Received: 4 August 2021 | Revised: 4 December 2021 | Accepted: 21 December 2021

DOI: 10.1111/tbed.14437

Transboundary and Emerging Diseases

WILEY

RAPID COMMUNICATION

Serological and molecular survey of hepatitis E virus in cats and dogs in Spain

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Funding information
Ministerio de Sanidad, Grant/Award Number: RD12/0017/0012; Fundación para la Investigación en Salud, Grant/Award Numbers: PI19/00864, PI21/00793; Spanish Ministry of Science, Innovation and Universities, Grant/Award Number: CP18/00111; Spanish Ministry of Science, Innovation and Universities, Grant/Award Number: INT20-00028; Spanish Ministry of Science, Innovation and Universities, Grant/Award Number: FPU17/01319; European Social Fund, Grant/Award Number: EJ20-AGR-149/AGR-013; Spanish Ministry of Science, Innovation, and Universities, Grant/Award Number: FPU19/06026; Instituto de Salud Carlos III, Grant/Award Numbers: PI19/00864, PI21/00793

Abstract

Hepatitis E virus (HEV) is an emerging zoonotic pathogen that is currently recognized as one of the major causes of acute human hepatitis worldwide. In Europe, the increasing number of hepatitis E cases is mainly associated with the consumption of animal food products or contact with infected animals. Dogs and cats have been suggested as a zoonotic source of HEV infection. The aim of this study was to assess *Orthohepevirus* circulation, including HEV-A, HEV-B and HEV-C species, in sympatric urban cats and dogs in southern Spain. Between 2017 and 2020, blood samples were collected from 144 stray cats and 152 dogs, both strays and pets. The presence of antibodies against HEV were tested using a double-antigen sandwich ELISA and seropositive samples were further analysed by western blot. A RT-PCR was performed to detect RNA of *Orthohepevirus* species (HEV-A, HEV-B and HEV-C). A total of 19 (6.4%; 95%CI: 3.6-9.2) of the 296 animals tested showed anti-HEV antibodies by ELISA. Seropositivity was significantly higher in dogs (9.9%; 15/152; 95%CI: 5.1-14.6) than in cats (2.8%; 4/144; 95%CI: 0.1-5.5). Ten of the 18 ELISA-positive animals that could be further analysed by western blot, reacted against HEV-3 and/or HEV-C1 antigens, which suggest circulation of both genotypes in urban cats and dogs in the study area. However, HEV-A, HEV-B and HEV-C RNA were not detected in any of the tested sera. This is the first study to assess HEV circulation in both stray cats and dogs in Europe. Our results provide evidence of HEV exposure in sympatric urban cat and dog populations in southern

Spain. Further studies are needed to determine the role of these species in the epidemiology of HEV.

KEYWORDS
cat, dog, Hepatitis E, Orthohepevirus A, Orthohepevirus C, survey, zoonoses

1 | INTRODUCTION

The *Orthohepevirus* genus includes single-stranded RNA viruses with four different species, designated *Orthohepevirus A*, *B*, *C* and *D* (henceforth HEV-A to D; Doceul et al., 2016). Of these, HEV-A is the most important in terms of public health concern, since it is considered one of the major causes of acute hepatitis worldwide (Aspinall et al., 2017). HEV-A is further subdivided into eight different genotypes (HEV-1 to HEV-8). HEV-1 and HEV-2 are restricted to humans and are mainly transmitted through contaminated drinking water in Central American, Asian and African countries. HEV-3 to HEV-8, however, have been detected in different animal species worldwide, and zoonotic transmission of HEV-3, HEV-4 and HEV-7 has been reported through the consumption of raw or undercooked animal products or contact with infected animals (Wang & Meng, 2021).

Circulation of HEV-B, HEV-C and HEV-D, on the other hand, has traditionally been limited to their main hosts (birds for HEV-B, rodents and wild carnivores for HEV-C, and bats for HEV-D) (Drexler et al., 2012; Huang et al., 2004; Purcell et al., 2011). However, our knowledge of the HEV-C host range has expanded in the last few years, since rat HEV-C1 infections have been detected in other mammal species, including human beings (Andonov et al., 2019; Spahr et al., 2017; Sridhar et al., 2018, 2020).

