DOCTORAL THESIS

EPIDEMIOLOGY OF HEPATITIS E IN SPAIN: ZOONOTIC IMPLICATION OF DOMESTIC AND WILD RESERVOIRS

TESIS DOCTORAL

EPIDEMIOLOGÍA DE LA HEPATITIS E EN ESPAÑA: IMPLICACIÓN ZOONÓTICA DE RESERVORIOS DOMÉSTICOS Y SILVESTRES

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TITULO: *EPIDEMIOLOGY OF HEPATITIS E IN SPAIN: ZOONOTIC IMPLICATION OF DOMESTIC AND WILD RESERVOIRS*

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TÍTULO DE LA TESIS: EPIDEMIOLOGY OF HEPATITIS E IN SPAIN: ZOONOTIC IMPLICATION OF DOMESTIC AND WILD RESERVOIRS

DOCTORANDO/A: JAVIER CABALLERO GÓMEZ

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La Tesis Doctoral titulada *Epidemiología de la Hepatitis E en España: Implicación zoonótica de reservorios domésticos y silvestres/Epidemiology of hepatitis E in Spain: Zoonotic implication of domestic and wild reservoirs*, que ha realizado el doctorando D. Javier Caballero dio comienzo en el curso académico 2017/2018. Durante este período, el doctorado ha realizado una estancia de tres meses en el Departamento de Biología Molecular de la Universidad de Princeton (Nueva Jersey, Estados Unidos de América).

El objetivo general de esta Tesis Doctoral es evaluar el papel de diferentes especies domésticas y silvestres en el ciclo epidemiológico del virus de la hepatitis E y el riesgo de transmisión del virus en la interfaz humano-doméstico-silvestre en España. Para la consecución de este objetivo general, se han establecido los siguientes objetivos específicos: 1) Evaluar la circulación de *Orthohepevirus*, incluido *Orthohepevirus A*, *B* y *C*, en perros y gatos urbanos simpátricos en el sur de España. 2) Determinar la prevalencia de VHE en équidos en Andalucía (sur de España) para conocer el papel de estas especies como potenciales reservorios del virus. 3) Establecer la prevalencia, distribución espacial y factores de riesgo asociados a la exposición al VHE en caprino y ovino en el sur de España. 4) Evaluar la circulación del VHE en el jabalí en el Parque Nacional de Doñana, así como 5) en conejo silvestre y liebre ibérica en

ecosistemas mediterráneos españoles. 6) Determinar la circulación del VHE en las poblaciones de lince ibérico, los potenciales factores de riesgo asociados a la exposición del VHE en esta especie y la dinámica de seropositividad en animales longitudinalmente muestreados. 7) Evaluar la infección por VHE en primates no humanos, así como 8) en carnívoros, perisodáctilos, artiodáctilos, roedores, y proboscidios de diferentes zoos de España. 9) Determinar la seroprevalencia y prevalencia del VHE en cetáceos de vida libre y cautivos en España, y evaluar la dinámica de seropositividad en individuales longitudinalmente muestreados durante el período de estudio.

Seis de los estudios realizados durante el desarrollo de la presente Tesis Doctoral ya han sido publicados en revistas indexadas en el JCR:

Caballero-Gómez, J., Rivero-Juárez, A., Jurado-Tarifa, E., Jiménez-Martín, D., Jiménez-Ruiz, E., Castro-Scholten, S., Ulrich, R., López-López, P., Rivero, A., García-Bocanegra, I. (2021). Serological and molecular survey of hepatitis E virus in cats and dogs in Spain. *Transboundary and Emerging Diseases*, *69*(2), 240-248. Factor de impacto (JCR 2020): 5,005. Posición de la revista (Categoría): 4/146, D1, Q1, T1 (Veterinary Sciences).

García-Bocanegra, I., Rivero, A., **Caballero-Gómez, J.**, López-López, P., Cano-Terriza, D., Frías, M., Jiménez-Ruiz, S., Risalde, MÁ., Gómez-Villamandos, JC., Rivero-Juárez, A. (2019). Hepatitis E virus infection in equines in Spain. *Transboundary and Emerging Diseases*, *66*(1), 66-71. Factor de impacto: 4,188. Posición de la revista (Categoría): 4/141, D1, Q1, T1 (Veterinary Sciences).

Caballero-Gómez, J., García-Bocanegra, I., Jiménez-Martín, D., Cano-Terriza, D., Risalde, M. A., López-López, P., Jiménez-Ruiz, S., Rivero, A., Rivero-Juárez, A. (2022). Epidemiological survey and risk factors associated with hepatitis E virus in small ruminants in southern Spain. *Zoonoses and Public Health, 69*(4), 387-393. Factor de impacto (JCR 2020): 2,702. Posición de la revista (Categoría): 24/146, Q1, T1 (Veterinary Sciences).

Caballero-Gómez, J., Jiménez-Ruiz, S., López-López, P., Vicente, J., Risalde, MÁ., Cano-Terriza, D., Frías, M., Barasona, JA., Rivero, A., Rivero-Juárez, A. (2019). Emergent subtype of hepatitis E virus genotype 3 in wild boar in Spain. *Transboundary and Emerging Diseases*, *66*(5), 1803-1808. Factor de impacto: 4,188. Posición de la revista (Categoría): 4/141, D1, Q1, T1 (Veterinary Sciences).

Caballero-Gómez, J., García Bocanegra, I., Gómez-Guillamón, F., Camacho-Sillero, L., Zorrilla, I., López-López, P., Cano-Terriza, D., Jiménez-Ruiz, S., Frías, M., Rivero-Juárez, A. (2020). Absence of Hepatitis E virus circulation in wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) in Mediterranean ecosystems in Spain. *Transboundary and Emerging Diseases*, *67*(4), 1422-1427. Factor de impacto: 5.005. Posición de la revista (Categoría): 4/146, D1, Q1, T1 (Veterinary Sciences).

Caballero-Gómez, J., Rivero-Juárez, A., Cano-Terriza, D., Risalde, MÁ., López-López, P., Frías, M., Jiménez-Ruiz, S., Rivero, A., García-Bocanegra, I. (2019). Survey for Hepatitis E virus infection in non-human primates in zoos in Spain. *Transboundary and Emerging Diseases*, *66*(4), 1771-1775. Factor de impacto: 4.188. Posición de la revista (Categoría): 4/141, D1, Q1, T1 (Veterinary Sciences).

Así mismo, actualmente tres de los trabajos incluidos en esta Tesis Doctoral se encuentra en revisión:

Caballero-Gómez, J., Rivero-Juárez, A., Zorrilla, I., López, G., Nájera, F., Ulrich, RG., Ruiz-Rubio, C., Salcedo, J., Rivero, A., Paniagua, J., García-Bocanegra, I. Hepatitis E virus in the endangered Iberian lynx (*Lynx pardinus*). *Transboundary and Emerging Diseases*. En revisión.

Caballero-Gómez, J., García Bocanegra, I., Cano-Terriza, D., Beato-Benítez, A., Ulrich, RG., Martínez, J., Guerra, R., Martínez-Valverde, R., Martínez-Nevado, E., Quevedo-Muñoz, MÁ., Sierra-Arqueros, C., Planas, J., de Castro-García, N., Rivero, A., Rivero-Juárez, A. Monitoring

of hepatitis E virus in zoo animals from Spain, 2007-2021. *Transboundary and Emerging Diseases. Transboundary and Emerging Diseases*. En revisión.

Caballero-Gómez, J., Rivero-Juárez, A., Beato-Benítez, A., Fernández-Maldonado, C., Domingo, M., García-Párraga, D., Sierra, E., Segura-Göthlin, S., Martínez-Nevado, E., Sierra, C., Canales, R., García-Bocanegra, I. Hepatitis E virus circulation in free-ranging and captive cetaceans in Spain, 2011-2022. *Transboundary and Emerging Diseases*. Under review.

Una vez redactada, la presente Tesis Doctoral ha sido revisada reuniendo a nuestro juicio todos los requisitos necesarios para su lectura y defensa.

Y para que conste, en cumplimiento de las disposiciones vigentes, se expide el presente informe y se autoriza la presentación de la tesis doctoral.

Córdoba, 27 de mayo de 2022

Firma de los directores

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Amor, Constancia y Disciplina

GENERAL INDEX

SUMMARY

Hepatitis E virus (HEV) is an emerging and zoonotic pathogen of worldwide distribution, which affects at least 20 million of humans annually, being recognized as the main cause of acute human viral hepatitis. In industrialized countries, HEV is mainly transmitted through the consumption of animal products or contact with faeces of infected animals. Although domestic pig and wild boar (*Sus scrofa*) are the main reservoirs of the zoonotic genotypes of the virus, the information about HEV circulation in animal species in the Iberian Mediterranean ecosystems is still very limited. In addition, despite the number of host and animal reservoirs has considerably increased since the first description of HEV in an animal host in 1997, the role of non-suid domestic and wildlife species in the epidemiology of HEV is poorly understood. Thus, the main general objective of this PhD thesis is to assess HEV circulation in domestic (Chapter 1) and wild (Chapter 2) animal species, including free-ranging and captive animals, in Spain. For this purpose, severalstudies were developed and are presented here under nine main headings.

In the first study (Chapter 1.1), *Orthohepevirus* circulation, including HEV-A, HEV-B and HEV-C species, was assessed in sympatric urban cats and dogs in southern Spain. The presence of anti-HEV antibodies was detected in a total of 19 (6.4%; 95%CI: 3.6-9.2) of the 296 animals 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) (p=0.011). Ten of the 18 ELISA-positive animals reacted against HEV-A and/or HEV-C, which suggests circulation of both *Orthohepevirus* species in urban cats and dogs in the study area. However, RNA from the *Orthohepevirus* species analyzed 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. The results detected

provide evidence of HEV exposure in sympatric urban cat and dog populations in southern Spain.

In the second study (Chapter 1.2), the objective was to determine the prevalence of HEV in equine species in Andalusia (southern Spain) in order to assess the role of equids as potential reservoirs of the virus. HEV RNA was detected in sera from 0.4% (3/692) of horses, 1.2% (1/86) of donkeys and 3.6% (3/83) of mules. Phylogenetic analysis identified the zoonotic genotype 3, being closely related to viral human and swine strains. This is the first report of HEV in equids in Europe and confirm the susceptibility of horses, donkeys and mules to HEV infection. The low prevalence detected indicates that equids may be considered spillover hosts rather than true reservoirs.

The aim of the third study (Chapter 1.3) 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. A total of 38 (7.9%; 95%CI: 5.5–10.3) out of 480 sampled animals showed anti-HEV antibodies. By species, the seroprevalence of 13.8% (33/240; 95%CI: 9.4–18.1) found in goats was significantly higher than the 2.1% (5/240; 95%CI: 0.3–3.9) detected in sheep. Anti-HEV antibodies were found on 19 (59.4%; 95%CI: 42.4–76.4) of the 32 sampled farms. The number of small ruminants in the farm (≤348 animals and ≥538 animals) was also a risk factor 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. The results obtained in this study 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.

The fourth study (Chapter 2.1) analyzed the circulation of HEV in free-ranging wild boar in the Doñana National Park, a study area characterized by very limited human activity and the absence of a pig industry. A total of 57 (57.6%; 95%CI: 47.8%–67.3%) of the 99 tested animals had anti-HEV antibodies, indicating that this virus is widespread in wild boar in the DNP. HEV RNA was detected in one animal and phylogenetic analysis showed that the sequence isolated belonged to subtype 3r (reassigned as 3m). The results suggest a potential risk of zoonotic transmission of this novel HEV-3 subtype and point the need to assess the role of wild boar in the epidemiology of HEV-3m and to determine the infectivity of this emergent HEV subtype in other species, including humans.

In the fifth study (Chapter 2.2) we assessed the circulation of HEV in European wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*), the most important lagomorph species in Spanish Mediterranean ecosystems. None of the 450 lagomorphs' livers tested positive for HEV infection. To the best of our knowledge, this is the first study to assess HEV circulation in wild rabbits in Spain and the first to evaluate HEV infection in Iberian hares. Our results indicate absence of HEV circulation in wild rabbits and Iberian hares in southern Spain during the study period, which suggests that the risk of transmission of HEV from wild lagomorphs to other species, including humans, is low.

The aims of the sixth study (Chapter 2.3) were to determine the seroprevalence and prevalence of HEV in both free-living and captive populations of the Iberian lynx (*Lynx pardinus*), the most endangered felid in the world, as well as to evaluate potential risk factors associated with HEV exposure in this species, and the dynamics of serological markers during the study period. A total of 50 (18.2%; 95%CI: 14.1-23.2) of the 275 animals analyzed had anti-HEV antibodies by ELISA. Exposure to HEV was confirmed by WB in most of the

ELISA-positive Iberian lynxes analyzed. The generalized estimating equation model identified "habitat status" (captivity) and "age" (senile, adult and subadult) in the captive population only as risk factors potentially associated with HEV exposure in the Iberian lynx. Thirteen (29.5%) of the 44 longitudinally surveyed individuals seroconverted against HEV during the study period. HEV RNA was detected in the faeces of one (1/364; 0.3%; 95% CI: 0.0-0.8) free-ranging adult animal sampled in 2021. Phylogenetic analysis showed that the sequenced strain belongs to HEV-3f subtype, which shared a high nucleotide sequence identity (97-99.6%) with human HEV-3f sequences from Spain and France. This is the first survey study on HEV in the Iberian lynx and the first molecular report of HEV-A infection in free-ranging felines. The results indicate a high exposure to HEV-3 in the Iberian lynx populations, particularly in those kept in captivity and suggest a possible role for this species as a potential spillover host of this virus. The serological results suggest the widespread but not homogeneous circulation of HEV in the Iberian lynx populations.

The seventh study (Chapter 2.4) assessed HEV infection in non-human primates (NHPs) housed in zoos in Spain. Anti-HEV antibodies were found in eight (4.4%; 95%CI: 1.4–7.4) of the 181 NHPs tested and at least one seropositive animal was detected in five of the 33 species sampled (15.2%). This is the first report of seropositivity in black-and-white ruffed lemurs (*Varecia variegata*), common chimpanzees (*Pan troglodytes*), and Barbary macaques (*Macaca sylvanus*). Anti-HEV antibodies were found in six (75.0%) of the eight zoos included in the study. Seroconversion was detected in one chimpanzee, which confirms HEV circulation in one zoo between 2015 and 2016. Seropositivity was significantly higher in hominids than in other NHP families. HEV-A RNA was not detected in any of the serum samples tested. This study increases the host range exposed to the virus and indicate susceptibility of NHPs to HEV

infection.

The aims of the eighth study (Chapter 2.5) were to assess HEV exposure in captive zoo animals in Spain and to determine the dynamics of seropositivity in individuals that were sampled longitudinally during the study period. Seropositivity to HEV were detected in 36 (8.5%; 95%CI: 5.8-11.1) of 425 sampled zoo animals and antibodies against HEV-3 and/or HEV-C1 were confirmed in ELISA-positive animals using WB. Two of 46 longitudinally surveyed animals seroconverted during the study period. Seropositivity was significantly higher in carnivores and perissodactyls than in artiodactyls, and also during the period 2012-2016 compared with 2007-2011. HEV RNA was not detected in any (0.0%; 95%CI: 0.0-1.4) of the 262 animals that could be tested by RT-PCR. This is the first large-scale, long-term surveillance on HEV in different orders of zoo mammals. Our results indicate exposure to HEV-A and HEV-C in zoo animals in Spain and, together with the results of Chapter 2.4, confirm a widespread but not homogeneous spatiotemporal circulation of HEV in captive species in this country.

In the ninth and last study (Chapter 2.6), the aims were to assess the seroprevalence and prevalence of HEV in both in both free-ranging and captive cetaceans in Spain and the dynamics of seropositivity in individuals sampled longitudinally during the study period. A total of 67 (49.3%; 95%CI: 40.9-57.7) out of 136 analyzed cetaceans by ELISA showed anti-HEV antibodies. Seropositivity was detected in six of the nine species sampled. Significantly higher seroprevalence was found in free-ranging (59.7%) than in captive (37.5%) cetaceans. Within the free-ranging population, the multivariate analyses identified “age” (adult) as a risk factor potentially associated with HEV exposure in cetaceans. Seroconversions were detected in two bottlenose dolphins (*Tursiops truncatus*) during the study period. None (0.0%; 95%CI:

0.0-1.2) of the 304 analyzed cetaceans were positive for active *Orthohepevirus* infection. To the best of the authors' knowledge this is the first study to assess *Orthohepevirus* circulation in cetaceans and to report exposure to HEV in Atlantic spotted (*Stenella frontalis*), Risso (*Grampus griseus*) and striped dolphins (*Stenella coeruleoalba*) as well as in Cuvier's Beaked (*Ziphius cavirostris*) and killer whales (*Orcinus orca*). Our results point high HEV exposure in cetacean populations in Spain, indicating widespread circulation of this virus in both free-ranging and captive over the last decade.

This PhD thesis adds novel knowledge about the epidemiological role of animal species to HEV in different scenarios, including households, farms, Mediterranean and marine ecosystems, zoos and captivity centres. The results obtained will allow to expand the knowledge about the epidemiology of this emerging virus and to prioritize HEV monitoring and surveillance on certain animal species and epidemiological contexts.

RESUMEN

El virus de la hepatitis E (VHE) es un patógeno emergente y zoonósico de distribución mundial que afecta a unos 20 millones de personas anualmente, siendo considerado como la principal causa de hepatitis aguda de origen viral. En países industrializados, el VHE se transmite principalmente a través del consumo de productos animales o el contacto con heces de animales infectados. Aunque el cerdo doméstico y el jabalí (*Sus scrofa*) son los principales reservorios de los genotipos zoonósicos del virus, la información acerca de la circulación del VHE en especies de los ecosistemas mediterráneos de la Península Ibérica es muy limitada. Asimismo, pese a que el número de hospedadores y reservorios animales ha aumentado significativamente desde su primera descripción en una especie animal en 1997, el papel en la epidemiología del VHE de especies domésticas y silvestres diferentes a los suidos es poco conocido. Por ello, la presente Tesis Doctoral tiene el objetivo principal de evaluar la circulación del VHE en especies domésticas (Capítulo 1) y silvestres (Capítulo 2), incluyendo animales de vida libre y mantenidos en cautividad, en España. Para ello se han diseñado nueve estudios que abordan cada uno de los objetivos específicos.

En el primero (Capítulo 1.1), se evalúa la circulación de las especies de *Orthohepevirus*, incluyendo VHE-A, VHE-B y VHE-C, en perros y gatos urbanos simpátricos en el sur de España. La presencia de anticuerpos frente al VHE se detectó en un total de 19 (6,4%; IC95%: 3,6-9,2) de los 296 animales analizados por ELISA. La seropositividad fue significativamente superior en perros (9,9%; 15/152; IC95%: 5,1-14,6) que en gatos (2,8%; 4/144; IC95%: 0,1-5,5) (p=0.011). Diez de los 18 animales positivos a ELISA que pudieron ser analizados por western blot (WB), reaccionaron frente a los antígenos del VHE-A y/o VHE-C, lo cual sugiere circulación de ambas especies de *Orthohepevirus* en gatos y perros urbanos en el área de

estudio. No se detectó ARN de ninguna especie de *Orthohepevirus* en ninguno de los sueros testados. Este es el primer estudio que evalúa la circulación de las diferentes especies de *Orthohepevirus* en gatos callejeros y perros simpátricos en Europa. Los resultados detectados muestran que las poblaciones de gatos y perros urbanos simpátricos del sur de España están expuestas al VHE.

El objetivo del segundo estudio (Capítulo 1.2) fue determinar la prevalencia al VHE en especies equinas en Andalucía (sur de España) para evaluar el papel de los équidos como potenciales reservorios del virus. Se detectó ARN del VHE en muestras de suero del 0,4% (3/692) de caballos, 1,2% (1/86) de burros y 3,6% (3/83) de mulos. Los análisis filogenéticos identificaron al genotipo zoonótico 3, estando estrechamente relacionado con cepas humanas y porcinas. Ésta es la primera detección del VHE en équidos en Europa, confirmando la susceptibilidad de caballos, burros y mulos a la infección. La baja prevalencia detectada indica que los équidos podrían ser considerados como hospedadores accidentales más que como verdaderos reservorios.

El tercer estudio (Capítulo 1.3) se centra en evaluar la prevalencia, distribución espacial y factores de riesgo asociados a la exposición por el VHE en ovino y caprino en el sur de España, el país con mayor censo de pequeños rumiantes en la Unión Europea. Un total de 38 (7,9%; IC95%: 5,5-10,3) de los 480 animales muestreados mostraron anticuerpos frente al VHE. Por especies, la seroprevalencia del 13,8% (33/240; IC95%: 9,4-18,1) encontrada en caprino fue significativamente superior que la del 2,1% (5/240; IC95%: 0,3-3,9) detectada en ovino. Se detectaron anticuerpos en 19 (59,4%; IC95%: 42,4–76,4) de las 32 granjas muestreadas. El censo de pequeños rumiantes en la granja (≤348 animales y ≥538 animales) fue también un factor de riesgo potencialmente asociado a la exposición por el VHE en

pequeños rumiantes en el área de estudio. No se detectó ARN del VHE en ninguno de los 480 (0.0%; IC95%: 0,0-0,8) animales testados. Los resultados obtenidos en el presente estudio confirman que el ovino y el caprino están natural, pero no igualmente expuestos al virus e indican la amplia distribución espacial del VHE entre la población de pequeños rumiantes en el sur de España.

El cuarto estudio (Capítulo 2.1) analiza la circulación del VHE en jabalíes en libertad en el Parque Nacional de Doñana (PND), un área caracterizada por una limitada actividad humana y la ausencia de industria porcina. Un total de 57 (57,6%; IC95%: 47,8%-67,3%) de los 99 animales testados presentaron anticuerpos frente al VHE, indicando que el virus está ampliamente distribuido en el PND. Se detectó ARN del VHE en un animal y el estudio filogenético mostró que la secuencia encontrada pertenecía al subtipo 3r (reasignado actualmente como 3m). Los resultados sugieren un potencial riesgo de transmisión zoonótica de este nuevo subtipo de VHE-3 y señala la necesidad de evaluar el papel del jabalí en la epidemiología del VHE-3m y determinar la capacidad infectiva de este subtipo emergente en otras especies, incluido seres humanos.

En el quinto estudio (Capítulo 2.2) se evalúa la circulación del VHE en conejo silvestre (*Oryctolagus cuniculus*) y liebre ibérica (*Lepus granatensis*), las dos especies de lagomorfos más importantes en los ecosistemas mediterráneos españoles. Ninguno de los 450 hígados de los lagomorfos analizados fueron positivos a la infección por VHE. Desde nuestro conocimiento, este es el primer estudio que evalúa la circulación del virus en conejo silvestre en España y el primero en liebre ibérica en todo el mundo. Nuestros resultados indican ausencia de circulación del VHE en conejo silvestre y liebre ibérica en el sur de España durante el período de estudio, sugiriendo que el riesgo de transmisión del VHE desde los lagomorfos

silvestres a otras especies, incluidos los humanos, es bajo.

Los objetivos del sexto estudio (Capítulo 2.3) fueron determinar la seroprevalencia y prevalencia del VHE en las poblaciones en libertad y cautividad de lince ibérico (*Lynx pardinus*), el felino más amenazado del mundo, así como evaluar los principales factores de riesgo asociados a la exposición al virus en esta especie y la dinámica de marcadores serológicos durante el período de estudio. Un total de 50 (18,2%; IC95%: 14,1-23,2) de los 275 animales analizados presentaron anticuerpos frente al VHE por ELISA. Se confirmó la exposición al virus por WB en la mayoría de los linces positivos a ELISA. El modelo de ecuación de estimación generalizada identificó el "hábitat" (cautividad) y la "edad" (senil, adulto y subadulto) dentro de la población cautiva como factores de riesgo potencialmente asociados a la exposición al VHE en lince ibérico. Trece (29,5%) de los 44 animales longitudinalmente muestreados seroconvirtieron durante el período de estudio. Se detectó ARN del VHE en las heces de un (1/364; 0,3%; IC95%: 0,0-0,8) lince adulto en libertad muestreado en 2021. El análisis filogenético identificó que la secuencia obtenida pertenece al subtipo VHE-3f, compartiendo una alta homología (97-99,6%) con cepas humanas de VHE-3f de España y Francia. Este es el primer estudio de VHE en lince ibérico y la primera detección molecular de VHE-A en felinos de vida libre. Los resultados indican una elevada exposición al VHE-3 en las poblaciones de lince ibérico, particularmente en las mantenidas en cautividad, y sugiere un posible papel como hospedador accidental. Los resultados serológicos sugieren una amplia pero no homogénea circulación del virus en las poblaciones de lince ibérico.

El séptimo estudio (Capítulo 2.4) evalúa la infección por VHE en primates no humanos (PNHs) de parques zoológicos en España. Se observaron anticuerpos frente al virus en ocho (4,4%; IC95%: 1,4-7,4) de los 181 PNHs testados y se encontró al menos un animal

seropositivo en cinco (15,2%) de las 33 especies muestreadas. Esta es la primera detección de seropositividad en lémur rufo blanco y negro (*Varecia variegata*), chimpancé común (*Pan troglodytes*), y macaco de Berbería (*Macaca sylvanus*). Seis (75,0%) de los ocho zoológicos incluidos en el estudio alojaban animales con anticuerpos frente al VHE. Se detectaron seroconversiones en un chimpancé, confirmando circulación del virus en un zoo entre 2015 y 2016. La seropositividad fue significativamente superior en homínidos que en otras familias de PNHs. No se encontró ARN del VHE-A en ninguna de las muestras de suero testadas. Este estudio aumenta el rango de hospedadores expuestos al virus y señala la susceptibilidad de PNHs a la infección por el VHE.

Los objetivos del octavo estudio (Capítulo 2.5) fueron evaluar la exposición al VHE en animales de zoológico en España y determinar la dinámica de seropositividad en individuos longitudinalmente muestreados durante el período de estudio. Se detectó seropositividad en 36 (8,5%; IC95%: 5,8-11,1) de los 425 animales analizados y se confirmaron anticuerpos frente al VHE-A y/o VHE-C en animales positivos a ELISA mediante WB. Dos de los 46 individuos longitudinalmente muestreados seroconvirtieron durante el periodo de estudio. La seroprevalencia fue significativamente superior en carnívoros y perisodáctilos que en artiodáctilos, y durante el período 2012-2016 comparado con el de 2007-2011. No se detectó ARN de *Orthohepevirus* en ninguno (0,0%; IC95%: 0,0-1,4) de los 262 animales que pudieron ser testados por RT-PCR. Este es el primer estudio a gran escala y largo plazo de VHE en diferentes órdenes de mamíferos en zoológicos. Nuestros resultados indican exposición al VHE-A y VHE-C en animales de zoo en España y, junto con los resultados del Capítulo 2.4, confirman una amplia pero no homogénea circulación espaciotemporal del VHE en especies cautivas en este país.

