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## Hepatitis E virus infection dynamics and organic distribution in naturally infected pigs in a farrow-to-finish farm

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1 **Hepatitis E virus infection dynamics and organic distribution in naturally**  
2 **infected pigs in a farrow-to-finish farm**

3  
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## 32 **Summary**

33           The objective of the present study was to determine the pattern of HEV  
34 infection in a naturally infected, farrow-to-finish herd. For that purpose, a prospective  
35 study was conducted in randomly selected 19 sows and 45 piglets. Blood samples  
36 were collected from sows at 1 week post-farrowing and from piglets at 1, 3, 6, 9, 12,  
37 15, 18 and 22 weeks of age. Furthermore 3 or 5 animals were necropsied at each  
38 bleeding day (but at 1 week of age), and serum, bile, liver, mesenteric lymph nodes  
39 and faeces taken. HEV IgG, IgM and IgA antibodies were determined in serum and  
40 viral RNA was analysed in all collected samples by semi-nested RT-PCR.  
41 Histopathological examination of mesenteric lymph nodes and liver was also  
42 conducted. From 13 analysed sows, 10 (76.9%) were positive to IgG, one to IgA (7.7%)  
43 and two to IgM (15.4%) antibodies specific to HEV. In piglets, IgG and IgA maternal  
44 antibodies lasted until 9 and 3 weeks of age, respectively. IgG seroconversion occurred  
45 by 15 weeks of age while IgM and IgA at 12. On individual basis, IgG was detectable  
46 until the end of the study while IgM and IgA antibody duration was of 4-7 weeks. HEV  
47 RNA was detected in serum at all analysed ages with the highest prevalence at 15  
48 weeks of age. HEV was detected in faeces and lymph nodes for the first time at 9  
49 weeks of age and peaked at 12 and 15 weeks of age. This peak coincided with the  
50 occurrence of hepatitis as well as with HEV detection in bile, liver, mesenteric lymph  
51 nodes and faeces, and also with highest IgG and IgM OD values at 15 weeks. Finally,  
52 different HEV sequences from this farm were obtained, which they clustered within 3  
53 different groups, together with other Spanish sequences, all of them of genotype 3.  
54 Moreover, the present study also indicates that the same pig can be infected with at  
55 least two different strains of HEV during its productive life. This is the first study  
56 characterizing HEV infection in naturally infected pigs with chronological virus detection  
57 and its relationship with tissue lesions throughout the productive life of the animals.

58

59 Key words: Swine, HEV, longitudinal study, serology, ELISA, IgG, IgM, IgA, RT-PCR,

60 phylogenetic analyses.

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## 62        **1. Introduction**

63        Hepatitis E virus (HEV) is a non-enveloped, single stranded, positive sense RNA  
64        virus of 27-34 nm in diameter (Emerson and Purcell, 2003). HEV is the sole member of  
65        the *Hepeviridae* family, genus *Hepevirus*, which includes all HEV strains detected in  
66        human and animals (Emerson *et al.*, 2004).

67        HEV is the causative agent of hepatitis E in humans, a disease mainly  
68        characterized by jaundice, abdominal pain, vomiting and fever, with relatively low  
69        mortality (Emerson and Purcell, 2003; Krawczynski *et al.*, 2000). However, the mortality  
70        can reach up to 25% in pregnant women in underdeveloped countries (Aggarwal and  
71        Krawczynski, 2000; Panda *et al.*, 2007).

72        After the first detection and characterization of swine HEV in USA (Meng *et al.*,  
73        1997), the virus has also been detected in pigs from many developed countries,  
74        including Spain (Banks *et al.*, 2004b; Clemente-Casares *et al.*, 2003; van der Poel *et al.*,  
75        2001). In addition, high prevalence of antibodies in the pig population has been  
76        reported in such countries, which suggests an enzootic nature of the infection  
77        (Seminati *et al.*, 2008; Takahashi *et al.*, 2005). Swine and human HEV sequences from  
78        those regions are usually clustered within the same genotype, providing indications that  
79        hepatitis E may be a potential zoonosis (Banks *et al.*, 2004a; Meng *et al.*, 1997).

80        Like in humans, HEV in pigs is mainly transmitted by faecal-oral route or by  
81        direct contact with infected animals (Kasorndorkbua *et al.*, 2004). However, the  
82        pathogenesis of HEV in pigs is different from humans and not still well characterized  
83        (Halbur *et al.*, 2001; Meng *et al.*, 1998a; Meng *et al.*, 1997). Most HEV studies of  
84        naturally infected pigs have been focused on the viral infection prevalence and  
85        seroprevalence to HEV in animals from different age groups, by means of cross-  
86        sectional approaches (Meng *et al.*, 1999; Takahashi *et al.*, 2005). Only one study has  
87        focused on the epidemiology of the infection including serologic longitudinal analysis  
88        and circulation of the virus through out the productive life of pigs in an enzootic herd  
89        (Meng *et al.*, 1997). Therefore, the main objective of the present work was to determine

90 the dynamics of HEV infection in an enzootically HEV infected farrow-to-finish farm,  
91 using serology, RT-PCR and sequencing. Also, hepatic lesion evolution was correlated  
92 with HEV detection and serologic results throughout the study.

