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1 **EVIDENCE OF WIDESPREAD INFECTION OF AVIAN HEPATITIS E VIRUS**
2 **(AVIAN HEV) IN CHICKENS FROM SPAIN**

3

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23 **Short title:** Avian HEV infection in Spain

24 **Key words:** Hepatitis E virus (HEV); Avian HEV; chickens; antibody prevalence;
25 sequence

26 **Abstract**

27 In the present work, 262 serum samples and 29 faeces pools from chicken coming from
28 29 healthy flocks were analysed by RT-PCR for detection of avian HEV and by ELISA
29 using an aHEV derived antigen for detection of anti-HEV IgG. Additionally, other 300
30 randomly selected serum samples were also analysed by RT-PCR and by ELISA. Seven
31 serum samples were positive to RNA detection. Sequence analysis of both the helicase
32 and the capsid genes revealed that the Spanish isolates were clustered together and close
33 related to those strains from the United States isolated from farms with HSS. On the
34 serology study, 26/29 flocks had at least one positive animal (89.7%) and chickens older
35 than 40 weeks were found to have higher seropositivities compared to the rest of age
36 groups. Within positive farms, the proportion of positive animals ranged from 20% to
37 80%. This is the first report of aHEV sequences in chickens from Europe. Further studies are
38 needed to elucidate the clinical significance of avian HEV infections in Europe.

39

40

41 **INTRODUCTION**

42 *Hepeviruses* are non-enveloped, single-stranded, positive-sense RNA viruses (Emerson
43 et al., 2004). Taxonomy of *hepeviruses* is not fully elucidated but, up to now, one
44 species, Hepatitis E virus (HEV), has been recognized. A second species, avian hepatitis
45 E virus (avian HEV), has been proposed (Emerson et al., 2004). HEV strains are
46 classified in four distinct genotypes of which genotypes 1 and 2 HEV strains are usually
47 associated with large epidemics of acute hepatitis in humans (Huang *et al.*, 1992;
48 Arankalle *et al.*, 1994; Wang *et al.*, 1999). In contrast, genotypes 3 and 4 HEV strains
49 are more often associated with sporadic cases of hepatitis in humans (Schlauder *et al.*,
50 1999; Zanetti *et al.*, 1999; Pina *et al.*, 2000; Takahashi et al., 2001; Clemente-Casares *et*
51 *al.*, 2003) and can also infect other animal species, particularly pigs (Meng et al., 1997;
52 Clemente-Casares et al., 2003; Chobe et al., 2006).

53 Avian HEV was firstly reported by Haqshenas *et al.* (2001) from chickens with
54 hepatitis-splenomegaly syndrome (HSS) in the United States and later it was described
55 in chickens with the same disease in Canada (Agunos *et al.*, 2006). Avian HEV shared
56 about 50%-60% nucleotide sequence identity with human and swine HEV strains
57 (Huang *et al.*, 2004), and an approximately 80% nucleotide identity with the big liver
58 and spleen disease (BLSV) virus identified in Australian chickens (Payne et al., 1999;
59 Haqshenas et al., 2001). The Avian HEV and BLSV are apparently variant strains of the
60 same virus (Haqshenas et al., 2002; Huang et al., 2004; Guo et al., 2006). The genome
61 of avian HEV consists of a sequence of 6,654 nt, excluding the poly A tail, and as
62 described for human and swine HEV there is a 5' non-coding region followed by three
63 partially overlapping open reading frames (ORFs) and a 3' non-coding region (Huang *et*
64 *al.*, 2004). It has been shown that a considerable proportion of the chicken flocks (71%)
65 in the United States were seropositive to avian HEV infection (Huang et al., 2002),

66 although seropositive flocks did not necessarily suffer from HSS or BLSD (Meng *et al.*,
67 2008).