En el noveno y último estudio (Capítulo 2.6), se determinó la seroprevalencia y prevalencia del VHE en las poblaciones de vida libre y cautivas de cetáceos en España, así como la dinámica de seropositividad en individuos longitudinalmente muestreados durante el periodo de estudio. Un total de 67 (49,3%; IC95%: 40,9-57,7) de los 136 cetáceos analizados por ELISA presentaron anticuerpos frente al VHE. Se detectó seropositividad en seis de las nueve especies analizadas serológicamente. Los cetáceos en libertad (59,7%) presentaron una seroprevalencia significativamente superior que los mantenidos en cautividad (37.5%). Dentro de la población de cetáceos en libertad, se identificó la "edad" (adulto) como un factor de riesgo potencialmente asociado a la exposición por el VHE. Se observaron seroconversiones en dos delfines mulares (*Tursiops truncatus*) durante el período de estudio. Ninguno (0,0%; IC95%: 0,0-1,2) de los 304 cetáceos analizados fue positivo a infección activa por *Orthohepevirus*. Desde el conocimiento de los autores, este es el primer estudio que evalúa la circulación de *Orthohepevirus* en cetáceos y en detectar exposición al VHE en el delfín moteado del Atlántico (*Stenella frontalis*), delfín listado (*Stenella coeruleoalba*), calderón gris (*Grampus griseus*), ballenato de Cuvier (*Ziphius cavirostris*) y orca (*Orcinus orca*). Nuestros resultados señalan una elevada exposición al VHE en las poblaciones de cetáceos en España e indican una circulación espaciotemporal endémica de este virus en cetáceos de vida libre y cautividad durante la última década.

Esta Tesis Doctoral aporta nuevos conocimientos acerca del papel epidemiológico de especies animales en diferentes escenarios, incluidos casas, explotaciones, ecosistemas mediterráneos y marinos, zoológicos, así como en otros centros de cautividad. Los resultados obtenidos permitirán mejorar el conocimiento sobre la epidemiología de este virus emergente y priorizar la monitorización y vigilancia del VHE en ciertas especies animales y en

determinados contextos epidemiológicos.

LIST OF ABBREVIATIONS AND ACRONYMS

AESAN: Agencia Española de Seguridad Alimentaria y Nutrición.

BC: Breeding centre

BLAST: Basic Local Alignment Search Tool

BSL-2: Biosafety level 2

CAD: Center for Analysis and Diagnosis of Wildlife

CIBER: Centro de Investigación Biomédica en Red

Ct: Threshold cycle

DNP: Doñana National Park

dNTPs: Deoxynucleotide triphosphates

ELISA: Enzyme Linked Immunosorbent Assay

EU: European Union

eHEV: Enveloped hepatitis E virus

FEDER: Fondo Europeo de Desarrollo Regional

FIS: Fundación para la Investigación en Salud

FWD: Forward

GEE: Generalized estimating equation

HA: Hepatitis A

HE: Hepatitis E

HEV: Hepatitis E virus

HEV-A: *Orthohepevirus A*

HEV-C: *Orthohepevirus C*

HEV-C1: Genotype C1

HEV-3: Genotype 3

HRP: Horse-radish peroxidase

ISCIII: Instituto de Salud Carlos III

IUCN: International Union for Conservation of Nature

IU: International unit

Kb: Kilobase

MAPA: Ministerio de Agricultura, Pesca y Alimentación

MAPAMA: Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente

MINECO: Ministerio de Economía y Empresa

NAT: Nucleic acid amplification technique

NHPs: Non-human primates

Nm: Nanometer

OR: Odds ratio

ORF: Open reading frame

Pb: Pairbase

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PCV-2: Porcine circovirus type 2

PRRSV: Porcine reproductive and respiratory virus

PVDF: Polyvinylidene difluoride

RdRp: RNA-Dependent RNA Polymerase

RNA: Ribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction

RVS: Reverse

SCS: South-central Spain

SPSS: Statistical package for the social sciences

USA: United States of America

WB: Western Blot

WHO: World Health Organization

Wpi: Weeks post-infection

ZC: Zoological park/Conservation centre

95%CI: 95% confidence intervals

µl: Microliter

µM: Micromolar

INTRODUCTION

1. The discovery of the virus

Between 1978-1979, a waterborne epidemic affecting over 200 villages in Kashmir (northern India) was reported, causing over 1,700 deaths and approximately 52,000 clinical cases (Khuroo, 1980; Khuroo & Khuroo, 2016). Jaundice, anorexia, dark-colored urine, nausea and vomiting were most of the recorded symptoms, being compatible with hepatitis A (HA) clinical signs. Nevertheless, serological analyses evidenced absence of antibodies against HA and also hepatitis B viruses (Khuroo, 1980). Similar findings were retrospectively described in a large outbreak in Delhi between 1955-1956 (Wong et al., 1980), which argued for the existence of non-A, non-B hepatitis agent (Khuroo, 1980). However, this pathogen was not identified until a hepatitis outbreak occurred in Soviet soldiers in Afghanistan in 1981. Fecal samples from the infected patients were famously, controversially but volunteered ingested by Mikhail Balayan, a Russian virologist, who finally evidenced a novel virus in his faeces by immune electron microscopy. These virus-like particles in stool were also experimentally confirmed to be able to infect non-human primates (Balayan et al., 1983). The non-A non-B hepatitis virus was designated as hepatitis E virus (HEV) in 1989 (Wang & Wang, 2016) and its viral genome was full-length sequenced in 1991 (Tam et al., 1991).

2. Molecular biology

HEV is an icosahedral single-stranded and positive-sense RNA virus of 7.2 kb and has 27-32 nm of diameter (Nimgaonkar et al., 2017; Wang & Meng, 2021). The genome is organized in three open reading frames (ORF1-3), although an additional ORF, named ORF4, has recently been described in one of the genotypes of this virus (HEV-1) (Figure 1) (Kamar et al., 2017). ORF1 is the largest open reading frame, and it encodes a non-estructural polyprotein of 1,700 amino acids that contains functional and essential domains for viral

replication, such as RNA dependent RNA polymerase (RdRp), RNA helicase, and methyltransferase (Kamar et al., 2012). ORF2 encodes for the single capsid protein of 660 amino acids, which is highly immunogenic (Yin & Feng, 2019), whereas ORF3 is an ion channel that release infectious HEV virions (Ding et al., 2017). Finally, the ORF4 expressed a protein that increases the activity of the RdRp (Nair et al., 2016).

It is considered to be a "quasi-enveloped" virus since both enveloped (eHEV) and non-enveloped forms can be detected in the same infected individual (Yin et al., 2016; Yin & Feng, 2019). eHEV is frequently detected in the bloodstream whereas non-enveloped particles, which are considered more infectious, can be found in bile and faeces (Wißing et al., 2021). The target organ of HEV is the liver, which is usually reached through the portal vein after gut infection (Marion et al., 2019). Within liver, HEV has been observed in hepatocytes, Kupfer cells and sinusoidal endothelial cells (Choi & Chae, 2003; Risalde et al., 2017) and its persistence in this organ appears to be immune mediated (Krain et al., 2014). Beyond liver, the virus has also been detected in several extrahepatic sites, including lymph nodes, intestines, bile, serum, spleen, kidney, placenta, muscle and central nervous system among others (Williams et al., 2001; Bose et al., 2014; Zhou et al., 2017).

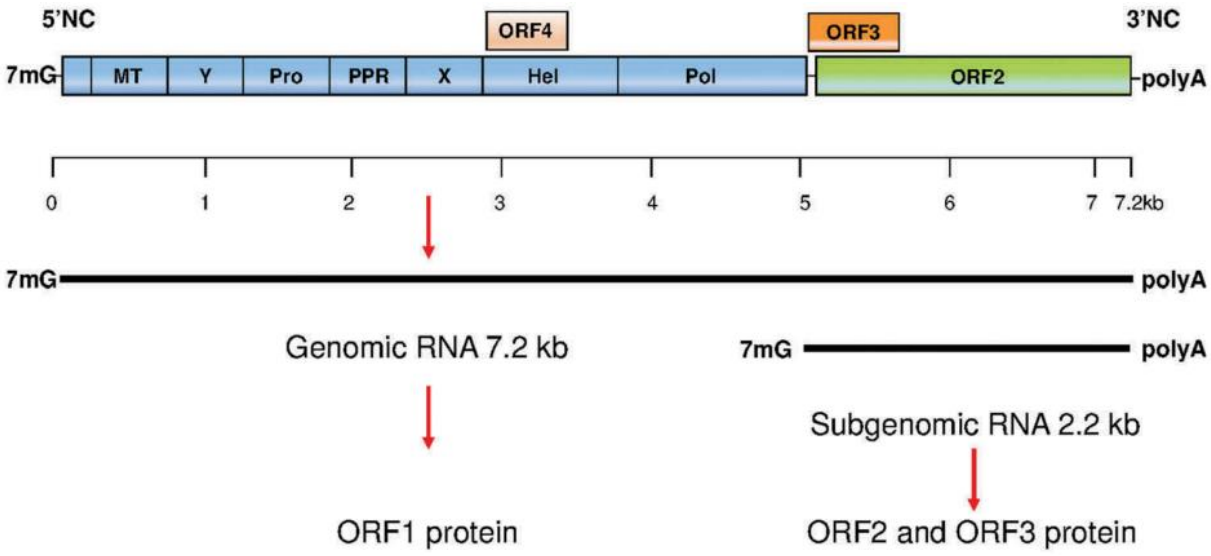

Figure 1. Molecular organization of the hepatitis E virus. Obtained from Lhomme et al. (2019).

The virus is classified within the family *Hepeviridae*, which gathers the genera *Orthohepevirus* and *Piscihepevirus*. While the last comprises only the cutthroat trout virus, the genus *Orthohepevirus* is further divided into four different species, named *Orthohepevirus* A-D (henceforth HEV-A to HEV-D) (Figure 2) (Smith et al., 2016).

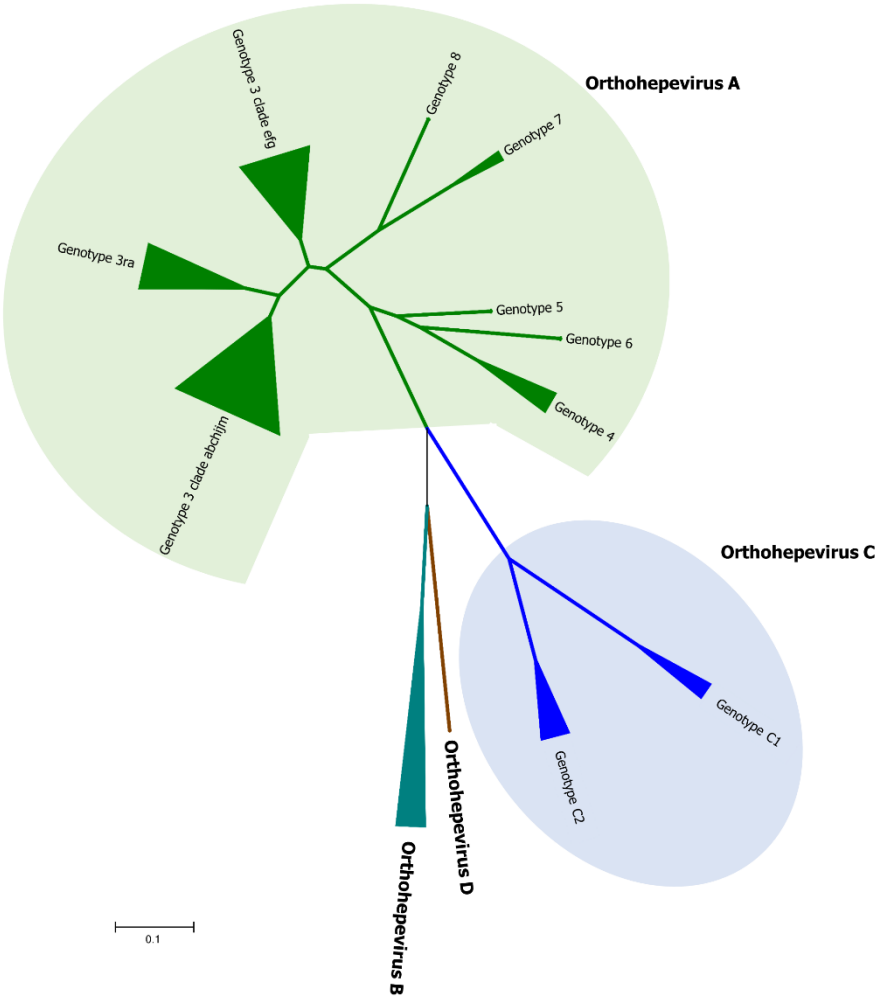

Figure 2. Phylogenetic tree of representative members of the *Orthohepevirus* genus.

HEV-A is the most important species in terms of public health concern and eight different genotypes (HEV-1 to HEV-8) of this species have been described so far (Figure 3). HEV-1 and HEV-2 are widely recognized that only infect humans (Doceul et al., 2016). HEV-3 and HEV-4 have been detected in a wide range of animal species, including humans, and harbor the major number of HEV subtypes. At present, fourteen (HEV-3a to HEV-3m and HEV-3ra) and nine (HEV-4a to HEV-4i) subtypes have been identified (Smith et al., 2020). HEV-5 and HEV-6 have only been detected to date in wild boar whereas HEV-7 and HEV-8

mainly affect camels (Wang & Meng, 2021). However, human cases by HEV-7 and experimental infection in cynomolgus monkeys by HEV-8 have been confirmed (Lee et al., 2017; Wang et al., 2019).

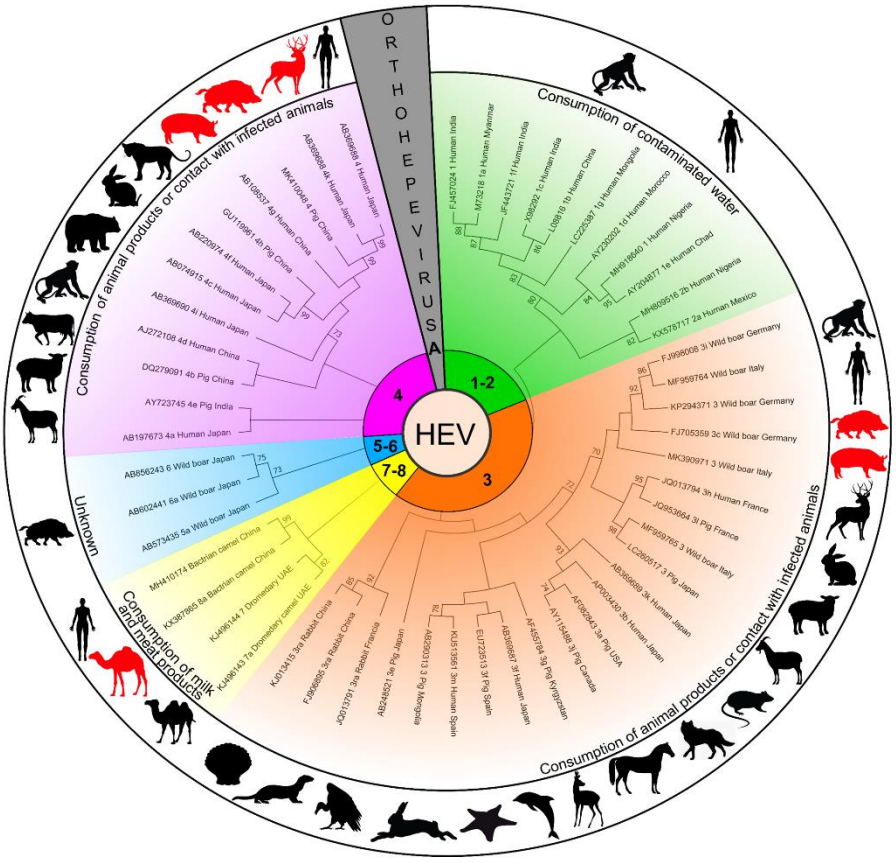

Figure 3. Genotypes and host range of *Orthohepevirus* A and the main transmission route to humans. The animal species colored in red have been confirmed as a zoonotic source of HEV by molecular studies. Phylogenetic tree was constructed by the neighbour-joining method using reference sequences of HEV-A described by Smith et al. (2020).

The species HEV-B is separated into at least four different genotypes and mostly affects chicken, but HEV-B infection has been detected in a wide range of domestic and wild avian species worldwide, including common buzzard (*Buteo buteo*), ducks (*Anas platyrhynchos*),

feral pigeon (*Columba livia domestica*), geese (*Anser anser domesticus*), little egret (*Egretta garzetta*), little owl (*Athene noctua*), rabbit New Zealand White (*Oryctolagus cuniculus*), song thrush (*Turdus philomelos*), sparrow (*Passer domesticus*) and turkey (*Meleagris gallopavo*) (Kenney, 2019). HEV-C is currently divided into two genotypes, named HEV-C1 and HEV-C2. To date, HEV-C1 infection has been confirmed in brown (*Rattus norvegius*), black (*Rattus rattus*), oriental house (*Rattus tanezumi*), lesser rice field (*Rattus losea*), yellow-breasted (*Rattus flavipectus*) and greater bandicoot (*Bandicota indica*) rats, in Asian musk shrew (*Suncus murinus*) and recently also in humans and carnivores, such as Syrian brown bear (*Ursus arctos syriacus*), whereas the susceptibility to HEV-C2 infection have been shown in wild carnivore species, including Western polecats (*Mustela putorius*), ferrets (*Mustela putorius*) and American mink (*Neovison vison*) (Wang et al., 2020). However, an increasing number of novel HEV-C variants and genotypes are being reported in wild birds of prey (Reuter et al., 2016), common voles (Kurucz et al., 2018), red fox (Bodewes et al., 2013) and African giant white-toothed shrews (Onyuok et al., 2019), among others. Finally, HEV-D has been detected in various bat species worldwide and variations of sequences within this *Orthohepevirus* species have been evidenced (Drexler et al., 2012). Nevertheless, the information regarding the genetic diversity of HEV-D is still scarce. As shown, animals are susceptible to the four species of *Orthohepevirus*, HEV-A to HEV-D (Doceul et al., 2016). However, HEV-A is considered the most important in terms of public health concern and HEV-C is currently recognized as an emerging cause of acute human hepatitis. Therefore, the present introduction is focused on these two zoonotic *Orthohepevirus* species.

3. Epidemiology

3.1. Hepatitis E virus in humans

Shortly after the first human cases reported in the late 1970's, epidemic HEV outbreaks were observed in different countries of Africa and Asia (Purcell & Emerson, 2008). HE is nowadays an endemic disease in these regions whereas small outbreaks and sporadic human cases are usually observed in several industrialized countries (Kamar et al., 2017). In different European countries, the number of HE human cases have considerably increased over the last decades (Aspinall et al., 2017). At present, HE is considered as an emerging disease of worldwide distribution of great relevance for public health (Figure 4). It has been estimated that one third of the human population have been infected by the virus (WHO, 2009) and that 20 million of infections occur annually (WHO, 2021).

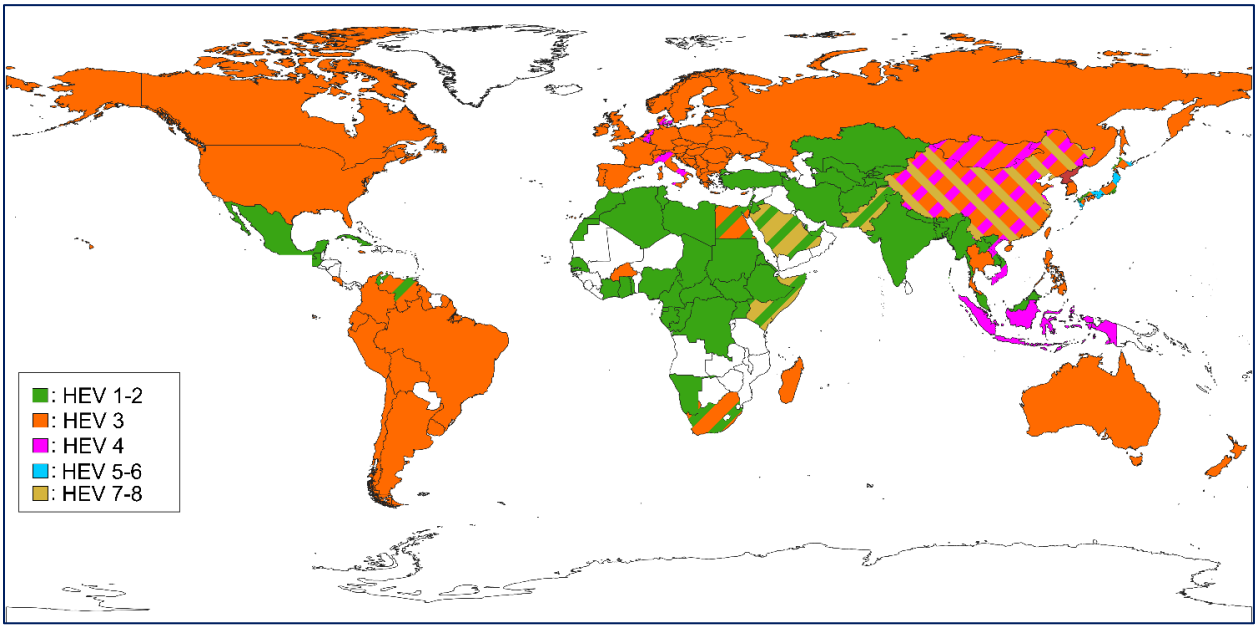

Figure 4. Main geographical distribution of the eight genotypes of *Orthohepevirus A*. Data obtained from Kamar et al. (2017) and Forni et al. (2018).

3.1.1. *Orthohepevirus A*, genotypes HEV-1 and HEV-2

HEV-1 and HEV-2 are endemic in developing countries and are associated with large waterborne hepatitis E (HE) outbreaks in humans. The first genotype is mainly distributed in

Asia and Africa while the second has been reported in Africa and Central America (Figure 4) (Khuroo et al., 2016). In industrialized countries, human HE cases caused by these strains are usually travel-associated (Kamar et al., 2017). The main source of HEV-1 and HEV-2 infection is the consumption of contaminated water frequently associated with heavy rainfall or flooding. However, vertical transmission to fetus and infant from the infected mothers and blood transfusions has also been reported (Khuroo et al., 2016).

Natural HEV-1 and HEV-2 infections have been almost observed in humans. However, in 2007 Saad et al. (2007) detected HEV-1 infections in horses in Egypt although independent confirmation of this report is still lacking. Experimental infections showed that non-human primates are also susceptible to these strains (Bradley et al., 1987; Tsarev et al., 1994) but not other mammal species, including goat, pig, rabbit and rat, denoting that HEV-1 and HEV-2 host ranges are probably limited to primates (Wang & Meng, 2020).

3.1.2. *Orthohepevirus A*, genotypes HEV-3 and HEV-4

HEV genotypes 3 and 4 are mainly distributed in high-income regions worldwide. HEV-4 is mainly found in Asia although circulation of this genotype has sporadically been detected in European countries, such as Belgium, Denmark, Italy and The Netherlands (Garbuglia et al., 2011; Hakze-van der Honing et al., 2011; Midgley et al., 2014). By contrast, HEV-3 is globally distributed, being frequently reported in the five continents (Figure 4) (Casares-Jiménez et al., 2021). In Europe, the seroprevalences in human general populations range from 4.7% to 29.5% (Figure 5), being described certain areas of this continent as hotspots for HEV exposure. In southeastern and southwestern regions of France, such as Ariège or Corsica, frequencies of seropositivity of 82% and 56.1% were reported (Izopet et al., 2019;

Capai et al., 2020). Similarly, hyperendemic areas (seroprevalences above 30.0%) have been observed in central Italy (Spada et al., 2018), southern Switzerland (Niederhauser et al., 2018) and western/central Poland (Bura et al., 2017). In Spain, the seroprevalence is around 20.0% (Sauleda et al., 2014), although it ranges from 0.6% to 38.7% in 2-5 years old and 70-80 years old, respectively (MS, 2021).

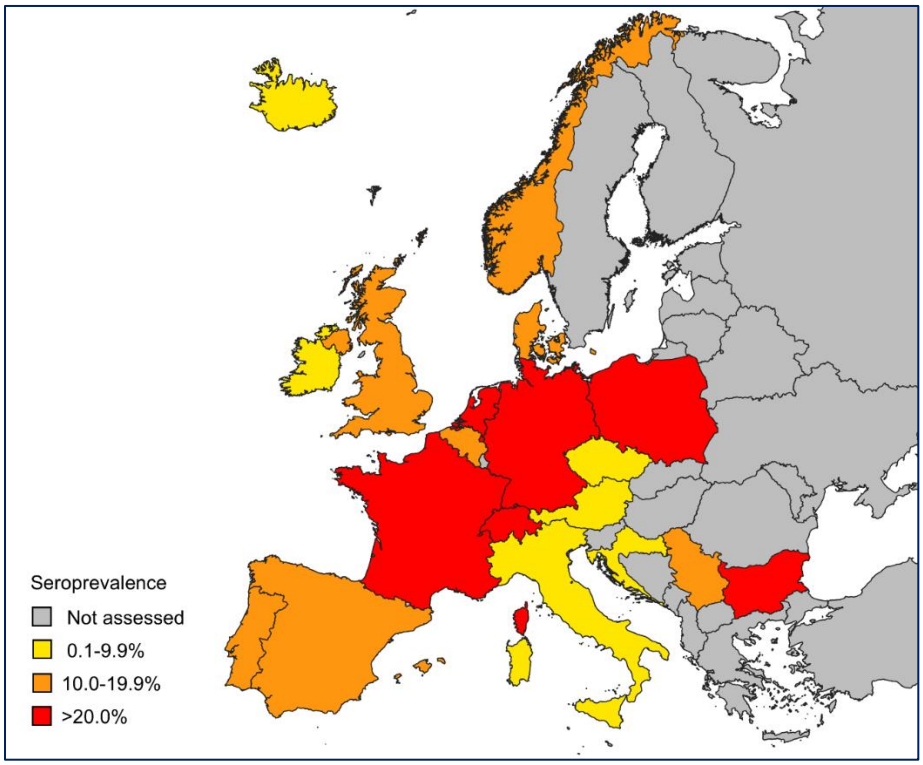

Figure 5. Seroprevalences against HEV in humans from different European countries. Data obtained from Van Hoecke et al. (2012), Lange et al. (2016), Grabarczyk et al. (2018), Löve et al. (2018), Izopet et al., (2019), Mesquita et al. (2020) and Baymakova et al. (2021).

