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Short Communication**Detection of Hepatitis E Virus in Archived German Wild Boar Serum Samples**

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Abstract. Hepatitis E is a rare human disease in Central Europe commonly imported from endemic regions. For autochthonous infections a zoonotic transmission from pigs, deer and wild boar is assumed. Using three different RT-PCR protocols, hepatitis E virus (HEV) RNA was detected in 10 out of 189 (5.3 %) serum samples collected in 1995/1996 from wild boars in Germany. Sequence analysis indicates a close relationship with genotype 3 isolates of pigs and humans from the Netherlands and Japan. The results indicate that HEV is present in Germany since more than 10 years and that wild boar may function as a reservoir for HEV.

Keywords: hepatitis E virus; wild boar; zoonotic transmission; phylogenetic analysis; archived samples

Summary: 102 words
Text: 1832 words
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1. Introduction

Infection with hepatitis E virus (HEV) could lead to a severe human disease with acute hepatitis as the major clinical symptom. In overall, the case/fatality rates of HEV are low, however, for pregnant women rates up to 25% have been recorded due to fulminant hepatitis (Khuroo et al., 1981). Epidemics of hepatitis E have been recorded in Central and South East Asia, North and West Africa, and Mexico (Worm et al., 2002). Most of the hepatitis E cases in North America and Central Europe could be traced to imported infections from endemic regions. However, there is increasing evidence for several autochthonous HEV infections in these non-endemic regions (Dalton et al., 2007; Herremans et al., 2007; Preiss et al., 2006; Schlauder et al., 1998).

The detection of strains closely related to human HEV in pigs, deer and wild boar have led to the assumption that zoonotic transmission of HEV is possible (Michitaka et al., 2007; Takahashi et al., 2004, Tei et al., 2003; Worm et al., 2002). Reports on human hepatitis E cases after consumption of uncooked meat from deer and wild boar strengthened the hypothesis of zoonotic food-borne HEV infection of humans (Masuda et al., 2005; Tei et al., 2001). Recently, HEV strains isolated from pigs in the Netherlands have been shown to be very closely related to HEV strains from human cases of hepatitis E of the same region indicating that autochthonous HEV infections are acquired from pigs in Central Europe (Herremans et al., 2007). In Japan, wild boars have been suggested to serve as a reservoir for HEV infections as a broad variety of strains including those closely related with human HEV strains has been detected in this animal species (Michitaka et al., 2007).

HEV is a non-enveloped virus with a single-stranded RNA genome. It is the only member of the unassigned genus *Hepevirus*. Until now, four genotypes have been defined. Genotypes 1, 2 and 4 are found only in distinct geographical regions of the world whereas genotype 3 seems to have a worldwide distribution and is found in humans as well as in pigs (Lu et al., 2006). The avian HEV-like viruses are only distantly related to the mammalian HEV types and a grouping into a new genotype 5 has been suggested (Huang et al., 2004).

In order to determine whether animals from Germany could also be a reservoir for HEV, samples of wild boars were chosen for examination. Due to availability, archived serum samples originally collected in 1995/1996 for serological surveillance purposes in Northern Germany (Mecklenburg-Western Pomerania) have been used. The obtained results indicate that HEV is present in Germany since more than 10 years and that wild boar may function as a reservoir for HEV in Central Europe.

2. Materials and methods

2.1. Samples

A total of 189 sera of wild boar were collected in 1995/1996 for serological surveillance purposes in Northern Germany (Mecklenburg-Western Pomerania). For all of the samples, data on gender, geographic region and date of sampling were available (Tab. 1 shows the data for the positive tested samples). The sera had been stored at -20°C until use. An RNA extract of a HEV-positive pig liver sample from the Netherlands (kindly provided by W. van der Poel, Wageningen University, The Netherlands) served as positive control for RT-PCR analyses.

2.2. RNA extraction and (real-time) RT-PCR

RNA was isolated from the sera using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol, however, using the two-fold volume of serum and reagents before application to the column. The higher amount of starting material was because of the expected low virus concentration in the serum samples. The extracted RNA was tested in parallel by real-time RT-PCR according to Jothikumar et al. (2006), RT-PCR according to Schlauder et al. (1999) and modified by Herremans et al. (2007), and nested RT-PCR according to Huang et al. (2002) amplifying PCR products of 69, 197 and 348 base pairs, respectively. The real-time PCR was performed in an ABI PRISM 7700 cycler using the Quantitect Probe RT-PCR Kit (Qiagen, Germany). Conventional RT-PCR was performed using the One-Step RT-PCR Kit (Qiagen, Germany) and nested PCR using the TITANIUM Taq Polymerase (Clontech, France). PCR products obtained by conventional RT-PCR

were separated on ethidium bromide-stained 1.5% agarose gels and visualized by UV light.

