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Detection of Hepatitis E Virus (HEV) in Italian pigs displaying different pathological lesions

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ABSTRACT

In this study we investigated the HEV prevalence in Italian pigs displaying different pathological lesions, possible risk factors related to the infection, and the possible relations occurring between HEV and other concomitant pig pathogens. Genetic characterization of some of the identified strains was also performed. Detection of HEV RNA was accomplished using a nested reverse-transcription polymerase chain reaction on bile samples from 137 pigs of 2–4 months of age submitted for diagnostic purposes. Forty-one of the 137 examined pigs (29.9%) tested positive for HEV RNA. Animals of 80–120 days of age showed a higher prevalence of HEV infection (46.9% against 20% of younger animals). No statistically significant correlations between HEV positivity and the presence of other pathological conditions detected at necropsy, or concomitant coinfections with PCV2 and/or PRRSV were detected. All identified strains belonged to genotype 3, and were similar to other HEV subtypes 3e, 3f, 3c circulating in Europe.

1. Introduction

Hepatitis E Virus (HEV) is a single-stranded, positive sense RNA virus now classified as the sole member of the genus *Hepevirus*, belonging to the recently created family *Hepeviridae* (Okamoto, 2007). HEV in humans is a common cause of enterically transmitted non-A non-B hepatitis outbreaks in many developing countries (Banks et al., 2004; Rutjes et al., 2007), but there are increasing evidences that also in developed countries locally acquired HEV infections sporadically occur (Rutjes et al., 2007). Swine has been demonstrated to be susceptible to the infection (Meng et al., 1997), and several lines of evidence suggest that it can represent an important reservoir of the virus (Lu et al., 2006; Zheng et al., 2006). A number of direct and indirect evidences (Meng et al., 1998; Hsieh et al., 1999; Drobeniuc et al., 2001; Meng et al., 2002; Nishizawa et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Sonoda et al., 2004; Lu et al., 2006; Dalton et al., 2007; Okamoto, 2007; Renou et al., 2007; Pavio et al., 2008) have also demonstrated the zoonotic nature of HEV, and that pigs are potentially able to transmit the virus to humans through the direct contact with contaminated secretes, excretes and organs, and through the food-chain, by the ingestion of raw or undercooked pork products.

HEV encompasses at least four genotypes, which differ with respect to geographic distribution, host range and pattern of infection, although they are serologically similar (Tei et al., 2003; Banks et al., 2004; Lu et al., 2006; Okamoto, 2007).

Genotype 1 is mainly responsible of epidemic outbreaks in humans in Asia and Africa, genotype 2 has been described in Mexico and in several African countries. Genotype 3 causes sporadic cases in both humans and pigs worldwide, while genotype 4 contains strains identified in pigs and humans in Asian countries (Okamoto, 2007). Recently, a classification of HEV into subtypes has been also proposed (Lu et al., 2006) and genotypes 3 and 4 that include a big range of different human and animal strains, have been divided, respectively in 10 and 7 subtypes.

Epidemiologically, since the first detection in pigs in 1997 (Meng et al., 1997), the virus has been identified in swine farms of many geographical areas, Europe included (van der Poel et al., 2001; Banks et al., 2004; Fernandez-Barredo et al., 2006; Caprioli et al., 2007; Rutjes et al., 2007; Vasickova et al., 2009; Xia et al., 2008). HEV prevalence in European pigs vary between 22% and 55% (van der Poel et al., 2001; Banks et al., 2004; Fernandez-Barredo et al., 2006; de Deus et al., 2007; Martin et al., 2007; Rutjes et al., 2007). Pigs of 2–4 months of age seem to be more susceptible to the infection (Huang et al., 2002; Banks et al., 2004), but HEV RNA has been also detected in finishers and breeders (Fernandez-Barredo et al., 2006). HEV seems to be asymptomatic in pigs of any age (Meng et al., 1998; Kasorndorkbua et al., 2002; Leblanc

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et al., 2007; Dos Santos et al., 2009), although some studies have highlighted a possible correlation of the infection with liver damages (Lee et al., 2007; Dos Santos et al., 2009) and histopathological lesions have often been detected in the liver of experimentally and naturally infected animals (Meng et al., 1998; Halbur et al., 2001; Martin et al., 2007; Lee et al., 2007; de Deus et al., 2008; Lee et al., 2009). Moreover, the possible interaction between HEV and other pig pathogens has also to be fully clarified. In particular, it has been suggested that HEV infection could act in synergy with Porcine Circovirus type 2 (PCV2), a virus also able to cause hepatitis in pigs (Segalés et al., 2005).

