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1 **Pigs orally inoculated with swine hepatitis E virus are able to infect contact**
2 **sentinels**

3
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20 Key words: hepatitis E virus (HEV), swine, experimental infection, oral inoculation, contact
21 sentinels

27 **Abstract**

28 The purpose of the present study was to explore the most likely natural route of infection of
29 swine hepatitis E virus (HEV) by oral inoculation of pigs and to investigate the potential
30 infection by direct contact exposure. A preliminary experiment was performed to assess
31 the infectiousness of the bile used as source of virus. Once confirmed, sixteen pigs were
32 inoculated via oral drop with an HEV positive bile suspension containing 2×10^5 genome
33 equivalents per pig. Nine animals were kept as contact sentinels and twelve more pigs
34 were used as negative controls. A number of pigs from the three groups were euthanized
35 at 16, 32 and 64 days post-inoculation. From the HEV inoculated group, three pigs shed
36 virus in faeces, two had virus RNA in bile at necropsy and two seroconverted. In the
37 contact group, two animals showed presence of HEV RNA in bile. This study demonstrates
38 that pigs orally inoculated with a single HEV dose got infection, although few animals had
39 evidence of infection. Moreover, the virus was successfully transmitted to direct contact
40 exposed pigs.

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53 **1. Introduction**

54 Hepatitis E virus (HEV) infection in humans is one of the most important causes of acute
55 clinical hepatitis in developing countries. Transmission of the virus occurs primarily by the
56 faecal-oral route through contaminated drinking water in areas with poor sanitary
57 conditions. Although the case fatal rate is generally low (<1%), it can reach up to 25% in
58 pregnant women (Kumar et al., 2004).

59 The existence of sporadic HEV infection cases in individuals of industrialized countries
60 with no history to travel to endemic areas has suggested the possibility of an animal
61 reservoir (Pina et al., 2000; Pavio et al., 2008). After the first description of swine HEV in
62 USA (Meng et al., 1997), sequence analyses have shown high nucleotide identity between
63 swine and human isolates from the same geographic area (Huang et al., 2002). The facts
64 that human clinical hepatitis E has been associated with consumption of undercooked
65 meat products (Tei et al., 2003; Li et al., 2005) and swine HEV strains can infect non-
66 human primates (Meng et al., 1998b) provide more evidence that HEV should be
67 considered an agent with zoonotic potential.

68 The lack of a practical animal model has hindered a better knowledge of human HEV
69 infection mechanisms. Different experimental infections have been done in swine, most of
70 them using the intravenous (IV) inoculation as the route of exposure. In an experiment
71 performed in 1998 (Meng et al., 1998a), pigs got infection after IV inoculation of a swine
72 strain of HEV and were able to infect non-inoculated contact animals. In the same study,
73 pigs could not become infected by some human HEV strains (Mex-14 and Sar-55).
74 However, in a subsequent study from the same research group, another human strain
75 (US-2) was infectious for pigs (Meng et al., 1998b). Following these studies, a comparison
76 of pathogenesis of pigs infected with swine and human strains of HEV was performed
77 (Halbur et al., 2001). When pigs are inoculated IV, liver seems to be the primary site of
78 virus replication (Lee et al., 2008). Then, the virus can be accumulated into the gallbladder,

79 probably as a consequence of HEV liver replication in large amounts. The virus seems to
80 be excreted into the bile duct and finally shed in faeces. Williams et al. (2001) showed the
81 existence of extrahepatic sites of HEV replication, including the small intestines, colon, and
82 hepatic and mesenteric lymph nodes, as the most important tissues.

