Open Access

Berl Münch Tierärztl Wochenschr 125, 281–289 (2012)
DOI 10.2376/0005-9366-125-281

ISSN 0005-9366

Korrespondenzadresse:
doris.zwettler@ages.at

Eingegangen: 19.07.2011
Angenommen: 19.01.2012

Online first: 24.02.2012

http://vetline.de/zeitschriften/bmtw/open_access.htm

Austrian Agency for Health and Food Safety (AGES), Institute for Veterinary Disease Control, Moedling, Austria[1]
Institute for Veterinary Public Health, University of Veterinary Medicine Vienna, Vienna, Austria[2]

First detection of Hepatitis E virus in Austrian pigs by RT-qPCR

Erster Nachweis des Hepatitis E-Virus in österreichischen Schweinen mittels RT-qPCR

Doris Zwettler[1], Maria Fink[1], Sandra Revilla-Fernández[1], Adolf Steinrigl[1], Petra Winter[1], Josef Köfer[1, 2]

Summary

Long known to cause disease outbreaks in man in countries with poor sanitary conditions, an increasing number of autochthonous HEV genotype 3 infections have been reported in industrialised countries. Genotype 3 poses an important potential zoonotic threat, with infected pigs functioning as the main reservoir. This study reports the first detected emergence of HEV in Austrian pigs. Five Austrian strains were partially sequenced and phylogenetic analysis demonstrates that they cluster within genotype 3. In addition, a reverse transcription quantitative real-time PCR (RT-qPCR) method using a MGB-hydrolysis probe was developed offering the possibility to detect the HEV genotype 3 in faeces, liquid- and tissue-samples from domestic pigs. The method was adapted to the strains found in Austria. Sensitivity of the assay was tested with different pig organs (liver, mesenteric lymph nodes and kidney) as well as with serum, bile and faeces samples. Within the dynamic range of the assay, a quantitative determination of virus loads was performed. For specificity testing several common swine pathogens were used. Results demonstrated that the proposed method allows implementation of reliable high-throughput screening of Austrian swine samples in the future.

Keywords: swine, phylogenetic analysis, genotype 3

Zusammenfassung

In Ländern mit unzureichenden sanitären Bedingungen kann das Hepatitis E-Virus (HEV) Krankheitsausbrüche bei Menschen verursachen, aber auch in industrialisierten Ländern mehren sich die Meldungen über autochthone HEV Genotyp 3-Erkrankungen. Dieser Genotyp stellt eine potenzielle zoonotische Gefahr dar, wobei die Schweinepopulation als größtes Virus-Reservoir gilt. In dieser Studie wurde HEV erstmals in österreichischen Hausschweinen nachgewiesen. Fünf Stämme wurden teilweise sequenziert und durch phylogenetische Analyse dem Genotyp 3 zugeordnet. Zusätzlich wurde eine Real-Time RT-PCR (RT-qPCR) entwickelt, mit der, adaptiert an die gefundenen österreichischen Stämme, HEV Genotyp 3 sowohl in festem und flüssigem Probenmaterial von Schweinen als auch in Schweinekot detektiert werden kann. Die Sensitivität wurde mit verschiedenen Schweineorganen (Leber, mesenteriale Lymphknoten und Niere) und auch mit Serum, Gallenflüssigkeit und Kotproben getestet. Im Rahmen des dynamischen Bereiches erfolgte die quantitative Bestimmung der Virusmenge. Um die Spezifität der Methode zu bestimmen wurden verschiedene Erreger von Schweinekrankheiten getestet. Auf Grund der Ergebnisse bietet sich diese Methode für das Screening der österreichischen Schweinepopulation auf das Vorkommen des Hepatitis E-Virus an.

Schlüsselwörter: Hausschwein, phylogenetische Analyse, Genotyp 3

Introduction

While some genotypes of Hepatitis E virus (HEV) show regional preferences, i. e. genotype 1 is endemic in Asian and African countries, genotype 2 in Mexican and African countries and genotype 4 is found in Asian countries, genotype 3 is encountered worldwide (with the exception of the African continent so far). Whereas genotypes 1 and 2 are restricted to humans, genotype 3 and 4 also infect other mammalians and are therefore, by definition, zoonotic pathogens.

While originally found as a cause for disease outbreaks in tropical and subtropical regions, HEV is now increasingly detected in European countries also. Since its first confirmation as the principal agent behind the hepatitis outbreak in India 1955/56 (Balayan et al., 1983) and its first isolation in infected pigs (Meng et al., 1997) HEV and HEV antibodies have been increasingly detected in many European countries in human and swine. The first human case in Austria was detected in 1998 (Worm et al., 1998).

Originally thought to be introduced by infected travellers returning to Europe from abroad, a systematic literature review by Lewis et al. (2010) suggested that none-travel-associated human HEV infections in Europe could apparently be linked to zoonotic transmission, most probably originating from pigs, though multiple routes of transmission certainly exist. The establishment of a thorough and scientifically sound linkage between HEV carrying pigs and humans is mandatory for determining the emergent zoonotic threat to public health and to justify necessary disease control measures in Europe.

A fundamental prerequisite for establishing this link is the availability of a sensitive and labour and cost effective detection method that allows rapid analysis of large samples from swine matrices. Sensitive methods to detect HEV in swine faeces-, tissue- or fluid-samples are often based on classical reverse transcription PCR (RT-PCR) assays (Huang et al., 2002; Preiss et al., 2006; de Deus et al., 2007; Leblanc et al., 2007; Martelli et al., 2008, 2010; Vasickova et al., 2009; Kanai et al., 2010). These methods however are prone to contamination and they are costly in terms of time and work and therefore are not suitable for high-throughput screening of pig populations. Furthermore they do not allow quantification of the viral load. Although the number of RT-qPCR is rising, we were endeavoured to develop an in-house RT-qPCR assay especially optimized for Austrian HEV of genotype 3 enabling rapid screening of large numbers of swine samples. Five Austrian HEV strains of domestic pig origin could be partially sequenced within the ORF3/ORF2 genomic virus region and phylogenetical analysis demonstrated that the Austrian strains belong to genotype 3.