HEV-3 and HEV-4 are mainly transmitted zoonotically through the consumption of raw or undercooked animal products (EFSA, 2017). Several studies have evidenced foodborne zoonotic transmission of HEV-3 and HEV-4 from suid and deer species (EFSA, 2017). Direct evidence of human cases due to the consumption of pig sausages and wild boar meat

contaminated with HEV-3 or deer meat with HEV-4 have been reported in France and Japan (Tei et al., 2003; Li et al., 2005; Renou et al., 2014). Similarly, Rivero-Juárez et al. (2017) confirmed a familiar outbreak of HEV-3 through the consumption of wild boar meat in Spain and Riveiro-Barciela et al. (2015) reported an acute HE case, being detected the same HEV-3 sequence in both patient and consumed domestic pig meat in this country. At the same time, several surveys conducted worldwide have linked sporadic cases or outbreaks of HEV with the consumption of raw or undercooked pork or wild boar products, including meat, liver or liver-derived products (Doceul et al., 2016). The high homology between HEV-3 and HEV-4 isolates from humans and swine species have been reported in many countries, which supports zoonotic transmission of this virus (Casares-Jimenez et al., 2021). In this regard, these genotypes have been evidenced in several pig products, such as liver pate, liver sausages, or salami, for human consumption in supermarkets worldwide, including different European countries (Kulkarni & Arankalle, 2008; Pavio et al., 2014; Szabo et al., 2015).

The contact with infected animals is also considered a zoonotic transmission route of both genotypes (EFSA, 2017). There have been a few number cases in which HEV transmission from an infected animal was supported by molecular evidence. In France, Renou et al. (2007) reported a possible zoonotic transmission between a pet pig and its owner whereas Colson et al. (2007) described a case of HEV-3 infection associated with exposure to pig blood during surgical training. In Spain, an autochthonous HEV-3 human case was observed in a slaughterhouse worker whose source of infection might be the manipulation of infected pig organs (Pérez-Gracia et al., 2007). Consistent with the above, farmers, veterinarians, slaughterers, or hunters have generally been recognized to have an increased risk of acquiring HEV infection (EFSA, 2017). Although less commonly, blood transfusions and organ

transplants have also been confirmed as a source for HEV-3 and HEV-4 infection (Dalton & Izopet, 2018).

3.1.3. *Orthohepevirus A*, other genotypes

The susceptibility of human beings to other genotypes of the species HEV-A, such as HEV-7, have recently been confirmed. Despite the limited number of cases, HEV-7 has been suggested to be transmitted through the consumption of camel products (Lee et al., 2017).

3.1.4. *Orthohepevirus C*, genotype HEV-C1

HEV-C has traditionally been limited to rodents and carnivores. However, HEV-C1 was recently confirmed as a cause of hepatitis in humans in China (Sridhar et al., 2018). The patient lived in a house estate with evidence of rat infestation, being identified HEV-C1 circulation in these rodent populations. During the subsequent years, a few additional human HEV-C1 cases were reported in China, Canada (possibly acquired in Africa) and also in Spain (Andonov et al., 2019; Sridhar et al., 2021; Rivero-Juárez et al., 2022). Despite this, little is known so far about the biology, ecology, epidemiology and pathogenesis of these zoonotic strains.

3.2. Hepatitis E virus in animals

The first description of HEV in animals was in domestic pigs from the USA in 1997, where HEV-3 infection was evidenced in most of the analyzed individuals older than 3-months old (Meng et al., 1997). Since then, a growing number of animal species susceptible to HEV have been confirmed (Kenney, 2019). In this regard, understanding the host range of HEV is key to identify potential zoonotic transmission routes, reservoir species that serve of viral

persistence in the environment, and potential hosts where HEV can mutate and become even more virulent (Kenney, 2019).

3.2.1. *Orthohepevirus A*, genotypes HEV-3 and HEV-4

3.2.1.1. Swine

Domestic pig and wild boar are the main reservoirs of the zoonotic HEV-3 and HEV-4. The virus is mainly transmitted among animals through the faecal-oral route by direct contact with infected individuals or ingestion of faeces-contaminated feed or water (Bouwknegt et al., 2008, 2009; Van der Poel, 2014). Approximately twelve days later after infection, which courses subclinical in animals, HEV can be found in sera of pigs and the mean duration of persistence in blood is eleven days. By contrast, HEV shedding in faeces last between 19 and 28 days in this animal species (Bouwknegt et al., 2009). Even though swine are one of the primary and most studied animal HEV models, many aspects about viral infection and transmission are still poorly understood. In this regard, chronic infection in two wild boar with a persistent viral shedding in faeces along 16 weeks was detected in Germany (Schlosser et al., 2015).

Surveillance on swine species reported seroprevalences that range widely from 8.6% to 92.8% in pigs (Salines et al., 2017) and from 1.6% to 71.4% in wild boars (Pavio et al., 2017) (Figure 6). In Spain, the seroprevalence values reported in these species are usually above 40.0% (Kukielka et al., 2015; Seminati et al., 2018).

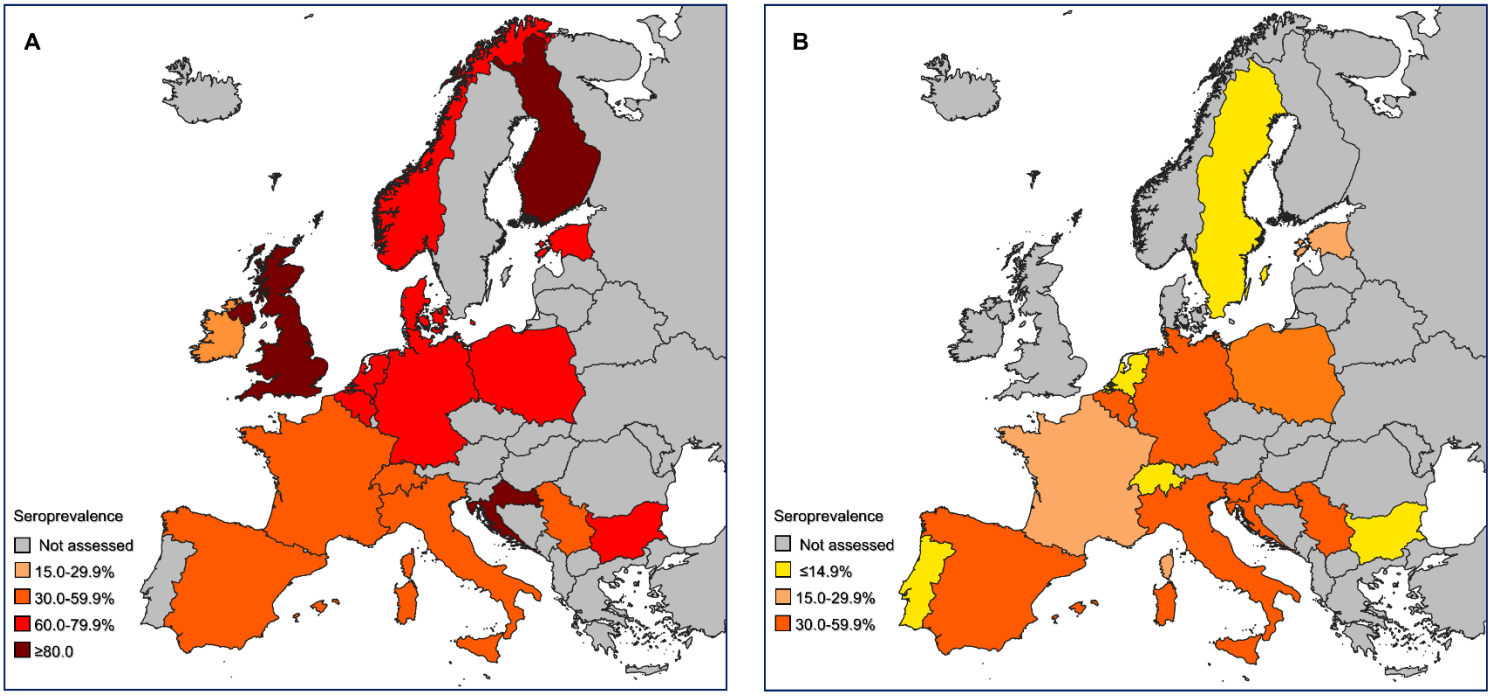

Figure 6. Seroprevalences against HEV in domestic pig (A) and wild boar (B) in Europe. Data obtained from Lupulovic et al. (2010), Weiner et al. (2016), Jemersic et al. (2017), Pavio et al. (2017), Salines et al. (2017), Gonçalves et al. (2018), Milojevic et al. (2019) and Tsachev e al. (2019).

Several risk factors have been associated with HEV infection in these two swine species. In pigs, HEV infection is more frequently detected between one and four months-old, gradually decreasing towards slaughter age (de Deus et al., 2008). However, quite a few reports of active HEV infection have also been reported in pigs at slaughterhouses (García et al., 2020; Sooryanarain et al., 2020; Chelli et al., 2021). By contrast, HEV RNA can be found indistinctly in all age groups of wild boars (Pavio et al., 2017). Additionally, the census of pigs within the farm, coinfections with immunosuppressive viruses, such as porcine circovirus type 2 (PCV-2) or porcine reproductive and respiratory virus (PRRSV), management systems, the contact with domestic or wildlife species, early slaughtering or the lack of biosecurity measures in the farm have been associated as risk factors for HEV infection in domestic pigs (Di Bartolo

et al., 2008; Walachowski et al., 2013; Salines et al., 2015, 2017; López-López et al., 2018; Salines et al., 2019). Similarly, the density and aggregation of animals increase the risk of HEV exposure in wild boar populations. In line with this, wild boar from fenced and intensively managed hunting estates showed higher HEV seroprevalences than in open areas (de Deus et al., 2008; Boadella et al., 2012). In addition, a potential seasonal pattern, and the region (urban or rural) where wild boars are located were also found as risk factors for HEV infection in this species (Forgách et al., 2010; Rivero-Juárez et al., 2018).

3.2.1.2. Other animal species

Knowledge of the host range of HEV-3 and HEV-4 have expanded over the last two decades (Kenney, 2019). At present, these strains have the widest host range among HEV genotypes, being detected during the last few years in several animal species besides suids (Figure 1) (Spahr et al., 2017a). The susceptibility to natural HEV-3 and/or HEV-4 infection has been confirmed in other artiodactyls as well as in carnivore, primate, lagomorph, rodent, mollusc, echinoderm, and avian species (Table 1). Even though several survey studies have assessed HEV circulation in some of these species, including red and sika deer (van der Poel, 2014; Kenney, 2019), as well as in rabbit, which is considered the main reservoir of the emerging and zoonotic HEV-3ra (Wang et al., 2017), the information about the role of non-suid species in the epidemiology of the virus is still very limited.

Table 1. Host range of HEV-3 and HEV-4.

Genotype	Order	Species	Reference
HEV-3	*Accipitriformes*	Himalayan griffon (*Gyps himalayensis*)	Li et al., 2015
	Artiodactyla	Bottlenose dolphin (*Tursiops truncatus*)	Montalvo-Villalba et al., 2017
		Domestic pig (*Sus scrofa domesticus*)	Meng et al., 1997

		Goat (*Capra aegagrus hircus*)	Di Martino et al., 2016
		Red deer (*Cervus elaphus*)	Kukielka et al., 2015
		Roe deer (*Capreolus capreolus*)	Anheyer-Behmenburg et al., 2017
		Sheep (*Ovis aries*)	Sarchese et al., 2019
		Sika deer (*Cervus nippon*)	Tei et al., 2003
		White-collared peccary (*Pecari tajacu*)	Ferreiro et al., 2020
		Wild boar (*Sus scrofa*)	Takahashi et al., 2004
	Camarodonta	Sea urchin (*Paracentrotus lividus*)	Santos-Ferreira et al., 2020
	Carnivora	Javan mongoose (*Herpestes javanicus*)	Nakamura et al., 2006
		Mink (*Neovison vison*)	Xie et al., 2018
		Wolf (*Canis lupus*)	Sarchese et al., 2021
	Lagomorpha	European brown hare (*Lepus europaeus*)	Corman et al., 2019
		Rabbit (*Oryctolagus cuniculus*)	Izopet et al., 2012
	Mytiloida	Mediterranean mussels (*Mytilus galloprovinciallis*)	Mesquita et al., 2016
		Blue mussels (*Mytilus edulis*)	O'Hara et al., 2018
	Perissodactyla	Horse (*Equus caballus*)	Zhang et al., 2008a
		Donkey (*Equus asinus*)	Rui et al., 2020
	Primates	Japanese monkey (*Macaca fuscata*)	Yanamoto et al., 2012
	Rodentia	Black rat (*Rattus rattus*)	Lack et al., 2012
		Norway rat (*Rattus norvegicus*)	Lack et al., 2012
	Veneroida	Japanese basket clam (*Corbicula japonica*)	Li et al., 2007
	Arcoida	Blood cockle (*Tegillarca granosa*)	Gao et al., 2016
		Half-crenated ark (*Scapharca subcrenata*)	Gao et al., 2016
	Artiodactyla	Black muntjac (*Muntiacus crinifrons*)	Zhang et al., 2008b
		Cattle (*Bos taurus*)	Huang et al., 2016
		David's deer (*Elaphurus davidianus*)	Zhang et al., 2008b
		Domestic pig (*Sus scrofa domesticus*)	Pavio et al., 2017
HEV-4		Goat (*Capra aegagrus hircus*)	Long et al., 2017
		Revees' muntjac (*Muntiacus reevesi*)	Zhang et al., 2008b
		Sheep (*Ovis aries*)	Wu et al., 2015
		Sika deer (*Cervus nippon*)	Zhang et al., 2008b
		Tufted deer (*Elaphodus cephalophus*)	Zhang et al., 2008b
		Wild boar (*Sus scrofa*)	Matsuda et al., 2003
		Yak (*Bos mutus*)	Xu et al., 2014

Carnivora	Asiatic black bear (*Selenarctos thibetanus*)	Zhang et al., 2008b
	Clouded leopard (*Neofelis nebulosa*)	Zhang et al., 2008b
Galliformes	Silver pheasant (*Lophura nycthemera*)	Zhang et al., 2008b
Gruiformes	Crowned crane (*Balaerica regulorum*)	Zhang et al., 2008b
Lagomorpha	Rabbit (*Oryctolagus cuniculus*)	Wu et al., 2017
Veneroida	Japanese carpet shell (*Ruditapes philippinarum*)	Gao et al., 2016

Despite the limited information regarding the zoonotic potential of non-suid species, a few zoonotic foodborne transmission cases have already been reported or suggested. As commented above, high nucleotide identity was found between HEV-3 sequences retrieved from infected patients and sika deer meat in Japan (Tei et al., 2003; Takahashi et al., 2004). Additionally, the consumption of shellfish was associated to a HEV-3 outbreak in humans (Said et al., 2009) and the subtype HEV-3ra has been isolated from humans in Belgium, France, Spain and Switzerland (Izopet et al., 2012; Rivero-Juárez et al., 2019; Sahli et al., 2019; Suin et al., 2019). In addition, the contact with other animals rather than swine and wild boar could be a source for HEV infection. Thus, people occupationally exposed to rabbits and ruminants, such as cattle and sheep, have been associated to be at higher risk for HEV infection (Tritz et al., 2017; Geng et al., 2019; Mesquita et al., 2020) and Kuno et al. (2009) suggested that pet cats could have transmitted HEV-4 to humans. All these reports indicate that non-suid species can be a zoonotic source of HEV. However, the risk of zoonotic transmission from these species is scarcely known.

3.2.2. *Orthohepevirus A*, genotypes HEV-5 and HEV-6

In 2010 and 2011, Takahashi et al. (2010, 2011) described divergent genotypes of HEV in wild boar in Japan, which were named HEV-5 and HEV-6. Circulation of these genotypes

seems to be restricted to Japan and wild boar is the single animal species described so far as susceptible to HEV-5 and HEV-6. However, one recent study has evidenced that a HEV-5 derived-virus could cause infections in cynomolgus monkeys, which suggest a potential risk of zoonotic transmission of this novel strain (Li et al., 2018).

3.2.3. *Orthohepevirus A*, genotypes HEV-7 and HEV-8

The genotypes HEV-7 and HEV-8 were discovered for first time in dromedary and Bactrian camels from Middle East countries and China, respectively (Woo et al., 2014, 2016). Contrary to HEV-7, whose zoonotic potential has already been evidenced, zoonotic cases of HEV-8 have not been reported until date although cynomolgus macaques were successfully experimentally infected by this genotype (Wang et al., 2019). In addition, experimental studies have been shown that rabbits are also susceptible to HEV-8 infection (Zhang et al., 2021).

3.2.4. *Orthohepevirus C*, genotypes HEV-C1 and HEV-C2.

A novel but distant hepevirus from HEV-A, named as *Orthohepevirus* C, was described for the first time in Norway rats from Germany in 2010 (Johne et al., 2010). During the subsequent years, rodents were evidenced to be the main reservoirs of the genotype HEV-C1, being already detected in rats and shrews from different American, Asian and European countries, including Spain (Reuter et al., 2020). Besides the recent evidence of the risk of zoonotic transmission of this genotype (Sridhar et al., 2018; Rivero-Juárez et al., 2022), HEV-C1 has also been described in other animal species, such as a Syrian brown bear (*Ursus arctos syriacus*) from a zoo in Germany (Spahr et al., 2017b). The detection of HEV-C1 in non-rodent species opens up other potential reservoirs and transmission routes of this emerging and zoonotic genotype.

The first report of HEV-C2 infection was in pet ferrets in The Netherlands in 2010 (Raj et al., 2012). At present, wild carnivores are considered the only reservoirs of this genotype (Wang et al., 2020), being detected in ferrets and American minks from Asia, Europe and North America (Krog et al., 2013; Li et al., 2014; Wang et al., 2018)

3.2.5. Unassigned or unknown orthohepevirus

The presence of anti-HEV antibodies, but not HEV RNA, have also been observed in an increasing number of animal species, including carnivores and artiodactyls among others (Kenney, 2019). However, given the existence of only a single serotype of HEV and the cross-reactivity of anti-HEV antibodies observed among *Orthohepevirus* species and genotypes (Sridhar et al., 2021), the recognition of the strain causing the seropositivity in these species is challenging. In this regard, several studies have identified novel HEV strains in seropositive animals. In 2014, Lin et al. (2014) reported a new strain of HEV in moose from Sweden that clusters within HEV-A but considerably differs with others HEV-A known genotypes. In that study, ten of the 67 (14.9%) seropositive animals were positive for active HEV infection. A novel hepevirus, similar to HEV-C, was also found in foxes from The Netherlands (Bodewes et al., 2013) and a divergent strain of HEV, which could potentially represent a novel *Orthohepevirus* species, was detected in common kestrel (*Falco tinnunculus*) in Hungary (Reuter et al., 2016). In that country, a novel hepevirus was also found recently in amphibians (Reuter et al., 2018). All these findings point the expanding host range of HEV and the need to increase the knowledge in the epidemiology of this emerging virus.

3.3. Hepatitis E virus in the environment

Taking into account that HEV is mainly shed in faeces of infected humans and animals,

the environment can also play a relevant role in HEV transmission (Van der Poel, 2014). Besides HEV-1 and HEV-2, contaminated water has been suggested to be a source of HEV-3 and HEV-4 infections in humans. Indeed, a study conducted in China described a tap water-mediated HEV-4 outbreak, probably contaminated with HEV-infected sewage (Chen et al. 2016). HEV sequences obtained from surface and waste waters frequently cluster with those found in human and animals from the same geographical region (Clemente-Casares et al., 2003; Beyer et al., 2020; Iaconelli et al., 2020). In this regard, water contamination with HEV may facilitate the entrance of the virus into food production chains, particularly through shellfish or irrigation waters. In line with this, a swine HEV-3 related strain was detected in mussels in Spain (Rivadulla et al., 2019) and on farm-grown strawberries in Canada (Brassard et al., 2012). The virus was also found in lettuce, rockets, spinaches, radicchio chicory, peppers and soft berries although the phylogenetic analyses were not performed (Kokkinos et al., 2012; Maunula et al., 2013; Loisy-Hamon & Leturnier, 2015; Terio et al., 2017; Santarelli et al., 2018). In addition, an outbreak caused by HEV-3 was related with the consumption of contaminated mussels in a cruise ship (Said et al., 2009). Although foodborne outbreaks of HEV from shellfish or vegetables consumption have not been confirmed so far, these reports indicate a potential risk of zoonotic transmission.

5. Diagnosis

5.1 Direct diagnosis

The first identification of the virus was performed using immune electron microscopy (Balayan et al., 1983). However, the routinely direct diagnosis techniques currently used includes the detection of HEV antigen or viral RNA by Enzyme Linked Immunosorbent Assay (ELISA) or conventional or real-time RT-PCR, respectively. Direct ELISAs are usually

commercially accessible and detect capsid antigens of HEV-A expressed from highly conserved region of ORF2, whereas there is no commercial nor in-house ELISA able to detect antigens from HEV-C so far.

Since the full-length genome description of HEV in 1991, several commercially and in-house RT-PCR assays for HEV detection have been developed (Lhomme et al., 2019). Given the genetic variability of HEV between the four different *Orthohepevirus* species, different authors established broad spectrum nested RT-PCRs able to amplify HEV-A to HEV-D viral genome (Johne et al., 2010; Drexler et al., 2012). There are also specific assays that detect HEV-A RNA. Several commercial and in-house assays able to detect HEV-1, HEV-2, HEV-3 and HEV-4 have been published (Inoue et al., 2006; Jothikumar et al., 2006; Abravanel et al., 2012). However, some of these assays are long since two consecutive PCRs are needed, and others may not detect all the novel genotypes and subtypes of HEV-A described during the last few years. Molecular assays should be, therefore, frequently reviewed or updated given this increasing genetic diversity. At present, there is only one reported real-time RT-PCR assay, that amplifies a 70-bp sequence of the ORF3 region, able to detect all described genotypes of HEV-A (Frias et al., 2021). Similar to this species, conventional and real-time in-house RT-PCRs able to amplify HEV-C1 and HEV-C2 are also available (Wang et al., 2020).

Although real-time PCR is a useful tool for screening the presence of HEV RNA, it is not considered appropriate for molecular characterization, especially genotyping (Lhomme et al., 2019). For that, nested RT-PCRs targeting the ORF-2 or the RdRp region of the ORF-1 are usually employed (Abravanel et al., 2009; Frias et al., 2021). In order to make genotyping and subtyping easier and consistent among studies, Smith et al. (2016, 2020) proposed reference

sequences of all the genotypes described of HEV. In addition, an international database of HEV sequence data, named HEVnet, was launched in 2019 to increase the knowledge about the molecular epidemiology of this emerging virus and to perform a standardized and routinely updated genotype and subtype assignment, even with a short genome fragment of HEV (Mulder et al., 2019). However, and thanks to the existence of novel molecular technologies, these authors encourage to sequence the whole genome of new positive HEV isolates in order to provide more accurate and complete strain comparisons.

5.2. Indirect diagnosis

The duration of HEV RNA in serum (between one- and two-weeks post infection (wpi)) or feces (usually between one and four wpi) in humans, suids and probably other animal species is limited (Figure 7) (Krain et al., 2014; Meester et al., 2021). This makes direct diagnosis, especially in alive individuals, challenging. By contrast, the duration of IgM and particularly IgG anti-HEV antibodies is higher, persisting months or years (Lhomme et al., 2019). Although the determination of both IgM antibodies and HEV RNA should be carried out in parallel in patients with suspicion of viral infection (Rivero-Juárez et al., 2021), serological assays have widely been recognized as a useful tool to get a global approach about exposure in human and/or animal populations (García-Bocanegra et al., 2011; Pollán et al., 2020).

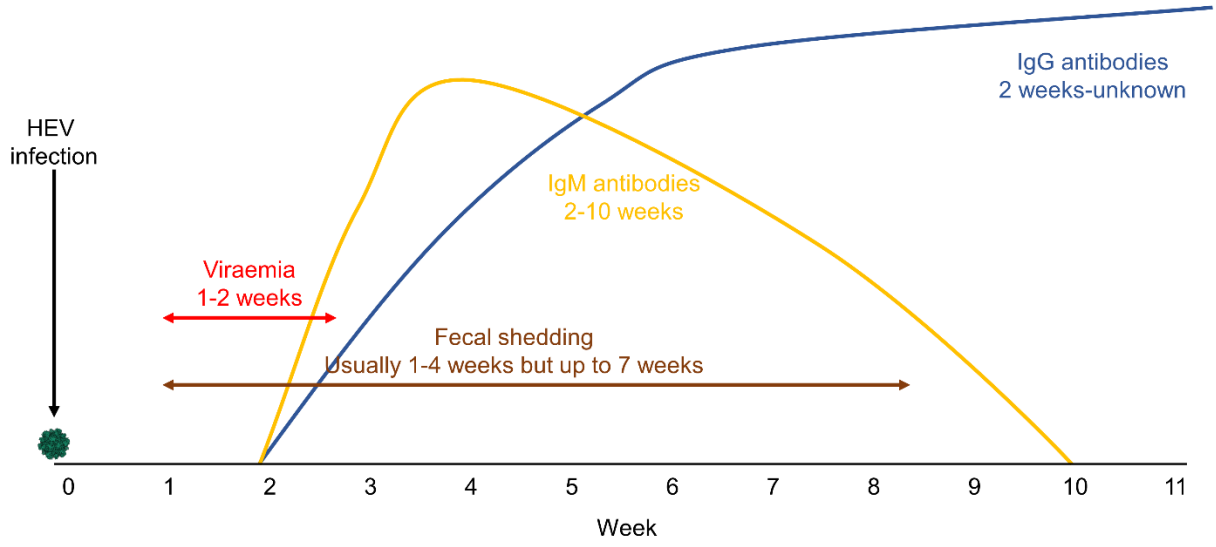

Figure 7. Course of HEV infection and antibodies response in humans and pigs. Adapted from Opriessnig, 2017. Data obtained from Meester et al. (2021) and Lhomme et al. (2019).

Among the serological methods used, rapid immune-chromatographic tests, ELISA and Western Blot are the most frequent. These assays are usually developed to detect the existence of anti-HEV antibodies against the capsid protein of the virus. Rapid immune-chromatographic tests are useful in hospital contexts or in studies with a limited number of samples whereas for epidemiological surveillance studies, which frequently analyze larger number of samples, ELISA and Western Blot are considered more suitable. For humans, several indirect ELISAs able to detect IgG or IgM antibodies are commercially available (Lhomme et al., 2019), although in-house assays have also been published (Krumbholz et al., 2014; Pandolfi et al., 2017). All these ELISAs have been developed to determine antibodies against HEV-A and, even though cross-reactivity among anti-HEV antibodies against genotypes of this and even other *Orthohepevirus* species have been confirmed (Kubickova et al., 2021), specificity and sensitivity for the detection of HEV-C antibodies of these ELISA assays should be assessed. In line with this, Sridhar et al. (2021) recently evidenced differences in the sensitivity of six

ELISA kits.