Cats and dogs are the main species kept as pets worldwide. In Europe, there are more than 195 million cats and dogs and approximately 25% of households own at least one of these two species (FEDIAF, 2020). The number of stray cats and dogs has also increased in recent decades (Tasker, 2007; Voslářvá & Passantino, 2012) and they are currently considered as potential sources for the transmission of zoonotic pathogens, including HEV (Peralta et al., 2009; Zeng et al., 2017).

The presence of anti-HEV antibodies has been reported in dogs and cats on different continents, with seroprevalence values ranging between 0.9% and 56.6% (Tables 1 and 2). Zoonotic HEV-A transmission from these species has previously been suggested (Kuno et al., 2003; Zeng et al., 2017). Despite their close contact with human beings, there is very little information about HEV infection in cats and dogs worldwide (Tables 1 and 2) and their role in the epidemiology of this virus is also still poorly understood. To date, only HEV-A exposure has been assessed in sympatric cat and dog populations. The aim of this study therefore was to assess HEV circulation, including HEV-A, HEV-B and HEV-C, in sympatric urban cats and dogs in Spain.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

This study was carried out in accordance with Spanish legislation guidelines (RD 8/2003) and with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for International Organization of Medical Sciences and the International Council for Laboratory Animal Science (RD 53/2013).

2.2 | Sampling

Between 2017 and 2020, blood samples from 144 feral cats, 53 stray dogs and 99 pet dogs were collected from 11 different urban sampling areas in the province of Córdoba (southern Spain; 37°53′5″ N, 4°46′45″ W; Figure 1). Samples from stray animals were collected taking advantage of the sanitary control undertaken by the regional government of Cordoba. Feral cats were part of catch-neuter-release (CNR) or population control programs, conducted by staff at the Córdoba Animal Health and Welfare Center (SBA, SADECO S.A.). Samples from cats were collected after being sedated with a combination of xylazine (Nerfasin®, 0.75 mg/kg) and ketamine (Ketamidor®, 15 mg/kg). Dog samples were collected during management programs for stray dogs, or from pets that had just been handed over to the SBA by their owners. Sera were obtained by centrifugation at 400 × *g* for 10 min and stored at −20°C until analysis. Epidemiological information included species, life condition (pet vs. stray), age (yearlings: < 1 year old; subadults: 1 to 3 years old; adults: >3 years old), sex, sampling date and location and was collected from each animal whenever possible.

2.3 | Serological analysis

All animals included in the study were tested in parallel for both anti-HEV antibodies by ELISA and molecular analysis. The presence of antibodies against HEV was determined from individual serum samples, using a commercial double-antigen multi-species sandwich HEV ELISA 4.0v (MP Diagnostics, Illkirch, France), following the manufacturer's instructions. This assay is based on the recombinant protein ET2.1, which is highly conserved in HEV-A genotypes (Hu et al., 2008), and detects the presence of total antibodies against this *Orthohepevirus*

TABLE 1 Prevalence of anti-HEV antibodies in cats worldwide

Life condition	Country	Sampling period	No. Seropositives/No. Analyzed (Seroprevalence)	No. Positives/No. Analyzed (HEV RNA prevalence)	Sample	Reference
Pet	China	2012–2013	12/191 (6.3%)	NA*	NA	Liang et al. (2014)
NA	Germany	2002–2005	21/65 (32.3%)	0/65 (0.0%)	Body cavity transudate	Dähnert et al. (2018)
Pet	Italy	2017–2018	10/324 (3.1%)	0/324 (0.0%)	Serum	Capozza et al. (2021)
Pet	Japan	2000–2004	4/202 (2.0%)	0/74 (0.0%)	Rectal swabs and serum	Mochizuki et al. (2006)
Pet	Japan	NA	44/135 (32.6%)	0/135 (0.0%)	Serum	Okamoto et al. (2004)
Pet	Korea	2007–2008	8/99 (8.1%)	NA	NA	Song et al. (2010)
Pet	The Netherlands	2017	7/47 (14.9%)	0/47 (0.0%)	Pools of sera	Li et al. (2020)
Shelter	Spain	NA	6/54 (11.1%)	NA	NA	Peralta et al. (2009)
Stray	Spain	2017–2019	4/144 (2.8%)	0/144 (0.0%)	Serum	Present study
Pet	The United States of America	NA	0/177 (0.0%)	NA	NA	Dong et al. (2011)
Stray	The United States of America	NA	0/22 (0.0%)	NA	NA	Dong et al. (2011)

*Not available.

species (IgM, IgG and IgA) in sera or plasma from all animal species. The sensitivity and specificity of this multi-species assay were set at 99.2%.

