High HEV presence in four different wild boar populations in East and West Germany
Cornelia Adlhoch, Alexander Wolf, Helga Meisel, Marco Kaiser, Heinz Ellerbrok, Georg Pauli

To cite this version:
Cornelia Adlhoch, Alexander Wolf, Helga Meisel, Marco Kaiser, Heinz Ellerbrok, et al.. High HEV presence in four different wild boar populations in East and West Germany. Veterinary Microbiology, Elsevier, 2009, 139 (3-4), pp.270. 10.1016/j.vetmic.2009.06.032. hal-00526941

HAL Id: hal-00526941
https://hal.archives-ouvertes.fr/hal-00526941
Submitted on 17 Oct 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
High HEV presence in four different wild boar populations in East and West Germany

Cornelia Adlhoch¹*, Alexander Wolf¹, Helga Meisel², Marco Kaiser¹, Heinz Ellerbrok¹,
and Georg Pauli¹

¹Robert Koch-Institut, Center for Biological Safety, Nordufer 20, 13353 Berlin; ²Institute of Medical Virology, Charité Medical School, Charitéplatz 1, 10117 Berlin, Germany

*Author’s address: Robert Koch-Institut, Center for Biological Safety ZBS 1, Nordufer 20, 13353 Berlin, Germany, telephone: +49 30 18754 2591, fax: +49 30 18754 2605, E-mail: adlhochc@rki.de

Running Title: HEV in German wild boars from 4 regions

Word count
Abstract: 243 (max 250)
Total number of words: ----
Number of figures/tables: 4 (max 8)
Number of references: 32 (max. 35)
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.

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

Four major genotypes can be differentiated in the genus Hepatitis E virus (HEV) within the family of Hepeviridae (Emerson and Purcell, 2007). Europe has long been considered to be non-endemic for HEV. However, in recent years an increasing number of autochthonous HEV cases were reported across Europe, and a close genetic relationship between these human-derived HEV sequences and porcine HEV isolates was demonstrated (Banks et al., 2004; van der Poel et al., 2001). Genotypes 3 and 4 are mainly found in industrialized countries. HEV isolates from sporadic acute human cases in these countries show a high genetic similarity to isolates from animals, especially from pigs (Goens and Perdue, 2004; Meng et al., 1997). For the years 2001 to 2007 311 human cases of acute hepatitis E were reported in Germany, 142 (45.7%) of these infections were most likely acquired in Germany (Robert Koch-Institut, 2008). A case-control study about the risk of acquiring an HEV infection in Germany determined that the main risk factor was consumption of offal and meat from wild boars (Wichmann et al., 2008). While several studies across Europe detected a high prevalence of anti-HEV IgG antibodies and HEV RNA in wild boars and domestic pigs within all age groups (de Deus et al., 2008; Martelli et al., 2008; McCreary et al., 2008; Rutjes et al., 2007; Seminati et al., 2008), an HEV RNA prevalence of only 5% was found in serum samples collected from wild boars in north-eastern Germany in the late 1990s (Kaci et al., 2008). The main object of this study was to determine the actual prevalence of HEV genome and anti-HEV antibodies in wild boar populations in four different regions in Germany. Together with data on the geographical distribution of the particular hunting spots, a comparison of the endemic situation in several different wild boar populations becomes possible. Bile, liver and serum samples were collected and
analysed by quantitative PCR for HEV virus load. Sequencing and phylogenetic analysis were performed to determine the respective HEV geno- and subtype, and data obtained were compared to isolates found in other European countries. Investigations on the molecular epidemiology of HEV in different wild boar populations might help to assess the potential risk of zoonotic transmission of HEV from wild boars to humans.
2. Materials and methods

