Retrospective serological study on hepatitis E infection in pigs from 1985 to 1997 in Spain

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ABSTRACT

The objective of the present work was to ascertain the date in which hepatitis E virus (HEV) was introduced in the Spanish pig population. For this, a serological retrospective study was carried out using archived sera. A total of 2871 serum samples gathered between 1985 and 1997 and collected in 208 farms of Spain were tested for anti-HEV IgG by an in-house ELISA. Of the 2871 sera analyzed by ELISA, 1390 were positive for anti-HEV antibodies (48.4%, 95% CI: 46.9–49.9%) and that corresponded to 204/208 farms (98%, 95% CI: 96.1–99.9%) having at least one positive pig. Our results show that HEV was present and widespread in Spanish swine farms at least since 1985. Any significant changes in prevalence were detected from 1 year to another and therefore, HEV infection in swine should be considered endemic in Spain.

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1. Introduction

The first evidence that acute hepatitis outbreaks in humans can be caused by an agent different from hepatitis A or hepatitis B viruses is dated in 1980 when Wong et al. demonstrated that none of the abovementioned agents were involved in the large epidemic of acute hepatitis in India in 1955–1956. Later on, in the 1980 and 1990 decades that new agent was shown to be a virus that was called hepatitis E virus (reviewed by Purcell and Emerson, 2008).

Hepatitis E virus (HEV) is a non-enveloped positive-sense single-stranded RNA virus of 27–34 nm in diameter which has been classified as Hepevirus genus (Emerson et al., 2004), and proposed as family Hepeviridae (Meng et al., 2008). In humans, HEV is responsible of major outbreaks of acute viral hepatitis in developing countries that, generally, are associated with poor sanitary conditions. In contrast, in industrialized countries HE outbreaks are not reported and cases are mostly diagnosed in individuals with no history of previous travel to endemic countries (Emerson and Purcell, 2003). Sporadic or endemic, the disease usually appears as a self-limiting acute hepatitis with a low (<1%) fatality rate. However, fatality in pregnant women can reach up to 25% (Kumar et al., 2004).

In 1997 a novel virus closely related to the known human HEV isolates was detected in pigs (Meng et al., 1997). Different serological studies reported that swine HEV infection is widespread in pigs regardless of whether HEV is endemic in the respective human population or not (reviewed by Meng and Halbur, 2006). Sequence analysis
have shown that swine HEV isolates are more similar to human HEV isolates from the same geographic area than to swine HEV isolates from other regions (Huang et al., 2002). Experimental infections have demonstrated that swine HEV can infect non-human primates and that pigs can be infected with the human strain US-2 (Halbur et al., 2001; Meng et al., 1998). Direct evidence of human hepatitis E associated with consumption of undercooked or uncooked boar and deer products have been reported (Li et al., 2005; Tei et al., 2003). These findings suggest that, potentially, boar and deer products have been reported (Li et al., 2005; Meng et al., 1998). Direct evidence of human hepatitis E infected with the human strain US-2 (Halbur et al., 2001; Meng et al., 1998). Experimental infections have demonstrated that swine hepatitis E is a zoonotic disease.

In Spain, HEV RNA has been detected in human cases of hepatitis, urban sewage and swine samples, confirming thus that HEV circulates in both human and animal populations in Spain (Clemente-Casares et al., 2003; Pina et al., 2000). A recent study showed that HEV is present in Spanish pig farms since, at least 1998, being 97% farms positive for anti-HEV IgG antibodies (Seminati et al., 2008).

The objective of the present retrospective study was to determine the presence of HEV antibodies in archived porcine sera (1985–1998) in order to elucidate when HEV appeared in Spain.

2. Materials and methods

2.1. Sample collection

Spain is an important swine-raising country of Europe, with more than 22 million pigs (Anonymous, 2007). The study was conducted using serum samples collected between 1985 and 1997 by the Institut de Recerca i Tecnologia Agroalimentària (IRTA, Barcelona, Spain) and the Departament d’Agricultura, Alimentació i Acció Rural (DAR, Barcelona, Spain) of the Catalan Government for monitoring health of pig farms. Sera were obtained from randomly selected farms. It was assumed that a HEV positive farm would have at least a proportion of 20% positive sows (95% confidence) and, accordingly, 15 sera per farm were analyzed. In 60 farms where this number of samples was not available, at least eight sera were analyzed (detection limit: 30% prevalence). Therefore, a total of 2871 sera from 208 farms of Spain were examined. For 13 farms sera were available for 3 consecutive years (1995–1997).