Material and Methods

Samples

The study comprised 138 samples (42 sera, 43 livers, 28 biles, 22 mesenteric lymph nodes, two faeces, one kidney) from 81 pigs (Tab. 1) which were originally submitted for other routine diagnostic examinations at the Institute for Veterinary Disease Control, Austrian Agency for Health and Food Safety (AGES). Organ-, bile- and faeces-samples were collected after pathological examinations for different pathogenic diseases and blood samples after different microbiological examinations. Health conditions of the animals were not considered, all samples were taken independently of pathological findings. Samples were collected between August 2007 and June 2009 and examined for HEV between April 2008 and October 2009. All samples were stored at –20°C. The animals originated from different Austrian provinces and were of both sexes, without age specification.

RNA extraction

Hepatitis E viral RNA from lymph nodes, livers and kidney was extracted with the RNeasy® Mini Kit (Qiagen, Germany) as described by the manufacturer including an additional mechanical disruption and homogenisation step of tissue samples at 20 Hz for 4 min using the Mixer Mill 301 (Retsch, Germany). Faeces samples were collected with swabs which were suspended in 500 µl 1xPBS (phosphate buffered saline) and centrifuged for 5.5 min at 8000 rpm before extraction. The supernatant was used as sample material. RNA extraction from liquid sample material like serum, bile (diluted 1:10 in 1xPBS) and faeces supernatant was performed with the QIAamp® Viral RNA Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer.

Reverse transcription (RT)

RT was performed with SuperScript™ II Reverse Transcriptase (Invitrogen, Austria) according to the pro-

TABLE 1: *Summary of investigated samples including total number of investigated animals, the different matrices investigated as well as the number of positive and negative samples tested for HEV by semi nested RT-PCR and RT-qPCR. The names of the resulting five Austrian strains are shown in the last column*

Group of investigated animal	Total no. of investigated animals	Matrix						Resulting strain
		liver	bile	lymph node	serum	faeces	kidney	
A	11	10/1						sw7aAT
B	9	7/2	7/2					sw1aAT, sw11_4bAT
C	3	3/0		3/0				
D	13	12/1	12/1	13/0				sw10_4AT
E	4	4/0	4/0	4/0	4/0			
F	1	0/1					0/1*	sw7_1AT
G	2	2/0	2/0	2/0		2/0		
H	38				36/2**			

First column indicates the group of animal which was examined for a certain set of matrices (A for liver; B for liver and bile; C for liver and lymph node; D for liver, bile and lymph node; E for liver, bile, lymph node and serum; F for liver and kidney; G for liver, bile, lymph node and faeces and H for serum); second column the total number of investigated animals of this group; column three to eight the number of negative animals/number of positive animals investigated for a certain matrix. The two positive liver and bile samples from group B represent two positive animals, the positive liver and bile sample from group D and the positive liver and kidney sample from group F always one positive animal, respectively.

* This positive kidney sample was only detected by RT-qPCR.

** Both positive serum samples were detected by RT-qPCR but only one was detected by semi-nested RT-PCR (method described by de Deus et al. (2007).

TABLE 2: *Primer pairs for conventional sequencing RT-PCR (partially overlapping) and primer/probe for RT-qPCR*

	Primer name	5′ – sequence – 3′	Amplicon size (bp)
RT-PCR primer	ORF3-5125-F1.1	GTCTTKTGCATYGCCCATG	809
	ORF2-5914R1	ACAGAGCGCCAGCCTTGATT	
	ORF2-5721F2	ACGATCCGTTAYCGCCCG	654
	ORF2-6361R2	CGGCTCGCCATTGGC	
RT-qPCR primer	HEV3_5261_MGBF1	CCGGCRGTGGTTTCTG	109
	HEV3_5353_MGBR1	CCAGCCCCGGRTTGTGA	
RT-qPCR probe	HEV3-MGB1	FAM-CTCCCCTATATTCATCC-MGB-NFQ	

Primer positions are indicated in the name and refer to strain E088STM04C. The letters "F" and "R" in the primer names indicate the direction of the primer. All forward primers are degenerated in one or two, the RT-qPCR reverse primer in one position, respectively (shown in bold letters). The expected overall length of the RT-PCR product is 1 250 bp. FAM = fluorescent dye, MGB = minor groove binding site, NFQ = non fluorescent quencher.

tocol described by the manufacturer using 2.25 µl of the extracted RNA and 25 µM Oligo dT primer.

Semi-nested RT-PCR

To detect HEV RNA in tissues, bile, serum and faeces from Austrian pigs, all samples were screened by semi-nested RT-PCR as described by de Deus et al. (2007). Positive control samples from bile and faeces were obtained from the Centre de Recerca en Sanitat Animal, Barcelona, Spain.

Nucleotide sequencing

PCR amplicons were excised from the agarose gel and purified with the QIAquick® Gel Extraction Kit (Qiagen, Germany) following the manufacturer's instructions. Sequencing was performed with the BigDye® Terminator v1.1 Cycle Sequencing Kit or the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, GB) according to amplicon length. 5 µl of purified DNA were added to 5 µl of sequencing reaction mix. Standard sequencing was carried out using the primer pair U74F and L266RS in case of the semi-nested RT-PCR method described by de Deus et al. (2007) and both newly designed primer pairs for conventional sequencing RT-PCR (Tab. 2). PCR-products were directly used for sequencing without subcloning. Sequencing reactions were purified using the DyeEx™ 2.0 Spin Kit (Qiagen, Germany) following the protocol of the manufacturer, and resolved by capillary electrophoresis on the 3130*xl* Genetic Analyzer (Applied Biosystems, GB). Sequence analysis was performed with the use of Sequencing Analysis v5.2 software (Applied Biosystems, GB).

