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1 **Title: Anti-HEV antibodies in domestic animal species and rodents from Spain using a**
2 **genotype 3-based ELISA**

3

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18 **Short title:** Anti-HEV IgG in domestic animals and rodents

19 **Key words:** HEV, domestic animals, rodents, ELISA, genotype 3

20

21 **Abstract**

22 A truncated ORF2 capsid HEV antigen derived from a genotype 3 strain was developed in
23 insect cells and insect larvae, and compared with the Sar55 antigen and a commercial ELISA.
24 The antigen expressed in insect cells showed a better correlation with Sar55 ($kappa$ value
25 (k)=0.84) than the insect larvae antigen (k =0.69), and a better reproducibility as indicated by the
26 intra and interplate variation coefficients. Commercial ELISA designed for human diagnosis but
27 adapted to animal use using specific secondary antibodies demonstrated to have a very low
28 sensitivity. The insect cell expressed antigen was used to develop an ELISA to detect antiHEV-
29 IgG in serum samples of different domestic animal and rodents. Seropositivity in the studied
30 animal populations was of 71.4% for pigs, 0.60% for goats, 1.92% for sheep, and 11.11% for
31 cats. None of the 1170 cattle samples or 166 rodent samples analyzed was positive.

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42 1. INTRODUCTION

43 Hepatitis E virus (HEV) is a small non-enveloped virus belonging to the Genus *Hepevirus*
44 (Emerson et al., 2004), proposed as the *Hepeviridae* family. The virus is the causative agent of
45 hepatitis E in humans. Four HEV genotypes have been described so far (Lu et al., 2006) with
46 several sub-genotypes that seem to have a geographical distribution. However, only one
47 serotype has been identified. Genotype 1 is common in Asia, particularly in the Indian
48 subcontinent (Arankalle et al., 1999). Genotype 2 was originally detected in Mexico but later on
49 has been described in Africa (Huang et al., 1992; Buisson et al., 2000). Genotype 3 strains are
50 present in Europe, America, Asia and Oceania and genotype 4 strains seem to be autochthonous
51 of Asia (Nishizawa et al., 2003). Human hepatitis E is endemic in many developing countries
52 where epidemics caused by genotypes 1 and 2 are common and usually associated with
53 contaminated drinking water. In industrialized countries, hepatitis E appears most often as
54 sporadic cases either related to travelling to endemic areas or caused by autochthonous strains
55 (Péron et al., 2006). Several studies have suggested that HEV might be a zoonotic agent. Thus,
56 it has been shown that exposure to domestic pigs could be a risk factor for seropositivity in
57 humans (Meng et al., 2002), human and swine strains of genotypes 3 and 4 seem to be closely
58 related (Herremans et al., 2007; Wibawa et al., 2007) and some documented sporadic cases of
59 hepatitis E in humans have been related to the consumption of uncooked or undercooked meat
60 or viscera of wild boars or deers (Takahashi et al., 2004; Tei et al., 2004). In those cases,
61 genotype 3 was involved. In Europe sporadic cases of human hepatitis E are mostly related to
62 genotype 3 of the virus. Recent studies showed that up to 97% of the pig herds studied in some
63 European countries including Spain (Rutjes et al., 2007; Seminati et al., 2008) have HEV
64 seropositive animals and porcine HEV isolates in European pigs belong to genotype 3 (Van der
65 Poel et al., 2001; Clemente-Casares et al., 2003; de Deus et al., 2007).

66

67 In addition to pigs and deers, so far HEV has been detected only in horses (Saad et al., 2007);
68 mongooses (Nakamura et al., 2006) and chickens (Haqshenas et al., 2001); in the last case, the
69 avian strains seem to belong to a different HEV species or, at least, to a different genotype.

70 Antibodies against HEV have been also demonstrated in species other than the abovementioned
71 such as cows, sheeps, goats, dogs, cats and rodents (Tien et al., 1997; Arankalle et al., 2001;
72 Okamoto et al., 2004; Vitral et al., 2005; Mochizuki et al., 2006; Zhang et al., 2008) but the
73 epidemiological role of those domestic species is uncertain. Most of these serological studies in
74 domestic animals other than pigs have been carried out in Asia and information about Europe is
75 still lacking.

76 In the current study, we developed and applied an ELISA test based on the truncated ORF2
77 capsid protein from a genotype 3 strain for the screening of serological evidence of HEV
78 infection among various domestic animal species.

79

80 **2. MATERIALS AND METHODS**

81 **2.1. Expression of a truncated HEV ORF2**

82 2.1.1. Virus sample and ORF2 amplification

83 One HEV RT-PCR positive bile sample collected from a 14-weeks-old pig was selected and
84 stored at -80°C until used. Total viral RNA was extracted from 150 µl of bile with Nucleospin®
85 RNA virus kit (Macherey-Nagel GmbH & Co., Düren, Germany) and the full length HEV ORF2
86 was amplified by means of a two round PCR. Primers were primers F5000 (5'-
87 AATGTKGCKCAGGTYTGTG-3') and R7260 (5'-
88 TTTTTTTTTTTTTCCCKGGGRGCGCG-3') for the first round of amplification, and
89 primers F5160 (5'-MGGSTRGAATGAATAACATG-3') and R7260 were used for the
90 seminested amplification (Peralta et al., 2008). The second round PCR product was cloned
91 into pCR®II-Blunt-TOPO® (Invitrogen).