83 As previously said, almost all experimental studies focused on swine HEV have used the
84 IV inoculation as the route of infection. Taking into account that the most likely natural
85 route of transmission among pigs is the faecal-oral one (Meng and Halbur, 2006; de Deus
86 et al., 2008), the pathogenic events that occur under natural conditions are not really
87 known. Few experimental infections have used oral inoculation as the route of exposure.
88 The first one was reported in 1990 by Balayan et al. (1990), in which two young piglets
89 were inoculated with a 1:10 faecal suspension containing a human HEV strain of genotype
90 1. Even though they attributed the infection to the virus inoculated from the human stool,
91 further studies demonstrated that those pigs were already infected with a swine HEV strain
92 of genotype 3 (Lu et al., 2004). In 2002, Kasorndorkbua et al. tested the faecal-oral route
93 infecting two pigs, one inoculated with a stomach tube and the other using an oral drop
94 system. None of them got infection although the inoculum contained a virus titer of 10^6
95 genome equivalents-GEs/ml. In a subsequent study from the same research group using
96 the oral route of inoculation (Kasorndorkbua et al., 2004), three pigs were inoculated by an
97 oral gavage with 15 g of fresh swine faeces for 3 consecutive days. Only one animal shed
98 virus in faeces (from 21 to 35 days post-inoculation (dpi)) and seroconverted by 56 dpi. No
99 direct contact exposure was evaluated in this work. Besides, the oral route of transmission
100 has also been investigated using manure slurry as inoculum, although no evidence of
101 infection was obtained (Kasorndorkbua et al., 2005).

102 Taking into account the poorly documented infectiousness of HEV exposed orally to pigs,
103 the objectives of the present study were to explore the most likely natural route of infection

104 by oral inoculation of a larger group of pigs and to investigate the possibility of infection by
105 direct contact to sentinel animals.

106

107 **2. Materials and methods**

108 **2.1. Virus inoculum**

109 The swine HEV inoculum used in this study was prepared from bile of a naturally infected
110 pig. The bile was tested positive for HEV by a semi-nested RT-PCR as described
111 previously (Martín et al., 2007) and had a titer of 10^6 GEs/ml. The phylogenetic analysis
112 revealed that the HEV strain used belonged to genotype 3 (Peralta et al., 2008a). A
113 second bile, negative by semi-nested RT-PCR, was used as negative control. Biles were
114 kept at -80°C until used.

115

116 **2.2. Experimental design**

117 **2.2.1. Preliminary infectivity study**

118 In order to test the infectivity of the inocula, four 2-week-old piglets with very low values of
119 IgG antibody to HEV measured by an in-house ELISA (Martín et al., 2007) and free of
120 swine HEV in faeces and blood were housed in a biosecurity level 3 (BL-3) facility. After
121 one week of acclimatization, each piglet was inoculated IV with 1 ml of a 1:5 bile
122 suspension (infectious dose: 10^5 GEs/ml) in physiological saline solution. Clinical signs
123 and rectal temperature were recorded every 2 to 3 days through a 28 day-period. Serum
124 and rectal swabs were taken twice a week to be analyzed by a semi-nested RT-PCR
125 (Martín et al., 2007). Serum samples were also analyzed by ELISA to detect anti-HEV IgG
126 antibodies. Bile collected at the necropsy day (28 dpi) was also tested to detect the
127 presence of HEV RNA. All samples were stored at -80°C until analysis.

128

129 **2.2.2. Transmission study**

130 Thirty-seven 2-week-old piglets with no or very low HEV IgG antibody OD ELISA values
131 and negative by semi-nested RT-PCR to the virus in blood and faeces were included in the
132 study. Pigs were kept together for 1-week acclimatization period in the same experimental
133 box in a BL-3 facility until the moment of challenge (0 dpi). Then, animals were divided in
134 two groups (group A, n=25; group B, n= 12), taking into account the ELISA OD values of
135 each animal; therefore, no statistical significant difference in mean OD between groups
136 were observed. Sixteen animals of group A were inoculated orally using a syringe with 2
137 ml of a 1:10 suspension (infectious dose: 10^5 GEs/ml) of a RT-PCR positive bile in
138 physiologic saline serum. The remaining nine pigs were kept as sentinels (contact
139 controls) and were inoculated with 2 ml of 1:10 suspension of a RT-PCR negative bile in
140 physiologic saline serum. Pigs from group B (negative controls) received orally the same
141 inoculum as the sentinels.

