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**Abstract**

Swine Hepatitis E virus (HEV) can be transmitted from pigs to humans causing hepatitis. A high prevalence of HEV in wild boar populations is reported for several European countries, but actual data for Germany are missing. During the hunting season from October to December 2007 liver, bile and blood samples were collected from wild boars in four different German regions. The samples were tested for HEV RNA by quantitative PCR (qPCR) and anti-HEV IgG antibodies by two different ELISAs and a Line immunoassay. A seroprevalence of 29.9% using ELISA and 26.2% in the Line immunoassay was determined. The seroprevalence rate varied greatly within the analysed regions. However, qPCR analysis revealed a higher prevalence of 68.2% positive animals with regional differences. Surprisingly, also adult wild sows and wild boars were highly HEV positive by qPCR. Compared to liver and serum samples, bile samples showed a higher rate of positive qPCR results. Sequencing and phylogenetic analysis of a 969 nt fragment within ORF 2 revealed that all isolates clustered within genotype 3 but differed in the subtype depending on the hunting spot. Isolates clustered within genotypes 3i, 3h, 3f and 3e. Within one population HEV isolates were closely related, but social groups of animals in close proximity might be infected with different subtypes. Two full-length genomes of subtypes 3i and 3e from two different geographic regions were generated. The wild boar is discussed as one of the main sources of human autochthonous infections in Germany.

42

Keywords: swine Hepatitis E virus, qPCR, serology, phylogenetic analysis, full-length genome sequence

45

## 46 **1. Introduction**

47 Four major genotypes can be differentiated in the genus Hepatitis E virus (HEV) within the  
48 family of *Hepeviridae* (Emerson and Purcell, 2007). Europe has long been considered to be  
49 non-endemic for HEV. However, in recent years an increasing number of autochthonous  
50 HEV cases were reported across Europe, and a close genetic relationship between these  
51 human-derived HEV sequences and porcine HEV isolates was demonstrated (Banks et al.,  
52 2004; van der Poel et al., 2001). Genotypes 3 and 4 are mainly found in industrialized  
53 countries. HEV isolates from sporadic acute human cases in these countries show a high  
54 genetic similarity to isolates from animals, especially from pigs (Goens and Perdue, 2004;  
55 Meng et al., 1997). For the years 2001 to 2007 311 human cases of acute hepatitis E were  
56 reported in Germany, 142 (45.7%) of these infections were most likely acquired in  
57 Germany (Robert Koch-Institut, 2008). A case-control study about the risk of acquiring an  
58 HEV infection in Germany determined that the main risk factor was consumption of offal  
59 and meat from wild boars (Wichmann et al., 2008). While several studies across Europe  
60 detected a high prevalence of anti-HEV IgG antibodies and HEV RNA in wild boars and  
61 domestic pigs within all age groups (de Deus et al., 2008; Martelli et al., 2008; McCreary et  
62 al., 2008; Rutjes et al., 2007; Seminati et al., 2008), an HEV RNA prevalence of only 5%  
63 was found in serum samples collected from wild boars in north-eastern Germany in the late  
64 1990s (Kaci et al., 2008). The main object of this study was to determine the actual  
65 prevalence of HEV genome and anti-HEV antibodies in wild boar populations in four  
66 different regions in Germany. Together with data on the geographical distribution of the  
67 particular hunting spots, a comparison of the endemic situation in several different wild  
68 boar populations becomes possible. Bile, liver and serum samples were collected and

69 analysed by quantitative PCR for HEV virus load. Sequencing and phylogenetic analysis  
70 were performed to determine the respective HEV geno- and subtype, and data obtained  
71 were compared to isolates found in other European countries. Investigations on the  
72 molecular epidemiology of HEV in different wild boar populations might help to assess the  
73 potential risk of zoonotic transmission of HEV from wild boars to humans.

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## 75 **2. Materials and methods**

### 76 **2.1. Sample collection**

77 Samples from 132 wild boars were collected during the hunting season between October  
78 and December 2007 at different regional sites in Germany. Nineteen animals were sampled  
79 in the federal state of Brandenburg (BB), 33 animals in a region at the border between the  
80 federal states of Brandenburg and Saxony (SA), 53 animals in 2 locations in Rhineland  
81 Palatine (RP), and 27 animals in 2 locations in Baden-Württemberg (BW) (Table 1).

82 When possible, samples were collected immediately after the opening of the wild boars'  
83 bodies. Generally, sampling and age determination of the animals took place at special  
84 assembling places, but at some sites the hunters themselves collected the samples in the  
85 context of opening the bodies at the place of shooting. The age of the animals was  
86 estimated using the presence and rotation of teeth (Habermehl, 1985). The age distribution  
87 of wild boars was: 1 year (n=53), between 1 and 2 years (n=38), adult sows (n=21) and  
88 adult boars (n=9). The age of 11 animals could not be determined. Bile was squeezed out  
89 into 50 ml or 15 ml tubes (Nunc Thermo Fischer Scientific, Langenselbold, Germany), liver  
90 pieces were preserved in RNAlater® solution (Ambion, Austin, TX, USA) and blood was  
91 collected in serum tubes. Samples were stored at 4°C overnight, processed on the next day  
92 and aliquots were frozen at -70°C. Because of relatively long time intervals between  
93 shooting and transport of the wild boars' bodies to assembly points in the forest for  
94 sampling, blood was often coagulated and serum samples were haemolytic.