Primer/probe design and sequence alignment

Primer and primer/probe design was performed with the Primer Express® v2.0 software (Applied Biosystems, GB). Primers were ordered from Invitrogen, the probe from Applied Biosystems. Because of the high sequence diversity within the HEV genomic RNA, a sequence alignment was made to detect conserved sequences within the ORF3/ORF2 coding region suitable for primer binding sites. In order to design two primer pairs resulting in two overlapping PCR fragments, sequence alignment was performed with genotype 3 strains which were used for constructing the phylogenetic tree together with the eight positive PCR products resulting from the semi-nested RT-PCR method as well as the following European human, swine and wild boar isolates which are available in the National Center for Biotechnology Information (NCBI) GenBank were adducted: DQ315770, DQ315745, EU035816, AF336297, AF336294, AF336292, AF336290, EU526642, EU035813, EU035812, EU035811, AF279123, AF110390, DQ200293, DQ200284, EF530669, EF530662, AY626042, AY626041, AF110391 and AF110392. These European isolates aligned between position 5980 and 6310; the eight semi-nested RT-PCR products between position 6020 and 6234 and the other genotype 3 strains between position 1 and 7215 (all nucleotide positions mentioned in this work refer to the numbering of reference strain E088-STM04C (NCBI GenBank acc. no. AB369689) isolated 2004 in Japan from a human serum sample). In order to design primers and probe for the RT-qPCR resulting in a rather small PCR fragment within the ORF 2 coding region, the performed alignment included the five Austrian strains from this study, as well as published HEV genotype 3 partial or complete genome sequences available from GenBank NCBI. To detect possible secondary structures within the primer or probe region, primer and probe sequences were controlled with the DNA and RNA folding server (http://mfold.bioinfo.rpi.edu/).

Conventional RT-PCR for sequencing

Reverse transcription (RT) was performed as mentioned above, PCR amplification with the kit Phusion® Hot

TABLE 3: *Mismatches within the primer/probe binding region on the basis of the five Austrian strains and additional strains available from the NCBI GenBank*

Nucleotide position NCBI GenBank accession no.	Strain	5261 5276 HEV3_5261_MGBF1	5308 5324 HEV3-MGB1	5353 5369 HEV3_5353_MGBR1
HM623774	sw7_1AT	CCAGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCACAACCGGGGCTGG
HM623775	sw7aAT	CCGGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCACAACCGGGGCTGG
HM623773	sw1aAT	CCGGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCACAATCGGGGCTGG
HM623776	sw10_04AT	CCGGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCACAATCGGGGCTGG
HM623777	sw11_4bAT	CCGGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCGCAACCGGGGCTGG
AB369689	E088STM04C	CCGGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCACAATCGGGGCTGG
EU360977	swX07E1	CCGGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCACAATCGGGGCTGG
AB189070	JBOAR1Hyo04	CCAGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCACAATCGGGGCTGG
AF082843	swUS1	CCGGCGGTGGTTTCTG	CTCCCCTATATTCATCC	TCACAACCGGGGCTGG

The forward primer HEV3_5261_MGBF1 has one mismatch located proximal to the 5´end on the binding region; reverse primer HEV3_5353_MGBR1 two different mismatches, one of these were located near the 3´end of the reverse primer. We compared the five Austrian strains together with sequences available from the NCBI GenBank for this specific genomic region and selected those representing all possible combination of mismatches. As a consequence of these mismatches, degenerated primers were designed. Nucleotide positions refer to strain E088STM04C, GenBank accession no. AB369689.

TABLE 4: *NCBI GenBank accession numbers of Austrian HEV-isolates, host species and host matrix and date of sampling*

NCBI GenBank acc. no.	Strain	Host	Matrix	Sample Date
HM623773	sw1aAT	swine	liver	03.2008
HM623774	sw7_1AT	swine	liver	12.2007
HM623775	sw7aAT	swine	liver	04.2008
HM623776	sw10_04AT	swine	bile	12.2007
HM623777	sw11_4bAT	swine	bile	04.2008

Start High-Fidelity DNA Polymerase (Finnzymes, Finland), using 2.5 µl of cDNA and 0.5 mM of each RT-PCR primer (Tab. 2). MgCl2 concentration was adjusted to 2 mM. The PCR profile for both reactions was as follows: initial denaturation at 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 10 sec, primer annealing at 59°C for 30 sec and elongation at 72°C for 50 sec. The final elongation was performed for 10 min at 72°C.

Phylogenetic analysis

Partial HEV ORF2 sequences from five Austrian strains were aligned with a selection of Asian, American, African and European HEV sequences downloaded from NCBI GenBank. All four HEV genotypes were represented in this reference sequence dataset. Nucleotide sequences were aligned using the ClustalX2 (www.clustal.org) program and edited with BioEdit Sequence Alignment Editor v7.0.9 (Hall, 1999). All gaps were removed, resulting in an alignment of 969 nt length. Phylogenetic analysis was performed using Mega 4.0 (Tamura et al., 2007). A Neighbour-Joining tree was constructed based on the TN93 substitution model (Tamura and Nei, 1993). Statistical significance was assessed by performing 1000 bootstrap replicates. Subtyping followed the scheme proposed by Lu et al. (2006).

Gel electrophoresis

PCR amplicons were visualised on a 1.5% or 2% (v/w) 1 xTBE agarose gel depending on the size of the amplicon. Agarose gels were directly stained with 0.1 ng/µl ethidium bromide.

RT-qPCR

RT-qPCR was performed with the QuantiTect® Virus + ROX Vial Kit (Qiagen, Germany). Primers and MGB-hydrolysis probe (Tab. 2) for HEV genotype 3 were designed as described, under consideration of occurring mismatches within the calculated primer binding site (Tab. 3). The final reaction volume of 25 µl contained 1 µl of each primer (10 pmole/µl), 0.5 µl probe (10 pmole/µl) and 5 µl RNA extract. After RT at 50°C for 20 min, PCR amplification parameters included an initial denaturation step at 95°C for 5 min and 40 annealing-cycles at 95°C for 15 sec, followed by 58°C for 45 sec. Amplifications were performed in the Mx 3005P™ Real-Time PCR System (Stratagene, USA) together with the corresponding QPCR software.