Contrary to the wide variety of assays for humans, the number of commercial kits that detects anti-HEV antibodies in animals is more limited. Indirect in-house and commercial ELISAs from different manufacturers have been developed to test swine serum samples. However, given that most of them uses a specific swine conjugate, the presence of antibodies in many others susceptible animal species cannot be analyzed. Some authors have used these precoated plates with HEV antigens and used a specific conjugate depending on the species tested (Zhang et al., 2008a; Liu et al., 2009). However, taking into account the wide diversity of animal species susceptible to this virus, the fully characterization of species-specific protocols using this approach can be difficult, time consuming and expensive. To solve this issue, different commercial manufacturers have developed double-antigen sandwich HEV ELISAs, which can potentially detect anti-HEV antibodies in human and all animal species. This ELISA do not use species specific conjugate and, therefore, are species independent (Hu et al., 2008). Similar to the commented in the above paragraph, the detection of anti-HEV-C antibodies using this ELISAs, which has also been developed using HEV-A antigens, need further investigations.

Interestingly, Western Blot assays have recently been used as a diagnostic method not only to confirm HEV seropositivity but also to differentiate the presence of antibodies against HEV-A or HEV-C. In this regard, Dremsek et al. (2011), who used this diagnostic approach, suggested exposure to HEV-C in human beings for the first time already in 2011, seven years before Sridhar et al. (2018) confirmed HEV-C infection in transplant patients.

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OBJECTIVES

The general goal of the present PhD is to assess the role of different domestic and wildlife species in the epidemiology of HEV. Thus, to achieve this general objective different specific goals are considered:

1. Hepatitis E virus in domestic animals, a silent threat?

This objective has been addressed in **Chapter 1**, which includes:

Chapter 1.1., to assess HEV circulation, including HEV-A, HEV-B and HEV-C, in sympatric urban cats and dogs in Spain.

Caballero-Gómez, J., Rivero-Juárez, A., Jurado-Tarifa, E., Jiménez-Martín, D., Jiménez-Ruiz, E., Castro-Scholten, S., Ulrich, RG., López-López, P., Rivero, A., & García-Bocanegra, I. (2021). Serological and molecular survey of hepatitis E virus in cats and dogs in Spain. Transboundary and Emerging Diseases, 69(2), 240-248.

Chapter 1.2., to determine the prevalence of HEV in equine species in Andalusia (southern Spain).

García-Bocanegra, I., Rivero, A., Caballero-Gómez, J., López-López, P., Cano-Terriza, D., Frías, M., Jiménez-Ruiz, S., Risalde, MÁ., Gómez-Villamandos, JC. & Rivero-Juárez, A. (2019). Hepatitis E virus infection in equines in Spain. Transboundary and Emerging Diseases, 66(1), 66-71.

Chapter 1.3., to determine the prevalence, spatial distribution and risk factors associated with HEV exposure in sheep and goats in southern Spain.

Caballero-Gómez, J., García-Bocanegra, I., Jiménez-Martín, D., Cano-Terriza, D., Risalde, M. A., López-López, P., Jiménez-Ruiz, S., Rivero, A., & Rivero-Juárez, A. (2022). Epidemiological survey and risk factors associated with hepatitis E virus in small ruminants in southern Spain. Zoonoses and Public Health, 69(4), 387-393.

2. Insights on the role of wildlife in the epidemiology of HEV.

This objective has been addressed in **Chapter 2**, which includes:

Chapter 2.1., to evaluate the circulation of HEV in free-ranging wild boar in the Doñana National Park (DNP), Spain.

Caballero-Gómez, J., Jiménez-Ruiz, S., López-López, P., Vicente, J., Risalde, M. A., Cano-Terriza, D., Frías, M., Barasona, JA., Rivero, A., García-Bocanegra & Rivero-Juárez, A. (2019). Emergent subtype of hepatitis E virus genotype 3 in wild boar in Spain. Transboundary and Emerging Diseases, 66(5), 1803-1808.

Chapter 2.2., to assess the circulation of HEV in European wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*), the most important lagomorph species in Spanish Mediterranean ecosystems.

Caballero-Gómez, J., García Bocanegra, I., Gómez-Guillamón, F., Camacho-Sillero, L., Zorrilla, I., López-López, P., Cano-Terriza, D., Jiménez-Ruiz, S., Frías, M., & Rivero-Juárez, A. (2020). Absence of Hepatitis E virus circulation in wild rabbits (Oryctolagus cuniculus) and Iberian hares (Lepus granatensis) in Mediterranean ecosystems in Spain. Transboundary and Emerging Diseases, 67(4), 1422-1427.

Chapter 2.3., to determine the seroprevalence and prevalence of HEV in both free-living and captive populations of the Iberian lynx (*Lynx pardinus*), to determine potential risk factors associated with HEV exposure in this species; and to evaluate the dynamics of seropositivity in longitudinally sampled animals during the study period.

Caballero-Gómez, J., Rivero-Juárez, A., Zorrilla, I., López, G., Nájera, F., Ulrich, RG., Ruiz-Rubio, C., Salcedo, J., Rivero, A., Paniagua, J., García-Bocanegra, I. Hepatitis E virus in the endangered Iberian lynx (Lynx pardinus). Under review

Chapter 2.4., to assess HEV infection in NHPs housed in zoos in Spain.

Caballero-Gómez, J., Rivero-Juárez, A., Cano-Terriza, D., Risalde, MA., Lopez-Lopez, P., Frías, M., Jiménez-Ruiz, S., Rivero, A., & García-Bocanegra, I. (2019). Survey for Hepatitis E virus infection in non-human primates in zoos in Spain. Transboundary and Emerging Diseases, 66(4), 1771-1775.

Chapter 2.5., assess HEV exposure in captive zoo animals in Spain and to determine the dynamics of seropositivity in individuals that were sampled longitudinally during the study period.

Caballero-Gómez, J., García Bocanegra, I., Cano-Terriza, D., Beato-Benítez, A., Ulrich, RG., Martínez, J., Guerra, R., Martínez-Valverde, R., Martínez-Nevado, E., Quevedo-Muñoz, MÁ., Sierra-Arqueros, C., Planas, J., de Castro-García, N., Rivero, A., Rivero-Juárez, A. Monitoring of hepatitis E virus in zoo animals from Spain, 2007-2021. Under review.

Chapter 2.6., to determine the seroprevalence and prevalence of HEV in both free-ranging and captive cetaceans in Spain, the potential risk factors associated with HEV exposure in these

species, and the dynamics of seropositivity in individuals sampled longitudinally during the study period.

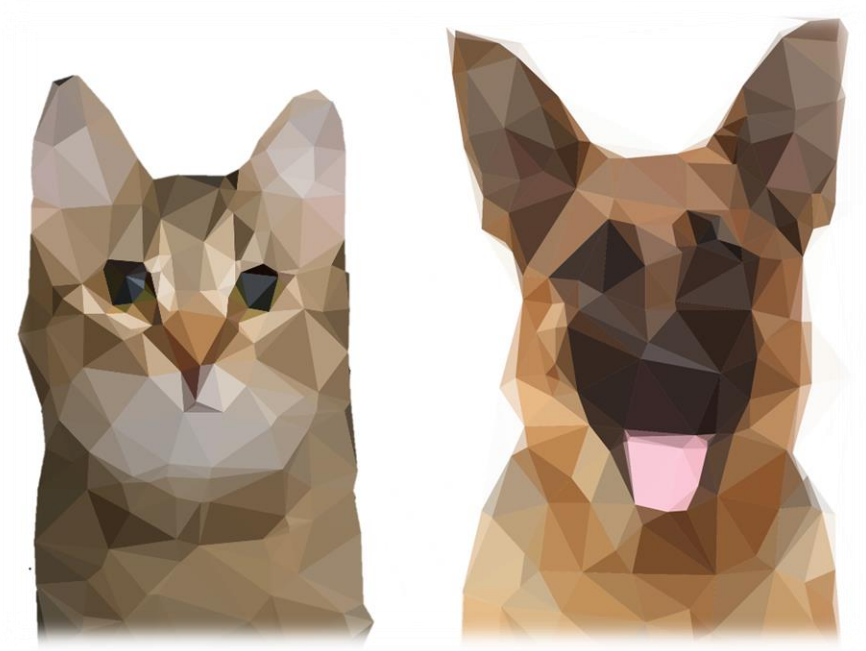
Caballero-Gómez, J., Rivero-Juárez, A., Beato-Benítez, A., Fernández-Maldonado, C., Domingo, M., García-Párraga, D., Sierra, E., Segura-Göthlin, S., Martínez-Nevado, E., Sierra, C., Canales, R., García-Bocanegra, I. Hepatitis E virus circulation in free-ranging and captive cetaceans in Spain, 2011-2022. Under review.

CHAPTER 1

Hepatitis E virus in domestic animals, a silent threat?

Chapter 1.1.

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



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Caballero-Gómez, J., Rivero-Juárez, A., Jurado-Tarifa, E., Jiménez-Martín, D., Jiménez-Ruiz, E., Castro-Scholten, S., Ulrich, RG., López-López, P., Rivero, A., & García-Bocanegra, I. (2021). **Serological and molecular survey of hepatitis E virus in cats and dogs in Spain**. *Transboundary and Emerging Diseases*, *69*(2), 240-248.

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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 analyzed 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 out of the 18 ELISA-positive animals that could be further analyzed 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 was 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: *Survey, hepatitis E, cat, dog, zoonoses, Orthohepevirus A, Orthohepevirus C.*

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, Bagdassarian, Demange, & Pavio, 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; Voslárorvá & 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.

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	United States of America (USA)	NA	0/177 (0.0%)	NA	NA	Dong et al., 2011
Stray	USA	NA	0/22 (0.0%)	NA	NA	Dong et al., 2011

*Not available

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., 2020
Pet	The Netherlands	2017	30/162 (15.5%)	0/162 (0.0%)	Faeces and pools of sera	Li et al., 2020
Pet	United Kingdom	2012-2013	2/92 (2.2%)	0/332 (0.0%)	Faeces and liver	McElroy et al., 2015
Pet	United States of America (USA)	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

Material and methods

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).

Sampling

Between 2017 and 2020, blood samples from 144 feral cats, 53 stray dogs and 99 pet dogs were collected from eleven 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.

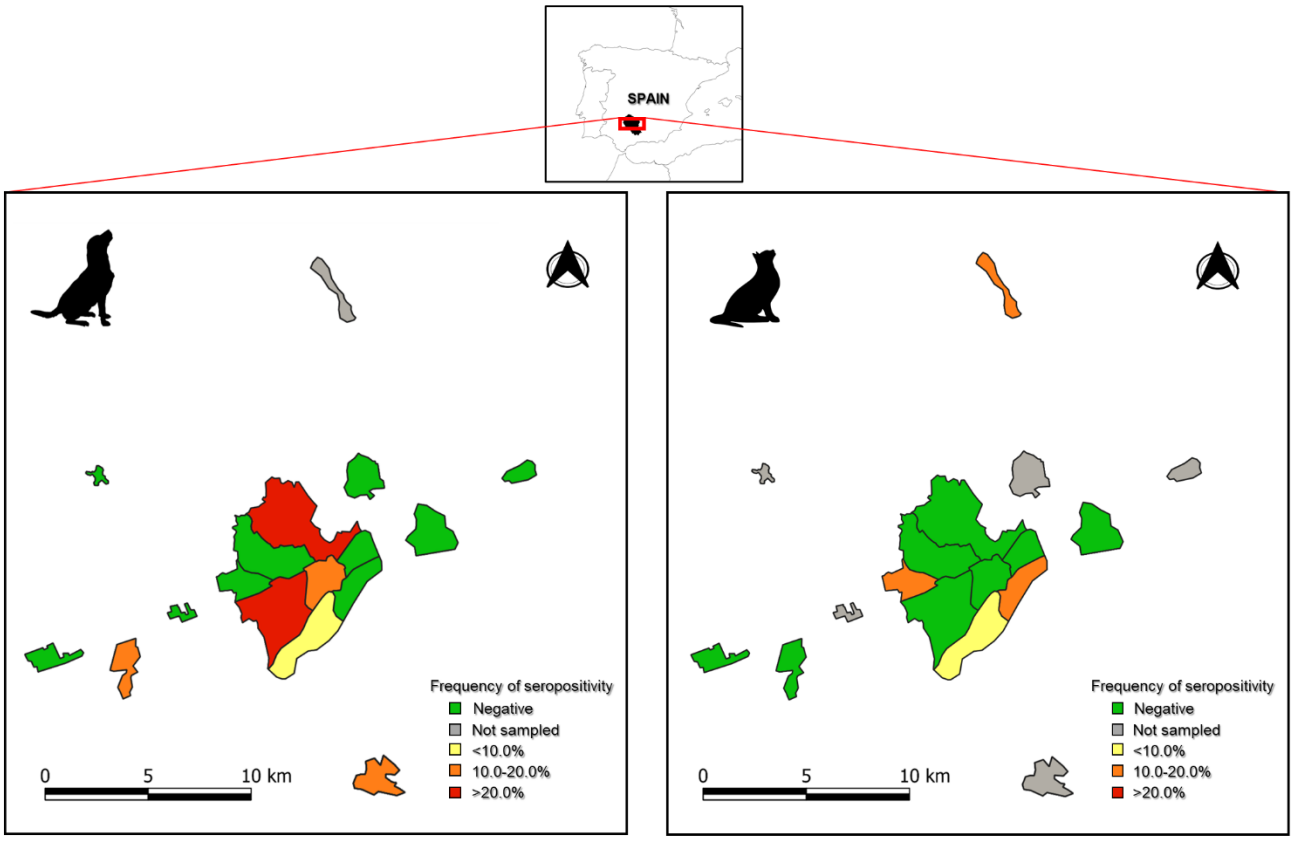

Figure 1. Spatial distribution of HEV seropositivity in dogs (left) and cats (right) in the province of Cordoba (southern Spain).

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* 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., 2011; 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 horse-radish peroxidase (HRP) labelled anti-cat IgG or anti-dog IgG (Jackson InmunoResearch, West Grove, Philadelphia, USA), diluted 1:2,500 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 five and 60 seconds.

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 30µL. 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 is 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 2X 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.

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 the explanatory variables (species, life condition, age, sex, and sampling year) were analyzed 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).

Results and discussion

A total of 19 (6.4%; 95%CI: 3.6-9.2) of the 296 analyzed animals showed antibodies against HEV (Table 3) and seropositive individuals were detected in eight (82.7%) of the eleven sampled areas (Figure 1). Ten out of the 18 ELISA-positive animals that could be

further analyzed 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 analyzed 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 (*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 last decade (Massei et al., 2015), becoming common the presence of wild boar in urban environments (Castillo-Contreras et al., 2021). In connection with this, fifty-two (19.7%) of 264 wild boars sampled in metropolitan areas of Barcelona (northeastern Spain) were positive for HEV RNA (Wang et al., 2018), 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-Juárez et al., 2018) and HEV exposure has also been detected in other mammals,

including zoo animals and humans (Caballero-Gómez et al., 2020; Rivero-Juárez 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.

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	
				Cats No. positives/No. analyzed (%)*	Dogs No. 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)	0.052	3/106 (2.8)	-
	2019	14/180 (7.8)		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)
	Subadult	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.

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) (Liang et al., 2014; Capozza et al., 2021; 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) (Okamoto, Takahashi, Nishizawa, Usui & Kobayashi, 2004; Li et al., 2020; Dähnert, Conraths, Reimer, Groschup, & Eiden, 2018). 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) out 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) (Vitral et al., 2005; Mazzei et al., 2015; Li et al., 2020; Liu et al., 2009; 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%) (Dähnert et al., 2018; Arankalle et al., 2001; Veronesi et al., 2020; Tien et al., 1997), while lower seroprevalence rates were observed in the United Kingdom (2.2%) and the USA (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 feces (Pavio, Doceul, Bagdassarian, & Johne, 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 six 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 analyzed, 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 of America, Japan and China (Spahr et al., 2017; Spahr, Knauf-Witzens, Vahlenkamp, Ulrich, & Johne 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 analyzed 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.

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Chapter 1.2.

Hepatitis E virus infection in equines in Spain

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Abstract

Hepatitis E (HE) is an important emerging disease in European countries. To analyze the role of equids as potential reservoirs for HE virus (HEV), we determined the prevalence of HEV infection in 861 equines from 464 herds in Spain. HEV RNA in serum was detected in 0.4% (3/692) of horses, 1.2% (1/86) of donkeys and 3.6% (3/83) of mules. Phylogenetic analysis identified the zoonotic genotype 3 as being closely related to viral human and swine strains. In this first report on HEV in equids in Europe, we confirm the susceptibility of horses, donkeys and mules to HEV infection. The low prevalence detected indicates that equids may be considered spillover hosts rather than true reservoirs.

Keywords: *Hepatitis E, horses, genotype 3, zoonoses, emerging disease, Spain*

Introduction

Hepatitis E virus (HEV) is a non-enveloped virus with a positive single-stranded RNA genome belonging to the genus *Orthohepevirus*, family *Hepeviridae*. HEV strains infecting humans are classified into 4 genotypes. Genotypes 1 and 2 have been detected only in humans, while genotypes 3 and 4 are zoonotic and predominant in domestic pigs and wild boar. Genotypes 3 and 4 have also been detected in a wide range of domestic and wild species (Spahr et al., 2018).

HEV infection is the main cause of acute hepatitis in humans in industrialized countries. Due to the risk of zoonotic transmission, hepatitis E (HE) is also considered an emerging disease of public health concern in Europe (Adlhoch et al., 2016). In developing countries, consumption of undercooked or raw infected pork and wild boar meat are major sources of exposure to HEV. Domestic pigs, wild boar and deer are the main species for foodborne transmission of HEV to humans (Tei et al., 2003; Colson et al., 2010; Rivero-Juárez et al., 2017a). Furthermore, HEV exposure has been confirmed in other species, including cattle, cats, rabbits, camels and shellfish (Kuno et al., 2003; Doceul et al., 2016; Huang et al., 2016; Lee et al., 2016), although the epidemiological role of these species is unclear.

Information about the prevalence of HEV in horses worldwide is still very scarce. Serological and molecular studies carried out in Asia and Africa suggest that horses are susceptible to HEV infection (Saad et al., 2007; Zhang et al., 2008). A previous study reported contact with horses as a risk factor for HEV seropositivity in humans (Christensen et al., 2008). The aim of this study was to determine the prevalence of HEV in equine species in Andalusia (southern Spain).

Materials and methods

Andalusia is the region with the largest number of both equines and herds in Spain, with about 223,696 individuals and 74,232 herds (mean=3 equines/herd), respectively (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2016).

Between April 2010 and March 2014, 692 blood samples were collected from horses (382 herds). Sampling was stratified by province according to the proportion of horses in each. The herds in each province were selected by simple random sampling using official records of herds obtained from the Regional Government of Andalusia. Based on the size of the selected herds (ranging from 1 to 180; median=16) and an estimated within-herd prevalence of 50% with confidence levels of 95% (95% CI), between one and 22 horses from each herd were randomly sampled for detection of HEV infection. Sampled horses were selected using systematic sampling. Further blood samples were obtained from 86 donkeys (42 herds) and 83 mules (40 herds) in the province of Cadiz, using convenience sampling for selection.

Blood samples were collected by jugular venipuncture using sterile collection tubes without anticoagulant (Vacutainer®, Becton-Dickinson, USA) and transported to the laboratory under refrigeration within 24 h of sampling. Samples were centrifuged for 15 min at 400g, and the sera separated and stored at −20 °C until required for analysis. Total RNA extraction was performed from 200uL of serum, using the automated MagNA Pure Compact RNA Isolation kit (Roche Diagnostics Corporation, Indianapolis, Indiana, USA), following the manufacturer's instructions. A negative control (200uL of RNAase-free water) was included in every RNA extraction run.

Serum samples were tested for RNA by quantitative real-time reverse-transcription

PCR (RT-PCR), following the protocol described by Abravanel et al. (2012). Two negative controls, randomly placed, were included in all RT-PCR runs. HEV viral load was calculated using the WHO HEV reference strain (code 6329/10) supplied by the Paul-Ehrlich-Institut, prepared from an HEV-3a strain. Viral load was expressed as copies/mL. All procedures were performed in the clinical virology and zoonoses biosafety level 2 (BSL-2) laboratory of our institution.

Nested RT-PCR of the ORF2 region was performed for sequencing and phylogenetic analysis, following the protocol described elsewhere (Muñoz-Chimeno et al, 2016), with primer modification in the second round (5´-AATTATGCYCAGTAYCGRGTTG-3´). The second amplification product of 649 bp was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed with SnapGene software (Version 3.1; GSL Bio-tech, snapgene.com). MUSCLE (Hinxton, UK) was used to create sequence alignments. This procedure amplifies a region of ORF2 located between 5699nt and 6401nt. Phylogenetic trees were constructed by the neighbor-joining method, with reference sequences for HEV genotypes/subtypes. Maximum Composite Likelihood method for evolutionary distances and a 1000-bootstrap procedure was conducted in MEGA Software (Version 7) to obtain the final tree.

Epidemiological information was obtained by interviewing the owners directly, and included data associated with the sampled animals (species, breed, gender and age), herd data (province), management (activity, type of housing, number of horses in the holding, transport of horses in the previous 6 months or two years) and biosecurity measures (contact with other domestic or wild species, disinfection and cleaning protocols, water sources and pest control

programs). The chi-squared and Fisher's exact test were used to establish associations between RT-PCR results (dependent variable) and explanatory variables. Variables that correlated $P< 0.20$ were included for further analysis. Biologically plausible confounding factors were assessed using Mantel-Haenszel analysis. Finally, multivariate logistic regression analysis was performed to identify risk factors potentially associated with HEV infection (likelihood ratio, Wald test, $P< 0.05$). Differences of $P\leq 0.05$ were considered statistically significant. Statistical analyses were performed using R software (R Development Core Team, 2013).

Results

HEV RNA was detected in 7 (0.8%; 95%CI: 0.2-1.4) of 861 equids tested. Individual prevalence was 0.4% (3/692) for horses, 1.2% (1/86) for donkeys and 3.6% (3/83) for mules. HEV was significantly more prevalent in mules compared with horses ($P = 0.019$). The age of the infected animals ranged from one to 18 years old (Table 1). Although the prevalence of HEV was higher in geriatric animals than in adults ($P = 0.003$) and young animals ($P= 0.005$), a high correlation was found between species and age ($P< 0.001$). No statistically significant differences between HEV infection and other explanatory variables were detected. Neither species nor age were retained in the multivariate logistic regression model.

Table 1. Epidemiological information of the HEV-positive equines detected in Andalusia (southern Spain).

Animal	Species	Age (years)	Gender	Breed	Province	Viremia levels (copies/mL)
1	Donkey	6	Male	Pure	Cádiz	90,000
2[#]	Horse	1	Female	Pure	Cádiz	2,570
3	Horse	5	Male	Crossbreed	Sevilla	1,640
4[#]	Horse	17	Male	Pure	Cádiz	20,200
5*	Mule	13	Female	Crossbreed	Cádiz	1,080
6*	Mule	16	Male	Crossbreed	Cádiz	1,950
7	Mule	18	Male	Crossbreed	Cádiz	5,570

[#]*Animals sampled in the same herd.

Five of 464 equine herds (1.1%) had at least one HEV-infected animal (Figure 1). One mule herd and one horse herd had two positive animals each. Six infected animals were detected in the provinces of Cadiz and one in the province of Sevilla (Table 1. Figure 1). Phylogenetic analysis could only be performed on one horse and showed that the sequenced isolate belonged to zoonotic genotype 3, clade 3efg (Figure 2). The strain (Genbank accession number: MH178352) was clustered with a viral strain that has been detected in human samples in France (Genbank accession number: EU495148) and Japan (Genbank accession number: AB850879) and in pigs in Spain (Genbank accession number: EU723516 and EU723514), sharing 94% nucleotide identity.

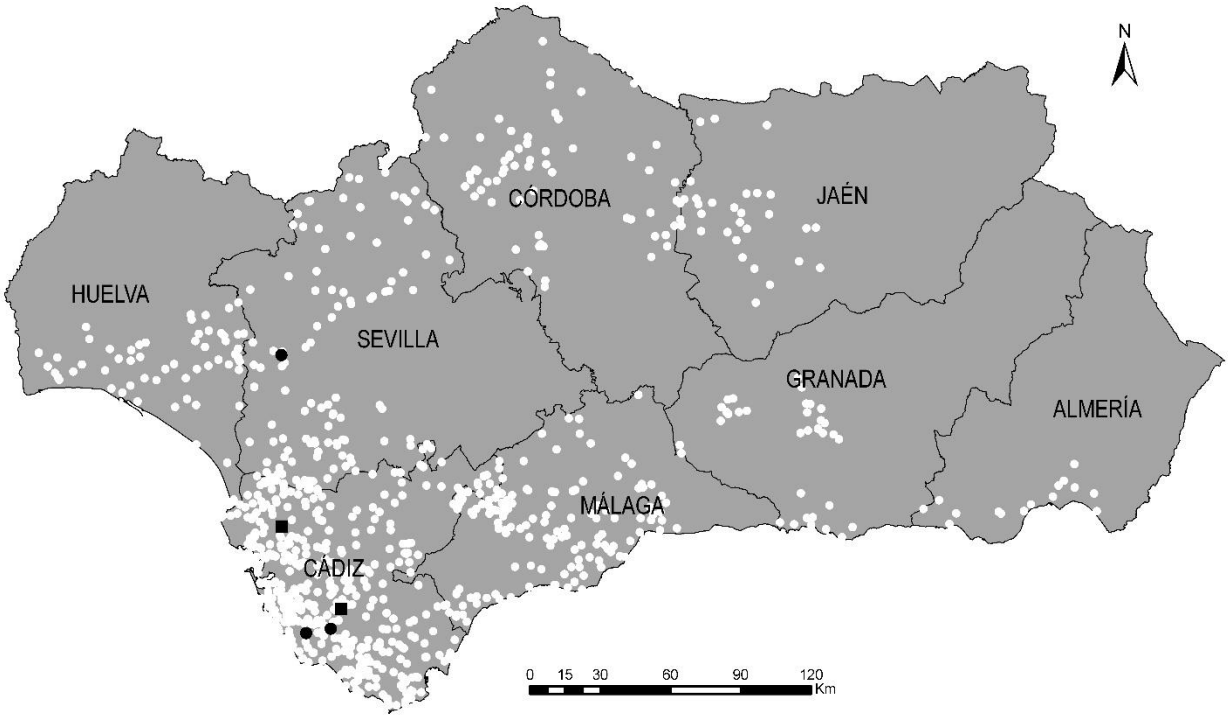

Figure 1. Map of Andalusia (southern Spain) showing the location of equid herds sampled. White dots indicate negative herds. Black dots and black squares represent equine herds with one and two HEV-infected animals, respectively.