## 95 **2.2. Isolation of RNA and cDNA synthesis**

96 A piece of 20 to 40 mg of liver samples preserved in RNAlater® (Ambion) was  
97 homogenized in 500 µl PBS using Precellys ceramic balls with a diameter of 1.4 mm  
98 (Peqlab Biotechnology, Erlangen, Germany) and the FastPrep® FP220A homogenizer  
99 (Qbiogene, MP Biomedicals, Solon, OH, USA). The supernatant was collected and 100 µl  
100 were taken for RNA extraction using the NucleoSpin® RNA II Mini preparation kit  
101 (Macherey-Nagel, Düren, Germany). Residual supernatant after homogenization was stored  
102 at -70°C. RNA was extracted from bile and serum following the manufacturer's protocol  
103 5.2 of the NucleoSpin® RNA II Mini kit (Macherey-Nagel). Additionally, RNA from bile  
104 samples tested negative was extracted, following the supplier's protocol on purification of  
105 viral RNA using QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany). DNase digestion  
106 was performed to reduce the DNA load in the sample, using Ambion® TURBO DNA  
107 free™ (Ambion). Briefly, 25 µl RNA were incubated with 2.5 µl 10xDNase buffer and 2 µl  
108 Turbo DNase for 30 min at 37°C. After adding 2 µl of inactivation reagent the sample was  
109 vortexed for 2 min and centrifuged at 6,800 x g in a Centrifuge 5417R (Eppendorf,  
110 Hamburg, Germany) for 3 min. A volume of 20.6 µl of the eluted RNA was reverse  
111 transcribed using the TaqMan® Reverse Transcription Reagents following the supplier's  
112 protocol (Applied Biosystems, Foster City, CA, USA). RNA was incubated with 2 µl  
113 primer mix R6/HEV R (Table 2) for 5 min at 65°C. The sample was cooled on ice to 4°C  
114 before adding the reverse transcription mix containing 4 µl 10xbuffer, 8.8 µl MgCl<sub>2</sub>  
115 (25 mM), 3.2 µl dNTP (25 mM), 0.8 µl RNase inhibitor and 0.6 µl reverse transcriptase.  
116 The reaction was run in a thermocycler (Mastercycler egradient, Eppendorf) for 60 min at  
117 42°C with a final 15 min incubation at 72°C before cooling to 4°C and storage at -20°C.



### 118 **2.3. Quantitative HEV detection**

119 A real-time PCR (qPCR) assay using 5' nuclease probes (TaqMan® probes) with primer  
120 combination HEV\_F/R/TM (Table 2, (Adlhoch et al., 2009)) was established in this study  
121 which should be able to detect all known HEV genotypes and to determine the copy  
122 numbers of HEV genomes. The reverse primer HEV\_R is the modified published primer  
123 HE040, and the probe HEV\_TM overlaps with HE041 (Mizuo et al., 2002). The Platinum®  
124 Taq DNA polymerase kit, MgCl<sub>2</sub> and dNTPs by Invitrogen (Carlsbad, CA, USA) and water  
125 (Molecular Biology Grade Eppendorf) were used in this assay. qPCR reactions were  
126 performed in a final volume of 25 µl with 10xbuffer, 4 mM of MgCl<sub>2</sub>, dNTP 0.2 mM each,  
127 0.3 µM of each primer, 0.1 µM of probe, ROX 0.1 µM and Platinum® Taq 0.5 U. General  
128 reaction conditions for the real-time PCR assay were 95°C for 15 min and 45 cycles with  
129 95°C for 15 sec, 60°C for 35 sec. Reactions were run in an ABI GeneAmp® 7500  
130 Detection System (Applied Biosystems). The PCR product was cloned into vector pCRII  
131 (Invitrogen) and purified using NucleoSpin® Plasmid (Macherey-Nagel) following the  
132 manufacturer's instructions. The generated plasmid pHEV\_RKI containing a 121 nt  
133 fragment of the related region from the isolate HEV\_RKI (FJ956757, (Adlhoch et al.,  
134 2009)) was quantified by measuring the DNA concentration at 260 nm. The plasmid was  
135 serially diluted in water containing λ-DNA (1 ng/µl) 10fold from 10<sup>6</sup> copies to 10 copies  
136 and used as standard for the quantification of viral genomes. Copy numbers were  
137 determined using a standard curve; the mean and standard deviation were calculated. The  
138 detection limit was calculated to be 10 copies of cDNA per reaction. For real-time PCR  
139 each sample was analysed in duplicate.

#### 140 **2.4. Amplification controls**

141 The porcine  $\beta$ -actin gene was used as internal preparation and amplification control  
142 (Chmielewicz et al., 2003). The plasmid pActin containing the corresponding fragment of  
143 porcine  $\beta$ -actin was used for standardisation. An additional control for RNA preparation,  
144 cDNA synthesis and PCR amplification was applied for all samples tested negative or  
145 showing low copy numbers of  $\beta$ -actin. Briefly: the original samples were spiked with 10  $\mu$ l  
146 of a Mammalian Reovirus 3 virus stock (MRV3, strain Dearing ATCC VR-824<sup>TM</sup>) with a  
147 titre of  $3.2 \times 10^7$  TCID<sub>50/ml</sub> prior to RNA preparation. These cDNA samples were tested  
148 with an established MRV3-specific qPCR using the published primers REOL3F and  
149 REOL3R (Spinner and Di Giovanni, 2001) in combination with the newly designed  
150 REO\_TM probe (6FAM-CCg CTg gCT CCT CCA CCT gAC-BHQ1). A plasmid pMRV3  
151 containing the corresponding region served as positive control. Plasmid preparation and  
152 general conditions of both real-time assays corresponded with the described HEV qPCR  
153 assay, but varied in the reaction times of the qPCR: 15 min at 95°C and 45 cycles at 95°C  
154 for 15 sec, at 60°C for 35 sec (MRV3) or at 72°C for 35 sec ( $\beta$ -actin).

