





Epidemiological survey and risk factors associated with hepatitis E virus in small ruminants in southern Spain

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Abstract

Autochthonous cases of hepatitis E (HE) associated with zoonotic genotypes HEV-3 and HEV-4 have significantly increased in industrialized countries over the last decade. Suidae are generally recognized as the main reservoirs of these genotypes. Susceptibility to HE virus (HEV) infection and zoonotic potential have also been confirmed in other species, including sheep and goat. However, the information about their role in the epidemiology of HEV remains very scarce. The objective of this study was to assess the prevalence, spatial distribution and risk factors associated with HEV exposure in sheep and goats in southern Spain, the country with the highest census of small domestic ruminants in the European Union. Blood samples from 240 sheep and 240 goats were collected between 2015 and 2017. Sera were analysed in parallel using a commercial double-antigen ELISA and real-time PCR. A total of 38 (7.9%; 95%CI: 5.5–10.3) out of 480 sampled animals showed anti-HEV antibodies. By species, the seroprevalences found in sheep and goats were 2.1% (5/240; 95%CI: 0.3–3.9) and 13.8% (33/240; 95%CI: 9.4–18.1) respectively. Anti-HEV antibodies were found on 19 (59.4%; 95%CI: 42.4–76.4) of the 32 sampled farms. The GEE model showed that species (goat) and number of small ruminants in the farm (≤ 348 animals and ≥ 538 animals) were risk factors potentially associated with HEV exposure in small ruminants in the study area. HEV RNA was not detected in any of the 480 (0.0%; 95%CI: 0.0–0.8) tested animals. Our results confirm that sheep and goats are naturally, but not equally exposed to HEV and indicate the widespread spatial distribution of HEV among small ruminant populations in southern Spain. Further studies are required to elucidate the role of sheep and goat in the epidemiology of HEV and their potential implications for public health.

KEYWORDS

Hepatitis E virus, livestock, public health, risk factors, zoonoses

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1 | INTRODUCTION

Hepatitis E virus (family *Hepeviridae*; *Orthohepevirus A* species) is an emerging pathogen of public health concern and is currently considered to be the main viral cause of acute human hepatitis worldwide (Wang & Meng, 2021). Of the eight different genotypes recognized so far, HEV-3, HEV-4 and HEV-7 are confirmed as zoonotic. In recent decades, hepatitis E cases, mainly associated with HEV-3 infections, have sharply increased in industrialized countries (Aspinall et al., 2017). This genotype is mainly transmitted through the consumption of raw or undercooked animal products or contact with infected animals (EFSA et al., 2017). Swine are the main reservoir of HEV-3 and cases of foodborne transmission from pigs and wild boar (*Sus scrofa*) to humans have been confirmed worldwide (Colson et al., 2010; Guillois et al., 2016; Renou et al., 2014; Rivero-Juarez et al., 2017). Susceptibility to this zoonotic genotype has also been demonstrated in other ungulates, including livestock species such as sheep and goats (Long et al., 2017; Sarchese et al., 2019). Indeed, contact with these small ruminant species has been found to be a risk factor for HEV infection in pigs (Lopez-Lopez et al., 2018).

The presence of anti-HEV antibodies in sheep and goats has been reported on different continents, with seroprevalences ranging from 1.4% to 100% (Peralta et al., 2009; Shukla et al., 2007). High homology between human and ruminant HEV sequences has also been confirmed (Di Martino et al., 2016; Long et al., 2017), raising concerns about zoonotic transmission of HEV from these animal species (Long et al., 2017; Mesquita et al., 2020). In connection with this, it has been suggested that the consumption of contaminated milk, meat and/or dairy products such as cheese from both sheep and goats could be a source of HEV infection in humans (Dziedzinska et al., 2020; El-Mokhtar et al., 2020). Nevertheless, information about the role of small ruminants in HEV epidemiology remains scarce.

Spain is the country with the largest census of small domestic ruminants in the European Union (EU), with more than 15.4 and 2.6 million sheep and goats respectively (Eurostat, 2020a,2020b). It is also the second largest supplier of milk from these species to the EU and the second largest producer of pure goat and sheep cheese in this region (MAPA, 2021). The aim of this study was to determine the prevalence, spatial distribution and risk factors associated with HEV exposure in sheep and goats in southern Spain.

