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

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

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

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

In addition to pigs and deers, so far HEV has been detected only in horses (Saad et al., 2007); mongooses (Nakamura et al., 2006) and chickens (Haqshenas et al., 2001); in the last case, the avian strains seem to belong to a different HEV species or, at least, to a different genotype.
Antibodies against HEV have been also demonstrated in species other than the abovementioned such as cows, sheeps, goats, dogs, cats and rodents (Tien et al., 1997; Arankalle et al., 2001; Okamoto et al., 2004; Vitral et al., 2005; Mochizuki et al., 2006; Zhang et al., 2008) but the epidemiological role of those domestic species is uncertain. Most of these serological studies in domestic animals other than pigs have been carried out in Asia and information about Europe is still lacking.

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

2. MATERIALS AND METHODS

2.1. Expression of a truncated HEV ORF2

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

2.1.2. Generation of HEV ORF2 recombinant baculovirus
Generation of the recombinant baculovirus containing the truncated HEV ORF2 was done using the Bac-to-Bac Baculovirus Expression System (Invitrogen) following manufacturers’s instructions. Briefly, a set of primers was designed to amplify a fragment of 1,491 bp: the
forward primer ForRsrIIHEV (5'-
ATATATTCCGWWCGATGGCTGGTTGCGCCTGCGCCTGATACGGCC-3') containing the
RsrII restriction site and the initiation codon, and the reverse primer RevHindIIIHEVHist (5'-
TATAAGCTTCTATTAGTGATGATTGATGATGATGATGGCTAAAGCAGAATGCGGGGC-3')
containing the HindIII restriction site, two stop codons and a 6xHis (H6+) tag. The N-terminal
and C-terminal truncated ORF2 expressed corresponded to the minimum sequence necessary to
allow virus like particles (VLPs) formation as described by Li et al. (2005), from aa 112 to aa
607. This means that the expressed product had an N'-terminus 111 aa deletion and a C'-
terminus 53 aa deletion. The PCR product was gel purified with Nucleospin® ExtractII
(Macherey-Nagel), digested with restriction enzymes, and ligated into the pFASTBAC vector
(Invitrogen) to yield the construction pFBAC-ORF2H6+. Then, ElectroMAX™DH5α E. coli
cells (Invitrogen) were transformed, and the recombinant plasmids were extracted, and
confirmed by PCR and fully sequencing of the insert. Competent cells MAX Efficiency®
DH10Bac™ were transformed with pFBAC-ORF2H6+ for production of a recombinant bacmid
BAC-ORF2H6+. Then, BAC-ORF2H+ was extracted following standard methods and
transfected into Sf9 cells using Lipofectamine™ (Invitrogen). Cells were cultured in Grace’s
insect media (Gibco BRL) supplemented with 10% foetal bovine serum (FBS), 3% non-
essential amino acids, 100 µg/ml of streptomycin and 100 U/ml of penicillin and 20 µg/ml
gentamycin and grown at 27ºC. At 96 hours post-transfection the supernatant containing the
recombinant baculovirus was recovered and stored at 4ºC.

2.1.3. Truncated ORF2 expression in insect cells and insect larvae
The recombinant baculovirus was used to infect Sf9 cells using a multiplicility of infection
(MOI) of 0.05. Infected cells were incubated for 6 days (the optimal conditions for recovery of
the HEV protein as determined in previous assays). Total protein was extracted by collecting the
infected cells and incubating them in milliQ water for 15 min in ice followed by a
centrifugation at 2500xg for 10 min. The recombinant protein ORF2H6+ contained in the
supernatant was purified using the kit Protino Ni-IDA packed columns (Macherey-Nagel) following manufacturer’s instructions. The recovered protein was stored at 4°C. Alternatively, *Trichoplusia ni* larvae were grown and inoculated as previously described (Medin et al., 1995; Pérez-Filgueira et al., 2006). The inocula consisted in 5x10^5 recombinant virus particles (BAC-ORF2H6+) or wtBAC (control baculovirus). Three days post infection, the larvae were frozen and homogenized in an extraction buffer to obtain total soluble proteins (TSP), including our recombinant ORF2H6+ protein, as previously described (Pérez-Filgueira et al., 2006). The amount of TSP contained in the supernatant was quantified by Bradford assay (Bradford, 1976), and lyophilised for long time storage (Pérez-Filgueira et al., 2006). The same protocol was followed for the control protein extract (Ni), obtained from the Bac-Ni infected larvae. (Pérez-Filgueira et al., 2006).