92

93 2.1.2. Generation of HEV ORF2 recombinant baculovirus

94 Generation of the recombinant baculovirus containing the truncated HEV ORF2 was done using
95 the Bac-to-Bac Baculovirus Expression System (Invitrogen) following manufacturers's
96 instructions. Briefly, a set of primers was designed to amplify a fragment of 1,491 bp: the

97 forward primer For*Rsr*IIHEV (5'-
 98 ATATATTCGGWCCGATGGCTGTTTCGCCTGCGCCTGATACGGCC-3') containing the
 99 *Rsr*II restriction site and the initiation codon, and the reverse primer Rev*Hind*IIIHEVHist (5'-
 100 TATAAGCTTCTATTAGTGATGGTGATGGTGATGGGCTAAAGCAGAATGCGGGGC-3')
 101 containing the *Hind*III restriction site, two stop codons and a 6xHis (H⁶⁺) tag. The N-terminal
 102 and C-terminal truncated ORF2 expressed corresponded to the minimum sequence necessary to
 103 allow virus like particles (VLPs) formation as described by Li *et al.* (2005), from aa 112 to aa
 104 607. This means that the expressed product had an N²-terminus 111 aa deletion and a C²-
 105 terminus 53 aa deletion. The PCR product was gel purified with Nucleospin® ExtractII
 106 (Macherey-Nagel), digested with restriction enzymes, and ligated into the pFASTBAC vector
 107 (Invitrogen) to yield the construction pFBAC-ORF2H⁶⁺. Then, ElectroMAX™DH5α *E. coli*
 108 cells (Invitrogen) were transformed, and the recombinant plasmids were extracted, and
 109 confirmed by PCR and fully sequencing of the insert. Competent cells MAX Efficiency®
 110 DH10Bac™ were transformed with pFBAC-ORF2H⁶⁺ for production of a recombinant bacmid
 111 BAC-ORF2H⁶⁺. Then, BAC-ORF2H⁶⁺ was extracted following standard methods and
 112 transfected into Sf9 cells using Lipofectamine™ (Invitrogen). Cells were cultured in Grace's
 113 insect media (Gibco BRL) supplemented with 10% foetal bovine serum (FBS), 3% non-
 114 essential amino acids, 100 µg/ml of streptomycin and 100 U/ml of penicillin and 20 µg/ml
 115 gentamycin and grown at 27°C. At 96 hours post-transfection the supernatant containing the
 116 recombinant baculovirus was recovered and stored at 4°C.

117

118 2.1.3. Truncated ORF2 expression in insect cells and insect larvae

119 The recombinant baculovirus was used to infect Sf9 cells using a multiplicity of infection
 120 (MOI) of 0.05. Infected cells were incubated for 6 days (the optimal conditions for recovery of
 121 the HEV protein as determined in previous assays). Total protein was extracted by collecting the
 122 infected cells and incubating them in milliQ water for 15 min in ice followed by a
 123 centrifugation at 2500xg for 10 min. The recombinant protein ORF2H⁶⁺ contained in the

124 supernatant was purified using the kit Protino Ni-IDA packed columns (Macherey-Nagel)
125 following manufacturer's instructions. The recovered protein was stored at 4°C.

126 Alternatively, *Trichoplusia ni* larvae were grown and inoculated as previously described (Medin
127 et al., 1995; Pérez-Filgueira et al., 2006). The inocula consisted in 5×10^5 recombinant virus
128 particles (BAC-ORF2H⁶⁺) or wtBAC (control baculovirus). Three days post infection, the larvae
129 were frozen and homogenized in an extraction buffer to obtain total soluble proteins (TSP),
130 including our recombinant ORF2H⁶⁺ protein, as previously described (Pérez-Filgueira et al.,
131 2006). The amount of TSP contained in the supernatant was quantified by Bradford assay
132 (Bradford, 1976), and lyophilised for long time storage (Pérez-Filgueira et al., 2006). The same
133 protocol was followed for the control protein extract (Ni), obtained from the Bac-Ni infected
134 larvae. (Pérez-Filgueira et al., 2006).

135

136 2.1.4. Analysis and quantification of recombinant protein production

137 The purified ORF2H⁶⁺ and the protein extract obtained from *T. ni* larvae were run in duplicate
138 in NuPAGE® Novex 4-12% Bis-Tris gels (Invitrogen). One of the gels was stained with
139 Coomassie blue and after confirmation of the presence of the protein, the other was used for
140 Western Blot analysis (WB). After transfer, nitrocellulose membranes were blocked for 1h at
141 room temperature with PBS added with 0.02% Tween-20 and 2% skim milk. A swine anti-HEV
142 hyperimmune sera obtained from an experimentally infected pig was used (provided by Dr. X.J.
143 Meng, CMMID, Virginia Tech). The antigen-antibody reaction was revealed by using a protein
144 A-peroxidase conjugate (Sigma) and 4-chloronaftol solution (Sigma) as a substrate. Protein
145 quantification was performed by means of the BCA protein Assay Kit (Pierce) following
146 manufacturer's directions. The autochthonous truncated ORF2H⁶⁺ was compared with the Sar55
147 strain derived protein at nucleotide and aminoacid level and the hydrophobicity profile was
148 predicted by means of the bioinformatic program BioEdit (Hall, 1999) using the Kyte and
149 Doolittle scale.

