from 66 mammal species derived from three zoos in Germany using a commercial double antigen sandwich ELISA. HEV-specific antibodies were detected in 16 animal species, with the highest detection rates in suids (33.3%) and carnivores (27.0%). However, RNA of the human pathogenic HEV genotypes 1–4 was not detected in the serum samples from suids or carnivores. Using a broad spectrum RT-PCR, a ratHEV-related sequence was identified in a sample of a female Syrian brown bear (Ursus arctos syriacus). Subsequent serum samples within a period of five years confirmed a HEV seroconversion in this animal. No symptoms of hepatitis were recorded. In a follow-up investigation at the same location, closely related ratHEV sequences were identified in free-living Norway rats (Rattus norvegicus), whereas feeder rats (Rattus norvegicus forma domestica) were negative for HEV-specific antibodies and RNA. Therefore, a spillover infection of ratHEV from free-living Norway rats is most likely. The results indicate that a wide range of zoo animals can be naturally infected with HEV or HEV-related viruses. Their distinct role as possible reservoir animals for HEV and sources of HEV infection for humans and other animals remains to be investigated.

1. Introduction

Hepatitis E virus (HEV) infections represent the most common cause of acute hepatitis in humans worldwide (Rein et al., 2012). In several European countries, the number of recorded human hepatitis E cases steadily increased during the past ten years (Adlhoch et al., 2016). The disease is mostly characterized by mild to moderate acute hepatitis; subclinical infections appear to be frequent. However, pregnant women in endemic regions with HEV-1 and persons with underlying liver disease portray a risk group for severe acute hepatitis including lethal outcomes. In addition, chronic infections, which can develop to liver cirrhosis, have been identified in immunosuppressed transplant patients (Kamar et al., 2012).

HEV belongs to the family *Hepeviridae* and possesses an RNA genome containing three open reading frames (ORFs). ORF1 encodes a non-structural polyprotein, ORF2 the capsid protein and ORF3 a small phosphoprotein. The human-pathogenic genotypes (GT) HEV-1 to HEV-4 are classified together with additional GT from wild boars and camels into the species *Orthohepevirus A* (Smith et al., 2014). The species *Orthohepevirus B* contains avian HEV strains, whereas mainly strains from rats and ferrets are found in *Orthohepevirus C* and batHEV strains in *Orthohepevirus D* (Smith et al., 2014).

The sources of infection with human-pathogenic HEV are GT-dependent (Johne et al., 2014). HEV-1 and HEV-2 are restricted to humans and mainly transmitted by fecally contaminated water. In contrast, HEV-3 and HEV-4 are zoonotic viruses, with pigs and wild boars representing the main animal reservoirs. These animals do not show any clinical symptoms due to HEV infection. Direct contact between humans and animals and ingestion of virus-containing food are the main transmission routes of these genotypes.

RNA of HEV-3 or HEV-4 as well as HEV-specific antibodies have also been detected in a considerable variety of other wildlife, farmed and pet

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animal species (Spahr et al., 2017b; Doceul et al., 2016; Pavio et al., 2010). To gain knowledge about the distribution of HEV infections in different animal species, zoo-like locations with a large diversity of mammal species represent interesting sites. However, only a few studies analysing zoo animals have been published yet (Spahr et al., 2017a; Li et al., 2015; Zhang et al., 2008).

To analyse HEV infections in zoo animals, a serological survey on HEV-specific antibodies was performed with animals from three zoos in Germany. Animals of taxa showing comparably high seroprevalences were additionally analysed by RT-PCR for the presence of HEV RNA. Follow-up investigations in free-living and feeder Norway rats should identify the source of HEV infections in zoo animals. The results of the investigation should contribute to further clarify the role of zoo animals as susceptible hosts of HEV.

2. Materials and methods

2.1. Sampling

In total, 244 individual sera from 66 mammal species were collected in three zoos (A–C) in Germany (Suppl. Table 1), though most sera were obtained from zoo A. The sera were obtained between 2006 and 2016 during animal immobilizations for different purposes, e.g. routine health checks, and stored at $-20\,°C$. Additionally, liver samples from 12 animals were taken during routine dissections of died zoo animals between 2015 and 2016 and stored at $-20\,°C$. No animal was sampled for the sole profit of this study. All animals in the zoos were routinely checked by their keepers for physical health, which was documented daily. 73 free-living Norway rats were collected between 2009 and 2016 from two zoos (A and D) and stored at $-20\,°C$ (Suppl. Table 2). These rats were collected routinely for use in the network "Rodent-borne pathogens" and standard protocols of the network were used for preparation of liver samples and extraction of transudates from the thoracic cavity (Ulrich et al., 2008). Additionally, 20 randomly selected feeder rats from zoo A were killed for internal stock control, using CO_2 inhalation in accordance with animal welfare regulations. All liver and transudate samples were stored at $-20\,°C$ until further investigation.

2.2. Serological analysis

The serum samples were analysed for HEV-specific antibodies using the Axiom® HEV-Ab EIA (Axiom Diagnostik, Bürstadt, Germany) and the results were evaluated according to the recommendations of the manufacturer. This assay is based on HEV-1 capsid protein antigens and uses the test principle of a double antigen sandwich ELISA. By this, it is species-independent and can detect all immunoglobulin classes.

2.3. RNA isolation

RNA was extracted from serum samples using the NucleoMag®VET kit (Macherey-Nagel, Düren, Germany) in a King Fisher 96 Flex Workstation (Thermo Fisher Scientific GmbH, Schwerte, Germany), following the manufacturer's instructions. Liver samples were homogenized using a TissueLyser (Qiagen GmbH, Hilden, Germany) and QIAzol®Lysis Reagent (Qiagen GmbH), and RNA was extracted by a modified QIAzol protocol method as described before (Schmidt et al., 2016). The RNA pellets were resolved in 100 µl DEPC-treated water and stored at $-80\,°C$ until further use.

2.4. Real-time RT-PCR (RT-qPCR)

RNA samples were tested for the presence of HEV-1 to HEV-4 using a previously described RT-qPCR protocol (Jothikumar et al., 2006). The QuantiTect® Probe RT-PCR Kit (Qiagen GmbH) was used in 20 µl reactions with conditions as previously described (Schielke et al., 2011). The limit of detection of this RT-qPCR as determined by dilution series

of in vitro transcribed RNA was seven genome equivalents per PCR reaction (Schielke et al., 2011).

2.5. Nested broad-spectrum RT-PCR (NBS-RT-PCR)

The NBS-RT-PCR was performed according to Johne et al. (2010). This assay amplifies a conserved region within the RNA-dependent RNA polymerase (RdRp)-encoding region of OFR1 and has been demonstrated to be capable of detection of HEV strains from the species Orthohepevirus A, B and C (Johne et al., 2010). The RT-PCR was performed using the One-Step RT-PCR kit (Qiagen GmbH) and the nested PCR using the TaKaRa ExTaq kit (TaKaRa Bio, Japan) as described before (Johne et al., 2010). The nested PCR products were separated by agarose gel electrophoresis and bands according to a length of 331–334 nucleotides (nt) were excised and purified using the QIAquick Gel Extraction Kit® (Qiagen GmbH).

2.6. SW-RT-PCR

The SW-RT-PCR targets a similar genomic region of the HEV genome like the NBS-RT-PCR, but is designed as one-step RT-PCR (Wolf et al., 2013). It has been shown to efficiently detect ratHEV, but should also be able to detect strains of the species Orthohepevirus A based on the primer sequences. This RT-PCR was performed using the Super-ScriptIII with PlatinumTaq Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) in a 25 µl reaction (Wolf et al., 2013). RT-PCR products with a length of 282 bp were purified using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel).

2.7. Sequence analyses

Purified amplification products were either sequenced by a commercial company (Eurofins GmbH, Hamburg, Germany) or sequenced in-house using the BigDye® Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) in an HITACHI 3130 Genetic Analyser (Applied Biosystems, Darmstadt, Germany). For sequence comparisons and phylogenetic analyses, a sequence fragment of the RdRp-encoding region with a length of 279 nt (nt 4108–4387; numbering according to ratHEV reference strain R63, acc. no. GU345042), derived from the products of the NBS-RT-PCR and/or the SW-RT-PCR, was used. The newly generated HEV sequences were deposited at GenBank (sequence from the Syrian brown bear: acc. no. MF480313, sequences from rats: acc. nos. MF480314–480320). Sequence alignments were performed using BioEdit 7.2.0 (Hall, 1999) and MEGA 7 (Kumar et al., 2016). The GTR + G model was used as it was identified as the best suited substitution model by MEGA 7. The phylogenetic analyses were performed by Bayesian algorithms via the CIPRES online portal (Ronquist et al., 2012) with 8 million generations and by Maximum likelihood algorithm performed via MEGA7 (Kumar et al., 2016) with 1.000 bootstrap replicates and a consensus tree was generated. Reference sequences for phylogenetic reconstructions were taken from Smith et al. (2014).

3. Results

3.1. HEV-specific antibodies are mainly detected in zoo animals of the family Suidae and the order Carnivora

A total of 244 serum samples from mammalian zoo animals, belonging to 66 species, were tested for the presence of HEV-specific antibodies (Table 1 and Suppl. Table 1). In total 28/244 (11.5%) turned out to be anti-HEV antibody-positive. Animals from 16 species in three orders (Artiodactyla, Carnivora, Perissodactyla) were tested positive. The highest seroprevalence was found in animals from the family Suidae with 9/27 (33.3%) positive samples originating from three different species. A high seroprevalence was also recorded for animals of

Table 1
Prevalence of HEV-specific markers in zoo animals from Germany using serological and molecular detection methods.

Order	Family	Axiom® HEV-Ab EIA		RT-qPCR		NBS-RT-PCR		SW-RT-PCR		Sequencing
		pos./total	%	pos./total	%	pos./total	%	pos./total	%	pos./total
Afrosoricida		0/1	N/A	–	–	–	–	–	–	–
	Tenrecidae	0/1	N/A	–	–	–	–	–	–	–
Artiodactyla		**16/167**	**9.6**	0/98	0	–	–	0/8[a]	0	–
	Suidae	**9/27**	**33.3**	0/27	0	–	–	0/2[a]	0	–
	Tayasuidae	0/1	N/A	0/1	N/A	–	–	–	–	–
	Hippopotamidae	0/1	N/A	–	–	–	–	–	–	–
	Camelidae	0/15	0	–	–	–	–	–	–	–
	Cervidae	**2/25**	**8**	0/16	0	–	–	–	–	–
	Giraffidae	0/4	0	–	–	–	–	0/1[a]	N/A	–
	Bovidae	**5/94**	**5.3**	0/54	0	–	–	0/5[a]	0	–
Carnivora		**10/37**	**27**	0/37	0	**3/37**	**8.1**	**2/37**	**2.7**	**1/3**
	Canidae	**2/8**	**25**	0/7	0	**1/7**	0	0/7	0	0/1
	Hyaenidae	**1/1**	N/A	0/1	N/A	0/1	0	0/1	N/A	–
	Otariidae	**1/1**	N/A	0/1	N/A	0/1	0	0/1	N/A	–
	Phocidae	0/1	N/A	0/1	N/A	0/1	0	0/1	N/A	–
	Ursidae	**1/12**	**8.3**	0/12	0	**2/12**	**16.6**	**2/12**	**8.3**	**1/2**
	Felidae	**5/10**	**50**	0/10	0	0/11	0	0/11	0	–
	Herpestidae	0/4	0	0/3	0	0/4	0	0/4	0	–
Chiroptera		0/4	0	0/4	0	–	–	–	–	–
	Pteropodidae	0/4	0	0/4	0	–	–	–	–	–
Diprotodontia		0/2	0	0/1	N/A	–	–	–	–	–
	Macropodidae	0/2	0	0/1	N/A	–	–	–	–	–
Perissodactyla		**2/24**	**8.3**	0/20	0	–	–	0/2[a]	0	–
	Equidae	**2/20**	**10**	0/20	0	–	–	0/2[a]	0	–
	Rhinocerotidae	0/3	0	–	–	–	–	–	–	–
	Tapiridae	0/1	N/A	–	–	–	–	–	–	–
Proboscidea		0/6	0	–	–	–	–	–	–	–
	Elephantidae	0/6	0	–	–	–	–	–	–	–
Rodentia		0/2	0	0/3	0	–	–	0/2[a]	0	–
	Castoridae	0/2	0	0/2	0	–	–	0/2[a]	0	–
	Chinchillidae	0/1	N/A	0/1	N/A	–	–	–	–	–
	Total	**28/244**	**11.5**	0/161	0	**3/37**	**8.1**	**2/49**	**4.1**	**1/3**

pos., positive; total, total number of individual samples analysed; –, not determined.
N/A, not applicable (only 1 sample analysed).
RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NBS-RT-PCR, nested broad-spectrum RT-PCR; SW-RT-PCR, (rat)HEV-specific RT-PCR.
Results printed in bold are positive results.
[a] Liver samples.

the order Carnivora with 10/37 (27.0%) positive samples originating from six different species. Table 1 gives an overview on the findings, whereas individual data are listed in the Suppl. Table 1.

3.2. RatHEV RNA is detected in a Syrian brown bear sample by RT-PCR

A subset of 161 serum samples, which were selected according to the availability of sample material, was analysed by the RT-qPCR for detection of RNA of HEV-1 to HEV-4 (Table 1). None of the investigated samples was positive in the assay. To allow the detection of hepeviruses from other species than *Orthohepevirus A*, two broadly reactive RT-PCR assays were applied to 37 individual serum samples belonging to animals of the order Carnivora and to 12 liver samples originating from different animal species, obtained during necropsy (Table 1). Three serum samples originating from a South American coati (*Nasua nasua*), a bush dog (*Speothos venaticus*) and a Syrian brown bear (*Ursus arctos syriacus*) showed bands of the expected lengths in the NBS-RT-PCR. The South American coati and the Syrian brown bear were also positive in the SW-RT-PCR. Attempts to sequence the amplicons were only successful for the products of the NBS-RT-PCR and the SW-RT-PCR of the Syrian brown bear sample, whereas the fainter bands of the other animals could not be sequenced. The analysis of the sequences indicated the closest relationship to ratHEV strain R68 from the species *Orthohepevirus C* (Fig. 1A). The serum sample of the female Syrian brown

bear of zoo A was taken in 2011, when the animal was 22 years old. This serum sample was negative for HEV-specific antibodies. However, a second serum sample taken in 2016, before the age-related death of the animal at 27 years, was positive for HEV-specific antibodies (Suppl. Table 1).

3.3. RatHEV RNA sequences from wild rats of the same zoo are closely related to that of the Syrian brown bear

To investigate the source of infection of the bear with ratHEV, rat samples from the same geographic location were analysed. 20 feeder rats from zoo A, hold in 2017, were tested negative in the Axiom® HEV-Ab EIA assay as well as in the SW-RT-PCR (Suppl. Table 2). Additional samples of 73 wild Norway rats, trapped between 2009 and 2016 in zoos A and D (located in a distance of 16 km from each other), were available. HEV-specific antibodies could not be demonstrated in transudates from the thoracic cavity of these rats using the EIA (Suppl. Table 2). HEV-RNA could be detected in 8/73 (10.9%) liver samples using the SW-RT-PCR (Suppl. Table 2). Sequencing of the RT-PCR products revealed the presence of ratHEV in 7/8 of the RNA-positive samples (1 positive sample from zoo D could not be sequenced). A phylogenetic tree was set up for the obtained 279 nt sequences from the RdRp-encoding region of the HEV ORF1 together with other available ratHEV sequences, also including previously published sequences from
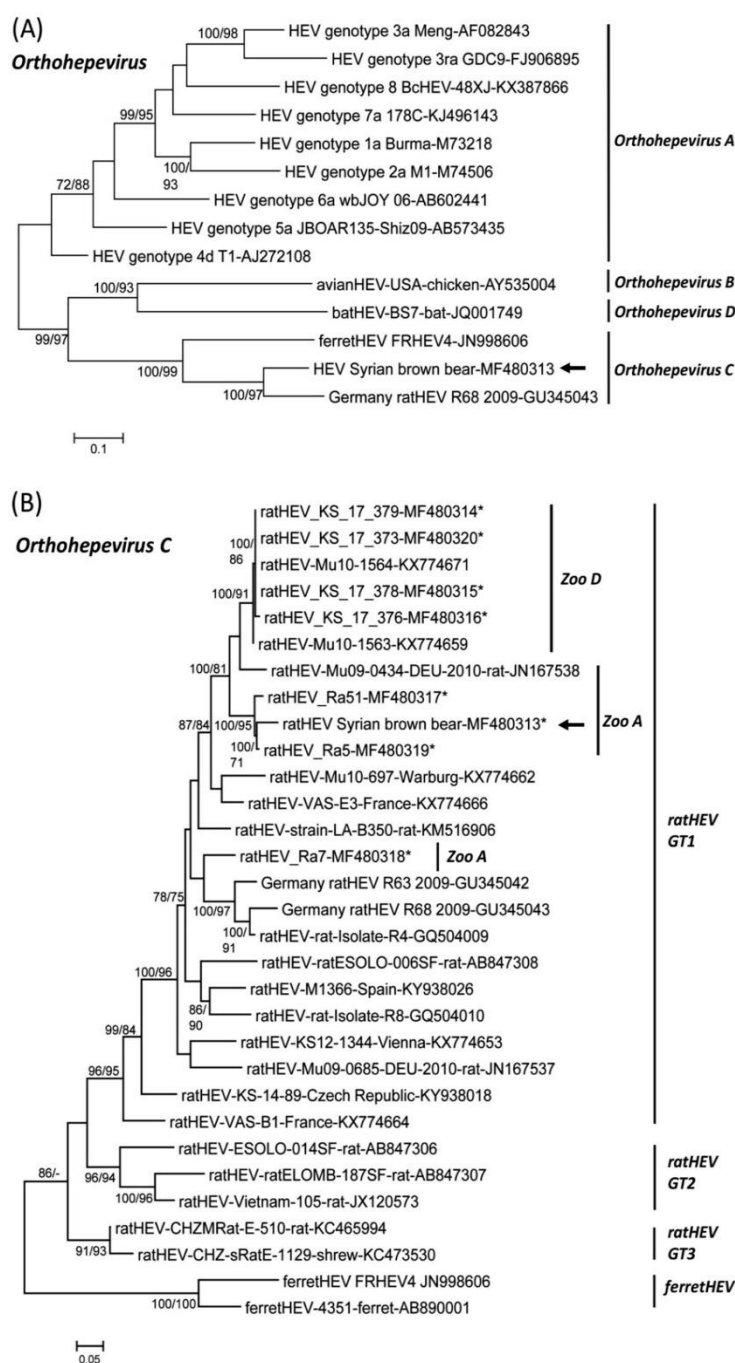
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Fig. 1. Phylogenetic relationship between the sequences from animals of zoos A and D and other HEV strains. (A) Comparison of reference sequences from the Genus *Orthohepevirus* with the sequence derived from the Syrian brown bear of zoo A (marked by an arrow). The species *Orthohepevirus A, B, C* and *D* are indicated right and the established genotypes are implemented into the strain designations. (B) Comparison of sequences from ratHEV and ferretHEV strains within species *Orthohepevirus C* with the sequence from the Syrian brown bear of zoo A (marked by an arrow). Sequences newly established in this study are marked by asterisks. The origin of sequences from zoo A or D as well as the proposed genotypes of ratHEV according to Mulyanto et al. (2014) are indicated.

zoo A and D. The sequences of two rats from zoo A and the one of the Syrian brown bear showed nt sequence identities of 94.6% to 97.8% to each other and define a well separated cluster within the ratHEV-clade (Fig. 1B). A sister cluster is formed by the sequence of an already published strain from the same location and published strains from zoo D. One of the newly determined ratHEV sequences (Ra7) from zoo A clusters differently but still within the proposed ratHEV GT1 clade (Mulyanto et al., 2014), which is typical for European ratHEV strains

(Ryll et al., 2017). Asian ratHEV sequences and ferretHEV strains are found in other branches of the tree.

4. Discussion

HEV infections in zoo animals have been investigated scarcely so far. In a previous study, 38 faecal samples belonging to 22 animal species from a zoo-like centre in Eastern China were analysed for the presence of HEV-RNA, revealing a positive result of 28.9% (Zhang et al., 2008). All detected sequences belonged to HEV-4 and were derived from three deer and two carnivore species. A larger study involving 244 sera from 66 mammal species from zoos in Germany was initiated here. HEV-reactive antibodies were detected in 11.5% of the animals belonging to 16 mammalian species out of nine families, indicating that infections with HEV or HEV-like viruses occur in a wide range of different zoo animal species. These data confirm that from other published studies: markers of HEV infection have been identified in suids and cervids, bovids, canids, felids, ursids and equids (Spahr et al., 2017b). In addition, we demonstrated for the first time the presence of HEV-specific antibodies in antelopes, hyenas and otariids. The host spectrum of HEV should be investigated in future studies involving a broader range of animal species and geographical areas.

Domestic pigs and wild boars are well known reservoirs for zoonotic HEV-3 and HEV-4. Therefore, the high seroprevalence of 33.3% obtained for the zoo-housed pigs is in line with the expectations. Reported anti-HEV-IgG seroprevalences in domestic pigs range between 23% (Argentina) and 100% (USA) (Doceul et al., 2016; Pavio et al., 2010) and in wild boars between 3% (USA) and 42.7% (Spain) (De Deus et al., 2008; Dong et al., 2011). Despite the high seroprevalence, we did not detect HEV RNA in the zoo-housed pigs. Productive infection commonly occurs in young pigs and viral excretion decreases with the appearance of antibodies (McCreary et al., 2008). Zoo-housed pigs usually have a long lifetime leading to a high median age of the study group, which could explain the high antibody prevalence and the absence of HEV RNA. In addition, serum may not represent the best sample material for HEV RNA detection as viremia during HEV infection is usually short (Grierson et al., 2015). Studies investigating younger animals and other sample types like faeces or liver should increase the chance to detect HEV RNA and to identify the involved HEV type.

