IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1726177 since 2022-02-01T16:19:09Z

Published version:

DOI:10.1016/j.vetmic.2017.07.001

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

03 May 2024

1 **Detection of rat hepatitis E virus in wild Norway rats (*Rattus norvegicus*) and**
2 **Black rats (*R. rattus*) from 11 European countries**
3

4 René Ryll[a] , Samuel Bernstein[a], Elisa Heuser[a], Mathias Schlegel[a,1], Paul Dremsek[a,2],
5 Maxi Zumpe[a,3], Sandro Wolf[b,4], Michel Pépin[c], Daniel Bajomi[d], Gabi Müller[e], Ann-
6 Charlotte Heiberg[f], Carina Spahr[g], Johannes Lang[h], Martin H. Groschup[a], Hermann
7 Ansorge[i], Jona Freise[j], Sebastian Guenther[k], Kristof Baert[l], Francisco Ruiz-
8 Fonscisco[m], Jiri Pikula[n,o], Nataša Knap[p], Ioannis Tsakmakidis[q], Chrysostomos
9 Dovas[r], Stefania Zanet[s], Christian Imholt[t] , Gerald Heckel[u,v], Reimar Johne[g], Rainer
10 G. Ulrich[a,w,*]
11

12 [a] Friedrich-Loeffler-Institut, Institute of Novel and Emerging Infectious Diseases,
13 Südufer 10, 17493 Greifswald-Insel Riems, Germany
14 [b] Institute for Microbiology, Technische Universität Dresden, Zellescher Weg 20b,
15 01217 Dresden, Germany
16 [c] VetagroSup (Microbiologie/Immunologie/Pathologie infectieuse), Coordinateur de
17 l'Unité d'Enseignement de MEDECINE PREVENTIVE, USC INRA-VAS 1233 /
18 Equipe PERS ("Pathogènes Emergents et Rongeurs Sauvages"), 1 avenue
19 Bourgelat, 69280 Marcy l'étoile, France
20 [d] Bábolna Bio Ltd., Szállás u. 6, H-1107 Budapest, Hungary
21 [e] Stadt Zürich, Umwelt- und Gesundheitsschutz Zürich, Walchestrasse 31, 8021
22 Zürich, Switzerland
23 [f] AC Heiberg Rådgivning, Åmosevej 69, Skellingsted, 4440 Mørkøv, Denmark

24 [g] German Federal Institute for Risk Assessment, Max-Dohrn-Str. 8-10, 10589 Berlin,
25 Germany
26 [h] Working Group for Wildlife Biology, Justus Liebig University Giessen, Leihgesterner
27 Weg 217, 35392 Giessen, Germany
28 [i] Senckenberg Museum of Natural History, Am Museum 1, 02826 Görlitz, Germany
29 [j] Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit,
30 Fachbereich Schädlingsbekämpfung, Task-Force Veterinärwesen, Röverskamp 5,
31 26203 Wardenburg, Germany
32 [k] Institute of Microbiology and Epizootics, Veterinary Faculty, Freie Universität Berlin,
33 Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany
34 [l] Research Institute for Nature and Forest (INBO), Research Group Wildlife
35 Management, Brussels, Belgium
36 [m] Spanish Wildlife Research Institute IREC (CSIC-UCLM-JCCCM), Ronda de Toledo
37 12, 13071 Ciudad Real, Spain
38 [n] Department of Ecology and Diseases of Game, Fish and Bees, University of
39 Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic
40 [o] CEITEC - Central European Institute of Technology, University of Veterinary and
41 Pharmaceutical Sciences Brno, Brno, Czech Republic
42 [p] Institute of Microbiology and Immunology, Faculty of Medicine, Zaloška 4, 1000
43 Ljubljana, Slovenia
44 [q] Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine,
45 Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece
46 [r] Diagnostic Laboratory, School of Veterinary Medicine, Faculty of Health Sciences,
47 Aristotle University of Thessaloniki, Greece

48 [s] Università degli Studi di Torino Dipartimento di Scienze Veterinarie Largo Paolo
49 Braccini, 2 (già Via L. DaVinci, 44)- 10095 Grugliasco (TO), Italy
50 [t] Julius Kühn-Institute, Federal Research Centre for Cultivated Plants, Institute for
51 Plant Protection in Horticulture and Forestry, Vertebrate Research, 48161 Münster,
52 Germany
53 [u] University of Bern, Institute of Ecology and Evolution, Baltzerstrasse 6, 3012 Bern,
54 Switzerland
55 [v] Swiss Institute of Bioinformatics, Quartier Sorge - Batiment Genopode, 1015
56 Lausanne, Switzerland
57 [w] German Center for Infection Research (DZIF), partner site Hamburg-Luebeck-
58 Borstel-Insel Riems, Germany
59
60 Current addresses:

[1] Seramun Diagnostica GmbH, 15754 Heidesee, Germany

[2] Labor Doz. DDr. Stefan Mustafa, 1030 Wien, Austria

[3] University Medicine Greifswald, Pediatric Hematology and Oncology, Ferdinand-Sauerbruch-Straße, 17475 Greifswald, Germany

[4] Labor Ostsachsen, Bremer Str. 57, 01067 Dresden, Germany

61 *Corresponding author at: Institute of Novel and Emerging Infectious Diseases,
62 Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10,
63 D-17493 Greifswald-Insel Riems, Germany. Tel. +49 38351 7 1159; fax +49 38351 7
64 1192.
65 *E-mail address:* rainer.ulrich@fli.de (R.G. Ulrich)