150

151 **2.2. Development of an ELISA for IgG detection using the protein obtained from insect**
152 **cells and insect larvae as an antigen**

153 2.2.1. Porcine sera samples

154 Three hundred and sixty-seven pig sera were used in this study; of them, two hundred and fifty-
155 two samples were obtained from nine different fattening farms located in the North-East Spain.
156 Other additional 85 samples were obtained from previous studies (de Deus et al., 2007, 2008)
157 and corresponded to pigs with known HEV viremia status. Finally, other 30 samples kindly
158 provided by Dr. X.J. Meng (CMMID, Virginia Tech) corresponded to experimentally infected
159 pigs or negative controls.

160

161 2.2.2. ELISA procedure

162 Ninety-six well polystyrene plates were coated with 100 µl of either the purified protein
163 recovered from Sf9 cell cultures at a concentration of 0.125µg/ml (final protein amount 12.5
164 ng/well) or with the protein extract obtained from the infected larvae at 3.5µg/ml (final protein
165 amount 350 ng/well) diluted in coating buffer (0.015M Na₂CO₃, 0.035M NaHCO₃, pH 9.6.
166 Those concentrations were determined to be optimal in previous titration experiments (not
167 shown). In order to minimize the effect of unspecific binding to the plates or to the antigen, sera
168 were analysed in duplicate in antigen-coated and mock-coated wells. For the ELISA procedure,
169 after coating the plates for 18h, wells were washed with PBS added with 0.02% Tween-20 (PBS
170 -T) and blocked for 1h at 37°C with blocking buffer (BB) (140µl/well; PBS, 0.035 M NaCl plus
171 0.5% gelatine and 10% FBS). After washing the BB, serum samples were diluted 1:100 in BB
172 and 100µl were dispensed per well. After 45 min of incubation at 37°C, plates were washed 5
173 times with PBS-T and a HRP-conjugated goat anti-swine IgG (Serotec Ltd., Oxford, UK) was
174 added at a dilution 1:100,000. Plates were incubated for 45 min at 37°C and washed as
175 described above. The reaction was revealed by adding 100µl of TMB (Sigma Chemical, St.
176 Louis, MO., USA) and stopped with 100 µl of H₂SO₄ 2M. Plates were read in an ELISA reader
177 at 450nm. The specific absorbance value for each sample was calculated by subtracting the
178 value of the mock-coated wells from the values of the plates coated with the specific protein.

179 Cut-off was set at 0.3 that was about 4 standard deviations above the mean OD value of the
180 negative control sera.

181

182 2.2.3. Validation of the genotype 3 ELISA antigens and comparison with a commercial kit

183 A truncated ORF2 obtained from the HEV genotype 1 Sar55 strain (Robinson et al., 1998) was
184 used in order to validate the genotype 3 ELISA antigen. Three hundred and sixty seven swine
185 samples were analyzed in coated and uncoated wells using the Sar55 protein at a concentration
186 of 0.25µg/ml (25 ng of protein per well) following the procedure described above. Also thirty
187 selected samples were tested by the test Bioelisa HEV IgG (Biokit) (intended for diagnosis in
188 humans) following manufacturer's instructions but modified for the analysis of porcine samples
189 by using an anti-swine IgG conjugate as explained above.

190

191 **2.3. Application of genotype 3 antigens for screening HEV seroprevalence in different**
192 **domestic species and rodents.**

193

194 2.3.1. Immunization of different animal species to obtain hyperimmune serum

195 In order to obtain a positive serum to be used as a positive control in the developed ELISA for
196 different species, an immunization assay was performed in cows, sheeps, goats, and rodents. For
197 that purpose, two 3-months-old cows, two adult goats, two adult sheep and five ICR-CD1 6-
198 months-old mice were housed in the Veterinary School facilities of the Universitat Autònoma de
199 Barcelona. One week after their arrival animals were injected with the HEV ORF2H⁶⁺ purified
200 recombinant protein using the dosages, adjuvants and inoculation routes summarized in Table 1.
201 For each group one animal was kept as a negative control inoculated with sterile saline solution
202 with the adjuvant. Blood samples were periodically obtained by jugular puncture in the case of
203 the ruminants and by tail incision in mice. The procedures were performed in accordance with
204 the guidelines of the Good Experimental Practices (GEP), under the supervision of the Ethical
205 Welfare Committee on Human and Animal Experimentation of the Universitat Autònoma de

206 Barcelona (CEEAH), Spain. Blood samples were centrifuged for 5 min at 2,500 xg, and sera
207 were stored at -80°C until used.

208

209 2.3.2. Sampling in domestic animals and rodents

210 Sera from ruminants (cows, sheeps, goats) were collected in farms of Catalonia (Spain).
211 Sampling was adjusted to detect a 3% of infected herds. Only counties accounting for $\geq 2.5\%$ of
212 the livestock census were taken into account. At the end, serum samples were collected from
213 242 herds accounting for 1,170 cows, 1,357 sheeps and 1,143 goats. Cat samples (n=54) were
214 collected from seven cat shelters located in urban areas near Barcelona. Sera from wild rodents
215 (n=166) were collected in rural areas in central Spain and belonged to the species *Apodemus*
216 *sylvaticus*, *Apodemus flavicolis*, *Mus musculus*, *Myodes glaerolus* and *Rattus norvergicus* .