A high seroprevalence of 27% was also identified for zoo-housed carnivores. For mongooses, which are small wild carnivores, seroprevalences of 21% were reported from Japan (Nakamura et al., 2006). Seroprevalences up to 21% for pet dogs (Liang et al., 2014) and 30% for pet cats (Mochizuki et al., 2006) have also been described. The distinct reasons for the high seroprevalence in carnivores are not known yet. However, virus transmission by ingestion of infected animals seems to be a reasonable source of infection.

So far, different HEV GT have been identified in carnivores: HEV-3 in mongooses, HEV-4 in leopards and bears and *Orthohepevirus C* carnivore strains in minks and ferrets (Spahr et al., 2017b). Attempts to detect RNA of HEV-3 and HEV-4 in our carnivore samples failed. In contrast, RNA of ratHEV was identified in a Syrian brown bear. This sample was seronegative for HEV, whereas a second serum sample taken 5 years later was antibody-positive. This might indicate seroconversion due to a ratHEV infection. During this time, no clinical symptoms were reported by the animal keepers performing daily routine checks. The distribution of ratHEV infections in carnivores and its clinical consequences for the animals should be investigated in further studies.

Different sources for the ratHEV infection of the bear can be imagined. Infections with ratHEV seem to be common in free-living Norway rats (*Rattus norvegicus*) in Germany, but also in other parts of the world (Ryll et al., 2017; Mulyanto et al., 2014). In addition, ratHEV has been detected in Black rats (*Rattus rattus*), Bandicoot rats (*Bandicota indica*) and Asian musk shrews (*Suncus murinus*) (Spahr et al., 2017b). No HEV-specific antibodies were detected in free-living and feeder Norway rats

in our study. However, ratHEV-RNA was detected in free-living Norway rats and the identified ratHEV sequences from zoo A were highly similar to the sequence obtained from the bear. A geographical clustering of ratHEV sequences from different locations in Germany has been previously described (Johne et al., 2012). Taken together, the results indicate that free-living Norway rats might have served as a source of ratHEV infection for this bear. Generally, the distinct host range of ratHEV is largely unknown. The experimental infection of rhesus macaques (*Macaca mulatta*) with ratHEV did not result in seroconversion or virus excretion (Purcell et al., 2011). In contrast, antibodies from healthy German forestry workers showed a higher reactivity to ratHEV than to HEV-3 (Dremsek et al., 2012) and ratHEV-reactive antibodies were recently identified in febrile patients with mild liver dysfunction from Vietnam (Shimizu et al., 2016). The zoonotic potential and the spillover potential of ratHEV therefore deserve more attention in future studies.

5. Conclusion

Our study indicates, that infection of various zoo animals of different mammal species with HEV or HEV-related viruses occurs. The observed seroprevalences were considerably low, except for suids and carnivores, which showed rather high antibody detection rates. Whereas pigs are commonly considered as reservoir animals for HEV, the reason for the high seroprevalence in carnivores remains unclear. The identification of ratHEV in a bear indicates that this virus is also able to infect non-rodent animal species under certain conditions. In the presented case, an accidental spillover infection from the infected wild rats to the bear is most likely. Control of pest animals and feed used for carnivores should be considered in zoos in order to prevent virus transmissions. Further investigations are needed to prove the role of zoo animals and especially carnivores as potential reservoirs for HEV or HEV-related viruses.

Conflicts of interest statement

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetmic.2017.11.005.

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Spahr et al.: "Serological evidence of hepatitis E virus infection in zoo animals and identification of a rodent-borne strain in a Syrian brown bear"

Supplementary Table 1. Detailed data on the prevalence of HEV-specific markers in zoo animals in Germany.

Order	Family	Species (race)	Scientific name	Axiom® HEV-Ab (EIA)					RT-qPCR		NBS-RT-PCR		SW-RT-PCR	
				Zoo A	Zoo B	Zoo C	total		pos. / total	%	pos. / total	%	pos. / total	%
				antibody positive / animals per zoo			pos. / total	%						
Afrosoricida														
	Tenrecidae	Lesser Madagascar hedgehog tenrec	*Echinops telfairi*	0/1	-	-	0/1	N/A	-	-	-	-	-	-
Artiodactyla				11/146	2/4	3/17	16/167	9.6	0/98	0	-	-	0/8	0
	Suidae	African bush pig	*Potamochoerus porcus pictus*	-	2/4	1/2	3/6	50	0/6	0	-	-	0/1	N/A
		Babirusa	*Babyrousa babyrussa*	0/6	-	-	0/6	0	0/6	0	-	-	-	-
		Common warthog	*Phacochoerus africanus*	-	-	1/1	1/1	N/A	0/1	N/A	-	-	-	-
		Domestic pig (Kunekune pig)	*Sus scrofa scrofa kunekune*	0/2	-	-	0/2	0	0/2	0	-	-	-	-
		Domestic pig (Schwäbisch-Hall)	*Sus scrofa forma domestica*	5/11	-	-	5/11	45.5	0/11	0	-	-	0/1	N/A
		European wild boar	*Sus scrofa*	0/1	-	-	0/1	N/A	0/1	N/A	-	-	-	-
	Tayasuidae	Collared peccary	*Pecari tajacu*	0/1	-	-	0/1	N/A	0/1	N/A	-	-	-	-
	Hippopotamidae	Pygmy hippopotamus	*Choeropsis liberiensis liberiensis*	0/1	-	-	0/1	N/A	-	-	-	-	-	-
	Camelidae	Alpaca	*Lama pacos domestic*	0/11	-	-	0/11	0	-	-	-	-	-	-
		Bactrian camel	*Camelus bactrianus*	0/2	-	-	0/2	0	-	-	-	-	-	-
		Vicugna	*Vicugna vicugna*	0/2	-	-	0/2	0	-	-	-	-	-	-

Supplementary Table 1 (*cont.*). Detailed data on the prevalence of HEV-specific markers in zoo animals in Germany.

Family	Common name	Scientific name										
Cervidae	Mesopotamian fallow deer	*Dama mesopotamica*	2/25	-	-	2/25	8	0/16	0	-	-	-
Giraffidae	Reticulated giraffe	*Giraffa camelopardalis reticulata*	0/1	-	-	0/1	N/A	-	-	-	0/1	N/A
	Okapi	*Okapia johnstoni*	0/3	-	-	0/3	0	-	-	-	-	-
Bovidae	Domestic sheep (Cameroon sheep)	*Ovis aries aries cameroon*	0/4	-	-	0/4	0	-	-	-	-	-
	Barbary sheep	*Ammotragus lervia*	0/8	-	-	0/8	0	-	-	-	0/2	0
	Congo dwarf goat	*Capra hircus domestic congo dwarf*	1/4	-	-	1/4	25	0/4	0	-	-	-
	Alpine ibex	*Capra ibex*	0/4	-	-	0/4	0	-	-	-	-	-
	Bezoar goat	*Capra hircus domestic bezoar*	0/8	-	-	0/8	0	0/8	0	-	-	-
	Domestic goat (Damara goat)	*Capra hircus domestic damara*	-	1/4	-	1/4	25	0/4	0	-	-	-
	Markhor	*Capra falconeri*	0/18	-	-	0/18	0	-	-	-	0/1	N/A
	Mishmi takin	*Budorcas taxicolor taxicolor*	0/2	-	-	0/2	0	-	-	-	-	-
	Rocky Mountain goat	*Oreamnos americanus*	2/5	-	-	2/5	40	0/5	0	-	-	-
	Domestic sheep (Skudde sheep)	*Ovis aries aries skudde*	-	-	-	-	-	-	-	-	0/1	N/A
	Anoa	*Bubalus depressicornis*	0/1	-	-	0/1	N/A	-	-	-	-	-
	Bongo	*Tragelaphus euryceros*	0/5	-	-	0/5	0	0/5	0	-	-	-
	Dorcas gazelle	*Gazella dorcas*	0/2	-	-	0/2	0	-	-	-	-	-
	Greater Kudu	*Tragelaphus strepsiceros*	-	0/1	-	0/1	N/A	0/1	N/A	-	-	-
	Lesser Kudu	*Tragelaphus imberbis*	1/12	0/2	-	1/14	7	0/14	0	-	0/1	N/A

Supplementary Table 1 (cont.). Detailed data on the prevalence of HEV-specific markers in zoo animals in Germany.

Family	Common name	Scientific name										
	Addax	*Addax nasomaculatus*	0/1	-	0/1	N/A	0/1	N/A	-	-	-	-
	Common waterbuck	*Kobus ellipsiprymnus*	0/1	-	0/1	N/A	0/1	N/A	-	-	-	-
	Domestic cattle	*Bos taurus taurus*	0/1	-	0/1	N/A	0/1	N/A	-	-	-	-
	(Hinterwald cow)	*hinterwald*										
	Domestic cattle (Limpurger cow)	*Bos taurus taurus limpurger*	0/4	-	0/4	0	0/4	0	-	-	-	-
	African buffalo	*Syncerus caffer nanus*	-	0/2	0/2	0	0/2	0	0/2	0	-	-
	European wisent	*Bison bonasus bonasus*	-	0/5	0/5	0	0/5	0	0/5	-	-	-
Carnivora			9/33	1/4	10/37	27	0/35	0	3/37	8.1	2/37	5.4
Canidae	Fennec fox	*Vulpes zerda*	0/1	-	0/1	N/A	0/1	N/A	0/1	0	0/1	N/A
	Maned wolf	*Chrysocyon brachyurus*	2/2	0/1	2/3	66.7	0/3	0	0/3	0	0/3	0
	Bush dog	*Speothos venaticus*	0/3	-	0/3	0	0/3	0	1/2	50	1/2	50
	European grey wolf	*Canis lupus lupus*	-	0/1	0/1	N/A	0/1	0	0/1	0	0/1	0
Hyaenidae	Spotted hyena	*Crocuta crocuta*	-	1/1	1/1	N/A	0/1	0	0/1	0	0/1	0
Otariidae	California sea lion	*Zalophus californianus*	1/1	-	1/1	N/A	0/1	0	0/1	0	0/1	0
Phocidae	Harbor seal	*Phoca vitulina*	-	0/1	0/1	N/A	0/1	N/A	0/1	0	0/1	N/A
Ursidae	Syrian brown bear	*Ursus arctos syriacus*	1/4*	-	1/4**	25	0/4	0	1/4**	25	1/4**	25
	Spectacled bear	*Tremarctos ornatus*	0/2	-	0/2	0	0/2	0	0/2	0	0/2	0
	Polar bear	*Ursus maritimus*	0/1	-	0/1	N/A	0/1	0	0/1	0	0/1	N/A
	South American coati	*Nasua nasua*	0/5	-	0/5	0	0/5	0	1/5	20	1/5	20
Felidae	Jaguar	*Panthera onca*	0/1	-	0/1	N/A	0/1	0	0/1	0	0/1	N/A
	Persian leopard	*Panthera pardus saxicolor*	2/3	-	2/3	66.7	0/3	0	0/3	0	0/3	0
	Snow leopard	*Uncia uncia*	3/5	-	3/5	60	0/5	0	0/5	0	0/5	0
	Serval	*Leptailurus serval*	0/1	-	0/1	N/A	0/1	N/A	0/1	0	0/1	N/A

Supplementary Table 1 (*cont.*). Detailed data on the prevalence of HEV-specific markers in zoo animals in Germany.

Family / Order	Common name	Scientific name	n/N	%	n/N	%	n/N	%
	Sumatran tiger	*Panthera tigris sumatrae*	-	-	0/1	0	0/1	N/A
Herpestidae	Slender-tailed meerkat	*Suricata suricatta*	0/3	0	0/3	0	0/3	0
	Banded mongoose	*Mungos mungo*	0/1	0	0/1	0	0/1	N/A
Chiroptera			-	-	-	-	-	-
Pteropodidae	Indian flying fox	*Pteropus giganteus*	0/4	0	0/4	0	0/4	0
Diprotodontia			-	-	-	-	-	N/A
Macropodidae	Red kangaroo	*Macropus rufus*	0/2	0	0/2	0	0/2	N/A
Perissodactyla			**2/24**	**8.3**	**2/24**	**8.3**	0/20	0
Equidae	Persian onager	*Equus hemionus onager*	0/3	0	0/3	0	0/3	0
	Poitou donkey	*Equus asinus domestic Poitou*	**1/3**	**33.3**	**1/3**	**33.3**	0/3	0
	Somali wild ass	*Equus africanus somaliensis*	**1/3**	**33.3**	**1/3**	**33.3**	0/3	0
	Dulmen pony	*Equus caballus caballus dulmen*	0/1	0	0/1	0	0/1	N/A
	Grevy's zebra	*Equus grevyi*	0/6	0	0/6	0	0/6	0
	Przewalski's wild horse	*Equus caballus przewalskii*	0/4	0	0/4	0	0/4	0
Rhinocerotidae	One-horned rhinoceros	*Rhinoceros unicornis*	0/3	0	0/3	0	-	-
Tapiridae	Malayan tapir	*Tapirus indicus*	0/1	0	0/1	0	0/1	N/A
Proboscidea			0/6	0	0/6	0	0/3	0
Elephantidae	African elephant	*Loxodonta africana*	-	-	0/3	0	0/3	-
	Indian elephant	*Elephas maximus indicus*	0/3	0	0/3	0	0/3	0

Supplementary Table 1 (*cont.*). Detailed data on the prevalence of HEV-specific markers in zoo animals in Germany.

Rodentia													
Castoridae	American beaver	*Castor canadensis*	0/3	-	-	0/3	0	0/3	0	-	-	0/2	0
Chinchillidae	Plains viscacha	*Lagostomus maximus*	0/1	-	-	0/1	N/A	0/1	0	-	-	-	-
total positive / investigated samples			22/216	2/4	4/24	28/244	11.5	0/161	0	3/37	8.1	2/49	4

pos., positive; total, total number of samples analysed; -, not determined;
*, seroconversion from 2011-2016; **, Sequence from a Syrian brown bear (GenBank Acc.-No. MF480313) was identified as ratHEV.
N/A, not applicable (only 1 sample analysed);
RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NBS-RT-PCR, nested broad-spectrum RT-PCR; SW-RT-PCR, (rat)HEV-specific RT-PCR.

Spahr et al.: "Serological evidence of hepatitis E virus infection in zoo animals and identification of a rodent-borne strain in a Syrian brown bear"

Supplementary Table 2. Prevalence of HEV-specific markers in free-living Norway rats (*Rattus norvegicus*) and feeder Norway rats (*Rattus norvegicus forma domestica*) from two zoos in Germany and detailed information for positive animals.

Animal	Sample No.	Zoo	Year of capture	Axiom® HEV-Ab EIA		SW-RT-PCR		Sequencing		Acc. No.
				pos. / total	%	pos. / total	%	pos. / total	%	
free-living rats	Sub-total	A	2010-2016	0/57	0	3/57	5.3	3/3	100	-
	Ra5	A	2013	neg.		pos.		pos.		MF480319
	Ra7	A	2014	neg.		pos.		pos.		MF480318
	Ra51	A	unknown	neg.		pos.		pos.		MF480317
free-living rats	Sub-total	D	2009-2010	0/16	0	5/16	31.3	4/5	80	-
	KS/17/373	D	2010	neg.		pos.		pos.		MF480320
	KS/17/376	D	2010	neg.		pos.		pos.		MF480316
	KS/17/378	D	2010	neg.		pos.		pos.		MF480315
	KS/17/379	D	2010	neg.		pos.		pos.		MF480314
	KS/17/382	D	2009	neg.		pos.		neg.		-
feeder rats	Sub-total	A	2017	0/20	0	0/20	0	0/0	0	-
Total	-	-	-	0/93	0	8/93	8.6	7/8	87.5	-

pos., positive; neg., negative; total, total number of individual samples analysed; SW-RT-PCR, (rat)HEV-specific RT-PCR.

2.4 Paper IV

Ryll, R., Eiden, M., Heuser, E., Weinhardt, M., Ziege, M., Hoper, D., Groschup, M.H., Heckel, G., Johne, R., Ulrich, R.G., 2018. Hepatitis E virus in feral rabbits along a rural-urban transect in Central Germany. Infection, genetics and evolution 61, 155-159.

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Infection, Genetics and Evolution

Short communication

Hepatitis E virus in feral rabbits along a rural-urban transect in Central Germany

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ARTICLE INFO

ABSTRACT

Rabbit associated genotype 3 hepatitis E virus (HEV) strains were detected in feral, pet and farm rabbits in different parts of the world since 2009 and recently also in human patients. Here, we report a serological and molecular survey on 72 feral rabbits, collected along a rural-urban transect in and next to Frankfurt am Main, Central Germany. ELISA investigations revealed in 25 of 72 (34.7%) animals HEV-specific antibodies. HEV derived RNA was detected in 18 of 72 (25%) animals by reverse transcription-polymerase chain reaction assay. The complete genomes from two rabbitHEV-strains, one from a rural site and the other from an inner-city area, were generated by a combination of high-throughput sequencing, a primer walking approach and 5′- and 3′-rapid amplification of cDNA ends. Phylogenetic analysis of open reading frame (ORF)1-derived partial and complete ORF1/ORF2 concatenated coding sequences indicated their similarity to rabbit-associated HEV strains. The partial sequences revealed one cluster of closely-related rabbitHEV sequences from the urban trapping sites that is well separated from several clusters representing rabbitHEV sequences from rural trapping sites. The complete genome sequences of the two novel strains indicated similarities of 75.6–86.4% to the other 17 rabbitHEV sequences; the amino acid sequence identity of the concatenated ORF1/ORF2-encoded proteins reached 89.0–93.1%. The detection of rabbitHEV in an inner-city area with a high human population density suggests a high risk of potential human infection with the zoonotic rabbitHEV, either by direct or indirect contact with infected animals. Therefore, future investigations on the occurrence and frequency of human infections with rabbitHEV are warranted in populations with different contact to rabbits.

1. Introduction

Hepatitis E virus (HEV) is the causative agent of acute hepatitis in humans and belongs to family *Hepeviridae*, genus *Orthohepevirus*, species *Orthohepevirus A* (Smith et al., 2014). The small, non-enveloped virus contains a single-stranded RNA genome of positive polarity with three major open reading frames (ORF; Fig. S1). HEV was subdivided into seven major genotypes: Genotypes 1 and 2 are only found in humans, transmitted via fecal-oral route. Genotypes 5 and 6 were exclusively detected in wild boar, whereas genotypes 3 (HEV-3), 4 (HEV-4) and 7

(HEV-7) are found to cause zoonotic infections in humans. The reservoir of HEV-7 is the camel, whereas HEV-3 and HEV-4 have been found in different mammals like pig, deer, rabbit and wild boar (Smith et al., 2014, 2016).

Rabbit-associated HEV strains were first described in farmed rex rabbits, a breed of European rabbit (*Oryctolagus cuniculus*) in China (Zhao et al., 2009) and thereafter in rabbit breedings in Mongolia (Jirintai et al., 2012), USA (Cossaboom et al., 2011), The Netherlands (Burt et al., 2016), Korea (Ahn et al., 2017) and in pet rabbits in Italy (Caruso et al., 2015) and The Netherlands (Burt et al., 2016).

RabbitHEV strains belong to zoonotic HEV-3 genotype, but form a clade that is well separated from other HEV-3 subtypes and HEV genotypes (Smith et al., 2016). Their zoonotic character was shown by experimental infection of non-human primates (Liu et al., 2013), and detection of related rabbitHEV sequences in rabbits and several acute and chronically infected humans in France (Izopet et al., 2012; Abravanel et al., 2017).

The average seroprevalence of the human population in Germany was found to be 16.8% (Faber et al., 2012) and the number of recorded hepatitis E cases per year is increasing since 2001 (Robert Koch-Institute, 2017). Zoonotic HEV-3 strains have been detected with high prevalence in domestic pig, wild boar, red and roe deer (Bächlein et al., 2013; Anheyer-Behmenburg et al., 2017). HEV infections were serologically also detected in primates and other zoo animals in Germany (Spahr et al., 2017a, 2017b). In addition, ratHEV was found to be broadly distributed in Norway rats in Germany (Johne et al., 2010; Ryll et al., 2017). Furthermore, rabbitHEV RNA has been detected in feral rabbits from Germany (Eiden et al., 2016; Hammerschmidt et al., 2017).

Here we describe a serological and molecular HEV survey of rabbits collected along a transect in Central Germany including an inner-city area with high human density.

2. The study

Seventy-two feral European rabbits were collected at three rural (R) and eight urban (U) sites (Fig. 1) during October 2012–March 2013 as part of a regular hunting (V54-19c 20/15 –F 104/59), organized by the city of Frankfurt (for urban sites) and conducted by local hunters (hunting license ID 1000250221).

Serological screening by commercial antibody ELISA (HEV Ab-ELISA kit; Axiom, Bürstadt, Germany) revealed 25 of 72 (34.7%) rabbits from ten sites being anti-HEV antibody positive (Tables 1 and 2).

Table 1

Results of the serological and RT-PCR investigations of rabbits collected in and around Frankfurt am Main, Germany.

Habitat	Site[*]	No of animals per site	Sex (m/f)	Results		
				No of positive/total no of tested animals		
				Antibody ELISA	SW-RT-PCR[1]	rt RT-PCR[2]
Rural	1	17	4/13	7/17	1/17	0/17
Rural	2	9[3]	6/2	4/9	4/9	4/9
Rural	3	8	5/3	2/8	4/8	4/8
Subtotal		34[3]	15/18	13/34	9/34	8/34
Urban	4	2	1/1	2/2	1/2	0/2
Urban	5	9	2/7	1/9	0/9	0/9
Urban	6	1	0/1	0/1	1/1	1/1
Urban	7	6	1/5	1/6	0/6	0/6
Urban	8	4	1/3	2/4	3/4	1/4
Urban	9	6	2/4	4/6	0/6	0/6
Urban	10	3[3]	2/0	1/3	2/3	2/3
Urban	11	7	3/4	1/7	2/7	2/7
Subtotal		38[3]	12/25	12/38	9/38	6/38
Total		72[4]	27/43	25/72 (34.7%)	18/72 (25%)	14/72

m, male; f, female.

