Short communication

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

René Ryll[a], Martin Eiden[a], Elisa Heuser[a], Markus Weinhardt[b], Madlen Ziege[c,i], Dirk Höper[d], Martin H. Groschup[a], Gerald Heckel[e,f], Reimar Johne[g], Rainer G. Ulrich[a,h,*]

[a] Friedrich-Loeffler-Institut, Institute of Novel and Emerging Infectious Diseases, Greifswald-Insel Riems, Germany
[b] Department of Zoology, State Museum of Natural History, Stuttgart, Germany
[c] Department of Ecology and Evolution, University of Frankfurt, Frankfurt am Main, Germany
[d] Friedrich-Loeffler-Institut, Institute of Diagnostic Virology, Greifswald-Insel Riems, Germany
[e] University of Bern, Institute of Ecology and Evolution, Bern, Switzerland
[f] Swiss Institute of Bioinformatics, Quartier Sorge - Batiment Genopode, Lausanne, Switzerland
[g] German Federal Institute for Risk Assessment, Berlin, Germany
[h] German Center for Infection Research (DZIF), Partner Site Hamburg-Luebeck-Borstel-Insel Riems, Germany
[i] University of Potsdam, Plant Ecology and Nature Conservation, Potsdam (Golm), Germany

ABSTRACT

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

1. Introduction

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

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

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

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

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

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

2. The study

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

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

Table 1

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

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

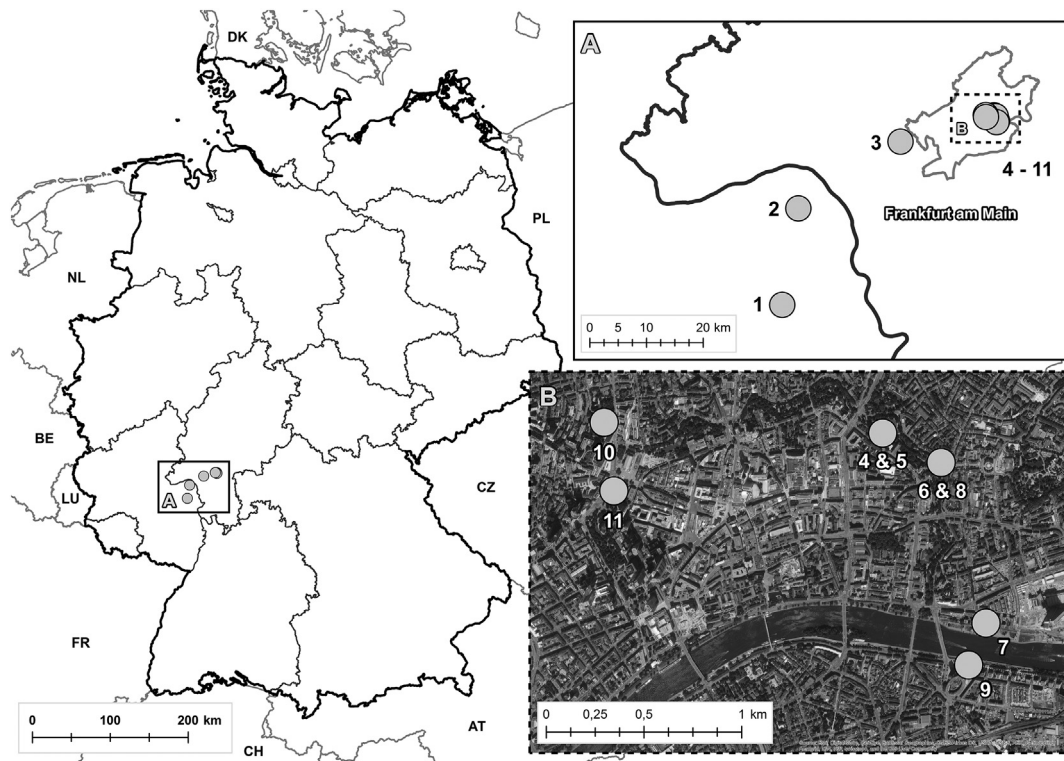m, male; f, female.