2.2. Serological analysis

Sera were tested for anti-HEV IgG antibodies by means of a previously described in-house enzyme-linked immunosorbent assay (ELISA) (Meng et al., 1997). Oldest sera (1985–1987) with optical densities (OD) close to the cut-off (that is between 0.25 and 0.4) were also tested by dot-blot analysis to confirm the ELISA results. Both serologic assays (ELISA and dot-blot) were based on the detection of a purified recombinant ORF2 HEV protein from Sar55 strain (genomeotype 1) (Robinson et al., 1998) provided by Dr. R.H. Purcell and Dr. S.U. Emerson (National Institutes of Health, Bethesda, MD). This antigen have been shown to be efficient for the detection of anti-HEV antibodies in pigs infected by HEV genotype 3 (Engle et al., 2002; Meng et al., 2002).

2.2.1. Enzyme-linked immunosorbtent assay (ELISA)

The test was performed as described by Meng et al. (1997) with minor modifications. Briefly, sera were initially diluted 1:100 and 100 μl were dispensed in wells of a 96-well plate previously coated with the ORF2 truncated protein (100 μl/well of a 0.25 μg/ml dilution of the ORF2 protein in 0.05 M carbonate–bicarbonate buffer pH 9.6). To minimize false positive reactions caused by the test background, each serum was tested simultaneously in ORF2-coated and un-coated wells. After 30 min of incubation of the sera at 37 °C, an anti-swine IgG conjugated with peroxidase (Bethyl) was added (100 μl, dilution 1:80,000) and, finally, test was revealed by adding tetramethylbenzidine as a substrate (TMB, Sigma). The reaction was stopped with H₂SO₄ 2 M and ODs were read at 450 nm. For each sample a corrected OD was calculated by subtracting the value obtained in uncoated wells from the value of HEV-coated wells. Samples having a corrected OD equal or higher than 0.300 were considered positive (Meng et al., 1997). In each plate one positive control, one negative control and two blank samples were included. Control sera were obtained from conventional pigs identified as positive or negative as previously described (Meng et al., 1997).

2.2.2. Dot-blot analysis

Dot-blot was performed only with the older sera (1985–1987) that yielded OD values close to the cut-off. Briefly, nitrocellulose membrane (GE Healthcare) strips were spotted with 1 μl of ORF2 protein in lysis buffer 2 × (30 mM Na₂HPO₄, 18 mM NaCl, 2% SDS, 200 mM DTT, 0.4 mM pefabloc-Sigma) at 20 and 10 ng/μl. A spot of the lysis buffer was included as a blank. Blots were blocked by shaking the strips overnight at 4 °C with TBS-T (2% Tris–HCl pH 7.5, 140 mM NaCl, 0.1% Tween 20) containing 2% blocking reagent (ECL Advance™ Western Blotting Detection kit, GE Healthcare). Each plate one positive control, one negative control and two blank samples were included. After incubation, blots were washed six times with TBS-T and incubated with each serum diluted 1:250 in blocking solution (1 h at room temperature with constant shaking). Positive and negative controls were included at dilutions 1:1000 and 1:250, respectively. After incubation, blots were washed six times with TBS-T, and incubated with a horseradish peroxidase-conjugated goat anti-pig IgG (Bethyl) at a 1:125,000 dilution. After 1 h of incubation at room temperature, blots were washed six times with TBS-T and color development was carried out using a chemiluminescence detection reagent (ECL Advance™ Western Blotting Detection kit, GE Healthcare). For the image capture and analysis, developed spots were photographed with a FluorChem™ HD2 Imaging System’s (Alpha Innotech). Cut-off was determined by comparing the spot produced by each serum to the reaction of the negative control.