217

218 2.3.3. ELISA for anti-HEV IgG detection

219 The ELISA was done as described above , but the secondary antibodies were HRP-conjugated
220 sheep anti-bovine IgG at a 1:5,000 dilution (Serotec Ltd., Oxford, UK) for cow samples; HRP-
221 conjugated donkey anti-sheep/goat IgG (Serotec Ltd., Oxford, UK) at 1:24,000 dilution for
222 sheep and goats, a protein A-peroxidase conjugate for cat samples (Sigma Chemical, St. Louis,
223 MO., USA) at 1:2,000 dilution, and a goat anti-mouse IgG (Fc specific) peroxidase conjugated
224 antibody (Sigma Chemical, St. Louis, MO., USA) at 1:80,000 for rodents. Samples were tested
225 in duplicate using the purified antigen expressed in cell culture and in both coated and uncoated
226 wells as detailed for pig samples. Cut-off were set to the mean OD plus 4x standard deviations
227 of the negative controls. Thus, the cut-offs were 0.13 for cattle, 0.24 for sheep, 0.20 for goats,
228 0.50 for cats and 0.40 for rodents.

229

230 2.3.4. Western blot and dot blot analysis

231 Confirmation of positive ELISA results was made by WB analysis using the truncated protein
232 expressed in insect cells. One μg of purified protein was run in a single well protein

233 electrophoresis gel (NuPAGE 4-12% Bis-Tris Gel 1.0mm x 2D, Invitrogen) for 1 h at 170V and
234 transferred to a nitrocellulose membrane Amersham Hybond™ ECL™ (GE Healthcare). Each
235 membrane was cut into 4mm strips and stored at 4°C until use. The Amersham™ ECL
236 Advance™ Western Blotting Detection Kit (GE Healthcare) was used following manufacturer's
237 instructions. Strips were treated with the blocking solution at 4°C overnight. After washing with
238 PBS 0.02% Tween 20, sera from cats, goats and sheep were diluted 1:1,000 in blocking
239 solution and added to the strips. Following 1 hour incubation, the strips were washed as
240 mentioned and the secondary antibody was added at the dilution determined in previous assays,
241 which resulted in 1:100,000 for goats and sheep and 1:5,000 for cats. Pig results were not
242 confirmed by WB since the infection in Spanish swine population has been reported before.
243 Chemiluminescence was detected using the fluorescence imager FluorChem® HD2 Imaging
244 System (Alpha Innotech). Sera positive in WB were re-confirmed in dot blot using both the
245 Sar55 and the ORF2-truncated protein expressed in insect cells adsorbed in native conformation
246 or after denaturation. Finally, in order to warrant specificity of the assay, we only considered as
247 positive the sera yielding positive results with both Sar55 and the ORF2H⁶⁺ protein.

248

249 2.3.5. Statistical analysis

250 Statistical analyses were done using Epi-Info 2000 v 3.4.1. Kappa value was calculated using
251 WinEpiscope software.

252

253 3. RESULTS

254 3.1. Standardization of an ELISA test for IgG anti-HEV based on the truncated ORF2H⁶⁺ 255 of genotype 3

256 3.1.1. Protein production

257 After six days of incubation, 1.65 mg of HEV ORF2H⁶⁺ were recovered from 3.6×10^7 Sf9 cells.
258 For the insect larvae expression, the amount of total protein recovered was 13 mg per larva, of
259 which about 10% was the specific protein (determined by OD comparison with the purified

260 protein). In both cases the truncated HEV ORF2H⁶⁺ had a molecular weight of 55kDa. The
261 protein produced in larvae was little soluble in water making very difficult to further purify the
262 larva extracts by the column method used in this study probably due to the formation of
263 aggregates with other molecules present in the larvae extract. In addition, storage of this extract
264 at 4°C or -20°C rapidly produced a loss of antigenic reactivity as revealed in ELISA (data not
265 shown). This did not occur with the cell culture-expressed protein.

266 Both recombinant proteins, the ORF2H⁶⁺ and the Sar55 protein, were located between
267 aminoacid positions 112 and 607, so the length was of 495 aminoacids for the Sar55 truncated
268 protein and 501 aminoacids for the ORF2H⁶⁺ protein, since a 6x histidine tail was added to the
269 3' end. Although at nucleotide level the identity was of 78%, only a difference of 5% at
270 aminoacid level was observed. The sequence of the strain used for the protein expression is
271 available at GenBank under the accession number EU723512. Moreover, the hydrophobicity
272 analysis performed revealed almost identical patterns for both proteins (data not shown).