#### 155 **2.5. Amplification of partial and full-length HEV sequences**

156 Long-range PCRs were run using the Roche FastStart High Fidelity PCR System, dNTPack  
157 (Roche Diagnostics, Mannheim, Germany). The final PCR volume was 25  $\mu$ l adjusted with  
158 water, 5  $\mu$ l 5xbuffer, dNTPs 10 mM 1.25  $\mu$ l, primer each 0.75  $\mu$ l 10  $\mu$ M, DMSO 1  $\mu$ l final  
159 4%, 0.2  $\mu$ l ET SSB 600 ng/ $\mu$ l (New England Biolabs, Ipswich, MA, USA), DNA  
160 polymerase 0.35  $\mu$ l final 1.75 U and 2  $\mu$ l cDNA. The reaction was run in a thermocycler  
161 (Mastercycler egradient, Eppendorf) at 95°C for 2 min, 10 cycles at 95°C for 10 sec, at  
162 X°C for 15 sec, at 68°C for 1.5 min, 35 cycles at 95°C for 10 sec, at X°C for 15 sec, at

163 68°C for 1.5 min and an elongation of 20 sec for every cycle. The final extension was 68°C  
164 for 5 min. The annealing temperature X was dependent on the melting temperature of the  
165 primers. Published primers HEV366F, HEV363R (Inoue et al., 2006), HE044F (Mizuo et  
166 al., 2002), HEVgenF2 (Li et al., 2005), HEV\_F and HEV\_R were used in combination with  
167 newly designed primers (Table 2) to amplify long fragments within ORF1 and ORF2 for  
168 subtyping and generation of the full-length genomes.

## 169 **2.6. Serological analysis**

170 107 serum samples were tested for the presence of anti-HEV IgG antibodies using two  
171 different commercially available HEV ELISA kits (Genelabs (GL) Diagnostics, Illkirch,  
172 France and *recomWell* HEV, Mikrogen (MG), Neuried, Germany) and the newly  
173 established Line immunoassay (*recomLine* HEV IgG, Mikrogen (MG)). The ELISAs and  
174 the Line immunoassay were performed essentially as described for the determination of  
175 anti-HEV IgG in human serum or plasma by the manufacturers except that the second  
176 antibody was replaced with peroxidase labelled goat anti-swine IgG (H+L) antibody  
177 (Kirkegaard&Perry Laboratories KPL, Gaithersburg, MD, USA) in a final concentration of  
178 0.04 µg/ml (ELISA) and 0.25 µg/ml (*recomLine* HEV). The cut-off serum for *recomWell*  
179 HEV was a sample from a domestic pig weakly reactive in *recomBlot* HEV (MG). The cut-  
180 off values were >1 OD for the GL ELISA, >0.244 OD in the MG ELISA and >15 points in  
181 the *recomLine* immunoassay. The *recomLine* points were determined using a grey scale  
182 value scheme which calculates the reactivity as point values for every band separately with  
183 a 4-fold weighing of the O2C band.

## 184 **2.7. Sequencing and phylogenetic analysis**

185 Plasmid and amplified products of the samples were sequenced directly or after cloning  
186 into vector pCR II (Invitrogen) on both strands using the Big Dye3.1 protocol. The  
187 sequence of PCR products was analyzed in an automated sequencer (Genetic Analyzer  
188 3130 xl, Applied Biosystems). Nucleotide sequences were analyzed with the ABI PRISM  
189 DNA Sequencing Analysis Software (Version 3.7, Applied Biosystems), MEGA 4 (Tamura  
190 et al., 2007) program, BioEdit v7.0.9 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and  
191 BLAST network program (National Center for Biotechnology Information, Bethesda, MD,  
192 USA). The wild boar full-length sequences generated were aligned with published complete  
193 sequences of all four genotypes and with human as well as porcine isolates from Europe.  
194 For subtyping, the isolates were compared to available European sequences and selected  
195 sequences of ORF1 and ORF2 (Lu et al., 2006).

## 196 **2.9. Accession numbers of generated wild boar HEV sequences**

197 The wild boar sequences determined in this study were deposited in the GenBank database  
198 with accession numbers:

199 **SHEV\_BB02** (FJ998008); **SHEV\_BW05** (FJ998009); **SHEV\_RP1647** (FJ998010);  
200 **SHEV\_RP1658** (FJ998011); **SHEV\_RP1752** (FJ998012); **SHEV\_SA11** (FJ998013);  
201 **SHEV\_SA13** (FJ998014); **SHEV\_SA21** (FJ998015); **SHEV\_SA28** (FJ998016);  
202 **SHEV\_SA29** (FJ998017); **SHEV\_BB19** (FJ998018); **SHEV\_SA34** (FJ998019)

203

## 204 **3. Results**

### 205 **3.1. Quantitative polymerase chain reaction (qPCR) analysis**

206 The qPCR assay established was designed to detect all known HEV isolates independent of  
207 the genotype. Samples from 132 animals were analysed for HEV genome copy numbers by  
208 qPCR. In total, 126 liver, 119 bile and 115 serum samples were screened. HEV RNA was  
209 detectable in 38.1% of the livers, 56.3% of the bile but in only 15.7% of the serum samples.  
210 Altogether, the HEV prevalence among the 132 animals was 68.2% when considering HEV  
211 detection in at least one sample per animal (serum, liver or bile, Table 3A). High copy  
212 numbers with  $C_T$ -values of 30 and below were detected in 16.7% of the animals, indicating  
213 approximately  $\geq 10^6$  genome equivalents in 1 g of liver tissue or  $\geq 10^5$  genome copies in  
214 1 mL of bile or serum, respectively. Eleven of the highly positive samples were collected in  
215 RP and 11 in BB or SA. One-third of the HEV RNA-positive animals were  $\leq 1$  year of age,  
216 17.4% between 1 and 2 years of age and 25.0% were adult animals with high copy numbers  
217 mainly in liver and bile samples. Table 3B shows the age distribution of the HEV genome-  
218 positive animals with the respective region of hunting. A clear difference in the prevalence  
219 of PCR-positive animals was observed: Whereas in samples from SA, RP and BB the HEV  
220 prevalences were high (between 57.6% and 100%), only 22.2% of the animals from Baden-  
221 Württemberg (BW) were tested positive (Table 3A).