93

## 94 **2. Material and methods**

### 95 **2.1. Farm and animals**

96 A 240-sow, farrow-to-finish farm with continuous flow production system (in  
97 nursery and finishing units) located in North-eastern Spain was studied. Forty-five  
98 piglets from 19 sows from the same weekly farrowing batch were randomly selected  
99 and ear-tagged at 1 week post-farrowing. All animals (pigs or sows) were housed  
100 under same conditions, received same feed and subjected to same management  
101 practices. Piglets were weaned at 17-23 days of age and transferred to nursery units,  
102 where litters from different sows were mixed. At 9 weeks of age, pigs were moved to  
103 the growing-finishing units and they were distributed according to the body condition,  
104 following the farmer criterion.

105

### 106 **2.2. Sample collection**

107 Blood samples were collected from sows at 1 week post-farrowing although  
108 only 13 serum samples were finally available for the study. Piglets were serially bled at  
109 1, 3, 6, 9, 12, 15, 18 and 22 weeks of age.

110 A number of pigs were randomly euthanized with an intravenous overdose of  
111 sodium pentobarbital at different time points (3 pigs at 3 weeks of age and 5 animals at  
112 6, 9, 12, 15 and 18 weeks of age). A complete necropsy was performed in each pig  
113 and samples of serum, liver, bile, mesenteric lymph node and faeces were taken and  
114 stored at -80°C until used. Also, samples from mesenteric lymph node and liver were  
115 taken and fixed by immersion in 10% neutral-buffered formalin for 24h for  
116 histopathological studies. To avoid HEV cross-contamination during necropsy  
117 sampling, sterile disposable materials were used to collect the samples from the



118 carcass and scissors were cleaned and disinfected with 96° ethanol between  
119 samplings. The needles used to collect serum and bile were used once and discarded.

120

### 121 **2.3. Serological analyses**

122 Sow and piglet serum samples were tested for specific anti-HEV IgG, IgA and  
123 IgM antibodies by means of an in-house ELISA as described elsewhere (de Deus et  
124 al., 2007a). Cut-off values of 0.300, 0.380 and 0.320 (OD values + 4SD) were  
125 considered for IgG, IgM and IgA, respectively. Serum samples with OD values equal to  
126 or greater than cut-off values were considered positive.

127

### 128 **2.4. Reverse transcription–polymerase chain reaction (RT-PCR)**

129 Viral RNA from serum, faeces suspension and bile were extracted with  
130 Nucleospin® RNA virus kit (Macherey-Nagel GmbH & Co., Düren, Germany), while  
131 RNA from lymph nodes and liver were extracted with Rneasy mini Kit (Qiagen, Hilden,  
132 Germany), following manufacturer's instructions. HEV detection was done by means of  
133 a semi-nested RT-PCR as previously described (de Deus et al., 2007b).

134

### 135 **2.5. Sequencing and phylogenetic analysis**

136 All resulting PCR products were excised from the agarose gel and purified by  
137 using the NucleoSpin® Extract II kit (Macherey-Nagel GmbH & Co, Düren, Germany),  
138 following manufacturer's instructions. Both strands of the purified DNA amplicon were  
139 sequenced using the ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City,  
140 USA). HEV sequences obtained in this study were compared with others from  
141 genotype 3 from humans, domestic swine and wild boar available at the GenBank and  
142 the European Molecular Biology Library by using the BLAST utility (available from:  
143 <http://www.ncbi.nlm.nih.gov/BLAST>). Obtained HEV sequences were also compared  
144 with other genotype sequences. Alignments were carried out by using ClustalX 1.8

145 program (available from: ftp://ftp-igbmc.u-stras/pub/clustalX) and the alignments were  
146 analyzed with MEGA version 3.1 (Kumar et al., 2004b) to calculate the distances  
147 between sequences. A phylogenetic tree was constructed by the neighbour-joining  
148 method using a 1000-bootstrap procedure, based on the partial nucleotide sequence of  
149 the ORF2 region (168 bp). Bootstrap values greater than 70% were considered to  
150 provide significant evidence for phylogenetic grouping (Baldauf, 2003). The sequences  
151 reported in this study have been deposited in the GenBank database under accession  
152 numbers EU372699-EU372718.