No, number.

[1] SW-RT-PCR (Wolf et al., 2013).

[2] SYBR-Green rt. RT-PCR (Vina-Rodriguez et al., 2015).

[3] Total number of animals including one animal with unknown sex.

[4] Including two animals with unknown sex.

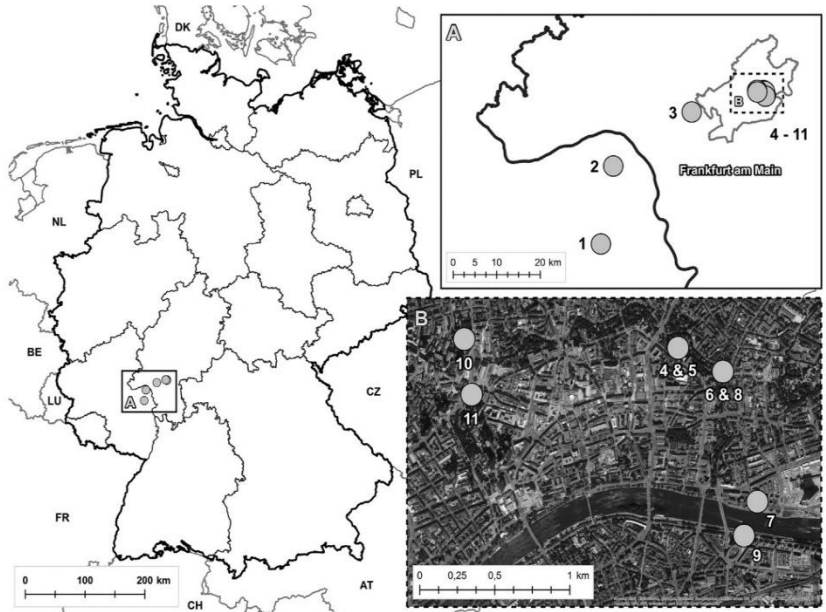[*] For additional information see Fig. 1.

Fig. 1. Location of trapping sites 1 to 11 along a transect next to Frankfurt am Main, Germany. Trapping sites 1–3 were defined as "rural" trapping sites and sites 4 to 11 as "urban" trapping sites (consequently, rabbits from these sites were designated as "R" or "U" animals, see Table 2). (A) shows a more detailed map of the trapping sites around Frankfurt am Main and (B) shows the trapping sites within the inner-city area of Frankfurt am Main.

DK, Denmark; NL, The Netherlands; BE, Belgium; LU, Luxemburg; FR, France; CH, Switzerland; AT, Austria, CZ, Czech Republic; PL, Poland.

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Table 2
Results of antibody ELISA, SYBR-Green based real-time RT-PCR (rt RT-PCR), SW-RT-PCR and the corresponding accession numbers for partial and complete genome sequences for all rabbits found to be positive in at least one assay.

Sample	Site	Antibody ELISA	rt RT-PCR[1]	SW-RT-PCR	Acc.no.
R 3	1	pos	neg	neg	–
R 6	1	pos	neg	neg	–
R 8	1	pos	neg	neg	–
R 9	1	pos	neg	neg	–
R 12	1	pos	neg	neg	–
R 14	1	pos	neg	neg	–
R 15	1	pos	neg	neg	–
R 17	1	neg	neg	pos	MF480300
R 30	2	neg	pos	pos	MF480301
R 31	2	neg	pos	pos	MF480302
R 33	2	neg	pos	pos	MF480303
R 36	2	pos	neg	neg	–
R 37	2	pos	neg	neg	–
R 38	2	neg	pos	pos	MF480304
R 40	3	pos	neg	neg	–
R 41	3	neg	pos	pos	MF480305
R 42	3	neg	pos	pos	MF480297[2]
R 44	3	pos	pos	pos	MF480306
R 46	3	neg	pos	pos	MF480307
U 1	4	pos	neg	neg	–
U 2	5	pos	neg	pos	MF480309
U 11	5	pos	neg	neg	–
U 12	6	neg	pos	pos	identical to U 19
U 16	7	pos	neg	neg	–
U 19	8	neg	neg	pos	MF480308
U 20	8	pos	neg	neg	–
U 22	8	neg	pos	pos	identical to U23
U 23	8	pos	neg	pos	MF480299
U 30	9	pos	neg	neg	–
U 31	9	pos	neg	neg	–
U 32	9	pos	neg	neg	–
U 33	9	pos	neg	neg	–
U 37	10	pos	neg	pos	MF480310
U 39	10	neg	pos	pos	MF480311
U 40	11	pos	neg	pos	MF480312
U 46	11	neg	pos	pos	MF480298[2]

Acc.no., accession number at GenBank; neg, negative; pos, positive.

[1] Samples with threshold cycle (Ct) values > 35 were counted as negative, samples with Ct values < 35 as positive; samples R42 and U46 were selected for complete genome determination due to a high viral RNA load.

[2] Complete genomes determined.

RNA was extracted from liver tissue by Qiazol reagent (QIAGEN, Hilden, Germany) and screening by a conventional RT-PCR, targeting a RNA-dependent RNA-polymerase (RdRp)-encoding region between nucleotides 4367 to 4649 (Wolf et al., 2013; see Table S1; numbering according rabbitHEV reference strain 3ra GDC9, accession number FJ906895), detected HEV-RNA in 18 of 72 (25%) animals (Tables 1 and 2). Seven of 18 RT-PCR-positive rabbits were also positive in antibody ELISA (Table 2).

RT-PCR products were sequenced using BigDye Terminator 1.1 Cycle Sequencing-Kit (Applied Biosystems, Darmstadt, Germany) and sequences were deposited to GenBank (for accession numbers see Fig. 2A). Phylogenetic analyses, including reference sequences for HEV genotypes and other hepeviruses (Smith et al., 2014, 2016), were done by maximum-likelihood- and Bayesian-methods via CIPRES portal (Miller et al., 2010) and subsequent generation of consensus trees. The phylogenetic tree for the partial RdRp-encoding nucleotide sequence shows a clade for the rabbitHEV-sequences within HEV-3 cluster, but well separated from sequences of other HEV-3 subtype strains and other HEV genotypes (Fig. 2A). The rabbitHEV nucleotide and amino acid sequences from rabbits collected in the inner-city area showed a high similarity to each other (94.3–98.6% and 95.7–100%, respectively; Table S2). This high similarity is also reflected in the phylogenetic tree (Fig. 2A, clade U). Sequences from rural sites were more divergent as documented in their positions in the phylogenetic tree (Fig. 2A, clades

RI – RIV) and the similarity values (Table S3; 80.1–99.6%; 82.8–100%).

To generate the complete rabbitHEV genomes from one urban animal and one rural animal, a SYBR-Green based real-time RT-PCR (rt RT-PCR), targeting the RdRp-encoding sequence between nucleotides 4402 and 4684 (Vina-Rodriguez et al., 2015) was used to select animals with the highest viral RNA load. Positive samples were identified by melting curve analysis and sequencing of the amplicon. Thirteen of 72 samples from six trapping sites were rt. RT-PCR positive, indicating a lower sensitivity of the rt. RT-PCR as compared to the conventional RT-PCR (Tables 1 and 2). Similar discordant results between a rt. RT-PCR and a conventional RT-PCR were previously observed in a molecular survey on ratHEV (Ryll et al., 2017). These discrepancies might be explained by the high divergence of the HEV sequences in the primer binding region of the rt. RT-PCR assay. The rabbitHEV-strains R42 (site 3) and U46 (site 11) were selected due to the high viral RNA load and a high-throughput sequencing approach was performed as described previously (Juozapaitis et al., 2014). This resulted in four consensus sequences around positions 500–1000 and 6000–6500 (numbered according to reference strain 3ra-GDC9, accession number FJ906895). Thereafter, the complete genome sequences were generated by 5′- and 3′ Rapid Amplification of cDNA Ends (RACE) analysis (5′/3′ RACE System, Invitrogen, Carlsbad, CA, USA) and primer-walking approach (for primers see Table S1). Both complete sequences have a length of 7263 nucleotides and a nucleotide sequence identity of 86.4% to each other. The nucleotide and amino acid sequence similarities to the reference strain and further 16 rabbitHEV sequences were 75.6–86.4% and 89.0–93.1%, respectively.

Prediction of potential ORFs resulted in the identification of ORFs 1, 2 and 3 in the expected regions of the genome, in the expected reading frames and with the expected overlapping pattern (Fig. S1). Simplot analysis revealed that most parts of the concatenated ORF1/ORF2 region of the two novel strains R42 and U46 share a nucleotide and amino acid sequence similarity of 62–89% and 69–99%, respectively, to the other rabbitHEV, HEV-3 and HEV genotype sequences (Fig. S2A and B). Nucleotide (and amino acid) sequences of both strains showed a lower level of similarity within ORF1 at the X-domain and the helicase protein-encoding (helicase) region (Fig. S2; regions I and II). A 93-nucleotide insertion, compared to other HEV 3 strains, was found in the X-domain region of all rabbitHEV strains, including the two novel strains R42 and U46. In contrast, for three regions the two novel strains showed a different level of sequence similarity to the other sequences (Fig. S2; regions a, b and c).

Phylogenetic analysis of the complete genomes and ORF1 and ORF2 nucleotide sequences separately as well as the amino acid sequences deduced from concatenated ORF1/ORF2 and separate ORF1 and ORF2 of rabbitHEV showed a clustering of the novel sequences (R42 and U46) with a previously determined sequence from Germany, other rabbit- and human patient-derived rabbitHEV-sequences as a separate sub-cluster within the HEV-3 clade (Figs. 2B and S3A–F). The ORF2- and ORF3-based amino acid sequence phylogenetic trees showed slightly different positions of the R42- and U46-derived sequences (Figs. S3D versus S3F).

3. Conclusions

The serological and molecular survey indicated a high prevalence of rabbitHEV in rabbits from Central Germany. Frequent detection and geography-based clustering of rabbitHEV sequences suggest a virus circulation in the local rabbit populations. The close similarity of sequences detected in the inner-city area of Frankfurt am Main may indicate a bottleneck in the rabbit population caused by immigration. The zoonotic potential of rabbitHEV warrants future investigations in human populations with increased risk of exposure due to contact to rabbits.
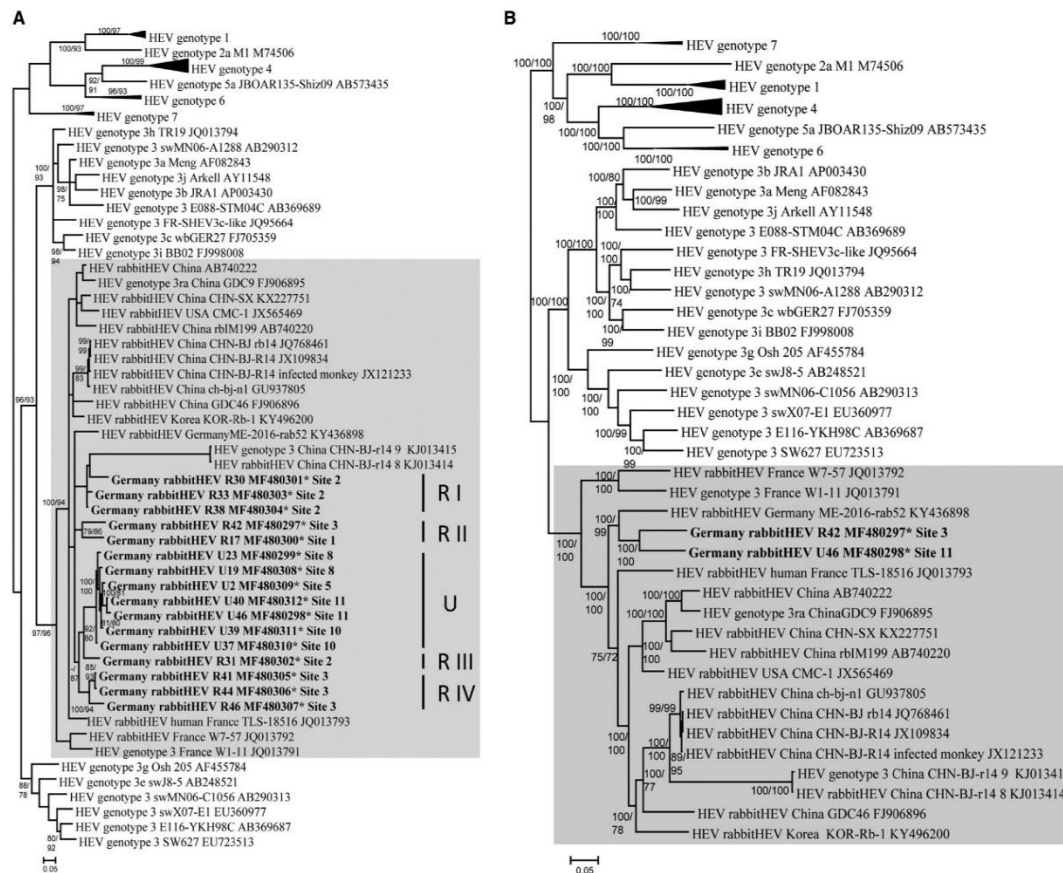
2. Publications

Fig. 2. Consensus phylogenetic trees of the novel rabbitHEV sequences, *Orthohepevirus A* reference sequences proposed by Smith et al., 2016, and additional rabbitHEV-sequences based on Bayesian analyses with 10,000,000 generations and a burn-in of 25%, and Maximum-Likelihood analysis with 1000 bootstraps and 50% cut-off. In (A) the tree for the RdRp-encoding ORF 1 screening fragment, nucleotide positions 4341–4623 (numbering according to rabbitHEV reference strain 3ra GDC9, accession number FJ906895), and in (B) the tree of the concatenated complete coding part of ORF1 and ORF2 are shown. Sequences of the rabbitHEV cluster are highlighted by a grey square. Posterior probability values/bootstrap values > 50 are given at the supported nodes. Novel sequences are given in bold and labeled by an asterisk. For location information, see Fig. 1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.03.019.

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Table S1: Primers used for screening SW-RT-PCR and generation of complete rabbitHEV genome sequences.

Strain	Primer/-pair	Primer sequence	Annealing temperature	Reference
R42/U46	5'-RACE-100as	5'-GCC AAG GCA GAA TTG GCC G-3'	60°C	This paper
R42/U46	5'-RACE-200as	5'-AAA GAT CTC CGG GCG AAA G-3'	58°C	
R42/U46	5'-RACE-300as	5'-CCT CCC AAC GGG TCG AAG A-3'	50°C	
R42/U46	15s	5'-ATG TGG TCG ATG CCA TGG AGG CCC A-3'	58°C	Izopet et al., 2012
R42/U46	15as	5'-CTC ATT ATG TAT AAC ACG TTG AAT AG-3'		
R42	152s	5'-GCC GCA AGT AAC ACG GGC GGC CGT GTG AGG TGT GAA-3'	58°C	Izopet et al., 2012
R42	152as	5'-AGA CAG ATA TTC TTA TCA ATT TAA TGC AAC CCC GC-3'		
R42	R42-1080s	5'-CTT GTT GCT AAC ACG GGC TGG AA-3'	58°C	This paper
R42	R42-2000as	5'-TGT AAC CAY AGC CCR CCR ACA A-3'		
R42	R42-2000s	5'-AGA TAC AAC AGG ACT ATC CAG C-3'	58°C	This paper
R42	R42-2600as	5'-TCA GGG GCA ACT GCA TGR ATG AT-3'		
R42	R42-2400s	5'-CAC TTC TCA GGC TAG GGT TCG-3'	58°C	This paper
R42	R42-3100as	5'-GTC ACC AAG GAG ATG CAC AGA-3'		
R42/U46	3207s	5'-AAG TCT AGG TCT ATA CAG CAG GG-3'	58°C	Izopet et al., 2012
R42/U46	rHEV-SWas	5'-GGY TCA CCR GAR TGY TTY TTC CA-3'	56°C	Wolf et al., 2013
R42	R42-3900s	5'-GCC TGC TGT ACA TGC CAC AGG A-3'	58°C	This paper
R42	R42-4200as	5'-CGT TAT TCG GGA CAC ATC TCG G-3'		
R42/U46	rHEV-SWs	5'- GCN CTG TTY GGC CCN TGG TT-3'	56°C	Wolf et al., 2013
R42/U46	rHEV-SWas	5'- GGY TCA CCR GAR TGY TTY TTC CA-3'		
R42/U46	R42-4626s	5'-TGG ATT CTA CAG GCC CCA AAG GA-3'	58°C	This paper
R42/U46	R42-5500as	5'-GGC AGC GGR GGG GCG CTG GGA CA-3'		
R42/U46	R42-5500s	5'-CCC CCT TGG CTC CTC TTG GCG-3'	58°C	This paper
R42/U46	R42-6560as	5'-CTG GTT ATC ATA GTC CTG GAT G-3'		
R42	R42-6272s	5'-TTT ACC GGG ATG AAT GGG GT-3'	58°C	This paper
R42/U46	R42-7268as	5'-TTT TTC CAG GGG AGC GCG-3'		
R42/U46	3'-RACE-240s	5'-ATT TCT GCA GTC GGT GTC CT-3'	52°C	This paper
R42/U46	3'-RACE-100s	5'-GCA GGG TTG TGC TTT CCA AT-3'		

Table S1 (continued)

Strain	Primer/-pair	Primer sequence	Annealing temperature	Reference
U46	U46-155s	5'-CAA TCT TAT GCA ACC CCG CC-3'	58°C	This paper
U46	U46-884as	5'-ACG GAC AAA CAC CTC TGT CG-3'		
U46	U46-859s	5'-CCC GTT CGA CAG AGG TGT TT-3'	58°C	This paper
U46	U46-1167as	5'-AGC GCT GAT GGC AGA TAG TG-3'		
U46	U46-1151s	5'-TAT CTG CCA TCA GCG CTA CC-3'	58°C	This paper
U46	U46-2147as	5'-AGA AAA GCC GGA CGT AGA CC-3'		
U46	U46-2000s	5'-AGA TAC AAC AGG RCT ATY CAG C-3'	58°C	This paper
U46	U46-2600as	5'-TCA GGM GCR CTG CAT GAA TRA T-3'		
U46	U46-2300s	5'-ATT CGG GGT CCC TGT TTG AG-3'		
U46	U46-3031s	5'-GGT AAG TCG AGG TCT ATA CAG CAG-3'	58°C	This paper
U46	U46-3679as	5'-AAA AGT TAC TGA CTA TAG CGT CCG A-3'		
U46	3207s	5'-AAG TCT AGG TCT ATA CAG CAG CAG GG-3'	58°C	Izopet et al., 2012
U46	3207as	5'-GCC GGT GGC GCG GGC AGC ATA GGC A-3'		
U46	U46-4626s	5'-TGG ATT YTR CAG GCC CCW AAG GA-3'	58°C	This paper
U46	R42-5500as	5'-GGC AGC GGR GGG GCG CTG GGA CA-3'		
U46	R42-5500s	5'-CCC CCT TGG CTC CTC TTG GCG-3'	58°C	This paper
U46	U46-6350as	5'-CCG MCG AAA TCA ATT CTG TCG G-3'		
U46	U46-6328s	5'-CGW GGY ATA GCC CTS ACG CTG TT-3'	58°C	This paper
R42/U46	R42-7268as	5'-TTT TTC CAG GGG AGC GCG-3'		

Table S2: Nucleotide and amino acid sequence similarity (in %) of the novel rabbitHEV sequences at each trapping site in Germany.