142 Clinical signs and rectal temperature were monitored every 2 to 3 days until the end of the
143 experiment at 64 dpi. Five or 6 pigs from the inoculated group, 3 from the contact group
144 (sentinels) and 4 from the control group were randomly euthanized at 16, 32 and 64 dpi.
145 Serum and faecal samples were collected twice a week to be analyzed by semi-nested
146 RT-PCR; bile obtained at necropsy was also tested for the mentioned technique. Serum
147 samples were studied for anti IgG HEV antibodies by ELISA. Faecal samples were
148 collected directly from the rectum and diluted 1:10 in PBS. After vigorous vortexing, faecal
149 samples were centrifuged at $16.000 \times g$ for 30 min and supernatants were taken to
150 perform RT-PCR. All samples were stored at -80°C until further use. Liver samples
151 obtained at necropsy were also taken and fixed by immersion in 10% neutral-buffered
152 formalin for histopathological studies.

153 Both experiments were performed in accordance with the guidelines of the Good
154 Experimental Practices (GEP), under the supervision of the Ethical Welfare Committee on

155 Human and Animal Experimentation of the Universitat Autònoma de Barcelona (CEEAH),
156 Spain.

157

158 **2.3. Detection of anti-HEV antibodies**

159 Serum and bile used as inoculum samples were tested for specific anti-HEV IgG
160 antibodies by an in-house enzyme-linked immunosorbent assay (ELISA) previously
161 described (Martín et al., 2007). This assay was performed using a purified ORF2 truncated
162 protein as antigen, which was obtained from a local strain of swine HEV genotype 3 that
163 was expressed into recombinant baculovirus (Peralta et al., 2008b). To minimize the
164 background on the ELISA results, each serum was analyzed both in coated and un-coated
165 wells. The working solution for the anti-IgG conjugate was 1:40.000 (Serotec). The
166 reaction was stopped with H₂SO₄ 2M and the optical density (OD) of each plate was read
167 at 450 nm. Samples that had an OD value (*antigen-coated well OD value less uncoated*
168 *well OD value*) equal or greater than the cut-off value (0.30) were considered positive
169 (Martín et al., 2007). All positive ELISA results were further tested by a Dot-blot test as
170 previously described (Casas et al., 2008).

171

172 **2.4. Detection of HEV RNA**

173 Total RNA from serum, bile, rectal swabs and faecal suspension was extracted with
174 Nucleospin® RNA virus kit (Macherey-Nagel), reverse-transcribed, and subjected to a
175 semi-nested RT-PCR as previously described (Martín et al., 2007). Sequencing analysis
176 was performed on all resulting positive samples to verify that the HEV strain found in pigs
177 was the same as the inoculated one. The resulting sequences were also compared with
178 the one used as positive control in the RT-PCR to check that no cross contamination
179 occurred. Serial dilutions of serum, faeces and bile were used to determine the titer of viral
180 genomes by semi-nested RT-PCR. Viral concentrations were expressed as GE per

181 microliters of sample (serum, faecal suspension or bile) as described elsewhere (Meng et
182 al., 1998a).

183

184 **2.5. Histopathological studies**

185 Fixed liver samples were dehydrated, embedded in paraffin and stained with hematoxylin
186 and eosin. Microscopic analysis was performed with special emphasis for the presence or
187 absence of hepatitis, the main lesion found in HEV infected pigs (Meng et al., 1997; de
188 Deus et al., 2007). Hepatic lesions were scored following a previously described staging
189 system (from I to IV) proposed by Rosell et al. (2000) to evaluate liver inflammation
190 associated to porcine circovirus type 2 infection.

191

192 **3. Results**

193 **3.1. Preliminary infectivity study**

194 All four pigs inoculated IV with the swine HEV inoculum remained clinically healthy during
195 the whole experimental period. Rectal temperatures remained within the normality rank
196 throughout the study with an average (avg) of 39.7°C (standard deviation (SD): 0.22).

197 RT-PCR results of HEV RNA detection in faeces and bile are shown in Table 1. Three out
198 of 4 animals became infected. HEV was firstly detected in faeces on 3 dpi in animal A and
199 on 7 dpi in animal B. Faecal shedding of pig A lasted for 1 week and only 4 days for pig B.
200 Viraemia was not detected in any studied animal. HEV RNA was not detected in faeces of
201 pig C, although virus genome was detected in bile at necropsy (28 dpi). The sequencing of
202 all RT-PCR products demonstrated a 100% nucleotide identity with respect to the
203 inoculated virus. Pig D did not show any evidence of HEV infection by RT-PCR.