2.1.4. Analysis and quantification of recombinant protein production

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

2.2.1. Porcine sera samples

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

2.2.2. ELISA procedure

Ninety-six well polysterene plates were coated with 100 µl of either the purified protein recovered from Sf9 cell cultures at a concentration of 0.125µg/ml (final protein amount 12.5 ng/well) or with the protein extract obtained from the infected larvae at 3.5µg/ml (final protein amount 350 ng/well) diluted in coating buffer (0.015M Na$_2$CO$_3$, 0.035M NaHCO$_3$, pH 9.6). Those concentrations were determined to be optimal in previous titration experiments (not shown). In order to minimize the effect of unspecific binding to the plates or to the antigen, sera were analysed in duplicate in antigen-coated and mock-coated wells. For the ELISA procedure, after coating the plates for 18h, wells were washed with PBS added with 0.02% Tween-20 (PBS –T) and blocked for 1h at 37°C with blocking buffer (BB) (140µl/well; PBS, 0.035 M NaCl plus 0.5% gelatine and 10% FBS). After washing the BB, serum samples were diluted 1:100 in BB and 100µl were dispensed per well. After 45 min of incubation at 37°C, plates were washed 5 times with PBS-T and a HRP-conjugated goat anti-swine IgG (Serotec Ltd., Oxford, UK) was added at a dilution 1:100,000. Plates were incubated for 45 min at 37°C 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$_2$SO$_4$ 2M. Plates were read in an ELISA reader at 450nm. The specific absorbance value for each sample was calculated by subtracting the value of the mock-coated wells from the values of the plates coated with the specific protein.
Cut-off was set at 0.3 that was about 4 standard deviations above the mean OD value of the negative control sera.

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

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

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

2.3.1. Immunization of different animal species to obtain hyperimmune serum

In order to obtain a positive serum to be used as a positive control in the developed ELISA for different species, an immunization assay was performed in cows, sheeps, goats, and rodents. For that purpose, two 3-months-old cows, two adult goats, two adult sheep and five ICR-CD1 6-months-old mice were housed in the Veterinary School facilities of the Universitat Autònoma of Barcelona. One week after their arrival animals were injected with the HEV ORF2H^{6+} purified recombinant protein using the dosages, adjuvants and inoculation routes summarized in Table 1. For each group one animal was kept as a negative control inoculated with sterile saline solution with the adjuvant. Blood samples were periodically obtained by jugular punction in the case of the ruminants and by tail incision in mice. The procedures were performed in accordance with the guidelines of the Good Experimental Practices (GEP), under the supervision of the Ethical Welfare Committee on Human and Animal Experimentation of the Universitat Autònoma de
Barcelona (CEEAH), Spain. Blood samples were centrifuged for 5 min at 2,500 xg, and sera were stored at -80°C until used.

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

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

2.3.4. Western blot and dot blot analysis
Confirmation of positive ELISA results was made by WB analysis using the truncated protein expressed in insect cells. One µg of purified protein was run in a single well protein
electrophoresis gel (NuPAGE 4-12% Bis-Tris Gel 1.0mm x 2D, Invitrogen) for 1 h at 170V and transferred to a nitrocellulose membrane Amersham Hybond™ ECL™ (GE Healthcare). Each membrane was cut into 4mm strips and stored at 4°C until use. The Amersham™ ECL Advance™ Western Blotting Detection Kit (GE Healthcare) was used following manufacturer’s instructions. Strips were treated with the blocking solution at 4°C overnight. After washing with PBS 0.02% Tween 20, sera from cats, goats ans sheeps were diluted 1:1,000 in blocking solution and added to the strips. Following 1 hour incubation, the strips were washed as mentioned and the secondary antibody was added at the dilution determined in previous assays, which resulted in 1:100,000 for goats and sheep and 1:5,000 for cats. Pig results were not confirmed by WB since the infection in Spanish swine population has been reported before.