Plasmid cloning and preparation of a HEV RNA standard

A plasmid was constructed by cloning the 809 bp RT-PCR product resulting from the conventional sequencing RT-PCR into the cloning vector pPCR-Script™ Amp SK (+) (Stratagene) provided in the kit PCR-Script™ Amp Cloning Kit (Stratagene). For PCR amplification we used the cDNA from strain sw7_1AT (Tab. 4) as template and plasmid amplification was performed in competent Top10F' cells (Invitrogen) according to instructions of kit PCR-Script™ Amp Cloning Kit (Stratagene). Plasmid DNA from ampicillin-resistant clones was purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany) as described by the manufacturers. The presence of the HEV insert was screened by restriction enzyme analysis followed by gel electrophoresis (not shown). Midiprep of one positive clone containing the appropriate size of the HEV insert was performed using the Jetstar 2.0 Plasmid Kit (Genomed, Germany) according to the manufacturer's instructions. A HEV RNA standard was generated by in vitro transcription of the linearised plasmid (Not I, New England Biolabs, Germany) using the T3 Transcription Kit (MBI Fermentas, Germany), according to the manufacturer's instructions. The DNA template (linearised plasmid) was digested for 15 min at 37°C using 5 units DNAse I from MBI Fermentas. The reaction was stopped with 0.5 M EDTA pH 8 according

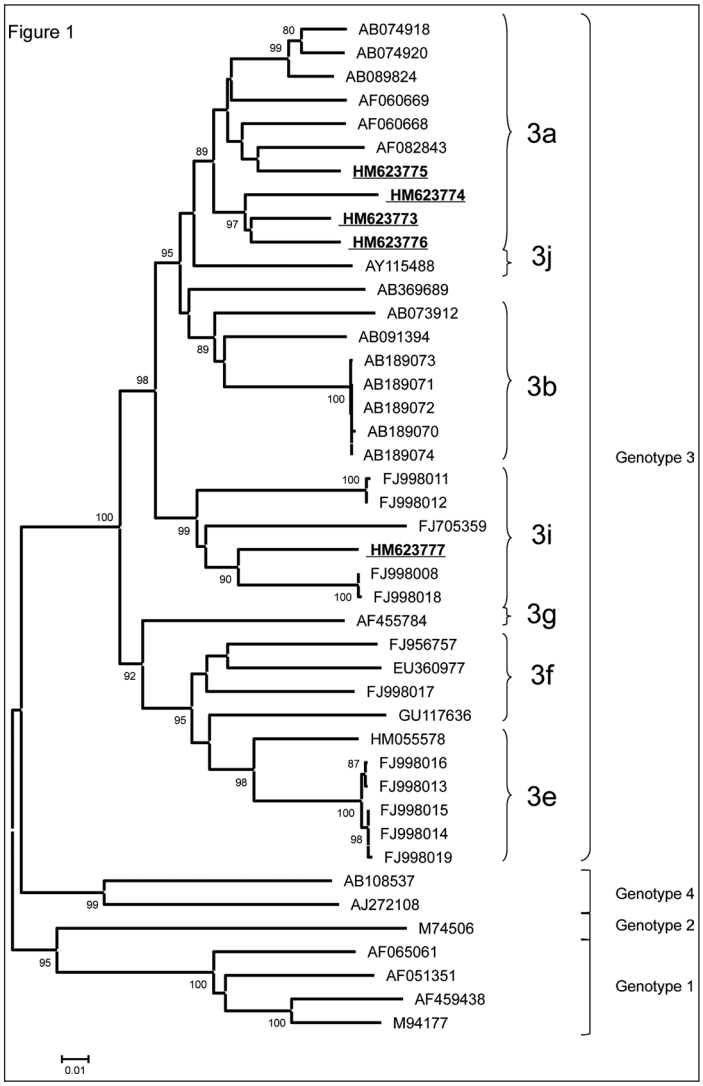

FIGURE 1: *Neighbour-Joining tree based on the 969-nucleotide alignment within the HEV ORF 2 region resulting from the conventional sequencing PCR. Numbers along the branches indicate the percentage of 1000 bootstrap replicates supporting this branch. Only bootstrap values of at least 80% are shown. The scale bar represents an evolutionary distance of 0.01 nucleotides per sites. Austrian isolates are in bold and underlined.*

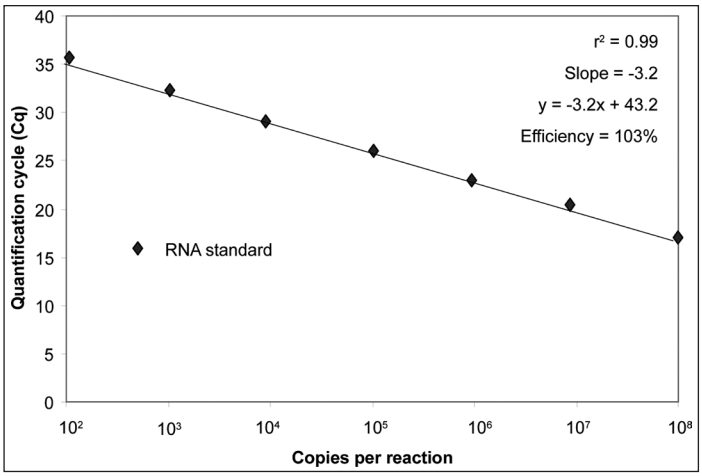

FIGURE 2A: *Dynamic range of the RT-qPCR assay with HEV RNA standard. A 10-fold dilution series of this HEV RNA standard was tested at least in triplicates. The copy numbers of the target sequences are indicated with the corresponding Cq-values ranging from 17 to 36. The regression coefficient (r2) was 0.99. Slope was –3.2.*

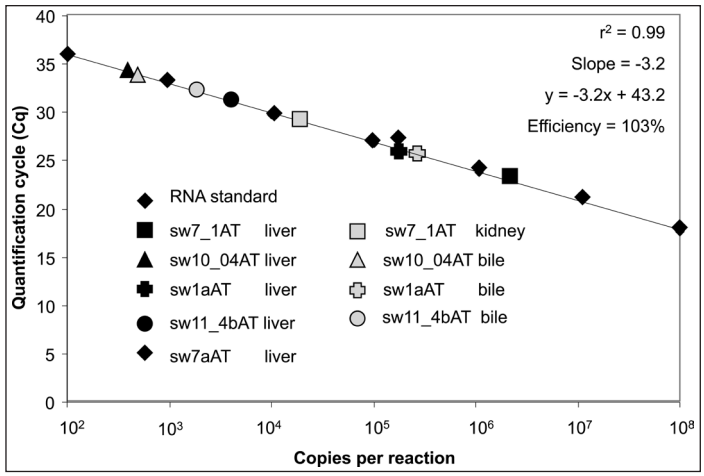

FIGURE 2B: *Dynamic range of the RT-qPCR assay with HEV RNA standard in addition to the cq-values of all field isolates within the detected dynamic range. Field samples correspond to liver, bile and kidney samples from the five sequenced Austrian strains sw7aAT, sw1aAT, sw11_4bAT, sw10_04AT and sw7_1AT.*

to the manufacturer's recommendations. The resulting in vitro transcribed RNA was precipitated by the addition of 0.3 M ammonium acetate and 2.5 volumes of ice cold ethanol followed by an over night incubation at –70°C. The pellet was centrifuged at 11 000 x g for 30 min, washed with ice cold ethanol (70%) and resuspended in 20 µl RNase free water. The concentration of this RNA standard was determined using program ImageJ (http://rsbweb.nih.gov/ij/). The calculated molarity of this RNA standard was 12 mM or 24 x 10^9 copy numbers per µl.