68 It has been demonstrated that swine HEV can infect non-human primates (Meng
69 *et al.*, 1998) but avian HEV cannot (Huang *et al.*, 2004), pointing at the fact that while
70 swine HEV is a zoonotic virus, avian HEV may be not. However, in contrast to swine
71 HEV infection in pigs, in which animals are completely asymptomatic (Meng *et al.*,
72 1998 a, b), avian HEV seems to have some pathogenic importance for chickens (Billam
73 *et al.*, 2005; Agunos *et al.*, 2006) as production and economic losses can be observed in
74 flocks with HSS (Ritchie & Riddell, 1991; Meng *et al.*, 2008). In Europe there is no
75 epidemiologic study on aHEV performed so far although there could be serologic
76 evidence of BLSDV infection (Todd *et al.*, 1993). In Italy, and very recently in
77 Hungary, outbreaks of HSS have been reported although information on the strains
78 related to these outbreaks is not given (Massi *et al.*, 2005; Morrow *et al.*, 2008).

79 The aims of the present study were to determine the possible circulation of avian
80 HEV in Spanish chicken flocks by means of serology and RT-PCR, and to genetically
81 characterize HEV strains detected.

82

83 **METHODS**

84 **Samples.** For the detection of avian HEV RNA and antibodies in Spanish chicken
85 flocks, 29 farms of layers and breeders located in the north-east region of Spain were
86 selected. No particular health problems had been reported in the examined farms. For
87 each farm 8-10 chickens (ranging from 26-54 weeks of age) were bled (n=262), and a
88 sample of pooled faeces from the floor was also taken for RT-PCR detection of avian
89 HEV RNA. After collection, blood was left to clot and was then centrifuged at 2,500xg
90 5 min to collect serum, which was stored at -80°C until use. To obtain a 10% (w/v)

91 faecal suspension, 1g of faeces was vigorously mixed with 9 ml of PBS buffer; the
92 suspension was then centrifuged at 3,000xg at 4°C for 30 min and the supernatants were
93 stored at -80°C until use.

94 Additionally, 300 random chicken sera were obtained from a bank of convenient
95 sera available at CESAC (Centro de Sanidad Avícola de Cataluña y Aragón, Spain).
96 These 300 serum samples were used for detection of avian HEV RNA by RT-PCR.

97

98 **ELISA to detect anti-HEV antibodies in chicken sera.** The recombinant avian HEV
99 antigen expressed in *E.coli* and purified by affinity chromatography (Haqshenas *et al.*,
100 2002; Sun *et al.*, 2004) was used as determined by Haqshenas *et al.*, (2002) with minor
101 modifications. Briefly, after coating the plates for with 100 µl of a 2 µg/ml protein
102 solution 18h, 140µl/well of blocking solution (PBS, 0.035 M NaCl, 0.5% gelatine, 10%
103 FBS) were added and incubated for 1h at 37°C. The samples were diluted 1:100 in the
104 blocking buffer and 100µl of the dilution were transferred to the plate. After 45 min
105 incubation at 37°C, the plates were washed 5 times with PBS 0.02% Tween-20 and a
106 HRP-conjugated goat anti-chicken IgG (Serotec Ltd., Oxford, UK) used as the
107 secondary antibody was added at a dilution 1:150,000. Plates were incubated for
108 another 45 min period at 37°C and washed as described above. The reaction was
109 revealed by adding 100µl of TMB (Sigma Chemical, St. Louis, MO., USA) and stopped
110 with 100 µl of H₂SO₄ 2M. Plates were read in an ELISA reader at 450 nm. To avoid
111 background or unspecific binding, samples were analyzed in duplicate in both coated
112 and uncoated wells. Sera from chickens experimentally-infected with the avian HEV
113 were used as positive controls while sera from non-infected control chickens were used
114 as negative controls (Billam *et al.*, 2005). The specific absorbance value for each sample
115 was calculated after subtracting the value of the uncoated wells from the values of the

116 coated wells. Cut-off was set at 0.3; this threshold corresponded approximately to the
117 mean plus four standard deviations of the OD value of negative control sera (Billam et
118 al., 2005).

119

120 **RT-PCR to detect avian HEV RNA in serum and fecal samples.** The RT-PCR
121 procedure used in this study was previously described by Sun *et al.*, (2004) with some
122 modifications. Total RNA was extracted from 100 μ l of chicken serum or fecal
123 suspensions using TriReagent (Molecular Research Center, Inc.). Total RNA was
124 resuspended in DNase, RNase and proteinase-free water and reverse-transcribed at 42°C
125 for 60 minutes. Reverse transcription mix contained 1x first strand buffer (50 mM Tris–
126 HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂), 5 mM DTT (Invitrogen), 2 mM of each
127 dNTPs, 10 pmol of the Helic R or AHEV ORF2/R-1/SD primers, 20 U of RNase OUT
128 and 200 U of Superscript II reverse transcriptase (Invitrogen).