153

## 154 **2.6. Histopathological studies**

155 Formalin fixed liver and mesenteric lymph node were dehydrated, embedded in  
156 paraffin, sectioned at 4  $\mu$ m, and stained with haematoxylin and eosin. Tissues were  
157 studied with special emphasis to the liver for presence or absence of hepatitis, which is  
158 the main lesion attributed to HEV infection both in humans and pigs (Meng et al., 1997;  
159 Panda et al., 2007). Hepatitis lesions were scored from I to IV according to a previous  
160 classification (Rosell et al., 2000).

161 In addition, an *in situ hybridisation* (ISH) technique to detect porcine circovirus  
162 type 2 (PCV2) in liver and mesenteric lymph nodes was performed as previously  
163 described (Rosell et al., 1999). PCV2 is the causative agent of postweaning  
164 multisystemic wasting syndrome (PMWS) and an hepatitis inducing agent (Rosell et  
165 al., 2000).

166

## 167 **2.7. Statistical analyses**

168 Statistical analyses were performed using the SAS system v. 9.1 (SAS institute  
169 Inc., Cary, North Carolina, USA). On the one hand, frequency of seropositive pigs  
170 among weeks was analysed by using proc GENMOD of SAS. An analysis of variance  
171 according to the GLM procedure of SAS and LSMEANS follow-up test was used to

172 compare mean ELISA OD values (IgG, IgA and IgM) between different weeks. Mean  
173 ELISA OD values were also compared between viraemic and non-viraemic pigs.  
174 Besides, Pearson correlation was used to analyze differences between OD values of  
175 sows and their offspring at 1 and 3 weeks of age. On the other hand, contingency  
176 tables with corresponding chi-square statistics were used to evaluate the relationship  
177 between the presence of microscopic lesions and HEV or PCV2 presence in tissue  
178 samples, and with ELISA positivity. Association between presence of PCV2 and HEV  
179 in tissue samples was also analysed. The level of significance was established at  
180  $\alpha=0.05$  for all analyses.

181

### 182 **3. Results**

#### 183 **3.1. Antibody dynamics**

184 The dynamics of HEV infection in studied pigs from 1 to 22 weeks of age using  
185 serology and RT-PCR in serum is displayed in Fig. 1.

186 From 13 sows from which serum was available, 10 (76.9%) were positive to  
187 IgG, one to IgA (7.7%) and two to IgM (15.4%) antibodies to HEV. Overall, piglet IgG  
188 seropositivity was associated with sow serological status ( $p<0.05$ ), although 8  
189 seronegative piglets were born from HEV seropositive sows and one seropositive piglet  
190 was born from a seronegative sow.

191 Percentage of positive HEV IgG piglets at 1, 3, 6 and 9 weeks of age were 54.7  
192 (23 out of 42), 34.9 (15 out of 43), 21.9% (9 out of 41) and 5.6% (5 out of 36),  
193 respectively. HEV IgG antibody duration was related to sow positivity ( $p<0.05$ ); IgG  
194 antibodies lasted until 9 weeks of age in animals born from strong serologically positive  
195 sows, while piglets born from weak seropositive sows were positive only at 1 or 1 and 3  
196 weeks of age. HEV IgA antibodies were detected exclusively in 4 of the piglets from the  
197 same litter at 1 week of age and 3 of them remained positive until 3 weeks of age.  
198 None of the piglets were positive to IgM antibody before 12 weeks age.

199           When maternal antibodies disappeared, piglets became infected by HEV and  
200 developed a detectable serologic response (Fig. 1). After natural infection, IgG was  
201 firstly detected by 15 weeks of age and the number of positive animals increased until  
202 the end of the study. IgM and IgA antibody dynamics were very similar. Both antibody  
203 types were detected firstly at 12 weeks of age; on individual basis, IgA lasted 4 weeks  
204 while IgM between 5-7 weeks (data not shown). The highest percentage of pigs with  
205 IgA and IgM antibodies was observed at 18 weeks of age, which decreased afterwards.

206           Mean optical densities (OD) values for all antibody types among weeks  
207 presented a dynamics curve similar to that of percentage of positive animals (Fig. 1)  
208 and showed statistical significant differences. IgG OD values were significantly higher  
209 at 18 ( $0.927\pm 0.587$ ) and 22 ( $1.126\pm 0.458$ ) weeks of age compared to the rest of  
210 sampling times ( $p<0.001$ ). On the other hand, 15 ( $0.745\pm 0.494$ ) and 18 ( $0.618\pm 0.681$ )  
211 week-old pigs had significantly higher IgM OD values compared to other ages  
212 ( $p<0.001$ ). Finally, IgA OD values were statistically higher at 22 ( $0.409\pm 0.497$ ) weeks of  
213 age compared to rest of sampling points ( $p<0.001$ ). Furthermore, IgG and IgM OD  
214 values were significantly higher in viraemic pigs than non-viraemic ones ( $0.936$  vs  
215  $0.123$ ;  $p<0.05$  for IgG) and ( $1.081$  vs  $0.543$ ;  $p<0.001$  for IgM) at 15 weeks of age.  
216 Moreover, at this age IgM antibody OD values were statistically related to viraemia in  
217 the studied animals ( $p<0.05$ ).

