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## Hepatitis E virus infection in wild rabbit (*Oryctolagus cuniculus*) in Italy and in the United Kingdom: a serological, molecular and pathological study --Manuscript Draft--

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<b>Abstract:</b>	<p>A novel animal strain of HEV demonstrating zoonotic potential rabbit HEV (rHEV) has been recently identified in farmed rabbits in China. To investigate the presence of rHEV in wild rabbit population, serum and tissue samples were taken from 65 rabbits, 35 Italian and 30 from United Kingdom. Sera were submitted to a double antigen sandwich ELISA, whereas hepatic tissues and other organs to molecular and pathological investigations. Sixteen serum samples (24.6%) scored positive for anti-HEV antibodies and six samples (9%) of hepatic tissue were positive for HEV RT-PCR, while the other organs were negative. Sequencing and phylogenetic analysis of HEV RNA-positive samples indicated that while two Italian rabbits were infected with strains closely related to wild boar and swine strains (HEV-3), the other four (2 Italian and 2 English) were clustered within rHEV clade. Megalocytosis and multifocal areas of hepatocytes degeneration and necrosis with a pericentrilobular pattern was observed in rHEV-infected rabbits. In those infected by the strain analogous to HEV- 3, the lesions were mainly localized in the periportal areas and were less severe. In both cases inflammatory infiltrates were predominantly composed of CD3-positive lymphocytes and a reduced number of macrophages. By immunohistochemistry only liver samples taken from HEV RNA-positive rabbits scored positive for viral antigen. Our results indicated that HEV infection is present in rabbit population with different clades and is endemic in the Italian and English wild rabbit population, suggesting the</p>

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Hepatitis E virus infection in wild rabbit (*Oryctolagus cuniculus*) in Italy and in the  
United Kingdom: a serological, molecular and pathological study

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## Abstract

A novel animal strain of HEV demonstrating zoonotic potential rabbit HEV (rHEV) has been recently identified in farmed rabbits in China. To investigate the presence of rHEV in wild rabbit population, serum and tissue samples were taken from 65 rabbits, 35 Italian and 30 from United Kingdom. Sera were submitted to a double antigen sandwich ELISA, whereas hepatic tissues and other organs to molecular and pathological investigations. Sixteen serum samples (24.6%) scored positive for anti-HEV antibodies and six samples (9%) of hepatic tissue were positive for HEV RT-PCR, while the other organs were negative. Sequencing and phylogenetic analysis of HEV RNA-positive samples indicated that while two Italian rabbits were infected with strains closely related to wild boar and swine strains (HEV-3), the other four (2 Italian and 2 English) were clustered within rHEV clade. Megalocytosis and multifocal areas of hepatocytes degeneration and necrosis with a pericentrilobular pattern was observed in rHEV-infected rabbits. In those infected by the strain analogous to HEV-3, the lesions were mainly localized in the periportal areas and were less severe. In both cases inflammatory infiltrates were predominantly composed of CD3-positive lymphocytes and a reduced number of macrophages. By immunohistochemistry only liver samples taken from HEV RNA-positive rabbits scored positive for viral antigen. Our results indicated that HEV infection is present in rabbit population with different clades and is endemic in the Italian and English wild rabbit population, suggesting the possibility that this species may be infected with rHEV or swine HEV-3 strains.

**Keywords:** Hepatitis E Virus, Histopathology, Serology, PCR, Phylogenetic analysis, Wild rabbit (*Oryctolagus cuniculus*).

## Introduction

Hepatitis E virus (HEV) is a non-enveloped single-stranded, positive-sense RNA virus causative agent of hepatitis E. The virus belongs to genus *Orthohepevirus* in the family *Hepeviridae* which contains 4 major genotypes closely related to human infection. Genotypes 1 (HEV-1) and 2 (HEV-2) only infect humans, while genotypes 3 (HEV-3) and 4 (HEV-4) infect a wide range of animal species and are zoonotic (Smith et al. 2015). HEV-1 and 2 are primarily present in epidemic forms in developing countries where outbreaks are mainly due to water contamination via fecal-oral route. HEV-3 and 4 are defined as zoonotic with numerous animal hosts: including domestic, pet and wild mammals (Spahr et al. 2018). In regard to these genotypes the consumption of raw or undercooked contaminated meat is the main source of the infection for humans (Van der Poel 2014). Novel genotypes of HEV (5, 6 and 7) are consecutively being recognised, and recently an eight genotype has been proposed (Sridhar et al. 2017). In 2009, a novel HEV strain (rHEV) was isolated in farmed rabbits in Gansu province in China (Zhao et al. 2009). rHEV is genetically similar to zoonotic HEV-3 strains, but genetically distinct from other HEV genotypes (Caruso et al. 2015).

