

The prevalence of hepatitis E virus in piglets on Czech pig production farms and phylogenetic analysis of recovered isolates

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ABSTRACT: The aim of our study was to determine the prevalence of hepatitis E virus (HEV) in domestic pigs and to investigate the genetic divergence of swine HEV in the Czech Republic. To this end, a one-step real time RT-PCR assay was introduced as a screening method while nested RT-PCR was used as an additional method to obtain specific sequences from the HEV genome and thus to perform sequence analysis. A total of 63 piglets originating from 14 farms were examined. Bile and intestinal contents were collected from each animal. At least one HEV RNA positive piglet was found in ten (71.4%) of the monitored farms. HEV RNA was most frequently detected in bile samples (34.9%) compared to intestinal content samples (22.2%). In nine piglets (14.3%), both biological samples were HEV RNA-positive. Based on these results sequence and phylogenetic analysis of one randomly selected HEV isolate originating from each HEV RNA-positive farm was performed. Analysis of 287 bp PCR products of the ORF1 gene showed that all the studied HEV isolates could be classified into genotype 3 and subgenotypes 3f and 3g. The failure to find any 100% homology between our isolates and HEV isolates deposited in the GenBank confirms the significant variability within the HEV genome.

Keywords: swine hepatitis E virus; real time RT-PCR; nested RT-PCR; genotype 3

Hepatitis E virus (HEV) is small (27–34 nm in diameter) non-enveloped RNA virus belonging to the genus *Hepevirus* of family *Hepeviridae* (Carstens 2010). HEV has a positive-sense, single-stranded RNA genome approximately 7.2 kb in length which is composed of a short 5' untranslated region followed by three partly overlapping open reading frames (ORFs; ORF1, ORF3 and ORF2) and a short 3' end terminated by a poly(A) tail (Aggarwal and Krawczynski 2000; Emerson and Purcell 2003; Lu et al. 2006). ORF1 encodes a non-structural polyprotein participating in virus replication and modifications of the structural protein; ORF2 encodes the only glycoprotein of the viral capsid, and ORF3 encodes a short phosphoprotein which takes part in virus replication and virion morphogenesis (Panda et al. 2007).

HEV is the major causative agent of hepatitis non-A non-B outbreaks in humans worldwide.

Transmission of HEV occurs primarily by the faecal-oral route via contaminated water. This mode of transmission is usually associated with large outbreaks and epidemics in developing countries. In both developed and developing countries, hepatitis E has also been detected sporadically. Due to the zoonotic potential of the virus, risk factors for these cases include consumption of uncooked or insufficiently heat treated meat or offal originating from domestic pigs, wild boar or deer, consumption of contaminated shellfish and direct contact with infected animals (Meng 2010). Direct transmission from human to human is rare (Aggarwal and Krawczynski 2000; Smith 2001).

Since the first characterisation of HEV in swine (Meng et al. 1997), HEV isolates have been described in different animal species worldwide and inter-species transmission has been demonstrated

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(Meng et al. 1998; Huang et al. 2002; Nishizawa et al. 2003; Takahashi et al. 2003; Lu et al. 2006). The genome organisation of both human and animal strains is identical. Geographically distinct HEV isolates from mammals show a high degree of sequence similarity and currently are segregated into four major genotypes and at least 24 subtypes. Each genotype and their subtypes predominate in specific geographical areas, but are not limited to them (Lu et al. 2006).

Most of the isolates from Asia and Africa cluster in genotype 1 (Emerson and Purcell 2003). Genotype 2, which predominantly circulates in the human population together with genotype 1, has been described in Mexico and Africa (Huang et al. 1992). In contrast, genotypes 3 and 4 were isolated from different animal species (e.g. pigs, rodents, deer); isolates of genotype 3 have been identified in Europe, the USA and other developed countries, and those of genotype 4 in sporadic cases in Asia (Schlauder and Mushahwar 2001; Lu et al. 2006; Okamoto 2007; Vasickova et al. 2007).

The objective of our study was to introduce a highly specific and sensitive real time RT-PCR (qRT-PCR) assay for the detection of HEV RNA, examine biological samples of domestic pigs (*Sus scrofa f. domestica*) originating from farms and perform sequence and phylogenetic analysis to identify epidemiological divergences of swine HEV in the Czech Republic.