218

### 219 **3.2. Detection of swine HEV RNA by RT-PCR**

220           Two out of 13 (15.4%) sows had a positive HEV RT-PCR result; however none  
221 of their offspring were positive at 1 week of age. HEV was detected in piglet serum  
222 samples at all analysed ages (Fig. 1). The highest ( $p<0.05$ ) percentage of RT-PCR  
223 positive animals in serum was at 15 weeks of age, in which 11 out of 26 (42.3%) were  
224 positive and this percentage decreased from then onwards. By the end of the study at  
225 22 weeks, 2 animals (12.5%) remained RT-PCR positive in serum.

226 HEV RT-PCR detection in serum, bile, liver, mesenteric lymph node and faeces  
227 from chronologically necropsied pigs is summarized in Table 1. HEV was detected for  
228 the first time by week 9 in mesenteric lymph nodes (1/5) and faeces (1/5). HEV  
229 detection in bile, liver, mesenteric lymph nodes and faeces was higher at 12 and 15  
230 weeks ( $p < 0.001$ ). At the last necropsy, performed at 18 weeks of age, HEV was also  
231 detected in liver (2/5) and faeces (2/5).

232

### 233 3.3. Histopathological studies

234 Seventeen (60.7%) and 6 (21.4%) out of 28 pigs had lesions classified as  
235 lesional stage I and II, respectively. Stage I lesions included slight, multifocal,  
236 lymphohistiocytic infiltrates in the portal tracts and/or irregularly distributed in the liver;  
237 stage II lesions comprised intermediate to intense lymphohistiocytic inflammation of the  
238 portal tracts often accompanied by clusters of mononuclear inflammatory cells in the  
239 liver parenchyma (Fig. 2). Occurrence of hepatic lesions varied between ages  
240 ( $p < 0.001$ ); moderate hepatitis (stage II lesions) were mainly observed at 12 (4/5) and  
241 15 (2/5) weeks. In addition, moderate hepatic lesions at 12 and 15 weeks of age were  
242 correlated with HEV detection in bile, liver, mesenteric lymph nodes and faeces  
243 ( $p < 0.05$ ) but not with its detection in serum ( $p > 0.05$ ). Mesenteric lymph nodes did not  
244 have relevant lesions.

245 PCV2 was detected in low amount in 3 mesenteric lymph nodes (in 2/5 and 1/5  
246 animals from 12 and 15 weeks of age, respectively) but not in liver of the tested  
247 animals. No statistical association was observed between PCV2 detection and hepatic  
248 lesions ( $p > 0.05$ ).

249

### 250 3.4. Sequencing and phylogenetic analysis of HEV

251 Positive nested RT-PCR samples were sequenced to confirm the presence of  
252 HEV. Paired sequences from the same animal were 100% identical. Nevertheless,  
253 some sequences from the same animal collected at different ages were not identical.

254 An HEV sequence from one 1 week-old pig (SWSP597-1) had 84% nucleotide identity  
255 with the sequence from the same animal at 15 weeks of age (SWSP597-15). On the  
256 other hand, the sequence of another week-old pig (SWSP357-1) was 94.6% identical  
257 to the HEV from the same animal at 18 weeks of age (SWSP357-18). Finally, a  
258 sequence from one pig at 9 week of age (SWSP545-9) was 84.6% identical to the  
259 sequence from the same animal at 15 weeks of age (SWSP545-12). As consequence,  
260 those sequences were grouped in three main different clusters (Fig. 3); one containing  
261 mainly sequences from animals with more than 12 weeks of age, including the  
262 sequences from the two positive sows (SWSPM623 and SWSPM769) and the other  
263 two with sequences from animals from 1 to 9 weeks of age.

264 Phylogenetic analysis based on the ORF2 gene region revealed that all swine  
265 strains of the studied farm were in the same cluster together with other Spanish human  
266 and swine HEV sequences within the genotype 3 (Fig. 3). Sequences detected in  
267 animals older than 9 week of age, including sows, were mostly grouped in the same  
268 cluster separated from those detected in 1 to 9 week-old pigs. Analyses revealed that  
269 swine HEV sequences from this farm shared 84 to 100% nucleotide sequence identity  
270 to each other and 81.8 to 100% nucleotide sequence identity to other Spanish HEV  
271 isolates from either human, porcine or wild boar origins. The swine strains from this  
272 study, shared 78.9 to 93.4% nucleotide identity with other genotype 3 HEV isolates.