2.1. Sample collection

Samples from 132 wild boars were collected during the hunting season between October and December 2007 at different regional sites in Germany. Nineteen animals were sampled in the federal state of Brandenburg (BB), 33 animals in a region at the border between the federal states of Brandenburg and Saxony (SA), 53 animals in 2 locations in Rhineland Palatine (RP), and 27 animals in 2 locations in Baden-Württemberg (BW) (Table 1). When possible, samples were collected immediately after the opening of the wild boars’ bodies. Generally, sampling and age determination of the animals took place at special assembling places, but at some sites the hunters themselves collected the samples in the context of opening the bodies at the place of shooting. The age of the animals was estimated using the presence and rotation of teeth (Habermehl, 1985). The age distribution of wild boars was: 1 year (n=53), between 1 and 2 years (n=38), adult sows (n=21) and adult boars (n=9). The age of 11 animals could not be determined. Bile was squeezed out into 50 ml or 15 ml tubes (Nunc Thermo Fischer Scientific, Langenselbold, Germany), liver pieces were preserved in RNAlater® solution (Ambion, Austin, TX, USA) and blood was collected in serum tubes. Samples were stored at 4°C overnight, processed on the next day and aliquots were frozen at -70°C. Because of relatively long time intervals between shooting and transport of the wild boars’ bodies to assembly points in the forest for sampling, blood was often coagulated and serum samples were haemolytic.
2.2. Isolation of RNA and cDNA synthesis

A piece of 20 to 40 mg of liver samples preserved in RNAlater® (Ambion) was homogenized in 500 µl PBS using Precellys ceramic balls with a diameter of 1.4 mm (Peqlab Biotechnology, Erlangen, Germany) and the FastPrep® FP220A homogenizer (Qbiogene, MP Biomedicals, Solon, OH, USA). The supernatant was collected and 100 µl were taken for RNA extraction using the NucleoSpin® RNA II Mini preparation kit (Macherey-Nagel, Düren, Germany). Residual supernatant after homogenization was stored at -70°C. RNA was extracted from bile and serum following the manufacturer’s protocol 5.2 of the NucleoSpin® RNA II Mini kit (Macherey-Nagel). Additionally, RNA from bile samples tested negative was extracted, following the supplier’s protocol on purification of viral RNA using QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany). DNase digestion was performed to reduce the DNA load in the sample, using Ambion® TURBO DNA free™ (Ambion). Briefly, 25 µl RNA were incubated with 2.5 µl 10xDNase buffer and 2 µl Turbo DNase for 30 min at 37°C. After adding 2 µl of inactivation reagent the sample was vortexed for 2 min and centrifuged at 6,800 x g in a Centrifuge 5417R (Eppendorf, Hamburg, Germany) for 3 min. A volume of 20.6 µl of the eluted RNA was reverse transcribed using the TaqMan® Reverse Transcription Reagents following the supplier’s protocol (Applied Biosystems, Foster City, CA, USA). RNA was incubated with 2 µl primer mix R6/HEV R (Table 2) for 5 min at 65°C. The sample was cooled on ice to 4°C before adding the reverse transcription mix containing 4 µl 10xbuffer, 8.8 µl MgCl₂ (25 mM), 3.2 µl dNTP (25 mM), 0.8 µl RNase inhibitor and 0.6 µl reverse transcriptase. The reaction was run in a thermocycler (Mastercycler epgradient, Eppendorf) for 60 min at 42°C with a final 15 min incubation at 72°C before cooling to 4°C and storage at -20°C.
2.3. Quantitative HEV detection