Anti-rHEV antibodies and rHEV-RNA were also found in farmed rabbits in USA (Cossaboom et al. 2011; (Birke et al. 2014), France (Izopet et al. 2012) and Netherlands (Burt et al. 2016). In addition, rHEV strains closely related to human patient isolates were identified in a pet house rabbit in Italy (Caruso et al. 2015). Epidemiological studies were performed in wild rabbit population in France (Izopet et al. 2012), in Germany (Hammerschmidt et al. 2017), but lesions associated with HEV infection in these subjects were not described.

The purpose of this study was to further investigate HEV presence in the wild rabbit population in two different European countries (Italy and UK), to characterize the viral strains involved, and to describe the lesions associated with this viral infection.

## Materials and methods

### Sample estimation

To determine the necessary sample size for the study (Italian rabbits), two accuracy factors were considered: I) the expected prevalence in the selected geographical area (Pisa province, Tuscany, Italy) based on previous serological and virological studies in Europe and similar ecosystems, II) the predicted number of free ranging wild rabbits in the study area.

Previous studies assessed the HEV serological prevalence in wild rabbits in Europe between 23% and 37.3% (Izopet et al. 2012); (Hammerschmidt et al. 2017). Virological data reported different values in relation to geographical areas of study: France (23%) (Izopet et al. 2012) , Netherlands (60%) (Burt et al. 2016) and Germany (37.3%) (Hammerschmidt et al. 2017). Based on this data an expected virological prevalence of 20%, with a confidence of 95% and a precision of 10% were used to calculate the sample size (Macfarlane 1997). The estimated wild rabbit population size in the study area was calculated, according to hunting data of the previous six years (about 150 free ranging animals shot per year). The proper sample size to conduct the study was calculated with “EpiTolls epidemiological calculators” ( <http://epitools.ausvet.com.au>), and resulted in 15 units. Sample size is less than 5% of the population and therefore no adjustment for finite population was needed. No hunting data and HEV serological information are available for wild rabbits in Merseyside County (UK) and therefore a sample of 30 subjects was deemed appropriate for this study and consistent with the number of Italian rabbits sampled.

### Animals examined

Thirty-five Italian wild rabbit samples were collected during 2014-2015 (18 subjects) and 2017-2018 (17 subjects) hunting season in the hunting reserve of Torre a Cenaia, Pisa province (Italy; 43°36'24.36"N 10°32'11.44"E). Thirty sample were collected from the UK - Merseyside county

(53°10'N 2°35'W), during 2018-2019 hunting season. For each rabbit ear, hind foot lengths and body mass were measured, and sex was determined. Three age classes were determined according to the body mass: newborn (< 300 g), juvenile (300–900 g) and adult (> 900 g) (Villafuerte et al. 2017). The 35 wild rabbits sampled in Italy were 19 females (4 juvenile and 15 adult subjects) and 16 males (5 juvenile e 11 adult subjects), while the 30 wild rabbit samples in UK were 9 females, 5 juvenile and 4 adult subjects and 21 males, 7 juvenile and 14 adult subjects. Immediately after shooting, all the rabbits underwent a complete post mortem examination and from each animal blood and representative samples of livers, lungs, kidneys, spleens, lymph nodes, stomach, duodenum, jejunum, ileum, sacculus, appendix and brain were collected in duplicate. Tissue samples were immediately fixed in 10% buffered formalin (pH 7.4) for histopathological and immunohistochemical investigations, within one-two hours from the subject's death one sample from each organ was stored at -80°C for PCR investigation.

### **Serology**

Blood samples were collected from the cardiac cavity as soon as possible after shooting by a 20 ml syringe and allowed to clot at room temperature. Serum samples were obtained by centrifugation and stored at -20 °C until used. All sera samples were analyzed by HEV Ab ULTRA (Dia.Pro, Italy)®, a double antigen sandwich ELISA detecting total antibodies to HEV in human or animal serum and plasma. The ELISA assay consists of microplates coated with highly specific synthetic antigen encoding for conservative and immunodominant determinants of HEV, unrelated to genotypes since HEV results in a unique serotype. In addition, the ELISA solid phase capture anti HEV total antibodies, irrespective to animal species, and then detected by the addition of the same HEV highly specific synthetic antigen labelled with peroxidase. Therefore, due to the assay configuration the ELISA kit, despite it is not formerly proven to analyse rabbit serum samples, the kit can be used for multiple species serological analysis.