2 | MATERIALS AND METHODS

2.1 | Study design and sampling

A cross-sectional study of small ruminants was conducted in Andalusia (southern Spain; 36°N–38° 60'N, 1°75'W–7°25'W) (Figure 1) between 2015 and 2017. The sample size was calculated at 196 sheep and 196 goats, assuming a seroprevalence of 15% (Mesquita et al., 2020; Palombieri et al., 2020), with a 95% confidence level (95%CI) and desired precision of $\pm 5\%$ (Thrusfield &

Impacts

- Sheep and goat are naturally, but not equally exposed to HEV. Significantly higher seroprevalence was found in goats compared to sheep.
- High (≥ 538 animals) and low (≤ 348 animals) numbers of small ruminants on farm was a risk factor potentially associated with HEV exposure in small ruminants.
- A wide spatial distribution of HEV was detected in small ruminant populations in southern Spain.

Christley, 2018). Farms were selected by simple random sampling from official flock registers provided by the Regional Government of Andalusia. Samples were collected from 15 animals per farm, selected by systematic random sampling, in order to detect HEV exposure with a 95% probability and a minimum expected seroprevalence of 20%.

A total of 480 animals (240 sheep and 240 goats) from 32 farms (16 sheep and 16 goats) were included in the study (Figure 1). The sampled farms were located in 15 different municipalities in the eight provinces of Andalusia. Median (Q1–Q3) flock size was 430 (172–689) for sheep and 378 (107–537) for goats. In terms of production system, 40 intensive, 6 semi-extensive and 12 extensive farms were sampled. On intensive farms, the animals are housed throughout the year and usually with a small open pen, whereas animals in semi-extensive and extensive management systems graze in natural pastures. Small ruminants on semi-extensive farms frequently receive additional feed as supplements and sleep on the farm, depending on the season and the physiological state of the animal. Ten of the 32 sampled farms were focused on dairy production, 16 on meat production and the remaining 6 on small ruminant's milk and meat.

Blood samples were collected by jugular vein puncture. Sera were obtained after centrifugation at 400 g for 10 min and stored at -20°C until laboratory analysis.

Epidemiological data related to the animals and farms sampled were collected by means of personal interviews with the farmers using a standardized questionnaire. A total of 48 explanatory variables (Supplementary Material, Table S1) were collected to obtain information on levels of exposure to possible on-farm potential risk factors associated with HEV.

2.2 | Laboratory analysis

The presence of anti-HEV antibodies was assessed using a commercial double-antigen multi-species ELISA (HEV ELISA 4.0v; MP Diagnostics, Illkirch, France), in accordance with the manufacturer's instructions. This assay is based on the recombinant ET2.1 protein, which is highly conserved among HEV genotypes (Hu et al., 2008), and detects the presence of total antibodies (IgM, IgG and IgA) against the virus in sera or plasma from all animal species. This

