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Keywords: *Hepeviridae*, hepatitis E virus, Genotype 3, Norway rat, Black rat, Europe

ABSTRACT

Rat hepatitis E virus (ratHEV) 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 and Black rats 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 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 et al., 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-Splenomegaly 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*, batHEV into *Orthohepevirus D* and the carnivore and ratHEV into *Orthohepevirus C* (http://ictvonline.org/virusTaxonomy.asp, accessed 07.04. 2017).

The hepevirus genome is a positive stranded RNA of approximately 6.7 to 7.3 kilobases (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., 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 contains 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; Johne et al., 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; Li et al., 2013d; Mulyanto et al., 2013; Mulyanto et al., 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 lengthrevealed 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 *R. rattus*, whereas ratHEV sequences of G3 originated exclusively from *R. rattus.* In contrast, genotype G2 was detected in *R. rattus*, *R. tanezumi*, *R. rattoides losea* and the shrew *Suncus murinus* (Li et al., 2013b; Li et al., 2013d; Mulyanto et al., 2013).

The zoonotic potential of ratHEV is currently controversially discussed. Serological studies in forestry workers 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 (*R. rattus*) were collected in Italy, Slovenia, Greece and Spain (Fig. 1).

The dissection and collection of tissue and chest cavity fluid 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; Johne et al., 2010a; Johne et al., 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 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. 3A).

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) 4,105-4,387 (numbering based on strain R63, acc. no. GU345042) and nucleotides 4,105-5,226. 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., 2016). 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 ($\leq$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 (>1,500 inhabitants/km^2), small town (300-1,500 inhabitants/km^2), rural (<300 inhabitants/km^2)) (database: Geostat, 2012). Rats collected in zoological gardens were put in a separate category. 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) to 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 4,105-4,387, prototype strain R63, accession number GU345042, see Fig. 2) and NBS RT-PCR (nt positions 4,000-4,423, 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 1,122/1,125-base pair (bp) long region including parts of ORF1, ORF2 and partial or entire ORF3 (see Fig. 2) was RT-PCR amplified and sequenced (see Table 4). 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 bp 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 C1,* well separated from sequences of species *Orthohepevirus C2* (Figs. 3A and 3B). In one Norway rat sample from Belgium (KS/16/825) 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 1,122/1,125 bp 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, 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 (Figs. 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 bp or 1,222 bp products were analyzed (Figs. 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).

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; Johne et al., 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 Norway 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 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; Li et al., 2013c; Purcell et al., 2011) and results from earlier field studies in Norway rats (Johne et al., 2012; Johne et al., 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 Bandicoot rats (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 1 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 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 1 of ratHEV (Figs. 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 1. 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.

Conflict of Interest

The authors declare that they have no competing interests.

Authors´ contributions

Designed the study: RGU, RJ, GH. Performed the experiments: SB, RR, EH, MS, PD, MZ, SW. Analyzed the data: SB, RR, CI, RJ, GH, RGU. Contributed materials: MP, DB, GM, ACH, JL, 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.

Acknowledgments

The authors are very grateful to Dörte Kaufmann and Günter Strebelow for excellent technical assistance, Patrick Wysocki for generation of Figure 1 and Hanan Sheikh Ali, Kathrin Baumann, Angele Breithaupt, Thomas Büchner, Stephan Drewes, Ulrike Duve, Julie Elkins, Bärbel Hammerschmidt, Dörte Kaufmann, Robert Klopfleisch, Nastasja Kratzmann, Christian Kretzschmar, Denny Maaz, Grit Möwert, Josephin Schröder, Ulrike M. Rosenfeld, Josephine Schlosser, Sabrina Schmidt, Ramona Spließ, Annemarie Steiner, André Schütte, Kerstin Tauscher, Franziska Thomas, Konrad Wanka and Ute Wessels for support of the dissections. The authors kindly acknowledge the collection of rats by Marianne Jäger (Vienna) and support of the rat trapping by Zoltán Kiss (Báblona Bio Ltd. Budapest, Hungary), Ezio Ferroglio (Università degli Studi di Torino Dipartimento di Scienze Veterinarie Largo Paolo Braccini, Grugliasco, Italy) and Diego Bünter (Anticimex, Zurich, Switzerland). Additional rats were kindly provided by Anne Balkema-Buschmann, Dan Balkema, Lothar Stitz, Lieselotte Minke (Friedrich-Loeffler-Institut, Greifswald-Insel Riems and

Wusterhausen, Germany), Marion Saathoff (LAVES, Wardenburg, Germany), Richard Kruczewski (Dortmund, Germany), Tomi Trilar (Slovenian Museum of Natural History, Slovenia), Diana Žele (Veterinary Faculty, University of Ljubljana, Slovenia) and the Flanders Environment Agency (VMM, Belgium). The investigations in the laboratory of Rainer G. Ulrich were supported by the German Center for Infection Research (DZIF).

Figure legends

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., 2010, 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., 2016).

Fig. 2. Genome organization of rat HEV, 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.

Fig. 3. Consensus phylogenetic trees based on Bayesian and Maximum-Likelihood analyses of a part of ORF 1 with all species within genus *Orthohepevirus* (A) and a

zoom-in for species *Orthohepevirus C1* and *Orthohepevirus C2* (B) and *Orthohepevirus A* (C), and the concatenated region of ORF1 and partial ORF2/ORF3 overlapping region (D).

Consensus phylogenetic trees based on Bayesian analyses were done with 8,000,000 or 6,000,000 generations and a burn-in of 25%, and Maximum-Likelihood analysis with 1,000 bootstraps and 50% cut-off, of a part of ORF 1 (nt positions 4,105-4,387, counting according prototype strain R63, accession number GU345042) (A-C) and the concatenated region of ORF1 (4,105-4,921) and partial ORF2/ORF3 overlapping region (nt positions 4,949-5,226) (D) of ratHEV. Posterior probability/bootstrap values of >50 are given at the supported nodes.

The ratHEV genotypes G1, G2 and G3 were defined previously (Mulyanto et al., 2014); the two sequences of clade ND (not designated) were found in this previous study, based on a partial region of ORF2, to be clustering with G1.

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).

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