Hepatitis E Virus in Wild Boars and Spillover Infection in Red and Roe Deer, Germany, 2013–2015

Helena E. Anheyer-Behmenburg, Kathrin Szabo, Ulrich Schotte, Alfred Binder, Günter Klein, Reimar Johne

To determine animal hepatitis E virus (HEV) reservoirs, we analyzed serologic and molecular markers of HEV infection among wild animals in Germany. We detected HEV genotype 3 strains in inner organs and muscle tissues of a high percentage of wild boars and a lower percentage of deer, indicating a risk for foodborne infection of humans.

Hepatitis E is an infection of public health concern, leading to an estimated global disease burden of 3.4 million acute cases, 70,000 deaths, and 3,000 stillbirths per year (*1*). Large disease outbreaks in nonindustrialized countries are mainly caused by drinking water contaminated with hepatitis E virus (HEV) (*2*). In industrialized countries, most cases of hepatitis E are sporadic and suspected to be a result of zoonotic HEV transmission from animals to humans (*3*). The numbers of notified hepatitis E cases have sharply increased in several European countries during recent years (*4*,*5*). Chronic HEV infections among recipients of solid organ transplants pose novel public health concerns (*6*).

HEV belongs to the family *Hepeviridae*, genus *Orthohepevirus,* Its RNA genome comprises 3 open reading frames (ORFs). ORF1 encodes a multifunctional nonstructural polyprotein with methyltransferase and RNA-dependent RNA polymerase genes often used for molecular typing. Human pathogenic HEVs are mainly classified into genotypes 1–4 (*2*,*3*). The camelid HEV genotype 7 was recently detected in a human (*7*), however. Although genotypes 1 and 2 infect only humans, genotypes 3 and 4 are zoonotic and infect different animal species and humans (*2*,*3*,*8*). HEV infection in animals is generally not associated with clinical disease.

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The main animal reservoirs for genotype 3 are domestic pigs and wild boars, although infections among other mammals have been described (*2*,*3*,*8*). However, whether these animal species represent true HEV reservoirs or are accidental infections due to spillover events is unclear. In this study, we investigated serologic and molecular evidence of HEV infection in wild boars and different deer species during 2 hunting seasons in a hunting area in Germany.

The Study

We obtained serum samples from wild boars, roe deer, red deer, and fallow deer during 2 hunting seasons (season A, 2013–2014; season B, 2014–2015) and analyzed them by using an ELISA (ID Screen Hepatitis E Indirect; ID Vet, Grabels, France) for HEV-specific IgG (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/23/1/16-1169-Techapp1.pdf). Of 339 serum samples, 81 (23.9%) were positive for HEV IgG; results from 1 sample (0.3%) were questionable. Although all wild deer samples tested negative, the proportion of antibody-positive wild boars increased significantly ($p = 0.018$) from 13 (27.1%; 95% CI 16.55–37.65) of 48 in season A to 68 (51.5%; 95% CI 44.34–58.66) of 132 samples in season B, with a mean antibody prevalence of 45.0% (Table 1). The capability of the ELISA for detection of HEV-specific antibodies in field serum samples from deer was demonstrated by testing of 153 deer serum samples from another hunting area, which led to 3 positive results (data not shown).

We also tested liver and serum samples from 415 animals for the HEV genome by using real-time reverse-transcription PCR (RT-PCR) (online Technical Appendix). HEV RNA was detected in 46 (11.1%) animals: 39 (16.8%) of 232 wild boars (6/95 [6.3%], from season A and 33/137 [24.1%] from season B); 5 (6.4%) of 78 roe deer; and 2 (2.4%) of 83 red deer (Table 1). Testing of all available organs from the HEV-positive wild boars revealed HEV RNA in >89% of the samples. HEV RNA was detected in all tested muscle samples and in most of the other organ samples of HEV-positive deer (Table 2). Comparison of viral loads in the organs revealed significantly higher genome copy numbers in wild boar liver (median 2.26×10^7 genome equivalents[GE]/g]) compared with those for wild boar musculature (median 4.37×10^3 GE/g) or for deer liver (median 2.22×10^3 GE/g) and deer musculature