### 222 **3.2. Serology**

223 Anti-HEV IgG was determined using a modification of two ELISA HEV kits (GL and MG)  
224 and the newly established Line immunoassay (MG). Table 4 illustrates the results of the  
225 serological assays for anti-HEV IgG. Anti-HEV reactivity was detected in 29.9% of the 107

226 serum samples using the ELISA assays, and 26.2% were reactive in the Line immunoassay.  
227 Isolated reactivity was seen in 15 sera using GL (7 samples also positive for HEV RNA)  
228 and in 4 sera (3 samples showing HEV RNA) using MG ELISA, 2 samples were reactive  
229 only in the Line immunoassay (no HEV RNA). No sample was reactive only in GL ELISA  
230 and Line immunoassay. Isolated anti-HEV reactivity without HEV RNA was seen in 8 of  
231 32 sera using GL and 1 of 32 sera in the MG ELISA. Comparison of the two IgG ELISAs  
232 showed that only 17 (53%) sera were reactive in both tests and 14 (82%) of these samples  
233 were concurrently HEV RNA positive. The Line immunoassay confirmed 15 out of these  
234 17 ELISA reactive sera. 12 (75%) of these 15 samples were also qPCR positive, with 5 of  
235 them (4 animals <1 year, 1 adult wild boar) indicating copy numbers of approximately  $10^9$   
236 or more per g of liver or ml of bile. There was a high degree of correlation between the  
237 ELISA and Line immunoassay from MG, 81% of the MG ELISA reactive sera were  
238 confirmed by the Line immunoassay, in contrast to the ELISA from GL where only 47% of  
239 the reactive sera were also reactive in the Line immunoassay. 15 of 26 (57.7%) animals  
240 showing anti-HEV IgG reactivity in both MG assays were simultaneously RNA positive (7  
241 animals <1 year, 2 animals 1-2 years, 4 adult sows, 1 adult wild boar, 1 without age  
242 determination), 7 of them showing high copy numbers (4 <1 year, 1 1-2 years, 1 sow, 1  
243 wild boar). Eight of the 10 animals that were IgG reactive in MG without detectable copy  
244 numbers were between 1 and 2 years old.

245

### 246 **3.3. Sequence analysis**

247 Samples containing high copy numbers of viral genome were selected for sequence  
248 analysis. Isolates from different hunting sites were sequenced and analysed to determine the  
249 genome subtypes within different geographic regions of Germany. Additionally, an

250 assortment of samples from each region was analysed to determine the variation within the  
251 social groups of the particular hunting spot. There was a mean homology of the wild boar  
252 isolates of 89.3% to genotype 1 (Burma M73218), 88.5% to genotype 2 (Mexico M74506),  
253 94.0% to genotype 3 (Meng AF082843) and 90.3% to genotype 4 (T1 AJ272108).  
254 Accordingly all HEV sequences from wild boars clustered within genotype 3 when  
255 comparing a 969 nt fragment with the reference strains of the different genotypes. For  
256 subtyping a 242 nt fragment within ORF1 and a 300 nt fragment within ORF2 were used  
257 (Lu et al., 2006). The wild boar samples clustered within different subtypes (Figures 1 and  
258 2). Isolates BB02 and BB19 and RP1647, RP1658 and RP1752 clustered in the ORF2 at the  
259 subtype 3c branch, but isolate BB02 showed homology to subtype 3i in the analysis of  
260 ORF1. Due to the lack of 3i isolate sequences within ORF2, all of these isolates most  
261 probably belong to subtype 3i, showing a mean homology of only 85.1% (range 89.2-  
262 81.8%) to swine isolates of subtype 3c from the Netherlands. Although the isolates from  
263 BB and RP clustered within one subtype, there were 105 divergent nucleotides between the  
264 isolates when comparing a longer 998 nt fragment within ORF2. Five of the SA isolates  
265 (SA11, SA13, SA21, SA28 and SA34) clustered within subtype 3e, showing a mean  
266 homology of 88.5% (range 90.2-85.5%) to European isolates of this subtype. SA29  
267 clustered within subtype 3f, showing a homology of 89% (range 91.3-85.3%) to European  
268 isolates. One sequence from BW belonged to subtype 3h. Comparing 1043 nt between  
269 isolates BB02 and BB19, only two divergent nucleotides were present (identity of 99.9%).  
270 Isolates RP1658 and RP1752 also differed only in two nucleotides when comparing 998 nt.  
271 The analysis of a 969 nt fragment showed a maximum identity of 99.4% (1-5 nucleotides  
272 difference) between SA11, SA13, SA21, SA28 and SA34. Surprisingly, isolate SA29  
273 showed a nucleotide sequence diversity of 14.8% (124 nucleotides) to all other isolates