129 For the amplification of the helicase gene region of avian HEV, a nested PCR was done.
130 Primers AHEV F-1/SD and Helic R were used in the first round PCR and primers
131 AHEV F-2/SD and Helic R2 in the second round, and the primer sequences were
132 described previously (Sun *et al.*, 2004). The composition of the PCR mixture (50 μ l
133 final volume) was 26.5 μ l of RNase/DNase free water, 1xPCR buffer (10 mM Tris–HCl
134 (pH 8.3), 40 mM KCl), 2 mM of MgCl₂, 2 mM of each dNTP, 10 pmol of each primer,
135 2.5 Unit of AmpliTaq1 gold DNA polymerase (Applied Biosystems) and 10 μ l of
136 cDNA. The PCR reaction was done under the following parameters: initial incubation at
137 95°C for 6 min, followed by 35 cycles of 94°C for 50 sec, 42°C for 50 sec and 72°C for
138 1 min, and a final incubation at 72°C for 7 min. The size of the expected PCR product
139 was 386 bp. This RT-PCR was used for detection of avian HEV RNA in serum and
140 fecal samples.

141 Additionally, in order to amplify another segment of the avian HEV genome for
142 analyses and confirmation, a partial fragment of the ORF2 region of avian HEV was
143 also amplified and sequenced. To amplify the ORF2 fragment, the primers AHEV
144 ORF2/F-1/SD and AHEV ORF2/R-1/SD were used as external primer set and primers
145 AHEV ORF2/F-2/SD and AHEV ORF2/R-2/SD as internal primer set. Again, the
146 primer sequences were described previously (Sun *et al.*, 2004). Approximately 5 µl of
147 the first-round products were added to a new batch of 50 µl PCR reaction mix
148 containing 10 pmol of each primer for a new nested-PCR amplification cycle with the
149 internal set of primers. PCR conditions were essentially the same as described above.
150 The expected final PCR product was 242 bp. A bile sample from a chicken positive to
151 avian HEV (Sun *et al.*, 2004) was used as the positive control, and blank (water)
152 samples were also included in each RT and PCR reaction.

153

154 **Sequence and phylogenetic analyses.** PCR products were examined on a 0.8% agarose
155 gel, excised and purified using the GeneClean kit (Bio 101 Inc). Sequencing was
156 performed directly on both strands at the Virginia Bioinformatics Institute (Blacksburg,
157 VA, USA). The resulting avian HEV sequences were compared with other avian HEV
158 and mammalian HEV strains available at the GenBank and the European Molecular
159 Biology Library by using the BLAST utility (available from:
160 <http://www.ncbi.nlm.nih.gov/BLAST>). Alignments were carried out by using ClustalX
161 1.8 program (available from: <ftp://ftpigbmc.u-stras/pub/clustalX>) and phylogenetic trees
162 were constructed by the neighbour-joining method using MEGA4.0 (Tamura *et al.*,
163 2007). Bootstrap test of 1,000 replicates was done to evaluate the reliability of the
164 different groups (Felsenstein, 1985). The Genbank accession numbers of the HEV
165 sequences used in the phylogenetical analysis were: AY671803, AY671802,

166 AY870832, AY871083, AY871081, AY870811, AY870810, AY870809, AY870808,
167 EF206691, AY535004, AB080575, M80581, M74506 and AF082843. The sequences
168 reported in this paper have been deposited in the GenBank database under the accession
169 numbers EU919186-EU919196.

170

171 **Statistical analysis.** Statistical analyses were done using Epi-Info 2000 v 3.4.1. Kappa
172 value was calculated using WinEpiscope software.

173

174 **RESULTS**

175 **Detection of anti-avian HEV IgG antibodies in chickens.** When using the avian HEV
176 antigen in ELISA, 26/29 analyzed chicken flocks had at least one seropositive animal
177 (89.7%, Confidence interval 95%: 71.5%-97.3%). Within positive farms, the proportion
178 of positive animals ranged from 20% to 80%. Considering all the examined animals by
179 rank of age, seropositive animals were detected at any age, but chickens older than 40
180 weeks were found to have higher seropositivities compared to the rest of age groups
181 (Relative risk= 1.7 (1.2-2.4; $p < 0.05$) (Figure 1).