In this study we investigated the HEV prevalence in Italian pigs displaying different pathological lesions, possible risk factors related to HEV infection, and possible relations occurring between HEV and other concomitant pig pathogens. Genetic characterization of some of the identified strains was also performed.

2. Material and methods

2.1. Sample collection

One hundred and thirty-seven pigs which had died or had been euthanized for different pathological conditions between January and June 2006 were enrolled for this study. The animals were selected from those arrived for diagnostic purposes at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna and originated from 45 different Italian pig herds. For each animal, information regarding age, type of herd (farrow-to-weaning, farrow-to-finish, fattening, and genetic centre), macroscopic lesions observed at necroscopy and presence of PCV2 and/or PRRSV infections were recorded. Detection of PCV2 and PRRSV nucleic acids were performed on organs of submitted animals using 2 PCR protocols previously described, respectively by Ouardani et al. (1999) and Oleksiewicz et al. (1998).

For the detection of HEV RNA, from each animal a bile sample was withdrawn with a sterile syringe (used once and then discarded) through the gall-bladder wall during necroscopy examination, collected and stored at −80 °C until processing.

Although HEV RNA can be detected in several pig tissues, excretes and secretes (Williams et al., 2001; de Deus et al., 2007), we chose to collect the bile because it has been recently reported to be the most reliable specimen for the detection of HEV in pigs (de Deus et al., 2007).

2.2. RNA extraction and HEV RT-nested-PCR

Each bile sample was diluted 1:10 in diethyl pyrocarbonate (DEPC) water. Total RNA was extracted from 140 µl of the bile solution using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Measures to asses and exclude the possible presence in the bile of PCR inhibitors interfering with our extraction and amplification protocols were evaluated. In particular, procedures such as spiking and testing of negative bile samples and dilution of a number of positive and negative extracted RNA prior amplification were preliminary conducted, demonstrating no significant interference by such inhibitors with the test protocols implemented.

For the detection of HEV RNA two distinct reverse-transcription-PCR in one tube format (RT-PCR one tube) followed each by a nested PCR and amplifying two different ORF2 regions were used. Protocol 1 used degenerate primers con-a1/con-s1 for the first RT-PCR, and con-a2/con-s2 for the nested PCR, amplifying a 160 bp and 145 bp region, respectively (Erker et al., 1999). Protocol 2 used primers 3156–3157 (first run) and 3158–3159 (nested) (Huang et al., 2002) to amplify 710 bp and 348 bp regions, respectively. RT-PCR reactions were conducted using Superscript III One-step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-PCR amplification was conducted on an ICycler (Biorad, Hercules, CA, USA) thermal cycler, using 3 µl of total RNA, under the following conditions: 45 °C for 30 min for RT, 94 °C for 2 min, followed by 39 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for 90 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. Two microliters of DNA obtained from the RT-PCR reactions were used as a template for the nested PCRs. These were performed using a recombinant Taq DNA polymerase (Fermentas, Burlington, Canada) according to the manufacturer's instructions, following the subsequent thermal conditions: initial denaturation at 95 °C for 3 min, followed by 39 cycles of denaturation at 94 °C for 45 s, annealing at 49 °C for 1 min, elongation at 72 °C for 2 min, and a final elongation at 72 °C for 7 min.

At each stage of the procedure (extraction, RT-PCR and nested PCR amplification), a negative (DEPC water) and a positive control (swine HEV positive bile) were included. To further minimize the possibility of cross-contamination, strict anti-contamination procedures, including the use of separate areas, safety hoods, frequent changing of disposable gloves and sanitization of surfaces and instruments using appropriate cleaning agents such as Virkon-S (Antec International, UK), sodium hypochlorite, 80% ethyl alcohol, followed by the use of UV lights at the end of every working phase, were implemented.

Amplified DNA products were visualized in a 2% agarose gel stained with ethidium bromide.