274 from SA. The full-length sequences of isolate SA 21 (subtype 3e) and BB02 (subtype 3i)  
275 were generated to investigate the relationship to published European partial and full-length  
276 sequences of porcine and human origin for a detailed molecular epidemiological analysis.  
277 A genome sequence with a length of 7153 nt was generated for isolate SA21, whereas the  
278 sequence for isolate BB02 was 7205 nt in length. Aligning these sequences with all full-  
279 length sequences from the database, isolate BB02 was most closely related to a Mongolian  
280 pig isolate (swMN06-A1288), two human isolates from Japan (HE-JA10 and E097-  
281 OSA05C) and a mongoose isolate from Japan (JMNG-Oki02C). Furthermore, isolate BB02  
282 was more closely related to the reference strain of Meng from US swine (AF082843,  
283 (Meng et al., 1997)) than to the pig strain from Sweden (Xia et al., 2008) and to the human  
284 isolate HEV\_RKI (FJ956757, (Adlhoch et al., 2009)) of subtype 3f from Germany. Isolate  
285 SA21 matched with pig sequences of genotype 3 from Japan (swJ8-5, swJ12-4) and  
286 Mongolia (swMN06-C1056) as well as with a human isolate from Japan (E116-YKH98C).  
287 Furthermore, SA21 was more closely related to the European isolates than to the reference  
288 strain from the US.  
289



#### 290 **4. Discussion**

291 Limited information on the prevalence of HEV in pigs and wild boars in Germany is  
292 available. In European countries an HEV seroprevalence of 40 to 80% and a genome  
293 prevalence of 20 to 50% were reported for pigs in commercial farms and wild boars (de  
294 Deus et al., 2008; de Deus et al., 2007; Di Bartolo et al., 2008; Martelli et al., 2008; Reuter  
295 et al., 2009; Seminati et al., 2008). Using the highly sensitive qPCR developed for the  
296 investigations presented here, a high prevalence of HEV RNA-positive animals (68%) was  
297 determined in wild boars hunted in different parts of Germany, while only 24.3% of the  
298 samples were reactive in serological tests (ELISA and Line immunoassay). Serological data  
299 showed a very high prevalence of HEV infections in SA, a low prevalence in RP and BB  
300 and no reactivity for the wild boar population in BW. No age dependency was observed.  
301 Only 3% of the samples with isolated reactivity in the MG ELISA were negative for HEV  
302 RNA, whereas 25% of the samples revealed isolated reactivity without detectable HEV  
303 RNA in the GL ELISA. Although the two different ELISAs contain only HEV antigens of  
304 the genotype (gt) 1 or 1 and 2 (GL: gt1 and gt2, MG: gt1), the MG ELISA exhibited higher  
305 specificity than the GL ELISA when analyzing the corresponding results with the Line  
306 immunoassay as confirmation test, which in addition to gt1 contains gt3 epitopes. It is  
307 unclear whether the differences between the two IgG ELISAs can be explained by  
308 differences in the strain-specific antigenic domains present in the assays and need further  
309 validation. Although the commercial test systems were developed for the detection of  
310 human antibodies, an efficient cross-detection of swine and human anti-HEV antibodies  
311 was previously shown (Engle et al., 2002). The lower seroprevalence in contrast to the  
312 qPCR results determined in this study might be due to a lower sensitivity of the antibody

313 detection systems or reflects acute infections raging in the wild boar populations at the time  
314 of sampling with incomplete seroconversion. However, a persistence of HEV or HEV  
315 genomes in liver or bile without detectable levels of serum antibodies or missing  
316 seroconversion cannot be ruled out. Although the sensitivity and detection limit of the  
317 qPCR and the ELISA test systems seem to be critical diagnostic factors (Bouwknegt et al.,  
318 2008; Rutjes et al., 2007), the regional different prevalence rates were seen correspondingly  
319 in serological as well as in qPCR analyses. For a good qPCR performance a series of steps  
320 e.g. RNA extraction of the different samples, cDNA synthesis and qPCR conditions were  
321 optimised, and for the exclusion of influencing inhibitors on the assay system two  
322 independent extraction and amplification controls ( $\beta$ -actin and MRV) were included in the  
323 qPCR regime. Accordingly, multiple differing serological test systems were applied in the  
324 study. Surveying the four randomly selected independent regions within Germany, different  
325 genetic subtypes (3e (SA), 3f (SA), 3h (BW) and 3i (BB and RP)) were found to be present  
326 among German wild boar populations. The prevailing heterogeneity in the wild boar  
327 population seems to correspond to the heterogeneity of HEV observed in domestic pigs in  
328 Japan (Takahashi et al., 2003). Within a given population the circulating HEV appears to be  
329 stable, but animals in close proximity can carry different subtypes, as seen for the isolate  
330 SA29. There was no information about the distance between the respective shooting places.  
331 It can therefore be hypothesized that animal SA29 belonged to a different social group. As  
332 also reported by others (de Deus et al., 2007), bile samples were more frequently genome  
333 positive and generally showed the highest genome titres. Interestingly, animals exhibiting  
334 high copy numbers were found in all age groups. It can be assumed that HEV genome-  
335 positive animals are virus carriers. HEV genome prevalence associated with anti-HEV IgG  
336 antibodies was mostly seen in animals younger than 1 and older than 2 years, which