204 Pig A seroconverted to anti-HEV IgG by 21 dpi (confirmed by ELISA and Dot-blot). No
205 other pig seroconverted at any time of the experimental period. Bile used as inoculum did
206 not contain specific anti-HEV IgG antibodies.

207 None of the animals had significant macroscopic and microscopic lesions.

208

209 **3.2. Transmission study**

210 Control animals remained healthy during the whole experimental period. Pigs from the
211 inoculated and contact group did not show clinical signs attributable to HEV during the
212 experiment; however five animals, three from the inoculated group and two from the
213 contact group, showed pyrexia and different clinical signs (prostration, difficult breathing
214 and shivers), dying during the first 3 weeks of experiment. Rectal temperatures remained
215 within normality in all the remaining animals: inoculated group, avg: 39.48°C / SD: 0.27;
216 sentinel group, avg: 39.52°C / SD: 0.35; control group: avg: 39.54°C / SD: 0.4.
217 Bacteriological culture of different swabs (from cerebrospinal fluid and pericardium,
218 thoracic and abdominal cavities) obtained at necropsy, revealed the presence of colonies
219 compatibles with common pathogens of swine farms such as *Haemophilus parasuis*,
220 *Streptococcus suis* and *Escherichia coli* (data not shown).

221 No significant gross lesions were observed in any of the pigs from all three experimental
222 groups (besides those that died because of systemic bacterial infections, which showed
223 fibrino-purulent meningitis, polyserositis and/or polyarthritis). Slight perilobular or periportal
224 hepatic fibrosis was histologically seen in animals 16 (inoculated) and 59 (contact),
225 respectively. Slight or mild periportal hepatitis (stage I of Rosell et al., (2000)) was
226 observed in pigs 17 and 89 (inoculated), 59 (contact) and 82 (control). No microscopic
227 hepatic lesions were seen in the remaining pigs.

228 Some animals had antibody titers of IgG at the beginning of the study: 8 from the HEV
229 inoculated group; 1 from the contact group and 3 from the control group. Most of these
230 animals lost maternal antibodies after 8 dpi.

231 A total of 4 out of 16 pigs presented one or more evidences of HEV infection (Table 2).

232 Three pigs from the HEV inoculated group shed virus in faeces, none of them had

233 detectable viraemia, two had virus RNA in bile at the day of necropsy and two of them
234 seroconverted to IgG anti-HEV antibodies (confirmed by ELISA and Dot-blot).
235 Nevertheless, these animals were able to infect contact sentinels; two contact pigs got
236 infection, with presence of HEV in bile. None of the contact pigs had virus faecal shedding,
237 viraemia or seroconversion at 64 dpi. Finally, control pigs remained seronegative
238 throughout the study. None of them had HEV RNA in faeces, blood or bile during all the
239 experimental period. Positive RT-PCR products showed a 100% nucleotide identity with
240 respect of the inoculated virus. Faecal shedding was detected between 22 and 36 dpi
241 (Table 2). Only one animal (pig 87) had HEV RNA detectable in faeces during different
242 time points of the experiment. Pig 94 (euthanized at 16 dpi) had detectable virus in bile,
243 but it was never recorded as positive in faeces. Pig 16 died at 22 dpi and was HEV RT-
244 PCR positive both in bile and faeces at necropsy; serial dilutions of positive bile and faeces
245 of this pig revealed that the genomic titer of HEV in bile (10^3 - 10^4 GEs/ml) was higher than
246 that in faeces (10 GE/ml). Seroconversion of inoculated pigs appeared 43 days after
247 challenge onwards. In the contact group of pigs, virus was only detected in bile of two
248 animals euthanized at 64 dpi.