273

274 3.1.2. Sensitivity, specificity and variability of the genotype 3 antigens

275 When the 30 sera from experimental pigs were analysed, the ORF2H⁶⁺ ELISA diagnosed
276 correctly 13/13 positive and 17/17 negative samples. Considering Sar55 ELISA results as the
277 golden standard (100% of relative sensitivity and specificity) and using the 252 field sera, the
278 truncated ORF2H⁶⁺ protein-ELISA expressed in cell cultures had a relative sensitivity of 98.9%
279 (180/182; CI_{95%}: 97.4-100%), whereas the relative specificity was 78.8% (55/70; CI_{95%}: 68.9-
280 88.2%). The ELISA set with the protein expressed in larvae showed a sensitivity of 97.8%
281 (178/182, CI_{95%}: 95.0-99.1%) and a specificity of 61.4% (43/70, CI_{95%}: 50.0-72.8%) compared
282 with the Sar55 protein ELISA. In order to know if the ELISA developed with the Spanish strain
283 of HEV genotype 3 was similar in performance to the Sar55 ELISA in terms of global
284 agreement of results, the *kappa* value was calculated. Thus, *kappa* was 0.82 (CI_{95%} 0.69-0.94%)
285 for the cell culture-expressed protein and 0.66 (CI_{95%} 0.54-0.78) for the antigen expressed in
286 insect larvae.

287

288 To further characterize the ELISA test developed, an analysis of intra- and interplate variability
289 was performed. This assay was conducted using only the 252 samples from commercial farms.
290 Samples were analyzed twice in the same plate and in different ELISA plates as described
291 above. For the cell culture expressed antigen, the results showed a 2.7% and 6.0% of intra- and
292 interplate variation, respectively and these values were 6.0% and 9.0% for the antigen expressed
293 in insect larvae.

294

295 Finally, a comparison of results with a commercially available HEV-antibody ELISA kit was
296 done. In this case, the commercial ELISA only recognized as positive pig sera yielding
297 ODs \geq 1.0 in our ELISA. Relative sensitivity and specificity were 31.25% (8.5-53.9%) and 100%
298 respectively.

299

300 **3.2. Serological survey of HEV in domestic species and rodents**

301 Positive control sera obtained by hyperimmunization with the truncated ORF2H⁶⁺ protein had
302 the following titre sin ELISA: sheep and goat sera: 1:16,000; cow and mouse sera titers were
303 1:1,000 and $>1:128,000$, respectively (Figure 1).

304 Two hundred and fifty-two pig samples collected in nine farms were examined; all nine herds
305 had HEV-seropositive pigs with an average within farm prevalence of 79.45% (\pm 19.31%). For
306 sheeps a total of 1357 samples from 89 different herds were analysed by ELISA, being positive
307 36 samples belonging to 27 farms. For goats the number of samples was 1143 form 76 herds.
308 and the ELISA revealed 18 positive sera from 16 farms. For cows, after analysing 1170 animals
309 from 77 herds, no positive results were observed. Twenty cats of 5 catteries were also
310 seropositive but none of the rodents presented antibodies.

311 Positive samples were tested in WB. For sheep, 28/33 animals of 26 herds produced a positive
312 result in this test; for goats, 14/17 positive sera (14 farms) in ELISA were confirmed in WB.
313 Regarding cat samples, due to the scarce amount of serum final confirmation was only done by
314 dot blot. Thus, final confirmation of WB positive sera by dot blot using Sar55 and the ORF2H⁶⁺
315 truncated protein either under native and denaturing conditions produced the following results:

316 for sheep, 26/28 WB positive sera were confirmed (22 herds); for goats 7/14 WB positive sera
317 were also positive with Sar55 (7 herds) and 6 cats reacted positively in WB (table 2 summarizes
318 these results). Interestingly, reactivity of sera was decreased by denaturation of Sar55 or
319 ORF2H⁶⁺ protein (figure 2) indicating that antibodies recognised primarily conformational
320 epitopes.

321

322 4. DISCUSSION

323 Several tools have been developed for HEV diagnosis. Molecular biology techniques such as
324 PCR produce the more certain results since they give proof of the presence of HEV in a tissue,
325 fluid or excreta of an animal. The main problem for the PCR detection of HEV is that, usually,
326 viremia or shedding in faeces is of short duration and thus, the chance to find a positive animal
327 is limited. In animals, other samples where the virus could be found easily, such as liver or bile,
328 are most often only available post-mortem. For this reason, serological tests such as ELISA are
329 widely used. Commercially available HEV ELISA kits for human diagnosis are usually based
330 on HEV genotype 1 or 2 peptides. Cross-reactivity among antigens obtained from different
331 genotypes seems to exist (Engle et al., 2002; Arankalle et al., 2007) and based on this property,
332 most serological studies in animals have been done using genotype 1 peptides, particularly from
333 the Sar55 strain (Meng et al., 1997; Arankalle et al., 2001; Seminati et al., 2008) However,
334 genotype 3 has demonstrated to be the most common in animals, particularly in pigs (Huang et
335 al., 2002; Ning et al., 2008). Thus, and although differences between ORF2 proteins of
336 genotype 1 and 3 seem not to be large, minor changes could affect sensitivity and specificity of
337 the test when applied to animals.

338

339 One of the aims of this study was to develop an antigen based on a genotype 3 European strain.
340 Two different expression systems were used: insect cells (a method reported to be optimal for
341 HEV (Mast et al., 1998) and insect larvae (a large scale production system). In our hands,
342 protein recovery was higher in insect larvae (about 1mg/larva) compared to insect cells;
343 however the larva protein was not possible to be purified and was little stable in cold storage.