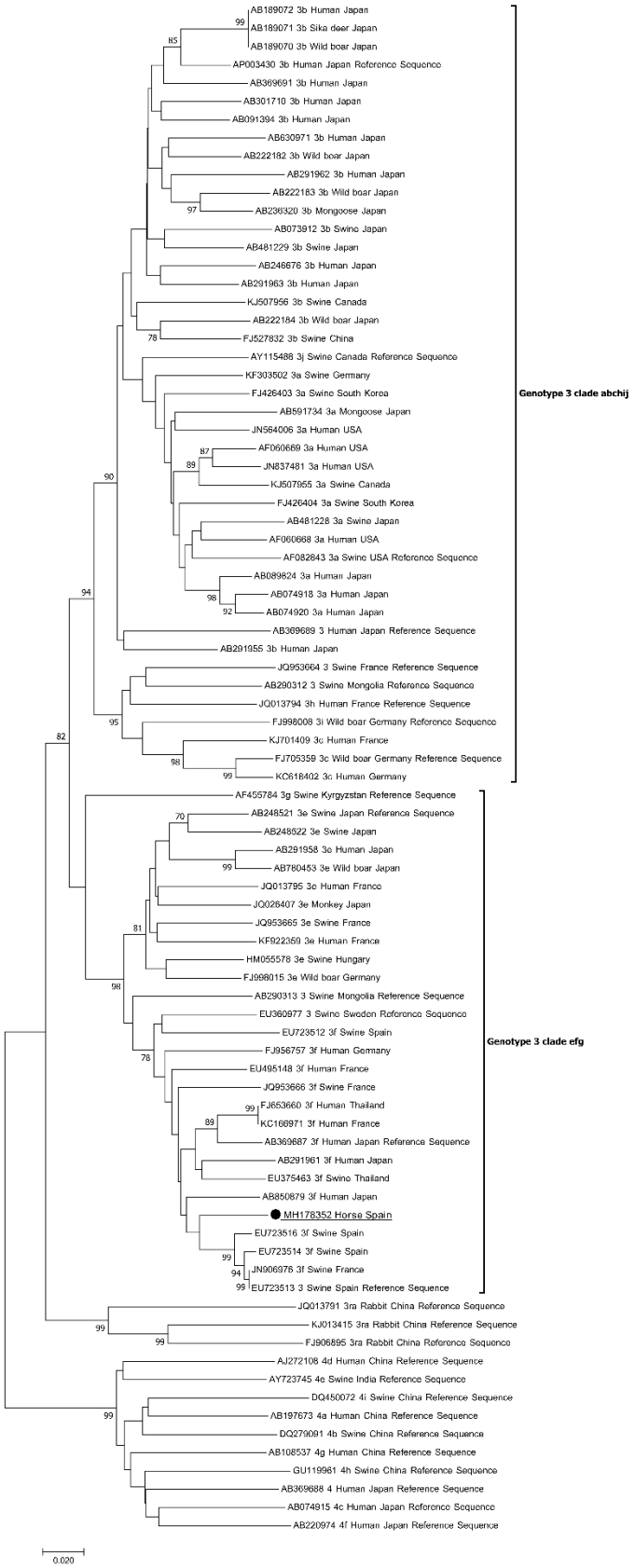

Figure 2. Phylogenetic tree constructed by the neighbor-joining method, based on 649 nt of ORF2 of 93 human and swine isolates. The optimal tree with the sum of branch length = 4.55376796 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7. Sequences proposed by Smith et al. (2016) as HEV reference sequences are referred to in the tree as Ref Seq.

Discussion

Over the last decade, the number of cases of HEV has sharply increased in humans in Europe (Adlhoch et al., 2016). In Spain, the reported prevalence (range 12%–26%) and incidence (range 4%–7%) of HEV in humans is relatively high (Pineda et al., 2014; Adlhoch et al., 2016; Rivero-Juárez et al., 2017b) and cases of fulminant HEV have been reported (Lapa et al., 2015). Identifying all potential animal reservoirs is a critical step in the control of this zoonotic disease. Although serologic and molecular evidence for HEV infection has been detected in a wide variety of wild and domestic species, the role of these species as reservoirs is still unclear, except for members of the *Suidae*.

Information about HEV infection in equids is limited strictly to reports suggesting that horses can act as HEV hosts. In Egypt, 13% (26/200) of horses had antibodies against HEV and the virus genome was detected in four of 100 (4.0%) animals (Saad et al., 2007). In China, 16.6% (8/49) of horses were positive for anti-HEV antibodies and HEV RNA was confirmed in one (2.0%) seropositive animal (Zhang et al., 2008). The genotypes detected in the studies of horses in Egypt (genotype 1) and China (genotype 3) were closely related with other HEV strains isolated previously in human and swine. The overall prevalence in our large survey coincides with figures reported for horses in endemic countries and suggests limited prevalence of HEV infection in equids in southern Spain.

To the best of our knowledge, this is the first survey study of HEV in equids in Europe and the first to report HEV infection in donkeys and mules worldwide. Our results confirm the susceptibility of these three equid species to HEV infection. Even though a significantly higher HEV prevalence was detected in mules compared to horses, the “species” variable was not

retained in the multivariate model. The absence of risk factors associated with HEV infection may be associated with the low number of positive animals. Further serosurvey studies should be carried out to identify potential risk factors affecting HEV exposure in equines.

HEV is endemic in the study area and widely distributed among domestic pigs (range 20%–71%), wild boar (range 26%– 43%) and deer species (10%) (de Deus et al., 2008; Peralta et al., 2009; Boadella et al., 2010; de Oya et al., 2011; Boadella et al., 2012; Rivero-Juárez et al., 2017a). The low prevalence detected in the present study indicates that equines can be considered spillover hosts rather than true reservoirs. Nevertheless, the presence of HEV RNA indicates active infection, so that handling live infected animals and eating raw or undercooked products made from horsemeat may be potential sources of HEV transmission to humans. Of the 8,940 tons of horsemeat products produced in Spain each year (MAPAMA, 2016), 40.1 tons could be contaminated by HEV. Consequently, approximately 183 of the 22,000 equids exported annually from Spain to other countries could be HEV-infected animals. Further research is required to elucidate HEV infectivity in equids and the zoonotic risk associated with the consumption of horsemeat products.

In Europe, human infections have been associated with HEV-3 subtypes -a, -c, -g, -e, -f, while genotypes 1 and 4 are reported more sporadically (Lapa et al., 2015). The genotype detected in the horse was identified as genotype 3, clade 3efg. The strain is clustered with the dominant type detected in swine species in Spain (de Deus et al., 2008; de Oya et al., 2011; Rivero-Juárez et al., 2017a). Unfortunately, only one viral strain could be amplified. In order to confirm the positive samples, two independent qPCR methods were used: i) the Altona RealStar HEV RT-PCR kit; and ii) a protocol elaborated by Vina-Rodriguez et al. (2015). All these samples were reactive in these two qPCR procedures. We think the reason we could not

sequence the strain may have been due to a relatively low viral load, or possibly, the presence of various mutations at the primer-binding site that were unable to amplify the sequences.

In conclusion, our results provide evidence of HEV infection in horses, donkeys and mules in Spain and suggest a possible role for these species as reservoirs or spillover hosts. Additional studies are required to assess the infectivity of equids in other species, including humans.

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Chapter 1.3.

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 analyzed in parallel using a commercial double antigen ELISA and real-time RT-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, zoonoses, public health, risk factors.*

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 and 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, 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; Renou et al., 2014; Guillois et al., 2016; Rivero-Juárez 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 (López-López 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% (Shukla et al., 2007; Peralta et al., 2009). 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,b). 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.

Materials and methods

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., 2016; Palombieri et al., 2020), with a 95% confidence level (95%CI) and desired precision of ±5% (Thrusfield & 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, forty intensive, six semi-extensive, and twelve 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 six on small ruminant's milk and meat.

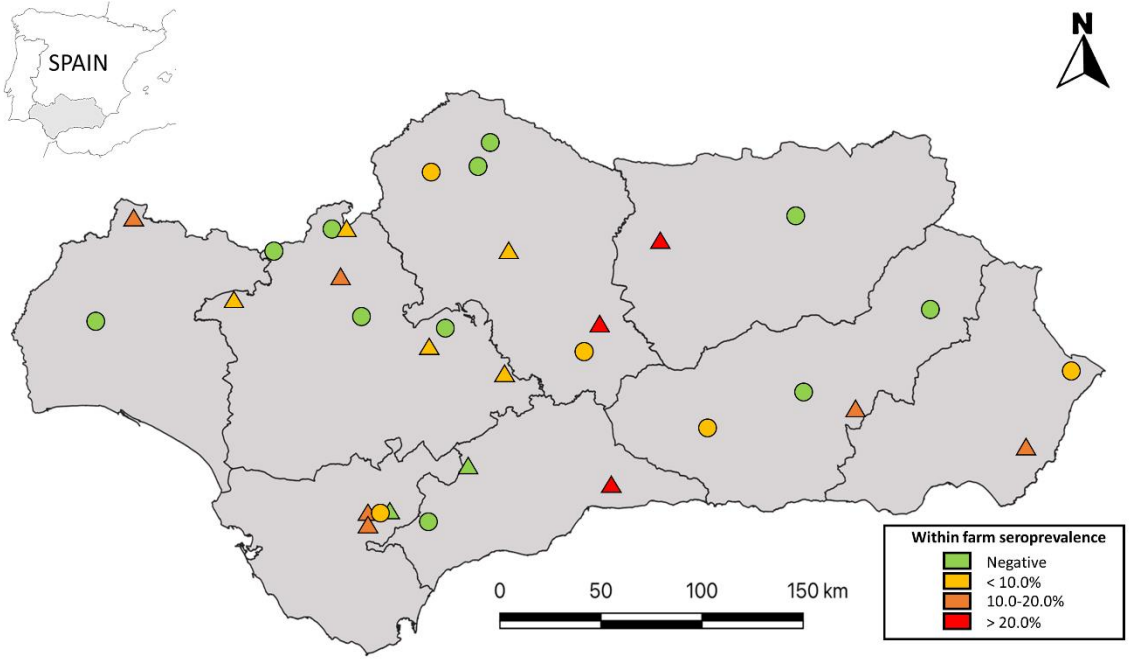

Figure 1. Distribution of sampled small ruminant farms. Triangles and circles represent goat and sheep flocks sampled, respectively. Color graduation shows within-farm seropositivity.

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.

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 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 µL of serum from four different individuals (100 µl 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 µL. The presence of HEV RNA was determined by real-time RT-PCR (CFX Connect Real Time PCR System), which detects all genotypes of *Orthohepevirus* A using 10 µL 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 was spiked in. 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.

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 $p < 0.10$ 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 analyzed 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 ($p < 0.05$) 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).

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 northeastern 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 and 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 northeastern 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 <0.001; 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 feces (Di Martino et al., 2016; Sarchese et al., 2019), with behavioral 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 feces. 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; Walachowski et al., 2014; López-López et al., 2018; Pavia et al., 2021). 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 favors HEV circulation within the farm.

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	<0.001	7.3 (3.2-16.4)
	Sheep	[a]	[a]
Number of small ruminants in the farm	≤348	0.012	2.6 (1.2-5.4)
	≥538	0.001	4.5 (1.9-10.6)
	349-537	[a]	[a]

[a]Reference category

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 (Wu et al., 2015; Long et al., 2017; Sarchese et al., 2019; El-Mokhtar et al., 2020). The presence of viral RNA in liver and milk from both sheep and goats, with prevalence values ranging between 2.8% and 18.5% (Wu et al., 2015; Demirci et al., 2019; Dziedzinska et al., 2020; El-Mokhtar et al., 2020), 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 liters 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 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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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.

Conflict of interest statement

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.

Supplementary material. Table S1. Distribution of explanatory variables associated with HEV seropositivity in small ruminants in southern Spain.

Variable	Categories	N/total*	%	*P*
Species	Sheep	5/240	2.1	<0.001
	Goat	33/240	13.8	
Sex	Female	33/434	7.6	0.128
	Male	3/16	18.8	
Breed	Purebreed	5/130	3.8	0.026
	Crossbred	33/345	9.6	
Province	Almeria	4/30	13.3	0.167
	Cadiz	5/60	8.3	
	Cordoba	8/90	8.9	
	Granada	3/60	5.0	
	Huelva	2/30	6.7	
	Jaen	6/30	20.0	
	Malaga	4/45	8.9	
	Sevilla	6/135	4.4	
Production system	Meat	11/240	4.6	0.015
	Milk	19/150	12.7	
	Mixed	5/60	8.3	
Management system	Extensive	6/180	3.3	0.002
	Intensive	27/210	12.9	
	Semiextensive	5/90	5.6	
Type of replacement	External	17/135	12.6	0.017
	Internal	21/345	6.1	
Number of small ruminants in the farm	≤ 348	14/165	8.5	0.013
	349-537	5/150	3.3	
	≥ 538	19/150	12.7	
Presence of dogs	No	2/45	4.4	0.365
	Yes	36/435	8.3	
Presence of cats	No	5/165	3.0	0.002
	Yes	33/315	10.5	
Presence of cattle	No	25/345	7.2	0.219
	Yes	12/120	10.0	
Presence of pigs	No	28/315	8.9	0.282
	Yes	9/150	6.0	
Presence of equines	No	22/345	6.4	0.038
	Yes	16/135	11.9	
Presence of poultry	No	10/195	5.1	0.042
	Yes	28/285	9.8	
Presence of wild boar (*Sus scrofa*)	No	23/225	10.2	0.056
	Yes	15/255	5.9	
Presence of red deer (*Cervus elaphus*)	No	28/285	9.8	0.042
	Yes	10/195	5.1	

Presence of fallow deer (*Dama dama)*	No	37/450	8.2	0.292
	Yes	1/30	3.3	
Presence of mouflon (*Ovis orientalis musimon*)	No	37/450	8.2	0.292
	Yes	1/30	3.3	
Presence of badger (*Meles meles*)	No	32/405	7.9	0.563
	Yes	6/75	8.0	
Presence of rodents	No	8/75	10.7	0.227
	Yes	23/315	7.3	
Transhumance in the last three years	No	34/435	7.8	0.672
	Yes	1/15	6.7	
Disinfection baths (entry)	No	24/270	8.9	0.200
	Yes	8/135	5.9	
Perimeter fencing	No	3/30	10.0	0.430
	Yes	29/375	7.7	
Dung removal system	No	3/60	5.0	0.265
	Yes	28/330	8.5	
Use of manure as fertilizer	No	4/30	13.3	0.206
	Yes	27/360	7.5	
Carcass container	No	20/240	8.3	0.228
	Yes	9/75	12.0	
Parking outside the farm	No	21/270	7.8	0.519
	Yes	11/135	8.1	
Livestock transport vehicles enter the farm perimeter	No	7/120	5.8	0.215
	Yes	25/285	8.8	
Carcass vehicle is allowed to enter the farm perimeter	No	11/150	7.3	0.184
	Yes	18/165	10.9	
Contact with wild species in feeders	No	31/390	7.9	0.579
	Yes	7/90	7.8	
Contact with wild species in drinkers	No	30/330	9.1	0.173
	Yes	8/135	5.9	
Contact with wild species in ponds	No	29/285	10.2	0.018
	Yes	9/195	4.6	
Contact with wild species in pastures	No	28/285	9.8	0.042
	Yes	10/195	5.1	
Contact with cattle from neighbouring farms	No	33/345	9.6	0.084
	Yes	3/75	4.0	
Contact with cattle from neighbouring farms	No	32/330	9.7	0.080
	Yes	4/90	4.4	
Contact with goats from neighbouring farms	No	29/315	9.2	0.279
	Yes	7/105	6.7	
Contact with sheep from neighbouring farms	No	31/300	10.3	0.027
	Yes	5/120	4.2	
Indirect contact with other farms (machinery)	No	29/360	8.1	0.514
	Yes	3/45	6.7	

Indirect contact with other farms (staff)	No	19/165	11.5	0.029
	Yes	18/300	6.0	
Indirect contact with other farms (pastures)	No	29/375	7.7	0.427
	Yes	8/90	8.9	
Indirect contact with other farms (troughs)	No	34/420	8.1	0.509
	Yes	3/45	6.7	
Rodent control	No	13/105	12.4	0.045
	Yes	17/270	6.3	
Cleaning protocol	No	2/30	6.7	0.566
	Yes	29/360	8.1	
Disinfection protocol	No	2/45	4.4	0.278
	Yes	29/345	8.4	
Animal insecticide treatment	No	8/105	7.6	0.527
	Yes	22/270	8.1	
Altitude (masl)	< 400	24/195	23.3	0.008
	400-1,000	1/60	1.7	
	>1,000	13/225	5.8	
Mean annual rainfall (l/m^2)	< 740	7/90	7.8	0.240
	740-1,140	11/195	5.6	
	<1,140	20/195	10.3	
Mean annual temperature (ºC)	<14	4/75	5.3	0.621
	14-16	23/285	8.1	
	>16	11/120	9.2	

* Missing values omitted.

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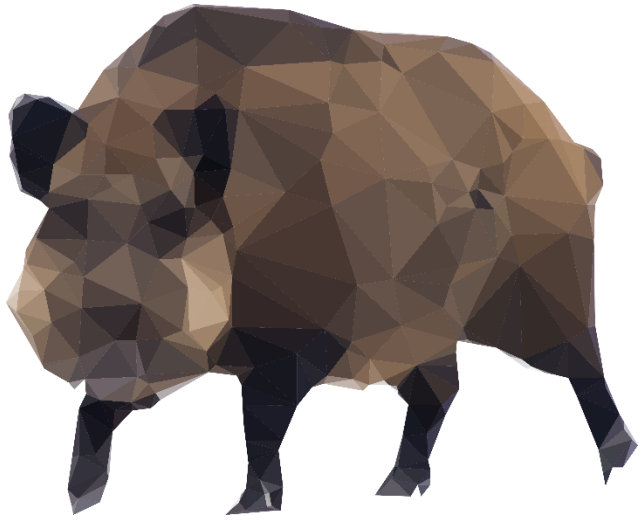
CHAPTER 2

Insights on the role of wildlife in the epidemiology of HEV

Chapter 2.1.

Emergent subtype of hepatitis E virus genotype 3 in wild boar in Spain



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Abstract

Wild boar (*Sus scrofa*) is considered the main wildlife reservoir of zoonotic hepatitis E virus (HEV) genotypes. The aim of this study was to evaluate the circulation of HEV in free-ranging wild boar in the Doñana National Park (DNP), Spain. Blood samples were collected from 99 wild boar in the DNP during 2015. Sera were analyzed in parallel using indirect ELISA and real-time RT-PCR. A total of 57 of the 99 tested animals (57.6%; 95%CI: 47.8-67.3%) had anti-HEV antibodies, indicating that this virus is widespread in wild boar in the DNP. HEV RNA was detected in one animal and phylogenetic analysis showed that the sequence isolated belonged to subtype 3r. The results suggest a potential risk of zoonotic transmission of this novel HEV-3 subtype, which could be of public health concern. Further studies are required to assess the role of wild boar in the epidemiology of HEV-3r and to determine the infectivity of this emergent HEV subtype in other species, including humans.

Keywords: *Hepatitis E virus, public health, genotype 3, zoonosis*.

Introduction

Hepatitis E (HE) is an emerging disease worldwide and the leading acute viral hepatitis in humans (Clemente-Casares, Ramos-Romero, Ramirez-Gonzalez, & Mas, 2016; Aspinall et al., 2017). At least 20 million of people are infected annually with the HE virus (HEV), which can cause up to 30% mortality in pregnant women and lead to chronicity in immunocompromised individuals (Nimgaonkar, Ding, Schwartz, & Ploss, 2018). HEV is a positive, single-stranded RNA virus belonging to the family *Hepeviridae*, genus *Orthohepevirus*, which includes the *Orthohepevirus A* species, comprising eight different genotypes (HEV-1 to 8) (Kenney & Meng, 2019).

HEV-1 and HEV-2 infections are restricted to humans, whereas HEV-3 and HEV-4 are zoonotic and their main reservoirs are the domestic pig (*Sus scrofa domesticus*) and wild boar (*Sus scrofa*) (Lapa, Capobianchi, & Garbuglia, 2015). In industrialized countries, there have been documented cases of human HEV infections transmitted by eating pork and game products (Tei, Kitajima, Takahashi, & Mishiro, 2003; Riveiro-Barciela et al., 2015). In Europe, foodborne transmission is considered to be the major zoonotic route of HEV infection (EFSA, 2017). In this regard, the high homology between HEV-3 isolates from humans and swine species reported in European countries (Vasickova et al., 2011; EFSA, 2017) supports zoonotic transmission of this virus. In Spain, Rivero-Juárez et al. (2017) recently provided evidence of a familial outbreak of HEV-3 infection linked to the consumption of wild boar meat.

In recent decades, wild boar densities have increased drastically in the Mediterranean ecosystems of south-central Spain (SCS) where this species coexists with other sympatric species. The circulation of HEV-3 has been reported in wild boar populations in these regions,

with prevalences ranging between 5.2% and 57.4% (Kukielka, Rodríguez-Prieto, Vicente, & Sanchez-Vizcaíno, 2016; Risalde et al., 2017). The differences between regions indicate the need to determine the role of wild boar in HEV transmission in each epidemiological context. Here, we assess HEV circulation in wild boar in the Doñana National Park (DNP, Spain), a study area characterized by very limited human activity and the absence of a pig industry.

Material and methods

The present study was conducted in the Doñana National Park (south-west Spain; 37°0′ N, 6°30′ W) (Figure 1), a region with the highest level of environmental protection in Spain and one of the most important natural reserves in Europe in terms of biodiversity. Sera from a total of 99 wild boar were collected during 2015. Blood was extracted by endocranial venous sinuses puncture (Arenas-Montes et al., 2013) during the annual wild boar population control program. Individual data of age (yearlings: < 1 year old; subadults: between 1 and 3 years old; adults: > 3 years old), gender and sampling location were recorded for each animal. Samples were centrifuged at 400 *g* for 10 min to obtain the serum, then conserved at -80ºC until analysis.

The presence of anti-HEV antibodies was determined using a commercial indirect enzymatic immunoassay (ELISA; PrioCHECK™ HEV Antibody ELISA Kit, porcine. Thermo Fisher Scientific, Massachusetts, USA) based on recombinant ORF2 and ORF3 antigens derived from genotypes 1 and 3, following the manufacturer's instructions. This ELISA has been widely used in wild boar studies (Ivanova et al., 2015; Risalde et al., 2017). According to the information provided by the manufacturer, it has sensitivity and specificity of 91% and 94%, respectively.

RNA was extracted from pools of 400 μl 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 animals. For detection of HEV RNA, real-time RT-PCR (CFX Connect Real Time PCR System) was performed using the iTaq Universal Probes One-Step Kit (Biorad, Hercules, California, USA). The primers, probes and thermal profile used were as previously described by Abravanel et al. (2012). Positive pools were then individually analyzed.

Phylogenetic analysis of the ORF2 region was performed using nested RT-PCR, following the protocol described elsewhere (Muñoz-Chimeno et al., 2016), with modified primers in the second round (5´-AATTATGCYCAGTAYCGRGTTG-3´). The second amplification product of 649 bp was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The consensus sequence was obtained using Seqman Software SeqMan NGen® Version 12.0 (DNASTAR. Madison, WI). Subtype assignment and phylogenetic analyses were performed using the HEVnet genotyping tool (https://www.rivm.nl/mpf/typingtool/hev/) (Mulder et al., 2019) and confirmed by BLAST. Sequence alignments were generated by the MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Phylogenetic trees were constructed using the maximum likelihood method using HEV genotype/subtype standard reference strains reported by Smith et al. (2016) including 156 viral sequences. Reference sequences of genotype 4 were included as an outgroup to root the tree. The final tree was obtained with MEGA Software (Version 7) using the bootstrap method (bootstrapped with 1000 replicates).

Prevalence was determined as the coefficient of positive animals / total animals tested, using two-sided exact binomial 95% confidence intervals (95%CI). Multiple logistic

regression analysis was used to explore associations between the prevalence of anti-HEV antibodies and HEV RNA results and explanatory variables (age and gender). The level of significance was established at $P<0.05$. Analyses were carried out using SPSS v.22.0 (Statistical Package for Social Sciences Inc., Chicago, IL, USA).

Results and discussion

A total of 57 of 99 wild boar (57.6%; 95%CI: 47.8-67.3%) presented anti-HEV antibodies (Figure 1). This seroprevalence is consistent with that found in Italy (56.3%; 36/64) but higher than that detected in other European countries, such as Belgium (34.0%; 130/383), France (29.2%; 101/346), Germany (45.0%; 81/180) and Poland (41.6%; 47/113) (reviewed in Pavio, Doceul, Bagdassarian, & Johne, 2017). In different regions of south-central Spain, where fencing and artificial feeding have been identified as risk factors for HEV infection in wild boar (de Deus et al., 2008; Boadella et al., 2012), seroprevalence values of 5.2% (3/58) (Risalde et al., 2017), 26.5% (195/735) (Boadella et al., 2012), 28.0% (42/150) (de Deus et al., 2008) and 57.4% (62/108) (Kukielka et al., 2016) have previously been detected in this species. The seropositivity found in the DNP is in accordance with that observed by Wang et al. (2019) in the Barcelona metropolitan area (northeastern Spain), where up to 58.9% (112/190) of wild boar tested presented anti-HEV antibodies. Since wildlife management is not allowed in the DNP and there is limited human activity and no pig industry, the high seroprevalence found there highlights the importance of wild boar in the epidemiological cycle of HEV in this national park.