249

250 **4. Discussion**

251 Porcine HEV was firstly described in 1997 (Meng et al., 1997); since then, several
252 experimental studies have been performed to clarify the pathogenesis of this viral infection.
253 Most of them used IV inoculation although oral HEV exposure would be the most natural
254 route of infection. Few experimental infections have been performed using the latter route
255 of exposure, clearly indicating the difficulty to cause HEV infection (Kasorndorkbua et al.,
256 2002, 2004, 2005). The present report further investigated the oral route of administration
257 of HEV, demonstrating its feasibility, and describes for the first time the successful
258 transmission of HEV from orally inoculated pigs to sentinel ones. Other experiments

259 showed that pigs with direct contact exposure to positive controls got also infection, but
260 those controls were inoculated IV (Meng et al., 1998a; Kasorndorkbua et al., 2004;
261 Bouwknecht et al., 2008).

262 Most of HEV experimental infections have mainly used faeces from infected pigs as
263 inocula (Meng et al., 1998b; Kasorndorkbua et al., 2002, 2004; Lee et al., 2008). In
264 contrast, this study used bile from a naturally infected pig; this animal had higher amount
265 of HEV genome copies in bile than faeces (data not shown). The used bile was previously
266 tested by ELISA to ensure that specific IgG antibodies that may hinder HEV infection
267 progress were not present.

268 The preliminary study performed demonstrated that the bile recovered from a naturally
269 infected animal was infectious for pigs when inoculated IV. Two of the pigs (A and B) got
270 infection because of the IV inoculation of the bile; however, a third pig only showed
271 evidence of infection by RT-PCR at the end of the experiment, suggesting that infection
272 probably occurred by oro-faecal transmission due to contact with the other two. The lack of
273 virus detection in faeces and blood of this animal previous to its detection in bile may
274 suggest that liver is the primary site of HEV replication, as described in other IV
275 experimental infection (Lee et al., 2008); therefore, bile would be one of the most important
276 samples to find the virus, as has also been suggested under natural infection conditions
277 (de Deus et al., 2007). It is worthy to note that a fourth animal did not show evidence of
278 infection during the whole experimental period, which suggests an inadequate IV
279 inoculation but also an apparent difficulty of horizontal transmission from infected pigs.

280 Faecal-oral route is believed to be the natural route of HEV transmission among pigs
281 (Meng and Halbur, 2006; de Deus et al., 2008). In fact, a recent study demonstrated the
282 potential of HEV to cause epidemics in populations of pigs since the basic reproduction
283 ratio (R_0) for contact-exposure was estimated to be 8.8 (Bouwknegt et al., 2008). In the
284 present study, pigs could be infected orally and they were able to infect animals in contact.

285 A previous study demonstrated that faeces containing a high titer of HEV (10^6 GEs/g) was
286 able to infect animals when they were inoculated orally, but three inoculations of 15 g of
287 faeces each were necessary to infect only one out of 3 animals (Kasorndorkbua et al.,
288 2004). Although the present work has reproduced HEV infection successfully by oral
289 inoculation, the infection rate was less efficient than when using IV inoculation, and orally
290 inoculated animals showed faecal shedding and seroconversion later compared with IV
291 inoculations (Tsarev et al., 1994; Kasorndorkbua et al., 2004, 2005). Moreover, in the
292 study cited above, pigs inoculated IV with an HEV dose of 10^6 GEs shed the same
293 concentration of viral particles in faeces (Kasorndorkbua et al., 2004). In contrast, pigs
294 from the present study inoculated orally with an HEV dose of 10^5 GEs excreted much
295 lower concentrations of virus in faeces (data not shown).

296 McCaustland et al. (2000) described that multiple variables may influence the outcome of
297 HEV infection: virus titer, the ratio of infectious to defective particles, the route of infection
298 and host factors such as immune status and age of exposure. The present results showed
299 that a single dose of 10^5 GEs is insufficient to induce a large infection rate, since only four
300 out of 16 inoculated pigs got measurable infection. Moreover, these four pigs could only
301 infect two contact animals. Thus, it remains unclear whether oral route explains the high
302 incidence of HEV infection in pig population (Meng and Halbur, 2006; Leblanc et al., 2007;
303 Satou and Nishiura, 2007; Seminati et al., 2008). Some studies attributed this difference
304 between experimental and natural conditions to the repeated exposure of animals to HEV
305 that is probably needed for a successful transmission (Kasorndorkbua et al., 2002, 2004,
306 2005). On the other hand, pigs kept under experimental facilities imply box cleaning every
307 day and, therefore, pigs probably do not have the same HEV infectious pressure as pigs in
308 conventional farms where slurry is not often removed during all the growing period.
309 Another point that could influence the success of infection is a possible strain-dependent

310 infectivity, since many different subtypes have been described within genotype 3. Even
311 though, this question has not yet been clarified (Chobe et al., 2005).

