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

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Short title: Avian HEV infection in Spain

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

Abstract

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

INTRODUCTION

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

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

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

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

ELISA to detect anti-HEV antibodies in chicken sera. The recombinant avian HEV antigen expressed in *E.coli* and purified by affinity chromatography (Haqshenas *et al*., 2002; Sun *et al*., 2004) was used as determined by Haqshenas et al., (2002) with minor modifications. Briefly, after coating the plates for with 100 µl of a 2 µg/ml protein solution 18h, 140µl/well of blocking solution (PBS, 0.035 M NaCl, 0.5% gelatine, 10% FBS) were added and incubated for 1h at 37ºC. The samples were diluted 1:100 in the blocking buffer and 100µl of the dilution were transferred to the plate. After 45 min incubation at 37ºC, the plates were washed 5 times with PBS 0.02% Tween-20 and a HRP-conjugated goat anti-chicken IgG (Serotec Ltd., Oxford, UK) used as the secondary antibody was added at a dilution 1:150,000. Plates were incubated for another 45 min period at 37ºC and washed as described above. The reaction was revealed by adding 100µl of TMB (Sigma Chemical, St. Louis, MO., USA) and stopped with 100 µl of H_2SO_4 2M. Plates were read in an ELISA reader at 450 nm. To avoid background or unspecific binding, samples were analyzed in duplicate in both coated and uncoated wells. Sera from chickens experimentally-infected with the avian HEV were used as positive controls while sera from non-infected control chickens were used as negative controls (Billam et al., 2005). The specific absorbance value for each sample was calculated after subtracting the value of the uncoated wells from the values of the

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

RT-PCR to detect avian HEV RNA in serum and fecal samples. The RT-PCR procedure used in this study was previously described by Sun *et al*., (2004) with some modifications. Total RNA was extracted from 100 µl of chicken serum or fecal suspensions using TriReagent (Molecular Research Center, Inc.). Total RNA was resuspended in DNase, RNase and proteinase-free water and reverse-transcribed at 42ºC for 60 minutes. Reverse transcription mix contained 1x first strand buffer (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$), 5 mM DTT (Invitrogen), 2 mM of each dNTPs, 10 pmol of the Helic R or AHEV ORF2/R-1/SD primers, 20 U of RNase OUT and 200 U of Superscript II reverse transcriptase (Invitrogen).

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

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

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

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

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

RESULTS

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

Detection of avian HEV RNA, sequence and phylogenetic analyses

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

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

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

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

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

DISCUSSION

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

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

The data presented in this study demonstrated that avian HEV infection is widespread in Spain since the vast majority of the farms examined in this study had seropositive animals. This is in agreement with what has been reported in the United 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 that 20 weeks and thus this

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

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

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385

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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