273

#### 274 **4. Discussion**

275 Swine HEV was discovered nearly ten years ago, although little is known about its  
276 infection dynamics in naturally infected pigs. This is the first study characterizing HEV  
277 infection in naturally infected pigs with chronological virus detection and tissues lesions  
278 throughout out the productive life of the animals.

279 In the present study it was observed that not only IgG but also IgA maternal  
280 antibodies can be transferred to the piglets. IgG maternal derived antibody duration in  
281 piglets were related to sow titres, as previously described (Meng et al., 1997).

282 However, one seropositive piglet came from a seronegative sow, probably due the  
283 usual cross-fostering practised at the farm during the first 48 hours post-farrowing. To  
284 date, little is known on HEV IgA antibody passive transfer to piglets, and the only  
285 previous study reporting IgA antibody in newborns (Nakai et al., 2006) described faecal  
286 IgA detection in suckling pigs less than 13 days of age. In contrast, in our study, serum  
287 IgA antibody lasted until 3 weeks.

288 The pattern of seroconversion here observed for HEV is similar to that of typical  
289 antibody dynamics to other swine viruses (Gelberg et al., 1991; Rodriguez-Arriola et  
290 al., 2002). Seroconversion to IgG was mainly observed at 15 weeks of age preceded  
291 by IgM and IgA at 12 weeks of age. Similar results for IgM and IgG antibodies were  
292 observed in a prospective study made in USA (Meng et al., 1997). In all performed  
293 studies to date, like in the present one, once pigs seroconvert to HEV IgG, those  
294 antibodies remain until slaughter age (Meng et al., 1997; Takahashi et al., 2003b). On  
295 the other hand, IgM duration varied between 5-7 weeks and was related with viraemia,  
296 as previously described in human and pigs (Chauhan et al., 1993; Meng et al., 1998a).  
297 Nevertheless, this relation was not observed with the other antibody types. Moreover,  
298 we describe here for the first time the dynamics of IgA antibody during the productive  
299 life of pigs. After maternal antibodies waned, IgA antibodies appeared again at 12  
300 weeks of age, with the highest percentage of seropositive pigs at 18 weeks of age. This  
301 observation is consistent with a previous cross-sectional study in which IgA antibody  
302 was detected to a high proportion at 2-3 months of age (Takahashi et al., 2005).

303 Interestingly, one week-old suckling piglets (n=3) were positive to HEV in serum.  
304 HEV sequences from positive sows were different from those of positive piglets at 1 or  
305 3 weeks of age; therefore, no direct evidence of HEV transmission from sow to piglets  
306 was confirmed. However, the fact that 2 of the sows were positive to HEV in serum is  
307 at least an indication that sows can potentially be a source of HEV infection for the  
308 piglets, at least during lactation. An interesting debate, not able to be answered with  
309 the present study, is whether HEV infection in very young piglets came from

310 transplacental or horizontal sow-to-piglet transmission. Previously, HEV transmission to  
311 offspring was not observed in pregnant gilts experimentally inoculated, although sow  
312 infection was successful with viral shedding in faeces (Kasorndorkbua et al., 2003). On  
313 the contrary, in humans, HEV transplacental transmission has been reported in 33 to  
314 100% of cases by detection of the virus in umbilical cord blood (Kumar et al., 2004a;  
315 Kumar et al., 2001).

316 Like in humans, HEV in pigs is mainly transmitted by faecal-oral route (Halbur et al.,  
317 2001; Meng et al., 1998a). Once an animal is infected, virus can be detected in faeces  
318 after 2-3 weeks and virus shedding can last up to 7 weeks (Meng et al., 1998b). In the  
319 present study, HEV was detected in faeces for the first time at 9 weeks of age, when  
320 piglets were moved to growing-finishing units. By that time, pigs were more susceptible  
321 to infection due to lack of detectable maternally derived antibodies. As soon as animals  
322 started to shed virus in faeces, it was quickly disseminated; by 12-15 weeks of age, all  
323 analysed animals shed virus in faeces and had HEV in different tissues such as liver  
324 and mesenteric lymph nodes. In a cross-sectional study, similar results were recorded  
325 with the maximum prevalence of HEV shedding during the first month of fattening  
326 (around 13-16 weeks of age) (Fernandez-Barredo et al., 2006).