A real-time PCR (qPCR) assay using 5’ nuclease probes (TaqMan® probes) with primer combination HEV_F/R/TM (Table 2, (Adlhoch et al., 2009)) was established in this study which should be able to detect all known HEV genotypes and to determine the copy numbers of HEV genomes. The reverse primer HEV_R is the modified published primer HE040, and the probe HEV_TM overlaps with HE041 (Mizuo et al., 2002). The Platinum® Taq DNA polymerase kit, MgCl₂ and dNTPs by Invitrogen (Carlsbad, CA, USA) and water (Molecular Biology Grade Eppendorf) were used in this assay. qPCR reactions were performed in a final volume of 25 µl with 10x buffer, 4 mM of MgCl₂, dNTP 0.2 mM each, 0.3 µM of each primer, 0.1 µM of probe, ROX 0.1 µM and Platinum® Taq 0.5 U. General reaction conditions for the real-time PCR assay were 95°C for 15 min and 45 cycles with 95°C for 15 sec, 60°C for 35 sec. Reactions were run in an ABI GeneAmp® 7500 Detection System (Applied Biosystems). The PCR product was cloned into vector pCRII (Invitrogen) and purified using NucleoSpin® Plasmid (Macherey-Nagel) following the manufacturer’s instructions. The generated plasmid pHEV_RKI containing a 121 nt fragment of the related region from the isolate HEV_RKI (FJ956757, (Adlhoch et al., 2009)) was quantified by measuring the DNA concentration at 260 nm. The plasmid was serially diluted in water containing λ-DNA (1 ng/µl) 10fold from 10⁶ copies to 10 copies and used as standard for the quantification of viral genomes. Copy numbers were determined using a standard curve; the mean and standard deviation were calculated. The detection limit was calculated to be 10 copies of cDNA per reaction. For real-time PCR each sample was analysed in duplicate.
2.4. Amplification controls

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

2.5. Amplification of partial and full-length HEV sequences

Long-range PCRs were run using the Roche FastStart High Fidelity PCR System, dNTPack (Roche Diagnostics, Mannheim, Germany). The final PCR volume was 25 µl adjusted with water, 5 µl 5xbuffer, dNTPs 10 mM 1.25 µl, primer each 0.75 µl 10 µM, DMSO 1 µl final 4%, 0.2 µl ET SSB 600 ng/µl (New England Biolabs, Ipswich, MA, USA), DNA polymerase 0.35 µl final 1.75 U and 2 µl cDNA. The reaction was run in a thermocycler (Mastercycler epgradient, Eppendorf) at 95°C for 2 min, 10 cycles at 95°C for 10 sec, at 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
68°C for 1.5 min and an elongation of 20 sec for every cycle. The final extension was 68°C for 5 min. The annealing temperature X was dependent on the melting temperature of the primers. Published primers HEV366F, HEV363R (Inoue et al., 2006), HE044F (Mizuo et al., 2002), HEVgenF2 (Li et al., 2005), HEV_F and HEV_R were used in combination with newly designed primers (Table 2) to amplify long fragments within ORF1 and ORF2 for subtyping and generation of the full-length genomes.

2.6. Serological analysis

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

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

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

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

- **SHEV_BB02** (FJ998008);
- **SHEV_BW05** (FJ998009);
- **SHEV_RP1647** (FJ998010);
- **SHEV_RP1658** (FJ998011);
- **SHEV_RP1752** (FJ998012);
- **SHEV_SA11** (FJ998013);
- **SHEV_SA13** (FJ998014);
- **SHEV_SA21** (FJ998015);
- **SHEV_SA28** (FJ998016);
- **SHEV_SA29** (FJ998017);
- **SHEV_BB19** (FJ998018);
- **SHEV_SA34** (FJ998019)
3. Results

3.1. Quantitative polymerase chain reaction (qPCR) analysis

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

3.2. Serology

Anti-HEV IgG was determined using a modification of two ELISA HEV kits (GL and MG) and the newly established Line immunoassay (MG). Table 4 illustrates the results of the serological assays for anti-HEV IgG. Anti-HEV reactivity was detected in 29.9% of the 107
serum samples using the ELISA assays, and 26.2% were reactive in the Line immunoassay. Isolated reactivity was seen in 15 sera using GL (7 samples also positive for HEV RNA) and in 4 sera (3 samples showing HEV RNA) using MG ELISA, 2 samples were reactive only in the Line immunoassay (no HEV RNA). No sample was reactive only in GL ELISA and Line immunoassay. Isolated anti-HEV reactivity without HEV RNA was seen in 8 of 32 sera using GL and 1 of 32 sera in the MG ELISA. Comparison of the two IgG ELISAs showed that only 17 (53%) sera were reactive in both tests and 14 (82%) of these samples were concurrently HEV RNA positive. The Line immunoassay confirmed 15 out of these 17 ELISA reactive sera. 12 (75%) of these 15 samples were also qPCR positive, with 5 of them (4 animals <1 year, 1 adult wild boar) indicating copy numbers of approximately $10^9$ or more per g of liver or ml of bile. There was a high degree of correlation between the ELISA and Line immunoassay from MG, 81% of the MG ELISA reactive sera were confirmed by the Line immunoassay, in contrast to the ELISA from GL where only 47% of the reactive sera were also reactive in the Line immunoassay. 15 of 26 (57.7%) animals showing anti-HEV IgG reactivity in both MG assays were simultaneously RNA positive (7 animals <1 year, 2 animals 1-2 years, 4 adult sows, 1 adult wild boar, 1 without age determination), 7 of them showing high copy numbers (4 <1 year, 1 1-2 years, 1 sow, 1 wild boar). Eight of the 10 animals that were IgG reactive in MG without detectable copy numbers were between 1 and 2 years old.