344 Although specific experiments to clarify these facts were not conducted, the reasons for this
345 may be the formation of protein aggregates so there is no free protein with exposed epitopes
346 which antibodies can recognize or the presence of proteases in the larva extract that even at low
347 temperatures can degrade the antigenic protein. These were two serious disadvantages for that
348 protein to be used in serological tests.

349 Regarding the performance of the commercial kit, it was inefficient for the serodiagnosis in
350 animals due to poor sensitivity.

351 The performance of the two expressed proteins (cell culture and larvae-produced) was assessed
352 by examining sera from experimental infections and by comparing results with the Sar55-
353 ELISA. The protein expressed in insect cells always exceeded in performance the larvae-
354 produced protein and thus it was the protein selected for the epidemiological study. The ELISA
355 set with this cell culture-produced protein recognized accurately positive and negative sera from
356 experimental pig and had sensitivity and specificity of 98.9% and 78.8% relative to the Sar55-
357 ELISA. It is worth to comment the apparent discrepancy in specificities between the
358 recombinant ORF2H⁶⁺ ELISA and the Sar55-ELISA. Both proteins had 497 aminoacids located
359 between aminoacid positions 112 and 607, with minor differences (5% at the aminoacid level).

360 The predicted hidrofobicity of the two proteins was quite similar as well and therefore, the
361 causes for this discrepancy were not evident. If we take into account that antibodies recognised
362 primarily conformational epitopes as evidenced by the Dot Blot analysis there is the possibility
363 that the 6x histidine tail is interfering in the protein natural conformation leading to a loss of
364 sensitivity and specificity. Experiments with a truncated protein without the histidine tag were
365 not performed. The fact that some react differently with the different proteins looked
366 unimportant for pigs since in this species most animals are reported to be seropositive (Seminati
367 et al., 2008) with seroprevalences that can reach up to 90% or higher. In contrast, a minor lack
368 of specificity could lead to an overestimation of the prevalence in other species. Thus a stringent
369 strategy for diagnosis was adopted: only sera reacting positively in ELISA, western blot and
370 finally the dot blot using both Sar55 and our protein would be considered to be truly positive.

371 With this approach, none of the cows was positive but 1.92% (1.29-2.84%) and 0.60% (0.26-

372 1.28%) of sheep and goat, respectively were found to be positive. In the case of cows, other
373 authors reported seroprevalences ranging from 1.42% to 6.9% (Arankalle et al., 2001; Wang et
374 al., 2002; Vitral et al., 2005). For sheep and goats, reports are controversial. However, viral
375 genome has never been detected in any of the domestic ruminant species. In our case, the
376 seroprevalence in sheep and goats was very low and the fact that in most cases a single reactor
377 per herd was found, opens the question of whether or not those could be false positive results in
378 spite of the stringency of the conditions required in the present study to be considered
379 seropositive. Our opinion is that extreme caution should be applied to the interpretation of
380 serological results in species where HEV has not been detected directly, particularly when a
381 very large number of sera are examined since this increases the chance of finding false positives
382 in spite a high specificity. In any case, true or false positive, our results indicate that HEV is
383 either not present or present at a very low frequency in domestic ruminants of Spain.

384 Six out of 54 cats analysed resulted positive (11%, 4.6-23.32%). In this case, HEV seropositive
385 cats were present in four out of seven studied catteries with several seropositive individuals per
386 cattery suggesting transmission among animals in the cattery. As carnivores, as well as dogs,
387 there is the possibility that cats get infected via the food chain. It is a common practice to feed
388 cats and dogs with raw meat, that could probably be contaminated with swine HEV. A
389 seroprevalence study performed on dog samples collected in the same area in our lab revealed
390 that more than 20% of the animals tested positive for anti-HEV IgG detection supporting this
391 hypothesis (Peralta et al., 2006). If this was true it does not necessarily mean that cats and dogs
392 suffer the infection, the antibodies found in this species could only be the result of repeatedly
393 contact with the virus. The percentage of positive cats found in our study is in agreement with
394 other previous studies (Okamoto et al., 2004; Mochizuki et al., 2006) that reported that the 33%
395 of the tested cats had anti-HEV antibodies. Those studies were done in an area with the same
396 epidemiological situation, sporadic cases in humans and endemic situation in pigs. This fact
397 may have a very important role in HEV transmission, since there are evidences of direct
398 transmission from a cat to its owner (Kuno et al., 2003).

399 Finally, all examined rodents analyzed in this work were negative. Studies made in other
400 countries reported seroprevalences varying from 0 to 90% in different rodent species depending
401 on the country and the species examined (Tien et al., 1997; Kabrane-Lazizi et al., 1999; Favorov
402 et al., 2000; Arankalle et al., 2001; He et al., 2002; Withers et al., 2002). The species analyzed
403 in this work were *A.sylvaticus*, *A. flavicolis*, *M. musculus*, *R. norvegicus* and *M. glaerolus*, and
404 few samples of each were tested. Seropositive results had been previously reported in *M.*
405 *musculus* (Favorov et al., 2000), *A. sylvaticus* (Karetnyi et al., 1993) and *R. norvegicus*
406 (Kabrane-Lazizi et al., 1999; Favorov et al., 2000), but *M. glaerolus* and *A. flavicolis* have been
407 never analyzed before. So far, the species that have shown to be more prevalent are *R. rattus*
408 and *R. norvegicus*, which were not present or present in a little quantity in our sampling.
409 Before assuring that the infection is not present in rodents in Spain, a more accurate analysis
410 should be made on species that have proved to be positive in other studies such as species of the
411 genera *Rattus*, preferably caught in rural areas close to pig farms.