Whenever possible, the presence of antibodies against the capsid proteins of HEV-A and HEV-C was assessed in the seropositive samples by western blot analysis as previously described (Kubickova et al., 2021). For this purpose, carboxy-terminal segments of the capsid proteins of rat HEV-C1, HEV-3 and a nucleocapsid protein derivative (amino acid residues 1-39/213-433) of *Puumala orthohantavirus* strain Vranica/Hällnäs, as negative control, were produced as His-tagged recombinant proteins in *Escherichia coli* and purified by nickel-chelate affinity chromatography (Dremsek et al., 2012; Lundkvist et al., 2002). Purified proteins were run in a 12% SDS-PAGE and transferred to a PVDF membrane and analysed for control by anti-His tag and HEV capsid protein cross-reactive monoclonal antibodies. Serum samples were diluted 1:100 in 5% skimmed milk in PBS-Tween 20 (PBS-T) and the antigen–antibody reaction was detected by adding horseradish peroxidase (HRP) labelled anti-cat IgG or anti-dog IgG (Jackson InmunoResearch, West Grove, Philadelphia, USA), diluted 1:2500 in 5% PBS-T. The immunoreaction was detected using Claritytm Western ECL Substrate (Biorad, California, USA) and documented in a VersaDoc 4000MP (Bio-Rad) with an exposure time between 5 and 60 s.

2.4 | Molecular analysis

For the molecular evaluation, RNA was extracted from 400 μl pools of serum, using the QIAamp MinElute virus spin kit and the QIAcube system (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Each pool contained sera from four different individuals (100 μl of each sample). The purified RNA was eluted in a total volume of 30 μl. For the detection of HEV-A RNA, real-time RT-PCR (CFX Connect Real Time PCR System) was performed using 10 μl of RNA template and the QIAGEN One-Step RT-PCR kit, as previously described (Frias et al., 2021). As a positive extraction control, the HEV-3a strain Kernow-C1 was spiked in a subset of pools of serum samples.

All samples were also tested by a broad-spectrum nested PCR that target the viral ORF1 and capable to detect HEV-A, HEV-B and HEV-C as previously described Johne et al. (2010). For the first round, the primers HEV-cs and HEV-cas and the QIAGEN One-Step RT-PCR kit were used, whereas for the second round the primers HEV-csn and HEV-casn and the premixed 2× solution of Taq DNA Polymerase, dNTPs and Reaction Buffer kit (Promega) were used. The WHO HEV-3a reference strain (code 6329/10) supplied by the Paul-Ehrlich-Institut, was included as a positive control in each run of RT-PCR. The amplicons of the second PCR were examined on 1.5% agarose gels stained with RedSafe™ Nucleic Acid Staining solution.

2.5 | Statistical analyses

The estimated prevalence of anti-HEV antibodies and HEV RNA was calculated by dividing the number of positive animals by total animals tested, using two-sided exact binomial tests, with 95% confidence intervals (95%CI). Associations between seroprevalence of HEV and