Standard curve

In order to determine the dynamic range of the developed RT-qPCR, a standard curve was generated. Therefore, a 10-fold serial dilution of the in vitro transcribed RNA was performed. The reproducibility of this dilution was tested by running each dilution in multiple independent reactions. The calculated regression curve allowed the determination of the virus loads, the reaction efficiency as well as the regression coefficient (Fig. 2A, B)

Results

Samples

All 138 samples resulting from 81 pigs including different sample materials or sample matrices were initially screened for detection of HEV RNA by semi-nested RT-PCR (de Deus et al., 2007). Amplicons from positive samples were expected to be 212 bp long. By this method HEV could be detected in five liver samples, two bile samples and one serum sample (5.8% of the investigated samples) originating from six different animals (7.4% of the investigated pigs) (Tab. 1).

Conventional RT-PCR and sequencing

To be able to perform genomic sequence comparison with published HEV sequences and subsequently to search for conserved regions within the genomic RNA as well as to perform classification of the HEV viruses by phylogenetic analysis, sequence information of a longer stretch of genomic RNA was necessary. We therefore generated a conventional sequencing RT-PCR with two newly designed, partially overlapping primer pairs amplifying within the most conserved ORF3/ORF2 coding regions. The combined length of both RT-PCR products was 1250 bp (Tab. 2). Because of the high sequence diversity of the virus and the lack of conserved regions, a partial ORF3 and ORF2 sequence alignment was performed. To adjust for observed differences in the primer binding site, both forward primers had to be degenerated (Tab. 2). The first forward primer binds at 5´ position at nucleotide 5125 within the ORF3 and the second reverse primer at 5´ position at nucleotide 6375 within ORF2. For the following conventional sequencing RT-PCR, we selected the five positive pigs, which showed one or two positive results for the matrix liver or bile and liver in the semi-nested RT-PCR (Tab. 1). In cases where both matrix samples, liver and bile, showed a positive signal, we performed conventional sequencing PCR whether with the cDNA resulting from liver or from bile (Tab. 4). The obtained ORF3/ORF2 nucleotide sequences informations generate strain sw1aAT, strain sw7_1AT, strain sw7aAT, strain sw10_04AT and strain sw11_4bAT. All five Austrian HEV strains were registered as"partially sequenced HEV genomic RNA" in the NCBI GenBank (Tab. 1, 4).

Phylogenetic analysis

Partial ORF2 sequences from five Austrian strains were compared by phylogenetic analysis with 38 HEV reference sequences, representing genotypes 1, 2, 3 and 4. Reference sequences were from different host species, and originated from different Asian, African, American and European countries. All five Austrian strains grouped within genotype 3, which had 100% bootstrap support (Fig. 1). Within this genotype, four Austrian strains clustered with subtype 3a: three of those, all originating from different political districts, formed a highly supported group (97% bootstrap support). The fourth Austrian subtype 3a strain was distinct from this cluster. Instead, it was more closely related to the prototype swine HEV

genotype 3 strain "Meng" (AF082843; Meng et al., 1997). A single Austrian strain clearly grouped within subtype 3i. All other subtype 3i sequences included in the phylogenetic tree shown in Figure 1 were isolated from wild boars from the German districts Brandenburg and Rhineland-Palatinate (Adlhoch et al., 2009; Schielke et al., 2009).

RT-qPCR

RT-qPCR is an improved PCR system with numerous advantages. Main improvements are the higher specificity, the quantitative determination of pathogen loads, the shorter running time and the significantly reduced risk of contamination allowing implementation of high-throughput screening-methods (Gibson et al. 1996; Heid et al., 1996). Because of the high sequence diversity of the virus, primers and probe were designed under consideration of possible mismatches within the calculated binding sites (between position 5261 and 5369 of ORF2), the sequences from our five Austrian strains obtained by conventional sequencing RT-PCR were aligned against each other and together with additional sequences obtained from the NCBI GenBank (Tab. 3). The sequences from the NCBI GenBank were selected according to their occurrence of mutations within the calculated primer and probe binding sites. Because of mismatches within both primer binding sites, each primer contains one degenerated nucleotide; the MGB-probe does not contain any degenerated nucleotide (Tab. 2, 3).

Sensitivity

The sensitivity of the assay was tested by investigating all 138 samples. By comparing the results with those obtained by semi-nested RT-PCR (de Deus et al., 2007), it could be demonstrated, that the newly developed RT-qPCR is more sensitive, because in addition to those matrices already tested positive for the semi-nested RT-PCR, one kidney sample and one more serum sample showed a HEV specific fluorescence signal (Tab. 1).

Quantification of virus load

To allow quantification, the dynamic range of the assay was determined by a standard curve. Therefore a linear regression curve was generated demonstrating the correlation between the serial dilution of the in vitro transcribed RNA ranging from 10^1 to 10^8 and the measured Cq-values. Due to a lower reproducibility of dilution 10^1 if compared to the other dilutions, dilution 10^1 was excluded from the standard curve. The optimal reaction efficiency within this range which corresponds to 10^2 to 10^8 copies per reaction was calculated with 103% (ranging from Cq-value 17 to 36). In order to perform quantification by determining the virus loads of the positive matrices, the received Cq-values were extrapolated against the regression curve resulting from the standard curve. The virus loads of strain sw10_4AT were $10^{2.7}$ and $10^{2.5}$ copy numbers for bile and liver, $10^{5.4}$ and $10^{5.3}$ copy numbers for bile and liver of strain sw1aAT and $10^{3.6}$ and $10^{3.2}$ copy numbers for liver and bile of strain sw11_4bAT, respectively. In contrast, virus loads of strain sw7_1AT detach from each other and were $10^{6.2}$ copy numbers for liver and $10^{4.1}$ copy numbers for kidney. The virus load for the liver of strain sw7aAT was $10^{5.3}$ copy numbers and could not be compared with any other matrices because it belongs to investigation group A (Tab. 1, Fig. 2A, B). The virus loads of the positive reference samples, a bile and a faeces sample, could be determined with $10^{5.7}$ and 10^5 copy numbers, respectively. Both positive serum samples showed viral loads close to the detection limit (copy numbers of $10^{0.7}$) outlying the determined dynamic range.

