

Accepted Manuscript

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PII: S0378-1135(08)00450-1
DOI: doi:10.1016/j.vetmic.2008.09.075
Reference: VETMIC 4216

To appear in: *VETMIC*

Received date: 17-6-2008
Revised date: 15-9-2008
Accepted date: 22-9-2008

Please cite this article as: Casas, M., Pujols, J., Rosell, R., de Deus, N., Peralta, B., Pina, S., Casal, J., Martín, M., Retrospective serological study on hepatitis E infection in pigs from 1985 to 1997 in Spain, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.075

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

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Key words: HEV, swine, epidemiology, ELISA, dot-blot

Running title: Retrospective study on hepatitis E virus in pigs in Spain

Abstract

The objective of the present work was to ascertain the date in which HEV was introduced in the Spanish pig population. For this, a serological retrospective study was carried out using archived sera. A total of 2,871 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 2,871 sera analyzed by ELISA, 1,390 were positive for anti-HEV antibodies (48.4%, CI_{95%}: 46.9%-49.9%) and that corresponded to 204/208 farms (98%; CI_{95%}: 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 one year to another and therefore, HEV infection in swine should be considered endemic in Spain.

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, 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 2,871 sera from 208 farms of Spain were examined. For 13 farms sera were available for three consecutive years (1995-1997).

2.2. Serological analysis

111 Sera were tested for anti-HEV IgG antibodies by means of a previously
 112 described in-house enzyme-linked immunosorbent assay (ELISA) (Meng et al., 1997).
 113 Oldest sera (1985-1987) with optical densities (OD) close to the cut-off (that is between
 114 0.25 and 0.4) were also tested by dot-blot analysis to confirm the ELISA results. Both
 115 serologic assays (ELISA and dot-blot) were based on the detection of a purified
 116 recombinant ORF2 HEV protein from Sar55 strain (genotype 1) (Robinson et al., 1998)
 117 provided by Drs. R.H. Purcell and S.U. Emerson (National Institutes of Health,
 118 Bethesda, MD). This antigen have been shown to be efficient for the detection of anti-
 119 HEV antibodies in pigs infected by HEV genotype 3 (Engle et al., 2002; Meng et al.,
 120 2002).

121 **2.2.1. Enzyme-linked immunosorbent assay (ELISA)**

122 The test was performed as described by Meng et al. (1997) with minor
 123 modifications. Briefly, sera were initially diluted 1:100 and 100 µl were dispensed in
 124 wells of a 96 wells plate previously coated with the ORF2 truncated protein (100 µl/well
 125 of a 0.25 µg/ml dilution of the ORF2 protein in 0.05M carbonate-bicarbonate buffer pH
 126 9.6). To minimize false positive reactions caused by the test background, each serum
 127 was tested simultaneously in ORF2-coated and un-coated wells. After 30 min of
 128 incubation of the sera at 37°C, an anti-swine IgG conjugated with peroxidase (Bethyl)
 129 was added (100µl, dilution 1:80,000) and, finally, test was revealed by adding
 130 tetramethylbenzidine as a substrate (TMB-Sigma). The reaction was stopped with
 131 H₂SO₄ 2M and ODs were read at 450nm. For each sample a corrected OD was
 132 calculated by subtracting the value obtained in uncoated wells from the value of HEV-
 133 coated wells. Samples having a corrected OD equal or higher than 0.300) were
 134 considered to be positive (Meng et al., 1997). In each plate one positive control, one
 135 negative control and two blank samples were included. Control sera were obtained
 136 from conventional pigs identified as positive or negative as previously described (Meng
 137 et al., 1997).

138

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 (Amersham Biosciences Hybond-ECL) strips were spotted with 1 μ l of ORF2 protein in 2x-urea lysis buffer (30mM Na₂HPO₄, 18mM NaCl, 2% SDS, 200mM DTT, 0.4mM pefabloc-Sigma) at 20 ng/ μ l and 10 ng/ μ l. 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, 140mM NaCl, 0.1% Tween 20) containing 2% blocking reagent (ECL Advance™ Western Blotting Detection kit, GE Healthcare Amersham). Then, blots were washed six times with TBS-T and incubated with each serum diluted 1:250 in blocking solution (1h at room temperature with constant shaking). Positive and negative controls were included at dilutions 1:1,000 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 1h 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 Amersham). 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

167 (<http://www.brixtonhealth.com/epicalc.html>) was used to assess statistical differences
 168 by Chi-square test among years in a same farm. Significance level was set at $p < 0.05$.
 169 Confidence intervals ($CI_{95\%}$) for standard errors were estimated using the expression
 170 $C.I. = 1.96\sqrt{p(1-p)/n}$ (Martin et al., 1987).

171

172 **3. Results**

173 **3.1. Serological analysis**

174 **3.1.1. Enzyme-linked immunosorbent assay (ELISA)**

175 Of the 2,871 sera analyzed by ELISA, 1,390 were positive for anti-HEV IgG
 176 antibodies (48.4%, 95% $CI_{95\%}$: 46.9%-49.9%). Optical densities of positive sera ranged
 177 from 0.3 to 3.0, with an average OD of 0.81 (standard deviation: 0.53). Distribution of
 178 ODs is shown in figure 1. Two hundred and four out of 208 farms had at least one
 179 seropositive pig (98%, $CI_{95\%}$: 96.1%-99.9%). Seroprevalence per year ranged from
 180 25% to 71% (Table 1). Statistical analysis of the ELISA results revealed significant
 181 differences between groups of years. Thus, the earlier examined years (1985 to 1987)
 182 had the highest seroprevalence compared to the other groups ($p < 0.01$). The
 183 percentage of seropositive sows in farms varied greatly from herd to herd, ranging from
 184 0% to 100% with an average of 47.8% (standard deviation: 25.1). In the 13 farms
 185 where sera were available for three consecutive years (1995 to 1997) no significant
 186 differences were found among years.