312 How host factors influence HEV infection when pigs are inoculated orally is still not known
313 (Kasorndorkbua et al., 2004). In the present study, all 16 inoculated pigs received the
314 same dose, but a variety of responses was seen. Several facts might explain such
315 response variability to infection. On one hand, conventional pigs usually have remaining
316 humoral immunity of maternal origin. In the present study, half of these inoculated animals
317 had residual IgG antibodies at the beginning of the study. Although this fact would
318 apparently be detrimental to achieve infection, 3 out of the 4 pigs that got infection (No. 87,
319 94 and 95) had some residual HEV antibodies, while only one of them was seronegative to
320 the virus. On the other hand, it has been described that host mucosal immunity in the
321 alimentary tract could play a role in the susceptibility to HEV infection (Kasorndorkbua et
322 al., 2002). It is feasible that IgA may neutralize the virus on mucosal surfaces (Tizard,
323 2000), which would explain the highest efficiency of IV inoculations than oral ones. Recent
324 studies have demonstrated that IgA maternal antibodies can be transferred to piglets (de
325 Deus et al., 2008). On the other hand, at least in dogs, this immunoglobulin can be present
326 in bile to high amounts (German et al., 1998); it can not be ruled out that a strong IgA
327 positivity in bile used as inoculum might interfere the outcome of HEV infection. In the
328 present work, IgA antibodies were not determined, so it remains unknown the role of them
329 in the success of oral infections.

330 Data obtained on virus shedding and seroconversion agree with results showed in a
331 previous oral inoculation study in which only one animal got infection (Kasorndorkbua et
332 al., 2004). This animal started to shed virus in faeces from 21 dpi to 35 dpi and
333 seroconverted by 56 dpi; bile was not tested in the mentioned study. In contrast, some
334 differences are observed when comparing data from oral inoculations and IV ones. When
335 animals are inoculated IV, virus in faeces is firstly detected at 7 dpi and lasts up to 3

336 weeks. Virus in bile is firstly detected at 3 dpi and seroconversion appears only 2 weeks
337 after challenge (Halbur et al., 2001). Oral intake of HEV needs to pass through the
338 intestinal tract, which implies a delayed appearance of virus in faeces and bile as well as
339 seroconversion compared to IV inoculation (Kasorndorkbua et al., 2004; Bouwknecht et al.,
340 2008). Moreover, the same dose given by one or the other route show different
341 bioavailability, which means that the real amount of virus inoculated *per os* reaching the
342 systemic circulation, can be rather low.

343 It has been described that HEV RNA is more frequently detected in faeces and for longer
344 periods than in blood (Wu et al., 2002; de Deus et al., 2007; Leblanc et al., 2007).
345 Therefore, to certain degree, it should not be surprising that viraemia was not detected in
346 the present study. It is possible that a localized HEV replication in the intestines, low
347 amounts of HEV particles in serum as a consequence of the low infection pressure, or an
348 intermittent viraemia could explain the negative results of RT-PCR of studied pigs in sera.
349 Transmission of HEV infection to contact sentinels appeared approximately 1 month after
350 HEV-excretion occurrence in inoculated pigs. In an experimental infection performed to
351 quantify the transmission of HEV among pigs showed that this period was only 6 days for
352 contact sentinels exposed to pigs previously in contact with IV infected animals
353 (Bouwknegt et al., 2008). This result is in contrast with data from the present study, but the
354 source of infection (IV versus oral) may explain a much lower infection pressure for the
355 latter inoculation route, thus, reducing the probability of infection and prolonging the period
356 between exposure and infection.