327 HEV was apparently able to cause hepatitis based on the analysed animals, and  
328 this lesion was also related to virus detection in all non-hepatic samples studied, but  
329 serum. This result is not surprising, since similar results have been described in  
330 previous studies (de Deus et al., 2007b); in addition, it is known that small intestine,  
331 colon, lymph nodes and liver are places where virus replicates (Williams et al., 2001)  
332 and, therefore, HEV detection can better correlate with such locations, such liver, than  
333 serum. It was not possible to unequivocally associate the presence of hepatitis and  
334 HEV due to the concomitant infection with PCV2. Nevertheless, another study  
335 performed on PCV2 and HEV co-infected pigs showed relation between HEV detection  
336 and mild to moderate hepatitis while PCV2 was responsible for more severe hepatitis  
337 lesions (Martin et al., 2007). Contrary to HEV infection in humans, natural swine HEV



338 infection does not cause clinical disease and infection is subclinical with mild to  
339 moderate hepatitis (Halbur et al., 2001; Meng et al., 1998a). Although it has been  
340 described that mild hepatitis could be a natural background of pig liver (Halbur et al.,  
341 2001), the mild to moderate hepatitis lesions observed in the present study were  
342 remarkably detected at 12 and 15 weeks of age, in coincidence with the maximum  
343 detection of virus in bile, liver, mesenteric lymph nodes and faeces, and also with high  
344 IgM and IgG OD values.

345 In the present study, HEV RNA was still detected in liver and faeces of 18  
346 week-old animals as well as in serum of 22 week-old pigs. Considering that the animals  
347 are usually sent to slaughterhouse at 22-25 weeks of age in Spain, we cannot rule out  
348 that positive animals to HEV RNA are sacrificed and, consequently, infected meat and  
349 organs may reach groceries. In fact, swine HEV RNA has been detected in 2 and 11%  
350 of raw pig liver in Japanese and American grocery stores, respectively, being even  
351 infectious for pigs (Feagins et al., 2007; Yazaki et al., 2003).

352 Swine HEV sequences can belong to either genotype 3 or 4 (Okamoto, 2007).  
353 All sequences from this study were grouped within the genotype 3 together with other  
354 Spanish sequences from human, pig and wild boar origins (de Deus et al., 2007a;  
355 Martin et al., 2007; Pina et al., 2000). Apparently, at least 3 different HEV strains or  
356 clones circulated in the same farm; however, one principal clone was mainly present at  
357 the peak of infection, which, in turn, might be the one responsible for the hepatitis  
358 lesions observed. However, other HEV strains circulated at earlier ages (mainly  
359 between 1 and 9 weeks of age). These results are in accordance with Japanese  
360 studies that described variability of strains circulating in the same farm (Nakai et al.,  
361 2006; Takahashi et al., 2003a). Moreover, the present study also indicates that the  
362 same pig can be infected with at least two different strains of HEV during its productive  
363 life probably because animals are housed in different places according to their  
364 productive stage/age. In fact, this situation might also be an indication of absence or  
365 partial protection of antibodies elicited by different HEV strains. The possibility of one

366 pig being infected simultaneously with more than one HEV strain at the same time;  
367 was not investigated. Although the used RT-PCR apparently detects all genotype 3  
368 HEV strains, it cannot be ruled out that other yet unknown HEV strains of the same  
369 genotype might have been unnoticed. However, if this would be the case, it would not  
370 be negligible the possibility that more than two strains could be detected in a single  
371 animal.