412

413 In conclusion, this study shows the performance of different HEV antigens used for the
414 serological diagnosis in animals and the importance to adopt stringent criteria for the
415 serodiagnosis. The seroprevalence of HEV in domestic ruminants was very low or nil while
416 frequency of antibodies in cats of catteries was high. These results emphasize the need for
417 developing more accurate serological tools for HEV diagnosis in animals as well as the
418 importance of gaining understanding of the role of animals in this infection.

419

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- 580
581
582

583 **Figure legends**

584 Figure 1. Anti-HEV antibodies titre in inoculated animals measured by indirect ELISA.

585

586 Figure 2. Dot Blot analysis of ELISA positive samples. Lines 1 to 7 belong to cat samples, lines

587 8 to 14 belong to goat samples and lines 15 to 20 are sheep samples. C-, negative control sera;

588 C+, positive control sera. a) blank, b) denatured Sar55 protein, c) native Sar55 protein, d)

589 denatured ORF2H⁶⁺, e) native ORF2H⁶⁺. Only samples positives to ORF2H⁶⁺ and at least one of

590 the two conditions of the Sar55 protein were considered to be positive.

591

Figure 1

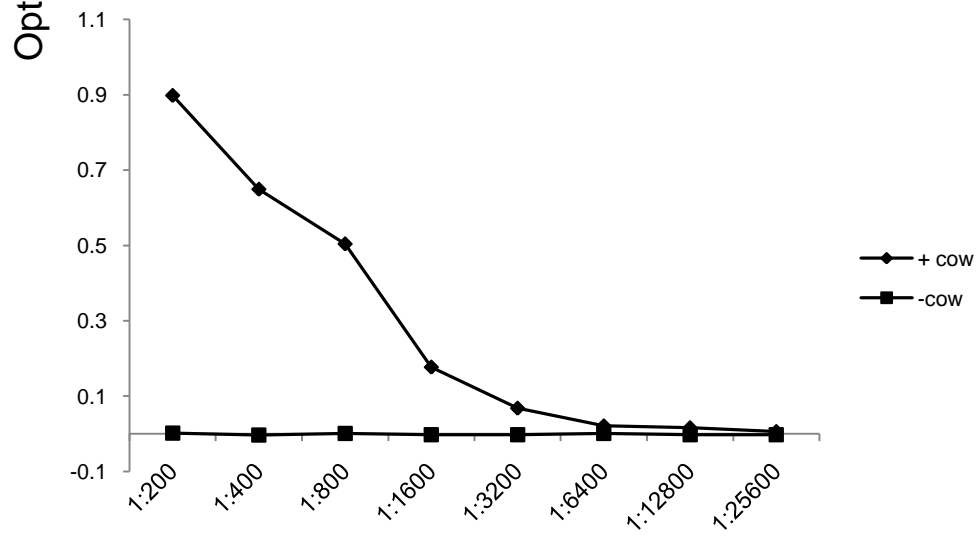
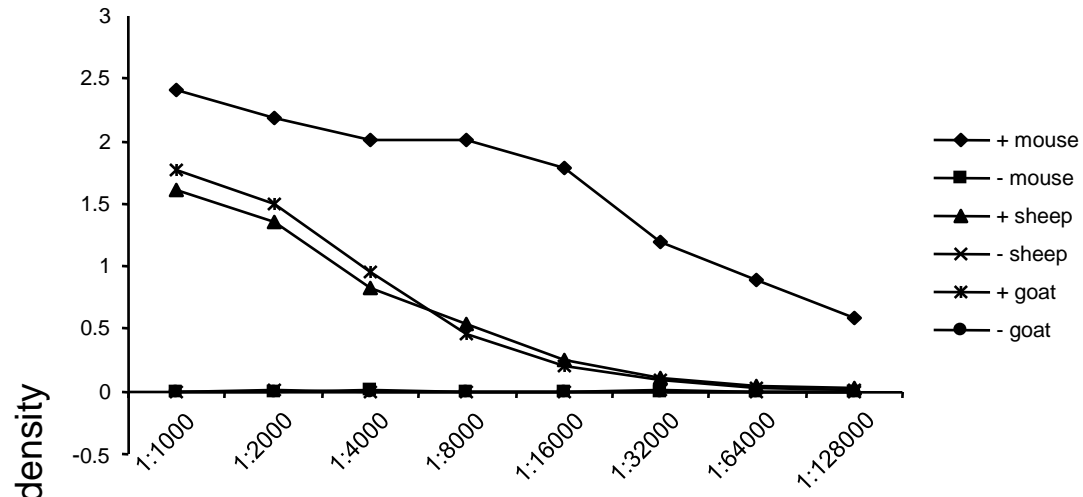