Chemiluminescence was detected using the fluorescence imager FluorChem® HD2 Imaging System (Alpha Innotech). Sera positive in WB were re-confirmed in dot blot using both the Sar55 and the ORF2-truncated protein expressed in insect cells adsorbed in native conformation or after denaturation. Finally, in order to warrant specificity of the assay, we only considered as positive the sera yielding positive results with both Sar55 and the ORF2H^6+ protein.

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

3. RESULTS
3.1. Standardization of an ELISA test for IgG anti-HEV based on the truncated ORF2H^6+
of genotype 3
3.1.1. Protein production
After six days of incubation, 1.65 mg of HEV ORF2H^6+ were recovered from 3.6x10^7 Sf9 cells. For the insect larvae expression, the amount of total protein recovered was 13 mg per larva, of which about 10% was the specific protein (determined by OD comparison with the purified
protein). In both cases the truncated HEV ORF2H\textsuperscript{6+} had a molecular weight of 55kDa. The protein produced in larvae was little soluble in water making very difficult to further purify the larva extracts by the column method used in this study probably due to the formation of aggregates with other molecules present in the larvae extract. In addition, storage of this extract at 4°C or -20°C rapidly produced a loss of antigenic reactivity as revealed in ELISA (data not shown). This did not occur with the cell culture-expressed protein.

Both recombinant proteins, the ORF2H\textsuperscript{6+} and the Sar55 protein, were located between aminoacid positions 112 and 607, so the length was of 495 aminoacids for the Sar55 truncated protein and 501 aminoacids for the ORF2H\textsuperscript{6+} protein, since a 6x histidine tail was added to the 3’ end. Although at nucleotide level the identity was of 78%, only a difference of 5% at aminoacid level was observed. The sequence of the strain used for the protein expression is available at GenBank under the accession number EU723512. Moreover, the hydrophobicity analysis performed revealed almost identical patterns for both proteins (data not shown).

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

When the 30 sera from experimental pigs were analysed, the ORF2H\textsuperscript{6+} ELISA diagnosed correctly 13/13 positive and 17/17 negative samples. Considering Sar55 ELISA results as the golden standard (100% of relative sensitivity and specificity) and using the 252 field sera, the truncated ORF2H\textsuperscript{6+} protein-ELISA expressed in cell cultures had a relative sensitivity of 98.9% (180/182; CI\textsubscript{95%}: 97.4-100%), whereas the relative specificity was 78.8% (55/70; CI\textsubscript{95%}: 68.9-88.2%). The ELISA set with the protein expressed in larvae showed a sensitivity of 97.8% (178/182, CI\textsubscript{95%}: 95.0-99.1%) and a specificity of 61.4% (43/70, CI\textsubscript{95%}: 50.0-72.8%) compared with the Sar55 protein ELISA. In order to know if the ELISA developed with the Spanish strain of HEV genotype 3 was similar in performance to the Sar55 ELISA in terms of global agreement of results, the \textit{kappa} value was calculated. Thus, \textit{kappa} was 0.82 (CI\textsubscript{95%}: 0.69-0.94%) for the cell culture-expressed protein and 0.66 (CI\textsubscript{95%}: 0.54-0.78) for the antigen expressed in insect larvae.
To further characterize the ELISA test developed, an analysis of intra- and interplate variability was performed. This assay was conducted using only the 252 samples from commercial farms. Samples were analyzed twice in the same plate and in different ELISA plates as described above. For the cell culture expressed antigen, the results showed a 2.7% and 6.0% of intra- and interplate variation, respectively and these values were 6.0% and 9.0% for the antigen expressed in insect larvae.

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

3.2. Serological survey of HEV in domestic species and rodents

Positive control sera obtained by hyperimmunization with the truncated ORF2H$_{6+}$ protein had the following titre sin ELISA: sheep and goat sera: 1:16,000; cow and mouse sera titers were 1:1,000 and >1:128,000, respectively (Figure 1).

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

Positive samples were tested in WB. For sheep, 28/33 animals of 26 herds produced a positive result in this test; for goats, 14/17 positive sera (14 farms) in ELISA were confirmed in WB.