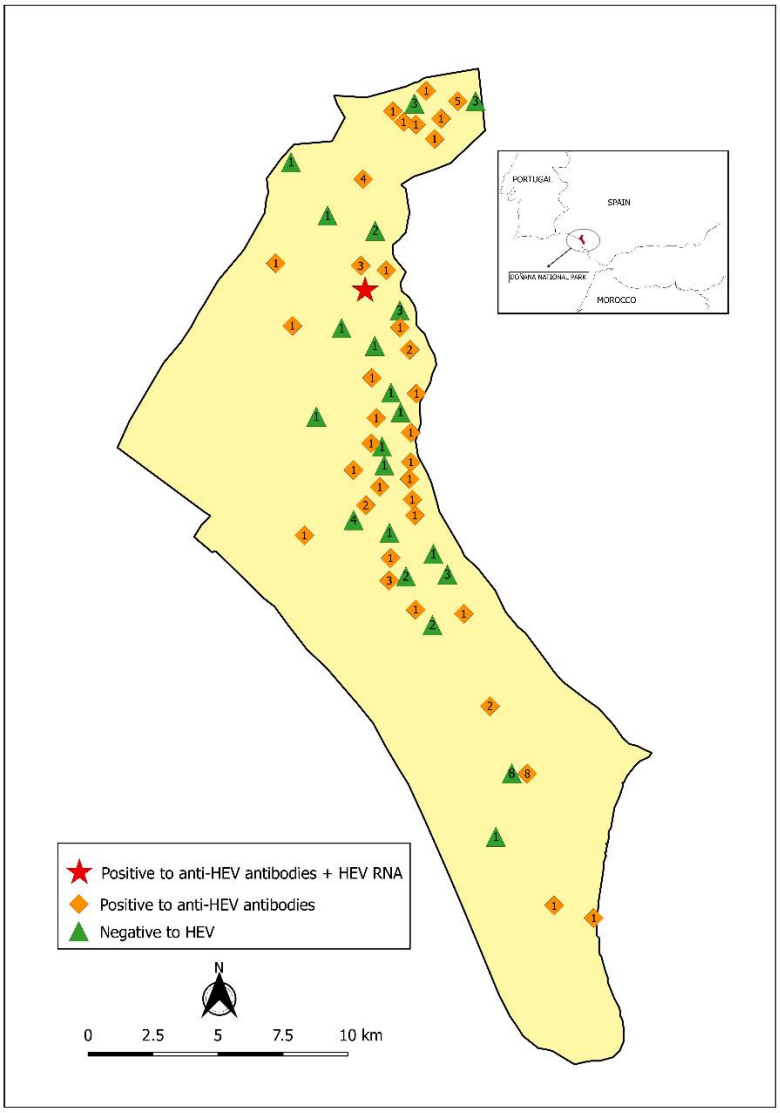

Figure 1. Seropositivity and HEV active infection in wild boar in the Doñana National Park (south-west Spain). Numbers within the symbols indicate the animals sampled at each sampling point.

Statistically significant differences between males (60.8%; 28/46) and females (53.8%; 28/52) were not found (P=0.310), which is consistent with previous observations (Rivero-Juárez et al., 2018; Weigand, Weigand, Schemmerer, Müller, & Wenzel, 2018). By contrast, the logistic regression model identified age as a risk factor associated with HEV exposure in wild boar. Significantly higher seropositivity was found among adults (68.5% (37/54); OR =

6.9; *P*<0.001; 95%CI: 2.3-20.4) and subadults (68.4% (13/19); OR = 6.9; *P*=0.005; 95%CI: 1.8-26.0) than in yearling animals (24.0% (6/19). This finding is consistent with other studies (Montagnaro et al., 2015; Thiry et al., 2015) and reflects the likelihood of cumulative exposure to HEV and lifelong persistence of antibodies in wild boar.

Only one young female of the 99 animals tested (1.0%) was positive for HEV RNA (Ct value: 28.3) (Figure 1), which indicates that there was limited circulation of HEV during the study period in the DNP. Low prevalence of active infection has also been reported in Belgium (5.8%; 4/69), the Netherlands (4.8%; 5/105), Northern Germany (5.3%; 10/189), Slovenia (0.4%; 1/288) and Sweden (5.2%; 7/134) (Kaci, Nöckler, & Johne, 2008; Rutjes et al., 2010; Roth et al., 2016; Thiry et al., 2017; Zele, Barry, Hakze-van der Honing, Vengust, & van der Poel, 2016). However, HEV RNA rates were higher in other European regions, such as East and West Germany (15.7%; 18/115) (Adlhoch et al., 2009) and Poland (25.8-11.6%; 42/163-19/163) (Dorn-In et al., 2016). Higher prevalence values have also been found in the south-central (10.1-23.2%; 16/158-33/142) and north-west (16.3%; 31/190) regions of Spain (de Deus et al., 2008; Rivero-Juárez et al., 2018; Wang et al., 2019). The considerable range in the duration of HEV viremia in swine species (between one and up to 16 weeks) (Schlosser, Vina-Rodriguez, Fast, Groschup, & Eiden, 2015; Salines, Andraud, & Rose, 2017), environmental conditions and the particular epidemiological context of the DNP are possible factors implicated in the low prevalence found in the present study. Further long-term studies are needed to assess HEV circulation in the DNP.

The sequenced isolate belongs to genotype 3, unassigned by Smith et al. (2016) but consistent with the proposed HEV subtype 3r (GenBank Accession Number: MH891834) according to the HEV genotyping tool (Figure 2). Although eleven subtypes (HEV-3a to 3j

and HEV-3ra) of HEV-3 were classified by Smith et al. (2016), molecular studies have recently identified novel strains in humans, domestic pigs and wild boar (Avellón, Muñoz-Chimeno, Arroyo, Morago, L., & Molina, 2018; De Sabato et al., 2018a,b). Analysis with the HEVnet genotyping tool showed minor phylo support of 99%, which indicates that the isolate belonged to the proposed HEV subtype 3r. This novel subtype was also recently found in the same species in north-eastern Spain (Wang et al., 2019). The strain detected in the present study is located in the same clade as other sequences isolated in humans in France and Spain in the last few years (GenBank Accession Number: KU17130, KU513561, MF444089 and MF444030), which indicates why it can be characterized as an emerging zoonotic subtype in Europe. Isolation of more sequences similar to this new proposed subtype will support the inclusion of this strain as a new subtype and facilitate its epidemiological investigation and traceability.

Figure 2. Molecular phylogenetic analysis used the maximum likelihood method, based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances

estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 156 nucleotide sequences. Sequences proposed by Smith et al., (2016) as reference sequences are indicated in the tree as Reference Sequence. Novel subtypes sequences proposed by HEVnet are referred in the tree as (p). All positions containing gaps and missing data were eliminated. There were a total of 649 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The accession number of wild boar isolate is MH891834.

In conclusion, the seropositivity obtained in the present study indicates widespread distribution of HEV in wild boar in the DNP. The results suggest a potential risk of zoonotic transmission of this novel HEV-3 subtype, which could be of public health concern. Further studies are required to assess the role of wild boar in the epidemiology of HEV-3r and to determine the infectivity of this emergent HEV subtype in other species.

Acknowledgements

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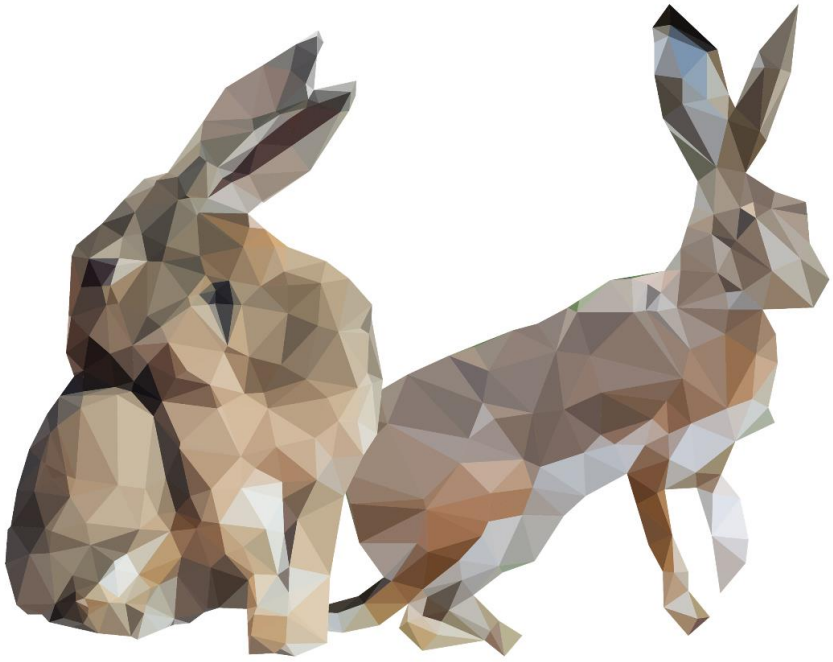
Chapter 2.2.

Absence of Hepatitis E virus circulation in wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) in Mediterranean ecosystems in Spain



Caballero-Gómez, J., García Bocanegra, I., Gómez-Guillamón, F., Camacho-Sillero, L., Zorrilla, I., López-López, P., Cano-Terriza, D., Jiménez-Ruiz, S., Frías, M., & Rivero-Juárez, A. (2020). **Absence of Hepatitis E virus circulation in wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) in Mediterranean ecosystems in Spain**. *Transboundary and Emerging Diseases*, *67*(4), 1422-1427.

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Abstract

In recent decades, cases of autochthonous hepatitis E (HE) have sharply increased in European countries where foodborne transmission is considered the main route of HE virus (HEV) transmission. Although rabbits are considered the main reservoir of the zoonotic HEV-3ra subtype, information on the role of wild lagomorphs in the epidemiology of HEV remains scarce. The aim of this study therefore was to assess the circulation of HEV in European wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*), the most important lagomorph species in Spanish Mediterranean ecosystems. Liver samples from 372 wild rabbits and 78 Iberian hares were analysed using a broad-spectrum RT-PCR that detects HEV genotypes 1 to 8. None of the 450 lagomorphs tested were positive for HEV infection. To the best of our knowledge, this is the first study to assess HEV circulation in wild rabbits in Spain and the first to evaluate HEV infection in Iberian hares. Our results indicate absence of HEV circulation in wild rabbits and Iberian hares in southern Spain during the study period, which suggests that the risk of transmission of HEV from wild lagomorphs to other species, including humans, is low.

Introduction

Hepatitis E virus (HEV; species *Orthohepevirus* A; genus *Orthohepevirus*; family *Hepeviridae*) is an emerging pathogen that is already considered the main cause of acute viral hepatitis worldwide (Kenney & Meng, 2019). This virus is composed of eight different genotypes (HEV-1 to HEV-8), of which HEV-3, HEV-4 and HEV-7 are zoonotic (Nimgaonkar, Ding, Schwartz, & Ploss, 2018). HEV-4 and HEV-7 are mainly distributed in Asia and the Middle East, respectively, whereas HEV-3 is spread worldwide, including Europe (Clemente-Casares, Ramos-Romero, Ramirez-Gonzalez, & Mas, 2016; Rasche et al., 2016; Sridhar, Teng, Chiu, Lau, & Woo, 2017). Even though domestic pigs and wild boar (*Sus scrofa*) are recognized as the main hosts of HEV-3, rabbits represent the major reservoir of the divergent but also zoonotic HEV-3ra subtype (Izopet et al., 2012; Doceul, Bagdassarian, Demange, & Pavio, 2016; Pavio, Doceul, Bagdassarian, & Johne, 2017).

Rabbits have been considered a useful model species for studying chronic HEV infection, extra-hepatic manifestations and HEV pathogenesis during pregnancy (Wang, Liu, & Wang, 2018; Kenney & Meng, 2019). In addition to HEV-3ra, this species has been shown to be susceptible to HEV-4 (Cheng et al., 2012) and more recently to wild boar-derived HEV-3 (Schlosser et al., 2019). Natural circulation of HEV-3-related strains has been detected in European rabbits (*Oryctolagus cuniculus*) and European brown hares (*Lepus europaeus*) in several European countries, including France, Germany, Italy, the UK and the Netherlands (Izopet et al., 2012; Caruso et al., 2015; Burt, Veltman, Hakze-van der Honing, Schmitt, & van der Poel, 2016; Hammerschmidt et al., 2017; Parisi et al., 2019). In Spain, the European wild rabbit and the Iberian hare (*Lepus granatensis*) are the most important lagomorph species in terms of abundance and hunting interest. About 5.3 million wild rabbits and 890 thousand

hares are harvested annually in this country (MAPA, 2019) and are commonly consumed without sanitary inspection, since hare is not usually intended for retail sale. Despite this, there is very little information on the role of wild lagomorphs in the epidemiology of HEV in Europe, and no studies have been carried out on these species in Spain. Here, we assess HEV circulation in European wild rabbits and Iberian hares in Mediterranean ecosystems in Spain.

Materials and Methods

A cross-sectional study was carried out in Andalusia (southern Spain; 36ºN–38º 60'N, 1º 75'W–7º 25'W). The study area represents different ecosystems of the Mediterranean habitat where wild rabbits and Iberian hares are keystone species, with different population densities, land use patterns and environmental characteristics. The sample size was calculated as 384 wild rabbits, assuming a prevalence of 50% (which provides the highest sample size in studies in which prevalence is unknown), with a 95% confidence level (95%CI) and a desired precision of ±5% (Thrusfield, 2001). Whenever possible, 49 rabbits were sampled in each province, in order to detect infection with a 95% probability, assuming a minimum within-province prevalence of 6%. Between 2013 and 2016, a total of 372 wild rabbits were sampled in 89 hunting areas distributed across the eight provinces of Andalusia (Figure 1). Liver samples were collected from all wild rabbits. In addition, 78 liver samples from Iberian hares were also collected in 2018 in 45 hunting areas in the same study region (Figure 1) using a convenience sampling. Information about each individual animal, including age, sex and location was gathered (Table 1). Liver samples were stored at -80°C until RNA extraction.

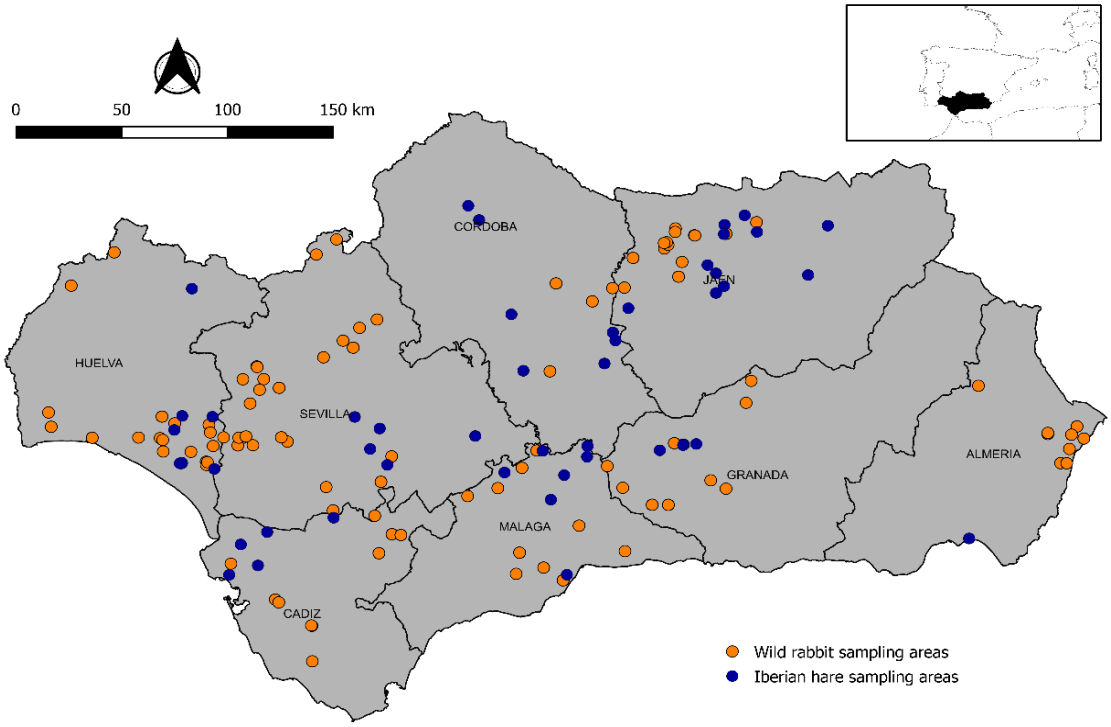

Figure 1. Geographical distribution of the sampling areas of European wild rabbits (orange dots) and Iberian hares (blue dots) for the presence of hepatitis E virus in Andalusia, southern Spain.

Table 1. Distribution of the samples by categories.

Variable	Categories	No. of wild rabbit samples* (relative frequency (%))	No. Iberian hare samples* (relative frequency (%))
Province	Almeria	53 (14.2)	2 (2.6)
	Cadiz	37 (9.9)	8 (10.3)
	Cordoba	22 (5.9)	23 (29.5)
	Granada	58 (15.6)	4 (5.1)
	Huelva	59 (15.9)	9 (11.5)
	Jaen	48 (12.9)	14 (17.9)
	Malaga	33 (8.9)	13 (16.7)
	Seville	62 (16.6)	5 (6.4)
Year	2013	14 (3.8)	-
	2014	176 (47.3)	-
	2015	171 (46.0)	-
	2016	11 (2.9)	-
	2018	-	78 (100)
Age	Young	41 (12.2)	3 (4.5)
	Subadult	34 (10.1)	17 (25.7)

	Adult	262 (77.7)	46 (69.7)
Sex	Female	148 (50.3)	31 (47.0)
	Male	146 (46.7)	35 (53.0)

Viral RNA was extracted from the wild rabbit liver samples with the Pure PCR Template Preparation Kit (Roche, Basel, Switzerland). The RNeasy Mini Kit (QIAgen, Hilden, Germany) was used for the Iberian hare liver samples, following automated procedures (QIAcube. QIAgen, Hilden, Germany). RNA was eluted in 50µL and stored at -80°C until analysis. For HEV RNA detection, a broad-spectrum real time RT-PCR (CFX Connect Real Time PCR System) was used, with the primers FWD (10µM) 5´-RGTRGTTTCTGGGGTGAC-3´ and RVS (10µM) 5´-AKGGRTTGGTTGGRTGA-3´ and the probe (15µM) 5´-FAM-TGAYTCYCARCCCTTCGC-TAMRA-3´. Both primers and probe were obtained by aligning all whole genome sequences of *Orthohepevirus* A species available in GenBank. The procedure was validated using the WHO Organization international reference panel for HEV RNA genotypes for nucleic acid amplification technique (NAT)-based assays (including genotypes HEV-1a, HEV-1e, HEV-2a, HEV-3b, HEV-3c, HEV-3e, HEV-3f, HEV-3ra, HEV-4c and HEV-4g) supplied by Paul-Ehrlich-Institute (code 8578/13) (Table S1). Reactions were conducted using 10µL of template and the QIAgen One step PCR Kit (QIAgen, Hilden, Germany). The thermal profile was 50°C for 30 min and 95°C for 15 min, followed by 45 cycles of 94°C for 10s, 51°C for 30s and 60°C for 20s.

Results and discussion

HEV is an emerging zoonotic pathogen that can cause up to 30% mortality in pregnant women or develop into chronic infection progressing to cirrhosis in immunocompromised humans (reviewed in Nimgaonkar et al., 2018). Autochthonous hepatitis E (HE) cases have

sharply increased in Europe, with more than 21,000 confirmed cases during the past decade (Aspinall et al., 2017). In Europe, foodborne transmission is considered the major route of HEV transmission to humans, although direct or indirect contact with infected animals has also been described as a zoonotic source of infection (EFSA, 2017; Faber, Askar, & Stark, 2018). In this context, HEV-3ra has been detected in both humans and rabbits (Izopet et al., 2012; Abravanel et al., 2017) and transmission of this subtype from rabbits to nonhuman primates has been experimentally confirmed (Liu et al., 2013). Moreover, there is evidence of occupational exposure to rabbits as a potential risk factor for HEV infection (Geng et al., 2019). These findings point to a potential risk of zoonotic transmission of this HEV subtype.

To the best of our knowledge, this is the first study to assess HEV circulation in wild rabbits in Spain and the first to evaluate HEV infection in Iberian hares. None of the 450 (0.0%; 95%CI: 0.00-0.82%) liver samples collected between 2013 and 2018 were positive for HEV RNA. Our results indicate the absence of HEV circulation in wild rabbit and Iberian hare populations in Mediterranean ecosystems in southern Spain during the study period. Absence of circulation or a low prevalence of HEV have also been found in wild rabbits in the UK (6.7%; 2/30) (Parisi et al., 2019), in farmed and laboratory domestic rabbits in China (1.3-4.8%; 5/396-16/332) (Xia et al., 2015; Wang, Zhang, Gong, Song, & Wang, 2016), in farmed and pet rabbits in Canada (0.9-4.8%; 1/114-3/63) (Xie, Bil, Shantz, Hammermueller, Nagy, & Turner, 2017) and Italy (0.0%; 0/7-0/122) (Di Bartolo et al., 2016) and in farmed rabbits in France (7.0%; 14/200) (Izopet et al., 2012) and the Netherlands (0.0%; 0/10) (Burt et al., 2016). By contrast, other studies have observed higher HEV RNA rates (reviewed in Wang et al., 2018), such as those detected in wild rabbits in Germany (17.1-25.0%; 28/264-18/72) (Hammerschmidt et al., 2017; Ryll et al., 2018), Italy (11.4%; 4/35) (Parisi et al., 2019), and

France (23.0%; 47/205) (Izopet et al., 2012) and in petting farm and wild rabbits in the Netherlands (22.9-37.1%; 8/35-23/62) (Burt et al., 2016). Despite the differences in HEV prevalence rates among European countries, human HEV-3ra cases have already been documented in Belgium (Suin et al., 2019), France (Izopet et al., 2012; Abravanel et al., 2017) and Switzerland (Sahli, Fraga, Semela, Moradpour, & Gouttenoire, 2019). Although HEV-3ra infection was not found in the present study and this subtype has not been detected in Spain so far, further surveillance studies should nevertheless be conducted to determine the risk of HEV-3ra infection in humans in this country.

The absence of HEV infection in Iberian hares is consistent with observations of European brown hares in Germany (0.00-0.04%; 0/669-1/2389) (Hammerschmidt et al., 2017; Corman et al., 2019) and Italy (0.0%; 0/47) (Serracca et al., 2015) and of black-tailed jackrabbits (*Lepus californicus*) in China (0.0%; 0/69) (Xia et al., 2015). These results suggest that hares play a limited role in the epidemiology of HEV. Nevertheless, since other hare species, such as the European brown hare and the broom hare (*Lepus castroviejoi*), are also present in the Iberian Peninsula, but not in the study area, additional studies on these wild lagomorph species should be carried out to determine their role in the epidemiology of HEV in Spain.

HEV-3 is endemic in the study area and has been detected in domestic and wild mammals, including domestic pigs, horses, wild boar and red deer (de Deus et al., 2008; Boadella et al., 2010; Jiménez de Oya et al., 2011; Kukielka, Rodríguez-Prieto, Vicente, & Sánchez-Vizcaíno, 2016; García-Bocanegra et al., 2019). Neither of the two lagomorph species analysed was positive for human/swine HEV-3 active infection. Further serosurvey studies are required to assess HEV exposure in the wild lagomorph populations in Spain. Our results are

consistent with previous experimental studies that failed to infect rabbits with human-derived HEV-3 strains (Cheng et al., 2012; Zhang et al., 2017), although rabbits were recently successfully infected with wild boar-derived HEV-3 strains (Schlosser et al., 2019), and pig-related strains of HEV-3 have also been detected in naturally infected wild rabbits in Germany and Italy (Hammerschmidt et al., 2017; Parisi et al., 2019). Nevertheless, there is no evidence of human HE cases linked to rabbits caused by subtypes different from HEV-3ra, so that the risk of zoonotic transmission of these strains appears to be limited.

In conclusion, our results indicate the absence of HEV circulation in European wild rabbit and Iberian hare populations during the study period and suggest a limited risk of transmission of HEV from these wild lagomorph species to other mammals, including humans, in Mediterranean ecosystems in Spain.

Supplementary Material

For HEV RNA detection, a broad-spectrum real time RT-PCR (CFX Connect Real Time PCR System) was used with primers (FWD (10µM) 5´-GTRGTTTCTGGGGTGAC-3´ and RVS (10µM) 5´-AKGGRTTGGTTGGRTGA-3´) and probe ((15µM) 5´-FAM-TGAYTCYCARCCCTTCGC-TAMRA-3´) designed by our research group. Conditions for a standard reaction are summarized in Table S1A.

Table S1A. Reagents and volume for a standard reaction

Reactive	Volume/reaction (µl)
FWD (10µM)	1
RVS (10µM)	1
Probe (15µM)	1
dNTPs	2
Buffer 5X	10
Enzyme	2

H_2O	8
Template	25
Total	50

To optimize the assay, PEI 6329/10 strain (genotype 3a) was used as template at different concentration levels using 16 replicates per condition (Table S1B). Moreover, the limit of detection (LOD) for each template was also calculated using probit analysis (Table S1C).

Table S1B. Optimization of template volume using PEI 6329/10 (16 replicates).

Concentration (IU/mL)	**Template quantity**				
	5 µl	**10 µl**	**15 µl**	**20 µl**	**25 µl**
976.5	16/16	16/16	16/16	16/16	16/16
488.2	15/16	16/16	16/16	16/16	16/16
244.1	10/16	16/16	16/16	16/16	16/16
122	10/16	16/16	16/16	16/16	16/16
61	9/16	13/16	15/16	16/16	16/16
30.5	4/16	5/16	4/16	13/16	16/16
15.25	3/16	3/16	5/16	9/16	11/16
7.62	0/16	0/16	0/16	3/16	5/16
3.81	0/16	0/16	0/16	0/16	1/16

Table S1C. Limit of detection for each template.

Template quantity	**Limit of detection (95% CI)**
5 µl	439.34 (285.34-1,225.13)
10 µl	74.16 (60.8-101.09)
15 µl	64.90 (53.1-88.53)
20 µl	35.78 (28.83-51.39)
25 µl	21.86 (17.38-34.30)

Following the standard conditions, the sensitivity of our assay was assessed using the WHO reference panel for HEV RNA genotypes (genotypes HEV-1a, HEV-1e, HEV-2a, HEV-3b, HEV-3c, HEV-3e, HEV-3f, HEV-3ra, HEV-4c and HEV-4g) and compared with the results obtained from the 24 laboratories designated by WHO for HEV detection (Baylis et al., 2015) (Table S1D).

Table S1D. Sensitivity for the WHO reference panel HEV genotypes (PEI8678/13).

