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

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

INTRODUCTION

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

HEV is classified as the only member of the genus Hepevirus. This genus is subdivided into four distinct genotypes and the avian HEV strains (Bilic et al., 2009), which are not included in any of the other genotypes. All mammalian HEV isolates described to date comprise the same serotype (Lu et al., 2006; Takahashi et al., 2005). The HEV virion appears as a non-enveloped icosahedral sphere of approximately 27–34 nm in diameter. The crystal structure of HEV-like particles has recently been solved (Yamashita et al., 2009). The particles are composed of a single capsid protein, which...
folds into three major domains: the shell (S) domain, the middle (M) domain and the protruding (P) domain. The outer surface of the particle, which is a target for antibodies, is mainly formed by the M and P domains. The genome of HEV is a single-stranded, positive-sense RNA of about 7.2 kb and contains three open reading frames (ORFs) (Aggarwal & Krawczynski, 2000; Purcell & Emerson, 2008). These three ORFs encode a non-structural polyprotein (ORF1), the capsid protein (ORF2) and a phosphoprotein (ORF3) associated with signal transduction. The genome is capped at the 5′ end and polyadenylated at the 3′ end (Smith, 2001; Xia et al., 2008).

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

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

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

**RESULTS**

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

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

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Primer designation</th>
<th>Sequence (5′→3′)*</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>HEV-cs</td>
<td>TGCCGGCATCACMTTYTTCARAA</td>
<td>469–472</td>
</tr>
<tr>
<td></td>
<td>HEV-cas</td>
<td>GCCATGTTCCAGACGDTRTCCTCA</td>
<td></td>
</tr>
<tr>
<td>Nested PCR</td>
<td>HEV-csn</td>
<td>TGGCTCCTGTTTGGCCCNCGTTTCYG</td>
<td>331–334</td>
</tr>
<tr>
<td></td>
<td>HEV-casn</td>
<td>CAGGCTCAGCRRGTYTCTTTCCA</td>
<td></td>
</tr>
</tbody>
</table>

*D=A, G or T; M=A or G; N=A, C, G or T; R=A or G; Y=C or T.
†According to a mismatch found in the described rat HEV sequences, C may be changed to M at this position.
length were amplified for both samples with additional slower-migrating bands of lower intensity, which most likely represented the remaining products of the initial RT-PCR.

**Screening of rat faecal samples for hepeviruses**

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

**Identification of virus particles by transmission electron microscopy**

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

**Cultivation trials of rat HEV in tissue culture**

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

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

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**Fig. 2.** Demonstration of HEV-like particles in the rat faecal sample R4. The transmission electron micrographs show (a) a filled particle with a diameter of 32 nm and (b) an empty particle with a diameter of 34 nm. (c) Solid phase immunoelectron microscopy using a human serum positively tested for HEV-specific antibodies and sample R4. The arrows indicate adsorbed HEV-like particles. Negative staining was performed with uranyl acetate. Bars, 50 nm (a, b); 200 nm (c).
shown). This culture supernatant and those from the fifth passages of all inoculated cell lines were tested using the nested broad-spectrum RT-PCR for detection of the hepevirus genome, as well as with an RT-PCR with the rat HEV-specific primers ORF2-s and ORF2-as (see Supplementary Table S2, available in JGV Online). However, a specific product could not be demonstrated (not shown).

**Genome sequence analysis of rat HEV**

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

The 4019 nt sequence of rat HEV strain R4 was most closely related to human genotypes 1 and 3 strains, but with only 58.4% nucleotide sequence identity. Comparison of the nucleotide sequences of the 1545 nt fragment of both rat HEV strains with the corresponding region of HEV strains from humans and different animal species revealed identities ranging from 49.9% (comparison of rat HEV R4 with avian HEV) to 59.9% (rat HEV R8 with human HEV genotype 1). The nucleotide sequence identity of the rat HEV strains R4 and R8 in this fragment was 90.4% with each other. A phylogenetic tree constructed on the basis of this sequence fragment showed that both rat HEV strains clustered together and represented a branch clearly separated from that represented by HEV genotypes 1–4 and the rabbit HEV isolate and that represented by HEV genotypes 5–7. The nucleotide sequence identity of the different viruses. However, two stretches of the rat HEV sequence in this region (aa 528–556 and 572–584) were nearly identical to the corresponding sequences of the other mammalian HEVs. The sequence identities in the remaining regions were low; however, a typical hydrophobic region in the signal sequence and an accumulation of arginine residues in the arginine-rich domain were evident. Tyrosine at position 288 (numbering according to genotype 3), which has been shown to be crucial for capsid formation in HEV genotype 3 (Yamashita et al., 2009), was the most conserved amino acid position among the highly variable regions among rat HEV and HEV genotypes 1–4 (Fig. 5). Most of the conserved amino acid positions were located in the S domain, which is known to have important functions in capsid assembly, and in the M domain, which has been shown to be tightly associated with the S domain and to form parts of the outer capsid surface. The P domain, which forms the protruding parts of the outer capsid surface, was more variable among the different viruses. However, two stretches of the rat HEV sequence in this region (aa 528–556 and 572–584) were nearly identical to the corresponding sequences of the other mammalian HEVs. The sequence identities in the remaining regions were low; however, a typical hydrophobic region in the signal sequence and an accumulation of arginine residues in the arginine-rich domain were evident. Tyrosine at position 288 (numbering according to genotype 3), which has been shown to be crucial for capsid formation in HEV genotype 3 (Yamashita et al., 2009), was mutated to phenylalanine in rat HEV. However, it was shown in the same study that phenylalanine can functionally replace tyrosine at this position.