Regarding cat samples, due to the scarce amount of serum final confirmation was only done by dot blot. Thus, final confirmation of WB positive sera by dot blot using Sar55 and the ORF2H$_{6+}$ truncated protein either under native and denaturing conditions produced the following results:
for sheep, 26/28 WB positive sera were confirmed (22 herds); for goats 7/14 WB positive sera were also positive with Sar55 (7 herds) and 6 cats reacted positively in WB (table 2 summarizes these results). Interestingly, reactivity of sera was decreased by denaturation of Sar55 or ORF2H^6+ protein (figure 2) indicating that antibodies recognised primarily conformational epitopes.

4. DISCUSSION

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

One of the aims of this study was to develop an antigen based on a genotype 3 European strain. Two different expression systems were used: insect cells (a method reported to be optimal for HEV (Mast et al., 1998) and insect larvae (a large scale production system). In our hands, protein recovery was higher in insect larvae (about 1mg/larva) compared to insect cells; however the larva protein was not possible to be purified and was little stable in cold storage.
Although specific experiments to clarify these facts were not conducted, the reasons for this may be the formation of protein aggregates so there is no free protein with exposed epitopes which antibodies can recognize or the presence of proteases in the larva extract that even at low temperatures can degrade the antigenic protein. These were two serious disadvantages for that protein to be used in serological tests.

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

The performance of the two expressed proteins (cell culture and larvae-produced) was assessed by examining sera from experimental infections and by comparing results with the Sar55-ELISA. The protein expressed in insect cells always exceeded in performance the larvae-produced protein and thus it was the protein selected for the epidemiological study. The ELISA set with this cell culture-produced protein recognized accurately positive and negative sera from experimental pig and had sensitivity and specificity of 98.9% and 78.8% relative to the Sar55-ELISA. It is worth to comment the apparent discrepancy in specificities between the recombinant ORF2H$_{6+}$ ELISA and the Sar55-ELISA. Both proteins had 497 aminoacids located between aminoacid positions 112 and 607, with minor differences (5% at the aminoacid level). The predicted hidrofobicity of the two proteins was quite similar as well and therefore, the causes for this discrepancy were not evident. If we take into account that antibodies recognised primarily conformational epitopes as evidenced by the Dot Blot analysis there is the possibility that the 6x histidine tail is interfering in the protein natural conformation leading to a loss of sensitivity and specificity. Experiments with a truncated protein without the histidine tag were not performed. The fact that some react differently with the different proteins looked unimportant for pigs since in this species most animals are reported to be seropositive (Seminati et al., 2008) with seroprevalences that can reach up to 90% or higher. In contrast, a minor lack of specificity could lead to an overestimation of the prevalence in other species. Thus a stringent strategy for diagnosis was adopted: only sera reacting positively in ELISA, western blot and finally the dot blot using both Sar55 and our protein would be considered to be truly positive. With this approach, none of the cows was positive but 1.92% (1.29-2.84%) and 0.60% (0.26-
1.28%) of sheep and goat, respectively were found to be positive. In the case of cows, other authors reported seroprevalences ranging from 1.42% to 6.9% (Arankalle et al., 2001; Wang et al., 2002; Vitral et al., 2005). For sheep and goats, reports are controversial. However, viral genome has never been detected in any of the domestic ruminant species. In our case, the seroprevalence in sheep and goats was very low and the fact that in most cases a single reactor per herd was found, opens the question of whether or not those could be false positive results in spite of the stringency of the conditions required in the present study to be considered seropositive. Our opinion is that extreme caution should be applied to the interpretation of serological results in species where HEV has not been detected directly, particularly when a very large number of sera are examined since this increases the chance of finding false positives in spite a high specificity. In any case, true or false positive, our results indicate that HEV is either not present or present at a very low frequency in domestic ruminants of Spain.