337 indicates acute HEV infections at the time of sampling. This might point to a naïve  
338 population under the age of 1 year and a loss of protective antibodies in old animals or long  
339 persisting infections of wild boars. Virus shedding in older animals that has also been  
340 shown previously for domestic sows (Fernández-Barredo et al., 2006) might play a crucial  
341 role in the persistent circulation of HEV in pig populations. It would be of interest to  
342 investigate whether HEV-infected wild boars excreted infectious HEV for a long time. The  
343 most important issue is to assess the risk of HEV transmission from pig to human via close  
344 contact to HEV-contaminated tissues as shown for the transmission of HEV from pig to pig  
345 (Bouwknegt et al., 2009). It can be assumed that hunters are at risk of acquiring an HEV  
346 infection from wild boars in view of the high copy numbers in a high percentage of  
347 animals, ranging from very young animals to adult wild boars. Transmission might occur  
348 when the body of the wild boar is opened and hunters remove organs with their unprotected  
349 hands. Although the full-length genomes of the two different HEV subtypes from wild boar  
350 matched with other porcine isolates, the isolate BB02 was more closely related to a human  
351 full-length sequence of subtype 3c (unpublished data) derived from a patient from  
352 Berlin/Brandenburg with an autochthonous HEV infection (le Coutre et al., 2009).  
353 Furthermore the results of the high prevalence of HEV genomes in wild boars support the  
354 hypothesis of a potential zoonotic transmission from wild boar to human (Wichmann et al.,  
355 2008). This indicates that the wild boars might be one of the main reservoirs of HEV in  
356 Germany and could be responsible for autochthonous human infections with different HEV  
357 subtypes. Further epidemiological studies of autochthonous cases are needed for the  
358 analysis of human HEV isolates circulating in Europe and the relationship to pig isolates as  
359 HEV reservoir. Studies on the prevalence of HEV and the distribution of different

360 genotypes in domestic pigs might give information about the role of wild boars as source of  
361 infection for the livestock or vice versa.

362

## 363 **5. Conclusions**

364 The present study revealed that the wild boars might represent one of the main reservoirs of  
365 HEV in Germany and therefore be responsible for autochthonous human infections with  
366 different HEV subtypes. At present, there is no information on the prevalence of HEV in  
367 domestic pigs in Germany. Studies on the molecular epidemiology of HEV in domestic pig  
368 populations should provide information on whether wild boars infect the livestock.  
369 Furthermore this will allow a better risk assessment of contracting HEV when handling raw  
370 pork.

371

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379

380

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- 481
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483 **Figure 1:** Subtyping of wild boar sequences using a 242 nt fragment within ORF1  
484 according to Lu et al. Samples presented in this report are circled. Human isolates from  
485 Germany are HEV\_RKI and isolates starting with V. Bootstrap values in percent.

486

487

488 **Figure 2:** Subtyping of wild boar sequences using a 300 nt fragment within ORF2  
489 according to Lu et al. Samples presented in this report are circled. HEV\_RKI: human  
490 isolate from Germany. Bootstrap values in percent.

491

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492 **Tables**

493 **Table 1:** Location of the hunting regions where wild boar samples were collected with  
494 dates of sampling and coordinates

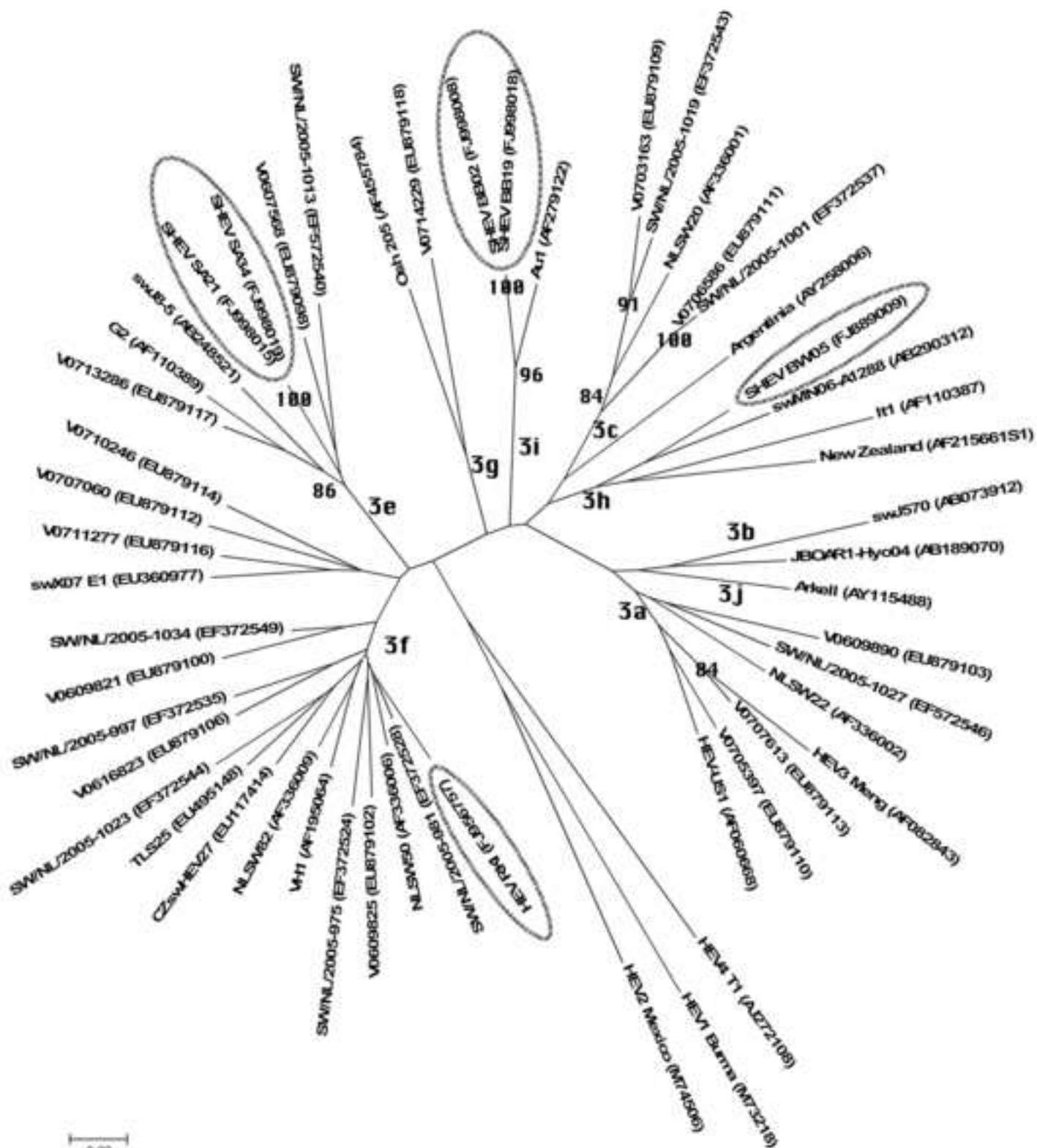
495 **Table 2:** Primer and probe sequences with nucleotide position numbers in isolate Meng  
496 (AF082843) used for qPCR, generation of partial and full-length sequences of HEV.