### 3.3. Sequence analysis

Samples containing high copy numbers of viral genome were selected for sequence analysis. Isolates from different hunting sites were sequenced and analysed to determine the genome subtypes within different geographic regions of Germany. Additionally, an
assortment of samples from each region was analysed to determine the variation within the social groups of the particular hunting spot. There was a mean homology of the wild boar isolates of 89.3% to genotype 1 (Burma M73218), 88.5% to genotype 2 (Mexico M74506), 94.0% to genotype 3 (Meng AF082843) and 90.3% to genotype 4 (T1 AJ272108). Accordingly all HEV sequences from wild boars clustered within genotype 3 when comparing a 969 nt fragment with the reference strains of the different genotypes. For subtyping a 242 nt fragment within ORF1 and a 300 nt fragment within ORF2 were used (Lu et al., 2006). The wild boar samples clustered within different subtypes (Figures 1 and 2). Isolates BB02 and BB19 and RP1647, RP1658 and RP1752 clustered in the ORF2 at the subtype 3c branch, but isolate BB02 showed homology to subtype 3i in the analysis of ORF1. Due to the lack of 3i isolate sequences within ORF2, all of these isolates most probably belong to subtype 3i, showing a mean homology of only 85.1% (range 89.2-81.8%) to swine isolates of subtype 3c from the Netherlands. Although the isolates from BB and RP clustered within one subtype, there were 105 divergent nucleotides between the isolates when comparing a longer 998 nt fragment within ORF2. Five of the SA isolates (SA11, SA13, SA21, SA28 and SA34) clustered within subtype 3e, showing a mean homology of 88.5% (range 90.2-85.5%) to European isolates of this subtype. SA29 clustered within subtype 3f, showing a homology of 89% (range 91.3-85.3%) to European isolates. One sequence from BW belonged to subtype 3h. Comparing 1043 nt between isolates BB02 and BB19, only two divergent nucleotides were present (identity of 99.9%). Isolates RP1658 and RP1752 also differed only in two nucleotides when comparing 998 nt. The analysis of a 969 nt fragment showed a maximum identity of 99.4% (1-5 nucleotides difference) between SA11, SA13, SA21, SA28 and SA34. Surprisingly, isolate SA29 showed a nucleotide sequence diversity of 14.8% (124 nucleotides) to all other isolates.
from SA. The full-length sequences of isolate SA 21 (subtype 3e) and BB02 (subtype 3i) were generated to investigate the relationship to published European partial and full-length sequences of porcine and human origin for a detailed molecular epidemiological analysis. A genome sequence with a length of 7153 nt was generated for isolate SA21, whereas the sequence for isolate BB02 was 7205 nt in length. Aligning these sequences with all full-length sequences from the database, isolate BB02 was most closely related to a Mongolian pig isolate (swMN06-A1288), two human isolates from Japan (HE-JA10 and E097-OSA05C) and a mongoose isolate from Japan (JMNG-Oki02C). Furthermore, isolate BB02 was more closely related to the reference strain of Meng from US swine (AF082843, (Meng et al., 1997)) than to the pig strain from Sweden (Xia et al., 2008) and to the human isolate HEV_RKI (FJ956757, (Adlhoch et al., 2009)) of subtype 3f from Germany. Isolate SA21 matched with pig sequences of genotype 3 from Japan (swJ8-5, swJ12-4) and Mongolia (swMN06-C1056) as well as with a human isolate from Japan (E116-YKH98C). Furthermore, SA21 was more closely related to the European isolates than to the reference strain from the US.
4. Discussion