2.3. Sequencing and phylogenetic analysis

To further confirm the identity of the viral strains detected by PCR, on randomly selected positive samples, a nucleotide sequence analysis was performed. On 15 of these sequences amplified by using the Huang PCR method (Huang et al., 2002), selected for their length, a phylogenetic analysis was also performed. The 348 bp DNA band was excised from a 2% agarose gel, purified with a High Pure PCR Product Purification Kit (Roche, Basel, Switzerland), and sequenced using the PCR primers with the BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Perkin Elmer, Applied Biosystems, Foster City, CA) in an automated sequencer (ABI Prism 310 DNA sequencer, Applied Biosystems, Foster City, CA). The sequences obtained were assembled and aligned with other swine and human HEV sequences present in NCBI GenBank (http://www.ncbi.nlm.nih.gov), using the DNASIS Max software (Hitachisoft). An avian HEV strain (Acc. No. EF206691) was used as out-group. Phylogenetic analyses were carried out with the Bionumerics software packages (Applied Maths, Kortrijk, Belgium), and the dendrogram was obtained with the UPGMA method. Reference strains used for the phylogenetic analysis were: Human HEV strains VH1 (Spain, Acc. No. AF195061) and Fr-26 (France, Acc. No. EU555183); swine strains NLSW15 and NLSW20 (The Netherland, Acc. No. AF332620; Acc. No. AF336290), strain P143 (UK, Acc. No. AF503512), strain J8–60 (Japan, Acc. No. AB094231).

2.4. Statistical analysis

A binary logistic regression was performed to determine possible differences in the HEV prevalence in function of the age of the sampled animals (< 80, or 80–120 days old), the herd typology (farrow-to-weaning, farrow-to-finish, fattening, genetic centre), the presence of pathological conditions detected at necropsy (liver disorders, intestine disorders, lung disorders, other disorders), and the presence of concomitant PCV2 and/or PRRSV infections at the time of death.

Preliminarily the proportion of positive animals was evaluated using the χ^2 test. The factors that screened through $p < 0.15$ were subsequently evaluated using the binary logistic regression. The model was based on the simultaneous entry of all variables, and its efficacy was assessed on the basis of the likelihood-ratio and the Hosmer–Lemeshow statistic. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated from the final binary logistic model.

All statistical analyses were performed using the software SPSS 12.0.0 (SPSS Inc., Chicago, IL, USA).

3. Results

HEV RNA was detected in 41 of the 137 bile samples analyzed (mean prevalence of 29.9%) with at least one of the two nested PCR protocols used. Twenty-seven samples yielded a visible DNA band of 145 bp (Erker et al., 1999), while twenty-eight tested positive using the second nested PCR technique (Huang et al., 2002) yielding a band of 348 bp. In fourteen cases, positivity was confirmed by both PCR tests. A significantly higher HEV prevalence ($\chi^2 = 10.77$; $p = 0.001$) was observed in swine of 80–120-day-old, with 23 positives out of 49 tested (46.9%), than in younger animals (<80 day-old), in which the prevalence was 20.0% (17 positives out of 85 tested) (Table 1). The odds of virus presence in bile samples was also 3.78 times higher in the formers than in the latters (95% CI 1.39–10.26; $p = 0.009$). The Hosmer–Lemeshow statistic showed a good fit for the final model ($\chi^2 = 10.81$; $p = 0.212$).

No statistically significant difference was observed evaluating the proportion of HEV positive animals in respect to the different herd typologies considered; prevalence of HEV infection was not significant higher in swine with or without gastrointestinal, hepatic or respiratory pathologies detected at necropsy.

No statistically significant association ($p < 0.05$) was observed relating the presence of HEV RNA with concomitant PCV2 and/or PRRSV infections detected at the time of death, although the HEV prevalence was slightly higher in PCV2/PRRSV positive swine (Table 1).

Following sequencing and comparative analysis of the 348 bp products from nested PCR (Huang et al., 2002), all 15 HEV strains examined appeared to belong to genotype 3 with a genomic identity one to each other ranging between 80.4% and 99.6%. When compared with other European strains, samples HEVSW/BO122/IT/06, HEVSW/BO/127/IT/06 and HEVSW/BO88/IT/06 presented a high percentage of identity (92.5%) with a swine Dutch strain belonging to subtype 3c. Samples HEVSW/BO87/IT/06, HEVSW/BO9/IT/06 and HEVSW/BO91/IT/06 had a nucleotide identity ranging from 87.6% to 92.8% with Spanish and France human strains of subtype 3f, while sample HEVSW/BO85/IT/06 had 88.7% identity with a UK swine strain belonging to subtype 3e (Fig. 1). The other strains showed 82% identity with either subtype 3f or 3e. Despite down to 82% identity at the nucleotide level, amino acid identity was high (97.4–100%). Differences, when present, were mostly located at the third base of the triplet, not affecting the amino acid sequence in the encoded protein.

Due to their short length, the 145 bp amplicons obtained by the RT-nested-PCR performed with primers con-a2/con-s2 were not included into comparative phylogenetic analysis, although these sequences also confirmed that all strains were genotype 3 HEV.