Figure 2

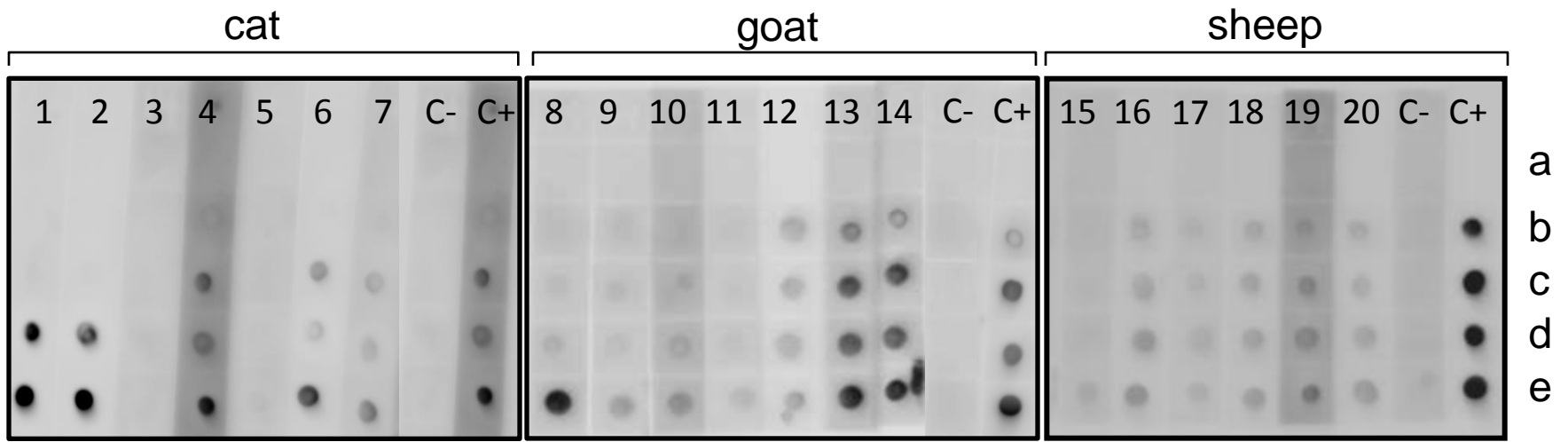


Table 1
Characteristics of the inoculations for the immunization of the different animal species in order to obtain hyper-immune sera

Specie	Num. of inoculations	Time between inoculations	Dosage per animal/adjuvant	Inoculation way
Mouse	3	2 weeks	35 $\mu\text{g}/\text{FA}^{\text{a}}$	Intraperitoneal
Goat	2	3 weeks	500 $\mu\text{g}/\text{FA}$	Subcutaneous
Sheep	2	3 weeks	500 $\mu\text{g}/\text{FA}$	Subcutaneous
Cow	2	3 weeks	750 $\mu\text{g}/\text{FA}$	Subcutaneous

^a Freund adjuvant, complete Freund adjuvant was used for the first inoculation and incomplete Freund adjuvant was used for the second and third immunizations.

Table 2
IgG anti-hepatitis E virus positivity rates in serum from different animal species using ELISA and Dot Blot confirmation

Species		ELISA results		Dot Blot results				Final results	
		ORF2H ⁶⁺		ORF2H ⁶⁺		Sar55			
		positives/tested	% (CI95%)	positives/tested	% (CI95%)	positives/tested	% (CI95%)	positives/tested	% (CI95%)
Pigs	individuals	180/252	71.4 (65.4-75.8)	NT ^a	NT	NT	NT	180/252	71.4 (65.4-75.8)
	herds	9/9	100	NT	NT	NT	NT	9/9	100
Sheeps	individuals	36/1357	2.6 (1.9-3.7)	32/1357	2.4 (1.6-3.4)	26/1357	1.9 (1.3-2.8)	26/1357	1.9 (1.3-2.8)
	herds	27/89	30.3 (21.3-41.1)	26/89	29.2 (20.3-39.9)	22/89	24.7 (16.5-32.2)	22/89	24.7 (16.5-32.2)
Goats	individuals	18/1143	1.6 (1-2.5)	16/1143	1.4 (0.8-2.3)	7/1143	0.6 (0.3-1.3)	7/1143	0.6 (0.3-1.3)
	herds	16/76	21.1 (12.9-32.2)	15/76	19.7 (11.8-30.8)	7/76	9.2 (4.1- 18.6)	7/76	9.2 (4.1- 18.6)
Cats	individuals	20/54	37 (24.6-51.3)	17/54	31.5 (19.9-45.7)	6/54	11.1 (4.6-23.3)	6/54	11.1 (4.6-23.3)
	shelters	5/7	71.4 (30.3-94.9)	5/7	71.4 (30.3-94.9)	3/7	42.9 (11.8-79.7)	3/7	42.9 (11.8-79.7)
Cows	individuals	0/1170	0	NT	NT	NT	NT	0/1170	0
	herds	0/77	0	NT	NT	NT	NT	0/77	0
Rodents	individuals	0/166	0	NT	NT	NT	NT	0/166	0
	locations	0/4	0	NT	NT	NT	NT	0/4	0

^aNT=not tested