No, number.

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

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

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

[4] Including two animals with unknown sex.

[*] For additional information see Fig. 1.

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

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

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

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

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

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

[2] Complete genomes determined.

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

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

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

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

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

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

3. Conclusions

The serological and molecular survey indicated a high prevalence of rabbitHEV in rabbits from Central Germany. Frequent detection and geography-based clustering of rabbitHEV sequences suggest a virus circulation in the local rabbit populations. The close similarity of sequences detected in the inner-city area of Frankfurt am Main may indicate a bottleneck in the rabbit population caused by immigration. The zoonotic potential of rabbitHEV warrants future investigations in human populations with increased risk of exposure due to contact to rabbits.
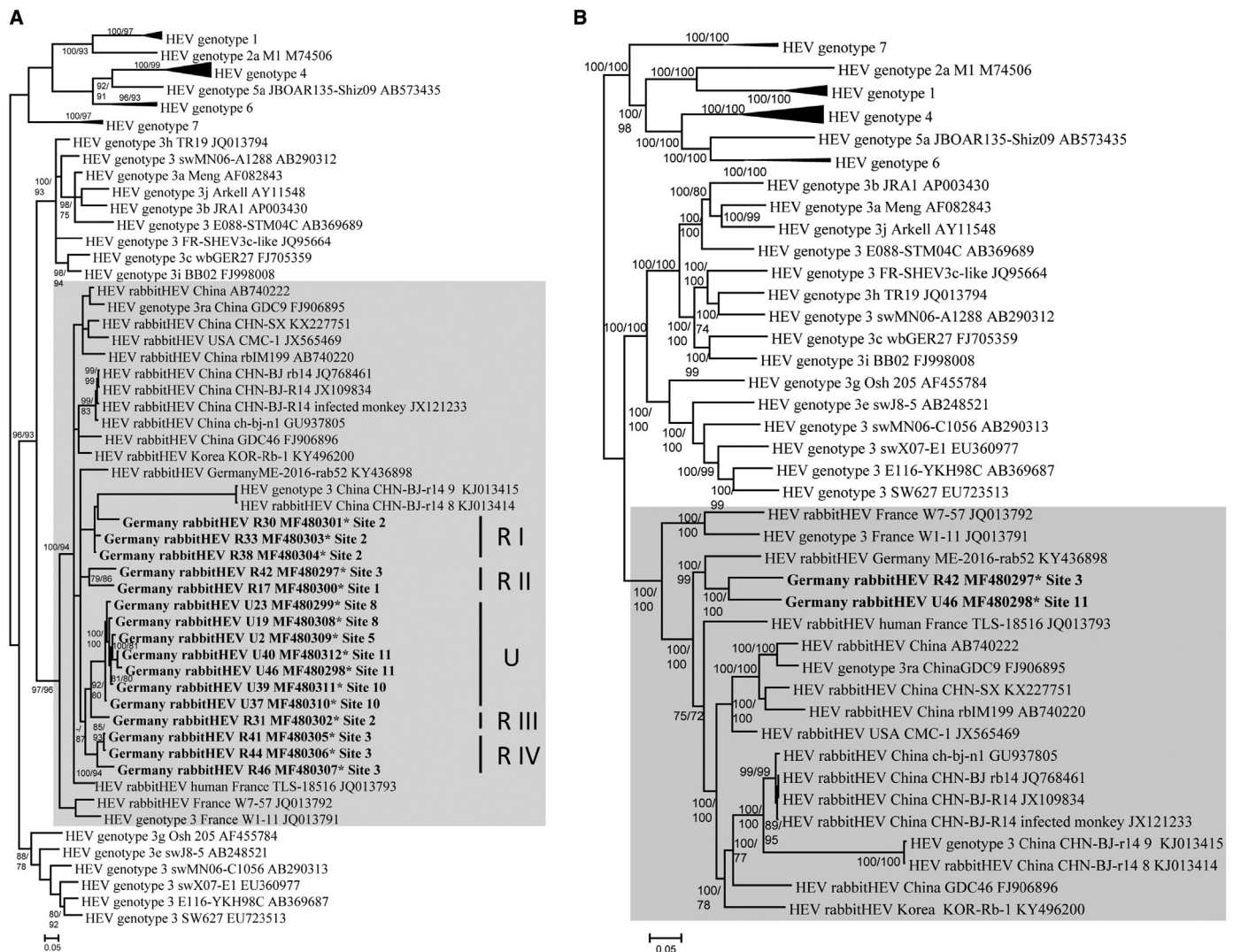
Fig. 2. Consensus phylogenetic trees of the novel rabbitHEV sequences, *Orthohepevirus A* reference sequences proposed by Smith et al., 2016, and additional rabbitHEV-sequences based on Bayesian analyses with 10,000,000 generations and a burn-in of 25%, and Maximum-Likelihood analysis with 1000 bootstraps and 50% cut-off. In (A) the tree for the RdRp-encoding ORF 1 screening fragment, nucleotide positions 4341–4623 (numbering according to rabbitHEV reference strain 3ra GDC9, accession number FJ906895), and in (B) the tree of the concatenated complete coding part of ORF1 and ORF2 are shown. Sequences of the rabbitHEV cluster are highlighted by a grey square. Posterior probability values/bootstrap values > 50 are given at the supported nodes. Novel sequences are given in bold and labeled by an asterisk. For location information, see Fig. 1.