Six out of 54 cats analysed resulted positive (11%, 4.6-23.32%). In this case, HEV seropositive cats were present in four out of seven studied catteries with several seropositive individuals per cattery suggesting transmission among animals in the cattery. As carnivores, as well as dogs, there is the possibility that cats get infected via the food chain. It is a common practice to feed cats and dogs with raw meat, that could probably be contaminated with swine HEV. A seroprevalence study performed on dog samples collected in the same area in our lab revealed that more than 20% of the animals tested positive for anti-HEV IgG detection supporting this hypothesis (Peralta et al., 2006). If this was true it does not necessarily mean that cats and dogs suffer the infection, the antibodies found in this species could only be the result of repeatedly contact with the virus. The percentage of positive cats found in our study is in agreement with other previous studies (Okamoto et al., 2004; Mochizuki et al., 2006) that reported that the 33% of the tested cats had anti-HEV antibodies. Those studies were done in an area with the same epidemiological situation, sporadic cases in humans and endemic situation in pigs. This fact may have a very important role in HEV transmission, since there are evidences of direct transmission from a cat to its owner (Kuno et al., 2003).
Finally, all examined rodents analyzed in this work were negative. Studies made in other countries reported seroprevalences varying from 0 to 90% in different rodent species depending on the country and the species examined (Tien et al., 1997; Kabrane-Lazizi et al., 1999; Favorov et al., 2000; Arankalle et al., 2001; He et al., 2002; Withers et al., 2002). The species analyzed in this work were *A. sylvaticus, A. flavicolis, M. musculus, R. norvegicus* and *M. glaerolus*, and few samples of each were tested. Seropositive results had been previously reported in *M. musculus* (Favorov et al., 2000), *A. sylvaticus* (Karetnyi et al., 1993) and *R. norvegicus* (Kabrane-Lazizi et al., 1999; Favorov et al., 2000), but *M. glaerolus* and *A. flavicolis* have been never analyzed before. So far, the species that have shown to be more prevalent are *R. rattus* and *R. norvegicus*, which were not present or present in a little quantity in our sampling. Before assuring that the infection is not present in rodents in Spain, a more accurate analysis should be made on species that have proved to be positive in other studies such as species of the genera *Rattus*, preferably caught in rural areas close to pig farms.

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

**Acknowledgements**

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samples and all the staff from DAR (Agricultural and Lifestock Department of the Generalitat de Catalunya) for providing ruminant samples. We also thank Dr. A. Bensaid for useful advice.

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Figure legends

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

Figure 2. Dot Blot analysis of ELISA positive samples. Lines 1 to 7 belong to cat samples, lines 8 to 14 belong to goat samples and lines 15 to 20 are sheep samples. C-, negative control sera; C+, positive control sera. a) blank, b) denatured Sar55 protein, c) native Sar55 protein, d) denatured ORF2H$^{6+}$, e) native ORF2H$^{6+}$. Only samples positives to ORF2H$^{6+}$ and at least one of the two conditions of the Sar55 protein were considered to be positive.
Figure 1

Optical density

+ mouse
- mouse
+ sheep
- sheep
+ goat
- goat

+ cow
- cow
Figure 2

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<th>6</th>
<th>7</th>
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<th>C+</th>
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Legend:
- a
- b
- c
- d
- e
Table 1
Characteristics of the inoculations for the immunization of the different animal species in order to obtain hyper-immune sera

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<tr>
<th>Specie</th>
<th>Num. of inoculations</th>
<th>Time between inoculations</th>
<th>Dosage per animal/adjuvant</th>
<th>Inoculation way</th>
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<td>Mouse</td>
<td>3</td>
<td>2 weeks</td>
<td>35 µg/FA &lt;sup&gt;a&lt;/sup&gt;</td>
<td>Intraperitoneal</td>
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<tr>
<td>Goat</td>
<td>2</td>
<td>3 weeks</td>
<td>500 µg/FA</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Sheep</td>
<td>2</td>
<td>3 weeks</td>
<td>500 µg/FA</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Cow</td>
<td>2</td>
<td>3 weeks</td>
<td>750 µg/FA</td>
<td>Subcutaneous</td>
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</table>

<sup>a</sup> 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

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<th>Species</th>
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<th>Dot Blot results</th>
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<th>Final results</th>
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<td></td>
<td>ORF2H&lt;sup&gt;6+&lt;/sup&gt;</td>
<td>Sar55</td>
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<td>% (CI95%)</td>
<td>positives/tested</td>
<td>% (CI95%)</td>
<td>positives/tested</td>
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<td>71.4 (65.4-75.8)</td>
<td>NT&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>NT</td>
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<td>NT</td>
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<sup>a</sup>NT=not tested