Briefly, 100 µl of each sample were loaded on microwell plates. After a first incubation of 30 min at 37°C the plate was washed to remove unbound antibodies and a second HEV recombinant antigen conjugated with HRP was added. A second incubation and further washing steps were performed. Chromogen solution was added to reveal the reaction between antibodies and antigens. The reaction was stopped after 15 minutes of incubation at 37°C in the dark and optical density (OD) was acquired by a plate reader at 450nm wavelength (Multiscan FC, Thermo Scientific). The mean OD values of negative control plus 0,12 were considered as a Cut Off value. Ratio between OD of each sample and the Cut Off value was calculated. Samples with a ratio less than 0.9 were considered as negative and those with a score more than 1.1 were considered as positive. Results included between 0.9 and 1.1 were considered as doubtful.

## **Molecular analysis**

Liver tissue samples of about 30 mg were used to extract total RNA using RNeasy mini kit (Qiagen). Template cDNAs were obtained using QuantiTect Reverse Transcription kit (Qiagen) according to standard protocols.

A 347 bp fragment of HEV ORF 2 was amplified from the prepared cDNAs by nested PCR (Hotstart Taq PCR mastermix, Qiagen), as described by Meng et al (1998). Products were visualised on a 2% agarose gel, and DNA of the correct size was purified for sequencing analysis by the MiniElute Gel Extraction Kit (Qiagen). Nucleotide sequencing on 6 positive PCR product belonging to different geographical areas were performed by BMR Genomics (Padova). Sequence data and phylogenetic analysis were performed by Bioedit software (Hall, 1999). and blast analysis was conducted. For phylogenetic analysis the neighbor-joining method using MEGA5 software was performed, the number of bootstrap replicates was 1000. Avian HEV sequence (AY043166) was used as an out-group.

## Histopathological and immunohistochemical investigations

Four-micrometer serial sections from paraffin- embedded organs were stained with H&E for general examination. For immunohistochemistry (IHC), serial sections of the different organs were cut, dewaxed in xylene for 5 min, and rehydrated through graded alcohols (100, 90 and 70%) and water. Antigen retrieval was achieved placing the slides in a bath of 10 mmol/L citric acid (pH 6) and boiling for 16 min in an 800-watt microwave oven. The slides were dried at room temperature and washed with running tap water. A peroxidase block was performed, and the slides were incubated with a mouse monoclonal antibody directed against HEV (clone 4B2, Chemicon International, Temecula, California, USA). The primary antibody was diluted 1:200 in a buffer solution (PBS) prior to incubation. In order to phenotype the lymphocytic infiltrates present in the livers, specific IHC was conducted with an anti-human CD3 mouse monoclonal antibody (clone F7.2.38, Dako, Glostrup, Denmark) diluted 1:200 in PBS, with an anti-human CD79a mouse monoclonal antibody diluted 1:100 in PBS (Clone HM57, Santa Cruz Biotechnology, CA, USA) and with an anti-MAC387 monoclonal antibody (Thermo Fisher Scientific, Rockford, Illinois, USA) diluted 1:100 for the detection of macrophages. A biotinylated goat anti mouse IgG polyclonal antibody (4plus, Biocare Medical, Pacheco, California, USA) was used as a secondary antibody for 30 min at room temperature. Antibody binding was detected using a streptavidin-biotin-peroxidase kit (Vector Laboratories, CA USA). The enzymatic reaction was developed by 3-1- diaminobenzidine as a substrate (Sigma Chemical, MO, USA). Stained slides were subsequently counter-stained in hematoxylin for 40 sec followed by a wash in tap water, dehydration in graded alcohols (70, 90, and 100%), and clearance with xylene. Sections were mounted in DPX (08600E; Surgipath Europe, UK). As a positive control a liver of a PCR and ELISA positive swine was used.

## Statistical analysis

Statistical analysis was performed using the statistical package SPSS Advanced Statistics 13.0 (SPSS Inc., Chicago, IL, USA).