2.3. Statistical analysis

Win Episcope 2.0 software (Thrusfield et al., 2001) was used for calculating sample size. Statistical analysis was carried out using SPSS 15.1 software (SPSS Inc., Chicago, IL, USA). A generalized linear model was used to evaluate the relationship between total positive sera (log transformed)
and years. In order to reduce variability, years were grouped as follows: (1) 1996–1997; (2) 1994–1995; (3) 1992–1993; (4) 1990–1991; (5) 1988–1989; and (6) 1985–1987. EpiCalc 2000 software (http://www.brixtonhealth.com/epicalc.html) was used to assess statistical differences by chi-square test among years in a same farm. Significance level was set at \( p < 0.05 \). Confidence intervals (95% CI) for standard errors were estimated using the expression CI = \( \frac{1.96}{\sqrt{n}} \frac{1}{\sqrt{p(1-p)}} \) (Martin et al., 1987).

3. Results

3.1. Serological analysis

3.1.1. Enzyme-linked immunosorbent assay (ELISA)

Of the 2871 sera analyzed by ELISA, 1390 were positive for anti-HEV IgG antibodies (48.4%, 95% CI: 46.9–49.9%). Optical densities of positive sera ranged from 0.3 to 3.0, with an average OD of 0.81 (standard deviation: 0.53). Distribution of ODs is shown in Fig. 1. Two hundred and four out of 208 farms had at least one seropositive pig (98%, 95% CI: 96.1–99.9%). Seroprevalence per year ranged from 25% to 71% (Table 1). Statistical analysis of the ELISA results revealed significant differences between groups of years. Thus, the earlier examined years (1985–1987) had the highest seroprevalence compared to the other groups (\( p < 0.01 \)). The percentage of seropositive sows in farms varied greatly from herd to herd, ranging from 0% to 100% with an average of 47.8% (standard deviation: 25.1%). In the 13 farms where sera were available for 3 consecutive years (1995–1997) no significant differences were found among years.

3.1.2. Dot-blot analysis

Sixty-four sera belonging to years 1985, 1986 and 1987 yielded ODs between 0.25 and 0.4. Of them 58 were positive in dot-blot, confirming that the samples recognized the Sar55 antigen (Fig. 2).

4. Discussion

This study proves that HEV infection has been present in the Spanish swine population since at least 1985, 13 years before any other report of HEV in pigs of this country (Pina et al., 2000; Seminati et al., 2008). In a previous study (Seminati et al., 2008) reported that HEV seroprevalence was increasing with years (1998: 32.1%; 1999: 57.5%; and 2000: 45.8%) and they had suggested that HEV infection in swine probably began to spread soon before 1998. The present study shows that HEV infection was widespread in Spanish pig livestock in 1985. The use of the dot-blot allowed the confirmation of the specific reactivity of the oldest examined sera and minimizes the chance for inclusion of false positive results.