Trapping site*	Animal number	Number of sequences	Nucleotide sequence similarity	Amino acid sequence similarity	Clade (Fig. 2A)
1	R17	1	-	-	RII
2	R30, R31, R33, R38	4	83.7-97.9	86.2-97.9	RI, RIII
3	R41, R42, R44, R46	4	86.2-99.6	90.4-100	RII, RIV
4	U4	1	-	-	U
5	U2	1	-	-	U
6	U12	1	-	-	U
7		-			
8	U19, U22, U23	3	96.8-98.2	97.9-98.9	U
9		-			
10	U37, U39	2	98.2	100	U
11	U40, U46	2	98.6	96.8	U

R, rural site; U, urban site

*For details see Fig. 1

Table S3: Nucleotide (below diagonal) and amino acid (above diagonal) sequence similarity (in %) for the RdRp-encoding region of ORF1 of the novel sequences from Germany (R17 – R46, U2 – U46), one additional German sequence (ME-2016-rab52; Hammerschmidt et al., 2017), and one human- and two rabbit-derived rabbitHEV sequences from France (human_TLS-18516, W1-11, W7-57; Izopet et al., 2012) to the rabbitHEV reference sequence (HEV_3ra; Smith et al., 2016).

nt/aa	3ra GDC9	ME-2016-rab52	Human TLS-18516	R17	R30	R31	R33	R38	R41	R42	R44	R46	U2	U19	U23	U37	U39	U40	U46	W1-11	W7-57
3ra	-	95.7	97.8	93.5	87.1	95.7	97.8	97.8	96.8	90.3	96.8	95.7	93.5	94.6	93.5	94.6	94.6	94.6	91.4	93.5	95.7
ME-2016-rab52	83.3	-	97.8	93.5	87.1	96.8	97.8	97.8	97.8	90.3	97.8	96.8	94.6	95.7	94.6	95.7	95.7	95.7	92.5	95.7	95.7
Human TLS-18516	85.4	86.8	-	95.7	89.2	97.8	100	100	98.9	92.5	98.9	97.8	95.7	96.8	95.7	96.8	96.8	96.8	93.5	95.7	97.8
R17	85.4	85.4	83.6	-	84.9	93.5	95.7	95.7	94.6	92.5	94.6	93.5	91.4	92.5	91.4	92.5	92.5	92.5	89.2	91.4	93.5
R30	82.6	81.5	82.6	80.1	-	87.1	84.9	89.2	88.2	82.8	88.2	90.3	86.0	87.1	86.0	87.1	87.1	87.1	83.9	87.1	89.2
R31	82.9	86.5	86.5	84.3	84.0	-	97.8	97.8	98.9	90.3	98.9	97.8	96.8	97.8	96.8	97.8	97.8	97.8	94.6	93.5	95.7
R33	86.5	87.2	86.5	86.8	91.1	85.8	-	100	98.9	92.5	98.9	97.8	95.7	96.8	95.7	96.8	96.8	96.8	93.5	95.7	97.8
R38	86.5	87.5	88.6	84.7	93.2	87.5	97.9	-	98.9	92.5	98.9	97.8	95.7	96.8	95.7	96.8	96.8	96.8	93.5	95.7	97.8
R41	82.9	87.2	85.8	86.1	84.7	89.7	87.9	89.3	-	91.4	98.9	98.9	96.8	97.8	96.8	97.8	97.8	97.8	94.6	94.6	96.8
R42	86.1	81.1	82.9	86.1	82.6	83.3	86.5	87.2	86.1	-	92.5	90.3	89.2	89.2	88.2	89.2	89.2	89.2	86.0	90.3	92.5
R44	82.9	86.8	85.4	86.1	84.3	89.3	87.5	89.0	91.4	82.9	-	98.9	95.7	96.8	96.8	96.8	96.8	97.8	96.0	94.6	96.8
R46	82.2	86.1	86.8	84.3	82.2	87.2	85.1	86.5	86.1	82.2	98.9	-	96.8	96.8	95.7	96.8	96.8	96.8	94.6	93.5	95.7
U2	83.3	85.8	84.7	83.6	83.3	87.5	86.8	88.3	87.5	82.6	96.8	95.7	-	98.9	98.6	98.6	98.6	98.6	95.7	91.4	93.5
U19	82.9	85.4	84.0	85.4	83.3	87.5	87.5	88.3	88.3	82.6	96.8	96.8	98.2	-	98.9	98.9	98.9	98.9	96.8	92.5	94.6
U23	83.6	85.8	83.6	85.8	83.6	87.9	87.9	88.6	88.6	82.2	96.8	95.7	98.6	98.9	-	98.6	98.6	98.6	95.7	91.4	93.5
U37	84.3	86.1	84.7	85.4	85.1	87.9	88.6	88.6	90.0	82.6	96.8	96.8	98.6	98.9	98.6	-	100	100	96.4	92.5	94.6
U39	83.3	85.8	85.4	84.3	84.0	88.3	87.5	89.0	89.0	82.9	96.8	96.8	98.6	98.9	98.6	100	-	97.5	96.1	92.5	94.6
U40	82.2	85.4	85.4	84.0	82.9	87.2	86.5	87.9	87.5	82.6	97.8	96.8	98.6	98.9	98.6	100	97.5	-	96.8	92.5	94.6
U46	81.1	84.3	84.3	82.9	81.9	87.2	85.4	86.8	86.5	81.5	96.0	94.6	95.7	96.8	95.7	96.4	96.1	98.6	-	89.2	94.6
W1-11	81.1	84.0	83.6	80.8	76.9	81.9	82.2	82.6	82.2	78.6	82.2	81.1	82.2	82.2	82.2	82.2	82.2	82.2	80.4	-	97.8
W7-57	81.5	83.6	83.3	83.3	78.6	81.9	84.7	84.7	82.9	82.9	82.9	82.2	82.9	82.2	82.6	82.9	82.9	82.2	81.5	87.2	-

Figure S1: Genome organization and localization of major predicted open reading frames (ORFs) for the two novel complete genomes of rabbitHEV (R42, MF480297 and U46, MF480298). Both genomes have the same length and positions of the three major ORFs.

Nucleotide numbering according to rabbitHEV reference strain 3ra GDC9, accession number FJ906895.

(NCR; Non coding region, Mt, methyltransferase; Y, Y-like domain; Prot?, papain-like cysteine protease; X?, X domain/ADP-ribose-binding module; Hel, Helicase; RdRp, RNA-dependent RNA polymerase; CP, capsid protein; VP, viroporin (Johne et al., 2014; Ding et al., 2017).
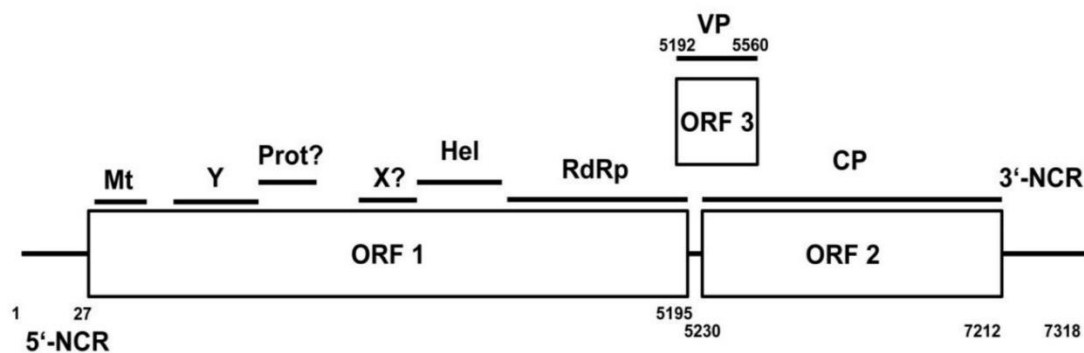
Figure S2: Simplot analysis of concatenated open reading frame (ORF) 1/ORF2 nucleotide (A) and amino acid sequences (B) of the novel rabbitHEV strains R42 (MF480297, red) and U46 (MF480298, blue) compared with rabbitHEV reference strain 3ra GDC9, accession number FJ906895, other rabbitHEV, HEV-3 and HEV genotype sequences.

The concatenated ORF1/ORF2 nucleotide and amino acid sequences were compared with rabbitHEV, other HEV-3 and HEV genotype strain sequences given in Figure 2B. For Simplot analysis with a window size of 100 nt and a step size of 25 nt, scripts were written in R (R Core Team, 2015). A schematic representation of ORF1 and ORF2 coding regions is shown with putative functional domains of nonstructural proteins encoded by ORF1 (Mt, methyltransferase; Y, Y-like domain; Prot?, papain-like cysteine protease; X?, X domain/ADP-ribose-binding module; Hel, Helicase; RdRp, RNA-dependent RNA polymerase), ORF2 (CP, capsid protein) and ORF3 (VP, viroporin). The overlapping ORF3 and the encoded proteins are indicated by dotted lines and a dotted square, respectively. Regions where the nucleotide or amino acid sequences of the two novel strains R42 and U46 show an almost identical low level similarity to all other sequences are labeled by "I" and "II", whereas regions with a different level of sequence similarity of the two novel strains to the other sequences were labeled by "a", "b" and "c".

A)
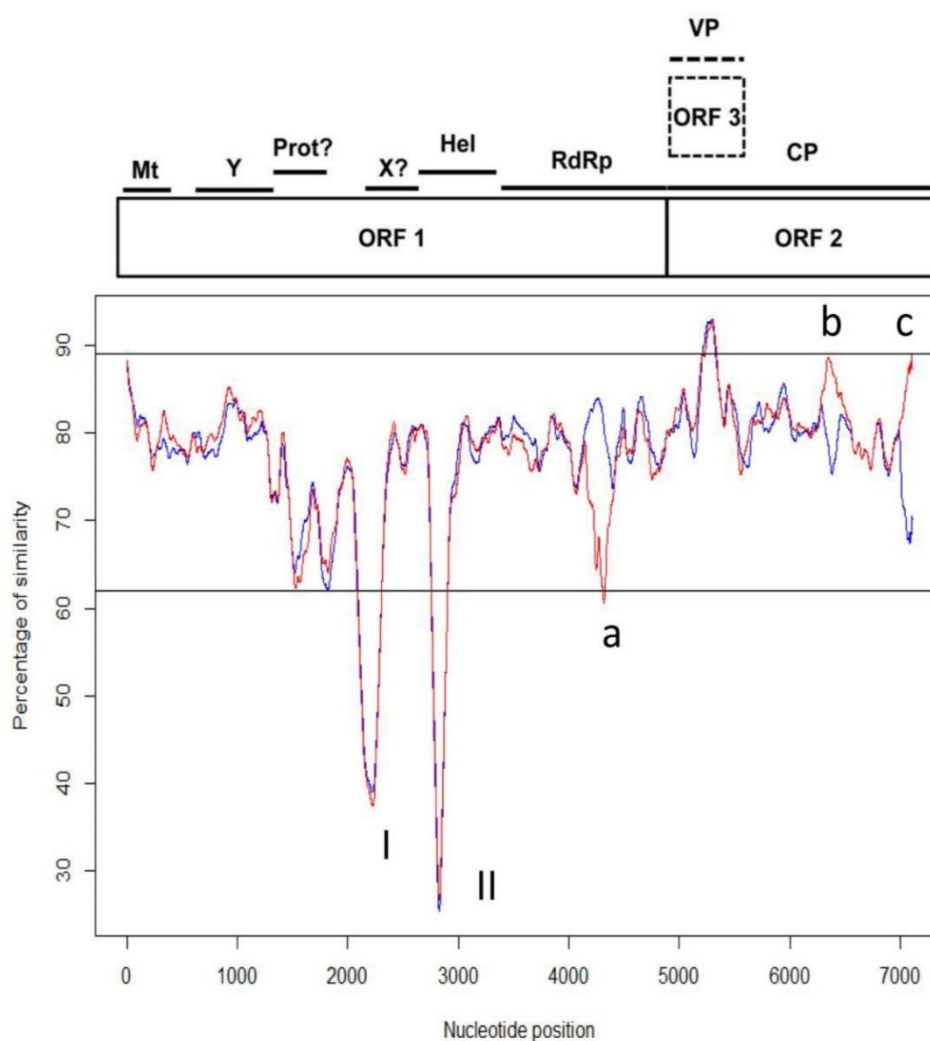
Figure S2 (continued)

B)
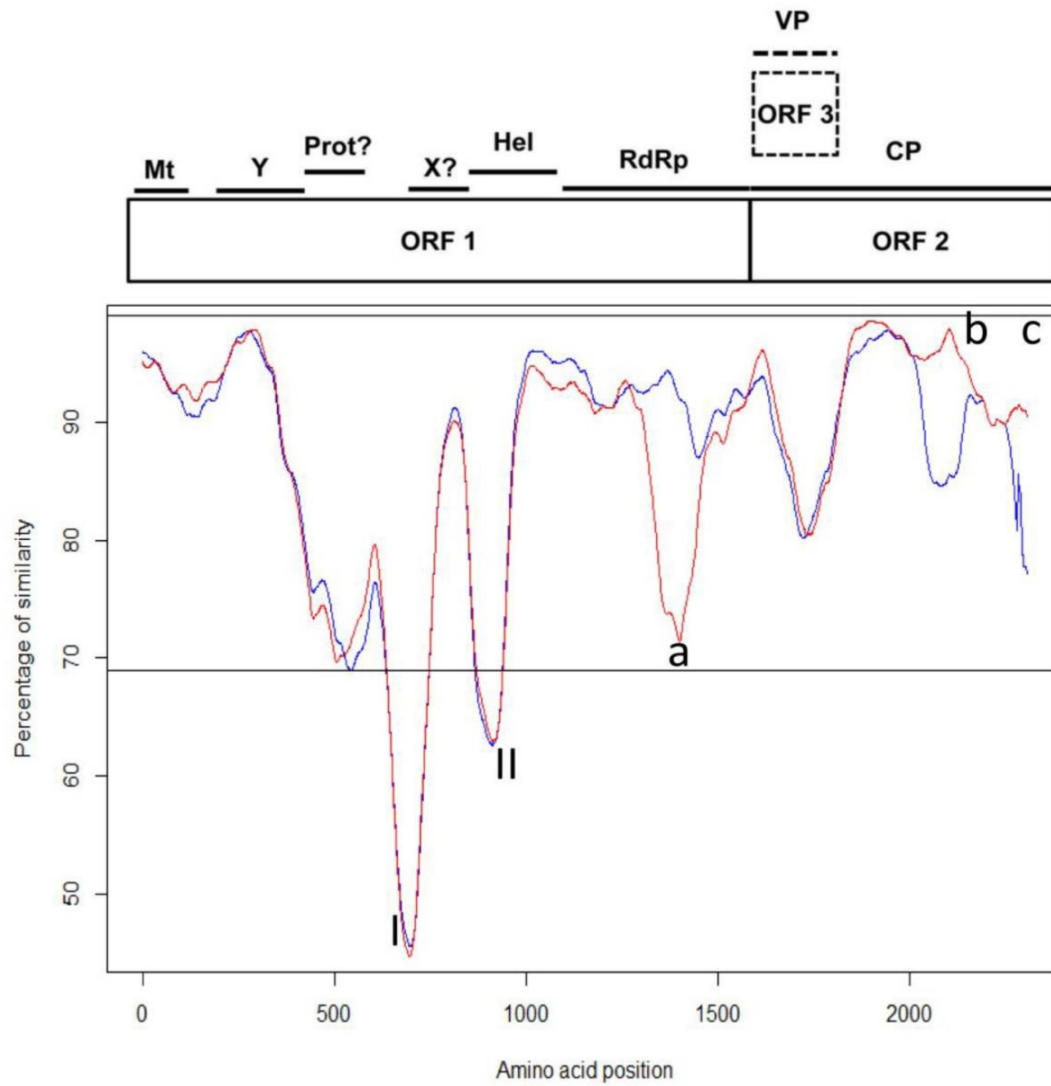
Figure S3: Consensus phylogenetic trees based on Bayesian analyses with 10,000,000 generations and a burn-in of 25%, and Maximum-Likelihood analysis with 1,000 bootstraps and 50% cut-off of the complete coding ORF1 on nucleotide (A) and amino acid (B) level, the complete ORF2 on nucleotide (C) and amino acid (D) level and the complete ORF3 on nucleotide (E) and amino acid (F) level of the two novel rabbitHEV genomes from Germany, additional rabbitHEV-sequences and HEV reference sequences taken from (Smith et al., 2014, 2016). The rabbitHEV-clade is highlighted by a grey square.

A)
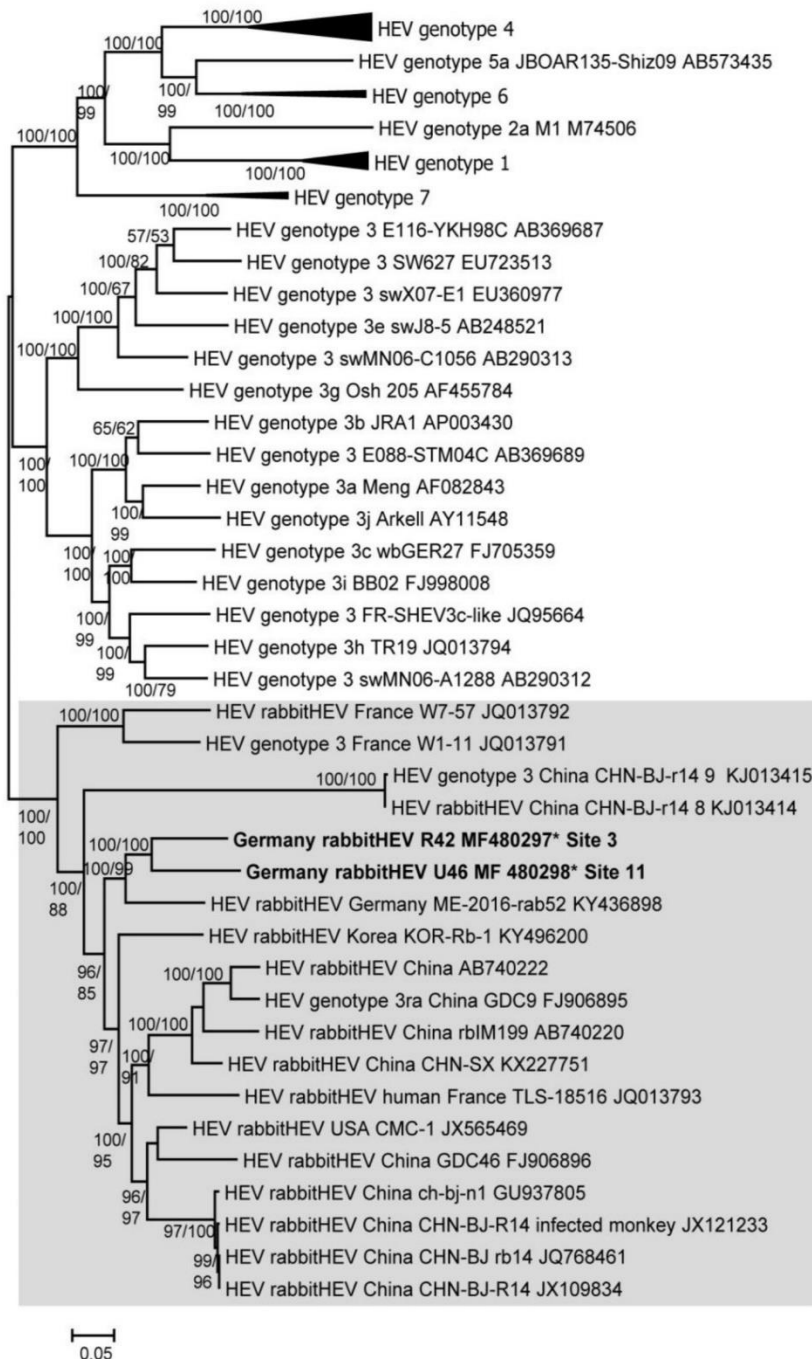
Figure S3 (continued)

B)
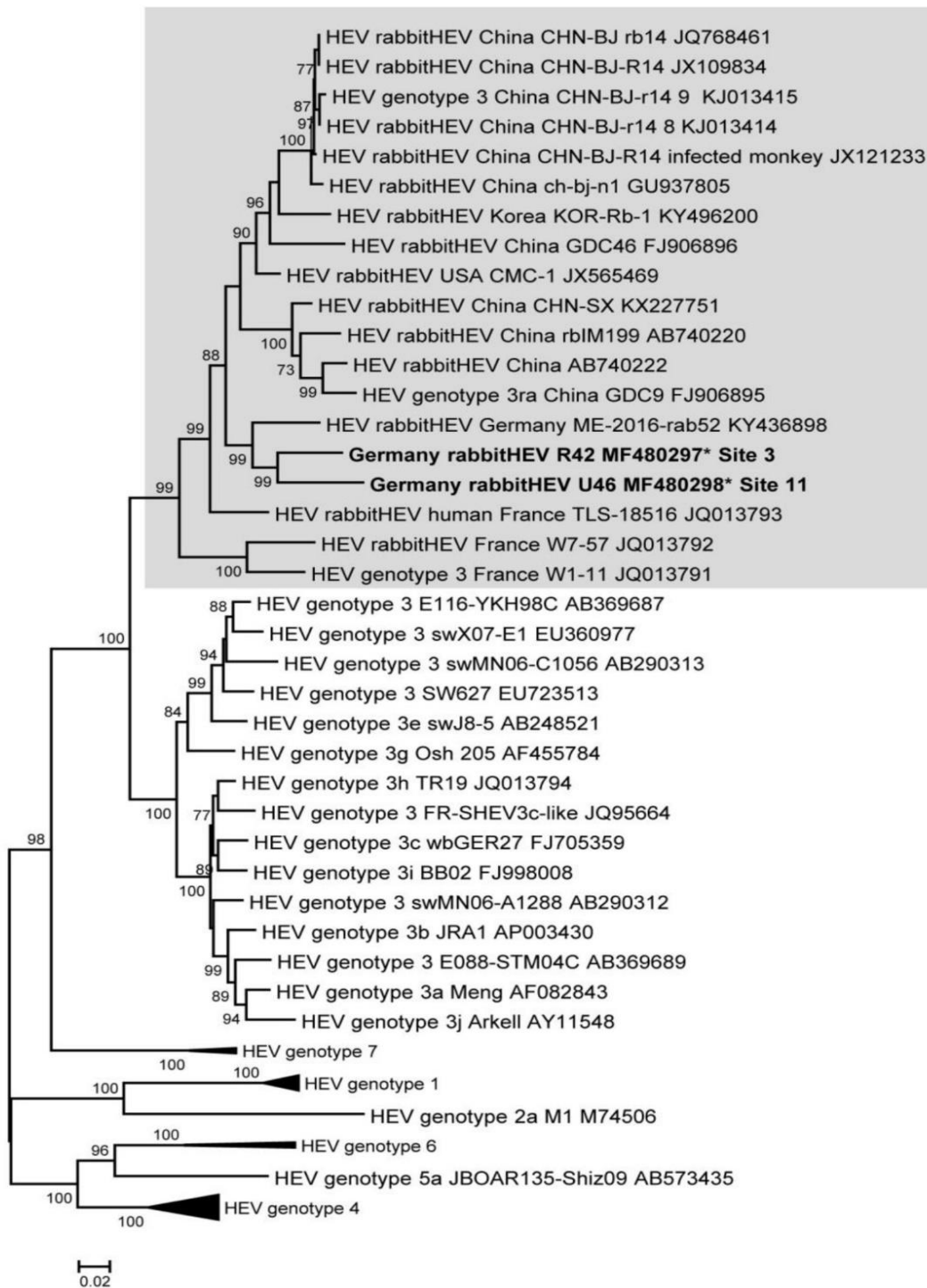
Figure S3 (continued)