Appendix A. Supplementary data

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

References

Abravanel, F., Lhomme, S., El Costa, H., Schvartz, B., Peron, J.M., Kamar, N., Izopet, J., 2017. Rabbit hepatitis E virus infections in humans, France. Emerg. Infect. Dis. 23, 1191.

Ahn, H.S., Park, B.J., Han, S.H., Kim, Y.H., Kim, D.H., Kim, B.S., et al., 2017. Prevalence and genetic features of rabbit hepatitis E virus in Korea. J. Med. Virol. 89, 1995–2002.

Anheyer-Behmenburg, H.E., Szabo, K., Schotte, U., Binder, A., Klein, G., Johne, R., 2017. Hepatitis E virus in wild boars and spillover infection in red and roe deer, Germany, 2013–2015. Emerg. Infect. Dis. 23, 130.

Bächlein, C., Seehusen, F., Nathues, H., Grosse Beilage, E., Baumgärtner, W., Grummer, B., 2013. Molecular detection of hepatitis E virus in German domestic pigs. Berl. Munch. Tierarztl. Wochenschr. 126, 25–31.

Burt, S.A., Veltman, J., Hakze-van der Honing, R., Schmitt, H., van der Poel, W.H., 2016. Hepatitis E virus in farmed rabbits, wild rabbits and petting farm rabbits in the Netherlands. Food Environ. Virol. 8, 227–229.

Caruso, C., Modesto, P., Prato, R., Scaglione, F.E., De Marco, L., Bollo, E., et al., 2015. Hepatitis E virus: first description in a pet house rabbit. A new transmission route for human? Transbound. Emerg. Dis. 62, 229–232.

Cossaboom, C.M., Córdoba, L., Dryman, B.A., Meng, X.J., 2011. Hepatitis E virus in rabbits, Virginia, USA. Emerg. Infect. Dis. 17, 2047–2049.

Eiden, M., Vina-Rodriguez, A., Schlosser, J., Schirrmeier, H., Groschup, M.H., 2016. Detection of hepatitis E virus in archived rabbit serum samples, Germany 1989. Food Environ. Virol. 8, 105–107.