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

67

68 **ABSTRACT**

69

70 Rat hepatitis E virus (ratHEV) is genetically only distantly related to hepeviruses
71 found in other mammalian reservoirs and in humans. It was initially detected in
72 Norway rats (*Rattus norvegicus*) from Germany, and subsequently in rats from
73 Vietnam, the USA, Indonesia, China, Denmark and France.
74 Here, we report on a molecular survey of Norway and Black rats from 12 European
75 countries for ratHEV and human pathogenic hepeviruses. RatHEV-specific real-time
76 and conventional RT-PCR investigations revealed the presence of ratHEV in 63 of
77 508 (12.4%) rats at the majority of sites in 11 of 12 countries. In contrast, a real-time
78 RT-PCR specific for human pathogenic HEV genotypes 1-4 and a nested broad-
79 spectrum (NBS) RT-PCR with subsequent sequence determination did not detect any
80 infections with these genotypes. Only in a single Norway rat from Belgium a rabbit
81 HEV-like genotype 3 sequence was detected. Phylogenetic analysis indicated a
82 clustering of all other novel Norway and Black rat-derived sequences with ratHEV
83 sequences from Europe, the USA and a Black rat-derived sequence from Indonesia
84 within the proposed ratHEV genotype 1. No difference in infection status was
85 detected related to age, sex, rat species or density of human settlements and
86 zoological gardens.
87 In conclusion, our investigation shows a broad geographical distribution of ratHEV in
88 Norway and Black rats from Europe and its presence in all settlement types
89 investigated.

90

91 ## 1. Introduction

92 The family *Hepeviridae* comprises an increasing number of viruses in mammals,
93 birds and fish (Johne et al., 2014, Pérez-Gracia et al., 2015). Initially, hepatitis E virus
94 (HEV) was the only member of this virus family, which was divided into four
95 genotypes. The genotypes 1 and 2 are supposed to exclusively infect humans,
96 whereas genotypes 3 and 4 are zoonotic with wild boar, domestic pig and deer
97 representing animal reservoirs (Meng et al., 2013). In chicken, additional divergent
98 genotypes were discovered and designated as Avian HEV, which can be associated
99 with the diseases Big Liver and Spleen Disease and Hepatitis-Splenomegaly
100 Syndrome (Handlinger and Williams 1988, Ritchie and Riddell 1991; Gerber et al.,
101 2015). The International Committee on Taxonomy of Viruses (ICTV) currently
102 classifies the human pathogenic HEV genotypes 1-4 into species *Orthohepevirus A*,
103 avian HEV into *Orthohepevirus B*, batHEV into *Orthohepevirus D* and the carnivore
104 and ratHEV into *Orthohepevirus C* (http://ictvonline.org/virusTaxonomy.asp,
105 accessed 07.04. 2017).

106 The hepevirus genome is a positive stranded RNA of approximately 6.7 to 7.3
107 kilobases (Meng et al., 2012). The genome contains the typical sequence elements
108 of an eukaryotic mRNA with a cap structure at its 5´-end and a polyadenylation at its
109 3´-end (Tam et al., 1991). For all hepeviruses, three major open reading frames
110 (ORF) were identified with almost the same organization, but differences in the
111 junction or overlapping region of ORF1 and ORF2/ORF3 (Johne et al., 2014). The
112 ORF1 of 4.6 to 5.2 kb is located at the 5´-end of the genome and codes for a
113 polyprotein comprising several nonstructural proteins including regions with similarity
114 to methyltransferases, papain-like proteases, helicases and RNA-dependent RNA

115 polymerases (Koonin et al., 1992). The capsid protein of 600-675 amino acid
116 residues is encoded by ORF2 and contains three domains with the carboxyterminal
117 domain being exposed on the surface of the virion (Yamashita et al., 2009). The
118 overlapping ORF3 codes for a small phosphoprotein of strongly varying length in
119 avian, mammalian and fish hepeviruses (Zafrullah et al., 1997; Holla et al., 2013;
120 Johne et al., 2014). This protein is essential for virus egress and found to be
121 associated with lipid membranes (Okamoto, 2013). Interestingly, ratHEV as well as
122 ferretHEV contains an additional putative open reading frame (ORF4), overlapping
123 ORF1 at its 5´-end, of still unknown function (Johne et al., 2010a, Raj et al., 2012).

124 Using a broad-spectrum RT-PCR assay, a novel, only distantly-related hepevirus was
125 identified in 2010 in Norway rats (*Rattus norvegicus)* from Hamburg, Germany
126 (Johne et al., 2010a; Johne et al., 2010b). This initial finding was confirmed by
127 detection of closely related sequences in Norway rats from other cities in Germany
128 (Johne et al., 2012). Detection of related sequences in rats from the USA, Vietnam,
129 Denmark, France, China and Indonesia suggests a host specificity of ratHEV for rats
130 of the genus *Rattus* and indicated its broad geographical distribution (Li et al., 2013b;
131 Li et al., 2013d; Mulyanto et al., 2013; Mulyanto et al., 2014; Purcell et al., 2011;
132 Widen et al., 2014; Wolf et al., 2013). The host specificity of this virus was also
133 demonstrated by infection experiments using laboratory rats and other mammals
134 (Cossaboom et al., 2012; Li et al., 2013c). However, recent studies in China
135 suggested a broader host range of the virus or frequent spillover infections of
136 bandicoot rats and even shrews (Guan et al., 2013; Li et al., 2013d). The genotypes
137 G1, G2 and G3 of ratHEV were previously defined on the basis of a complete
138 genome sequence comparison; a further comparison of 31 ORF 2-derived

139 sequences of 281-bp lengthrevealed two additional sequences of a non-designated
140 clade (ND), which clustered with G1 (Mulyanto et al., 2014). All G1 ratHEV
141 sequences in previous studies originated from *R. norvegicus* or *R. rattus*, whereas
142 ratHEV sequences of G3 originated exclusively from *R. rattus.* In contrast, genotype
143 G2 was detected in *R. rattus*, *R. tanezumi*, *R. rattoides losea* and the shrew *Suncus*
144 *murinus* (Li et al., 2013b; Li et al., 2013d; Mulyanto et al., 2013).