C)
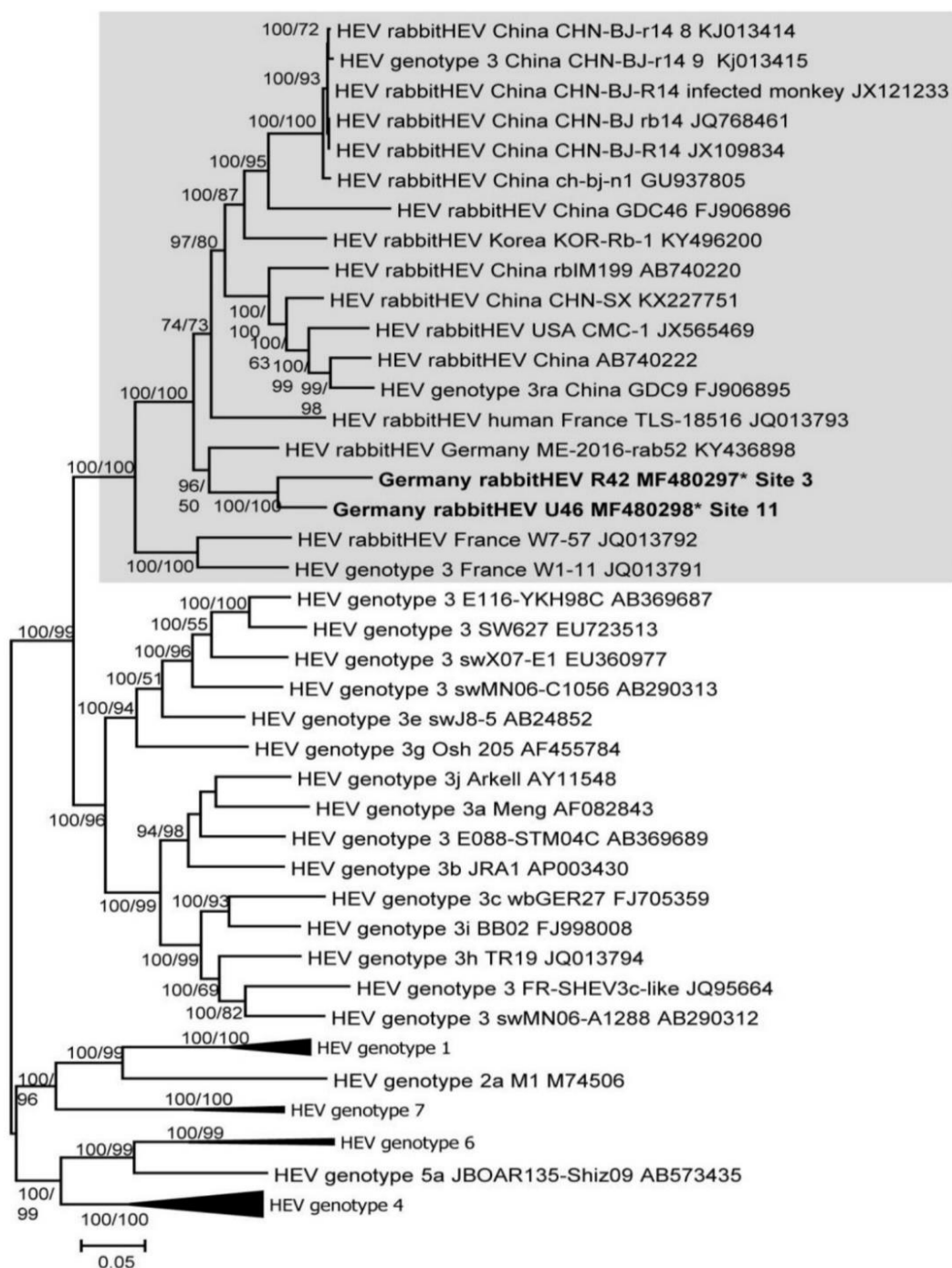
Figure S3 (continued)

D)
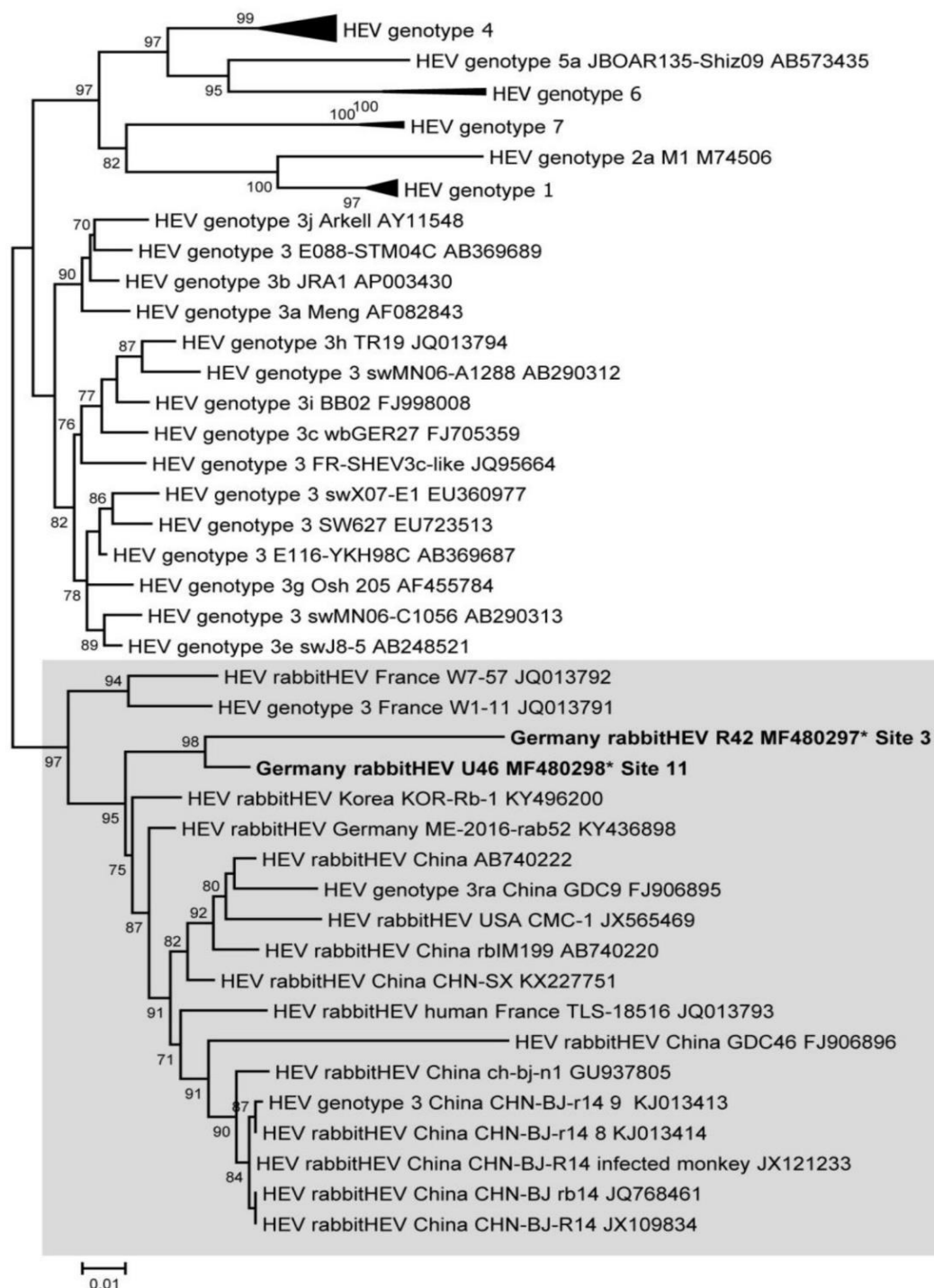
Figure S3 (continued)

E)
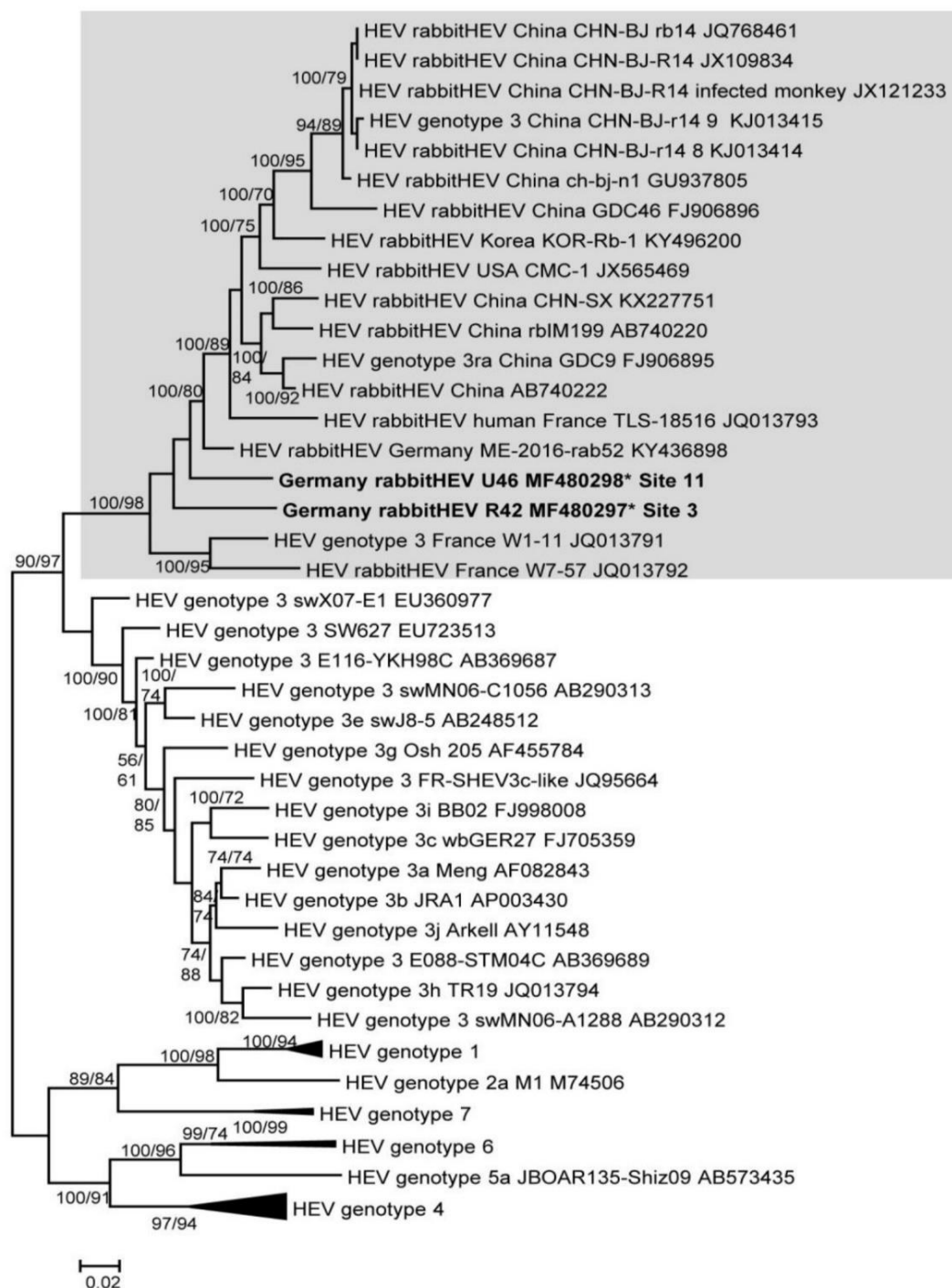
Figure S3 (continued)

F)
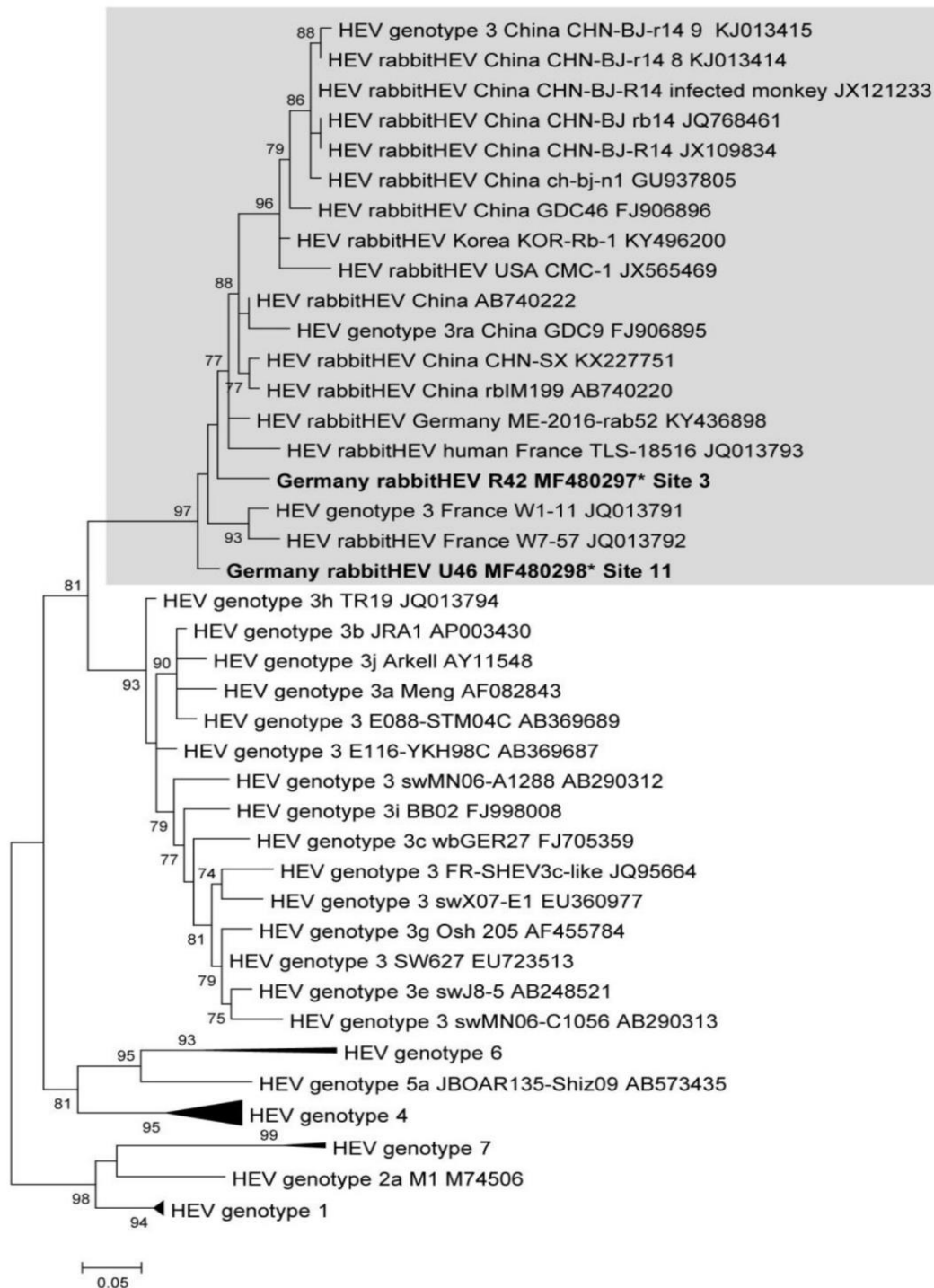
References:

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Own contribution to publications

Publication I

Ryll, R., Heckel, G., Corman, V. M., Drexler, J. F., Ulrich, R. G., 2019. Genomic and spatial variability of a European common vole hepevirus. Archives of Virology, submitted.

René Ryll:	Sample preparation (cutting liver samples), RNA extraction (pooled samples and liver samples), RT-PCR, molecular species determination by cytochrome *b* determination, sequencing, primer-walking approach (primer design and RT-PCR design), sequence analysis (generating of consensus sequences), statistical calculation, performed "isolation by distance", writing and proofreading of the manuscript, preparation of all figures and tables
Gerald Heckel:	Providing of vole samples, advise of "isolation by distance" and phylogenetic analyses, writing and proofreading of the manuscript,
Victor M. Corman:	Advice in RNA extraction and RT-PCR, proofreading of the manuscript
Jan Felix Drexler:	Design of the study, providing materials for RT-PCR and RNA extraction, advice in phylogenetic analysis, proofreading of the manuscript
Rainer G. Ulrich:	Design of the study, writing and proofreading of the manuscript

Publication II

Ryll, R., Bernstein, S., Heuser, E., Schlegel, M., Dremsek, P., Zumpe, M., Wolf, S., Pepin, M., Bajomi, D., Muller, G., Heiberg, A.C., Spahr, C., Lang, J., Groschup, M.H., Ansorge, H., Freise, J., Guenther, S., Baert, K., Ruiz-Fons, F., Pikula, J., Knap, N., Tsakmakidis, I., Dovas, C., Zanet, S., Imholt, C., Heckel, G., Johne, R., Ulrich, R.G., 2017. Detection of rat hepatitis E virus in wild Norway rats (*Rattus norvegicus*) and Black rats (*Rattus rattus*) from 11 European countries. Veterinary microbiology 208, 58-68.

René Ryll:	Coordination of rat collection and dissection, RNA preparation, RT-PCR, morphological and molecular species determination by cytochrome *b* determination, sequencing, phylogenetic and statistical analysis, writing and proofreading of the manuscript, preparation of all figures and tables
Samuel Bernstein:	Dissection of rats, RNA extraction, RT-PCR, sequence analysis, writing and proofreading of the manuscript
Elisa Heuser:	Coordination of rat collection and dissection, cytochrome b determination, proofreading of the manuscript
Mathias Schlegel:	Dissection of rats, proofreading of the manuscript
Paul Dremsek:	Dissection of rats, proofreading of the manuscript
Maxi Zumpe:	dissection of rats, RNA preparation, RT-PCR, proofreading of the manuscript
Sandro Wolf:	Establishment of novel RT-PCR protocol, proofreading of the manuscript
Michel Pépin:	Providing of rat samples, proofreading of the manuscript
Daniel Bajomi:	Providing of rat carcasses, proofreading of the manuscript
Gabi Müller:	Providing of rat carcasses, proofreading of the manuscript
Ann-Charlotte Heiberg:	Providing of rat carcasses, proofreading of the manuscript
Carina Spahr:	Collection and providing of rats and dissection, proofreading of the manuscript

Johannes Lang:	Collection and providing of rat carcasses, proofreading of the manuscript
Martin H. Groschup:	Proofreading of the manuscript
Hermann Ansorge:	Collection and providing of rat carcasses, proofreading of the manuscript
Jona Freise:	Collection and providing of rat carcasses, proofreading of the manuscript
Sebastian Guenther:	Collection and providing of rat carcasses, proofreading of the manuscript
Kristof Baert:	Collection and providing of rat carcasses, proofreading of the manuscript
Francisco Ruiz-Fons:	Collection and providing of rat samples, proofreading of the manuscript
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Stefania Zanet:	Collection and providing of rat samples, proofreading of the manuscript
Christian Imholt:	Statistical analysis, proofreading of the manuscript
Gerald Heckel:	Proofreading of the manuscript
Reimar Johne:	Proofreading of the manuscript
Rainer G. Ulrich:	Coordination of the project, providing of rat samples, writing and proofreading of the manuscript

Publication III

Spahr, C., **Ryll, R.**, Knauf-Witzens, T., Vahlenkamp, T. W., Ulrich, R. G., & Johne, R., 2017. Serological evidence of hepatitis E virus infection in zoo animals and identification of a rodent-borne strain in a Syrian brown bear. Veterinary microbiology, 212, 87-92.

Carina Spahr:	Serological investigations, providing of rat carcasses and dissection, writing and proofreading of the manuscript, preparation of all tables
René Ryll:	Dissection of rats, RNA extraction, RT-PCR, sequence analysis, phylogenetic analysis, writing and proofreading of the manuscript, preparation of figure
Tobias Knauf-Witzens:	Proofreading of the manuscript
Thomas W. Vahlenkamp:	Proofreading of the manuscript
Rainer G. Ulrich:	Writing and proofreading of the manuscript
Reimar Johne:	Coordination of the project, writing and proofreading of the manuscript

Publication IV

Ryll, R., Eiden, M., Heuser, E., Weinhardt, M., Ziege, M., Hoper, D., Groschup, M.H., Heckel, G., Johne, R., Ulrich, R.G., 2018. Hepatitis E virus in feral rabbits along a rural-urban transect in Central Germany. Infection, genetics and evolution 61, 155-159.

René Ryll:	RNA preparation, conventional RT-PCR, sequence analysis, phylogenetic analysis, writing and proofreading of the manuscript, preparation of all figures and tables
Martin Eiden:	Real-time RT-PCR investigation, proofreading of the manuscript
Elisa Heuser:	Serological analysis
Markus Weinhardt:	Trapping and dissection of rabbits, proofreading of the manuscript
Madlen Ziege:	Trapping and dissection of rabbits, proofreading of the manuscript
Dirk Höper:	High-throughput sequencing, proofreading of the manuscript
Martin H. Groschup:	Proofreading of the manuscript
Gerald Heckel:	Advice of phylogenetic analysis, proofreading of the manuscript
Reimar Johne:	Proofreading of the manuscript
Rainer G. Ulrich:	Coordination of the project, writing and proofreading of the manuscript

Obige Angaben werden bestätigt:

Unterschrift des wissenschaftlichen Betreuers

3. Results and Discussion

3.1. Detection and typing of HEV infections

3.1.1 Prevalence of anti-HEV antibodies and HEV-RNA detection rates

The majority of surveillance studies are based on serological investigations and indirectly reflect the occurrence of HEV (for review see (Spahr et al., 2018)). Serological investigations of several mammal species from Germany, including different zoo animals, and two rat species from European countries detected anti-HEV antibodies with different prevalences (**PAPER II-IV**). These studies were done by using a commercially available, broadly reactive double antigen sandwich anti-HEV antibody ELISA (Axiom Diagnostik, Bürstadt, Germany). This assay used a recombinant CP of HEV-1 and can be applied for different mammal species, but did not differentiate between IgM and IgG antibodies. HEV-RNA was detected in several small mammal populations from Germany (**PAPER I**, Figure 2; **PAPER II**, Figure 3 and **PAPER IV**, Figure 1) and Europe (**PAPER I**, Figure 2 and **PAPER II**, Figure 3). The in-house assay used for comparative studies is based on a truncated variant of ratHEV ORF2-encoded CP (Dremsek et al., 2012). Serological investigation of rabbits in other studies used antigens expressed by parts of the ORF2 and ORF3 of HEV-1 or HEV-3 (Table 1).