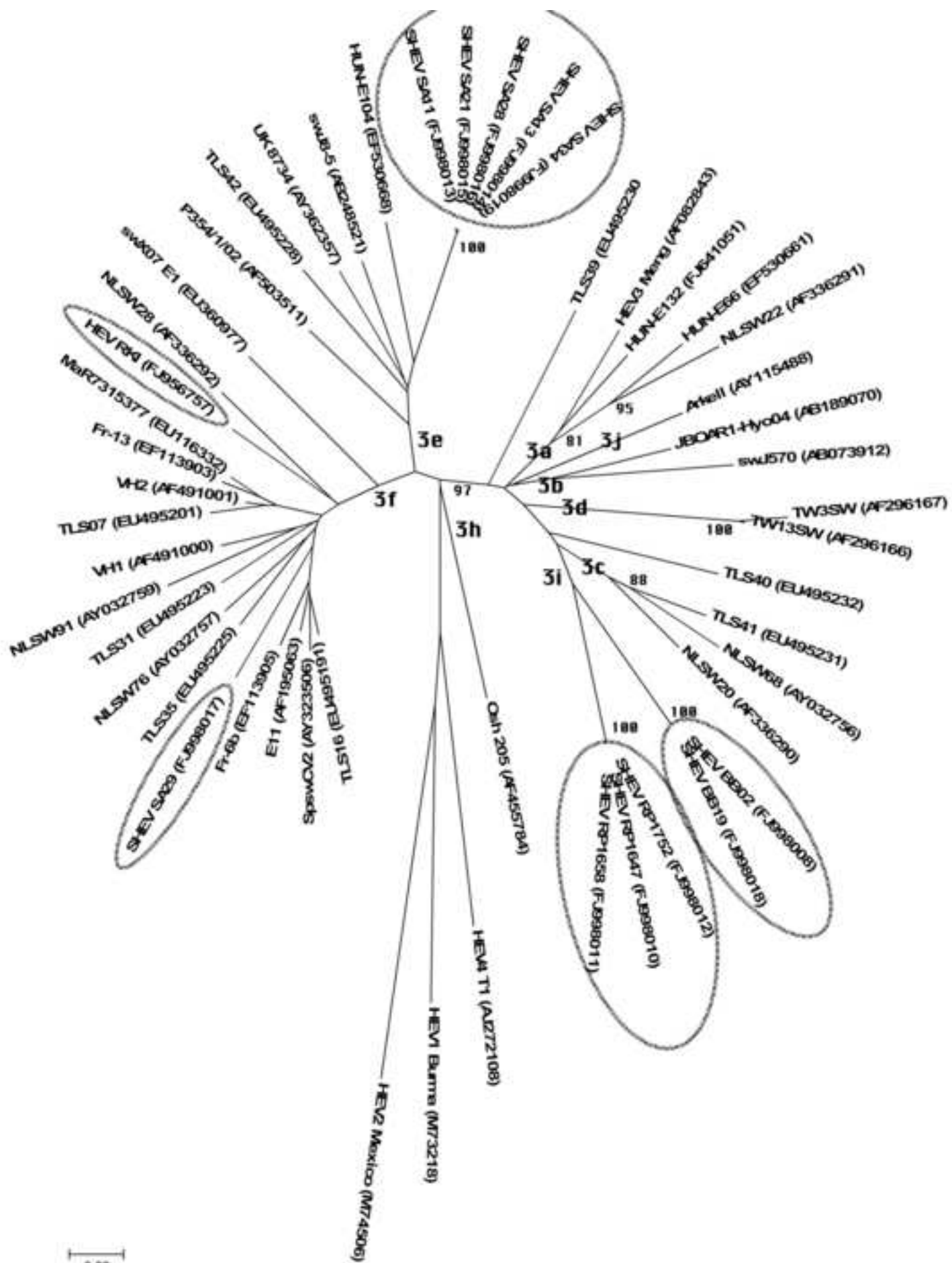
497 **Table 3:** Detection of HEV RNA by qPCR in different samples from wild boars (A) with  
498 age distribution (B) and region of collection.

499 **Table 4:** Detection of anti-HEV IgG by ELISAs from Genelabs (GL) and Mikrogen (MG-  
500 E) and a Line immunoassay (Mikrogen, MG-L) in serum samples from wild boars collected  
501 in different regions of Germany.