## Results

When shot the wild rabbits appeared asymptomatic. At post mortem examination, none of the subjects examined showed gross lesions and all were in good body condition as observed by the amount of subcutaneous and perirenal adipose tissue. Thirty out of the 65 examined subjects had gastric nematodes (*Graphidium strigosum*) and 22 rabbits showed the presence of intestinal tapeworms (*Andrya cuniculi*; Blanchard, 1891).

The results of serological and virological analyses are presented in Table 1. Sixteen of the 65 serum samples (35 from Italy and 30 from UK; 24.6%) resulted HEV positive. In particular, two of the 21 samples collected from juvenile rabbits and fourteen of the 44 samples collected from adult rabbits scored positive. Higher seroprevalence was observed in subjects sampled in Italy during the 2014-2015 hunting season (11/18) compared with those sampled in the 2017-18 (4/17;  $P < 0.02$ ) and with the UK subjects (1/30;  $p < 0.001$ ). No differences were detected when the tested subjects were divided based on age or gender. Biometric characteristic (weight and ear and hind foot length) were not statistically different in HEV-seropositive and HEV-seronegative subjects. A 347bp HEV specific product was amplified from 6 livers (9%), four from subjects sampled in Italy and 2 from the UK rabbits: two of the 21 juvenile rabbit and 4 out of the 44 adults. Both the juvenile PCR-positive rabbits were seronegative, while of the four PCR-positive adults three (one male and two female) were seropositive and one (a male) resulted negative to anti-HEV antibodies.

Despite the low number of positive animals, the evidence that positive animals are mostly adults and seroprevalence is higher than virological positivity in both class ages, we could confirm that in rabbits, HEV infection results in a self-limiting hepatitis. The nucleotide sequences obtained from positive HEV PCR amplicons were aligned using the CLUSTAL W program

(<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). BLASTn analysis confirmed the HEV identity for all the amplified products. Phylogenetic analysis showed that the rabbit HEV sequences clustered within the genotype 3 (Figure 1). In particular, two sequences belonging to two subjects hunted during 2014 (subjects # 1 and #5; accession number: MK524375, MK524376) clustered within the HEV-3 human/wild boar subtypes (wbHEV-3), and the other four (subjects # 16 and #17 hunted during 2017 in Italy MK524377, MK524378; and # 8 and # 10 hunted in UK MK524379, MK524380) clustered within rHEV-3 clade 3ra.

Histologically all PCR HEV-negative subjects did not show significant inflammatory infiltrates in periductal areas and no changes on hepatocytes architecture and morphology. No detectable immunostain was observed in hepatic ducts and in hepatocytes of all serological and PCR-negative subjects.

Liver alterations in rHEV-PCR positive wild rabbits (Figure 2) were characterized by multifocal areas of pericentrilobular necrosis with slight infiltration of mononuclear cells; megalocytic and binucleated hepatocytes were also present in these areas (Figure 2A). Sinusoid congestion was detected in these subjects associated, in one case, with mild fibrosis. Hepatocytes in these areas resulted positive with IHC for viral antigen (Figure 2C). Inflammatory infiltrates in affected areas were mainly comprised of CD3<sup>+</sup> lymphocytes (Figure 2E) and a reduced number of MAC387<sup>+</sup> mononuclear cells (Figure 2G), while few scattered CD79a<sup>+</sup> cells (up to 5% of the inflammatory infiltrating cells) were observed and interpreted as infiltrating B lymphocytes.

In the liver of subjects infected with wbHEV-3, a variable degree of periductular infiltration was evident (Figure 2B), sometimes associated with a mild periductular fibrosis, hepatocyte vacuolization and single cell hepatocyte necrosis in the periportal areas. Mild sinusoid congestion was detected in one subject and mild biliary duct proliferation associated with mild cholestasis in the other one.

In HEV-positive rabbits, histological examination of other organs did not allow to detect the presence of inflammatory lymphocytic infiltrates, characteristic of a viral infection.

Immunohistochemical investigation revealed the presence of viral antigen mainly in ductal epithelium and in the ductal wall (Figure 2D). Scattered hepatocytes in periportal areas were also positive for HEV. Inflammatory periductal infiltrates were mainly constituted by CD3+ lymphocytes (Figure 2F) and by scattered MAC-387+ mononuclear cells (Figure 2H). In these infiltrates the number of CD79a+ cells was also low.

Negative immunohistochemical results were obtained when extrahepatic tissues of HEV PCR-positive rabbits were tested.