Table 1. Detection of HEV-RNA and HEV-specific antibodies in wild boars and 3 deer species during 2 hunting seasons in a hunting area in Germany, 2013–2015*

Animal species	HEV-RNA determined by real time RT-PCR, no. positive/no. tested (%)	HEV-specific antibodies determined by ELISA, no. positive/no. tested (%)
Season A, 2013–2014,		
Wild boars	6/95 (6.31)	13/48 (27.08)
Roe deer	2/17 (11.76)	0/8
Red deer	0/25	0/21
Fallow deer	0/2	0/2
Total	8/139 (5.76)	13/79 (16.46)
Season B, 2014–2015		
Wild boars	33/137 (24.09)	68/132 (51.52)
Roe deer	3/61 (4.92)	0/51
Red deer	2/58 (3.45)	0/57
Fallow deer	0/20	0/20
Total	38/276 (13.77)	68/260 (26.15)
Total, 2013–2015	46/415 (11.08)	81/339 (23.89)

*HEV, hepatitis E virus; RT-PCR, reverse transcription PCR.

Table 2. Organ distribution of HEV RNA in positive-screened wild boars and deer during 2 hunting seasons in a hunting area in Germany, 2013–2015*

Animal species	Sample type, no. positive/no. tested (%)					All
	Liver	Musculature	Spleen	Kidney	Serum	
Wild boars	26/26 (100.0)	29/35 (82.9)	23/27 (85.2)	16/19 (84.2)	32/34 (94.1)	126/141 (89.4)
Deer	4/5 (80.0)	6/6 (100.0)	2/4 (50.0)	2/4 (50.0)	3/5 (60.0)	17/24 (70.8)
Total	30/31 (96.8)	35/41 (85.4)	25/31 (80.7)	18/23 (78.3)	35/39 (89.7)	143/165 (86.8)

*HEV, hepatitis E virus.

(median 5.25×10^2 GE/g) (Figure 1; online Technical Appendix Figure 2). However, the low number of positive deer samples limits the interpretation of the statistical results.

A total of 39 of 46 samples were positive in a nested RT-PCR assay targeting the RNA-dependent RNA polymerase gene in the ORF1 (online Technical Appendix)

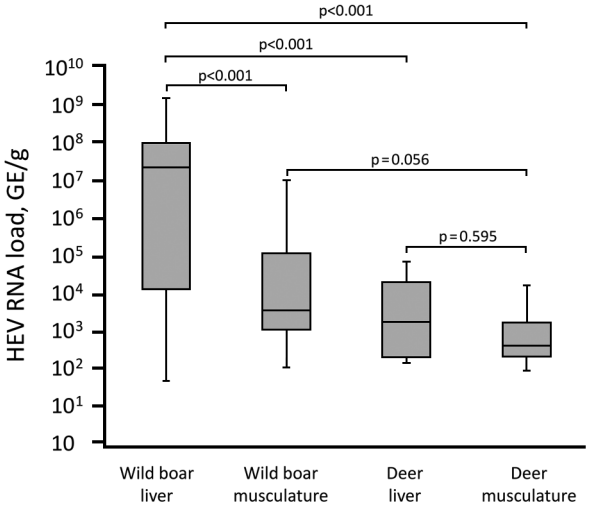

Figure 1. Box plot comparison of HEV RNA load in wild boar and deer specimens from Germany, 2013–2015. Boxes indicate first (bottom) and third quartile; horizontal line within boxes indicate median; error bars indicate minimum and maximum. p values for pairwise comparison of groups are shown. GE, genome equivalents; HEV, hepatitis E virus.

that were suitable for sequencing. The amplicons showed nucleotide sequence identities to each other ranging from 73.6% to 100.0%. A phylogenetic tree set up for the samples together with HEV subtype reference strains indicated that most sequences cluster in a clade containing subtypes 3c and 3i (Figure 2). Within this clade, HEV sequences from wild boar and deer from both hunting seasons clustered very closely together. Four sequences from wild boars of season B clustered in genotype 3f. HEV isolates from human hepatitis E cases from Germany clustered near the wild boar and deer HEV sequences (nucleotide sequence identities up to 86.1% to a German 3f strain and 88.2% to a German 3c strain).