2.3. Sequence analysis

RT-PCR products considered for sequence analysis were purified using the Qiaquick DNA purification kit (Qiagen, Hilden) and subsequently cloned into the vector pCR4-TOPO using the TOPO TA Cloning Kit for Sequencing (Invitrogen, The Netherlands). The inserts of the plasmids were sequenced using M13 Forward and M13 Reverse primers (Invitrogen, The Netherlands) in an ABI 377 DNA sequencer (Applied Biosystems, USA). Sequence similarity searches were performed using the BLAST 2.2.14 search facility (Altschul et al., 1997) and the GenBank database. A phylogenetic tree was constructed on the basis of the nucleotide sequences using the MegAlign module of the DNASTAR software package (LASERGENE, USA).

3. Results

3.1. Detection of HEV RNA in sera of wild boars from Germany

A total of 189 sera derived from wild boars originating from the North of Germany and sampled in 1995/1996 were analysed for the presence of HEV RNA. The detection of the HEV genome in sera of pigs using RT-PCR has been recently described (Halbur et al., 2002). Table 1 summarizes the results obtained with three different RT-PCR protocols. Using the real-time protocol according to Jothikumar et al. (2006), 10 out of the 189 sera (5.3 %) gave a positive result. Out of these, 6 samples were positive using the RT-PCR according to Schlauder et al. (1999) and one sample was positive using the nested RT-PCR according to Huang et al. (2002) resulting in detection rates of 3.2 % and 0.5 %, respectively. There was no indication with regard to sex ratio or a distinct geographic distribution of positives in comparison to negatives.

3.2. Sequence analysis of the HEV isolates

The six RT-PCR products and the one nested RT-PCR product were cloned and sequenced. The (nested) RT-PCR products of the used positive control (isolate

WvdP) were also cloned and sequenced. The sequence data have been deposited in the GenBank database (accession numbers EU035811 to EU035816). Regarding the four samples tested positive by real-time RT-PCR but negative by the other assays, a genotyping and confirmation of HEV infection was not possible since no PCR products of sufficient length were available for sequencing. Sequence analysis revealed that all of the sequences obtained from wild boars were clearly different from that of the positive control, thus excluding the possibility of a laboratory contamination with the positive control. A GenBank search using the BLASTN algorithm revealed a close relationship of the German wild boar sample sequences with HEV isolates of human and pig originating from Japan and South Africa as indicated in Table 1.

A phylogenetic tree was established on the basis of the amplified 148 bp region of the capsid protein-encoding region (excluding the primer sequences) using 25 HEV isolates from human, pig and wild boar of different regions of the world. As evident from Figure 1, all of the German wild boar samples clustered within one branch together with the genotype 3 isolate NLSW105, which was isolated in 1999 from pig in The Netherlands. Within this branch, two subgroups are evident with isolates 118E, 119E and NLSW105 in one subgroup and all of the other isolates in the other. As isolates 118E and 119E originated from the same geographical area, a local subgrouping may be postulated. It is evident from the tree that the only available human HEV sequence obtained from an autochthonous infection in Germany is more closely related to pig HEV strains from The Netherlands than to the German wild boar HEV sequences. From the phylogenetic tree, there is no evidence for a clustering of HEV genotype 3 isolates according to host species or time-point of sampling.

4. Discussion

The obtained data show that HEV is present in Germany since more than ten years. To our knowledge, this is the first demonstration of HEV infections in the animal population of Germany. Recently, HEV has been detected in wild boars in Italy (Martelli et al., 2007). Together with data obtained from pigs in the Netherlands (Herremans et al., 2007; van der Poel et al., 2003) and Spain (Seminati et al., 2007), our finding demonstrates the broad distribution of HEV in the animal population of

Central Europe. The close relationship of the obtained sequences with those of infected humans supports the hypothesis of a zoonotic transmission of this virus from animals to human and may be an explanation for autochthonous HEV infections in Central Europe. The first case of an autochthonous human HEV infection in Germany has been published in 2006 (Preiss et al., 2006), however, earlier autochthonous HEV infections may be remained undetected or unpublished. The different time-points of sampling and the different geographical origin of the German HEV isolates may explain the obvious sequence dissimilarities which rule out a direct connection between the wild boar isolates of 1995/1996 and the human isolate of 2005. Analysis of recent samples from pigs and wild boars derived from the same geographical region will help to identify the distinct source of infection for this human case of hepatitis E.