Presence of HEV-RNA was evaluated by different conventional and real-time RT-PCR protocols, namely three different conventional assays and two real-time RT-PCR assays (**PAPER I – PAPER IV**). The conventional assays are broad-spectrum RT-PCRs targeting regions within the ORF1 coding region (Drexler et al., 2012, Johne et al., 2012, Wolf et al., 2012), whereas the real-time assays are specific for genotypes HEV-1 to HEV-4 or ratHEV (Johne et al., 2012, Jothikumar et al., 2006).

*Table 1. **Rabbit HEV-RNA and anti-HEV antibody detection in wild, breeding and pet rabbits from different countries.***

Country	Type of population	RT-PCR results	HEV-RNA detection rate	HEV-genotype	Antibody reactivity	Seroprevalence	Reference
The Netherlands	pet	8/35	22.8%	HEV-3ra	n.d.	n.d.	Burt et al., 2016
	farm	0/10	0%	-	n.d.	n.d.	
	wild	23/62	37.1%	HEV-3ra	n.d.	n.d.	
Italy	farm	0/7	n.d.	-	7/206	3.4%	Di Bartolo et al., 2016
	pet	0/8	n.d.	-	8/122	6.5%	
Germany	wild	29/801	3.6%	HEV-3ra	51/139 (rabbit) 14/624 (hares)	36.7% (rabbit) 2.2% (hares)	Eiden et al., 2016; Hammerschmidt et al., 2017
France	farm	14/200	7%	HEV-3ra	n.d.	n.d.	Izopet et al., 2012
	wild	47/205	22.9%	HEV-3ra	n.d.	n.d.	
China	farm	212/2247	9.4%	HEV-3ra	546/1759	31%	J. Geng et al., 2011; Y. Geng et al., 2011; Jirintai et al., 2012; Xia et al., 2015b; Zhao et al., 2009
USA	farm	14/85	15.3%	HEV-3ra	31/85	36.4%	Cossaboom et al., 2011

n.d.: not determined

The investigation of different zoo animal species for anti-HEV antibodies by using the AXIOM ELISA revealed an average prevalence of 11.5% (28/244; **PAPER III**, Table 1). In contrast to the other investigated species, suid and carnivore species had a higher anti-HEV antibody prevalence of 33% and 27%, respectively (**PAPER III**, Table 1 and Supplementary Table 1). Different suid species most likely represent reservoir hosts for members of the species *Orthohepevirus A* and are therefore highly susceptible for HEV infection (Caruso et al., 2017; Johne et al., 2014a). In line with the high seroprevalence in carnivore species kept in zoos, investigations of different wild and domestic carnivore species from Brandenburg, Germany, including cats, dogs, raccoon and raccoon dogs, revealed an anti-HEV antibody prevalence of 53.8% (Daehnert et al., 2018; Table 2).

Table 2. Detection in different carnivore species populations from four countries.

Country	Species	Type of population	RT-PCR results	HEV-RNA detection rate	HEV-genotype	Antibody reactivity	Seroprevalence	Reference
The Netherlands	Red fox (*Vulpes vulpes*)	wild	2/13	15.4%	HEV-C2	n.d.	-	Bodewes et al., 2013
	Ferret (*Mustela putorius*)	pet	4/43	9.3%	HEV-C2	n.d.	-	Raj et al., 2012
Japan*	Ferret (*Mustela putorius*)	pet	6/85	7.1%	HEV-C2	n.d.	-	Li et al., 2015
	Mongoose (*Herpestes javanicus*)	wild	7/309	2.2%	HEV-3	21/100	21%	Nakamura et al., 2006, Nidaira et al., 2012
Denmark	Mink (*Neovison vison*)	farm	4/318	1.2%	HEV-C2	n.d.	-	Krog et al., 2013
	Mink (*Neovison vison*)	wild	0/89	0%	-	n.d.	-	
China	Dog** (not reported)	pet	0/101	0%	-	21/101	17.8%	Zhang et al., 2008

n.d.: not determined; *ferrets are nonindigenous in Japan and the investigated ferrets were imported from the USA, New Zealand and Canada or of unknown origin; ** specie of the investigated dogs were not reported by Zhang et al., 2008

In total, 8.1% (3/37) of the carnivore samples revealed detectable HEV-RNA (**PAPER III**, Supplementary Table 1). HEV-RNA was detected in one of two Bush dogs (*Speothos venaticus*), one of four Syrian brown bears and in one of five South American coati (*Nasua nasua*). The low detection rates in carnivore species from the zoos are in line with other studies, investigating the presence of HEV-RNA in carnivore species (Bodewes et al., 2013, Raj et al., 2012, Li et al., 2015, Nakamura et al., 2006, Nidaira et al., 2012, Krog et al., 2013, see Table 1). In addition to the detection of HEV-3 in wildlife Mongoose (*Herpestes javanicus*), HEV-4 was identified in an Asiatic black bear (*Selenarctos thibetanus*) and a Clouded leopard (*Neofelis nebulosa*) kept in zoos (for Review see Spahr et al., 2018).

The anti-HEV antibody prevalence in European rabbits from in and around Frankfurt am Main, Germany, reached approximately 35% (**PAPER IV**, Table 1). A slightly higher anti-HEV antibody prevalence was detected at the rural trapping site (38%, 13/34) in contrast to the urban trapping site (31.6%, 12/38) (**PAPER IV**, Table 1). The seroprevalence in

rabbits from the rural trapping site was similar to the seroprevalence from wildlife rabbits from the Netherlands (37.1%, 23/62), but the average seroprevalence was higher compared to other rabbits originating from wild life, breeding and pet populations (see Table 1). The analysis of rabbits by using a RT-PCR (Wolf et al., 2013) and a SYBR-green based RT-qPCR (Vina-Rodriguez et al., 2015) resulted in 25% (18/72; RT-PCR) and 19.4% (14/72; RT-qPCR) HEV-RNA positive animals (detection rate of 25%; **PAPER IV**, Table 1). All of the HEV-sequences were identified as rabbitHEV. The difference between the detection rate at the rural (26.5%, 9/34) and the urban (23.6%, 9/38) trapping sites was very low. The average HEV-RNA detection rate observed here is in line with a study in wild rabbits from the Netherlands, but differ to other studies with a larger number of investigated animals (see Table 1).

A subset of rats was investigated by the AXIOM ELISA and by ratHEV-specific ELISA. Only 3.1% (3/414) of Norway rat sera showed a specific reaction in the commercial ELISA (**PAPER II** and unpublished data). In contrast to these results, the analysis of Norway rats from different countries from Europe by an in-house ELISA, based on a ratHEV CP antigen, showed a prevalence of 28% (32/114, Bernstein, 2013). The AXIOM ELISA detected anti-HEV antibodies in only 5.2% (6/114) of the same rats. Only two animals showed a reactivity in both ELISAs. Thereby, the comparison of the results of the serological investigations suggested a higher sensitivity of the in-house ELISA than the AXIOM ELISA. In line, a serological study of Norway and Black rats from Lithuania by AXIOM ELISA detected no anti-HEV positive animals, whereas an in-house ELISA using a yeast expressed truncated ratHEV CP variant indicated a seroprevalence of 31.2% (Simanavicius et al., 2018). The reason for a lower sensitivity of the AXIOM ELISA (HEV-1 antigen) than that of the ratHEV antigen based ELISAs (HEV-C1 antigen) in detecting ratHEV-specific antibodies is the high aa sequence divergence of the CP between HEV-strains of the species *Orthohepevirus C* and *Orthohepevirus A* (40%, **PAPER I**, Supplementary Table 3). Therefore, the AXIOM ELISA is not suitable for highly sensitive serological detection of antibodies raised against non-*Orthohepevirus A* species strains in rodents.

HEV-RNA detection in rats (Norway and Black rats) was done by using two RT-PCR assays targeting the RdRP coding region of the ORF1 of HEV (**PAPER II**, Figure 2), i.e. a one-step RT-PCR (Wolf et al., 2013) and a broadly reactive nested RT-PCR (Johne et al., 2010b). In total, 12.4% (63/508) of the investigated rats were positive for HEV-RNA by at

least one of RT-PCR assays (**PAPER II**, Table 2). The detection rate of the nested RT-PCR (9%, 20/221) was slightly higher than that of the one-step RT-PCR (7.7%, 17/221) (**PAPER II**, Table 2). The detection rates of ratHEV-RNA observed here are in line with investigations of rats from eight other countries (Table 3). Similar results were obtained during the HEV-RNA testing of three carnivore species. The nested RT-PCR was able to detect HEV-RNA in one additional sample compared to the one-step RT-PCR (**PAPER III**, Supplementary Table 1). These discrepant results might be explained by the higher sensitivity of the nested RT-PCRs to amplify even low amounts of viral RNA.

*Table 3. **Results of ratHEV RT-PCR and anti-HEV antibody detection in rats from eight countries.***

Country	RT-PCR results	ratHEV-RNA detection rate	Antibody reactivity	Seroprevalence	Reference
China	12/59	20.3%	166/713	23.3%	W. Li et al., 2013
Vietnam	1/5	20%	n.d.	n.d.	T. C. Li et al., 2013b
Indonesia	99/611	16.2%	168/611	27.5%	Mulyanto et al., 2013; Mulyanto et al., 2014; Primadharsini et al., 2018
France	12/81	14.8%	n.d.	n.d.	Widen et al., 2014
Germany	19/183	10.4%	36/153	23.5%	Johne et al., 2012; Johne et al., 2010a; Johne et al., 2010b
Denmark	1/11	9.1%	n.d.	n.d.	Wolf et al., 2013
Lithuania	9/109	8.3%	13/109	11.9%	Simanavicius et al., 2018
USA	2/134	1.5%	105/134	78.3%	Purcell et al., 2011

n.d. not determined

Initial testing of nearly 3000 rodents by another broadly reactive, nested ORF1-specific RT-PCR (Drexler et al., 2012) resulted in the detection of HEV-RNA in 13 common voles (*Microtus arvalis*) and one bank vole (*Myodes glareolus*) (**PAPER I**, Table 1). The obtained HEV-sequences shared the highest similarities towards an unassigned kestrel-derived HEV-strain, obtained from a faecal sample of a bird of prey (Reuter et al., 2016a), and HEV-sequences obtained from common voles from Hungary (Kurucz et al., 2018). Phylogenetic analysis of the common vole associated HEV (cvHEV) showed that the cvHEV-sequences formed a well-separated clade, next to the proposed genotype HEV-C4. Investigation of a subset of 181 common voles from 19 different trapping sites, with HEV-

RNA positive common voles or from those close to a HEV-RNA positive trapping site, by the commercial ELISA revealed a seroprevalence of 5.5% (10/181) of the investigated common voles (**PAPER I**, unpublished data; see Table 4).

*Table 4. **Comparison of the detection rate of HEV-RNA and anti-HEV antibody seroprevalence in Common voles and Norway rats from Germany.***

Species	Trapping site	HEV-RNA detection rate	anti-HEV seroprevalence*	Reference
Common vole	Weissach	1/24 (4.2%)	4/24 (16.6%)	**PAPER I**
	Stuttgart	0/1	1/1 (100%)	
	Altdöbern	0/14	1/14 (7.1%)	
	Rutesheim	0/15	2/15 (13.3%)	
	Bergatreute	0/3	1/3 (33.3%)	
	Falkenstein	2/32 (5.3%)	1/32 (3.1%)	
Subtotal	6 sites	3/89 (3.3%)	10/89 (11.2%)	
Norway rat	Ahlen	0/21 (0%)	0/21 (0%)	Johne et al., 2012
	Hamburg	6/17 (35.3)	7/17 (41.2%)	
	Stuttgart	1/34 (2.9%)	5/34 (14.7%)	
	Esslingen	1/14 (7.1%)	3/14 (21.4%)	
	Berlin	7/61 (11.5%)	21/61 (34.4%)	
Subtotal	5 sites	15/147 (10.2%)	36/147 (24.5%)	

*: Common voles were investigated for anti-HEV antibodies by using a commercially available ELISA (AXIOM, HEV-1 antigen) and the Norway rats were investigated by an in-house ratHEV-specific ELISA (HEV-C1 antigen)

Commonly used ELISAs for serological detection of anti-HEV antibodies are using different antigens (truncated variant of ratHEV ORF2-encoded CP or parts of the ORF2 and ORF3 of HEV-1 or HEV-3). Thereby, cross-reactivities of the antigens were observed, like a high discrepancy between the broadly reactive AXIOM ELISA and an in-house ELISA for the detection of rodent-associated HEV-specific anti-HEV antibodies. Thus, the use of the commercially available AXIOM ELISA seems to be not suitable for a highly sensitive detection of anti-HEV antibodies among different rodent species.

The studies indicated differences in the sensitivity of the different types of RT-PCRs (one-step, two-step/nested and real-time) all targeting a similar region of the RdRP encoding region of the ORF1 of HEV for detecting HEV-RNA. In total, the nested RT-PCRs seems

to perform better by detecting more HEV-RNA positive animals but they are also more time consuming due to a second round of PCR step and more vulnerable to contamination. None of the used RT-PCRs were able to detect all HEV-RNA positive individuals, even by amplifying within a highly conserved region within the HEV genome.

3.1.2. Identification of the causative agent by serological and molecular methods

As mentioned above, the surveillance of HEV reservoirs and humans is mainly done by serological assays (Khudyakov and Kamili, 2011). These investigations frequently use assays with standard antigens, mainly ORF2-derived CP derivatives, exploiting the high cross-reactivity of CP-reactive antibodies. This high cross-reactivity of HEV-reactive antibodies is explained by the antigenic similarity of HEV genotypes, which represent a single serotype (Wang et al., 2013). As a consequence, the differentiation of antibody reactivities is rather complicated. For the differentiation of ratHEV and HEV-3 specific antibodies, two homologous ELISAs, that allowed the typing of antibody reactivities, were developed (Dremsek et al., 2012, 2013). A limitation of this typing is, that the antibody specificities can be typed only to the antigens used in the assays. As a prerequisite, the aa sequence divergence between the used antigens must be high enough to allow the differentiation. Comparing the CP encoding aa residues of representative members of the family *Hepeviridae* (Smith et al., 2014; Smith et al., 2016), including proposed genotypes (Wang et al., 2018; Woo et al., 2016), the differences range from 32.7% (*Orthohepevirus A* compared with *Orthohepevirus C*) to 53.6% (*Orthohepevirus B* compared with *Orthohepevirus C*). Therefore, the detection of ratHEV reactive antibodies in forestry workers (Dremsek et al., 2012) might be alternatively also explained by infection of the forestry workers with related, rodent- or carnivore-borne HEV, such as the novel cvHEV (Bodewes et al., 2013; Johne et al., 2012; Kurucz et al., 2018; Raj et al., 2012, **PAPER I**).

During acute infection or in persistently infected reservoirs a molecular identification ("typing") and even the molecular epidemiological evaluation of the source of infection might be possible. Using conventional RT-PCR assays targeting a highly variable region or by the use of highly specific real-time RT-PCR assays this aim could be reached. Thus, the differentiation of ratHEV and HEV-1 - HEV-4 infections of rats and zoo animals was evaluated by two RT-qPCR assays (**PAPER II** and **PAPER III**, Table 2). Alternatively, a broad spectrum (one-step) RT-PCR assay or two broad spectrum nested (two-step) RT-

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PCR assays, with subsequent sequence determination of the amplicons, were used (**PAPER I** and **PAPER II,** Table 2, and **PAPER III**, Table 1). By using two types of RT-PCRs (RT-qPCR and one-step RT-PCR) rabbitHEV-RNA in rabbits and ratHEV in rats were almost exclusively detected (**PAPER II**, Figure 3B, PAPER IV, Figure 2A). However, in addition to ratHEV sequences, the use of the broad-spectrum RT-PCR detected a rabbitHEV-like sequence in a Norway rat from Belgium, indicating the first finding of a rat being infected with a zoonotic HEV-strain (HEV-3ra) in Europe (**PAPER II**, Figure 3 C). The RT-qPCR failed to detect this strain in the Norway rat (**PAPER II**, Table 2). Additionally, by one-step and two-step RT-PCR assays, a spillover infection of a Syrian brown bear with ratHEV was detected, but not by the ratHEV-specific RT-qPCR.

The most sophisticated typing can be achieved by complete genome sequence determination. Initially, a shot-gun HTS approach was applied to determine the complete genomes of rabbitHEV in rabbit samples. Unfortunately, this approach resulted only in the identification of four regions with a coverage of 1000 nt (**PAPER IV**). Therefore, a primer-walking approach was used and allowed the determination of complete rabbitHEV sequences (**PAPER IV**) and of novel complete cvHEV sequences (**PAPER I**). A very low viral RNA copy number might cause the failure in the HTS approach. The complete genome determination of cvHEV strains from different common vole trapping sites allowed a precise molecular phylogenetic analysis. Based on this, a previously found kestrel-derived HEV sequence was concluded to be originated from an infected common vole (Reuter et al., 2016, **PAPER I**, Figure 2B). Interestingly this conclusion was not only possible with the complete genomes of kestrel-derived HEV and cvHEV, but also when analyzing an ORF1-derived short sequence (**PAPER I**, Figure 2A). Similarly, ratHEV trees with nearly the same topology were obtained by phylogenetic analysis of a 1122 nt fragment and the screening fragment of 280 nt of the genome (**PAPER II**, Figure 2 and **PAPER II**, Figure 3B and D). This observation is in line with a study on the topology of phylogenetic trees for HEV-3 strains. Analyzing fragments from the HVR (263 nt or 293 nt), RdRP (330 nt) or ORF2 (241 nt) resulted in phylogenetic trees with the same topology like using the whole genome (Vina-Rodriguez et al., 2015).

For sequence-based typing it is very important to exclude regions with overlapping reading frames, as here selection pressure on two coding sequences is different from that on a single non-overlapping reading frame and may thereby influence the phylogenetic relationships (Holmes, 2009). This was most obvious when comparing the topology of phylogenetic trees

of the complete ORF2 and ORF3 encoded aa sequences of HEV (see **PAPER IV**, Figures 3C-F).

Finally, the molecular characterization of the novel cvHEV genomes identified multiple insertions/deletions (InDels) that might be also used in the future for a molecular typing, at least for cvHEV (**PAPER I** Figure 4B and 4C).

3.2. Host association, persistence and spillover infections

The definition of a "host" or even a "reservoir host" is difficult and usually approached by field studies and/or experimental studies *in vitro* and *in vivo* and may differ between pathogens (Jones et al., 2008; Sawyer and Elde, 2012). A try to generally define a "reservoir host" was given by Olival et al, e.g. (i) isolation of the pathogen from the host, (ii) significant higher detection rates of nucleic acid in a proportion of individuals from a population or (iii) detection of antibodies in a significant number of individuals from the target population (Olival et al., 2012). The problems of "host" definition can be illustrated by two examples, Puumala orthohantavirus (PUUV), a member of the family *Hantaviridae* and rabies virus (RABV), a member of the family *Rhabdoviridae* (Walker et al., 2018). For hantaviruses the definition of a reservoir host is based on the molecular detection rate of the virus with higher frequency in the putative reservoir host than in sympatrically occurring other species (Hjelle and Yates, 2001). Furthermore, the pathogen must have the ability to cause a persistent infection in the reservoir host (Schonrich et al., 2008), with lifelong shedding (Villarreal et al., 2000), but should not cause obvious disease (Schonrich et al., 2008). This definition of a reservoir host for hantavirus is in line with a previous try to define a reservoir host in general (Olival et al., 2012). Thus, the bank vole represents the only reservoir of PUUV (Vapalahti et al., 2003). In contrast, RABV infection leads to the death of the host (World Health Organization, 2018). The RABV has a wide range of reservoir hosts among different mammal species (Rupprecht et al., 1995) and as potential, original reservoir host, bats were identified (Hurst and Pawal, 1931). Experimental infections of different bat species showed the susceptibility of the bats to RABV, the typical clinical symptoms and the ability of transmitting and even surviving a RABV infection (Baer and Bales, 1967, Jackson et al., 2008) in contrast to other mammal reservoir hosts (Rupprecht et al., 1995). A potential explanation of these observations is the assumption that the original reservoir of RABV are bats and the virus was more recently transferred to carnivores where it causes lethal cases (e.g. Ethiopian wolves) (Badrane and Tordo, 2001; Sillero-Zubiri et al., 1996).

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The definition of a reservoir association of hepeviruses was also tried by HEV analysis in field studies and cross-species infection experiments. For some members of the family *Hepeviridae* a narrow host range was determined, but for other members a wide host range is assumed (Johne et al., 2014a). Examples for host-specific hepeviruses are the genotypes HEV-1 and HEV-2, being restricted to humans as reservoir hosts. On the other side, members of the genotypes HEV-3 and HEV-4 were detected in humans, pigs, wild boar, deer, mongoose and rats (Meng, 2013).

The novel cvHEV was detected exclusively in common voles, but not in sympatrically occurring other rodents (**PAPER I**, Kurucz et al., 2018). Supporting this observation, multiple molecular detection in common voles at a geographical range of approximately 800 km were detected (**PAPER I**, Kurucz et al., 2018). Sequence comparison, phylogenetic analysis and "isolation-by-distance" investigation confirmed a persistence in local populations and evolution on a local scale (**PAPER I**).