As the assay should also be suitable for high-throughput screening of pig herds to possibly allow prevalence studies, it should be able to detect the presence of the HEV RNA in matrices that could be collected intra vitam from living animals. Such an intra vitam sample would be faeces as the end product from the digestive tract. Our sample collection contained two faeces samples from two animals which were tested together with bile, liver and lymph node samples from the same two animals. As all matrices from both animals were tested negative in the RT-qPCR as well as in the semi-nested RT-PCR, we hypothesized that both animals were negative with respect to HEV. Regardless to our results, we were able to investigate the presence of HEV in a positive faeces sample obtained from Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Spain. A dilution series (2^5, 2^7, 2^9 and 2^{11}) of the virus RNA extracted from this faeces sample was investigated and the calculated virus loads and observed Cq-values were within the dynamic range of the assay and within the calculated exponential distances to each other (data not shown). For further analyses and conclusions regarding the ability of the assay to detect HEV in faeces, more than one HEV-positive faeces sample would be necessary.

Specificity

Analytical specificity of the newly developed RT-qPCR was shown by the absence of cross-amplification when testing positive samples containing the nucleic acid of the following swine specific pathogens originating from field samples or cell isolates: Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Porcine Circovirus 2 (PCV2), Porcine Parvovirus, Swine Influenza Virus A, Pestivirus (CSFV), *Lawsonia intracellularis*, *Erysipelothrix rhusiopathiae*, Suid Herpesvirus 1 (Pseudorabies Virus), *Leptospira interrogans*, Porcine Enterovirus, Porcine Rotavirus A and Porcine Teschovirus (data not shown). All these pathogens were tested and cross-reactions were not observed, neither for viral nor for bacterial isolates.

Discussion

As virus caused hepatitis is notifiable in Austria the Austrian Federal Ministry of Health reported 107 human hepatitis E cases from 1995 to the end of 2010. The first scientifically discussed Austrian HEV-case occurred in 1998 in the province of Styria, where a 65-year-old man without travel history and without contact with travellers or food from abroad was affected (Worm et al., 1998). In January 2011, the Department of Virology from the Medical University of Vienna reported the first case of death related to an autochthonous HEV genotype 3 infection in Austria (http://www.virologie.meduniwien.ac.at/home/upload/vei/2010/2310.pdf). As zoonotic transmission from pigs seems likely for reported non-travel-associated HEV infections (Lewis et al., 2010), the aim of this study therefore was to detect, partially sequence and classify HEV in Austrian pigs.

Different swine samples from 81 animals from all Austrian provinces (with the exception of Vorarlberg and Vienna, which have together with the province of Salzburg the well-defined lowest pig density throughout Austria) were investigated. As the present work is meant as a pilot study for the occurrence of HEV in Austrian pig herds, the samples have not been selected on the bases of any predetermined criteria.

After having been known as a human pathogen for more than one decade in Austria, we were able to demonstrate for the first time the presence of HEV in Austrian domestic pigs.

Phylogenetic analysis based on a 969 nt alignment within the ORF2 region allowed assignment of all five Austrian HEV strains to genotype 3. This is in line with work by others, who showed that the multitude of European HEV sequences belong to this genotype (Lewis at a., 2010; Pavio et al., 2010). More specifically, the Austrian strains belonged to either subtype 3a or 3i (Lu et al., 2006). To our knowledge, there was neither geographical nor epidemiological linkage between the pigs that hosted these strains. Nevertheless, three sequences formed a highly supported cluster within subtype 3a, indicating common ancestry of these strains. Subtype 3a strains were recently detected in wild boar from Potsdam, Germany (Schielke et al., 2009) and in domestic swine, wild boar and roe deer from Hungary (Forgach et al., 2010) (data not shown). Subtype 3i appears to be present in wild boar from both western and eastern parts of Germany (Adlhoch et al., 2009; Schielke et al., 2009) (Fig. 1). Although there are many published European HEV sequences – even clustering to other subtypes like 3a, 3b or 3d – only sequences which were of comparable length to the newly described sequences from Austria were included in the phylogenetic tree (Fig. 1).

Screening of whole pig populations also in view to possible prevalence studies implies a method suitable for high-throughput screening. We therefore developed a new RT-qPCR optimized for the detection of HEV genotype 3 in Austrian swine. The assay allows in addition a screening of faeces samples with the advantage that this matrix can be collected intra vitam from living pigs.

The majority of published RT-qPCR methods for detecting HEV work within the ORF2 and the overlapping ORF3/ORF2 region of the HEV genome, whereas the 5′ end of the ORF2 seems more preserved than the 3′ end. Though there are some RT-qPCRs working in the very same region at the 5′ end of the ORF2 as our newly developed method, we found our primers and probe better matching for the HEV strains detected in Austrian pigs. RT-qPCR assays either use SYBR Green dye or TaqMan® probes. SYBR Green dye binds to double-stranded nucleic acids generated during the amplification, allowing sensitive detection of products in real time. However, unlike PCR assays using internal fluorescent probes, this approach does not avoid the quantitation of non-specific amplification products (Ririe et al., 1997). The probe-based TaqMan® assay versus the non-probe approaches like SYBR Green allows a higher annealing temperature and therefore a higher specificity and avoids non-specific amplification products. In addition, the usage of a TaqMan® MGB-hydrolysis probe further raises the annealing temperature and therefore the stringency of the reaction, making the reaction even more specific. A higher specificity reduces the amount of non-specific amplification products and enables quantitation of the viral load. Orrù et al. (2004) reported a SYBR Green RT-qPCR assay for detection of HEV which raises the potential for non-specific amplification of non-target nucleic acid due to the low annealing temperature of the primers. Many TaqMan® assays have been reported in the last years for detecting HEV genotype 3 (Mansuy et al., 2004; Enouf et al., 2006; Gyarmati et al., 2007). Due to a reasonable number of mutations within the primer-probe binding sites found in our Austrian sequenced strains, these assays seem not to be suitable for screening Austrian pig samples. Furthermore, two of these assays use the Light Cycler TaqMan® probe technology (Mansuy et al., 2004; Enouf et al., 2006). Another TaqMan® assay which shows only a single mutation, is validated for environment samples and does not allow a quantitation of the viral load from animal clinical samples (Jothikumar et al., 2006).