Limited information on the prevalence of HEV in pigs and wild boars in Germany is available. In European countries an HEV seroprevalence of 40 to 80% and a genome prevalence of 20 to 50% were reported for pigs in commercial farms and wild boars (de Deus et al., 2008; de Deus et al., 2007; Di Bartolo et al., 2008; Martelli et al., 2008; Reuter et al., 2009; Seminati et al., 2008). Using the highly sensitive qPCR developed for the investigations presented here, a high prevalence of HEV RNA-positive animals (68%) was determined in wild boars hunted in different parts of Germany, while only 24.3% of the samples were reactive in serological tests (ELISA and Line immunoassay). Serological data showed a very high prevalence of HEV infections in SA, a low prevalence in RP and BB and no reactivity for the wild boar population in BW. No age dependency was observed. Only 3% of the samples with isolated reactivity in the MG ELISA were negative for HEV RNA, whereas 25% of the samples revealed isolated reactivity without detectable HEV RNA in the GL ELISA. Although the two different ELISAs contain only HEV antigens of the genotype (gt) 1 or 1 and 2 (GL: gt1 and gt2, MG: gt1), the MG ELISA exhibited higher specificity than the GL ELISA when analyzing the corresponding results with the Line immunoassay as confirmation test, which in addition to gt1 contains gt3 epitopes. It is unclear whether the differences between the two IgG ELISAs can be explained by differences in the strain-specific antigenic domains present in the assays and need further validation. Although the commercial test systems were developed for the detection of human antibodies, an efficient cross-detection of swine and human anti-HEV antibodies was previously shown (Engle et al., 2002). The lower seroprevalence in contrast to the qPCR results determined in this study might be due to a lower sensitivity of the antibody
detection systems or reflects acute infections raging in the wild boar populations at the time of sampling with incomplete seroconversion. However, a persistence of HEV or HEV genomes in liver or bile without detectable levels of serum antibodies or missing seroconversion cannot be ruled out. Although the sensitivity and detection limit of the qPCR and the ELISA test systems seem to be critical diagnostic factors (Bouwknegt et al., 2008; Rutjes et al., 2007), the regional different prevalence rates were seen correspondingly in serological as well as in qPCR analyses. For a good qPCR performance a series of steps e.g. RNA extraction of the different samples, cDNA synthesis and qPCR conditions were optimised, and for the exclusion of influencing inhibitors on the assay system two independent extraction and amplification controls (β–actin and MRV) were included in the qPCR regime. Accordingly, multiple differing serological test systems were applied in the study. Surveying the four randomly selected independent regions within Germany, different genetic subtypes (3e (SA), 3f (SA), 3h (BW) and 3i (BB and RP)) were found to be present among German wild boar populations. The prevailing heterogeneity in the wild boar population seems to correspond to the heterogeneity of HEV observed in domestic pigs in Japan (Takahashi et al., 2003). Within a given population the circulating HEV appears to be stable, but animals in close proximity can carry different subtypes, as seen for the isolate SA29. There was no information about the distance between the respective shooting places. It can therefore be hypothesized that animal SA29 belonged to a different social group. As also reported by others (de Deus et al., 2007), bile samples were more frequently genome positive and generally showed the highest genome titres. Interestingly, animals exhibiting high copy numbers were found in all age groups. It can be assumed that HEV genome-positive animals are virus carriers. HEV genome prevalence associated with anti-HEV IgG antibodies was mostly seen in animals younger than 1 and older than 2 years, which
indicates acute HEV infections at the time of sampling. This might point to a naïve population under the age of 1 year and a loss of protective antibodies in old animals or long persisting infections of wild boars. Virus shedding in older animals that has also been shown previously for domestic sows (Fernández-Barredo et al., 2006) might play a crucial role in the persistent circulation of HEV in pig populations. It would be of interest to investigate whether HEV-infected wild boars excreted infectious HEV for a long time. The most important issue is to assess the risk of HEV transmission from pig to human via close contact to HEV-contaminated tissues as shown for the transmission of HEV from pig to pig (Bouwknegt et al., 2009). It can be assumed that hunters are at risk of acquiring an HEV infection from wild boars in view of the high copy numbers in a high percentage of animals, ranging from very young animals to adult wild boars. Transmission might occur when the body of the wild boar is opened and hunters remove organs with their unprotected hands. Although the full-length genomes of the two different HEV subtypes from wild boar matched with other porcine isolates, the isolate BB02 was more closely related to a human full-length sequence of subtype 3c (unpublished data) derived from a patient from Berlin/Brandenburg with an autochthonous HEV infection (le Coutre et al., 2009). Furthermore the results of the high prevalence of HEV genomes in wild boars support the hypothesis of a potential zoonotic transmission from wild boar to human (Wichmann et al., 2008). This indicates that the wild boars might be one of the main reservoirs of HEV in Germany and could be responsible for autochthonous human infections with different HEV subtypes. Further epidemiological studies of autochthonous cases are needed for the analysis of human HEV isolates circulating in Europe and the relationship to pig isolates as HEV reservoir. Studies on the prevalence of HEV and the distribution of different
genotypes in domestic pigs might give information about the role of wild boars as source of infection for the livestock or vice versa.