Using a nested RT-PCR assay targeting the methyltransferase gene in the ORF1 (online Technical Appendix), we sequenced a PCR product in 18/46 samples. The nucleotide identities of the sequences ranged from 72.7% to 99.6%. A phylogenetic tree again showed grouping into HEV subtype clade 3ci and subtype 3f (online Technical Appendix Figure 3). All sequences were deposited in GenBank (accession nos. KX455427–KX455478).

Conclusions

We detected HEV-RNA and HEV-specific antibodies in a high percentage of wild boars, with a significant difference between the 2 hunting seasons. The detection rates are consistent with previous reports of infection of wild boars in Germany (*9*–*11*). The data underline the high importance of this animal species in the epidemiology of HEV and indicate that wild boars likely represent a persistent reservoir

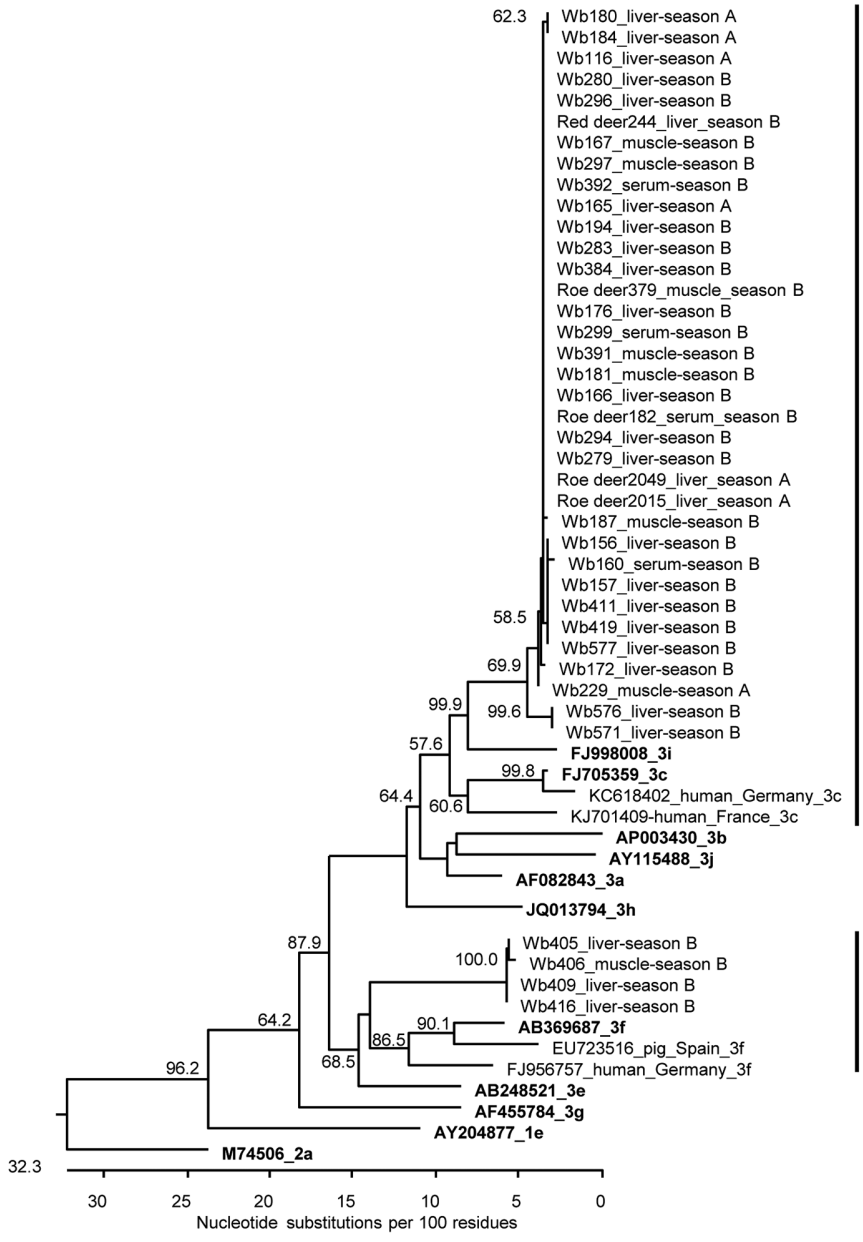

Figure 2. Phylogenetic relationship of HEV sequences derived from wild boars and deer from Germany, 2013–2015. The tree is based on a 280-bp fragment of the ORF1 (RNA-dependent RNA polymerase gene) region. The strain designations, animal species (Wb, wild boar), sample type, and sampling year (season A, 2013–2014; season B: 2014–2015) are indicated for the novel strains. The GenBank accession numbers, the corresponding hosts, the geographic origins and genotypes are indicated for selected additional strains. Reference strains are given in bold; the genotype of the novel strains is indicated at right. Bootstrap values >50% are shown. The tree is scaled in nucleotide substitution units and was constructed using MEGALIGN software (ClustalW [http://www.ebi.ac.uk/Tools/msa/clustalw2/], IUB residue weight table, 1,000 trials and 111 random seeds in bootstrap analysis). HEV, hepatitis E virus; ORF, open reading frame.

for this virus. The detection of high amounts of HEV RNA in wild boar liver, other organs, and especially in muscle tissue highlights the high risk that HEV can be transmitted to humans through the consumption of meat from these animals that has not been cooked properly.

In contrast, only low percentages of samples from roe deer and red deer tested positive for HEV in our study. Data about HEV infection in wild ruminants in Europe are rare, but some reports have demonstrated HEV infection in several deer species (*12*,*13*). Neumann et al. (*14*) reported serologic and molecular evidence for HEV infection of the indigenous deer species in Germany. We detected HEV RNA in liver, in several organs, and