The host association of rabbitHEV to the European rabbit is confirmed by a large number of field studies (Table 1) and experimental investigations (Cheng et al., 2012; Ma et al., 2010, see also 3.3). The field study in Frankfurt am Main and its surrounding (**PAPER IV**) and all other field studies, revealed exclusively rabbitHEV in the rabbits investigated (Table 1). Comparison of rabbit-associated sequences from different geographic origins indicated a well-separated clade of related rabbitHEV sequences (HEV-3ra) (Zhao et al., 2009). The successful infection of pigs with two strains of rabbitHEV, one obtained from China and one obtained from the USA, shows the ability of rabbitHEV to cross the species barrier. The infected pigs developed a transient viremia and sporadic fecal shedding (Cossaboom et al., 2012). Besides different animal experiments, also cell culture-based infection experiments were done with rabbitHEV. A successful propagation of rabbitHEV, obtained from farmed rabbits in China, was shown in human lung cancer cell line A549 and in the human hepatoma carcinoma cell line PLC/PRF/5 (Jirintai et al., 2012). The zoonotic character of rabbitHEV was shown by successful experimental infections of non-human primates (Liu et al., 2013) and the detection of rabbitHEV related sequences in both, rabbits and several acute and chronically infected humans in France (Abravanel et al., 2017; Izopet et al., 2012). Parallel analysis of anti-HEV antibodies and HEV-RNA revealed for the majority of rabbits the presence of antibodies or RNA, only in a small proportion of animals both was detected (**PAPER IV**, Table 2). The parallel detection of viral RNA and specific antibodies might indicate that a few animals become (at least temporally) persistently

infected; alternatively, this finding might be interpreted as a late acute phase of the infection, where RNA is still detected (Schlosser et al., 2018). The exclusive detection of rabbitHEV RNA or rabbitHEV-reactive antibodies might be seen as a non-persistent infection with virus clearance. *In vivo* studies showed a seroconversion of rabbits, being infected with rabbitHEV, after three months (Ma et al., 2010) and a clearance of the virus (Cheng et al., 2012; Zhang et al., 2015).

In contrast to the situation for cvHEV or rabbitHEV, the reservoir of ratHEV is more difficult to identify as ratHEV was found in different rat species, including Norway rat, Black rat, Lesser rice-field rat, Yellow-breasted rat and Bandicoot rat (**PAPER II**, and Supplementary Table 1). The detection of similar sequences in Norway and Black rats suggest a reservoir function of both and did not indicate a rat species specific separate evolution of ratHEV (**PAPER II** and Simanavicius et al., 2018). The subgenotypes defined by Mulyanto follows a spatial distribution, independently from the reservoir rat species (**PAPER II**, Mulyanto et al., 2014). Related sequences were identified in rats from different parts of the world, like in rats from USA (*Rattus norvegicus*), Vietnam (*R. norvegicus* or *Rattus tanezumi*), Denmark (*R. norvegicus*), France (*R. norvegicus*), China (*R. norvegicus*, *Rattus flavipectus*, *Rattus rattoides losea*), Indonesia (*Rattus rattus*) and Lithuania (*R. norvegicus*/ *R. rattus*), indicating a strong host association of ratHEV towards members of the genus *Rattus* and showing its broad geographical distribution (T. C. Li et al., 2013b; W. Li et al., 2013; Mulyanto et al., 2013; Mulyanto et al., 2014; Purcell et al., 2011; Simanavicius et al., 2018; Widen et al., 2014; Wolf et al., 2013, see, Supplementary Table 1). Successful experimental ratHEV infection to laboratory rats, but lacking replication in other mammals confirmed the natural host association of ratHEV (Cossaboom et al., 2012; Johne et al., 2014a; T. C. Li et al., 2013c). The multiple detection of ratHEV sequences in different rodent species makes the reservoir host identification even more complicated. Thus, the virus was detected in bandicoot rats (*Bandicota indica*) and even Asian musk shrew (*Suncus murinus*) in China suggesting an even broader host range or frequent spillover infections of ratHEV (Guan et al., 2013; W. Li et al., 2013). Prior to our study (**PAPER II**), it was reported only for Black rats from Indonesia to be infected with ratHEV, whereas in USA Norway and Black rat populations were found the be infected with a zoonotic HEV-3 strain, indicating the susceptibility of Black rats for members of the species *Orthohepevirus A* and *Orthohepevirus C* (Kanai et al., 2012; Lack et al., 2012; Mulyanto et al., 2013).

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In line with the latter assumption Norway rat faeces was found to contain human pathogenic norovirus and extended spectrum β-lactamase producing enterobacteria (Guenther et al., 2012; Wolf et al., 2013).

None of the seropositive common voles was HEV-RNA positive, suggesting a clearance of a cvHEV infection. Interestingly, the detection rates for HEV-RNA are in rat and vole species lower compared to the detection rate of anti-HEV antibodies suggesting a non-persistent infection with HEV in both species (Table 4). Currently, it is unclear if the virus is causing a persistent infection in common voles or if the persistence in populations is driven by a high stability of the pathogen in the environment. Thermal stability studies on other hepeviruses suggested a high stability and infectivity at different temperature – time points, helping to estimate the stability of hepeviruses in environment and food (Johne et al., 2016, Schielke et al., 2011). Additionally, infection experiments showed a potential route of transmission of HEV by the environment (Andraud et al., 2013).

An initial longitudinal study indicated a persistence of ratHEV in the Norway rat populations (Johne et al., 2012). Similarly, Norway rats at the zoo where the bear got infected were investigated during years 2009-2010 and 2010-2016 confirming the persistence of ratHEV in the local population (**PAPER III**, Supplementary Table 1). A comparison of the results of serological and RT-PCR investigations suggest, however, that the infection in Norway rats is not persistent (**PAPER II** and Johne et al., 2012). This conclusion is in line with results of *in vivo* studies, which showed that laboratory rats are susceptible for ratHEV, showing no persistence, but a clearance of the virus and a seroconversion (T. C. Li et al., 2013c; Purcell et al., 2011, Schlosser et al., 2018).

The detection of a cvHEV sequence in a bank vole might be explained by a spillover infection by common voles that occur sympatrically with the bank vole (**PAPER I**, Figure 1, Figure 2A, Table 1). Interestingly, a cvHEV-related sequence was found in a faecal sample of a kestrel earlier. A pairwise comparison of the complete genome of the kestrel-derived sequence with the five common vole derived complete genomes and phylogenetic analysis of sequences confirmed this high sequence similarity. This similarity is also evidenced by specific attributes common to all these sequences, i.e. a noncanonical start codon (UUG encoding for leucine) for the ORF3 encoded protein and the absence of a putative ORF4, previously found in other genera of the species *Orthohepevirus C* (Johne et al., 2014a). Therefore, it is most likely that the kestrel was affected by a spillover infection of cvHEV; common voles are the usual diet of birds of prey like the kestrel (Steen

et al., 2011). The authors describing this infection already hypothesized this dietary origin of the infection (Reuter et al., 2016a).

The high seroprevalence observed in carnivore species might indicate that they were (i) infected by consuming HEV-infected animals or alternatively (ii) a carnivore specific HEV-strain exists, which is suitable to be detected by a broadly reactive ELISA. In favor with the first assumption (i), the seroconversion of a Syrian brown bear was shown and (rat)HEV-RNA was detected, indicating a self-limiting, spillover infection with (rat)HEV in a carnivore species in captivity (**PAPER III**, Figure 1B and Supplementary Table 1). To find the possible source of this HEV infection, feeder rats and free-living pest rats from the zoo were investigated and ratHEV-RNA was only detected in three free-living wild rats (**PAPER III**, Supplementary Table 2). The potential route of transmission of ratHEV to the Syrian brown bear might be explained by the hunting and eating of a ratHEV-infected, free-living wild rat from the same zoo. Besides the detection of a rodent associated HEV-strain in a bird of prey (Reuter et al., 2016a), this is the first description of a carnivore species to be (spillover) infected by a rodent associated HEV-strain.

Recently, ratHEV spillover infections were documented in an immunocompromised and in an immunocompetent human. Therefore, the zoonotic potential of ratHEV seems to be evident, although experimental infections to non-human primates failed (Andonov et al., 2019; Sridhar et al., 2018, see 3.3).

In general, the investigations on ratHEV, cvHEV and rabbitHEV suggest a local evolution of these pathogens. This was evidenced by results of extensive phylogenetic investigations (**PAPER I**, Figure 2A, **PAPER II**, 3B, **PAPER III**, Figure 1B and **PAPER IV**, Figure 2A) and for cvHEV by an "isolation by distance" analysis (**PAPER I**, Figure 3). In addition, to the local evolution of ratHEV, there were indications for an incursion or invasion of rats in certain rat populations, e.g. single ratHEV positive rats originating from Austria, Belgium and France did not cluster together with other ratHEV sequences from rats from the same trapping site like the majority of ratHEV from Austria or Czech Republic (**PAPER II**, Figure 3B and 3D). To clarify the potential invasion of ratHEV-infected rats phylogeographic studies of the potential hosts are needed, e.g. by analysis of the mitochondrial DNA (mtDNA) (Avise et al., 1987) like the cytochrome *b* gene (cyt *b*) (Johns and Avise, 1998) or cytochrome *c* oxidase 1 (COX 1) (Hebert et al., 2003) and nuclear DNA (Shaw, 2002). Similarly, the results of the rabbitHEV investigations may suggest an

invasion of rabbits from the surroundings of Frankfurt am Main to the city area, which results in a founder population; the high sequence similarity of rabbitHEV strains in the city area might support this bottleneck event for the rabbit population (**PAPER IV**). Phylogenetic analysis of rabbitHEV complete genomes, using the concatenated ORF1/ORF2, showed a clustering of a "European rabbitHEV clade" and an "Asian rabbitHEV clade" (**Paper IV**, Figure 2 B). This might indicate a local evolution of different lineages of rabbitHEV.

Additionally, a high discrepancy between the detection rates of HEV-RNA and anti-HEV antibodies was observed. In all our studies, the detection rate of HEV-RNA was lower in comparison to the prevalence of anti-HEV antibodies (**PAPER I,** unpublished data and Table 1; **PAPER II,** unpublished data and Table 2, Bernstein 2013; **PAPER III**, Table 1 and Supplementary Table 1; **PAPER IV**, Table 1). This leads to the assumption, that HEV causes a non-persistent infection in their suspected reservoir hosts, although rarely persistent/chronic infections may occur (Schlosser et al., 2014).

3.3. Cell culture and animal models

Currently available cell culture models for HEV are based on tumor cell lines of human origin – PLC/PRF/5, HuH-7 and HepG2 (Jirintai et al., 2014; Tanaka et al., 2007). Rodent cell lines can be generated in different ways, by spontaneous immortalization or by SV-40 mediated immortalization (Eckerle et al., 2014a; Eckerle et al., 2014b). Thus, a vole-derived permanent cell line was generated (Essbauer et al., 2011).

Human PLC/PRF/5 and A549 cell lines were used to evaluate the replication of rabbitHEV by *in vitro* studies confirming its zoonotic potential (Jirintai et al., 2012). In addition, *in vitro* experiments showed an efficient replication of ratHEV (from Black rats) in human hepatoma carcinoma cell lines PLC/PRF/5, HuH-7 and HepG2 cells (Jirintai et al., 2014), but no replication was seen by inoculating rat liver cell lines and human lung cancer cell line A549 (Jirintai et al., 2014; Johne et al., 2010b).

Histopathological and immunohistochemistry investigations in ratHEV-infected rats and in HEV-3 infected wild boar or pig– revealed certain cell types as target for HEV, namely Kupffer stem cells, liver sinusoidal endothelial cells, lymphocytes, plasma cells and/or stellate cells (de Deus et al., 2008; Johne et al., 2010a; Lee et al., 2009; Schlosser et al., 2014; Williams et al., 2001). So far, these cell types have not been evaluated for their susceptibility for HEV infection *in vitro*.

Interestingly, HEV strains from chronically infected patients were found to replicate more efficiently in human tumor cell lines (Johne et al., 2014b; Shukla et al., 2012). Complete genome analysis of these strains indicated in-frame insertions within the ORF1 region; the place and length of the insertions were found to be similar (Johne et al., 2014b; Shukla et al., 2012). The rabbitHEV strains described here (**PAPER IV**) also have typical insertions within the ORF1 region (Zhao et al., 2009) and might be therefore interesting to be tested in the *in vitro* system. Similarly, the novel cvHEV strains demonstrated a genomic plasticity within the ORF1 region containing multiple InDels (**PAPER I**).

Animal models are an important tool to study the disease (animal disease model), the persistence and transmission of its causative agent (reservoir model) and to develop and evaluate anti-HEV vaccines and therapies (Krawczynski et al., 2011; Schlosser et al., 2018). Currently, different animal models were established to prove the zoonotic potential (see chapter 1.1.3), study pathogenesis or evaluate antiviral strategies.

For mammal associated HEV strains several animal models were established. A fish HEV (CTV)/ fish-based system was used to study antiviral therapies, but might be limited in its application (Debing et al., 2013). For zoonotic genotypes HEV-3, HEV-4 and HEV-7 pig, wild boar and non-human primate models were established (Krawczynski et al., 2011). Alternatively, to pigs and wild boars, minipigs were recently introduced as potential model animals (Schlosser et al., 2014). Non-zoonotic genotypes HEV-1 and HEV-2 do not replicate in rats and pigs, therefore here only non-human primates can be used for pathogenicity studies (T. C. Li et al., 2013c; Maneerat et al., 1996; Meng et al., 1998; Purcell et al., 2011; Purcell et al., 2003; Krawczynski et al., 2011).

Rabbits seems to be the most promising candidate animal model to study various aspects of HEV pathogenesis and evaluation of antiviral strategies. A successful infection of rabbits with rabbitHEV (Chinese strain) and with human derived HEV-4 was shown, but not with a human derived HEV-1 strain (Ma et al., 2010). To prove the transplacental/vertical transmission of human related HEV strains, pregnant rabbits were inoculated with rabbitHEV and the transplacental transmission of rabbitHEV from the infected females to their unborn babies was shown (Xia et al., 2015a). Furthermore, rabbits were used for evaluation of the protective potential of the only available vaccine for HEV, Hecolin against rabbitHEV and HEV-4 challenge (Zhang et al., 2015). Additionally, it was noted

that all unvaccinated rabbits developed hepatitis, with liver enzyme elevations, viremia and fecal virus shedding (Liu et al., 2014; Zhang et al., 2015).

Laboratory rats (*R. norvegicus*) are frequently used as animal model. Inoculation of Wistar rats (*R. norvegicus*) with a stool solution from a HEV-positive patient (HEV-1) demonstrated the susceptibility of rats for human derived HEV (T. C. Li et al., 2013c). Human derived HEV-RNA was found in various tissues of the inoculated rats indicating that the virus replicated efficiently in these rats (Maneerat et al., 1996). Moreover, the successful infection of Sprague-Dawley rats (*R. norvegicus*) with an infectious cDNA clone of swine-derived HEV-4 (strain SAAS-FX17) supports the potential role of rats as a model organism for HEV (Zhu et al., 2013). Under laboratory conditions rats were not susceptible to HEV-3 (T. C. Li et al., 2013a; T. C. Li et al., 2013c; Purcell et al., 2011) but under natural conditions, rats were identified to harbor zoonotic HEV-3 strains (Kanai et al., 2012; Lack et al., 2012; **PAPER II**).

Common voles are already in use as an animal model for the investigation of cowpox virus (Hoffmann et al., 2015), *Francisella tularensis* (Rossow et al., 2014) or tick-borne encephalitis virus (Achazi et al., 2011) and could serve therefore as an animal model for cvHEV as well. Different linages of common voles, as they are present in Europe (Beysard and Heckel, 2014), should be taken into account, when establishing an animal model for cvHEV by using common voles.

In conclusion, the strains of ratHEV, rabbitHEV and cvHEV detected here (**PAPER I – PAPER IV**) represent promising candidates for the development of suitable animal models. This includes the four clades of cvHEV (**PAPER I**, Figure 2 A), and the highly divergent ratHEV strains decribed here (**PAPER II** and **PAPER III**) as well as the divergent rabbitHEV strains described here (**PAPER IV**) and by others (Hammerschmidt et al., 2017). Furthermore, the detection of ratHEV in ratHEV-RNA positive populations could be very promising to gain ratHEV-RNA to allow future broad screening of strains with a higher *in vitro* replication capacity. In some rat populations a persistence of ratHEV was already detected (Johne et al., 2012, **PAPER II** and **III**).

4. Outlook

The recent finding of the zoonotic potential of ratHEV (Andonov et al., 2019; Sridhar et al., 2018) together with the here described broad geographical distribution of this virus in rats from Europe indicate the necessity of future molecular and serological surveillance and case control studies in the human population. As the HEV infection is usually rapidly cleared, the differentiation of the causative agent of a hepatitis E needs the improvement of existing diagnostic tools or the development of novel ones. Previous studies have indicated that antibodies against HEV-3 and ratHEV can be differentiated by the selection of the homologous and heterologous antigens (Dremsek et al., 2012; Shimizu et al., 2016). Results from real-time RT-PCR investigations illustrated the problems in the sensitivity of the assays for detection of selected HEV-3 or ratHEV strains. And finally, standardization and harmonization of HEV detection methods are still very important issues to get comparable seroprevalence and RNA detection rate data.

The detection of rabbitHEV in a high-human density area in Germany together with findings of rabbitHEV sequences in human patients (Abravanel et al., 2017; Izopet et al., 2012) indicate the necessity of further studies on the role of rabbits in the transmission of HEV to human and the development of hepatitis E in humans. For a risk assessment, further studies are needed on the occurrence of this virus in additional wildlife populations, pet animals and breeding colonies. Furthermore, it remains unclear why rabbitHEV was found exclusively in rabbits and seems to be absent in hares (Hammerschmidt et al., 2017). Therefore, molecular studies on the replication of rabbitHEV in rabbits of different breeds and *in vitro* assays to pinpoint potential factors differentiating the susceptibility of different lagomorphs for rabbitHEV are needed. Additionally, the investigation of environmental samples would improve our understanding of potential transmission routes.

The identification of a novel hepevirus in common voles (cvHEV) raised the question of its zoonotic potential, the possibility of crossing the species barrier, its origin and distribution in other parts of Europe. This is especially important as the common vole is a pest animal in agriculture with large population outbreaks that have been shown already also to cause disease outbreaks in humans, e.g. of leptospirosis (Desai et al., 2009) or tularemia (Luque-Larena et al., 2017). Therefore, further studies are warranted to prove the presence of this virus in common voles during different phases of the population dynamics. For this purpose, highly specific and sensitive serological and RT-PCR assays should be

developed. Furthermore, the large-scale screening of small mammals should be continued to understand the deep phylogeny of hepeviruses in general. An enrichment of the viral RNA, e.g. by a hybrid-capture approach, and subsequent HTS analysis could improve the generation of complete hepevirus genomes (Drewes et al., 2017; Ho et al., 1999).

The frequent detection of these three small mammal-associated hepeviruses, ratHEV, rabbitHEV and cvHEV, may allow in the future to isolate these viruses and the development of small mammal animal models for further studies on the transmission and pathogenicity of HEV in general. Interestingly, currently running *in vitro* systems for HEV replication are mainly based on HEV strains with insertions in ORF1 (Johne et al., 2014b; Shukla et al., 2012) – therefore it would be especially interesting to evaluate rabbitHEV and cvHEV for their replication capability *in vitro*.

5. Summary

Hepeviruses are small viruses with a RNA-genome of positive polarity that form the family *Hepeviridae*. The family includes two genera: members of the genus *Piscihepevirus* were detected in fish species and members of the genus *Orthohepevirus* were found in different mammal and bird species. The genus *Orthohepevirus* contains four different species, namely *Orthohepevirus A, B, C* and *D*. The species *Orthohepevirus A* contains five human pathogenic genotypes, with three of them being zoonotic. The species *Orthohepevirus C* contains mammal-associated pathogens, which were identified in rats and carnivores. The human pathogenic genotypes are responsible for a self-limiting acute hepatitis in humans, which could become chronically in immunocompromised individuals. The main route of transmission is the consumption of undercooked meat and direct contact with HEV-positive excreta or blood. In Germany, hepatitis E is a notifiable disease since 2001 with an increased number of cases per year. Rats are the reservoir of rat-associated HEV (ratHEV), but also the zoonotic HEV-3 genotype was detected in rats. The European rabbit (*Oryctolagus cuniculus*) was identified as a reservoir host of a subgenotype of human pathogenic HEV-3 (HEV-3ra).

For the development of small mammal animal models, the objective of this study was to evaluate different small mammal populations for novel hepeviruses and to study the presence of HEV and sequence divergence of ratHEV and rabbitHEV in rat and rabbit populations from Europe.

Approximately 3000 rodents from Germany and the Czech Republic were screened by broad spectrum HEV-RT-PCR. As a result, 13 common voles (*Microtus arvalis*) and one bank vole (*Myodes glareolus*) were detected to be HEV-RNA positive. Comparison of the obtained sequences, complete genome determination and phylogenetic analysis indicated the finding of a novel common vole-associated HEV (cvHEV), which shows a high sequence divergence towards other members of the species *Orthohepevirus C*, but shares a high sequence similarity to a HEV-genome derived from a kestrel (*Falco tinnunculus*). The finding of cvHEV-RNA in a bank vole might be caused by a spillover infection. The cvHEV genome shares the hepevirus-typical open reading frames, but also has unique cvHEV-specific attributes in its genome.

The investigation of 420 Norway rats (*Rattus norvegicus*) and 88 Black rats (*Rattus rattus*) identified HEV-RNA in Norway rats from eight of nine and Black rats from two of four

European countries. In a single Norway rat from Belgium, a HEV-3-strain with high sequence similarities to rabbitHEV (HEV-3ra), was detected. The investigation of zoo animals revealed a ratHEV spillover infection in a Syrian brown bear (*Ursus arctos syriacus*). This infection was most likely caused by ratHEV-infected free-living, wild rats from the same zoo.

Investigation of wild rabbit populations trapped in and around Frankfurt am Main, Germany, showed anti-HEV antibodies (34.7%) and rabbitHEV-RNA (25%). A high sequence similarity of rabbitHEV in the animals trapped at the urban site was observed, whereas a high sequence divergence was seen for the animals trapped at the rural trapping sites.

In conclusion, hepeviruses are widespread among different small mammal populations in Europe. The broad geographical distribution of these hepeviruses should be taken into account in further public health risk assessments. Further investigations are needed to characterize the presence of cvHEV in more detail, especially by taking the population dynamics of common voles into account. The detected HEV-strains could be taken as basis for the establishment of novel HEV-animal models, which might replace the so far used swine and non-human primate models.