145 The zoonotic potential of ratHEV is currently controversially discussed. Serological
146 studies in forestry workers showed a few seropositive individuals (Dremsek et al.,
147 2012). In addition, febrile patients from China showed a stronger reactivity with
148 ratHEV antigen than with genotype 1 and 3 antigens (Shimizu et al., 2016).
149 Furthermore, ratHEV was shown to replicate in a human-derived cell line (Jirintai et
150 al., 2014; Li et al., 2015). In contrast, experimental infection of monkeys and
151 domestic pigs with ratHEV failed (Cossaboom et al., 2012; Purcell et al., 2011).
152 Reproducible experimental infections of nude rats and Wistar rats with ratHEV (Li et
153 al., 2013c; Purcell et al., 2011) and the availability of a recently developed reverse
154 genetics system for ratHEV (Li et al., 2015) led to the suggestion to use ratHEV-
155 infected laboratory rats as an infection model for hepeviruses. On the other hand,
156 Norway rats were found to be infected with human pathogenic genotype 3 associated
157 strains, suggesting a potential role for zoonotic transmission (Lack et al., 2012; Kanai
158 et al., 2012).

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

162

163

164 ## 2. Material and methods

165 ### *2.1. Rat collection, dissection and sample collection*

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

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

176

177 ### *2.2. RNA isolation, real-time and conventional RT-PCR and sequencing*

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

184 the data were evaluated using 7500 Software v2.0.1 (Applied Biosystems Life
185 Technologies, Darmstadt, Germany).

186 A one-step RT-PCR (designated SW-RT-PCR; see Fig. 2) was then performed using
187 a SuperScriptIII One-Step RT-PCR with PlatinumTaq Kit (Invitrogen Life
188 Technologies, Carlsbad, CA, USA) in a C1000 Thermal Cycler (Bio-Rad
189 Laboratories, Munich, Germany). Reverse transcription was conducted at 42 °C for
190 50 min, followed by a denaturation step at 94 °C for 2 min. A total of 45 PCR cycles
191 each consisting of 30 s at 94 °C, 30 s at the primer-specific annealing temperature
192 (Table 1), 1 min at 68 °C and a final incubation at 68 °C for 10 min were performed.

193 Additionally, a slightly modified nested broad-spectrum (NBS) RT-PCR was
194 performed to test the samples for all possible HEV strains, including ratHEV and
195 human pathogenic genotypes as described (Johne et al., 2010b; see Fig. 2). A first
196 RT-PCR was performed using a One-Step RT-PCR kit (Qiagen) with primers HEV-cs
197 and HEV-cas in a 2720 thermal cycler (Applied Biosystems). The thermal profile
198 comprised 42 °C for 60 min and 95 °C for 15 min, followed by 40 cycles of 94 °C for
199 30 s, 50 °C for 30 s and 74 °C for 45 s, with a final incubation at 74 °C for 5 min. An
200 aliquot of the RT-PCR product (5 µl) was used in a nested PCR with a GoTaq kit
201 (Promega) and the primers HEV-csn and HEV-casn. The thermal profile consisted of
202 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,
203 with a final incubation at 72 °C for 5 min.

204 To generate a longer sequence stretch, overlapping the SW-/NBS-RT-PCR products
205 and including the 3'-end of ORF 1, the 5'-region of ORF 2 and a partial or complete
206 ORF 3, selected samples were analyzed by a primer walking-based attempt using

207 two different primer pairs and following the protocols of the SW-RT-PCR (see Fig. 2
208 and Table 1; Primer-walking RT-PCR-I/II).

209

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

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

221

222 *2.3. Phylogenetic analysis*

223 The General Time Reversible + discrete Gamma distribution (GTR+G) model was the
224 best suited substitution model determined by MEGA 7 for both regions spanning
225 nucleotides (nt) 4,105-4,387 (numbering based on strain R63, acc. no. GU345042)
226 and nucleotides 4,105-5,226. The phylogenetic analyses were performed by
227 Bayesian algorithms via MrBayes v.3.2.2 and CIPRES online portal (Ronquist et al.,
228 2012) and by Maximum likelihood algorithm performed via MEGA7 (Kumar et al.,
229 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,

253 156 rats), Denmark (1 site, 11 rats), Austria (1 site, 43 rats), Switzerland (3 sites, 29
254 rats), Czech Republic (3 sites, 58 rats), Belgium (2 sites, 60 rats), France (1 site, 28
255 rats), Slovenia (1 site, 1 animal) and Greece (3 sites, 16 rats) and 88 Black rats from
256 trapping sites in Italy (1 site, 17 rats), Slovenia (1 site, 17 rats), Greece (2 sites, 4
257 rats) and Spain (1 site, 50 rats). Initially, liver-derived RNA preparations of a Norway
258 rat sample subset were tested in parallel by real-time RT-PCR assays either targeting
259 ratHEV or HEV genotypes 1 to 4. The ratHEV-specific real-time RT-PCR (RTD)
260 resulted in the detection of 5 out of 145 (3.4%) samples from Germany (Table 2).
261 Norway rat samples from Hungary, Denmark, Switzerland and France were also
262 positive for ratHEV-RNA by ratHEV-specific real-time RT-PCR with a detection range
263 of 5.5% (1/18) to 18.1% (2/11; see Table 2). The Ct values of positive samples
264 ranged between 20 and 34. In the real-time RT-PCR targeting the human pathogenic
265 genotypes 1-4 none of the Norway rat samples showed a Ct value <35, used as cut-
266 off (Table 2).