Table 1

<table>
<thead>
<tr>
<th>Year</th>
<th>No. farms</th>
<th>No. analyzed sera</th>
<th>No. positive sera</th>
<th>Proportion (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1985</td>
<td>4</td>
<td>32</td>
<td>8</td>
<td>25% (10–40)</td>
</tr>
<tr>
<td>1986</td>
<td>13</td>
<td>179</td>
<td>116</td>
<td>65% (58.1–71.9)</td>
</tr>
<tr>
<td>1987</td>
<td>8</td>
<td>119</td>
<td>85</td>
<td>71% (62.9–79.1)</td>
</tr>
<tr>
<td>1988</td>
<td>8</td>
<td>105</td>
<td>54</td>
<td>51% (41.5–60.5)</td>
</tr>
<tr>
<td>1989</td>
<td>34</td>
<td>474</td>
<td>238</td>
<td>50% (45.6–54.4)</td>
</tr>
<tr>
<td>1990</td>
<td>32</td>
<td>422</td>
<td>177</td>
<td>41% (36.4–45.6)</td>
</tr>
<tr>
<td>1991</td>
<td>23</td>
<td>304</td>
<td>156</td>
<td>51% (45.5–56.5)</td>
</tr>
<tr>
<td>1992</td>
<td>3</td>
<td>41</td>
<td>19</td>
<td>46% (30.8–61.2)</td>
</tr>
<tr>
<td>1993</td>
<td>21</td>
<td>278</td>
<td>113</td>
<td>40% (34.3–45.7)</td>
</tr>
<tr>
<td>1994</td>
<td>15</td>
<td>223</td>
<td>104</td>
<td>46% (39.5–52.5)</td>
</tr>
<tr>
<td>1995</td>
<td>8</td>
<td>120</td>
<td>33</td>
<td>27% (19.1–34.9)</td>
</tr>
<tr>
<td>1996</td>
<td>18</td>
<td>270</td>
<td>165</td>
<td>61% (55.3–66.7)</td>
</tr>
<tr>
<td>1997</td>
<td>21</td>
<td>304</td>
<td>122</td>
<td>40% (34.6–45.4)</td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
<td>2871</td>
<td>1390</td>
<td>48.4% (46.9–49.9)</td>
</tr>
</tbody>
</table>
Our results support the notion proposed by Purcell and Emerson (2008) that HEV could have probably been present for many years but would not have been recognized until recently. Those authors suggested that HEV could have been the cause of epidemic and endemic hepatitis in adults – supposedly immune to hepatitis A virus – that occurred regularly in different areas of the world before the 20th century.

On the other hand, our data agree with retrospective studies performed in other countries, in which HEV antibodies and/or viral RNA were detected in swine before 1998. In India, anti-HEV antibodies have been detected in swine sera collected between 1985 and 1987 (Arankalle et al., 2003). Also, a study done in Korea reported that HEV was identified in archived porcine hepatic tissues of 1995 (Jung et al., 2007). Finally, a recent study has demonstrated that HEV was present in the wild boar population of Germany from at least 1995 (Kaci et al., 2008).

The statistical differences for prevalences in different years must be interpreted cautiously. Although seroprevalence of HEV in the first examined years seem to be very high, hidden biases related to sampling cannot be discarded and differences could be due to factors such as the geographic location of farms, hygienic conditions in positive farms, density of animals in the farm, etc. The present study did not find differences among consecutive years in the 13 farms in which samples for 3 consecutive years were available (data not shown). These findings probably indicate that the HEV infection was endemic in the Spanish pig farms at least since 1985.

A significant variation in the percentage of seropositive sows between herds was reported by Meng et al. (1999), although these authors attributed variations to the small sample size. Our results seem to indicate that differences between farms could be real. More studies are needed to know which variables can have influence in the distribution of the HEV infection in the farm and how they would affect the overall seroprevalence of sows.

Recent studies have shown that human and animal Spanish HEV isolates are genetically similar (de Deus et al., 2007; Pérez-Gracia et al., 2004; Pérez-Gracia and Rodríguez-Iglesias, 2003; Pina et al., 2000) a fact that could indicate a common origin for the viruses circulating in humans and pigs. Nevertheless, the role of HEV infection in industrialized countries remains unknown. Pérez-Gracia and Rodríguez-Iglesias (2003) suggested that, in an industrialized country, HEV variants were determined by an animal reservoir. In fact, some studies have shown that exposure to swine or to contaminated water could be a risk factor for seropositivity in humans (Galiana et al., 2008; Clemente-Casares et al., 2003; Meng et al., 2002; Withers et al., 2002; Pina et al., 2000). In agreement with these studies, Zheng et al. (2006) demonstrated that swine constitute a principal source of genotype 4 of HEV for human infection in eastern China by direct contact with swine and their wastes.

The high prevalence of HEV infection among pig livestock since 1985 detected in our study and the evidence that seroprevalence and clinical cases of HE among human population are relatively low (Buti et al., 2006; Buti et al., 1995), supports the notion that although the virus is endemic in Spanish pig population, transmission to humans is not very frequent. Therefore, more research is necessary to gain insight the role of swine in HEV transmission in industrialized countries, especially the sanitary risk that could suppose this virus in the food chain.

As a conclusion, we have demonstrated that HEV circulates endemically in Spanish pig farms at least since 1985.

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