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

Acknowledgments
The authors are grateful to U. Hohmann, D. Huckschlag and J. Hof at the FAWF, Trippstadt (RP), H.-J. Hormel at the forestry management of Baden-Württemberg in Tübingen and the forest officials J. P. Huber in Welzow-Proschim (SA) and F. Wehnert in Krausnick (BB) as well as all the collaborating hunters for the opportunity and the support in collecting wild boar samples. Thanks to Sigrid Kersten for excellent technical assistance and Ursula Erikli for copy-editing.
References


Figure 1: Subtyping of wild boar sequences using a 242 nt fragment within ORF1 according to Lu et al. Samples presented in this report are circled. Human isolates from Germany are HEV_RKI and isolates starting with V. Bootstrap values in percent.

Figure 2: Subtyping of wild boar sequences using a 300 nt fragment within ORF2 according to Lu et al. Samples presented in this report are circled. HEV_RKI: human isolate from Germany. Bootstrap values in percent.
Table 1: Location of the hunting regions where wild boar samples were collected with dates of sampling and coordinates.

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

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

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

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

<table>
<thead>
<tr>
<th>Location of sampling</th>
<th>Federal state</th>
<th>Date of sampling</th>
<th>Number of animals</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johanniskreuz</td>
<td>Rhineland Palatine (RP)</td>
<td>24 Oct 2007</td>
<td>13</td>
<td>49°16'54.77&quot;N 7°48'35.13&quot;E</td>
</tr>
<tr>
<td>Johanniskreuz</td>
<td>Rhineland Palatine (RP)</td>
<td>15 Nov 2007</td>
<td>12</td>
<td>49°16'54.77&quot;N 7°48'35.13&quot;E</td>
</tr>
<tr>
<td>Bebenhausen</td>
<td>Baden-Württemberg (BW)</td>
<td>20 Nov 2007</td>
<td>16</td>
<td>48°33'34.41&quot;N 9° 3'39.40&quot;E</td>
</tr>
<tr>
<td>Rottenburg</td>
<td>Baden-Württemberg (BW)</td>
<td>21 Nov 2007</td>
<td>11</td>
<td>48°28'35.90&quot;N 8°56'15.97&quot;E</td>
</tr>
<tr>
<td>Krausnick</td>
<td>Brandenburg (BB)</td>
<td>24 Nov 2007</td>
<td>19</td>
<td>52°1'20.68&quot;N 13°49'40.89&quot;E</td>