## Discussion

The results of our study confirmed the presence of HEV infection in wild rabbit population in Italy and highlighted for the first time the presence of the viral infection in the wild rabbit population in UK. Previous studies conducted in Italy demonstrated the presence of rHEV in a pet rabbit's liver in North Italy (Caruso et al. 2015) and recent serological surveys revealed an anti-HEV seroprevalence of 3.4% in 206 farmed rabbits and 6.6% in 122 rabbits kept as companion animals (Di Bartolo et al. 2016). In a preliminary serological survey carried on a reduced number of wild rabbits shot in Tuscany (Italy) 5/13 subjects (38.5%) resulted positive for HEV antibodies (Mazzei et al. 2015a). The present study confirmed that HEV infection is diffused among the Italian and UK wild rabbit population, with a seroprevalence of 24.6%. The seroprevalence in Italy was similar to HEV seroprevalence data obtained in previous investigations conducted in wild life rabbit population in France (Izopet et al. 2012) and Germany (Hammerschmidt et al. 2017), 23% and 37.3% respectively, while the seroprevalence observed in UK was lower (3.3%). On the other hand, the percentage of HEV RNA-positive rabbits obtained in our study (11.4% in Italy and 5.7% in UK) are lower than that reported in France (23%) (Izopet et al., 2012), Netherlands (60%) (Burt et al. 2016) and Germany (37.3%) (Hammerschmidt et al. 2017).

It appears, from this study, that neither gender nor age are related to HEV positivity obtained either by serological or virological studies. HEV PCR-positivity was detected in each classes of age tested, confirming previous studies that hypothesized that HEV infection in domestic swine (Meng et al. 1998) and wild boar may also occur in young subjects (Nardini *et al.*, 2014). The fact that most of the seropositive subjects were PCR negative adults, indicates how in rabbits HEV infection can be self-limiting as already described in swine, (Meng et al. 1998), moreover the higher number of serological and virological positivity scored in adult animals is indicative of rare occurrence of vertical transmission, a possible transmission route described in pigs (Morozov et al., 2015).

Of the samples resulted PCR-positive for the presence of viral RNA, three belonged to serologically positive subjects (two female and one adult rabbits), while a young female, a young male and one adult male were HEV PCR-positive and HEV seronegative. In humans it has been suggested that the presence of HEV seronegative but PCR-positive subjects could be related to a reduced immune response due to an immunodeficiency status (Geng et al. 2014). However, it is more likely that, as previously observed in wild boars, in wild these rabbits and particularly in young subjects, the lack of HEV antibodies can be attributed to an acute infection developed before an efficient immune response against the virus has been raised (Risalde et al. 2017).

The phylogenetic investigation allowed to demonstrate that HEV-strains isolated from rabbits # 1 and # 5 hunted in Italy during 2014 were genetically related to the strains of HEV—3 isolated in 2012 from wild boars belonging to the same sampling areas (Mazzei et al. 2015b). On the other hand, sequence analysis from rabbits # 16 and # 17 hunted in 2017 in Italy and #8 and #10 hunted in UK were related to the r-HEV strains isolated in China (Liu et al. 2012) (Wang et al. 2013) and in the USA in 2010 (Cossaboom et al. 2011) and to the human strains isolated in France in 2015 (Izopet et al. 2012). The presence of HEV genotype 3 strain related to wild boar confirms previous observations referring to wild rabbits infected with a HEV-3 strain related to pig (Hammerschmidt et al. 2017).

Pathological investigations demonstrated that HEV infection in wild rabbits, as in other animal species, is characterized by changes and necrosis of hepatocytes, presence of inflammatory infiltrates and fibrosis, with some differences related to different viral subtypes. The involvement of the small bile ducts seems to be present also in the wild rabbits, even if the biliary proliferation was observed in only one subject and it was never associated with cholestasis.

In both rabbits infected with r-HEV more severe lesions were detected, as previously described in this species (Ma et al. 2010) (Han et al. 2014) as well as in pigs (Schlosser et al. 2014) and wild boars infected with HEV-3 (Nardini et al. 2014). On the contrary, in HEV-3 infected rabbits the lesions appeared to be less severe, mainly localized in portal spaces with reduced hepatocytes necrosis and degeneration.