TABLE 2 Prevalence of anti-HEV antibodies in dogs worldwide

Life condition	Country	Sampling period	No. Seropositives/No. Analyzed (Seroprevalence)	No. Positives/No. Analyzed (HEV RNA prevalence)	Sample	Reference
NA	Brazil	NA	3/43 (7.0%)	NA	NA	Vitral et al. (2005)
Pet	China	2012–2013	139/658 (2.1%)	NA	NA	Liang et al. (2014)
NA	China	NA	0/21 (0.0%)	NA	Serum	Geng et al. (2010)
Pet	China	2004–2006	18/101 (17.8%)	0/101 (0.0%)	Serum	Zhang et al. (2008)
Pet	China	2007–2008	23/192 (12.0%)	0/192 (0.0%)	Serum	Liu et al. (2009)
Pet	China	NA	62/387 (16.0%)	0/387 (0.0%)	Serum	Wang et al. (2016)
Stray	China	NA	16/55 (29.1%)	0/55 (0.0%)	Serum	Wang et al. (2016)
Pet	China	2014–2016	1030/3101 (33.2%)	NA	NA	Zeng et al. (2017)
Farm	China	2014–2016	231/757 (30.5%)	NA	NA	Zeng et al. (2017)
Stray	China	2014–2016	380/632 (60.1%)	NA	NA	Zeng et al. (2017)
NA	Germany	2002–2005	47/83 (56.6%)	0/83 (0.0%)	Body cavity transudate	Dähnert et al. (2018)
Pet	India	1983	10/44 (22.7%)	NA	NA	Arankalle et al. (2001)
Hunting	Italy	2014	5/35 (14.3%)	NA	NA	Mazzei et al. (2015)
Pet	Japan	2000–2004	0/424 (0.0%)	0/110 (0.0%)	Rectal swabs and serum	Mochizuki et al. (2006)
Pet	Korea	2007–2008	0/213 (0.0%)	NA	NA	Song et al. (2010)
Pet	Spain	2019–2020	10/99 (9.9%)	0/99 (0.0%)	Serum	Present study
Stray	Spain	2019–2020	5/53 (9.4%)	0/53 (0.0%)	Serum	Present study
Pet	Switzerland	2019	32/84 (38.1%)	0/32 (0.0%)	Serum and plasma	Veronesi et al. (2021)
Pet	The Netherlands	2017	30/162 (15.5%)	0/162 (0.0%)	Faeces and pools of sera	Li et al. (2020)
Pet	The United Kingdom	2012–2013	2/92 (2.2%)	0/332 (0.0%)	Faeces and liver	McElroy et al. (2015)
Pet	The United States of America	NA	2/212 (0.9%)	NA	NA	Dong et al. (2011)
NA	Vietnam	NA	NA (27.0%)	NA	NA	Tien et al. (1997)

*Not available.

the explanatory variables (species, life condition, age, sex and sampling year) were analysed using the Fisher's exact test or Pearson's chi-square test, as appropriate. Values with $p < 0.05$ were considered statistically significant. Statistical analyses were performed using SPSS 25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA).

3 | RESULTS AND DISCUSSION

A total of 19 (6.4%; 95%CI: 3.6-9.2) of the 296 analysed animals showed antibodies against HEV (Table 3) and seropositive individuals were detected in eight (82.7%) of the 11 sampled areas (Figure 1). Ten of the 18 ELISA-positive animals that could be further analysed were confirmed by western blot. Of note, five sera (from one cat and four dogs) reacted against both HEV-3 and rat HEV-C1 antigens, whereas three (from one cat and two dogs) and two (one cat and one dog) samples reacted only against HEV-3 and rat HEV-C1 antigen, respectively. These findings suggest circulation of both genotypes in urban cats and dogs in southern Spain. Overall, our results indicate exposure to HEV in the pet species analysed and a widespread distribution in cat and dog populations in the study area. Ingestion of contaminated food is considered to be one of the main transmission routes of HEV in pigs and humans (Kamar et al., 2017; Meng, 2010), and probably also in cats and dogs (Liu et al., 2009; Peralta et al., 2009; Wang et al., 2016). Indeed, feeding on kitchen waste and/or animal offal has been identified as a risk factor associated with HEV seropositivity in both cats and dogs (Liang et al., 2014; Wang et al., 2016). Nevertheless, since cats and dogs can directly or indirectly come into contact with other susceptible domestic and wild sympatric species, such as rabbits