Faber, M.S., Wenzel, J.J., Jilg, W., Thamm, M., Höhle, M., Stark, K., 2012. Hepatitis E virus seroprevalence among adults, Germany. Emerg. Infect. Dis. 18, 1654.

Hammerschmidt, F., Schwaiger, K., Dähnert, L., Vina-Rodriguez, A., Höper, D., Gareis, M., et al., 2017. Hepatitis E virus in wild rabbits and European brown hares in Germany. Zoonoses Public Health 64, 612–622.

Izopet, J., Dubois, M., Bertagnoli, S., Lhomme, S., Marchandeau, S., Boucher, S., et al., 2012. Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France. Emerg. Infect. Dis. 18, 1274–1281.

Jirintai, S., Manglai, D., Takahashi, M., Nagashima, S., Kobayashi, T., Nishizawa, T.,

Okamoto, H., 2012. Molecular analysis of hepatitis E virus from farm rabbits in Inner Mongolia, China and its successful propagation in A549 and PLC/PRF/5 cells. Virus Res. 170, 126–137.

Johne, R., Plenge-Bönig, A., Hess, M., Ulrich, R.G., Reetz, J., Schielke, A., 2010. Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. J. Gen. Virol. 91, 750–758.

Juozapaitis, M., Aguiar Moreira, É., Mena, I., Giese, S., Riegger, D., Pohlmann, A., et al., 2014. An infectious bat-derived chimeric influenza virus harbouring the entry machinery of an influenza A virus. Nat. Commun. 5, 4448.

Liu, P., Bu, Q.N., Wang, L., Han, J., Du, R.J., Lei, Y.X., et al., 2013. Transmission of hepatitis E virus from rabbits to cynomolgus macaques. Emerg. Infect. Dis. 19, 559–565.

Miller, M.A., Wayne, P., Terri, S., 2010. Creating the CIPRES science gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop (GCE) 2010.

Robert Koch-Institute, 2017. Survstat@RKI 2.0.

Ryll, R., Bernstein, S., Heuser, E., Schlegel, M., Dremsek, P., Zumpe, M., et al., 2017. Detection of rat hepatitis E virus in wild Norway rats (*Rattus norvegicus*) and black rats (*Rattus rattus*) from 11 European countries. Vet. Microbiol. 208, 58–68.

Smith, D.B., Simmonds, P., International Committee on Taxonomy of Viruses Hepeviridae Study Group, Jameel, S., Emerson, S.U., Harrison, T.J., Meng, X.J., Okamoto, H., Van

der Poel, W.H., Purdy, M.A., 2014. Consensus proposals for classification of the family *Hepeviridae*. J. Gen. Virol. 95, 2223–2232.

Smith, D.B., Simmonds, P., Izopet, J., Oliveira-Filho, E.F., Ulrich, R.G., Johne, R., et al., 2016. Proposed reference sequences for hepatitis E virus subtypes. J. Gen. Virol. 97, 537–542.

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

Spahr, C., Knauf-Witzens, T., Dähnert, L., Enders, M., Müller, M., Johne, R., Ulrich, R.G., 2017b. Detection of HEV-specific antibodies in four non-human primate species, including great apes, from different zoos in Germany. Epidemiol. Infect. 146 (1), 119–124.

Vina-Rodriguez, A., Schlosser, J., Becher, D., Kaden, V., Groschup, M.H., Eiden, M., 2015. Hepatitis E virus genotype 3 diversity: phylogenetic analysis and presence of subtype 3b in wild boar in Europe. Viruses 7, 2704–2726.

Wolf, S., Reetz, J., Johne, R., Heiberg, A.C., Petri, S., Kanig, H., et al., 2013. The simultaneous occurrence of human norovirus and hepatitis E virus in a Norway rat (*Rattus norvegicus*). Arch. Virol. 158, 1575–1578.

Zhao, C., Ma, Z., Harrison, T.J., Feng, R., Zhang, C., Qiao, Z., et al., 2009. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. J. Med. Virol. 81, 1371–1379.