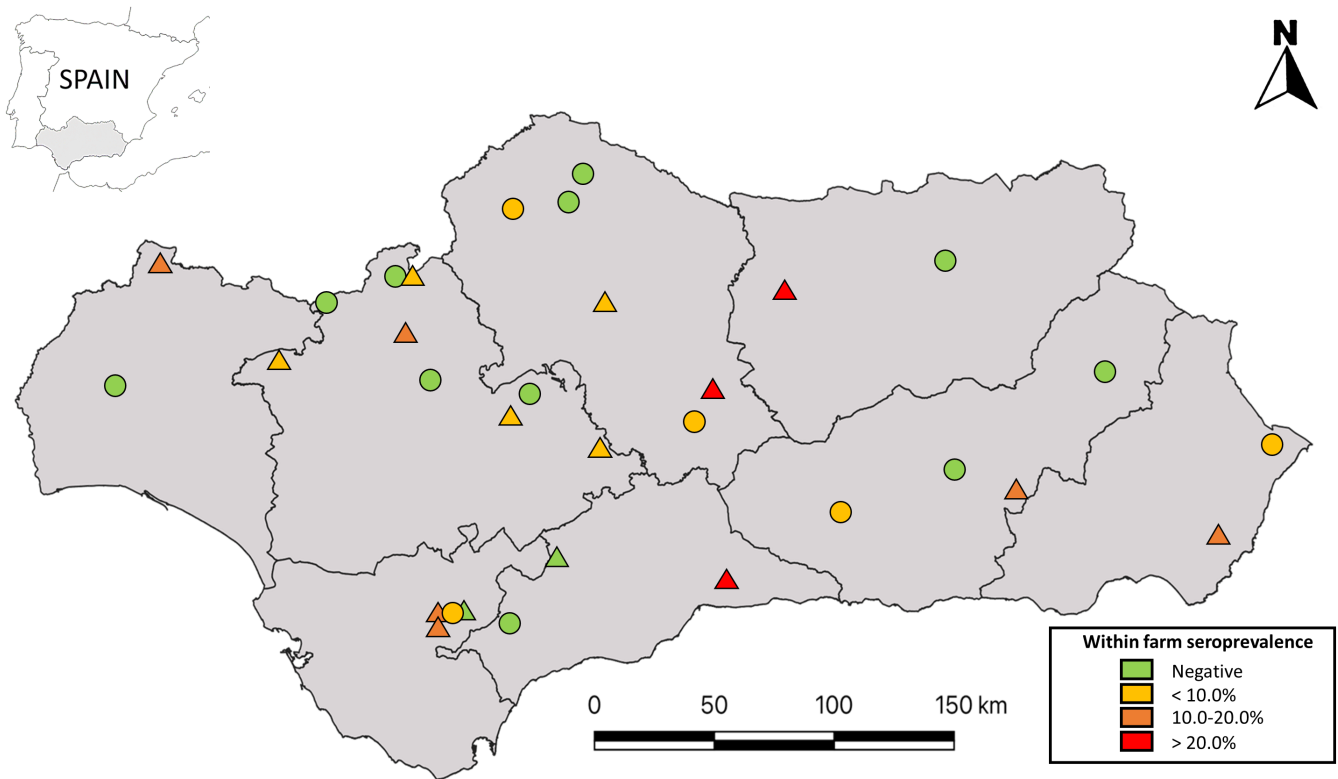


FIGURE 1 Distribution of sampled small ruminant farms. Triangles and circles represent goat and sheep flocks sampled respectively. Colour gradation shows within-farm seropositivity

multi-species ELISA has also been previously used in other ungulate species, including sheep and goats (Kukielka et al., 2015; Ouoba et al., 2019). The sensitivity and specificity of the assay have been established at 99.2%.

Pools of 400 μ l of serum from four different individuals (100 μ l from each animal) were prepared for molecular analysis. RNA was extracted from pools using the QIAamp MinElute virus spin kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. RNA was eluted in 30 μ l. The presence of HEV RNA was determined by real-time PCR (CFX Connect Real Time PCR System), which detects all genotypes of *Orthohepevirus A* using 10 μ l of RNA template and the QIAGEN One-Step RT-PCR kit as previously described (Frías et al., 2020). The detection limit was set at 74.1 IU/ml (95% confidence intervals (95% CI) = 60.8–101.2). A subset of pools of serum samples was spiked with the HEV-3a Kernow-C1 strain as a positive extraction control. The WHO HEV-3a reference strain (code 6329/10), supplied by the Paul-Ehrlich-Institut, was included as a positive control in every run of RT-PCR.

2.3 | Statistical analysis

The prevalence of HEV was determined as the coefficient of positive/total animals tested, using the two-sided exact binomial test, 95% CI. Continuous variables were transformed into qualitative variables with three categories, considering the 33rd and 66th

percentiles as cut-off points. Associations between results and explanatory variables were first screened using the Pearson's Chi-square or Fisher's exact test, as appropriate. Variables with $p < .10$ in the bivariate analysis were selected as possible risk factors. Collinearity between pairs of variables was tested by Cramer's V coefficient. Given the large number of explanatory variables, the four data subsets (A. Individual data; B. General farm production data; C. Biosecurity and health parameters; D. Climatological variables) were analysed separately. Finally, generalized estimating equation (GEE) analysis was used to study the effect of the variables selected based on the bivariate analysis. The number of seropositive animals was assumed to follow a binomial distribution, and 'farm' was included as a random factor. The model was re-run until all remaining variables showed statistically significant values ($p < .05$) and goodness of fit was assessed using the Quasi likelihood under independence model criterion. Statistical analyses were performed using SPSS 25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA).