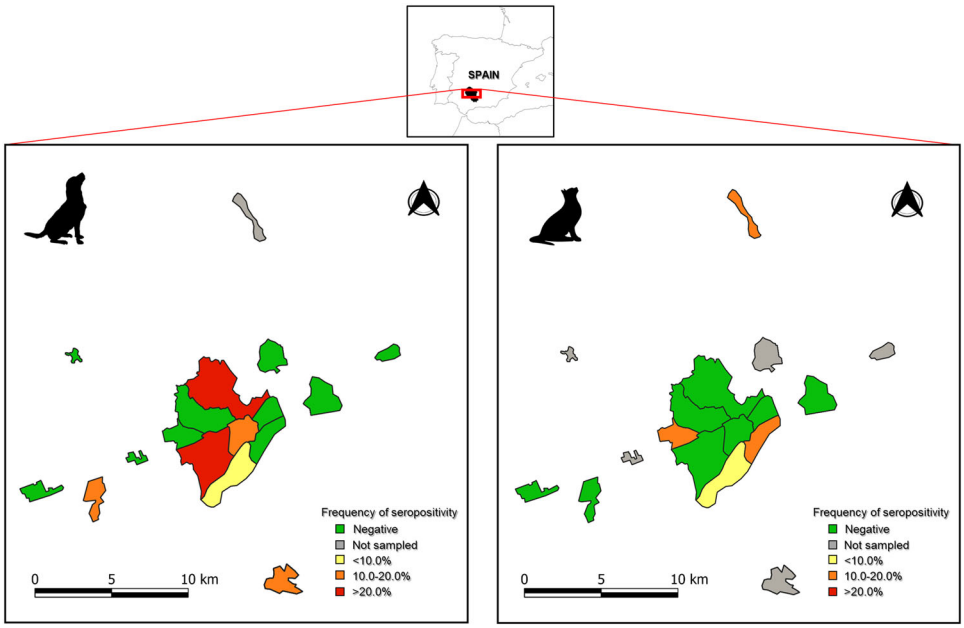

FIGURE 1 Spatial distribution of HEV seropositivity in dogs (left) and cats (right) in the province of Cordoba (southern Spain)

TABLE 3 Distribution by categories of HEV seropositivity in sympatric urban cats and dogs in southern Spain

Variable	Categories	No. positives/No. analyzed (%)*	*p*-Value	Distribution by species	
				CatsNo. positives/No. analyzed (%)*	DogsNo. positives/No. analyzed (%)*
Species	Cat	4/144 (2.8)	0.011**	4/144 (2.8)	–
	Dog	15/152 (9.9)		–	15/152 (9.9)
Year	2017	3/106 (2.8)		3/106 (2.8)	–
	2019	14/180 (7.8)	0.052	1/38 (2.6)	13/142 (9.2)
	2020	2/10 (20.0)		–	2/10 (20.0)
Life condition	Stray	9/197 (4.6)	0.06	4/144 (2.7)	5/53 (9.4)
	Pet	10/99 (10.1)		–	10/99 (10.1)
Age	Yearling	2/20 (10.0)	0.650	0/2 (0.0)	2/18 (11.1)
	Sub-adult	3/63 (4.8)		0/25 (0.0)	3/38 (7.9)
	Adult	13/169 (7.7)		3/76 (3.9)	10/93 (10.8)
Sex	Female	6/146 (4.1)	0.086	2/83 (2.4)	4/63 (6.4)
	Male	13/150 (8.7)		2/61 (3.3)	11/89 (12.4)

*Missing values excluded; ****p*-value < 0.05**.

(*Oryctolagus cuniculus*), rats (*Rattus* spp.) or wild boar, HEV transmission from these animal species should also be considered. In line, rat HEV-C1 has been detected in several European regions, including southern Spain (Ryll et al., 2017). Moreover, the abundance and distribution of wild boar populations have both sharply increased in Spain in the past decade (Massei et al., 2015), becoming common the presence of wild boar in urban environments (Castillo-Contreras et al., 2021). In connection with this, 52 (19.7%) of 264 wild boars sampled in metropolitan areas of Barcelona (northeastern Spain) were positive for HEV RNA (Wang et al., 2019), indicating a potential source of HEV transmission in urban areas. In our study region, HEV circulation has been reported in wild boar populations with prevalence values ranging between 6.8% and 23.2% (Risalde et al., 2017; Rivero-Juarez et al., 2018) and HEV exposure has also been detected in other mammals, including zoo animals and humans (Caballero-Gómez et al., 2019; Rivero-Juarez et al., 2015). In any case, additional studies are needed to determine the sources of HEV transmission in cat and dog populations in the study area.