Our newly developed assay uses a TaqMan® MGB-hydrolysis probe with an annealing temperature close to 60°C (58°C) which diminishes a non-target specific amplification and therefore makes this assay considered suitable for quantitation of the viral load from faeces and other highly contaminated animal samples.

Seven of 81 pigs were tested positive in one or more matrices (liver, bile, kidney and serum) by our newly developed RT-qPCR. This test enables a screening and as a consequence not only the determination of HEV-prevalence in the Austrian domestic pig population but also a comparison with other European countries. Hungary and Spain report a prevalence of 23% and Italy a prevalence of 30% in their domestic pig-populations, the prevalence in the wild boar population in Germany is reported with 15% (Fernández-Barredo et al., 2007; Schielke et al., 2009; Forgach et al., 2010; Martelli et al., 2010).

HEV primarily replicates in liver- and gall bladder cells (Tam et al., 1996, 1997; Kawai et al., 1999; Vasickova et al., 2007; Meng, 2010) but also in extrahepatic regions of the digestive tract such as small intestine, lymph nodes, colon or salivary glands; in contrast the virus was not found to replicate in spleen, tonsils and kidney (Williams et al, 2001; de Deus et al., 2007; Billam et al., 2008). Tissue and fluid such as bile from the lower digestive tract bear the highest viral load (Leblanc et al., 2010). Thus, faeces as the "end product" from the digestive tract might be used as a suitable matrix to test individual animals for the presence of HEV (Fernández-Barredo et al., 2006, 2007; Lee et al., 2009; Kanai et al., 2010). Additionally this matrix can be collected intra vitam in a non-harmful way for the living animal. Our newly developed RT-qPCR proofed to be suitable for the quantitative determination of pathogen loads and the obtained results are consistent with the hypothesis that tissues which support active viral replication show a higher virus load than non-replicating host cells. We were able to compare the virus loads of replicating host tissues (bile and liver) with non-replicating host tissue (kidney). Host cells, where the virus primarily replicates, showed nearly the same virus loads for different tissues from one and the same animal, while the virus load in kidney was distinctly lower (difference 10^2) than for liver from the same animal.

Conclusion

This study shows for the first time the occurrence of HEV genotype 3 in Austrian pigs. As HEV is postulated a zoonosis which can be transmitted by pigs, this method now provides the methodology for a thorough determination of the prevalence of HEV and furthermore will allow a risk assessment within the Austrian pig population.

Acknowledgement

The authors wish to thank Joaquim Segales I Coma and Nilsa de Deus, Centre de Recerca en Sanitat Animal, Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Spain, for kindly providing a HEV-positive bile sample as well as a HEV-positive faeces sample.

We also thank the Department for Pathology, Institute for Veterinary Medicine, AGES Moedling for providing organs, bile and faeces from examined pigs and the Department for Microbiology, Institute for Veterinary Medicine, AGES Moedling for providing serum samples.

Hermann Schobesberger, Division Veterinary Medicine, AGES Vienna, we thank for valuable advice and helpful discussions.

Conflict of interest: The authors have no conflict of interests to declare in regard to this manuscript.

References

Adlhoch C, Wolf A, Meisel H, Kaiser M, Ellerbrok H, Pauli G (2009): High HEV presence in four different wild boar populations in East and West Germany. Vet Microbiol 139: 270–278.

Balayan MS, Andjaparidze AG, Savinskaya SS, Ketiladze ES, Braginsky DM, Savinov AP, Poleschuk VF (1983): Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology 20: 23–31.

Billam P, Pierson FW, Li W, LeRoith T, Duncan RB, Meng XJ (2008): Development and Validation of a Negative-Strand-Specific Reverse Transcription-PCR Assay for Detection of a Chicken Strain of Hepatitis E Virus: Identification of nonliver replication sites. J Clin Microbiol 46: 2630–2634

Bouwknegt M, Rutjes SA, Reusken CB, Stockhofe-Zurwieden N, Frankena K, de Jong MC, de Roda Husman AM, Poel WH (2009): The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation. BMC Vet Res 5: 7.

De Deus N, Seminati C, Pina S, Mateu M, Segales J (2007): Detection of hepatitis E virus in liver, mesenteric lymph node, serum, bile and faeces of naturally infected pigs affected by different pathological conditions. Vet Microbiol 117: 105–114.

Enouf V, Dos Reis G, Guthmann JP, Guerin PJ, Caron M, Marechal V, Nicand E (2006): Validation of single real-time TaqMan PCR assay for the detection and quantitation of four major genotypes of hepatitis E virus in clinical specimens. J Med Virol 78: 1076–1082.

Fernández-Barredo S, Galiana C, García A, Vega S, Gómez MT, Pérez-Gracia MT (2006): Detection of hepatitis E virus shedding in feces of pigs at different stages of production using reverse transcription-polymerase chain reaction. J Vet Diagn Invest 18: 462–465.

Fernández-Barredo S, Galiana C, García A, Gómez-Muñoz MT, Vega S, Rodríguez-Iglesias MA, Pérez-Gracia MT (2007): Prevalence and genetic characterization of hepatitis E virus in paired samples of feces and serum from naturally infected pigs. Can J Vet Res 71: 236–40.

Forgách P, Nowotny N, Erdélyi K, Boncz A, Zentai J, Szucs G, Reuter G, Bakonyi T (2010): Detection of hepatitis E virus in samples of animal origin collected in Hungary. Vet Microbiol 143: 106–116.