267

268 *3.2. Conventional SW-RT-PCR and NBS-RT-PCR analysis*

269 A conventional RT-PCR approach using ORF1-specific SW-RT-PCR (nt positions
270 4,105-4,387, prototype strain R63, accession number GU345042, see Fig. 2) and
271 NBS RT-PCR (nt positions 4,000-4,423, see Fig. 2) resulted in the detection of HEV-
272 specific RNA in 17 of 156 (10.8%) samples from Germany (Table 2). The prevalence
273 for samples from the sites in the other ten countries reached from 4% (2/50) to 27.2%
274 3/11; Table 2). The prevalences in Norway rats and Black rats were 10%-27.2%
275 (2/20 and 3/11) and 4%-5.8% (2/50 and 1/17), respectively. None of the single
276 Norway rat and 17 Black rats from Slovenia was HEV-RNA positive (Table 2).

277 Using a primer-walking based approach for thirteen samples from nine sites in
278 Germany, France, Spain, Belgium, Austria and Denmark, a 1,122/1,125-base pair
279 (bp) long region including parts of ORF1, ORF2 and partial or entire ORF3 (see Fig.
280 2) was RT-PCR amplified and sequenced (see Table 4). The different lengths of the
281 sequences B1 and B4 from France were caused by a triplet indel, i.e.,
282 insertion/deletion of three nucleotides (data not shown).

283

284 *3.3. Sequence comparison and phylogenetic analysis*

285 Phylogenetic analysis of the 280 bp fusion-product of the SW-/NBS-RT-PCR assays
286 showed that almost all novel sequences, independently whether from Norway or
287 Black rats, clustered together with ratHEV sequences, species *Orthohepevirus C1,*
288 well separated from sequences of species *Orthohepevirus C2* (Figs. 3A and 3B). In
289 one Norway rat sample from Belgium (KS/16/825) a sequence with 88.8% sequence
290 similarity to genotype 3 HEV sequences was found (see below); in no other sample
291 human pathogenic genotype-related sequences were found. This HEV genotype 3 -
292 like sequence from the single Norway rat sample from Belgium clustered in the
293 phylogenetic tree with three rabbit HEV strains from China and a human rabbit HEV
294 sequence from France within species *Orthohepevirus A* (Fig. 3C); attempts to
295 generate a longer sequence failed. The phylogenetic analysis of the concatenated
296 1,122/1,125 bp product of the coding sequences revealed clustering of all novel
297 sequences within the ratHEV genotype G1 defined by Mulyanto et al. (2014), in sister
298 clade relationship with ratHEV genotypes G2 and G3 (Fig. 3D). Genotype G1
299 contains the prototype sequence R63 from a Norway rat from Hamburg, Norway rat-
300 derived sequences from different European countries and the USA, Black rat-derived

301 sequences from Spain and Italy and one sequence originating from a Black rat
302 collected in Solo, Indonesia (Figs. 3B and D).

303 A novel sequence from rats in Berlin, detected in five animals, clustered with a
304 previously determined sequence from Berlin and two novel sequences from rats in
305 Esslingen, with one found in four animals, clustered with a sequence detected
306 previously in Stuttgart, a site close to Esslingen (Johne et al., 2012; see Fig. 3B, and
307 legend to Fig. 3). Similarly, two sequences from Warburg formed a well-separated
308 subclade and all sequences from Czech Republic were highly related (Fig. 3B). Most
309 novel ratHEV sequences from Vienna formed a well-supported cluster but one
310 sequence (KS12-1338) was highly divergent. Both sequences from Spain are closely
311 related, independently if the 280 bp or 1,222 bp products were analyzed (Figs. 3B
312 and D). Interestingly, ratHEV sequences from three trapping sites close to Lyon (B
313 and E/A) formed two well-separated subclades and sequences from Zurich belonged
314 also to two subclades (Fig. 3B). Sequences from Norway rats from Belgium were
315 found at highly divergent positions within the tree (Fig. 3B).

316 Comparison of ORF1-derived sequences from the fusion product of SW-/NBS-RT-
317 PCR from the same site resulted in an intra-cluster sequence similarity of 79.6% to
318 100% for the nucleotide and 86.8% to 100% for the corresponding amino acid
319 sequences (Table 3). When analyzing the nucleotide sequence similarity within
320 partial ORF1 or the overlapping ORF1/ORF2/ORF3 regions between different sites,
321 the values reached similar levels of 81.0% to 96.1% and 87.2% to 91.5%,
322 respectively (Supplementary Table and Table 4). The corresponding aa sequence
323 similarities of ORF1-encoded protein and concatenated ORF1- and ORF2-encoded

324 proteins ranged between 93.4% and 100% and 95.9% and 98.6%, respectively
325 (Supplementary Table and Table 4).