The seroprevalence found in cats was 2.8% (4/144; 95%CI: 5.1-14.6; Table 3). This value is of the same magnitude as that found in this species in China (6.3%), Italy (3.1%), Japan (2.0%) and Korea (8.1%; Table 1; Capozza et al., 2021; Liang et al., 2014; Mochizuki et al., 2006; Song et al., 2010). Higher mean seropositivity was reported in the only previous study carried out in Spain, in which 11.1% of 54 shelter cats from Catalonia (northeastern Spain) were seropositive for HEV (Peralta et al., 2009). Higher seroprevalence rates were also observed in other countries including Germany (32.3%), the Netherlands (14.9%) and also Japan (32.6%; Table 1; Dähnert et al., 2018; Li et al., 2020; Okamoto et al., 2004). By contrast, Dong et al. (2011) failed to detect anti-HEV antibodies in cats from the USA.

With respect to dogs, 15 (9.9%; 95%CI: 0.1-5.5) of 152 dogs tested had anti-HEV antibodies (Table 3). The life condition of this species was not shown to be a risk factor for HEV exposure in our study, because statistically significant differences between strays (9.4%; 5/53) and pets (10.1%; 10/99) ($p = 0.570$) were not found. The seroprevalence obtained in dogs in the present study was similar to that reported in Brazil (7.0%) and slightly lower than that found in Italy (14.3%), the Netherlands (15.5%) and China (ranging between 12.0 and 17.8%; Table 2; Li et al., 2020; Liu et al., 2009; Mazzei et al., 2015; Vitral et al., 2005; Wang et al., 2016). A higher frequency of seropositivity was detected in other studies in China (29.1-60.1%), Germany (56.6%), India (22.7%), Switzerland (38.1%) and Vietnam (27.0%; Arankalle et al., 2001; Dähnert et al., 2018; Tien et al., 1997; Veronesi et al., 2021), while lower seroprevalence rates were observed in the United Kingdom (2.2%) and the United States (0.9%; Dong et al., 2011; McElroy et al., 2015). Similarly, antibodies against HEV were not found in this species in some Asian countries, including China, Japan and Korea (Table 2; Geng et al., 2010; Mochizuki et al., 2006; Song et al., 2010). Even though it is not possible to make accurate comparisons across studies, given the differences in numbers of animals tested, populations sampled and/or the different serological methods used, we would like to state that the seroprevalence of HEV in urban cats and dogs in the study area (Córdoba province, southern Spain) should be considered low and moderate, respectively.

Differences in seroprevalence between species were found, with significantly higher seropositivity in dogs compared to cats ($p = 0.011$; 95%CI: 1.2−10.5; Relative Risk = 3.6). This finding suggests that these species would not necessarily have the same susceptibility to HEV or another unknown HEV-related virus or that, even though both species shared the same habitat, they were not equally exposed to HEV in the study area, which is consistent with previous studies (Tables 1 and 2; Dähnert et al., 2018; Li et al., 2020; Peralta et al., 2009). Whereas stray cats usually live in groups, form colonies and have a limited range of movement, dogs can cover greater distances, frequently entering both urban and periurban areas, which may increase the risk of exposure to different sources of HEV. In addition, the fact that HEV is excreted in faeces (Pavio et al., 2017) and the common practice of canine coprophagia are other possible factors associated with the higher exposure to HEV in this species (Fahrion Schnyder, Wichert, & Deplazes, 2011). Of note, anti-HEV antibodies were also observed in two yearling (4 months old) stray dogs from the same sampling area in 2019. Although the presence of maternal antibodies in yearling mammals cannot be ruled out, this finding denotes HEV circulation in dog populations in the study area during that year.

This is the first study to assess the presence of HEV-A RNA in cats and dogs in Spain. None of the 296 (0.0%; 95%CI: 0.0−1.2) tested animals were positive for active HEV-A infection. Likewise, HEV-A RNA has not to date been found in any of the previous studies conducted in cats and dogs worldwide (Tables 1 and 2). Liu et al. (2009) also failed to detect active infection in sera from two dogs experimentally infected with HEV-4, which denotes absence or limited HEV-A viremia in this species. However, seroconversions in experimentally infected dogs occurred 14 days post-infection and the anti-HEV-A antibodies persisted for at least 6 months, which confirms their susceptibility to HEV-A infection. Cats have already been suggested as a potential zoonotic source for this *Orthohepevirus* species (Kuno et al., 2003). In this regard, previous studies have identified that the contact with cats and dogs could be a risk factor for HEV exposure in humans (Cong et al., 2015; Li et al., 2020).