182

183 **Detection of avian HEV RNA, sequence and phylogenetic analyses**

184 The helicase gene-based RT-PCR assay was used to detect the prevalence of
185 avian HEV RNA in chickens. Of the samples collected from the 29 farms, only two
186 were positive for avian HEV RNA RT-PCR, and the two positive samples were from
187 hens (41 weeks of age) belonging to the same flock. Of the 300 random sera tested by
188 the RT-PCR, five samples were found positive for avian HEV RNA. In four of the
189 positive samples, a second RT-PCR assay that amplifies the ORF2 region was also

190 performed to further confirm the results. All samples positive for avian HEV RNA
191 tested positive to anti-HEV IgG in the ELISA test.

192 Sequences analyses of the helicase gene region showed that avian HEV isolates
193 identified in Spanish chickens had 93.5% to 100% nucleotide sequence identities to
194 each other, 80.4%-92.1% to other avian HEV isolates in the United States and Canada
195 including the prototype avian HEV strain (AY535004), 75.0%-75.8% to a BLSV isolate
196 from Australia, and 52.3-57.8% to other known human or swine HEV strains.
197 Comparison of the predicted amino acid sequences showed that the helicase is
198 conserved among the Spanish avian HEV isolates (99.1%-100% identity) and was
199 similar to other avian HEV strains in North America (92.9%-99.1%), and showed an
200 83.1-84.2% sequence identity to BLSV, but only 49.1-53.5% identity to other strains of
201 human or swine origin.

202 When the ORF2 gene was examined, the Spanish avian HEV isolates share
203 93.0% to 99.5% nucleotide sequence identities to each other, 74%-93.5% to other avian
204 HEV isolates and 48.4%-51.7% to other known HEV strains of human and swine
205 HEVs. The predicted amino acid sequences of the Spanish avian HEV isolates were
206 similar to each other with 98.6%-100%, but is less conserved with other avian HEV
207 isolates in the USA and Canada (82.1%-100%), and had only 45-9%-48.6% amino acid
208 sequence identity with mammalian HEV strains (Supplementary tables 1 and 2).

209 Phylogenetic analyses based on the partial ORF1 helicase gene (Figure 2A) and
210 the partial ORF2 capsid gene (Figure 2B) revealed that the avian HEV strains detected
211 from chickens in Spain are related to the avian HEV strains in North America but
212 clearly the Spanish avian HEV isolates form a separate distinct cluster.

213

214 **DISCUSSION**

215 So far, avian HEV strains have been identified only in North America
216 (Haqshenas *et al.*, 2001; Agunos *et al.*, 2006), although a variant strain of avian HEV,
217 designated BLSV, was reported in chicken flocks in Australia (Payne *et al.*, 1999). This
218 is the first report genetically characterizing European avian HEV strains although there
219 are previous reports that describe what could be HSS in broiler breeder farms in Italy
220 (Massi *et al.*, 2005) and Hungary (Morrow *et al.*, 2008). In Italy, they detected what
221 could be HSS in two broiler breeder farms and they found that some of the analysed
222 samples tested positive in an ELISA using a human derived antigen and some of the
223 samples tested positive for avian HEV RT-PCR, although confirmation by sequencing
224 of the amplified region was never performed and nor was the genetic characterization of
225 the strains obtained. In Hungary, the authors observed a syndrome resembling that
226 reported for aHEV infection and samples were positive to aHEV RNA detection by
227 PCR.

228 Results presented in this study raise the question whether or not HSS cases may
229 occur in Europe more frequently than thought and pass unnoticed or masked as a fatty
230 liver syndrome. However, avian HEV has been also detected in the United States not
231 only from chickens with HSS but in healthy flocks as well (Haqshenas *et al.*, 2001;
232 Huang *et al.*, 2002; Meng *et al.*, 2008). Sequence analyses showed that an apparently
233 avirulent strain of avian HEV is closely related to those detected in HSS outbreaks
234 (Billam *et al.*, 2007). Therefore, in most cases, avian HEV infections are subclinical
235 (Meng *et al.*, 2008).