The used serum samples turned out to be appropriate for RT-PCR analysis even after storage for more than 10 years. Taking into account the relatively short viraemic period of 2 to 3 weeks as determined by experimental HEV infection of pigs (Halbur et al., 2001) and the long-term sample storage at -20°C , the obtained percentage of positives appears to be relatively high indicating a high incidence of HEV infections in wild boars in Germany. The detection rate of HEV-RNA in wild boars in Japan has been reported to be 2.3% (Nishizawa et al., 2005), which is slightly lower, but may be explained by the use of a different RT-PCR protocol amplifying a product of 458 bp (Mizuo et al., 2002). Different detection rates have also been observed in our study by the use of different RT-PCR protocols. The reasons for these differences are not known, however, it may be speculated that the RNA extracted from the serum samples was slightly degraded enabling a more efficient amplification of small PCR products.

The determined high percentage of infected wild boars suggests a role of this species as a reservoir for HEV even in Central Europe. The analysis of the HEV sequences did not reveal a distinct pattern of virus evolution with regard to the time of sampling. This may indicate that different HEV types co-circulate for a longer period of time and that a distinct evolution of predominant types did not occur within the last ten years. However, testing of a larger sample number obtained from different geographical regions as well as inclusion of older samples will be needed to assess the distinct

pattern of HEV distribution and evolution within Central Europe. Archived serum samples seem to be an appropriate substrate for analyses within this context.

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Figure Legends.

Table 1: Samples of wild boar from Germany tested positive for HEV by RT-PCR.

Figure 1: Phylogenetic relationship of the sequences amplified from wild boar sera of Germany with known HEV strains, based on a 148 base pair fragment of the capsid protein-encoding region (ORF-2). For each isolate the host species, geographical origin and the year of sampling is indicated in brackets. In the case of unknown sampling date, the year of sequence submission to GenBank is shown and indicated with “sub”. Genotype grouping is shown at the right (* grouping of the avian HEV into genotype 5 is provisional). The analysed samples and the used positive control are indicated by a bracket and an arrow, respectively. The human isolate obtained from an autochthonous infection in Germany is indicated with an open arrow.

Table 1. Samples of wild boar of Germany tested positive for HEV by RT-PCR.

Sample number	Gender	Geographic region (La: Latitude Lo: Longitude)	Date (month/year)	real-time RT-PCR (Jothikumar et al. 2006)	RT-PCR (Herremans et al., 2007) [max sequence identity by BLASTN]	nested RT-PCR (Huang et al., 2002) [max sequence identity by BLASTN]
64S	female	Zurow (La: 53 52' 00" Lo: 11 37' 00")	04/1995	+	-	-
65S	male	Neumühle (La: 53 42' 00" Lo: 11 07' 00")	04/1995	+	-	-
108S	male	Kaarz (La: 53 42' 00" Lo: 11 44' 00")	05/1995	+	+ [91% identity with JJT-Kan, human, Japan]	-
12044E	male	Gützkow (La: 53 57' 00" Lo: 13 25' 00")	12/1995	+	-	-
13464E	female	Tangrim (La: 54 02' 00" Lo: 12 43' 00")	12/1995	+	+ [90% identity with swRSA-1, swine, South Africa]	-

13474E	male	Millienhagen (La: 54 12' 00" Lo: 12 49' 00")	12/1995	+	-	-
118E	male	Wesselstorf (La: 53 58' 00" Lo: 12 29' 00")	01/1996	+	+	-
					[91% identity with JJT-Kan, human, Japan]	
119E	female	Wesselstorf (La: 53 58' 00" Lo: 12 29' 00")	01/1996	+	+	-
					[92% identity with JJT-Kan, human, Japan]	
149E	male	Stubbendorf (La: 54 05' 00" Lo: 12 31' 00")	01/1996	+	+	+
					[91% identity with JJT-Kan, human, Japan]	[90% identity with swJ16-1, swine, Japan]
922N	male	Ludorf (La: 53 23' 00" Lo: 12 40' 00")	01/1996	+	+	-
					[90% identity with JJT-Kan, human, Japan]	
WvdP	female	The Netherlands	2006	+	+	+
					[91% identity with SwMN06, swine, Mongolia]	[87% identity with JRA1, human, Japan]