372

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379

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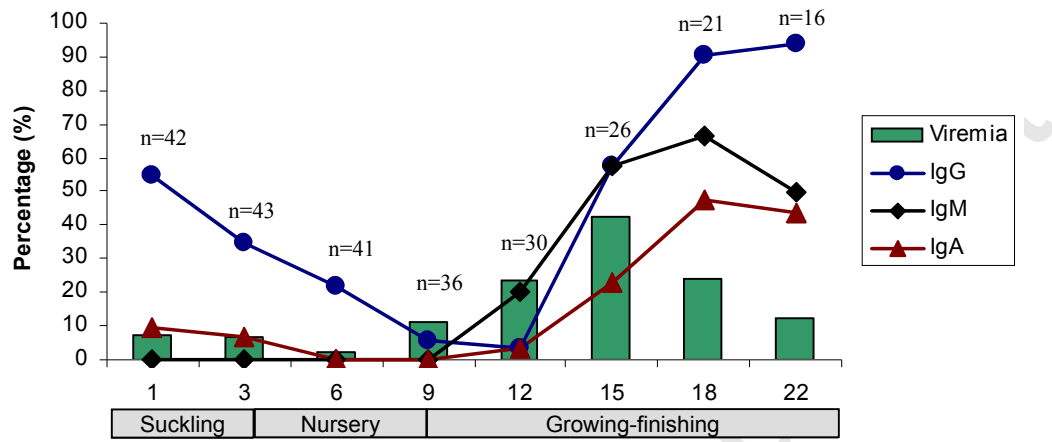
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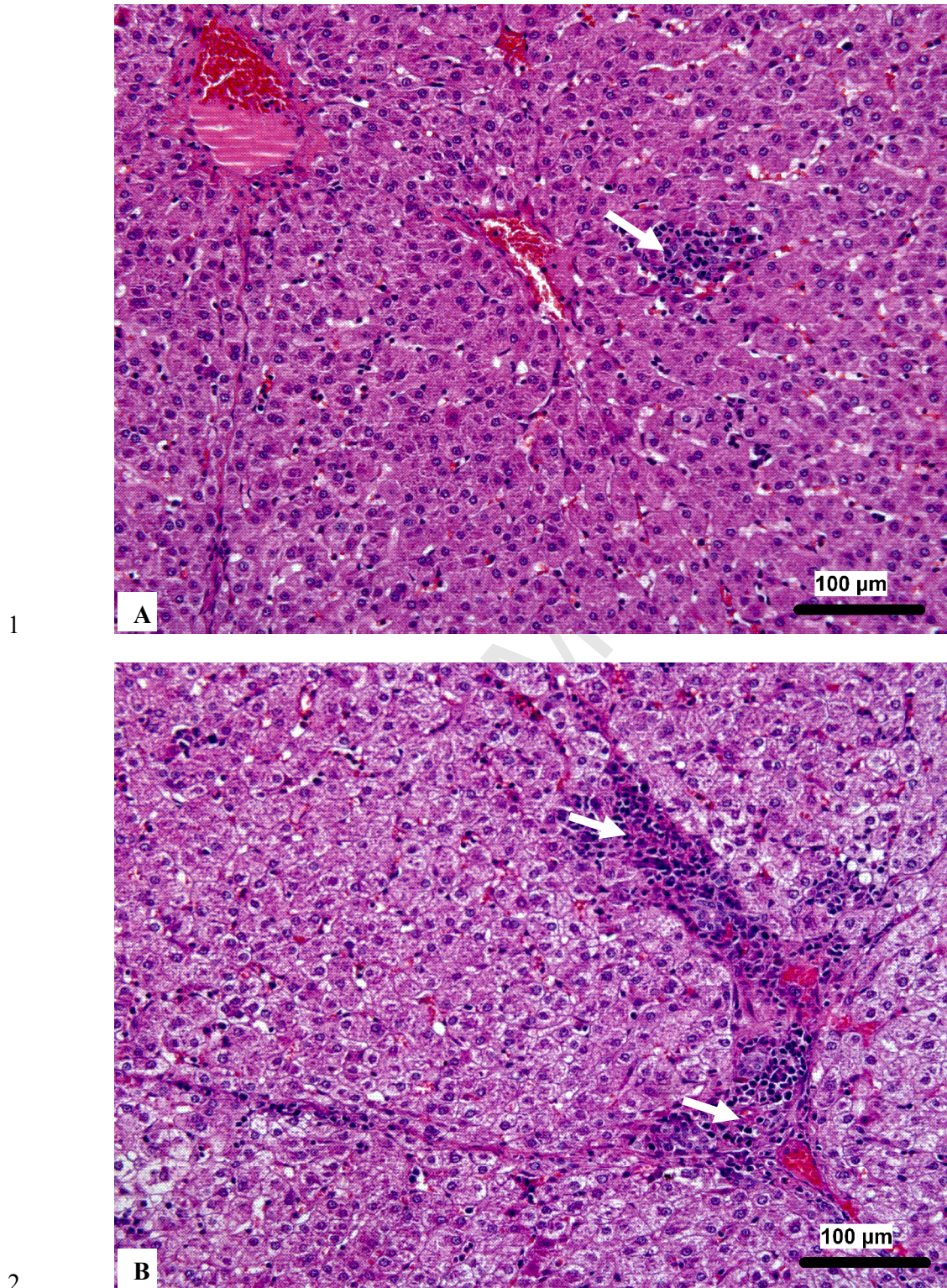
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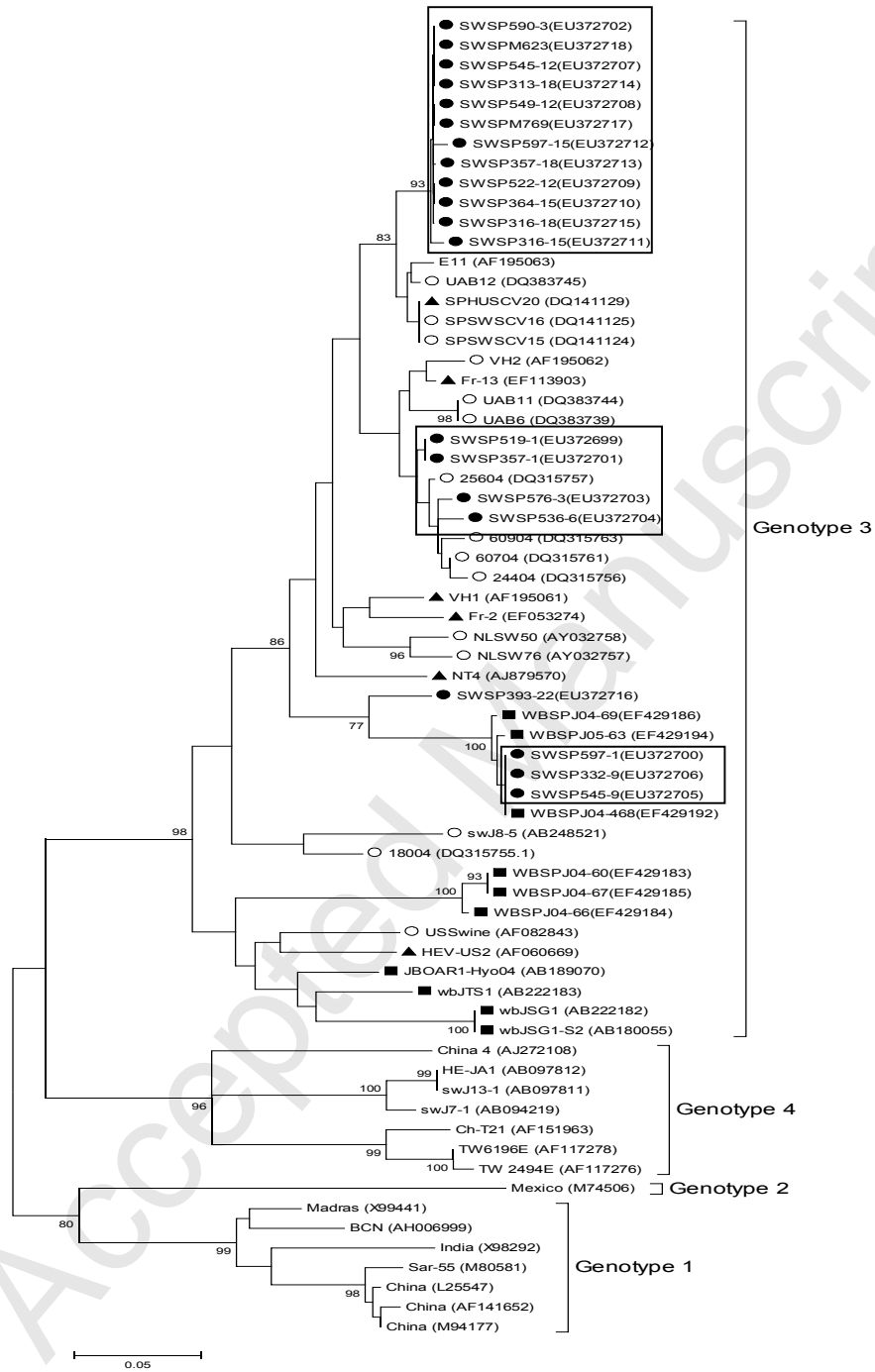
- 1 Figure 1. Antibodies (IgG, IgM and IgA) and viraemia kinetic curves to HEV.
- 2 Percentage of positive samples (n=number of animals analysed animals in each week).