In view of the absence of HEV-A infection, coupled with the rates of seropositivity observed in cats and dogs not only in the present study but also in previous surveys (Tables 1 and 2) and the possibility of HEV antibody cross-reactivity among *Orthohepevirus* species, we hypothesized that these species may be infected with other related hepeviruses. HEV-B, HEV-C and HEV-D RNA was not detected in any of the 144 cats and 152 dogs analysed, which indicates absence of active infection with these *Orthohepevirus* species in the sampled populations. Our results agree with those reported in 324 sera from cats in Italy (Capozza et al., 2021). To the best of our knowledge, this is the first study to assess active HEV-B, HEV-C and HEV-D infection in dogs worldwide. Previous studies confirmed the presence of hepeviruses clustered within the HEV-C group in as well as in other carnivore species, including a captive Syrian brown bear (*Ursus arctos syriacus*), European mink (*Mustela lutreola*) and European ferrets (*Mustela putorius*) from Germany, the Netherlands, the United States, Japan and China (Spahr et al., 2017; Spahr et al., 2018). Further studies are required to evaluate HEV-C circulation in sympatric carnivores in Spain.

This study has some limitations that should be taken into account. First, even though the high specificity of the ELISA used in the present study and that anti-HEV antibodies against HEV-3 and rat HEV-C1 were confirmed by western blot, cross-reactivity to other related hepevirus cannot be ruled out. Second, it was not possible to assess HEV excretion in faeces of the analysed animals. Although HEV RNA has not been detected either in stool samples from dogs and cats in previous studies (Tables 1 and 2), further studies testing serum and faecal samples should be carried out to increase the sensitivity of HEV detection in these species.

In conclusion, the serological results provide evidence of HEV exposure in sympatric urban cats and dogs in Spain. However, the absence of active infection suggests that these species play a limited role in the epidemiology of HEV in southern Spain. Given the results obtained in the present study and taking into consideration that these species may be exposed to HEV through the same source of contamination as

humans, cat and dog could be potential sentinels of environmental circulation of hepeviruses in urban and periurban areas. Additional studies are warranted to determine the risk of HEV transmission from cats and dogs to other sympatric species, including human beings.

ACKNOWLEDGEMENTS

This work was supported by the Ministerio de Sanidad (RD12/0017/0012) integrated in the Plan Nacional de I+D+I and cofinanced by the ISCIII-Subdirección General de Evaluación and the Fondo Europeo de Desarrollo Regional (FEDER), Fundación para la Investigación en Salud (FIS) del Instituto Carlos III (PI19/00864 and PI21/00793). A. Rivero-Juarez is the recipient of a Miguel Servet Research Contract by the Spanish Ministry of Science, Innovation and Universities (CP18/00111). A. Rivero is the beneficiary of Contratos para la intensificación de la actividad investigadora en el Sistema Nacional de Salud by the Spanish Ministry of Science, Innovation and Universities (INT20-00028). J. Caballero-Gómez is supported by an FPU grant of the Spanish Ministry of Science, Innovation and Universities (FPU17/01319). D. Jiménez-Martín holds a I+D+i contract from the University of Cordoba co-supported by the European Social Fund (EJ20-AGR-149/AGR-013). S. Castro-Scholten is supported by an FPU grant from the Spanish Ministry of Science, Innovation, and Universities (FPU19/06026). R.G. Ulrich acknowledges support by the Deutsches Zentrum für Infektionsforschung (DZIF), TTU "Emerging infections". We would like to thank Patrick Slowikowski for the support and Barbara Kubickova for providing recombinant HEV antigens. The funders did not play any role in the design, conclusions or interpretation of the study. Funding for open access charge: Universidad de Córdoba / CBUA.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the authors upon reasonable request.

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How to cite this article: Caballero-Gómez, J., Rivero-Juarez, A., Jurado-Tarifa, E., & Jiménez-Martín, D., Jiménez-Ruiz, E., Castro-Scholten, S., Ulrich, R. G., López-López, P., Rivero, A., & García-Bocanegra, I. (2022). Serological and molecular survey of hepatitis E virus in cats and dogs in Spain. *Transboundary and Emerging Diseases*, *69*, 240–248. https://doi.org/10.1111/tbed.14437