**DISCUSSION**

HEV is suspected to be a zoonotic virus, which could be transmitted from pigs, wild boars or Sika deer to humans...
In chickens (Bilic et al., 2001). In addition, HEV-like viruses have been identified in other animal species. However, as suggested above, a further adjustment of primer sequences based on the rat HEV sequences described here might improve the performance of the assay, i.e. by the inclusion of a wobble site (M instead of C) at nt 25 of primer HEV-csn (see Table 1).

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

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

For sensitive detection of such HEV-like viruses, a nested broad-spectrum RT-PCR was developed here. The binding sites of the primers were chosen within a highly conserved region of ORF1. This region shows strong similarities to conserved domains of RNA-dependent RNA polymerases and contains putative functionally important regions for nucleotide and template binding (Koonin et al., 1992). These essential functions may ensure the presence of such highly conserved sequences in many HEV-like viruses. A nested RT-PCR protocol was chosen due to its higher sensitivity compared with a single-step RT-PCR, as reported previously for other viruses (Johne et al., 2005). The general applicability of this novel RT-PCR approach was verified by detection of two only distantly related HEV strains from wild boar and chicken whose sequences were not included in the initial alignment for primer design. In addition, the nested broad-spectrum RT-PCR could readily identify the novel rat HEV and may therefore also be suitable for detection of other, so far unknown hepeviruses in other animal species.
Fig. 5. Alignment of the deduced amino acid sequences of the ORF2-encoded capsid protein of rat HEV strain R4 and representatives of genotypes 1–4 (GenBank accession nos X99441, M74506, AF060668 and AJ272108), as well as avian HEV (GenBank accession no. EF205691). Residues that are conserved among all HEV isolates are shown as a consensus above the sequences; residues that are conserved in all mammalian isolates are shaded grey. The structural domains of the HEV capsid protein according to Yamashita et al. (2009) are indicated in italics. Amino acid position 288 in genotype 3, which has been shown previously to exert an important function in capsid assembly, is marked with an asterisk.
with non-rodent HEV strains due to the presence of cross-reacting antibodies.

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

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

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

METHODS

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

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

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

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

Nested broad-spectrum RT-PCR. Primers for the nested broad-spectrum RT-PCR for the detection of hepeviruses (Table 1) were selected on the basis of an alignment of 22 full-length HEV sequences of genotypes 1–4 derived from humans, pigs and wild boars and one full-length sequence of an avian HEV isolate (GenBank accession numbers are listed in Supplementary Table S1). RNA was isolated.
from the faecal suspensions using a QIAamp Viral RNA Mini kit (Qiagen). For homogenization of the liver samples, QIAshredder columns (Qiagen) were applied prior to RNA extraction using an RNeasy Mini kit (Qiagen). A first RT-PCR was performed using a One-Step RT-PCR kit (Qiagen) with primers HEV-cs and HEV-cas in a 2720 thermal cycler (Applied Biosystems). The thermal profile comprised 42 °C for 60 min and 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 74 °C for 45 s, with a final incubation at 74 °C for 5 min. An aliquot of the RT-PCR product (5 µl) was used in a nested PCR with a TaKaRa ExTaq kit (TaKaRa Bio) and the primers HEV-csn and HEV-casn. The thermal profile consisted of 95 °C for 5 min and 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s, with a final incubation at 72 °C for 5 min. Nested PCR products were separated by electrophoresis on ethidium bromide-stained agarose gels.

**Amplification and sequencing of rat HEV genome.** Primers used for amplification of parts of the rat HEV genome were generated using the sequence alignment mentioned above, and primers were selected with binding sites on the rat HEV sequences obtained in this study. Primer sequences that successfully amplified parts of the rat HEV genome are listed in Supplementary Table S2. RNA isolated from the faecal suspension as described above was used together with a LongRange 2Step RT-PCR kit (Qiagen). Reverse transcription was performed in a 20 µl reaction at 42 °C for 90 min using two primers. PCR was subsequently performed with 5 µl cDNA, applying different thermal profiles depending on the primer sequence and the expected product length. For rapid amplification of cDNA ends (RACE) of the 3’ end of the rat HEV genome, reverse transcription was performed using the primer pA1 [5’-CCGATTCCGGGATCC(T)5] 3’, complementary to the poly(A) tail, followed by PCR with primers 5’-CCGAATTCGGGATCC3’ (binding site on primer pA1) and primer ratORF2-s. PCR products were separated by electrophoresis on ethidium bromide-stained agarose gels. Bands of the expected length were excised, purified using a QIAquick Gel Extraction kit (Qiagen) and subsequently cloned into the vector pCR4-TOPO using a TOPO TA Cloning kit for Sequencing (Invitrogen). The inserts of the plasmids were sequenced using M13 Forward and M13 Reverse primers (Invitrogen), as well as gene-specific primers, in an ABI 3730 DNA Analyser (Applied Biosystems).

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

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