</tr>
<tr>
<td>Johanniskreuz</td>
<td>Rhineland Palatine (RP)</td>
<td>26 Nov 2007</td>
<td>23</td>
<td>49°16'54.77&quot;N 7°48'35.13&quot;E</td>
</tr>
<tr>
<td>Proschim-Welzow</td>
<td>Brandenburg/Saxony (SA)</td>
<td>01 Dec 2007</td>
<td>33</td>
<td>51°33'6.11&quot;N 14°11'32.74&quot;E</td>
</tr>
<tr>
<td>Hinterweidenthal</td>
<td>Rhineland Palatine (RP)</td>
<td>17 Dec 2007</td>
<td>5</td>
<td>49°11'46.33&quot;N 7°48'19.69&quot;E</td>
</tr>
</tbody>
</table>
Table 2: Primer and probe sequences with nucleotide position numbers in isolate Meng (AF082843) used for qPCR, generation of partial and full-length sequences of HEV.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Oligonucleotide sequence 5’-3’</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEV_F</td>
<td>CGACAGAATTGATTTTCGTCGGC</td>
<td>6321-6342</td>
</tr>
<tr>
<td>HEV_R</td>
<td>CCYTTRTCYTGCTGNGCRTTCTC</td>
<td>6441-6419</td>
</tr>
<tr>
<td>HEV_TM</td>
<td>6FAM-TYGGCTCGCCATTTGCGGAGAC--BHQ1</td>
<td>6396-6374</td>
</tr>
<tr>
<td>HEV 27F</td>
<td>CGAYGCCATGGAGGCC</td>
<td>3-19</td>
</tr>
<tr>
<td>HEV 837F 10C</td>
<td>CTGAGCCGTCACCAATGCC</td>
<td>821-839</td>
</tr>
<tr>
<td>HEV 1749F</td>
<td>GACCTTCGCACATCA ATAACTG</td>
<td>1719-1741</td>
</tr>
<tr>
<td>HEV 1054R</td>
<td>CGGAGATAGGTACATAAGCGC</td>
<td>1043-1024</td>
</tr>
<tr>
<td>HEV 1054F</td>
<td>CGGCTTATGACCTATCTCCG</td>
<td>1024-1043</td>
</tr>
<tr>
<td>HEV 3012F</td>
<td>TGTYCATTTACAGTTTACTGCCG</td>
<td>2955-2978</td>
</tr>
<tr>
<td>HEV 3127F 10C</td>
<td>TCGTGTTGTGATTGATGAGGCC</td>
<td>3126-3147</td>
</tr>
<tr>
<td>HEV 3132F SA21</td>
<td>GATGTGTTGTGATTGAGGCC</td>
<td>3128-3149</td>
</tr>
<tr>
<td>HEV 3254R</td>
<td>CGANGAGGCCCCGCTGCAT</td>
<td>3181-3198</td>
</tr>
<tr>
<td>HEV 4653F</td>
<td>GGAAGAARCAYTCNNGGYGAGC</td>
<td>4592-4612</td>
</tr>
<tr>
<td>HEV 4704F SA21</td>
<td>GACTTGAGAGTGCGGCAGGCTTC</td>
<td>4678-4698</td>
</tr>
<tr>
<td>HEV 7321R</td>
<td>TTTTTTCCAGGGAGCGCG</td>
<td>7271-7253*</td>
</tr>
</tbody>
</table>

F: forward orientation; R: reverse orientation; * according to isolate HE-JA04-1911 (AB248520)
Table 3: Detection of HEV RNA by qPCR in different samples from wild boars (A) with age distribution (B) and region of collection.