MATERIAL AND METHODS

Sample collection

A total of 63 piglets (*Sus scrofa f. domestica*) between three and eight weeks of age originating from 14 Czech farms were examined. All tested pigs were suspected of postweaning multisystemic wasting syndrome (PMWS). The piglets were euthanized and examined by necropsy. Bile and intestinal content were collected from each of the piglets, immediately frozen at -80°C and stored until analysed.

Extraction of RNA

Total RNA from bile samples was extracted using a method based on silica particles described by Boom et al. (1990), while RNA from intestinal contents and the controls was isolated using Trizol[®] LS

Reagent (Invitrogen, USA) according to the manufacturer's instructions. Isolated RNA was eluted in 50 μl Nuclease-Free Water (Promega, USA) and stored at -80°C until further use. A positive control template was isolated from the bile of a HEV-infected pig (strain CZswHEV21, EU117413). To reduce the risk of cross-contamination, one water sample for every ten biological samples was used as a negative control of RNA isolation and qRT-PCR.

Real-time RT-PCR assay (qRT-PCR)

Detection of a highly conserved region of the HEV ORF3 was performed according to Jothikumar et al. (2006) with slight modifications. Briefly, one step qRT-PCR was carried out in a total volume of 20 μl and contained 2 \times QuantiTect Probe RT-PCR Master Mix, 0.2 μl QuantiTect RT Mix, 4 μl RNA, 6.0mM MgCl_2 , 250nM primers and 100nM probe modified by 5'-reporter dye (FAM) and 3'-quencher dye (BHQ1). The assay was performed using the LightCycler[®] 480 instrument (Roche Diagnostics, Germany) using 96-well PCR plates under the following conditions: reverse transcription at 50°C for 30 min, PCR initial activation step at 95°C for 15 min and 50 cycles of 94°C for 15 s, 55°C for 60 s and 76°C for 30 s. The subsequent analysis of results was carried out using the "Fit point analysis" option of the LightCycler 480 Software release 1.5.0 (version 1.5.0.39). All samples were analysed in triplicates.

Nested RT-PCR assay and sequence analysis

Based on the results of qRT-PCR one HEV isolate originating from each HEV RNA-positive farm was prepared for sequencing. For this purpose, nested RT-PCR specific for the 287 bp long conserved region of ORF1 was performed according to Vasickova et al. (2009). Obtained PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Germany) according to the manufacturer's instructions. Sequencing of both strands was carried out at the Eurofins MWG Operon (Manheim, Germany). Obtained HEV sequences were analysed using the Staden Package (<http://staden.sourceforge.net>) and subsequently were compared with sequences available in the GenBank database using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple

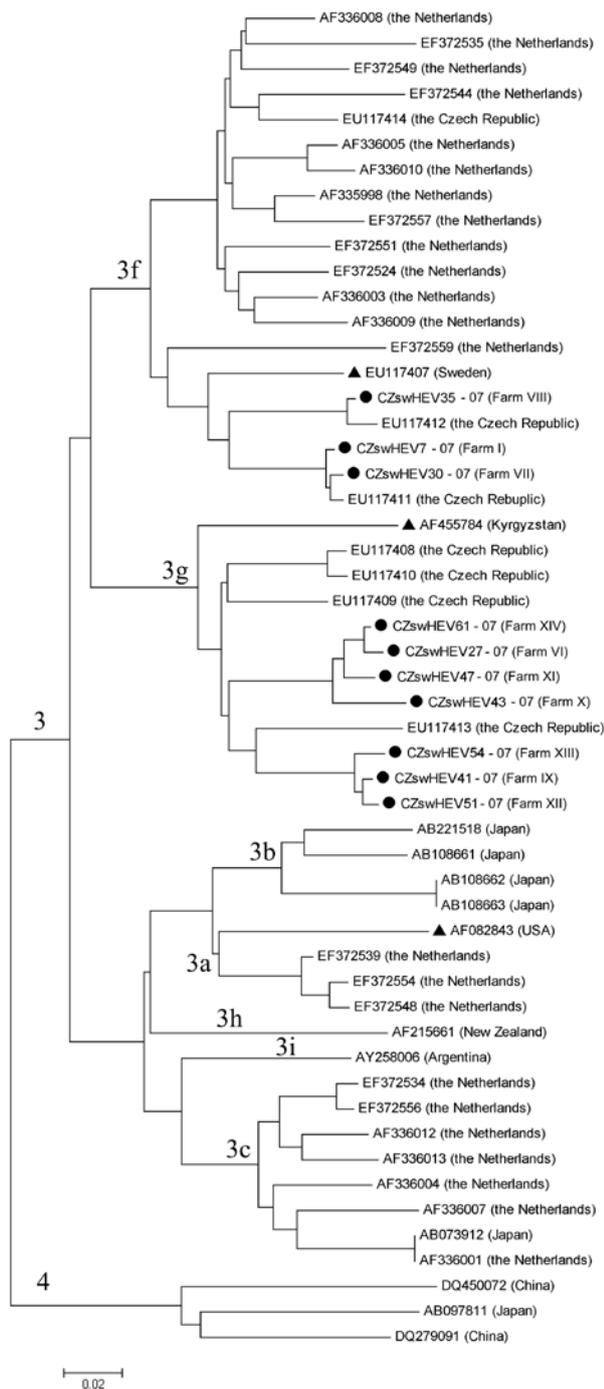


Figure 1. Phylogenetic tree constructed using the neighbour-joining method and mega 3.1 software based on the nucleotide sequences obtained from the orf1 region of ten CZswHEV isolates and selected representatives of swHEV genotypes 3 and 4. All identified sequences of swHEV isolates were in the submitted study marked by a black point and the number of their original farm. Representatives of subgenotypes 3a, 3f and 3g were marked by a black triangle. The designation of the identified CZswHEV isolates and the numbers of their farms correspond with the terms used in the text

sequential alignments and a non-rooted phylogram were created using MEGA 3.1 software (Kumar et al. 2004). The phylogenetic tree was constructed by the neighbour-joining method with nucleotide distance (p-distance) with 1000 replications in the bootstrap test and maximum likelihood. The reference HEV sequences were represented by members of swHEV genotype 3 as well as by representatives of swHEV genotype 4 (Figure 1).

RESULTS

At least one HEV RNA-positive piglet was found in ten (71.4%) of the monitored farms and HEV RNA was detected in at least one sample from 27 (42.8%) piglets. A positive fluorescence signal revealed a higher frequency of HEV RNA in bile samples (34.9%) compared to intestinal content samples (22.2%). In nine piglets (14.3%), HEV RNA was detected in both bile and intestinal contents (Table 1).

A total of ten samples determined HEV RNA-positive by qRT-PCR were subjected to conventional nested RT-PCR to carry out sequence analysis.

Table 1. Results of HEV RNA detection using the real time RT-PCR method in bile and intestinal content samples originating from 63 examined piglets (*Sus scrofa f. domestica*)

Farm	Number of tested animals	Number of HEV RNA positive animals	
		bile	intestinal contents
I	12	2	0
II	3	0	0
III	3	0	0
IV	3	0	0
V	3	0	0
VI	4	0	1
VII	5	3	3
VIII	5	3	5
IX	3	0	1
X	4	2	3
XI	4	4	1
XII	3	3	0
XIII	7	3	0
XIV	4	2	0
Total/%	63	22/34.9%	14/22.2%

The set predominantly included bile samples, with the exception of two intestinal content samples collected from farms VI and IX, from which no HEV RNA was found in the bile samples (Table 1).

Analyses of 242 bp sequences revealed that the recovered CZswHEV isolates possessed 81.8–99.2% nucleotide sequence homology with each other. Isolates CZswHEV7-07 and CZswHEV30-07 possessed the highest homology which was 99.2% (a difference of two nucleotides) with the previously described Czech isolate CZswHEV10 (EU117411), while isolate CZswHEV35-07 had 98.8% homology (a difference of three nucleotides) with the Czech isolate CZswHEV14 (EU117412). The CZswHEV41-07, CZswHEV51-07 and CZswHEV54-07 isolates showed 89.7–90.5% homology with the Czech isolate CZswHEV21 (EU117413).

Sequence comparison of the studied CZswHEV isolates with corresponding sequences of swHEV available in the GenBank did not reveal any swHEV isolate with a 100% homology. The highest homology (90.5%) was found for isolates CZswHEV7-07 and CZswHEV30-07 with the Swedish strain SWswHEV (EU117407). The ten sequences of the ten recovered CZswHEV isolates; CZswHEV27-07, CZswHEV30-07, CZswHEV35-07, CZswHEV41-07, CZswHEV43-07, CZswHEV47-07, CZswHEV51-07, CZswHEV54-07, CZswHEV61 and CZswHEV7-07 were deposited in GenBank under accession Nos. HM052801 to HM052810, respectively.