326

327 ### *3.4. Association of ratHEV infections with age, sex, rat species and human*
328 ### *settlement density*

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

339

340 ## **4. Discussion**

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

347 11 of 12 countries. This finding is in line with the previously demonstrated inability in
348 experimentally infecting Norway rats with human pathogenic genotypes ((Li et al.,
349 2013a; Li et al., 2013c; Purcell et al., 2011) and results from earlier field studies in
350 Norway rats (Johne et al., 2012; Johne et al., 2010a). Similar to previous studies
351 reporting the human pathogenic HEV genotype 3 in Norway rats (Lack et al., 2012;
352 Kanai et al., 2012), in one Norway rat from Belgium a short rabbit HEV-like genotype
353 3 sequence was detected. This might be explained by a spillover infection of this
354 strain from a rabbit reservoir. Rabbits and rats may share their habitats in this region
355 of Belgium, either in wildlife habitats or when wild (pest) rats search for food close to
356 private rabbit husbandry.

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

362 In addition, in our study ratHEV was not only detected in rats from urban areas, but
363 also in rats from small towns and rural areas. The detection of ratHEV in rural areas
364 complements our previous finding of a local absence of ratHEV in a rural area close
365 to Ahlen (Johne et al., 2012), which may suggest site-specific differences and a
366 heterogeneous distribution of ratHEV not primarily driven by human settlement. In
367 addition, ratHEV was identified in pest rats from zoological gardens raising questions
368 on the potential transmission of this virus to zoo animals. In fact, serological
369 investigations have detected HEV-specific antibodies in captive macaques and HEV-
370 RNA in different mammalian and avian species in a wildlife rescue center in China

371 (Korzaia et al., 2007; Zhang et al., 2008). The recently developed in-house ELISA
372 technology based on ratHEV- and HEV genotype 3-derived recombinant capsid
373 protein derivatives (Dremsek et al., 2012; Johne et al., 2012) may be used in the
374 future for differentiation of antibodies raised against these viruses in zoo animals.

375 The phylogenetic analysis of the novel ratHEV sequences showed for almost all a
376 high similarity to ratHEV genotype 1 defined recently (Mulyanto et al., 2014),
377 independently whether the sequences originated from Norway or Black rats. In line
378 with a previous investigation (Purdy and Sue, 2017), the resolution of the
379 phylogenetic analysis using the short-sized ORF1 region was lower than the
380 resolution for the larger segment of ORF1/ORF2/ORF3. The observed phylogenetic
381 clustering of many sequences from the same or neighbouring sites may indicate the
382 persistence of ratHEV strains within the local populations. The separate clustering of
383 sequences from the same geographical origin might be caused by an incursion (and
384 perhaps establishment) of additional, highly divergent ratHEV strains by invading
385 rats. In line with this assumption, sequences from the USA (strain LA-8350) and
386 Indonesia (strain SOLO-006SF) cluster also within genotype 1 of ratHEV (Figs. 3B
387 and D).

388 The previous finding of the majority of rats being only HEV RNA or anti-ratHEV
389 antibody positive suggested non-persistent infections in individual rats (Johne et al.,
390 2012). In line with this assumption, we did not find here a significantly higher RNA
391 prevalence in adult rats compared to juvenile animals. These findings of non-
392 persistent infections of rats are also in line with results of experimental infection
393 studies in Norway rats (Purcell et al., 2011). At this time we cannot exclude age-
394 dependent differences in susceptibility and mortality of rats for ratHEV infection,

395 possibly associated with co-infections with other pathogens or genetic or
396 environmental factors.

397

398 ## 5. Conclusion

399 The detection of ratHEV in Norway and Black rats from 11 European countries
400 indicates a broad geographical distribution of ratHEV suggesting an (almost)
401 continent-wide occurrence and no specific association with human population
402 density. Phylogenetic investigations indicated clustering of all European ratHEV
403 sequences within ratHEV genotype 1. Well-separated subclades of sequences from
404 the same or neighbouring sites might indicate the incursion of novel ratHEV strains
405 into local Norway rat populations with a parallel persistence of a local ratHEV strain.
406 This necessitates future studies on the population structure and potential invasion of
407 individuals into existing rat populations and their association with ratHEV incursion. In
408 addition, the finding of ratHEV infections in zoological gardens may allow future
409 studies on the zoonotic potential of ratHEV based on the investigation of putative
410 natural ratHEV transmission to non-human primates. Finally, the finding of a rabbit
411 HEV-like sequence in a single Norway rat necessitates further studies, especially in
412 habitats with sympatric occurrence of rabbits or pigs and rats, to evaluate potential
413 spillover infections of human pathogenic genotype(s) and their potential public health
414 impact.

415

416 ## Conflict of Interest

417 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).

448 **Figure legends**

449 **Fig. 1.** Geographical map representing the rat collection sites in Denmark (1,
450 Copenhagen), Germany (2, Hamburg; 3, Elmenhorst; 4, Stahlbrode; 5, Osnabrück; 6,
451 Wolbrechtshausen; 7, Magdeburg; 8, Kampehl; 9, Berlin; 10, Neschwitz; 11,
452 Königshain; 12, Görlitz; 13, Niederoderwitz; 14, Zittau; 15, Aachen; 16, Köln; 17, Oer-
453 Erkenschwick; 18, Münster; 19, Ahlen; 20, Warburg; 21, Heidelberg; 22, Stuttgart; 23,
454 Esslingen; 24, Möggingen), Switzerland (25, Gränichen, 26, Dübendorf, 27, Zurich),
455 Austria (28, Vienna), Hungary (29, Budapest), France (30, five sites close to Lyon),
456 Belgium (31 Dender, 32 Ijzer), Italy (33 Pianosa Island), Slovenia (34 close to
457 Ljubljana), Spain (35 Cadiz), Czech Republic (36 Prague, 37 Brno, 38 Northern
458 Moravia), Greece (39 Thessaloniki, 40 Kilkis, 41 Chalkidiki). All or some of the rats
459 from sites 1, 2, 9, 19, 22 and 23 were investigated for ratHEV previously (Johne et
460 al., 2010, 2012; Wolf et al., 2013; indicated by empty or half-filled circles,
461 respectively) and were included here for analysis of demographic, reservoir and
462 human settlement type association of ratHEV infections (see Heuser et al., 2016).