Genotype	Source	Cq mean	VL of our assay	Mean VL obtained by the WHO designated labs (95% CI)
1a	Plasma	37.22	520	436 (465-1,148)
1a	Fecal	32.12	42,720	17,782 (7,413-42,657)
1e	Stool	35.51	2,320	1,778 (812-3,890)
2a	Faecal	29,17	1,524	263,026 (125,892-549,540)
3b	Plasma	32.60	28,240	15,848 (13,182-19,054)
3c	Plasma	36.02	1,480	2,511 (1,862-3,388)
3e	Plasma	36.06	1,440	3,162 (2,290-4,365)
3f	Plasma	33.99	8,520	6,918 (3,981-11,748)
3ra	Faecal	29.29	448,840	95,499 (53,703-173,780)
4c	Plasma	33.32	15,200	11,748 (6,606-20,417)
4g	Plasma	35.57	2,200	5,888 (3,090-10,964)

VL: Viral load (IU/ml)

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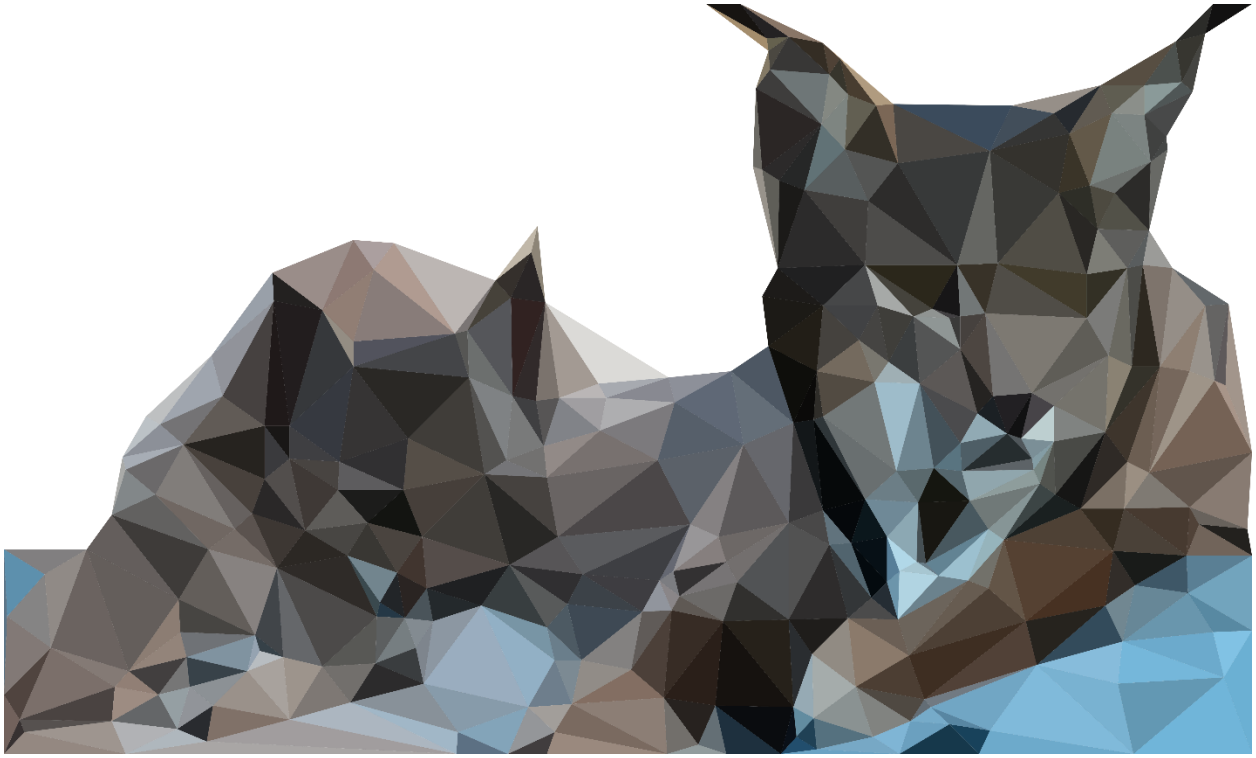
Chapter 2.3.

Hepatitis E virus in the endangered Iberian lynx (*Lynx pardinus*)



Caballero-Gómez, J., Rivero-Juárez, A., Zorrilla, I., López, G., Nájera, F., Ulrich, RG., Ruiz-Rubio, C., Salcedo, J., Rivero, A., Paniagua, J., García-Bocanegra, I. **Hepatitis E virus in the endangered Iberian lynx (*Lynx pardinus*)**. Transboundary and Emerging Diseases. *Under review*.

Abstract

Hepatitis E virus (HEV) is an emerging zoonotic pathogen in Europe. In the Iberian Peninsula, wild boar (*Sus scrofa*) is considered the main wildlife reservoir of HEV. This wild ungulate shares habitat and resources with other potential HEV carriers in Iberian Mediterranean ecosystems, although information about the role of such sympatric species in the HEV epidemiological cycle is still very limited. The aims of the present large-scale, long-term study were: (1) to determine the seroprevalence and prevalence of HEV in both free-living and captive populations of the Iberian lynx (*Lynx pardinus*), the most endangered felid in the world; (2) to determine potential risk factors associated with HEV exposure in this species; and (3) to evaluate the dynamics of seropositivity in longitudinally sampled animals during the study period. Between 2010 and 2021, serum samples from 275 Iberian lynxes were collected in free-ranging and captive populations across the Iberian Peninsula. Forty-four of the 275 lynxes were also longitudinally sampled during the study period. A double-antigen sandwich ELISA was used to test for the presence of antibodies against HEV. A subset of seropositive samples was analyzed by western blot assay (WB) to confirm exposure to HEV. In addition, serum, liver and/or faecal samples from 367 individuals were tested for orthohepevirus RNA by RT-PCR. A total of 50 (18.2%; 95% CI: 14.1-23.2) of the 275 animals analyzed had anti-HEV antibodies by ELISA. Exposure to HEV was confirmed by WB in most of the ELISA-positive Iberian lynxes analysed. Significantly higher seroprevalence was found in captive (33.6%) compared to free-ranging (7.4%) individuals. Within captive population, the GEE model identified “age” (senile, adult and subadult) as a risk factor potentially associated with HEV exposure in the Iberian lynx. Thirteen (29.5%) of 44 longitudinally surveyed individuals seroconverted against HEV during the study period. HEV RNA was detected in the faeces of one (1/364; 0.3%; 95% CI: 0.0-0.8) free-ranging adult animal sampled in 2021. Phylogenetic

analysis showed that the sequenced strain belongs to HEV-3f subtype and shared a high nucleotide sequence identity (97-99.6%) with human HEV-3f sequences from Spain and France. To the best of the authors' knowledge, this is the first survey study on HEV in the Iberian lynx and the first molecular report of HEV-A infection in free-ranging felines. Our results indicate high exposure to HEV-3 in Iberian lynx populations, particularly those kept in captivity. The serological results suggest widespread but not homogeneous circulation of HEV in Iberian lynx populations. Further studies are required to assess the epidemiological role of this endangered species as a potential spillover host of HEV.

Keywords: *Hepatitis E, HEV-3f, Iberian lynx, risk factors, epidemiology.*

Introduction

Hepatitis E virus (HEV; family *Hepeviridae*; genus *Orthohepevirus*) is an important emerging and zoonotic pathogen currently considered the main cause of acute viral human hepatitis worldwide (Lhomme et al., 2019). At present, four species of *Orthohepevirus* (henceforth *HEV-A* to *HEV-D*) have been confirmed, with HEV-A, particularly genotypes HEV-1 to HEV-4 of this species, being the most important in terms of public health concern. Although the domestic pig and wild boar (*Sus scrofa*) are the primary reservoirs (Pavio et al., 2017), susceptibility to HEV-A infection has been confirmed in an expanding range of mammal species (Meng, 2016; Sarchese et al., 2021), whose role in the epidemiology of this virus is still poorly understood.

The Iberian lynx (*Lynx pardinus*) is the most endangered felid species in the world (Nowell & Jackson, 1996) and one of the most endangered carnivores in Europe (IUCN, 2021). Iberian lynx populations declined drastically during the last decades of the twentieth century, and it was estimated that there were only around 100 individuals in 2002, distributed in two isolated areas of Andalusia (southern Spain) (Simón et al., 2012). The decline was associated with a reduction in the numbers of its staple prey, the European wild rabbit (*Oryctolagus cuniculus*), habitat destruction, illegal trapping and hunting, road kills and infectious diseases (Ferreras et al., 1992, 2001; López et al., 2014; Rodríguez & Delibes, 2004). Over the last two decades, clinical cases and mortalities in this endangered species have been reported as due to feline leukemia virus, Suid alphaherpesvirus 1 and *Mycobacterium bovis* infections, among others (Briones et al., 2000; Masot et al., 2016; Meli et al., 2010). Since 2000, a number of projects, including EU LIFE-Nature projects, have been launched to save the Iberian lynx from extinction, focusing on *in situ* and also *ex situ* conservation programs (Vargas et al., 2008). As

a result, the Iberian lynx census has soared during the last decade, reaching more than 1,100 free-ranging individuals in 2020 (MITECO, 2021). The monitoring of pathogens that could affect the Iberian lynx in the two different epidemiological habitats in which it is found (in captivity and in the wild) is a key component of the conservation programs of this endangered species, and health surveillance programs are being conducted in both free-living and captive populations (Nájera et al., 2021; Rivas et al., 2016). Nevertheless, information about the susceptibility of the Iberian lynx to pathogens that are not monitored but highly prevalent in their habitat, which may be important in terms of conservation and animal and public health, is still very limited.

In the Iberian Mediterranean ecosystems where the Iberian lynx is distributed, different studies have confirmed HEV-3 infection in extensively raised Iberian pig, a breed of domestic pig native to the Iberian Peninsula, wild boar (seroprevalence ranging from 5.2% to 57.6%), red deer (*Cervus elaphus*) (seroprevalence ranging from 10.2 to 12.9%) and horses (Caballero-Gómez et al., 2019; García-Bocanegra et al., 2019; Kukielka et al., 2015; López-López et al., 2018; Risalde et al., 2017). In addition, HEV exposure has been reported in other sympatric species that could act as potential reservoirs or spillover hosts, including wild rabbits (4.1%) and extensively raised goats (8.9%) and sheep (2.2%) (Boadella et al., 2010; Caballero-Gómez et al., 2022; Lopes & Abrantes, 2020; Kukielka et al., 2015). However, there is no information about the susceptibility of the Iberian lynx to HEV infection and its possible role in the transmission of the virus in these ecosystems. HEV infection or exposure has so far been detected in several felid species, including the Eurasian lynx (*Lynx lynx*), and the captive clouded (*Neofelis nebulosa*), Persian (*Panthera pardus saxicolor*) and snow leopards (*Uncia uncia*), as well as in domestic pet and stray cats (Caballero-Gómez et al., 2021; Song et al.,

2013; Spahr et al., 2017a; Zhang et al., 2007). Our hypothesis is that free-living and captive Iberian lynx populations may be exposed to HEV, in which case, this species could play a role in the epidemiology of this emerging virus. The objectives of the present large-scale, long-term study were therefore: (1) to determine the seroprevalence and prevalence of HEV in Iberian lynx populations, (2) to determine potential risk factors associated with HEV exposure in this species and (3) to evaluate the dynamics of seropositivity in longitudinally sampled animals during the study period.

Material and methods

Sampling

Blood samples were collected from 275 Iberian lynxes across the Iberian Peninsula between 2010 and 2021. Thus, a total of 162 were free-ranging animals in the three areas where the Iberian lynx population is already distributed (central, south and southwest Spain). In addition, 113 lynxes kept in captivity, including 106 from the four captive breeding centres (BC1-BC4) belonging to the Iberian lynx *ex-situ* conservation program and seven from four zoological parks/conservation centres (ZC1-ZC4), were sampled. A total of 44 (including 10 free-ranging, 26 captive and eight animals translocated from captivity to free-range areas or vice versa) of the 275 sampled individuals were also longitudinally surveyed (between two and four samplings per animal) during the study period. During follow-up, the median (Q1-Q3) interval between consecutive samplings was 48 months (24-60). Sera were obtained by blood centrifugation at 400x *g* for 15 min and stored at -80°C until laboratory analysis. Between 2017 and 2021, liver and/or faecal samples from 176 Iberian lynxes (including 131 free-ranging and 45 captive individuals) were also collected and stored at -20ºC until molecular analysis. Of

these, both liver and faecal samples were collected from fifty-one animals (46 free-ranging and five captive Iberian lynxes).

All available samples were used in the present study. Serum, liver and faecal samples collected from individuals subjected to health programs, medical check-ups or necropsy during the study period were obtained from serum/tissue banks at the Center for Analysis and Diagnosis of Wildlife (CAD, Andalusia, southern Spain). This study did not involve the intentional killing of animals. Iberian lynxes were sampled by authorized veterinarians and animal keepers following routine procedures on live and dead individuals before the design of this study, in compliance with Ethical Principles in Animal Research. Samples from a representative number of free-ranging individuals and almost all the captive population were analyzed in the present study.

Whenever possible, epidemiological information about each individual animal was recorded, including age (yearlings: <1 year old; subadults: 1 to 3 years old; adults: 3 to 10 years old; senile: >10 years old), sex, habitat status (free-ranging vs captivity), origin (free-range area, breeding centre, zoological park/conservation centre), sampling date and georeferenced location.

Serological analysis

The presence of total antibodies against HEV was assessed using a commercial double-antigen sandwich multi-species ELISA (HEV 4.0v; MP Diagnostics, Illkirch, France) following the manufacturer's instructions. This assay is based on the highly conserved recombinant protein ET2.1 of the HEV capsid (Hu et al., 2008), and detects anti-HEV antibodies in serum or plasma in a wide range of animal species, including felids such as cats

(Caballero-Gómez et al., 2021), the Eurasian lynx and the serval (*Leptailurus serval*) (unpublished data), in which the presence of anti-HEV antibodies in ELISA-positive animals was also confirmed by Western Blot assay (WB).

Serum samples from 34 Iberian lynx were randomly selected for WB analyses in order to confirm exposure to either HEV-A and/or HEV-C in Iberian lynx populations (Kubickova et al., 2021). Carboxy-terminal segments of the capsid proteins of HEV-3 and rat HEV-C1 and a nucleocapsid protein derivative (amino acid residues 1-39/213-433) of the *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., 2011; Lundkvist et al., 2002). Purified proteins were run in 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane and analyzed for control by anti-His tag and HEV capsid protein cross-reactive monoclonal antibodies (Merck, Darmstadt, Germany; Kubickova et al., 2021, Supplementary Figure 1). Serum samples were diluted 1:100 in 5% skimmed milk in phosphate-buffered saline (PBS)-0.1% Tween 20 (PBS-T) and the antigen–antibody reaction was detected by adding purified recombinant protein A/G conjugated with horse-radish peroxidase (HRP) (Thermo Scientific, Schwerte, Germany), diluted 1:50,000 in 5% PBS-T. The immunoreaction was detected using Clarity Western ECL Substrate (Biorad, Feldkirchen, Germany) and documented in a VersaDoc 4000MP system (Bio-Rad) with an exposure time between one and 60 seconds.

Molecular analysis

For the molecular analysis, RNA from serum (number of samples analyzed (n)=248 from 220 individuals), liver (n= 158) and faecal (n= 73) samples was extracted using the

QIAmp MinElute Virus Spin, RNeasy Mini, and QIAamp cador Pathogen Mini Kits (QIAGEN, Hilden, Germany), respectively, whenever possible. Seropositive sera as well as liver and stool samples were individually extracted whereas seronegative serum samples were randomly subjected to a pool testing approach (pools of eight samples; total volume: 400µl) and extracted. The sensitivity of RT-PCR in pools was set at 670 IU/mL (Rivero-Juárez et al., 2019).

The presence of HEV RNA was assessed using two RT-PCR assays in parallel. A real-time RT-PCR (CFX Connect Real Time PCR System), capable of detecting all HEV-A genotypes was performed using 10µl of RNA template and the QIAGEN One-Step RT-PCR kit as previously described (Frias et al., 2021). Briefly, the primers employed were FWD 5′-RGTRGTTTCTGGGGTGAC-3′ and RVS 5′-AKGGRTTGGTTGGRTGA-3′ and the probe was 5′-FAM-TGAYTCYCARCCCTTCGC-TAMRA-3′. In addition, a nested broad-spectrum RT-PCR (Fisher Scientific Applied Biosystems SimpliAmp™) able to detect HEV-A, HEV-B and HEV-C strains was performed using the QIAGEN One-Step RT-PCR kit and HEV-cs and HEV-cas primers for the first round, and the premixed 2X solution containing Taq DNA Polymerase, dNTPs and Reaction Buffer (Promega, Madison, WI, USA) and HEV-csn and HEV-casn primers for the second round (Johne et al., 2010). The amplicons of the nested RT-PCR were examined on 1.5% agarose gels stained with RedSafe™ Nucleic Acid Staining solution (iNtRON Biotechnology, Seongnam, Korea). Positive samples using this assay were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The consensus sequence was obtained using SeqMan Software NGen® Version 12.0 (DNASTAR. Madison, WI, USA). Subtype assignment and phylogenetic analyses were performed using the HEVnet

genotyping too1 (Mulder et al., 2019) and confirmed by Basic Local Alignment Search Tool (BLASTn). Sequence alignments were generated by the MAFFT online service (https://mafft.cbrc.jp/alignment/server/): multiple sequence alignment, interactive sequence choice and visualization. Phylogenetic trees were reconstructed with the maximum likelihood method using the proposed HEV-A genotype/subtype standard reference strains (Smith et al., 2016, 2020). The final tree was obtained with MEGA Software (Version 10) using the bootstrap method (with 1000 replicates).

Statistical analyses

Seroprevalence and prevalence were estimated by dividing the number of positive or seropositive animals by the total number of animals tested, using two-sided exact binomial 95% confidence intervals (95% CI). Associations between the presence of anti-HEV antibodies and the variable "habitat status" were analyzed using Pearson's chi-square test. To avoid a possible collinearity bias, free-ranging and captive populations were tested separately. Associations between the presence of anti-HEV antibodies and explanatory variables (age, sex and sampling period (categorized by terciles in each population)) were analyzed using Pearson's chi-square test or Fisher's exact test, as appropriate. Variables with $P<0.10$ in bivariate analyses were selected for inclusion in the multivariate analyses. Collinearity between pairs of variables was tested using Spearman's Rho test. Finally, a generalized estimating equation model (GEE) was used to assess the effect of the variables selected in bivariate analysis. "Origin" was included as a random factor, and the number of seropositive animals was assumed to follow a binomial distribution. A manual forward stepwise approach was used, starting with the variable with the lowest *P*-value in bivariate analysis. At each step, the confounding effect of the included variable was assessed by computing change in odds

ratios (OR) greater than 30%. Variables 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).

Results

A total of 50 (18.2%; 95% CI: 14.1-23.2) of 275 Iberian lynxes sampled had antibodies against HEV. Seroprevalence was significantly higher in captive (38/113; 33.6%; CI 95%: 25.6-42.8) compared to free-ranging (12/162; 7.4%; CI 95%: 4.3-12.5) individuals ($P<0.001$; Relative Risk: 6.3; CI 95%: 3.1-12.8). The frequency of anti-HEV antibodies in free-ranging and captive populations by age, sex, and sampling periods is shown in Table 1. The GEE model identified "age" as a risk factor potentially associated with HEV exposure in captive Iberian lynxes (Table 2). The seroprevalence was significantly higher in captive senile, adult and subadult individuals compared to captive yearling animals (50.0%, 42.9% and 22.7% *vs*. 5.6%; $P=0.005$, $P=0.001$ and $P=0.049$). Exposure to HEV was detected in two yearling animals sampled in 2017 and 2021: one captive three months-old and one free-ranging ten months-old lynxes, respectively. Antibodies against HEV-3 were confirmed by WB in 29 (85.3%) of 34 ELISA-positive animals, and 21 of these also reacted against rat HEV-C1 antigen (Supplementary Figure 1).

The distribution of seroprevalences by origin is shown in Table 3 and Figure 1. Seropositive Iberian lynx were detected in the three free-ranging areas sampled, with frequencies ranging from 5.5% in the south to 11.6% in southwest Spain. Animals with anti-HEV antibodies were also found in the four captive breeding centres, with within-centre rates ranging between 11.1% in BC2 and 51.4% in BC4. Statistically significant differences were

observed between captive breeding centres (P=0.019) but not between free-living areas (P=0.466) (Table 3). Two Iberian lynxes from zoological parks/conservation centres, both from the same zoo (ZC2), were also found to be seropositive to HEV.

Seropositive Iberian lynxes were observed in all years of the study period except for 2011, since no animals were sampled. Similar seroprevalence was found among sampling periods in free-ranging Iberian lynxes (9.5% in 2010-2016, 5.7% in 2017-2018 and 6.5% in 2019-2021), whereas fluctuations were observed in captive animals (50.0% in 2010-2014, 23.7% in 2015-2016 and 31.1% in 2017-2020) (Table 1). "Sampling period" was selected from bivariate analysis in the captive population but was identified as a confounding variable of "age" and removed from the final GEE model. Of the 44 longitudinally surveyed animals, 15 (34.1%) tested positive by ELISA at each sampling in the study period (Table 4) and 15 animals also remained seronegative at the different samplings. Of note, 13 (29.5%) of the 44 individuals seroconverted against HEV during the study period. In addition, seroreversion was detected in two lynxes; the sampling intervals between the seropositive and first seronegative sampling in these animals ranged between six and seven years.

Table 1. Distribution of HEV seroprevalence in free-ranging and captive Iberian lynx populations and results of bivariate analysis.

	Free-ranging				Captive			
Variable	**Categories**	**No. Positives/ No. Analyzed***	**Seroprevalence (%) (CI95%)**	***P*-value**	**Categories**	**No. Positives/ No. Analyzed***	**Seroprevalence (%) (CI95%)**	***P*-value**
Age	Yearling	1/27	3.7 (0.0-10.8)	0.269	Yearling	1/18	5.6 (0.0-16.1)	0.011
	Subadult	3/61	4.9 (0.0-10.3)		Subadult	5/22	22.7 (5.2-40.2)	
	Adult	6/59	10.2 (2.5-17.9)		Adult	27/63	42.9 (30.6-55.1)	
	Senile	2/10	20.0 (0.0-44.8)		Senile	5/10	50.0 (19.0-81.0)	
Sex	Female	7/81	8.6 (2.5-14.8)	0.313	Female	22/61	36.1 (24.0-48.1)	0.294
	Male	4/75	5.3 (0.3-10.4)		Male	15/51	29.4 (16.9-41.9)	
Sampling period	2010-2016**	6/63	9.5 (2.3-16.8)	0.705	2010-2014**	15/30	50.0 (32.1-67.9)	0.067
	2017-2018	3/53	5.7 (0.0-11.9)		2015-2016	9/38	23.7 (10.2-37.2)	
	2019-2021	3/46	6.5 (0.0-13.7)		2017-2020	14/45	31.1 (17.5-44.6)	

*Missing values excluded.; **No animals were sampled in 2011

Table 2. Results of the generalized estimating equation analysis of potential risk factors associated with HEV exposure in the Iberian lynx.

Variable	**Categories**	***β***	***P***	**OR (95%CI)**
Age	Senile	2.693	0.005	14.8 (2.2-98.5)
	Adult	2.348	0.001	10.5 (2.5-43.0)
	Subadult	1.499	0.049	4.5 (1.0-19.9)
	Yearling	[a]	[a]	[a]

[a]Reference category; OR, odds ratio; CI, confidence interval

Table 3. Distribution of HEV seroprevalence in Iberian lynx by origin and results of bivariate analysis.

Variable	Categories	No. Positives/No. Analyzed	Seroprevalence (%) (CI95%)	*P*-value
Free-range areas	Central Spain	3/53	5.7 (0.0-11.9)	0.466
	South Spain	4/66	6.1 (0.3-11.8)	
	Southwest Spain	5/43	11.6 (2.1-21.2)	
Breeding centres	BC1	10/31	32.3 (15.8-48.7)	0.019
	BC2	2/18	11.1 (0.0-25.6)	
	BC3	5/20	25.0 (6.0-44.0)	
	BC4	19/37	51.4 (35.3-67.5)	
Zoological parks/ Conservation centres	ZC1	0/2	0.0 (0.0-84.2)	NA*
	ZC2	2/2	100.0 (15.8-100.0)	
	ZC3	0/1	0.0 (0.0-97.5)	
	ZC4	0/2	0.0 (0.0-84.2)	

NA* Not analyzed because of the low number of individuals sampled.

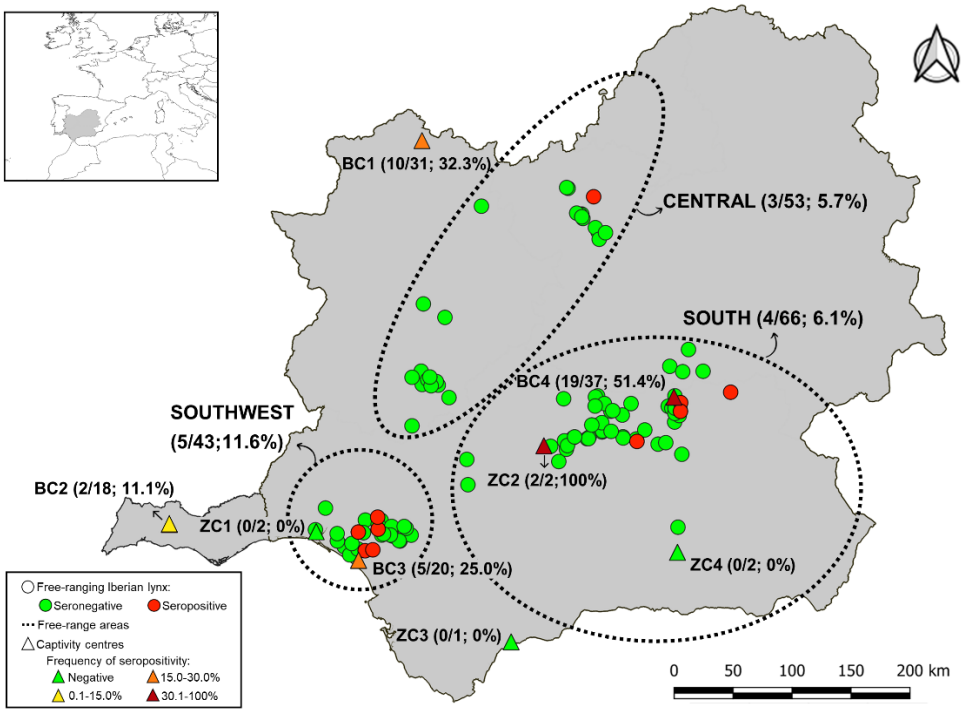


Figure 1. Spatial distribution and serological results of Iberian lynxes sampled in the Iberian Peninsula. The HEV seroprevalence, numbers of seropositive and total number of animals analysed in each free-range area and captivity centre are shown in brackets. The abbreviations

"BC" and "ZC" refer to Breeding centres and Zoological parks/ Conservation centres, respectively.

Table 4. Exposure to HEV in longitudinally sampled animals determined by ELISA. When two samplings were carried out in the same year, a superscript indicates the number of interval months.