3 | RESULTS AND DISCUSSION

Hepatitis E is an emerging but still underdiagnosed disease worldwide (Wang & Meng, 2021). Nevertheless, in Europe, where the zoonotic genotype HEV-3 is endemic (Izopet et al., 2019), a 10-fold increase in the number of human cases has been reported in the

last few years (Aspinall et al., 2017). Knowledge of the host range of this zoonotic genotype has also expanded considerably (Wang & Meng, 2021). In this context, the identification of all potential animal hosts and their role in the epidemiology of HEV are important key issues to control this emerging pathogen.

A total of 38 (7.9%; 95%CI: 5.5–10.3) out of 480 sampled animals showed anti-HEV antibodies (Supplementary Material, Table S1). These results confirm that small ruminants are naturally exposed to HEV in southern Spain. The seroprevalence detected in sheep was 2.1% (5/240; 95%CI: 0.3–3.9), which is similar to those previously observed in north-eastern Spain (2.6%) (Peralta et al., 2009) and in Egypt (4.4%) (El-Tras et al., 2013). Higher seropositivity values were found in China (9.8%) (Chang et al., 2009), Burkina Faso (12.0%) (Ouoba et al., 2019), Jordan (12.7%) (Obaidat & Roess, 2020), Portugal (16.6%) (Mesquita et al., 2020), Italy (21.3%–21.6%) (Palombieri et al., 2020; Sarchese et al., 2019), Nigeria (31.8%) (Shuaibu et al., 2016), Turkmenistan (42.0%) (Favorov et al., 1998) and India (77.6%–100%) (Shukla et al., 2007). Contrasting with this, previous studies failed to detect anti-HEV antibodies in both sheep and goats from Brazil and India (Arankalle et al., 2001; Vitral et al., 2005). In the present study, 33 (13.8%; 95%CI: 9.4–18.1) of the 240 goats sampled showed anti-HEV antibodies. This seroprevalence is of the same magnitude as those found in Italy (11.4%) (Palombieri et al., 2020) and the United States of America (16.3%) (Sanford et al., 2012), but higher than the frequencies observed in Laos (5.0%) (Tritz et al., 2018), Jordan (8.3%) (Obaidat & Roess, 2020), Egypt (9.4%) (El-Tras et al., 2013) and a previous study conducted in north-eastern Spain (1.4%) (Peralta et al., 2009). However, higher seropositivity values were detected in Burkina Faso (28.4%) (Ouoba et al., 2019), China (41.6%) (Li et al., 2017) and Turkmenistan (67.0%) (Favorov et al., 1998). While cross-study comparisons should be made with caution because of possible differences in epidemiological context, study designs, serological methods used and/or age of animals sampled, we think that it is possible to state that the seroprevalence of HEV in sheep and goats in the study area should be considered low and moderate respectively.

Anti-HEV antibodies were found in all the provinces of Andalusia and on 19 (59.4%; 95%CI: 42.4–76.4) of the 32 sampled farms (within farm seropositivity ranged between 6.7% and 40.0%) (Figure 1), indicating a wide distribution of HEV in small ruminants in southern Spain. According to species, at least one seropositive animal was detected in five (31.3%) and 14 (93.3%) of the sheep and goat farms sampled respectively. The GEE model showed that species and the number of small ruminants on the farm were risk factors potentially associated with HEV exposure in these species in the study area (Table 1). Significantly higher seropositivity was detected in goats ($p < .001$; OR = 7.3; 95%CI: 3.2–16.4) versus sheep in the study area, which could be associated with differences in susceptibility between species or, given that HEV is excreted in faeces (Di Martino et al., 2016; Sarchese et al., 2019), with behavioural or ethological differences. Unlike sheep, goats frequently jump inside or insert their feet into feeders and drinkers, which may increase