463

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

468

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

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

474 Consensus phylogenetic trees based on Bayesian analyses were done with
475 8,000,000 or 6,000,000 generations and a burn-in of 25%, and Maximum-Likelihood
476 analysis with 1,000 bootstraps and 50% cut-off, of a part of ORF 1 (nt positions
477 4,105-4,387, counting according prototype strain R63, accession number GU345042)
478 (A-C) and the concatenated region of ORF1 (4,105-4,921) and partial ORF2/ORF3
479 overlapping region (nt positions 4,949-5,226) (D) of ratHEV. Posterior
480 probability/bootstrap values of >50 are given at the supported nodes.

481 The ratHEV genotypes G1, G2 and G3 were defined previously (Mulyanto et al.,
482 2014); the two sequences of clade ND (not designated) were found in this previous
483 study, based on a partial region of ORF2, to be clustering with G1.

484 Novel sequences are given in bold and labeled by a star. Identical sequences were
485 omitted from the analysis and only different sequence types are presented (Berlin
486 KS11/573 = KS11/576, /578, /580, /587, Esslingen Mu10/1564= Mu10/1567, /1568,
487 /1571, Warburg Mu10/697= Mu10/698, Zurich KS12/1361=KS12/1363, Czech
488 Republic KS14/73 = KS14/75, /76, /99, and KS14/70 = KS14/80, /98).

489

490 **References**

491 Batts, W., Yun, S., Hedrick, R., Winton, J., 2011. A novel member of the family
492 *Hepeviridae* from cutthroat trout (*Oncorhynchus clarkii*). Virus Res 158, 116-
493 123.
494 Bodewes, R., van der Giessen, J., Haagmans, B.L., Osterhaus, A.D., Smits, S.L.,
495 2013. Identification of multiple novel viruses, including a parvovirus and a
496 hepevirus, in feces of red foxes. J Virol 87, 7758-7764.
497 Cossaboom, C.M., Cordoba, L., Sanford, B.J., Pineyro, P., Kenney, S.P., Dryman,
498 B.A., Wang, Y., Meng, X.J., 2012. Cross-species infection of pigs with a novel
499 rabbit, but not rat, strain of hepatitis E virus isolated in the United States. J
500 Gen Virol 93, 1687-1695.
501 Dremsek, P., Wenzel, J.J., Johne, R., Ziller, M., Hofmann, J., Groschup, M.H.,
502 Werdermann, S., Mohn, U., Dorn, S., Motz, M., Mertens, M., Jilg, W., Ulrich,
503 R.G., 2012. Seroprevalence study in forestry workers from eastern Germany
504 using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G
505 ELISAs. Med Microbiol Immunol 201, 189-200.
506 Drexler, J.F., Seelen, A., Corman, V.M., Fumie Tateno, A., Cottontail, V., Melim
507 Zerbinati, R., Gloza-Rausch, F., Klose, S.M., Adu-Sarkodie, Y., Oppong, S.K.,
508 Kalko, E.K., Osterman, A., Rasche, A., Adam, A., Muller, M.A., Ulrich, R.G.,
509 Leroy, E.M., Lukashev, A.N., Drosten, C., 2012. Bats worldwide carry hepatitis
510 E virus-related viruses that form a putative novel genus within the family
511 *Hepeviridae*. J Virol 86, 9134-9147.

512 Gerber, P. F., Trampel, D. W., Willinghan, E. M., Billam, P., Meng, X. J., &
513 Opriessnig, T. (2015). Subclinical avian hepatitis E virus infection in layer
514 flocks in the United States. Vet. J., 206(3), 304-311.
515 Geostat initiative, Eurostat. http://eceuropaeu/eurostat/web/gisco/gisco-
516 activities/integrating-statistics-geospatial-information/geostat-initiative 2012).
517 Guan, D., Li, W., Su, J., Fang, L., Takeda, N., Wakita, T., Li, T.C., Ke, C., 2013.
518 Asian musk shrew as a reservoir of rat hepatitis E virus, China. Emerg Infect
519 Dis 19, 1341-1343.
520 Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and
521 analysis program for Windows 95/98/NT. Nucleic acids symposium series Vol.
522 41.
523 Handlinger, J. H., & Williams, W. (1988). An egg drop associated with splenomegaly
524 in broiler breeders. Avian Dis, 32(4): 773-778.
525 Heuser, E., Fischer, S., Ryll, R., Mayer-Scholl, A., Hoffmann, D., Spahr, C., Imholt,
526 C., Alfa, D.M., Frohlich, A., Luschow, D., Johne, R., Ehlers, B., Essbauer, S.,
527 Nockler, K., Ulrich, R.G., 2016. Survey for zoonotic pathogens in Norway rat
528 populations from Europe. Pest Manag Sci, 73(2), 341-348.
529 Holla RP, Ahmad I, Ahmad Z, Jameel S. Molecular virology of hepatitis E virus.
530 Semin Liver Dis. 2013 Feb;33(1):3-14.
531 Jirintai, S., Tanggis, Mulyanto, Suparyatmo, J.B., Takahashi, M., Kobayashi, T.,
532 Nagashima, S., Nishizawa, T., Okamoto, H., 2014. Rat hepatitis E virus
533 derived from wild rats (*Rattus rattus*) propagates efficiently in human
534 hepatoma cell lines. Virus Res 185, 92-102.
535 Johne, R., Dremsek, P., Kindler, E., Schielke, A., Plenge-Bonig, A., Gregersen, H.,
536 Wessels, U., Schmidt, K., Rietschel, W., Groschup, M.H., Guenther, S.,