502

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1

1 **Tables**2 **Table 1:** Location of the hunting regions where wild boar samples were collected with dates of sampling and coordinates

<b>Location of sampling</b>	<b>Federal state</b>	<b>Date of sampling</b>	<b>Number of animals</b>	<b>Coordinates</b>	3
<b>Johanniskreuz</b>	Rhineland Palatine (RP)	24 Oct 2007	13	49°16'54.77"N 7°48'35.13"E	
<b>Johanniskreuz</b>	Rhineland Palatine (RP)	15 Nov 2007	12	49°16'54.77"N 7°48'35.13"E	
<b>Bebenhausen</b>	Baden-Württemberg (BW)	20 Nov 2007	16	48°33'34.41"N 9° 3'39.40"E	
<b>Rottenburg</b>	Baden-Württemberg (BW)	21 Nov 2007	11	48°28'35.90"N 8°56'15.97"E	
<b>Krausnick</b>	Brandenburg (BB)	24 Nov 2007	19	52°1'20.68"N 13°49'40.89"E	
<b>Johanniskreuz</b>	Rhineland Palatine (RP)	26 Nov 2007	23	49°16'54.77"N 7°48'35.13"E	
<b>Proschim-Welzow</b>	Brandenburg/Saxony (SA)	01 Dec 2007	33	51°33'6.11"N 14°11'32.74"E	
<b>Hinterweidenthal</b>	Rhineland Palatine (RP)	17 Dec 2007	5	49°11'46.33"N 7°48'19.69"E	

4

5 **Table 2:** Primer and probe sequences with nucleotide position numbers in isolate Meng  
6 (AF082843) used for qPCR, generation of partial and full-length sequences of HEV.

<b>Oligo name</b>	<b>Oligonucleotide sequence 5'-3'</b>	<b>Localisation</b>
HEV_F	CGACAGAATTGATTCGTCGGC	6321-6342
HEV_R	CCYTTRTCYTGCTGNGCRTTCTC	6441-6419
HEV_TM	6FAM-TYGGCTCGCCATTGGCYGAGAC--BHQ1	6396-6374
HEV 27F	CGAYGCCATGGAGGCC	3-19
HEV 837F 10C	CTGAGCCGTCACCAATGCC	821-839
HEV 1054R	CGGAGATAGGTCATAAGCCG	1043-1024
HEV 1054F	CGGCTTATGACCTATCTCCG	1024-1043
HEV 1749F	GACCTCCGCACATCAATAACTG	1719-1741
HEV 2034R	GGTWAGCGAGTGGCGCTG	1987-2004
HEV 2506F	GATTGTGATTGGCTGGTTAATGCG	2449-2472
HEV 2520R	GGCCAGGGTTAGACGCATTAACC	2463-2485
HEV 2663R	GCCTCAARCCTCTTNGGGTTCTGCTCAA	2633-2660
HEV 3012F	TGTYCATTATCAGTTTACTGCCGG	2955-2978
HEV 3127F 10C	TCGTGTTGTGATTGATGAGGCC	3126-3147
HEV 3132F SA21	GTGTTGTGATTGATGAGGCTCC	3128-3149
HEV 3254R	CGANGAGGCCCGCTGCAT	3181-3198
HEV 4653F	GGAAGAARCAYTCNGGYGAGC	4592-4612
HEV 4704F SA21	GACTTGAGAGTGGCAGCGTTC	4678-4698
HEV 7321R	TTTTTTCCAGGGAGCGCG	7271-7253*

7 F: forward orientation; R: reverse orientation; \* according to isolate HE-JA04-1911  
8 (AB248520)

9

10 **Table 3:** Detection of HEV RNA by qPCR in different samples from wild boars (A) with  
 11 age distribution (B) and region of collection.

12 (A)

No. of materials or animals positive/no. tested (%)				
Region	Serum	Bile	Liver	Wild boar
<b>BW</b>	4/27 (14.8)	2/27 (7.4)	0/26 (0.0)	6/27 (22.2)
<b>BB</b>	1/17 (5.9)	13/14 (92.9)	19/19 (100)	19/19 (100.0)
<b>SA</b>	10/32 (31.3)	11/26 (42.3)	11/28 (39.3)	19/33 (57.6)
<b>RP</b>	3/39 (7.7)	41/52 (78.8)	18/53 (34.0)	46/53 (86.8)
<b>Total</b>	18/115 (15.7)	67/119 (56.3)	48/126 (38.1)	90/132 (68.2)

13

14 (B)

No. of animals positive/no. tested					
Age/ Region	<1 y	1-2 y	Sows	Male boars	Age unknown
<b>BW</b>	1/10	2/7	1/3	0/1	2/6
<b>BB</b>	9/9	3/3	1/1	2/2	4/4
<b>SA</b>	6/8	5/14	6/7	2/4	0
<b>RP</b>	20/26	13/14	10/10	2/2	1/1
<b>Total</b>	36/53	23/38	18/21	6/9	7/11
<b>(%)</b>	(67.9)	(60.5)	(85.7)	(66.7)	(63.6)

15 No. = number; BW: Baden-Württemberg; BB: Brandenburg; SA: Brandenburg/Saxony;

16 RP: Rhineland Palatine

- 17 **Table 4:** Detection of anti-HEV IgG by ELISAs from Genelabs (GL) and Mikrogen (MG-E) and a Line immunoassay (Mikrogen, MG-L)  
 18 in serum samples from wild boars collected in different regions of Germany.

No. of samples reactive/no. tested (%)						
Region	GL	MG-E	GL & MG-E	MG-L	MG-E & MG-L	GL, MG-E & MG-L
<b>BW</b>	7/27 (25.9)	0/27 (0.0)	0/27 (0.0)	0/27 (0.0)	0/27 (0.0)	0/27 (0.0)
<b>BB</b>	3/17 (17.6)	4/17 (23.5)	3/17 (17.6)	4/17 (23.5)	4/17 (23.5)	3/17 (17.6)
<b>SA</b>	10/23 (43.5)	21/23 (91.3)	10/23 (43.5)	19/23 (82.6)	19/23 (82.6)	9/23 (39.1)
<b>RP</b>	12/40 (30.0)	7/40 (17.5)	4/40 (10.0)	5/40 (12.5)	3/40 (7.5)	3/40 (7.5)
<b>Total</b>	32/107 (29.9)	32/107 (29.9)	17/107 (15.9)	28/107 (26.2)	26/107 (24.3)	15/107 (14.0)

19