4. Discussion

The relatively high prevalence (29.9%) detected in this study confirms the presence and the wide spread of HEV genotype 3 infection in Italian pig herds (5.9%, Caprioli et al., 2007; 42%, Di Bartolo et al., 2008), independently from the herd typology considered (farrow-to-weaning, farrow-to-finish, fattening, genetic centre). The prevalence detected is similar to that reported in other European countries (van der Poel et al., 2001; Banks et al., 2004; de Deus et al., 2007; Fernandez-Barredo et al., 2006; Rutjes et al., 2007; Martin et al., 2007). However it should be pointed out that our present data may not represent an accurate estimation of HEV prevalence in Italy, since the sampling was focused on animals displaying different pathological conditions, not representative of the whole swine population. Nevertheless our results are consistent with those presented in a previous study (de Deus et al., 2007) where the same animal category was considered. In this study, HEV was detected in at least one sample in 26 out of 69 animals (37.7%); bile was the most frequently positive sample (19.1%), followed by mesenteric lymph nodes (17.4%), liver (14.5%), faeces (12.3%) and serum (10.1%).

The higher prevalence detected in swine of 80–120 days of age with respect to younger animals is also consistent with the results obtained in other studies (Huang et al., 2002; Banks et al., 2004), and supports the hypothesis that, as occurs for other infectious diseases, HEV prevalence probably increases as maternal immunity fades out (Meng et al., 1998).

Table 1
Logistic regression analysis of the HEV prevalence in function of swine age, presence of macroscopic lesions detected at necropsy, presence of PCV2 or PRRSV infections, herd typology.

Risk factor		HEV positive/examined (%)	OR	IC 95%	*P*
Age (days)	<80	17/85 (20.0)	–	–	–
	80–120	23/49 (46.9)	3.78	1.39–10.26	0.009
Liver disorders	Present	6/24 (25.0)	0.60	0.18–2.04	0.413
	Absent	35/113 (31.0)	–	–	–
Intestine disorders	Present	20/58 (34.5)	1.43	0.55–3.72	0.457
	Absent	21/79 (26.6)	–	–	–
Lung disorders	Present	28/81 (34.6)	2.03	0.74–5.53	0.167
	Absent	13/56 (23.2)	–	–	–
Other disorders	Present	19/68 (27.9)	0.74	0.30–1.81	0.505
	Absent	22/69 (31.9)	–	–	–
PCV2	Positive	14/36 (38.9)	1.02	0.36–2.92	0.966
	Negative	27/101 (26.7)	–	–	–
PRRSV	Positive	17/54 (31.5)	0.53	0.20–1.41	0.203
	Negative	24/83 (28.9)	–	–	–
Herd typology	Farrow-to-weaning	4/17 (23.5)	–	–	–
	Farrow-to-finish	22/72 (30.6)	1.77	0.30–10.40	0.525
	Fattening	5/8 (62.5)	1.19	0.34–4.11	0.787
	Genetic centre	8/23 (34.8)	1.76	0.24–12.99	0.581
Costant		–	0.05	–	0.003