537 Heckel, G., Ulrich, R.G., 2012. Rat hepatitis E virus: geographical clustering
538 within Germany and serological detection in wild Norway rats (*Rattus*
539 *norvegicus*). Infect Genet Evol 12, 947-956.
540 Johne, R., Dremsek, P., Reetz, J., Heckel, G., Hess, M., Ulrich, R.G., 2014.
541 *Hepeviridae*: an expanding family of vertebrate viruses. Infect Genet Evol 27,
542 212-229.
543 Johne, R., Heckel, G., Plenge-Bonig, A., Kindler, E., Maresch, C., Reetz, J., Schielke,
544 A., Ulrich, R.G., 2010a. Novel hepatitis E virus genotype in Norway rats,
545 Germany. Emerg Infect Dis 16, 1452-1455.
546 Johne, R., Plenge-Bonig, A., Hess, M., Ulrich, R.G., Reetz, J., Schielke, A., 2010b.
547 Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested
548 broad-spectrum RT-PCR. J Gen Virol 91, 750-758.
549 Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J., Hill, V.R., 2006. A
550 broadly reactive one-step real-time RT-PCR assay for rapid and sensitive
551 detection of hepatitis E virus. J Virol Methods 131, 65-71.
552 Kanai, Y., Miyasaka, S., Uyama, S., Kawami, S., Kato-Mori, Y., Tsujikawa, M.,
553 Yunoki, M., Nishiyama, S., Ikuta, K., Hagiwara, K. 2012. Hepatitis E virus in
554 Norway rats (Rattus norvegicus) captured around a pig farm. BMC research
555 notes, 5(1), 4.
556 Koonin EV, Gorbalenya AE, Purdy MA, Rozanov MN, Reyes GR, Bradley DW.
557 Computer-assisted assignment of functional domains in the nonstructural
558 polyprotein of hepatitis E virus: delineation of an additional group of positive-
559 strand RNA plant and animal viruses. Proc Natl Acad Sci U S A. 1992 Sep
560 1;89(17):8259-63.

561 Korzaia, L.I., Lapin, B.A., Keburia, V.V., Lazareva, I., 2007. [Hepatitis E virus
562 antibodies in the macaques and in the personnel serving the macaques of the
563 Adler apery]. Vopr Virusol 52, 36-40.
564 Krog, J.S., Breum, S.O., Jensen, T.H., Larsen, L.E., 2013. Hepatitis E virus variant in
565 farmed mink, Denmark. Emerg Infect Dis 19, 2028-2030.
566 Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics
567 Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33, 1870-1874.
568 Lack, J.B., Volk, K., Van Den Bussche, R.A., 2012. Hepatitis E virus genotype 3 in
569 wild rats, United States. Emerg Infect Dis 18, 1268-1273.
570 Li, T.C., Ami, Y., Suzaki, Y., Takeda, N., Takaji, W., 2013a. No evidence for hepatitis
571 E virus genotype 3 susceptibility in rats. Emerg Infect Dis 19, 1343-1345.
572 Li, T.C., Ami, Y., Suzaki, Y., Yasuda, S.P., Yoshimatsu, K., Arikawa, J., Takeda, N.,
573 Takaji, W., 2013b. Characterization of full genome of rat hepatitis E virus strain
574 from Vietnam. Emerg Infect Dis 19, 115-118.
575 Li, T.C., Yang, T., Yoshizaki, S., Ami, Y., Suzaki, Y., Ishii, K., Haga, K., Nakamura,
576 T., Ochiai, S., Takaji, W., Johne, R., 2015. Construction and characterization
577 of an infectious cDNA clone of rat hepatitis E virus. J Gen Virol 96, 1320-1327.
578 Li, T.C., Yoshizaki, S., Ami, Y., Suzaki, Y., Yasuda, S.P., Yoshimatsu, K., Arikawa, J.,
579 Takeda, N., Wakita, T., 2013c. Susceptibility of laboratory rats against
580 genotypes 1, 3, 4, and rat hepatitis E viruses. Vet Microbiol 163, 54-61.
581 Li, W., Guan, D., Su, J., Takeda, N., Wakita, T., Li, T.C., Ke, C.W., 2013d. High
582 prevalence of rat hepatitis E virus in wild rats in China. Vet Microbiol 165, 275-
583 280.
584 Lin, J., Norder, H., Uhlhorn, H., Belak, S., Widen, F., 2014. Novel hepatitis E like
585 virus found in Swedish moose. J Gen Virol 95, 557-570.