the risk of transmission of HEV contaminated faeces. However, our results contrast with those previously found in Spain, where frequencies of seropositivity between sympatric sheep (1.9%) and goats (0.6%) were similar (Peralta et al., 2009), and in Italy, where a higher seroprevalence was detected among sheep (21.6%) sampled than goats (11.4%) sampled (Palombieri et al., 2020). In any case, further studies are warranted to assess potential differences in susceptibility to HEV infection between these ruminant species. At the same time, the risk of being exposed to HEV was 2.6 and 4.5 times higher on farms where the animal census was ≤ 348 and ≥ 538 compared to farms with flocks of between 349 and 537 animals. This finding could be associated with the transmission dynamics of HEV, which depend on several factors such as herd size, animal density and hygiene practices (Di Bartolo et al., 2008; Hinjoy et al., 2013; Lopez-Lopez et al., 2018; Pavia et al., 2021; Walachowski et al., 2014). Consistent with the above, the biosecurity measures and sanitation practices for small flocks of sheep and goats in the study area are generally limited whereas in large flocks, animal housing density is high (Gazzonis et al., 2015), which favours HEV circulation within the farm.

This is the first study to assess the presence of HEV RNA in small ruminants in Spain. None of the 480 (0.0%; 95%CI: 0.0–0.8) animals tested were positive for active infection, which points to the absence of or limited HEV viremia in these species. Although Sanford et al. (2012) were not able to experimentally infect goats, several studies have confirmed that sheep and goat are susceptible to natural infection with zoonotic genotypes HEV-3 and HEV-4 (El-Mokhtar et al., 2020; Long et al., 2017; Sarchese et al., 2019; Wu et al., 2015). The presence of viral RNA in liver and milk from both sheep and goats, with prevalence values ranging between 2.8% and 18.5% (Demirci et al., 2019; Dziejzinska et al., 2020; El-Mokhtar et al., 2020; Wu et al., 2015), raises concerns about whether the edible by-products of these species are a potential zoonotic source of HEV for human beings. Indeed, HEV-4 contaminated cow milk was able to infect rhesus macaques (*Macaca mulatta*) even after low-temperature pasteurization (Huang et al., 2016), and the consumption of camel milk has been linked to a human case caused by HEV-7 in the United Arab Emirates (Lee et al., 2016). It should be noted that small ruminants produce more than one billion litres of milk each year in Spain, which is the second largest supplier of goat and sheep milk to the EU (MAPA, 2021), and pasteurized and even raw milk from these species is commercially available in this country (AESAN, 2021). Spain is also the second largest producer of pure sheep and goat cheese in the European Union (MAPA, 2021). The presence and persistence of HEV in cheese has not so far been evaluated, although previous studies have indicated that cheesemakers have an increased risk of acquiring HEV infection (Mesquita et al., 2020). Further, additional studies are needed to assess the risk of zoonotic transmission of the virus through the consumption of these small ruminant products.

In conclusion, the seropositivity found in the present study provides evidence of widespread but unequal HEV exposure in

TABLE 1 Generalized estimating equation analysis of the risk factors associated with HEV seropositivity in small ruminants in southern Spain

Variables	Category	p	OR (95%CI)
Species	Goat	<.001	7.3 (3.2-16.4)
	Sheep	^a	^a
Number of small ruminants in the farm	≤348	.012	2.6 (1.2-5.4)
	≥538	.001	4.5 (1.9-10.6)
	349-537	^a	^a

^aReference category.

small ruminant populations from southern Spain. Further studies are required to elucidate differences in HEV seroprevalence between sheep and goats and to determine the risk of zoonotic foodborne transmission of this emerging virus through small ruminant products.

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CONFLICT OF INTEREST

None of the authors of this study has any financial or personal relationship with other people or organizations that would inappropriately influence or bias the content of the paper.

ETHICAL APPROVAL


The collection of blood samples analysed in the present study was part of the official animal health campaigns of the Regional Government of Andalusia, Spain. Ethical approval was not therefore required for this study.

DATA AVAILABILITY STATEMENT

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

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