236 The data presented in this study demonstrated that avian HEV infection is
237 widespread in Spain since the vast majority of the farms examined in this study had
238 seropositive animals. This is in agreement with what has been reported in the United
239 States (Huang *et al.*, 2002). Huang *et al.* (2002) found that approximately 76% of the

240 flocks in the United States were positive for avian HEV and that within a positive farm,
241 the percentage of positive chickens could vary from 15% to 100%. The present study
242 also reflects that the likelihood of being seropositive increases with age as shown by the
243 relative higher risk of older animals as expected in a closed endemically infected
244 population. This is also in accordance with Huang *et al.* (2002) who found that
245 prevalence of avian HEV antibodies in chickens older than 18 weeks was higher than in
246 younger ones.

247 Phylogenetic analyses revealed that the Spanish avian HEV isolates form a
248 distinct genetic cluster different from the avian HEV isolates identified in North
249 American chicken flocks (Fig. 2). Additional sequences of avian HEV from European
250 chicken flocks, especially full-length genomic sequences, are needed to determine if the
251 European isolates represent a distinct genotype. Currently, the clinical significance of
252 these genetic clustering is unclear, largely due to the lack of clinical data.

253 Of the 562 serum samples tested by RT-PCR, only 7 sera were positive for avian
254 HEV RNA. The low detection rate may be due to the fact that the samples tested in this
255 study belonged to animals older than 24 weeks. Sun *et al.* (2004) showed that under
256 natural infection conditions, chickens generally seroconverted at 15-17 weeks of age.
257 Billam *et al.* (2005) conducted a study in which specific-pathogen-free animals were
258 experimentally infected by the oronasal route and they showed that chickens
259 seroconverted to avian HEV antibodies approximately 3 weeks after the inoculation,
260 virus excretion in faeces was observed from 1 to 8 weeks post-inoculation and that
261 viremia was present from 2 to 5 weeks after the inoculation. Therefore, a susceptible
262 flock may naturally get infected around 12 weeks of age; virus shedding in faeces could
263 be detected between 13 and 20 weeks of age and viremia only between 14 and 17 weeks
264 of age. All the animals examined in this work were older than 20 weeks and thus this

265 may explain why only 7 samples were positive for avian HEV RNA, despite a high
266 seroprevalence rate. It is likely that viremia and virus shedding in these older animals
267 were cleared at the time of sample collection. Future studies are warranted to investigate
268 the prevalence of avian HEV RNA in younger chickens in Spain.

269 It is believed that, unlike swine HEV, avian HEV is likely not zoonotic, since
270 attempts to infect Rhesus monkeys with avian HEV failed (Huang *et al.*, 2004).
271 However, avian HEV alone or coinfection with other avian pathogens is clinically
272 important in poultry industry. It has been reported that HSS affected animals show
273 reduced food consumption, reduced body weight gain and poor peak production, and at
274 necropsy acute periportal lymphoplasmacytic hepatitis is usually observed (Agunos *et*
275 *al.*, 2006; Meng *et al.*, 2008), leading to significant economical losses for the producers.
276 In summary, our work provides evidences of the circulation avian HEV strains in
277 chickens from Europe and also provides partial genomic sequences and a genetic
278 analysis of those strains. Moreover, this is the first description of avian HEV sequences
279 from Spain, where HSS has not been reported before. This finding raises the questions
280 of either the lack of diagnosis of HSS in the country or the possibility of subclinical
281 avian HEV infection in Spanish farms. Further studies are needed to elucidate the
282 clinical significance of avian HEV infections in Europe.

283

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289

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386 **Figure legends**

387 **Figure 1.** Prevalence of IgG anti-avian HEV antibodies in chickens of different age
388 groups.

389 **Figure 2.** (A) A phylogenetic tree based on a partial helicase gene region of a 343 bp
390 constructed by the neighbour joining method using avian HEV, BLSV and mammalian
391 HEV strains. (B) A phylogenetic tree based on a partial capsid gene region of a 235 bp
392 constructed by the neighbour joining method using avian HEV and mammalian HEV
393 strains. Bootstrap values >70% are indicated for the major nodes. Avian HEV
394 sequences from chickens in Spain in this study are marked as (●).

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