187 **3.1.2. Dot-blot analysis**

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

191

192 **4. Discussion**

193 This study proves that HEV infection has been present in the Spanish swine
 194 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 begun 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.

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 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 three 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.

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

229 Recent studies have shown that human and animal Spanish HEV isolates
 230 are genetically similar (de Deus et al., 2007; Perez-Gracia et al., 2004; Perez-Gracia
 231 and Rodriguez-Iglesias, 2003; Pina et al., 2000) a fact that could indicate a common
 232 origin for the viruses circulating in humans and pigs. Nevertheless, the role of HEV
 233 infection in industrialized countries remains unknown. Perez-Gracia and Rodriguez-
 234 Iglesias (2003) suggested that, in an industrialized country, HEV variants were
 235 determined by an animal reservoir. In fact, some studies have shown that exposure to
 236 swine or to contaminated water could be a risk factor for seropositivity in humans
 237 (Galiana et al., 2008; Clemente-Casares et al., 2003; Meng et al., 2002; Withers et al.,
 238 2002; Pina et al., 2000). In agreement with these studies, Zheng et al. (2006)
 239 demonstrated that swine constitute a principal source of genotype 4 of HEV for human
 240 infection in eastern China by direct contact with swine and their wastes.

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

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

250

5. Acknowledgements

This study was supported by the research grant AGL2004/06688 from the Spanish government. Maribel Casas and Bibiana Peralta have a fellowship from the Generalitat de Catalunya. Nilsa de Deus has a fellowship from CReSA. We thank Dr Purcell (HIH, Maryland, USA) for providing ORF2 protein used in serologic assays. We also thank Dr Albert Bensaid and Servei d'Estadística de la UAB (SEA) for useful advice.

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Figure 1. Optical densities intervals of positive sera (cut-off ≥ 0.300)

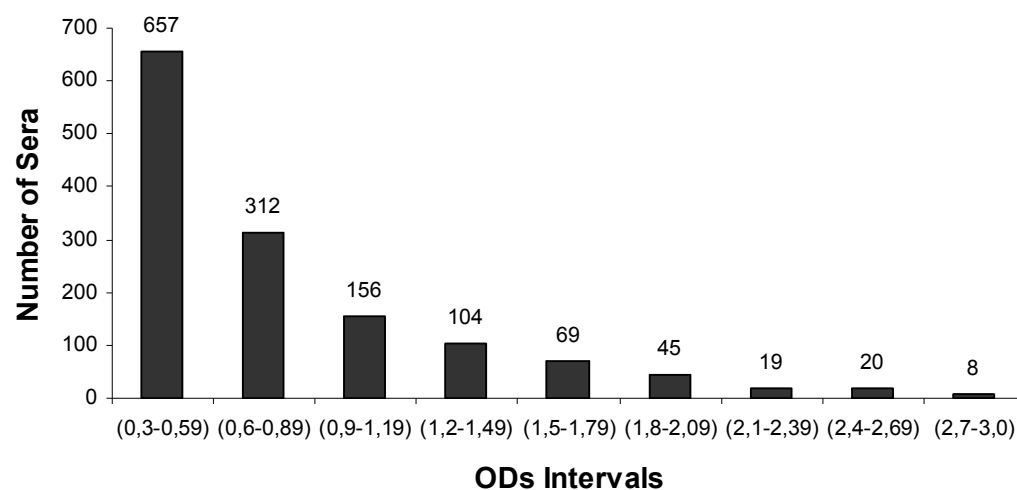


Figure 2. Dot-blot analysis of ELISA positive samples (1985-1987). Strips 1 and 2: positive and negative controls (recent sera); Strips 3 and 4: positive serums samples (old sera); Strips 5 and 6: negative serums samples (old sera)

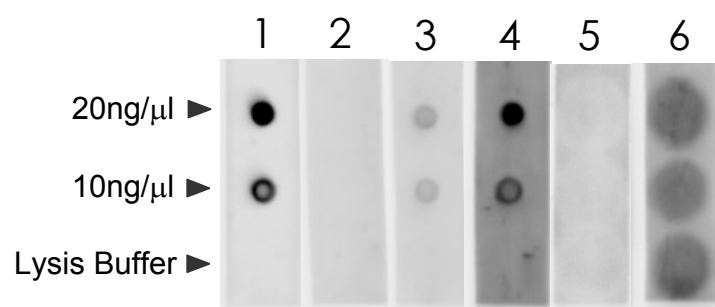


Table 1. Prevalence of HEV antibodies in pigs in different years (1985-1997)

Year	N° farms	N° analyzed sera	N° positive sera	Proportion (95% CI)
1985	4	32	8	25% (10-40)
1986	13	179	116	65% (58.1-71.9)
1987	8	119	85	71% (62.9-79.1)
1988	8	105	54	51% (41.5-60.5)
1989	34	474	238	50% (45.6-54.4)
1990	32	422	177	41% (36.4-45.6)
1991	23	304	156	51% (45.5-56.5)
1992	3	41	19	46% (30.8-61.2)
1993	21	278	113	40% (34.3-45.7)
1994	15	223	104	46% (39.5-52.5)
1995	8	120	33	27% (19.1-34.9)
1996	18	270	165	61% (55.3-66.7)
1997	21	304	122	40% (34.6-45.4)
Total	208	2871	1390	48.4% (46.9-49.9)