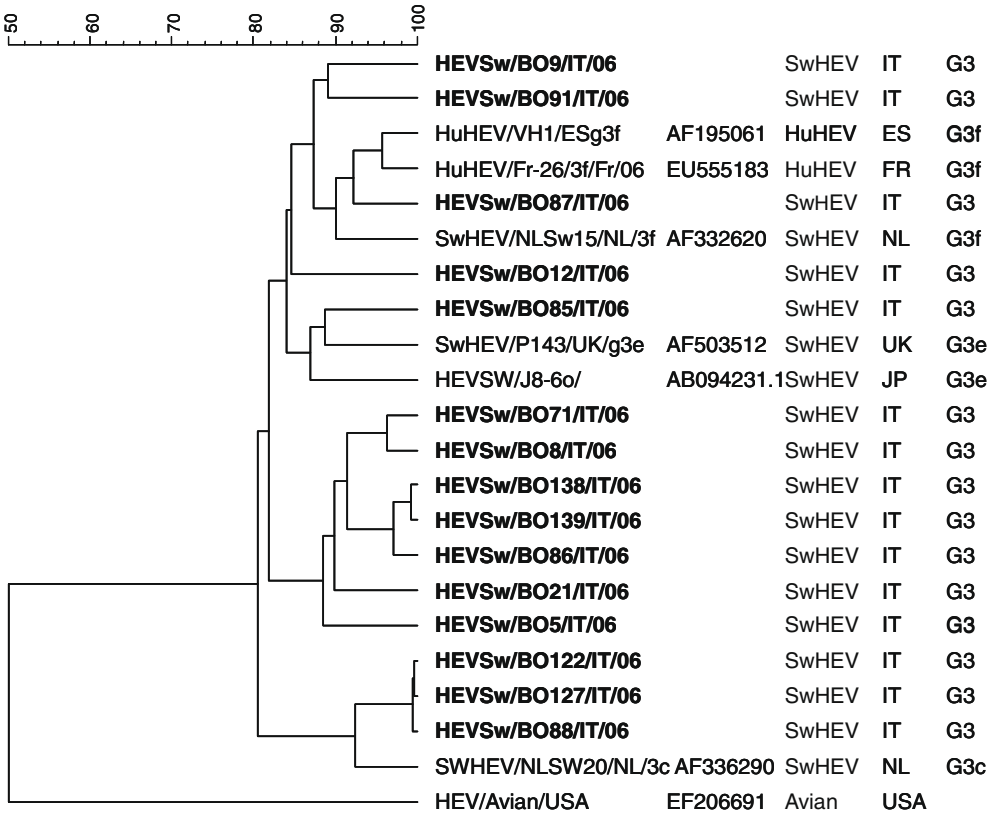

Fig. 1. Dendrogram, was based on 348 bp of the ORF2 fragment (Huang et al., 2002) of the swine positive bile HEV samples (boldface type). The dendrogram was drawn using UPGMA and an avian HEV strain (United States, Acc. No. AY535004) as out-group GenBank Acc. No., origin, country, and genotype are reported.

In our study no statistically significant associations were detected comparing the presence of HEV infection and the occurrence of any specific pathological lesion detected in the animals at necropsy, not even those affecting the liver. These results confirm the apparent asymptomatic nature of HEV infection in pigs, at least at macroscopic level (Meng et al., 1998; Kasorndorkbua et al., 2002; Leblanc et al., 2007). The significant increase of hepatic histopathological lesions constantly associated with HEV infection (Meng et al., 1998; Halbur et al., 2001; Williams et al., 2001; Lee et al., 2007; Martin et al., 2007), should anyway lead to better investigate and study the possible effects and damages of this apparent sub-clincal infection on the pig productivity and performances (e.g. growth rate, feed intake, nutrients digestion pig carcass quality, reproductive parameters), especially in intensive rearing system farms. This finding is nevertheless of some public health concern because of the possible transmission of the virus to human beings through direct contact with infected, but apparently healthy animals, or through the ingestion of contaminated undercooked meat or organs originating from animals normally processed at slaughtering. Moreover, as previously suggested (Meng et al., 2002; de Deus et al., 2007), our results confirm that veterinarians are probably frequently exposed to swine HEV, and should consequently be made more aware of the potential risks for professionally acquired zoonotic swine HEV.

Finally, no statistically significant correlations were observed between the presence of HEV and concomitant PCV2 and/or PRRSV infections, at least at the time of death of the animals, suggesting that HEV does not interact with those viruses. These data are consistent with a previous study (de Deus et al., 2007) reporting no statistically significant difference in the HEV prevalence between PCV2 and non-PCV2 infected pigs. Yet, further studies will be necessary to fully understand the possible role played by HEV in the genesis and evolution of multifactorial or conditioned diseases. Regarding the phylogenetic analysis, the finding that all HEV strains sequenced in our study belonged to genotype 3, confirms previous reports on swine HEV in Europe (van der Poel et al., 2001; Banks et al., 2004; Fernandez-Barredo et al., 2006; Fernandez-Barredo et al., 2007; Caprioli et al., 2007; Rutjes et al., 2007; Di Bartolo et al., 2008). Three different genotype 3 lineages containing sequences similar to subtypes 3f, 3c and 3e were identified. Subtypes 3e and 3f have been demonstrated to be common in European countries, while subtype 3c is very rare and since recently was considered a local strain circulating only in the Netherlands (Lu et al., 2006). This report confirms the presence of swine HEV subtypes 3f and 3e also in Italy, and supports the hypothesis that the spreading of other subtypes, such as 3c, can occur, probably favoured by international animal trades.

The amino acid profile deducted from the identified strains showed that the variations at nucleotide level were mostly silent mutations; this observation is of particular interest considering that the genomic region analyzed is within the gene coding for the capsid protein. These data further support the hypothesis that only a single serotype of HEV may be present worldwide (Lu et al., 2006), even if the range of nucleotide identity between distinct strains is very wide.

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