in muscle tissue of the infected deer species. Sequence analysis showed a relationship of HEV from deer with human hepatitis E cases from Germany. In Japan, consumption of deer meat could be linked to acute hepatitis E cases in humans (*15*). Taken together, deer are likely to represent a source of HEV for humans, and consumption of undercooked deer meat should be considered a risk for acquiring HEV infection.

Analysis of the detected HEV sequences indicated that the same strains of genotype clade 3ci circulated in wild boar and deer species. This finding argues against specific HEV strains exclusively circulating in deer species; however, longer sequence parts or whole virus genomes should be analyzed in future studies to support this finding

further. The consistently lower HEV RNA and antibody prevalence in deer than in wild boars indicates a primary circulation in wild boars and only accident transmission to deer. The hypothesis of spillover infections of deer is further supported by the consistent lower viral loads in tissues of infected deer. However, other authors classified deer as a true reservoir for HEV (*8*). Further studies investigating more geographic areas over longer time, including the parallel analysis of different animal species, are necessary to unravel the epidemiology and transmission dynamics of HEV in wildlife.

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Dr. Anheyer-Behmenburg is a veterinary specialist for microbiology at the University of Veterinary Medicine, Hannover, Germany. She is interested in the epidemiology of emerging zoonotic diseases in wildlife. Her recent scientific work focused on HEV in wild animals.

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Hepatitis E Virus in Wild Boars and Spillover Infection in Red and Roe Deer, Germany, 2013–2015

Technical Appendix

Materials

During the 2 hunting seasons (season A: November 2013–January 2014; season B: November 2014–January 2015), samples of 415 wild animals (wild boars [*Sus scrofa*], roe deer [*Capreolus capreolus*], red deer [*Cervus elaphus*], fallow deer [*Dama dama*]) were collected from liver, spleen, kidney, muscle, and serum (not all sample types were available in all animals). All animals belonged to free-ranging populations living in a military training area of the German armed forces (53°36′34 N, 14°02′36 E) in Mecklenburg–Western Pomerania, Germany (Figure 1). The sampling took place subsequently after the battue (game were driven toward hunters by beaters).