Gibson UE, Heid CA, Williams PM (1996): A novel method for real time quantitative RT-PCR. Genome Res 6: 995–1001.

Gyarmati P, Mohammed N, Norder H, Blomberg J, Belák S, Widén F (2007): Universal detection of hepatitis E virus by two real-time PCR assays: TaqMan and Primer-Probe Energy Transfer. J Virol Methods 146: 226–235.

Hall TA (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis programme for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95–98.

Heid CA, Stevens J, Livak KJ, Williams PM (1996): Real time quantitative PCR. Genome Res 6: 986–994.

Huang FF, Haqshenas G, Guenette DK, Halbur PG, Schommer SK, Person FW, Toth TE, Meng XJ (2002): Detection by reverse transcribtion-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. J Clin Microbiol 40: 1326–1332.

Jothikumar N, Cromeans TL, Robertson BH, Meng XJ, Hill VR (2006): A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J Virol Methods 131: 65–71.

Kanai Y, Tsujikawa M, Yunoki M, Nishiyama S, Ikuta K, Hagiwara K (2010): Long-term shedding of hepatitis E virus in the feces of pigs infected naturally, born to sows with and without maternal antibodies. J Med Virol 82: 69–76.

Kawai HF, Koji T, Iida F, Kaneko S, Kobayashi K, Nakane PK (1999): Shift of hepatitis E virus RNA from hepatocytes to biliary epithelial cells during acute infection of rhesus monkey. J Viral Hepatitis 6: 287–297.

Leblanc D, Ward P, Gagné MJ, Poitras E, Müller P, Trottier YL, Simard C, Houde A (2007): Presence of hepatitis E virus in a naturally infected swine herd from nursery to slaughter. Int J Food Microbiol 117: 160–166.

Leblanc D, Poitras E, Gagne MJ, Ward P, Houde A (2010): Hepatitis E virus load in swine organs and tissues at slaughterhouse determined by real-time RT-PCR. Int J Food Microbiol 139: 206–209.

Lee YH, Ha Y, Ahn KK, Chae C (2009): Localisation of swine hepatitis E virus in experimentally infected pigs. Vet J 179: 417–421.

Lewis HC, Wichmann O, Duizer E (2010): Transmission routes and risk factors for autochthonous hepatitis E virus infection in Europe: a systematic review. Epidemiol Infect 138: 145–166.

Lu L, Li C, Hagedorn CH (2006): Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Rev Med Virol 16: 5–36.

Mansuy JM, Peron JM, Abravanel F, Poirson H, Dubois M, Miedouge M, Vischi F, Alric L, Vinel JP, Izopet J (2004): Hepatitis E in the south west of France in individuals who have never visited an endemic area. J Med Virol 74: 419–424.

Martelli F, Caprioli A, Zengarini M, Marata A, Fiegna C, DiBartolo I, Ruggeri FM, Delogu M, Ostanello F (2008): Detection of Hepatitis E virus (HEV) in a demographic managed

wild boar (*Sus scrofa scrofa*) population in Italy. Vet Microbiol 126: 74–81.

Martelli F, Toma S, DiBartolo I, Caprioli A, Ruggeri FM, Lelli D, Bonci M, Ostanello F (2010): Detection of Hepatitis E Virus (HEV) in Italian pigs displaying different pathological lesions. J Vet Sci 88: 492–496.

Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsavera TS, Haynes JS, Thacker BJ, Emerson SU (1997): A novel virus in swine is closely related to the human hepatitis E virus. P Natl Acad Sci USA 94 (18): 9860–9865.

Meng XJ (2010): Hepatitis E virus: Animal reservoir and zoonotic risk. Vet Microbiol 140: 256–265.

Orrù G, Masia G, Orrù G, Romanò L, Piras V, Coppola RC (2004): Detection and quantitation of hepatitis E virus in human faeces by real-time quantitative PCR. J Virol Methods 118: 77–82.

Pavio N, Meng XJ, Renou C (2010): Zoonotic hepatitis E: anaimal reservoirs and emerging risk. Vet Res 41: 46.

Preiss JC, Plentz A, Engelmann E, Schneider T, Jilg W, Zeitz M, Duchmann R (2006): Autochthonous Hepatitis E Virus Infection in Germany with Sequence Similarities to Other European Isolates. Infection 34: 173–175.

Ririe KM, Rasmussen RP, Wittwer CT (1997): Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 245: 154–160.

Schielke A, Sachs K, Lierz M, Appel B, Jansen A, Johne R (2009): Detection of hepatitis E virus in wild boars of rural and urban regions in Germany and whole genome characterization of an endemic strain. Virol J 6: 58.

Tam AW, White R, Reed E, Short M, Zhang Y, Fuerst TR, Lanford RE (1996): In vitro propagation and production of hepatitis E virus from in vivo-infected primary macaque hepatocytes. Virology 215: 1–9.

Tam AW, White R, Yarbough PO, Murphy BJ, McAtee CP, Lanford RE, Fuerst TR (1997): In vitro infection and replication of the hepatitis E virus in primary cynomolgus macaque hepatocytes. Virology 238: 94–102.

Tamura K, Nei M (1993): Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512–526.

Tamura K, Nei M, Kumar S (2007): Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.

Vasickova P, Psikal I, Kralik P, Widen F, Hubalek Z, Pavlik I (2007): Hepatitis E Virus: a review. Vet Med-Czech 52: 365–384.

Vasickova P, Psikal I, Widen F, Smitalova R, Bendova J, Pavlik I, Kralik P (2009): Detection and genetic characterisation of Hepatitis E virus in Czech pig. Res Vet Sci 87: 143–184.

Williams TPE, Kasorndorkbua C, Halbur PG, Haqshenas G, Guenette DK, Toth TE, Meng XJ (2001): Evidence of extrahepatic sites of replication of the hepatitis E virus in a swine model. J Clin Microbiol 39: 3040–3046.

Worm HC, Wurzer H, Frösner G (1998): Sporadic Hepatitis E in Austria. New Engl J Med 339: 1554–1555.

Address for correspondence:
Doris Zwettler
Austrian Agency for Health and Food Safety (AGES)
Institute for Veterinary Disease Control
Robert Koch Gasse 17
2340 Moedling
Austria
doris.zwettler@ages.at