357 The histopathological liver lesions observed in only five animals were slight and could not
358 have any relation to virus detection since only one of these pigs got infection. In fact, it has
359 been described that mild, non-suppurative hepatitis can be considered as normal
360 background of pig liver (Halbur et al., 2001). In regards the five animals that died naturally
361 during the second experiment, they probably were carriers of common pathogens from the

362 farm origin and, when specific maternal immunity waned, bacteria were able to cause
363 disease in a limited number of pigs.

364 In conclusion the results from this study demonstrated that pigs orally inoculated with a
365 single HEV dose of 10^5 GEs got infection. Although few animals had evidence of infection,
366 thus confirming the more difficult reproduction of an oral HEV infection than IV one, those
367 animals were able to successfully transmit the virus to direct contact exposure sentinels.

368

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375

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1 TABLE 1. Viremia, fecal shedding and serological OD values in pigs inoculated
 2 intravenously from the preliminary infectivity study

Pig	Virus/antibody detection ^a		Days post inoculation						
			0	3	7	10	14	21	28
A	Virus	Serum	-	-	-	NT	-	-	-
		Feces	-	+	+	+	-	-	-
		Bile	NA	NA	NA	NA	NA	NA	-
	Antibodies		0.18	0.11	0.08	0.04	0.10	1.08	0.33
B	Virus	Serum	-	-	-	NT	-	-	-
		Feces	-	-	+	+	-	-	-
		Bile	NA	NA	NA	NA	NA	NA	-
	Antibodies		0.29	0.09	0.32	0.21	0.14	0.11	0.05
C	Virus	Serum	-	-	-	NT	-	-	-
		Feces	-	-	-	-	-	-	-
		Bile	NA	NA	NA	NA	NA	NA	+
	Antibodies		0.24	0.27	0.44	0.19	0.18	0.13	0.08
D	Virus	Serum	-	-	-	NT	-	-	-
		Feces	-	-	-	-	-	-	-
		Bile	NA	NA	NA	NA	NA	NA	-
	Antibodies		0.33	0.26	0.19	0.16	0.12	0.05	0.05

3 ^a -, negative; +, positive; NT, not tested; NA, non-available; cut-off value ≥ 0.30

4 TABLE 2. HEV RNA detection in feces and bile, and HEV IgG presence (OD values) in orally inoculated and contact
 5 sentinel pigs showing evidence of infection during the experimental period

Group	Pig	Virus/antibody detection ^a	Days post inoculation															
			0	3	8	11	16	22	25	29	32	36	43	50	57	64		
Inoculated	16	Virus	Feces	-	-	-	-	-	-	+								
			Bile	NA	NA	NA	NA	NA	+									
		87	Antibodies		0.02	NT	0.05	NT	0.01	0.01								
	Virus			Feces	-	-	-	-	-	-	+	-	+	+	-	-	-	-
				Bile	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-
			Antibodies		0.56	NT	0.39	NT	0.17	0.10	NT	0.04	NT	0.21	0.33	0.04	1.18	1.31
		94	Virus	Feces	-	-	-	-	-									
	Bile			NA	NA	NA	NA	+										
			Antibodies		0.36	NT	0.30	NT	0.09									
		95	Virus	Feces	-	-	-	-	-	+	-	-	-	-	-	-	-	
	Bile			NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-
			Antibodies		0.44	NT	0.14	NT	0.06	0.07	NT	0.04	NT	0.04	0.13	0.20	0.41	0.22
Sentinels	40	Virus	Feces	-	-	-	-	-	-	-	-	-	-	-	-	-		
			Bile	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	
			Antibodies		0.04	NT	0.03	NT	0.03	0.03	NT	0.03	NT	0.02	0.02	0.02	0.02	0.02
		99	Virus	Feces	-	-	-	-	-	-	-	-	-	-	-	-		
	Bile			NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	
			Antibodies		0.55	NT	0.33	NT	0.24	0.13	NT	0.08	NT	0.04	0.03	0.02	0.02	0.10

6 ^a -, negative; +, positive, NT, not tested; NA, non-available; cut-off value ≥ 0.30 .

7