Methods

ELISA

Serum samples were analyzed for hepatitis E virus (HEV)–specific antibodies by an indirect ELISA based on a recombinant HEV genotype 3 capsid protein and an anti-multispecies IgG–horseradish peroxidase conjugate (ID Screen Hepatitis E Indirect; ID Vet, Grabels, France). In addition to the control samples included in the kit, HEV-positive serum samples from deer were used as control samples. Test procedures were performed, and results were interpreted according to the manufacturer's instructions. The optical density was measured at 450-nm wavelength by a plate reader (Sunrise, Tecan Group Ltd, Männedorf, Switzerland).

RNA Extraction

Liver samples from all animals were screened by an HEV-specific real-time reverse transcription PCR (RT-PCR); if those were not available, serum samples were used. 120 mg

portions of the samples were suspended in 700 μL of phosphate-buffered saline (PBS) and homogenized by using a 5-mm-diameter steel ball in a Tissue Lyser (QIAGEN, Hilden, Germany) for 210 seconds at 30 Hz. After centrifugation at 2,500 × *g* for 15 min, 50 μL of supernatant was mixed with 350 μL of Buffer RLT (QIAGEN) before centrifugation through a QIA-Shredder (QIAGEN) at 16,000 × *g* for 180 s. RNA was extracted by using the QIAsymphony RNA kit by using a 2-fold concentrated DNase in a QIAsymphony device according to the manufacturer's protocol. The elution volume was 50 μL. Nucleic acids were extracted from 350 μl serum using the QIAsymphony DSP Virus/Pathogen Kit in a QIAsymphony device according to the manufacturer's instructions. The elution volume was 60 μL.

Real-Time RT-PCR

An HEV-specific RT-qPCR was performed with the QuantiTect Probe RT-PCR Kit (QIAGEN) by using 5 μL of sample RNA in a total volume of 20 μL. Primers and probe were used according to the method of Jothikumar et al. (*1*). Reverse transcription was performed for 30 min at 50°C, followed by denaturation for 15 min at 95°C and 45 cycles each with 30 s at 94°C, 30 s at 55°C and 30 s at 72°C. Quantification of all available organs from positive screened animals was performed by using a dilution series of 10^3 to 10^{14} copies of in vitro-transcribed RNA.

RT-PCR and Sequencing

Two nested RT-PCR assays targeting the RNA-dependent RNA polymerase region (RdRp) (*2*) and methyltransferase region (*3*) within open reading frame 1 were performed for subsequent sequence analysis. The One-Step RT-PCR Kit (QIAGEN) was used for the RT-PCR and the TaKaRa Ex Taq (Takara Bio Europe S.A.S., Saint-Germain-en-Laye, France) for the nested PCR. The nested PCR products with sizes of 331 bp (*2*) or 286 bp (*3*) were separated on ethidium bromide–stained 1.5% agarose gels and visualized by ultraviolet light. The amplicons were purified by using the QIAquick DNA purification kit (QIAGEN) and Sanger sequencing was carried out by an external company (Eurofins Genomics, Ebersberg, Germany) by using the nested PCR primers. Sequences (excluding primer sequences) were deposited in GenBank with accession nos. KX455427–KX455478.

Sequence Analysis

Nucleotide sequences were analyzed using the DNASTAR software package (Lasergene, Madison, WI, USA), and compared with HEV sequences available in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) by using the BLASTn facility as well as with HEV subtype reference strains (*4*). Phylogenetic trees were constructed on the basis of the nucleotide sequences by using a neighbor-joining method implemented in the MegAlign module of the DNASTAR software package (Lasergene) with the parameters: ClustalW method, IUB residue weight table and a bootstrap analysis with 1,000 trials and 111 random seeds.

Statistical Analysis

The median, minimum, maximum, and first and third quartiles were calculated and used for generation of boxplot figures using Excel (Microsoft Office Professional 2010, Microsoft Corp., Redmond, WA, USA). The differences of estimated prevalences per season were tested for significance with the 2-sample *z* test (http://epitools.ausvet.com.au/content.php?page = z-test-2), differences of HEV-RNA load in organ samples were tested with the logarithm of GE/g using 2-sample *t*test for summary data (http://epitools.ausvet.com.au/content.php?page = 2-sample-t-test). All analyses were done by using EpiTools epidemiologic calculators (Ausvet Pty Ltd., available at http://epitools.ausvet.com.au).