586 Meng, X. J., Anderson, D. A., Arankalle, V. A., Emerson, S. U., Harrison, T. J.,
587 Jameel, S., Okamoto, H. (2012). Hepeviridae. Virus Taxonomy 9th Report of
588 the ICTV, Elsevier Academic Press, London (2012), pp. 1021–1028.
589 Meng, X. J. (2013). Zoonotic and foodborne transmission of hepatitis E virus. In
590 Seminars in liver disease (Vol. 33, No. 01, pp. 041-049). Thieme Medical
591 Publishers.
592 Mulyanto, Depamede, S.N., Sriasih, M., Takahashi, M., Nagashima, S., Jirintai, S.,
593 Nishizawa, T., Okamoto, H., 2013. Frequent detection and characterization of
594 hepatitis E virus variants in wild rats (*Rattus rattus*) in Indonesia. Arch Virol
595 158, 87-96.
596 Mulyanto, Suparyatmo, J.B., Andayani, I.G., Khalid, Takahashi, M., Ohnishi, H.,
597 Jirintai, S., Nagashima, S., Nishizawa, T., Okamoto, H., 2014. Marked
598 genomic heterogeneity of rat hepatitis E virus strains in Indonesia
599 demonstrated on a full-length genome analysis. Virus Res 179, 102-112.
600 Okamoto, H., 2013. Culture systems for hepatitis E virus. J Gastroenterol 48, 147-
601 158.
602 Purcell, R.H., Engle, R.E., Rood, M.P., Kabrane-Lazizi, Y., Nguyen, H.T.,
603 Govindarajan, S., St Claire, M., Emerson, S.U., 2011. Hepatitis E virus in rats,
604 Los Angeles, California, USA. Emerg Infect Dis 17, 2216-2222.
605 Pérez-Gracia, M. T., García, M., Suay, B., & Mateos-Lindemann, M. L. (2015).
606 Current knowledge on hepatitis E. J Clin Transl Hepatol 3(2), 117.
607 R Foundation for Statical Computing, R: A language and environment for statistical
608 computing. https://www.R-projectorg/ 2015).

609 Raj, V.S., Smits, S.L., Pas, S.D., Provacia, L.B., Moorman-Roest, H., Osterhaus,
610 A.D., Haagmans, B.L., 2012. Novel hepatitis E virus in ferrets, the
611 Netherlands. Emerg Infect Dis 18, 1369-1370.
612 Ritchie, S. J., & Riddell, C. (1991). British Columbia."Hepatitis-splenomegaly"
613 syndrome in commercial egg laying hens. Can. Vet. J., 32(8), 500.
614 Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S.,
615 Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2:
616 efficient Bayesian phylogenetic inference and model choice across a large
617 model space. Syst Biol 61, 539-542.
618 Sachsenroder, J., Braun, A., Machnowska, P., Ng, T.F., Deng, X., Guenther, S.,
619 Bernstein, S., Ulrich, R.G., Delwart, E., Johne, R., 2014. Metagenomic
620 identification of novel enteric viruses in urban wild rats and genome
621 characterization of a group A rotavirus. J Gen Virol 95, 2734-2747.
622 Shimizu, K., Hamaguchi, S., Ngo, C. C., Tian-Cheng, L. I., Yoshimatsu, K., Yasuda,
623 S. P., Fujita, H., Pham, T.T., Le, M.Q., Dang, A.D., Nguyen, T.Q., Yoshida,
624 L.M., Ariyoshi, K. , Arikawa, J.. (2016). Serological evidence of infection with
625 rodent-borne hepatitis E virus HEV-C1 or antigenically related virus in
626 humans. J. Vet. Med. Sci.,, *78*(11), 1677-1681.
627 Smith, D.B., Simmonds, P., International Committee on Taxonomy of Viruses
628 *Hepeviridae* Study, G., Jameel, S., Emerson, S.U., Harrison, T.J., Meng, X.J.,
629 Okamoto, H., Van der Poel, W.H., Purdy, M.A., 2014. Consensus proposals
630 for classification of the family *Hepeviridae*. J Gen Virol 95, 2223-2232.
631 Tam, A. W., Smith, M. M., Guerra, M. E., Huang, C. C., Bradley, D. W., Fry, K. E., &
632 Reyes, G. R. (1991). Hepatitis E virus (HEV): molecular cloning and
633 sequencing of the full-length viral genome. Virology, 185(1), 120-131.

634 Webster, J.P., Ellis, W.A., Macdonald, D.W., 1995. Prevalence of *Leptospira* spp. in
635 wild brown rats (*Rattus norvegicus*) on UK farms. Epidemiol Infect 114, 195-
636 201.
637 Widen, F., Ayral, F., Artois, M., Olofson, A.S., Lin, J., 2014. PCR detection and
638 analysis of potentially zoonotic Hepatitis E virus in French rats. Virol J 11, 90.
639 Wolf, S., Reetz, J., Johne, R., Heiberg, A.C., Petri, S., Kanig, H., Ulrich, R.G., 2013.
640 The simultaneous occurrence of human norovirus and hepatitis E virus in a
641 Norway rat (*Rattus norvegicus*). Arch Virol 158, 1575-1578.
642 Yamashita, T., Mori, Y., Miyazaki, N., Cheng, R.H., Yoshimura, M., Unno, H., Shima,
643 R., Moriishi, K., Tsukihara, T., Li, T.C., Takeda, N., Miyamura, T., Matsuura,
644 Y., 2009. Biological and immunological characteristics of hepatitis E virus-like
645 particles based on the crystal structure. Proc Natl Acad Sci U S A 106, 12986-
646 12991.
647 Zafrullah M, Ozdener MH, Panda SK, Jameel S. The ORF3 protein of hepatitis E
648 virus is a phosphoprotein that associates with the cytoskeleton. J Virol. 1997
649 Dec;71(12):9045-53.
650 Zhang, W., Shen, Q., Mou, J., Yang, Z.B., Yuan, C.L., Cui, L., Zhu, J.G., Hua, X.G.,
651 Xu, C.M., Hu, J., 2008. Cross-species infection of hepatitis E virus in a zoo-like
652 location, including birds. Epidemiol Infect 136, 1020-1026.