(A)  

<table>
<thead>
<tr>
<th>Region</th>
<th>Serum</th>
<th>Bile</th>
<th>Liver</th>
<th>Wild boar</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW</td>
<td>4/27 (14.8)</td>
<td>2/27 (7.4)</td>
<td>0/26 (0.0)</td>
<td>6/27 (22.2)</td>
</tr>
<tr>
<td>BB</td>
<td>1/17 (5.9)</td>
<td>13/14 (92.9)</td>
<td>19/19 (100)</td>
<td>19/19 (100.0)</td>
</tr>
<tr>
<td>SA</td>
<td>10/32 (31.3)</td>
<td>11/26 (42.3)</td>
<td>11/28 (39.3)</td>
<td>19/33 (57.6)</td>
</tr>
<tr>
<td>RP</td>
<td>3/39 (7.7)</td>
<td>41/52 (78.8)</td>
<td>18/53 (34.0)</td>
<td>46/53 (86.8)</td>
</tr>
<tr>
<td>Total</td>
<td>18/115 (15.7)</td>
<td>67/119 (56.3)</td>
<td>48/126 (38.1)</td>
<td>90/132 (68.2)</td>
</tr>
</tbody>
</table>

(B)  

<table>
<thead>
<tr>
<th>Age/Region</th>
<th>&lt;1 y</th>
<th>1-2 y</th>
<th>Sows</th>
<th>Male boars</th>
<th>Age unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW</td>
<td>1/10</td>
<td>2/7</td>
<td>1/3</td>
<td>0/1</td>
<td>2/6</td>
</tr>
<tr>
<td>BB</td>
<td>9/9</td>
<td>3/3</td>
<td>1/1</td>
<td>2/2</td>
<td>4/4</td>
</tr>
<tr>
<td>SA</td>
<td>6/8</td>
<td>5/14</td>
<td>6/7</td>
<td>2/4</td>
<td>0</td>
</tr>
<tr>
<td>RP</td>
<td>20/26</td>
<td>13/14</td>
<td>10/10</td>
<td>2/2</td>
<td>1/1</td>
</tr>
<tr>
<td>Total</td>
<td>36/53</td>
<td>23/38</td>
<td>18/21</td>
<td>6/9</td>
<td>7/11</td>
</tr>
</tbody>
</table>

| (%)        | (67.9) | (60.5) | (85.7) | (66.7) | (63.6) |

No. = number; BW: Baden-Württemberg; BB: Brandenburg; SA: Brandenburg/Saxony; RP: Rhineland Palatine
Table 4: Detection of anti-HEV IgG by ELISAs from Genelabs (GL) and Mikrogen (MG-E) and a Line immunoassay (Mikrogen, MG-L) in serum samples from wild boars collected in different regions of Germany.

<table>
<thead>
<tr>
<th>Region</th>
<th>GL</th>
<th>MG-E</th>
<th>GL &amp; MG-E</th>
<th>MG-L</th>
<th>MG-E &amp; MG-L</th>
<th>GL, MG-E &amp; MG-L</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW</td>
<td>7/27 (25.9)</td>
<td>0/27 (0.0)</td>
<td>0/27 (0.0)</td>
<td>0/27 (0.0)</td>
<td>0/27 (0.0)</td>
<td>0/27 (0.0)</td>
</tr>
<tr>
<td>BB</td>
<td>3/17 (17.6)</td>
<td>4/17 (23.5)</td>
<td>3/17 (17.6)</td>
<td>4/17 (23.5)</td>
<td>4/17 (23.5)</td>
<td>3/17 (17.6)</td>
</tr>
<tr>
<td>SA</td>
<td>10/23 (43.5)</td>
<td>21/23 (91.3)</td>
<td>10/23 (43.5)</td>
<td>19/23 (82.6)</td>
<td>19/23 (82.6)</td>
<td>9/23 (39.1)</td>
</tr>
<tr>
<td>RP</td>
<td>12/40 (30.0)</td>
<td>7/40 (17.5)</td>
<td>4/40 (10.0)</td>
<td>5/40 (12.5)</td>
<td>3/40 (7.5)</td>
<td>3/40 (7.5)</td>
</tr>
<tr>
<td>Total</td>
<td>32/107 (29.9)</td>
<td>32/107 (29.9)</td>
<td>17/107 (15.9)</td>
<td>28/107 (26.2)</td>
<td>26/107 (24.3)</td>
<td>15/107 (14.0)</td>
</tr>
</tbody>
</table>