In rabbits infected with one or the other viral strain, the lesions did not present such a severity to induce clinical symptoms, as evident by the good body conditions of these subjects. These results confirm that also in wild rabbits as in domestic pigs (Meng et al. 1998) and wild boar (Nardini et al. 2014), the HEV infection may be subclinical, making it difficult to identify infected subjects.

Immunohistochemical investigations showed that the presence of the viral antigen was associated with the necrotic and degenerative lesions found in the hepatocytes and in the ducts affected by the inflammatory changes, confirming the correlation between the presence of these lesions and the viral infection. It was also possible, by means of immunohistochemistry, to demonstrate that T-lymphocytes (CD3+) were a predominant component of lymphocyte infiltrates in all cases examined, as previously described in liver biopsies of HEV-infected humans (Krain et al. 2014) and wild boars (Nardini et al. 2014). A reduced number of macrophages was also present while B-lymphocytes were rare or absent.

In our study, it was not possible to detect the presence of the viral antigen in extrahepatic tissues, as evidenced in rabbits experimentally infected by intraperitoneal route with r-HEV

(Mao et al. 2014). The absence of the virus in tissues other than in livers was also confirmed by the lack of viral RNA by PCR analysis. These different HEV localization could be related to the experimental conditions or to different sampling times of the subjects examined.

The presence of HEV-infected wild rabbits could represent an important route of infection for humans, particularly for rabbits infected with r-HEV. Although the consumption of raw rabbit meat is unusual in Europe, handling of shot wild rabbits could be an important source of infection for humans, as was already the case for a patient who had butchered a wild boar he had hunted in Barberino del Mugello (Tuscany) in March 2012 and he had never consumed the meat nor traveled outside Italy (Giordani et al. 2013). For this reason consumers and occupational groups such as hunters, veterinarians and forestry workers should minimize contact to animals that are known to be a reservoir for HEV and protective equipment (gloves etc.) should be used when handling rabbits after hunting or during slaughtering (Schielke et al. 2015).

In conclusion, this study confirms that HEV infection is endemic in wild rabbit population in Italy and in UK and that these wild species could be infected by both r-HEV and HEV-3 strains closely related to other species. The widespread presence of the virus throughout wild rabbit populations in Europe reinforces the evidence that this species can have an important role in the zoonotic transmission of the virus to humans.

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## Figure legends

Fig. 1. The evolutionary history was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 26 nucleotide sequences. There were a total of 235 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Fig. 2. Wild rabbit liver, lesions associated with HEV infection, HEV antigen distribution and cellular composition of inflammatory infiltrates. A) rHEV infected rabbit. Small areas of pericentrilobular necrosis associated with megalocytosis and binucleation of hepatocytes and with mild infiltration of monuclear cells (H-E, bar=150  $\mu$ m). B) rHEV infected rabbit. Anti-HEV antigen immunostaining revealed that the hepatocytes in pericentrilobular area resulted positive for viral antigen (IHC, bar=150  $\mu$ m). C) rHEV infected rabbit. Anti-CD3 immunostaining showing that T lymphocytes are associated with HEV infection (IHC, bar=150  $\mu$ m). D) rHEV infected rabbit. Several MAC387-positive macrophages are present in the same areas (IHC, bar=150  $\mu$ m). E) wbHEV-3 infected rabbit. Periductular infiltration associated with mild fibrosis (H-E, bar=150  $\mu$ m). F) wbHEV-3 infected rabbit. Anti-HEV antigen immunostaining revealed the presence of viral antigen in ductal epithelium (IHC, bar=150  $\mu$ m). G) wbHEV-3 infected rabbit. Anti-CD3-positive T lymphocytes are present in the inflammatory exudates targeting the ductal wall (IHC bar=150  $\mu$ m). H) wbHEV-3 infected rabbit. Scattered MAC387-positive macrophages are present in the inflammatory exudates targeting the ductal wall (IHC bar=150  $\mu$ m).

Table 1. Results of serological and virological analysis performed on the 65 wild rabbits examined.

Subjects		N° positive /examined		
		Rabbits sampled in Italy	Rabbits sampled in Italy	Rabbits sampled in UK
		in 2014-2015	in 2017-2018	in 2018-2019
		Total	Total	Total
Juveniles	ELISA positive	2/6	0/3	0/12
	PCR positive	1/6	0/3	1/12
Adults	ELISA positive	9/12	4/14	1/18
	PCR positive	3/12	0/14	1/18
Total	ELISA positive	11/18	4/17	1/30
	PCR positive	4/18	0/17	2/30