3 Figure 2. Liver from natural infected piglets showing multifocal lymphohistiocytic  
4 infiltrates irregularly distributed in the liver (A, mild) and cluster of mononuclear  
5 inflammatory cells in portal tract (B, moderate). Hematoxylin/eosin staining (x20).

1



2

3 Fig. 3. Phylogenetic tree constructed by neighbour-joining method based on the  
 4 nucleotide sequences (as bootstrap search option) with 1000 replications (mega 3.1  
 5 program). Bootstrap values higher than 70% are indicated for the major nodes as a  
 6 percentage of the data obtained. Sequences from this study (●) are compared to those

- 1 from human (▲), wild boar (■) and others from swine (○). The different clusters were
- 2 the sequences from this study were grouped are indicated by the boxes.

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1 Table 1. Results of HEV RNA detection in serum, bile, liver, mesenteric lymph nodes  
 2 and faeces of necropsied animals and hepatic lesions by weeks of age (number of  
 3 positive /number of tested samples).

Weeks	Number of positive samples/number of samples tested						Total
	3	6	9	12	15	18	
Serum	1/3	1/5	0/5	3/5	3/5	0/5	8/28
Bile	0/3	0/5	0/5	5/5	5/5	0/5	10/28
Liver	0/3	0/5	0/5	4/5	4/5	2/5	10/28
M. Lymph	0/3	0/5	1/5	4/5	4/5	0/5	9/28
Faeces	0/3	0/5	1/5	5/5	5/5	2/5	13/28
Hepatitis lesions							
Mild	0/3	5/5	3/5	1/5	3/5	5/5	17/28
Moderate	0/3	0/5	0/5	4/5	2/5	0/5	6/28

4