Phylogenetic analysis based on the neighbour-joining method clustered all Czech swHEV isolates into genotype 3. The isolates CZswHEV7-07, CZswHEV30-07, and CZswHEV35-07 were classified into the subtype 3f. These isolates were phylogenetically closely related to other Czech swHEV isolates (EU117411, EU117412) and to a representative Swedish strain SWswHEV (EU117407), but also to Dutch swHEV strains. The remaining seven isolates (CZswHEV27-07, CZswHEV41-07, CZswHEV43-07, CZswHEV47-07, CZswHEV51-07, CZswHEV54-07, and CZswHEV61-07) clustered into subgenotype 3g. A representative of subgenotype 3g, the Kyrgyzstani strain Osh 205 (AF455784), formed a closed independent branch in the phylogenetic tree together with these CZswHEV isolates (Figure 1).

DISCUSSION

Based on the results of the utilised qRT-PCR assay, at least one sample of bile or intestinal content

positive for HEV RNA was detected in 42.8% of examined piglets originating from ten farms. This prevalence of HEV RNA in piglets is comparable to a previous Czech study (Vasickova et al. 2009) as well as to reports from other European countries: 36.0% HEV prevalence in Hungary (Forgach et al. 2010), 37.7% and 42.3% HEV prevalence in Spain (de Deus et al. 2007, 2008) and 44.0% HEV prevalence in the United Kingdom (McCreary et al. 2008).

Most previous studies focused on the detection of HEV RNA in pigs were carried out on liver tissues, faeces and serum (Arankalle et al. 2002; Cooper et al. 2005). Choi and Chae (2003) detected HEV RNA in different tissues of naturally infected pigs using *in situ* hybridization; they found that liver tissue was most frequently positive for HEV RNA. Williams et al. (2001) obtained comparable results in experimentally infected pigs, and found that liver tissues were most frequently positive, followed by lymph nodes, faeces and sera. Halbur et al. (2001) found that HEV RNA is present in bile for a longer period compared to liver, faeces and serum, which was confirmed by subsequent studies (de Deus et al. 2007; Vasickova et al. 2009). This is in accordance with the results of our study, where 34.9% of bile samples were detected as HEV-positive compared to 22.2% of samples of intestinal content. Although the monitored piglets suffered from PMWS, the above studies in connection with health risk show that bile (if available) in combination with liver or faecal samples should be tested in HEV monitoring studies.

As minimal sequence variability (between 98.8 and 100%) of Czech swHEV within individual farms has so far been described (Vasickova et al. 2009), only one randomly selected CZswHEV isolate originating from each HEV RNA-positive farm was selected for sequence and phylogenetic analysis. Comparison of the obtained sequences revealed that these CZswHEV isolates possessed 81.8–99.2% nucleotide sequence homology with each other. This nearly 20% difference among the CZswHEV isolates can be ascribed to the great variability in the genomes of representatives of the genus *Hepevirus* and to the different origins of piglets. Based on phylogenetic analysis of a 242 bp long part of the ORF1 gene, all ten presented CZswHEV isolates (HM052801 to HM052810) were classified into subgenotypes 3g and 3f. A comparison of the obtained sequences with sequences of CZswHEV isolates previously described by Vasickova et al. (2009) together with their phylogenetic relation

reveal a circulation of subgenotypes 3f and 3g in the swine population of the Czech Republic.

According to Lu et al. (2006), subtypes 3c, 3e, 3f, 3h and 3i have been mainly identified in Europe and subtype 3g in Asia. Due to the subclinical form of HEV infection in pigs, different swHEV strains could be transmitted into the Czech pig population or *vice versa* and thus these strains could circulate not only within pig production farms. This possibility is supported by the phylogenetic relationship and higher homology among the recovered CZswHEV and HEV isolates originating from Sweden, the Netherlands and Kyrgyzstan (Figure 1).

In conclusion, we discovered a HEV prevalence in Czech piglets of 42.8% and the circulation of subgenotypes 3f and 3g in the swine population of the Czech Republic. These results confirm that using qRT-PCR and nested RT-PCR together is suitable for determining common diagnostic parameters and in epizootological studies of HEV.

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