6. Zusammenfassung

Hepeviren sind kleine Viren mit einem einzelsträngigen RNA-Genom positiver Polarität und bilden die Familie *Hepeviridae*. Die Familie beinhaltet zwei Gattungen: Zur Gattung *Piscihepevirus* gehört ein bei Fischen gefundenes Virus, während Vertreter der Gattung *Orthohepevirus* in Säugetieren und Vögeln nachgewiesen wurden. Die Gattung *Orthohepevirus* enthält die Arten *Orthohepevirus A, B, C* und *D.* Die Spezies *Orthohepevirus A* beinhaltet fünf humanpathogene Genotypen, von denen drei zoonotische Erreger sind (HEV-3, HEV-4, HEV -7). Die Spezies *Orthohepevirus C* beinhaltet ebenfalls Säugetier-assoziierte Viren, die bei Ratten und Karnivoren identifiziert worden sind. Die humanpathogenen Genotypen sind für eine akute selbstlimitierende Hepatitis beim Menschen, die bei immungeschwächten Individuen chronisch werden kann, verantwortlich. Als Hauptübertragungsweg wird der Verzehr von rohem oder ungenügend gegartem Fleisch sowie der direkte Kontakt mit HEV-positiven Ausscheidungen oder Blut angenommen. In Deutschland ist die Hepatitis E seit 2001 eine meldepflichtige Krankheit mit einer jährlich steigenden Zahl von gemeldeten Fällen. Ratten stellen das Reservoir des Ratten-Hepatitis E-Virus (ratHEV) dar, dessen zoonotisches Potenzial kontrovers diskutiert wird. Darüber hinaus wurde in Wanderratten (*Rattus norvegicus*) der humanpathogene Genotyp HEV-3 nachgewiesen. Kaninchen (*Oryctolagus cuniculus*) wurden als Reservoir eines Subgenotyps des humanpathogenen Genotyp HEV-3 (Kaninchen-assoziiertes HEV, rabbitHEV, HEV-3ra) identifiziert.

Im Rahmen der Entwicklung von Kleinsäuger-Tiermodellen für HEV sollte in der vorliegenden Studie in Kleinsäugerpopulationen nach neuen Hepeviren gesucht werden und das Vorkommen und die Sequenzvariation von ratHEV und rabbitHEV in Ratten- und Kaninchenpopulationen in Europa genauer charakterisiert werden.

Bei der Suche nach neuen Hepeviren wurden ca. 3000 Kleinsäuger aus Deutschland und der Tschechischen Republik mittels einer Breitspektrum HEV-RT-PCR untersucht. Dabei wurde in 13 Feldmäusen (*Microtus arvalis*) und einer Rötelmaus (*Myodes glareolus*) HEV-RNA nachgewiesen. Sequenzvergleiche und phylogenetische Untersuchungen anhand von partiellen und Komplettgenomen zeigten, dass es sich dabei um ein neues Feldmaus-assoziiertes HEV handelt (common vole HEV, cvHEV), das sich deutlich von den bisher bekannten Genotypen der Spezies *Orthohepevirus C* unterscheidet, aber eine große Ähnlichkeit zu einem bei einem Falken (*Falco tinnunculus*) gefundenen Virusgenom

zeigte. Der Nachweis von cvHEV-RNA in einer Rötelmaus könnte auf eine Spilloverinfektion zurückzuführen sein. Das cvHEV-Genom besitzt die für alle Hepeviren typischen offenen Leserahmen, zeigt aber auch einige cvHEV-spezifische Besonderheiten im Genom.

Im Rahmen der Untersuchungen wurden insgesamt 420 Wanderratten und 88 Hausratten (*Rattus rattus*) aus zwölf europäischen Ländern auf das Vorhandensein von HEV-RNA untersucht. In Wanderratten aus acht europäischen Ländern und Hausratten aus zwei Ländern konnte ratHEV-RNA nachgewiesen werden. In einer Wanderratte aus Belgien wurde ein HEV-3-Stamm mit großer Sequenzähnlichkeit zum rabbitHEV (HEV-3ra) identifiziert. Bei der Untersuchung von Zootieren wurde eine ratHEV-Spilloverinfektion in einem syrischen Braunbären (*Ursus arctos syriacus*) nachgewiesen, welche vermutlich durch Schadratten im gleichen Zoo verursacht worden ist.

Die Untersuchung von Wildkaninchenpopulationen aus der Stadt Frankfurt am Main und deren Umgebung zeigte das Vorkommen von anti-HEV Antikörpern (34.7%) und rabbitHEV-RNA (25%). Die rabbitHEV-Stämme der urbanen Population zeigten eine sehr große Sequenzähnlichkeit, während bei den Tieren aus der ländlichen Population eine starke Sequenzdivergenz des rabbitHEV beobachtet wurde.

Die hier vorgestellten Ergebnisse belegen das gleichzeitige Vorkommen verschiedener Hepeviren in Kleinsäugerpopulationen in Europa. Die weite geografische Verbreitung der Erreger sollte zukünftig bei einer Gefährdungsbeurteilung für die Bevölkerung berücksichtigt werden. Weitere Untersuchungen sollten das Vorkommen des cvHEV genauer charakterisieren, insbesondere auch im Zusammenhang mit Populationsveränderungen bei der Feldmaus. Auf der Basis der hier nachgewiesenen Erreger können zukünftig neue HEV-Tiermodelle entwickelt werden, die die bisher verwendeten Schweine- und Primatenmodelle möglicher Weise ersetzen könnten.

7. Literature

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8. Appendix

8.1 Supplementary Table

Appendix Supplementary Table 1. Detection of HEV-specific RNA or anti-HEV antibodies in different rodent and shrew species.

Order	Family	Species	Number of investigated animals	Antigen (Serological Results)	RT-PCR results (typing)	References
Rodentia	Muridae	Norway rat (*Rattus norvegicus*)	1162*	ratHEV truncated CP (pos), HEV-1 CP (neg), HEV-3 CP (pos), ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos), CP of HEV-3 (neg), SAR 55 HEV CP (pos), N-terminally truncated CP (recombinant ratHEV-VLPs) (pos)	partial ORF1 fragment (ratHEV), whole genome (ratHEV)	Arankalle et al., 2001; Favorov et al., 2000; Johne et al., 2012; Johne et al., 2010a; Johne et al., 2010b; Kabrane-Lazizi et al., 1999; Li et al., 2011; W. Li et al., 2013; Peralta et al., 2009; Purcell et al., 2011; Simanavicius et al., 2018; Widen et al., 2014; Wolf et al., 2013
		Black rat (*Rattus rattus*)	392	ratHEV truncated CP (pos), HEV-1 CP (neg), HEV-3 CP (pos), ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos), SAR 55 HEV CP (pos), N-terminally truncated CP (recombinant ratHEV-VLPs) (pos)	partial ORF1 fragment (ratHEV), whole genome(ratHEV)	Favorov et al., 2000; Kabrane-Lazizi et al., 1999; W. Li et al., 2013; Mulyanto et al., 2013; Simanavicius et al., 2018
		Lesser rice-field rat (*Rattus rattoides losea*)	121	N-terminally truncated CP (recombinant ratHEV-VLPs) (pos)	partial ORF1 fragment (ratHEV)	W. Li et al., 2013
		Yellow-breasted rat (*Rattus flavipectus*)	171	N-terminally truncated CP (recombinant ratHEV-VLPs) (pos)	partial ORF1 fragment (ratHEV)	W. Li et al., 2013
		Rattus rattus rufescens	58	SAR 55 HEV CP (pos)	n.d.	Arankalle et al., 2001
		Greater Bandicoot rat (*Bandicota indica*)	174	N-terminally truncated CP (recombinant ratHEV-VLPs) (pos)	partial ORF1 fragment (ratHEV)	W. Li et al., 2013
		Indochinese forest rat (*Rattus rattus hainanus*)	17**	N-terminally truncated CP (recombinant ratHEV-VLPs) (pos)	neg	W. Li et al., 2013
		Lesser bandicoot rat (*Bandicota bengalensis*)	22	SAR 55 HEV CP (pos)	n.d.	Arankalle et al., 2001

nd.: not done; pos: positive; neg: negative; ORF: open reading frame; VLPs: virus-like particles; CP: capsid protein; *: Peralta et al., investigated 166 rodents without specifying the number of individuals per species

Appendix Supplementary Table 1 (continued)

Order	Family	Species	Number of investigated animals	Antigen (Serological Results)	RT-PCR results (typing)	References
Rodentia	Muridae	Indochinese forest rat (*Rattus rattus andamanensis*)	55	SAR 55 HEV CP (pos)	n.d.	Arankalle et al., 2001
		Asian rat (*Rattus tanezumi*)	18	n.d.	partial ORF1 and ORF2 (ratHEV)	Li et al., 2011; B. Wang et al., 2018
		Polynesian rat (*Rattus exulans*)	18	SAR 55 HEV CP (pos)	n.d.	Kabrane-Lazizi et al., 1999
		Ryuku mouse (*Mus caroli*)	1	n.d.	neg	B. Wang et al., 2018
		Wood mouse (*Apodemus sylvaticus*)	-*	CP of HEV-3 (neg)	n.d.	Peralta et al., 2009
		Yellow-necked mouse (*Apodemus flavicollis*)	-*	CP of HEV-3 (neg)	n.d.	Peralta et al., 2009
		Chevrier's field mouse (*Apodemus chevrieri*)	202	n.d.	Complete genome (ratHEV)	B. Wang et al., 2018
		House mouse (*Mus musculus*)	26*	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos), CP of HEV-3 (neg), SAR 55 HEV CP (neg)	neg	Arankalle et al., 2001; de Souza et al., 2018; Favorov et al., 2000; Peralta et al., 2009
		Southern red-backed vole (*Myodes gapperi*)	6	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
	Cricetidae	Meadow vole (*Microtus pennsylvanicus*)	9	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		White-throated wood rat (*Neotoma albigula*)	22	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Mexican wood rat (*Neotoma mexicana*)	84	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000

n.d.: not done; pos: positive; neg: negative; ORF: open reading frame; VLPs: virus-like particles; CP: capsid protein; *: Peralta et al., investigated 166 rodents without specifying the number of individuals per species; **downgraded to *R. rattus andamanensis* (Musser and Carleton, 2005)

Appendix Supplementary Table 1 (continued)

Order	Family	Species	Number of investigated animals	Antigen (Serological Results)	RT-PCR results (typing)	References
Rodentia		Southern plains wood rat (*Neotoma micropus*)	8	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Grasshopper mouse (*Onychomys leucogaszer*)	1	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Golden mouse (*Ochrotomys nuttalli*)	11	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Marsh rice rat (*Oryzomys palustris*)	41	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Brush mouse (*Peromyscus boylii*)	24	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Zacatecan deer mouse (*Peromyscus difficilis*)	3	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Cactus mouse (*Peromyscus eremicus*)	7	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
	Cricetidae	Cotton mouse (*Peromyscus gossiypinus*)	4	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		White-footed mouse (*Peromyscus leucopus*)	53	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Deer mouse (*Peromyscus maniculatus*)	91	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Oldfield mouse (*Peromyscus polionotus*)	3	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Pinon mouse (*Peromyscus truei*)	15	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Woodland vole (*Microtus pinetorum*)	2	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Hispid cotton rat (*Sigmodon hispidus*)	113	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Nectomys spp.	4	Mosaic protein of the prototype HEV-strain SAR 55 and ORF2 of HEV-1 (Burma) (pos)	n.d.	Vitral et al., 2005
		Pratt's vole (*Eothenomys chinensis*)	13	n.d.	neg	B. Wang et al., 2018

n.d.: not done; pos: positive; neg: negative; ORF: open reading frame; VLPs: virus-like particles; CP: capsid protein; *: Peralta et al., investigated 66 rodents without specifying the number of individuals per species

Appendix Supplementary Table 1 (continued)

Order	Family	Species	Number of investigated animals	Antigen (Serological Results)	RT-PCR results (typing)	References
Rodentia	Cricetidae	Black-footed pygmy rice rat (*Oligoryzomys nigripes*)	63	n.d.	neg	de Souza et al., 2018
		Delicate vesper mouse (*Calomys tener*)	109	n.d.	Complete genome (ratHEV)	de Souza et al., 2018
		Hairy-taled bolo mouse (*Necromys lasiurus*)	252	n.d.	Complete genome (ratHEV)	de Souza et al., 2018
		Père David's vole (*Eothenomys melanogaster*)	55	n.d.	Complete genome (ratHEV)	B. Wang et al., 2018
		Montane grass mouse (*Akodon montensis*)	199	n.d.	neg	de Souza et al., 2018
		Bank vole (*Myodes glareolus*)	-*	CP of HEV-3 (neg)	n.d.	Peralta et al., 2009
Rodentia	Sciuridae	Mexican ground squirrel (*Citellus mexicanus*)	2	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Rock squirrel (*Citellus variegatus*)	1	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
		Eastern gray squirrel (*Sciurus carolinensis*)	2	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
Soricomorpha		Chinese mole shrew (*Anourosorex squamipes*)	4	n.d.	neg	B. Wang et al., 2018
Rodentia	Heteromyidae	Desert pocket mouse (*Perognathus penicillatus*)	10	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
Rodentia	Dipodidae	Meadow jumping mouse (*Zapus hudsonius*)	2	ORF2 and ORF3 of HEV-2 (Mexican) and HEV-1 (Burma) (pos)	n.d.	Favorov et al., 2000
Soricomorpha	Soricidae	Asian musk shrew (*Suncus murinus*)	260	N-terminally truncated CP (recombinant ratHEV-VLPs) (pos)	partial ORF1 fragment (ratHEV)	Guan et al., 2013

n.d.: not done; pos: positive; neg: negative; ORF: open reading frame; VLPs: virus-like particles; CP: capsid protein; *: Peralta et al., investigated 166 rodents without specifying the number of individuals per species

8.2 List of publications

Scientific articles

First authorship

Ryll, R., Heckel, G., Corman, V. M., Drexler, J. F., Ulrich, R. G., 2019. Genomic and spatial variability of a European common vole hepevirus. Archives of Virology, submitted.

Ryll, R., Bernstein, S., Heuser, E., Schlegel, M., Dremsek, P., Zumpe, M., Wolf, S., Pepin, M., Bajomi, D., Muller, G., Heiberg, A.C., Spahr, C., Lang, J., Groschup, M.H., Ansorge, H., Freise, J., Guenther, S., Baert, K., Ruiz-Fons, F., Pikula, J., Knap, N., Tsakmakidis, I., Dovas, C., Zanet, S., Imholt, C., Heckel, G., Johne, R., Ulrich, R.G., 2017. Detection of rat hepatitis E virus in wild Norway rats (*Rattus norvegicus*) and Black rats (*Rattus rattus*) from 11 European countries. Veterinary microbiology 208, 58-68.

Ryll, R., Eiden, M., Heuser, E., Weinhardt, M., Ziege, M., Hoper, D., Groschup, M.H., Heckel, G., Johne, R., Ulrich, R.G., 2018. Hepatitis E virus in feral rabbits along a rural-urban transect in Central Germany. Infection, genetics and evolution 61, 155-159.

Co-authorship

Obiegala, A., Heuser, E., **Ryll, R.**, Imholt, C., Furst, J., Prautsch, L. M., Plenge-Bonig, A., Ulrich, R. G., Pfeffer, M., 2019. Norway and black rats in Europe: Potential reservoirs for zoonotic arthropod-borne pathogens? Pest management science, doi 10.1002/ps.5323.

Spahr, C., **Ryll, R.**, Knauf-Witzens, T., Vahlenkamp, T. W., Ulrich, R. G., & Johne, R., 2017. Serological evidence of hepatitis E virus infection in zoo animals and identification of a rodent-borne strain in a Syrian brown bear. Veterinary microbiology, 212, 87-92.

Gertler, C., Schlegel, M., Linnenbrink, M., Hutterer, R., König, P., Ehlers, B., Fischer, K., **Ryll, R.**, Lewitzki, J., Sauer, S., 2017. Indigenous house mice dominate small mammal communities in northern Afghan military bases. BMC Zoology 2, 15.

Heuser, E., Fischer, S., **Ryll, R.**, Mayer-Scholl, A., Hoffmann, D., Spahr, C., Imholt, C., Alfa, D.M., Frohlich, A., Luschow, D., Johne, R., Ehlers, B., Essbauer, S., Nockler, K., Ulrich, R.G., 2016. Survey for zoonotic pathogens in Norway rat populations from Europe. Pest management science, 73(2), 341-348.

Oral presentations

Ryll, R., 26.06.2018, „Hepatitis E Virus in rabbits, rats and other rodents", Hausseminar FLI, Greifswald – Insel Riems, Germany.

Ryll, R., 06.06.2018, „Progress Report V – Summary of research topic: Hepatitis E Virus", Progress Report FLI, Greifswald – Insel Riems, Germany.

Ryll, R., 16.11.2017, „Progress Report IV - Hepatitis E Virus in carnivores", Progress Report FLI, Greifswald- Insel Riems, Germany.

Ryll, R., Eiden, M., Heuser, E., Weinhardt, M., Ziege, M., Groschup, M.H., Johne, R., Ulrich, R. G., 11./12.10.2017, "Hepeviruses in small mammals: RabbitHEV in two populations in and around Frankfurt/ Main, Germany", National Symposium on Zoonoses Research, Berlin, Germany.

Ryll, R., Eiden, M., Heuser, E., Weinhardt, M., Ziege, M., Groschup, M.H., Heckel, G., Johne, R., Ulrich, R. G., 25.-29.09.2017, "Zoonotic rabbit hepatitis E virus in two wild rabbit populations in and around Frankfurt/ Main, Germany", 11th European Vertebrate Pest Management Conference, Warsaw, Poland.

Ryll, R., Johne, R., Ulrich, R.G., 7.-9.6.2017, "Identification and genetic characterization of small mammal-associated hepeviruses", Junior Science Zoonoses Meeting, Langen, Germany.

Ryll, R., „Progress Report III - Hepatitis E Virus in small mammals", Progress Report FLI, Greifswald- Insel Riems, Germany, 29.03.2017.

Ryll, R., Bernstein, S., Heuser, E., Fischer, S., Ulrich, R. G., 28.-30.11.2016, " Die Wanderratte: Reservoirwirt unterschiedlicher Bakterien und Viren", 5. Workshop des Netzwerks "Nagetier-übertragene Pathogene", Gießen, Germany.

Ryll, R., 29.02.2016, „Progress Report II - Hepatitis E Virus in rats, rabbits and bats", Progress Report FLI, Greifswald - Insel Riems, Germany.

Heuser, E., Fischer, S., Ryll, R., Mayer-Scholl, A., Hoffmann, D., Spahr, C., Imholt, C., Alfa, D. M, Fröhlich, A., Lüschow, D., Johne, R., Ehlers, B., Essbauer, S., Nöckler, K.,

Ulrich, R. G., 10./11.2.2016, „Survey for zoonotic pathogens in Norway rat populations from Europe", Workshop „AKTimo- Alternative Kleinsäuger-Tierversuchsmodelle, Greifswald - Insel Riems, Germany.

Ryll, R., 16.11.2015, "Vorstellung Promotionsvorhaben: Small mammal associated hepeviruses", Hausseminar FLI, Greifswald – Insel Riems, Germany.

Heuser, E., Ryll, R., Fischer, S., Pépin, M., Müller, G., Heiberg, A.-C., Lang, J., Hoffmann, D., Drewes, S., Ansorge, H., Freise, J., Guenther, S., Johne, R., Mayer-Scholl, A., Nöckler, K., Essbauer, S., Ulrich, R. G., 15./16.10.2015, „Survey for zoonotic pathogens in Norway rat populations from European cities", National Symposium on Zoonoses Research, Berlin, Germany.

Ryll, R., 29.09.2015, „Progress Report I – Hepatitis E Virus in rats and rabbits", Progress Report FLI, Greifswald - Insel Riems, Germany.

Poster

Ryll, R., Bernstein, S., Heuser, E., Schlegel, M., Dremsek, P., Zumpe, M., Wolf, S., Pépin, M., Bajomi, D., Müller, G., Heiberg, A.-C., Spahr, C., Lang, J., Groschup, MH., Ansorge, H., Freise, J., Guenther, S., Imholt, C., Heckel, G., Johne, R., Ulrich, R. G., 18.-21.09.2017, „Rat hepatitis E virus in Europe: Looking for viral and bacterial co-infections", 91th Annual Meeting Deutsche Gesellschaft für Säugetierkunde, Greifswald, Germany.

Ryll, R., Johne, R., Ulrich, R. G., 7.-9.6.2017, "A survey for rat-associated hepevirus in European Norway and Black rat populations from 12 different European countries", Junior Scienctist Zoonoses Meeting, Langen, Germany.

Heuser, E., Fischer, S., Ryll, R., Mayer-Scholl, A., Hoffmann, D., Spahr, C., Imholt, C., Alfa, D. M., Fröhlich, A., Lüschow, D., Johne, R., Ehlers, B., Essbauer, S., Nöckler, K., Ulrich, R. G., 27.-29.4.2016, „Survey for zoonotic pathogens in Norway rat populations from Europe", Medical Biodefense Conference, Munich, Germany.

Ryll, R., Bernstein, S., Heuser, E., Schlegel, M., Dremsek, P., Zumpe, M., Wolf, S., Pépin, M., Bajomi, D., Müller, G., Heiberg, A.-C., Spahr, C., Lang, J., Groschup, M.H., Ansorge, H., Freise, J., Guenther, S., Imholt, C., Heckel, G., Johne, R., Ulrich, R.G.,

29./30.09.2016, „Rat hepatitis E virus in Europe: Looking for viral and bacterial co-infections", 1st Summer School, Greifswald, Germany.

8.3 Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Unterschrift des Promovenden

8.4 Danksagung

Unterschrift, Datum