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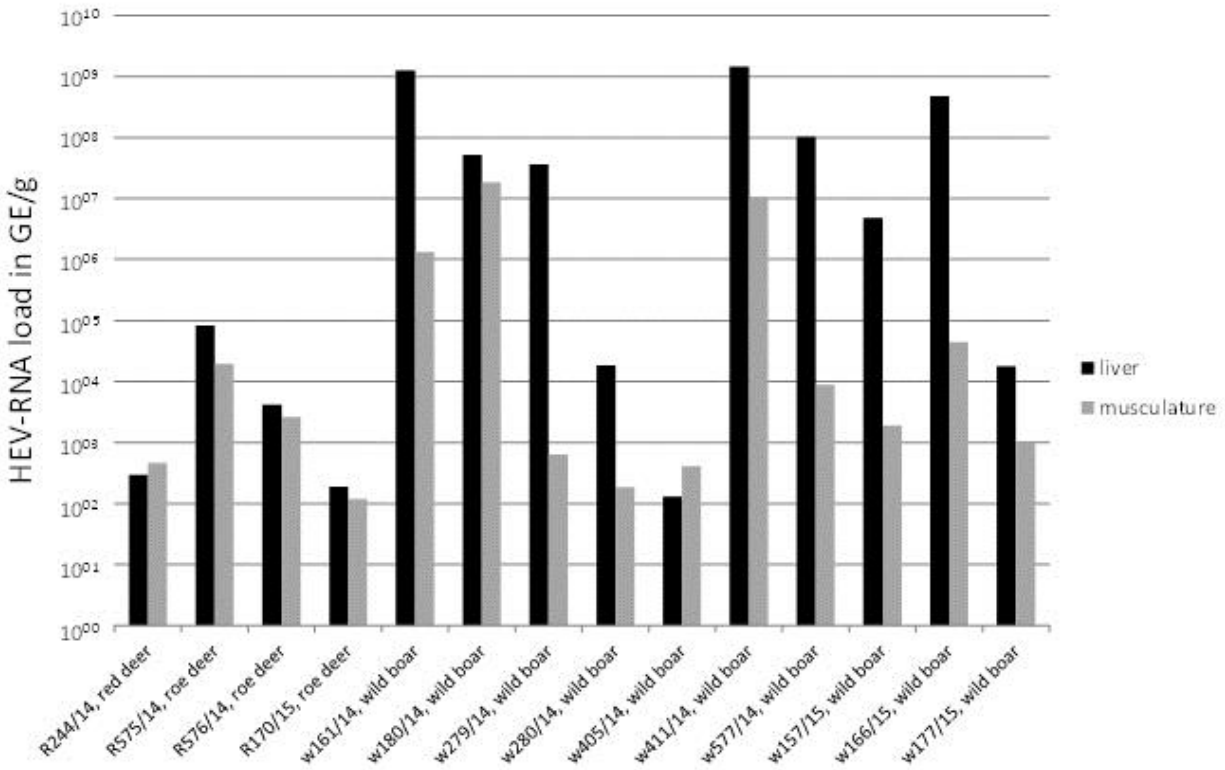
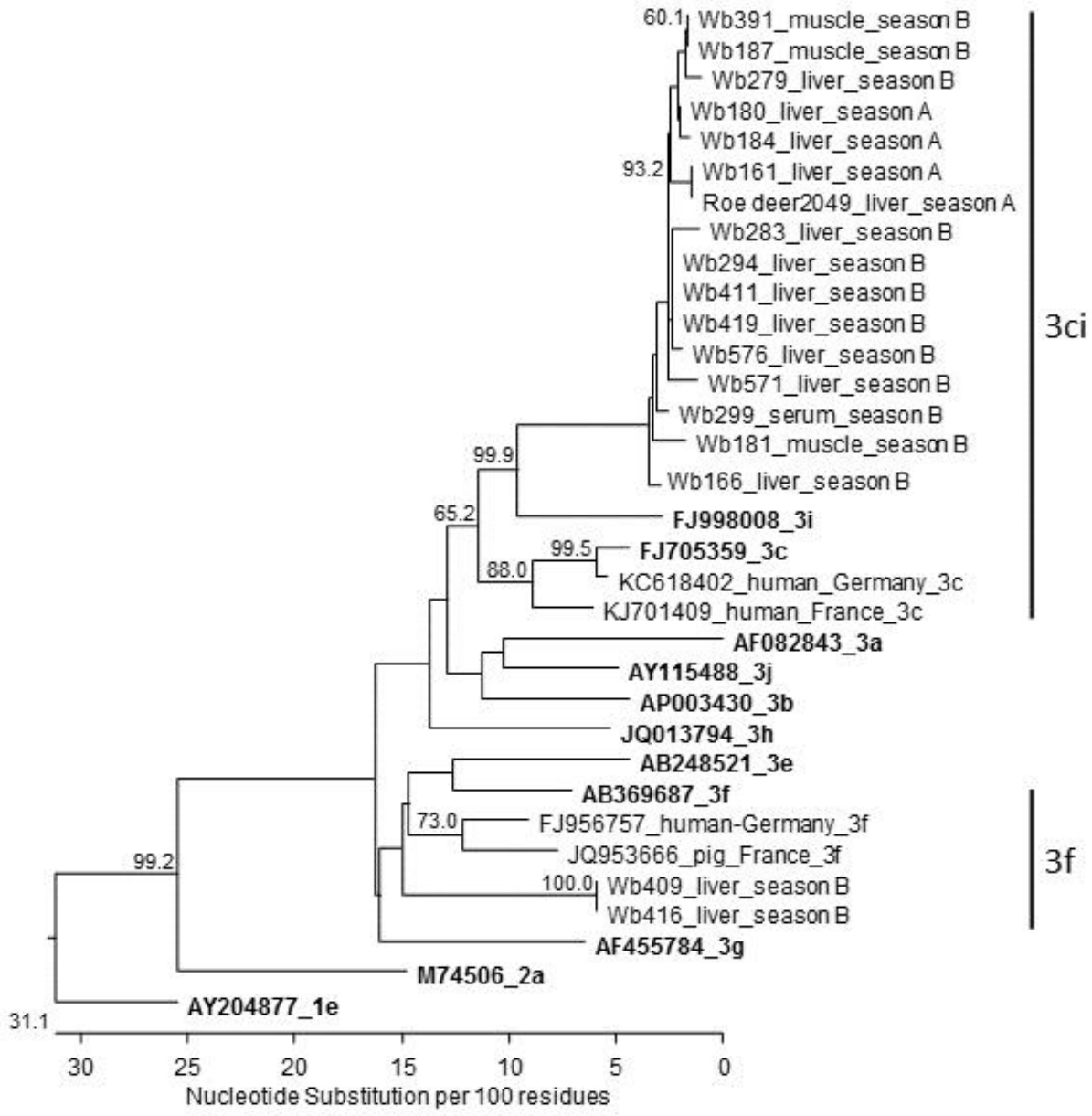
Technical Appendix Figure 1. Location of the hunting area within Germany where wild boars, roe deer, red deer, and fallow deer were sampled for hepatitis E virus, Germany, 2013–2015 (indicated by cross-hatching).



Technical Appendix Figure 2. Hepatitis E virus RNA load in liver and muscle specimens of individual deer and wild boars, Germany, 2013–2015.



Technical Appendix Figure 3. Phylogenetic relationship of hepatitis E virus sequences derived from wild boars (wb) and deer, Germany, 2013–2015. The tree is based on a 242-bp fragment of the open reading frame 1 (methyltransferase gene) region. The strain designations, animal species, sample type, and sampling year (season A: 2013–2014; season B: 2014–2015) are indicated for the novel strains. The GenBank accession numbers, the corresponding hosts, the geographic origins and genotypes are indicated for selected additional strains. Reference strains are given in bold, and the genotype of the novel strains is indicated on the right. Bootstrap values >50% are shown. The tree is scaled in nucleotide substitution units and was constructed by using the MEGALIGN module of the DNASTAR software package (Lasergene; parameters: ClustalW, IUB residue weight table, 1,000 trials and 111 random seeds in bootstrap analysis).