ID	Life condition	Origin	2010	2012	2013	2014	2015	2016	2017	2018	2019	2020
157	Free-ranging	South Spain	-	-	-	-	-	Pos	-	Pos	-	-
158	Free-ranging	South Spain	Pos	-	-	-	-	Neg	-	Neg	-	-
319	Free-ranging	South Spain	Neg	-	-	-	Neg	-	-	-	-	-
307	Free-ranging	South Spain/Southwest Spain	-	-	-	-	Neg	Neg	-	-	-	-
113	Free-ranging	South Spain	-	-	-	-	-	-	-	-	Neg	Neg
142	Free-ranging	South Spain	-	-	-	-	-	-	-	Neg	-	Neg
270	Free-ranging	South Spain	-	-	-	-	-	-	Neg	-	-	Neg
70	Free-ranging	Central Spain	-	-	-	-	-	-	-	Neg	Neg	-
239	Free-ranging	Central Spain	-	-	-	-	-	-	-	Neg	Neg	-
228	Free-ranging	Central Spain	-	-	-	-	-	-	Neg	-	Neg	-
145	Free-ranging/ Captivity	South Spain/BC1	-	-	-	-	-	-	-	Neg	-	Pos
337	Free-ranging/Captivity	South Spain/BC4	-	Neg	-	-	-	Pos	Pos	-	-	-
171	Free-ranging/Captivity	Central Spain/BC3	Pos	-	-	-	-	Pos	-	-	-	-
180	Captivity/Free-ranging	BC3/Central Spain	-	-	-	-	Neg	-	-	Pos	-	-
458	Captivity/Free-ranging	BC3/Central Spain	-	-	-	-	Neg	-	Neg	-	-	-
308	Captivity/Free-ranging	BC4/South Spain	-	-	-	-	-	Neg	-	-	-	Neg
267	Captivity/Free-ranging	BC2/South Spain	-	-	Neg	-	-	-	Neg	-	-	-
475	Captivity/Free-ranging	BC2/South Spain	-	-	Neg	-	-	-	Pos	-	-	-
432	Captivity	ZC2	Pos	-	-	-	-	-	Neg	-	-	Pos; Pos[7]
496	Captivity	ZC2	Pos	-	Pos	-	Pos	-	-	-	-	-
264	Captivity	BC3	-	-	-	-	-	Pos	Pos	-	-	-
254	Captivity	BC3/BC4	-	Neg	-	-	-	-	Pos	-	-	-
34	Captivity	BC4	-	Neg	-	-	-	-	Neg	-	-	-
369	Captivity	BC4	Pos	-	-	-	Pos	-	-	-	-	-
335	Captivity	BC4	Pos	-	-	-	-	-	Pos	-	-	-
568	Captivity	BC4	Pos	-	-	-	Pos	-	-	-	-	-
431	Captivity	BC4	Pos	-	-	-	Pos	-	Pos	-	-	-
259	Captivity	BC4	Neg	-	-	-	Pos	-	Pos	-	-	-
392	Captivity	BC4	Pos	Pos	-	-	-	-	Pos	-	-	-
465	Captivity	BC4	Pos	-	-	-	-	-	Pos	-	-	-
470	Captivity	BC4	Pos	-	-	-	Pos	-	-	-	-	-

HEV RNA was detected in one (0.3%; 95% CI: 0.0-0.8) of the 364 individuals tested by RT-PCR. One of 73 stool (individual prevalence: 1/73; 1.4%; 95% CI: 0.0-4.0) samples tested positive for HEV RNA. None of the 248 serum (individual prevalence: 0/220; 0.0%; 95% CI: 0.0-1.5) or 158 liver (0.0%; 95% CI: 0.0-1.9) samples were positive for active HEV infection (Figure 2). The infected Iberian lynx, in which both liver (negative) and faeces (positive) were analyzed, was an adult male sampled in the free-range area of southwestern Spain in 2021 (Figure 2). The sequenced ORF2 fragment of 285 base pairs belongs to genotype 3, subtype 3f (GenBank Accession Number: OL840902). BLAST analysis showed high nucleotide sequence identity (97-99.6%) with human HEV-3f sequences obtained from the same Spanish study area (GenBank Accession Numbers: OL741651, OL741653 and OL746155) and France (Figure 3).

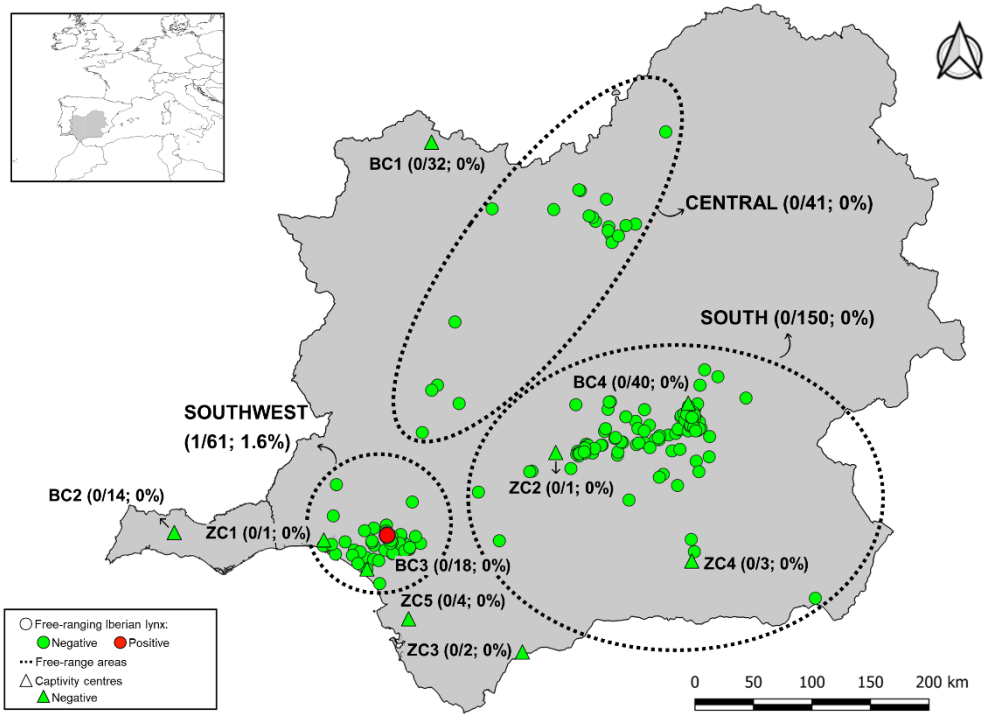


Figure 2. Spatial distribution and molecular results of Iberian lynx sampled in the Iberian Peninsula. Prevalence of HEV infection, number of RNA positive and total number of animals analysed in each free-range area and captivity centre are shown in brackets. The abbreviations

"BC" and "ZC" refer to Breeding centres and Zoological parks/ Conservation centres, respectively.

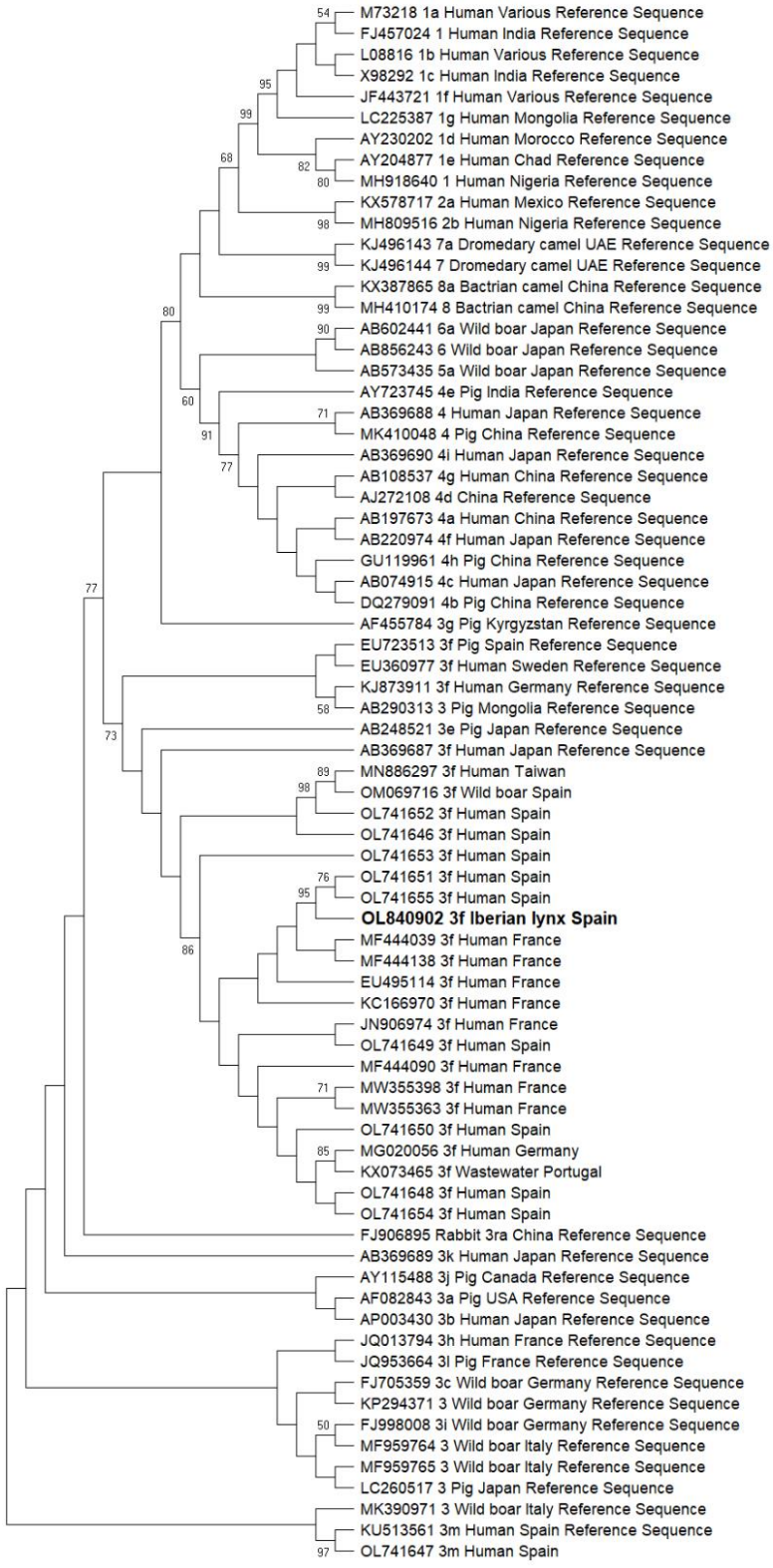


Figure 3. Phylogenetic tree constructed by the maximum likelihood method and Kimura 2-parameter model. The bootstrap consensus tree was inferred from 1,000 replicates and used to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches when values were larger than 70. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 74 nucleotide sequences. Sequences proposed by Smith et al. (2020) as reference sequences are shown as "Reference Sequence" in the tree. There was a total of 270 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. The sequence retrieved from the positive Iberian lynx is given in bold type (accession number OL840902).

Discussion

This is the first survey study on HEV in the Iberian lynx. The seroprevalences detected in the present study in free-ranging (7.4%) and captive (33.6%) populations indicate moderate and high circulation respectively of this emerging virus. In most ELISA-positive animals, antibodies against HEV-3 were confirmed by WB, showing circulation of this genotype in Iberian lynx populations. Some of these individuals also reacted against rat HEV-C1. Although cross-reactivity among hepeviruses has previously been observed (Sridhar et al., 2021; Wang et al., 2020), exposure to both genotypes or to a hitherto unknown virus of a putative novel genotype in the Iberian lynx population cannot be ruled out. In line with this, rat HEV-C1 exposure and infection have previously been reported in stray cats and humans, respectively, in the study area, and infection with this genotype has also been found in other carnivores, such as the Syrian brown bear (*Ursus arctos syriacus*), in a zoo in Germany (Caballero-Gómez et al., 2021; Rivero-Juárez et al., 2022; Spahr et al., 2017a).

The detection of seropositive lynxes in all free-range areas and captive breeding centers, with seroprevalence rates ranging from 5.7% to 51.4%, suggests widespread but not homogeneous HEV circulation among their populations. Indeed, animals kept in captivity had a more than six times higher risk of being exposed to the virus than free-ranging individuals. Statistically significant differences between the four captive breeding centres were also found. This finding may be associated with differences in food suppliers or management measures between centres. Consumption of products derived from infected animals is considered to be the main route of HEV transmission, not only for humans but also for other mammal species. The Iberian lynx is a trophic specialist that depends mainly on rabbits, which are the main reservoir of the HEV-3ra subtype but also susceptible to HEV-4 and other strains of HEV-3

(Spahr et al., 2017b). Limited circulation of HEV has been demonstrated in free-ranging wild rabbits in Iberian Mediterranean ecosystems (Caballero-Gómez et al., 2020; Lopes & Abrantes, 2020), which is consistent with the low seroprevalence found in free-ranging (6.5%) Iberian lynx populations in the present study. On the other hand, it should be noted that Iberian lynxes in captive breeding centres feed mainly on domestic farmed rabbits (Rivas et al., 2016) and that HEV circulation has been confirmed in farmed rabbits in other European countries (Bigoraj et al., 2020; Di Bartolo et al., 2016), although further studies are warranted to assess the role of wild and domestic rabbits in the epidemiology of HEV in Spain. In the event that its role as an HEV reservoir is confirmed, using domestic rabbits from HEV-free farms to feed Iberian lynxes kept in captive breeding centres could be a useful tool to limit HEV transmission in this species. Captive Iberian lynxes can also be fed sporadically with partridge, quail and veal (Rivas et al., 2016). However, orthohepevirus infection in partridge and quail has not been reported so far, and the susceptibility of cattle to this virus remains under debate (Yugo et al., 2018).

Interestingly, seroconversions were detected in a high percentage (29.5%) of the longitudinally sampled individuals. This, coupled with the detection of seropositive yearlings, indicates HEV circulation during the study period. In addition, fifteen animals were seropositive at all samplings, which could be related to repeated exposure to the virus or more likely to the lifelong persistence of antibodies in the Iberian lynx, as has previously been shown in other species (Pavio et al., 2017; Seminati et al., 2008). The significantly higher seroprevalence detected in older captive animals in multivariate analysis supports this hypothesis. There is no known information about the persistence of anti-HEV antibodies in the Iberian lynx, although experimental studies conducted on non-human primates have confirmed

that the persistence of antibodies against this virus can range from 100 days to more than seven years post-infection (Arankalle et al., 1999; Li et al., 1994). In our study, the sampling intervals for the two lynxes that seroreverted were six and seven years, and while some individuals remained seropositive with similar sampling periods, this finding suggest that, in the absence of re-exposure, anti-HEV antibodies may decline six or seven years after infection in this felid species. In any case, additional studies are needed to determine the duration of persistence of anti-HEV antibodies in the Iberian lynx.

None of the 250 serum and 161 liver samples were positive for HEV-A HEV-B or HEV-C infection. However, one of the 76 stool samples tested positive for HEV RNA, which confirm the susceptibility of the Iberian lynx to virus infection and increase the host range of HEV-A. To the best of the authors' knowledge, this is the first molecular report of HEV-A infection in free-ranging felines. Interestingly, HEV RNA was detected in the faeces, but not the liver, of the infected Iberian lynx. This could be related to an early or even late stage of acute infection as has previously been suggested for suid species (de Deus et al., 2008; Kozyra et al., 2020). Unfortunately, serum from this animal could not be obtained. The sequenced subgenotype in the positive lynx belonging to HEV-3f (GenBank Accession Number: OL840902) has previously been detected in different wild and domestic ungulate species in the study area, including domestic pigs, horses, red deer, and wild boar (García-Bocanegra et al., 2019; Kukielka et al., 2015; López-López et al., 2018; Rivero-Juárez et al., 2018). Besides lagomorphs, free-ranging Iberian lynxes may sporadically consume different ungulate species, such as red deer or wild boar (Masot et al., 2016; Rivas et al., 2016). There may also be direct or indirect contact between the Iberian lynx and these as well as other susceptible wild and domestic ungulates, including fallow deer, pigs, goats and sheep, can occur in the Iberian

Mediterranean ecosystems, which may increase the risk of HEV transmission among these sympatric species. In connection with this, the HEV-positive lynx was sampled in a region characterized by high densities of wild boar, Iberian pig and red deer populations (Gortázar et al., 2011; Jiménez-Ruiz et al., 2022) and where HEV-3r has previously been found in wild boar (Caballero-Gómez et al., 2019). This, coupled with the detection of HEV-3f in the present study, confirms the circulation of both subtypes in wildlife in southwest Spain. HEV-3f is also the main HEV subtype detected in humans in this country (Izopet et al., 2019). Detection of this subtype in both humans and the Iberian lynx and the strong similarity of HEV-3f sequences retrieved from them (Figure 3) points to HEV circulation in different epidemiological contexts and suggest a common epidemiological cycle. In this context, cross-species foodborne transmission of HEV-3 from suids to humans or carnivores, such as the wolf (*Canis lupus*), has been demonstrated and recently suggested, respectively, in European countries, such as Spain and Italy (Riveiro-Barciela et al., 2015; Rivero-Juárez et al., 2017; Sarchese et al., 2021). This finding may also suggest that there is a possible risk of zoonotic transmission of HEV from the Iberian lynx, although further studies are warranted to support this hypothesis.

In conclusion, the serological and molecular results obtained in the present study provide evidence of HEV infection in free-ranging and captive Iberian lynx populations and suggest a possible role for this species as spillover hosts of this virus in Iberian Mediterranean ecosystems. We also confirmed infection with the zoonotic genotype HEV-3f in the Iberian lynx, which may of public health concern. The serological results indicate the widespread but not homogeneous circulation of HEV in Iberian lynx populations. Additional studies are needed to determine the source of HEV infection in the Iberian lynx, particularly in animals kept in captivity, in order to implement control measures to limit exposure to this virus.

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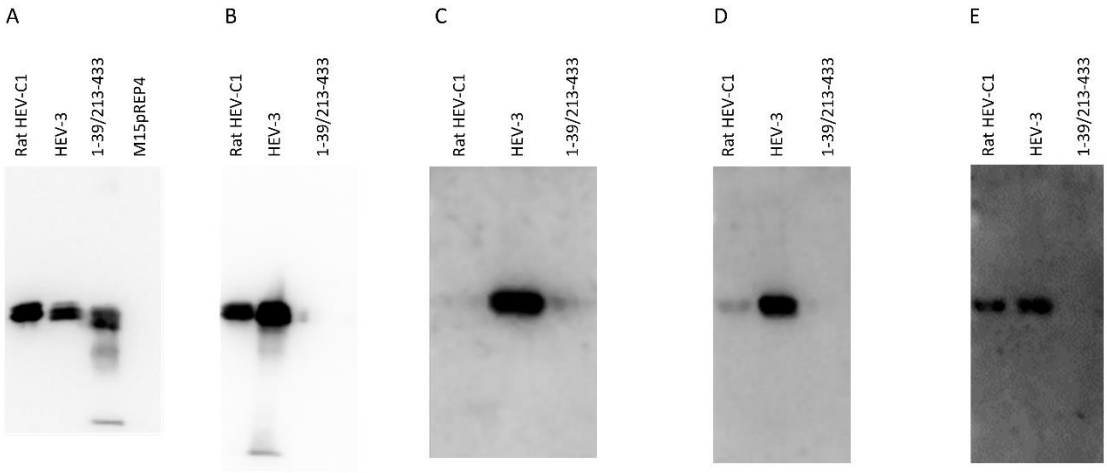
Supplementary material



Supplementary Figure 1. Detection of anti-HEV antibodies in three lynx sera by western blot assay illustrating the reactivity against HEV-3 (C) or against both HEV-3 and HEV-C1 (D and E). For control, the recombinant antigens and a total lysate of M15pREP4 cells were tested by anti-His tag antibody (A) and HEV capsid protein cross-reactive monoclonal antibody (B). Nickel-chelate affinity chromatography purified carboxy-terminal segments of the capsid proteins of HEV-3 and rat HEV-C1 (Dremsek et al., 2011) and a nucleocapsid protein derivative (amino acid residues 1-39/213-433) of Puumala orthohantavirus strain Vranica/Hällnäs, as negative control, were run in a 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with serum samples diluted 1:100, the antigen–antibody reaction was detected by incubation with purified recombinant protein A/G conjugated with horse-radish peroxidase and visualized using Clarity Western ECL Substrate.

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Chapter 2.4.

Survey for Hepatitis E virus infection in non-human primates in zoos in Spain

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Abstract

Hepatitis E virus (HEV) is an emerging zoonotic pathogen that has been detected in different animal species. A survey study was carried out to assess HEV infection in nonhuman primates (NHPs) housed in zoos in Spain. Anti-HEV antibodies were detected in eight of the 181 NHPs tested (4.4%; 95%CI: 1.4-7.4). At least one seropositive animal was detected in five of the 33 species sampled (15.2%). This is the first report of seropositivity in black-and-white ruffed lemurs (*Varecia variegata*), common chimpanzees (*Pan troglodytes*) and Barbary macaques (*Macaca sylvanus*). Anti-HEV antibodies were found in six of the eight zoos included in the study (75.0%). Seroconversion was detected in one chimpanzee, which confirms HEV circulation in one zoo between 2015 and 2016. Seropositivity was significantly higher in hominids than in other NHP families. HEV RNA was not detected in any of the serum samples tested. The results indicate susceptibility of NHPs to HEV infection. Further studies are required to elucidate the role of these species in the epidemiology of HEV.

Keywords: *Hepatitis E, nonhuman primates, Spain, zoonotic.*

Introduction

Hepatitis E virus (HEV; species *Orthohepevirus* A; genus *Orthohepevirus*; family *Hepeviridae*) is an important emerging pathogen and the most common cause of human acute hepatitis (Ricci et al., 2017). Genotypes 1 and 2 are restricted to waterborne human outbreaks in developing countries, whereas genotypes 3 and 4 are zoonotic and have been documented worldwide. Even though pig and wild boar (*Sus scrofa*) are recognized as the main reservoirs of genotypes 3 and 4 (Pavio, Doceul, Bagdassarian, & Johne, 2017), HEV RNA and anti-HEV antibodies have been detected in other mammal species, including captive wildlife animals (Zhang et al., 2008; Reviewed in Spahr, Knauf-Witzens, Vahlenkamp, Ulrich, & Johne, 2018a).

Nonhuman primates (NHPs) have been widely used as models in HEV studies, confirming their susceptibility to HEV infection by genotypes 1-4 (Reviewed in Spahr et al., 2018a; Kenney & Meng, 2019). Likewise, serological and molecular studies have evidenced natural exposure to HEV in NHPs (Hirano et al., 2003; Melegari et al., 2018; Spahr et al., 2018a, 2018b). Nevertheless, information on the role of these species in the epidemiology of HEV is still very limited. Hence, the aim of this study was to assess HEV infection in NHPs housed in zoos in Spain.

Materials and Methods

A total of 194 sera from 181 NHPs belonging to 33 different species were obtained from eight different zoos (A-H) in Spain between 2002 and 2018. Additionally, longitudinal samples were collected from nine of the 181 animals, including three common chimpanzees (*Pan troglodytes*), two De Brazza's monkeys (*Cercopithecus neglectus*), one Barbary macaque

(*Macaca sylvanus*), one mangabey (*Cercocebus atys*), one mongoose lemur (*Eulemur mongoz*) and one red-bellied lemur (*Eulemur rubriventer*) (Table 1). Epidemiological information related to the sampled animals (zoo, species**,** Hominidae family, Parvorder (New World monkeys *vs* Old World monkeys), age, sex and sampling date) was gathered whenever possible. Sera were tested for the presence of anti-HEV antibodies using a commercial indirect ELISA (Wantai HEV-IgG ELISA®; Beijing Wantai Biological Pharmacy Enterprise© Ltd, Beijing, China). Following the manufacturer's instructions, samples with mean optical densities (OD) > mean OD negative control + 0.16 were considered to be positive. This commercial ELISA has been widely used in NHP studies (Li et al., 2005; Huang et al., 2011; Wang et al., 2019).

HEV RNA was simultaneously extracted from pools of 400µl of serum using the QIAamp MinElute virus spin kit on the automated QIAcube platform (QIAGEN, Hilden, Germany). Sera from four different animals of the same species were included in each pool whenever possible. To detect HEV RNA, a real-time reverse transcription PCR (CFX Connect Real Time PCR System) was performed with the iTaq Universal Probes One-Step Kit (Biorad, Hercules, California, EEUU). The primers (15µMol) used were sense HEV5260 (5´-GGTGGTTTCTGGGGTGAC-3´) and antisense HEV5330 (5´-AGGGGTTGGTTGGATGAA-3´), and the probe employed (20µMol) was the HEV5283 (5´-FAM-TGATTCTCAGCCCTTCGC-TAMRA-3´), as described previously (Abravanel et al., 2012).

Associations between prevalence of anti-HEV antibodies and HEV RNA and explanatory variables (age, sex, Hominidae family and Parvorder) were analyzed using the Fisher's exact test or Pearson's chi-square test. Variables with *P*-values < 0.15 in bivariate

analysis were included for further analysis. Collinearity between pairs of variables was tested by Cramer's V coefficient. Finally, multiple logistic regression analysis was carried out. Values with $P < 0.05$ were considered statistically significant.

Results

Anti-HEV antibodies were detected in eight of the 181 NHPs tested (4.4%; 95% confidence intervals (95%CI): 1.4-7.4). At least one seropositive animal was detected in five of the 33 species sampled (15.2%), and seropositivity values ranged from 12.5% (1/8) in Western chimpanzees (*Pan troglodytes verus*) to 50.0% (1/2) in mona monkeys (*Cercopithecus mona*). Anti-HEV antibodies were also found in black-and-white ruffed lemurs (*Varecia variegata*) (1/7; 14.3%), common chimpanzees (3/17; 17.6%) and Barbary macaques (2/9; 22.2%) (Supplementary material). Of the nine NHPs sampled more than once, seven were seronegative and one showed seropositivity at all samplings. In addition, one chimpanzee was seronegative in March 2015 but showed seropositivity in both March and April 2016 (Table 1).

Table 1. Antibodies against hepatitis E virus detected in longitudinally sampled nonhuman primates.

ID number	Species	Zoos	2007	2008	2009	2010	2011	2012	2013	2015	2016
284	Common chimpanzee	C	-	-	-	-	-	-	-	NEG	†POS; POS
288	Barbary macaque	C	-	-	-	-	-	-	-	POS	POS
329	Mangabey	C	NEG	-	-	NEG	-	-	-	-	-
332	De Brazza's monkey	G	-	-	-	-	‡NEG; NEG	-	-	-	-
335	De Brazza's monkey	G	NEG	-	-	NEG	-	-	-	-	-
340	Mongoose lemur	G	-	NEG	-	-	-	-	NEG	-	NEG
342	Red-bellied lemur	G	-	-	-	-	§NEG; NEG	-	-	-	-
364	Common chimpanzee	G	NEG	-	NEG	-	NEG	-	-	-	NEG

365	Common chimpanzee	G	-	-	-	-	-	-	NEG	-	NEG

ID number: Identification number. Samples collected in: †March and April; ‡May and November; §February and August.

Anti-HEV antibodies were found in six of the eight zoos included in the study (75.0%) (Figure 1). HEV seropositivity by age, sex, Hominidae family and Parvorder is shown in Table 2. Multivariate regression analysis identified hominids as a risk factor associated with HEV seropositivity. Frequency of seropositive hominids (4/41; OR = 6.8; *P* = 0.031; 95%CI: 1.2-39.0) was significantly higher than in rest of primate families (4/140). HEV RNA was not detected in any of the 194 serum samples tested.

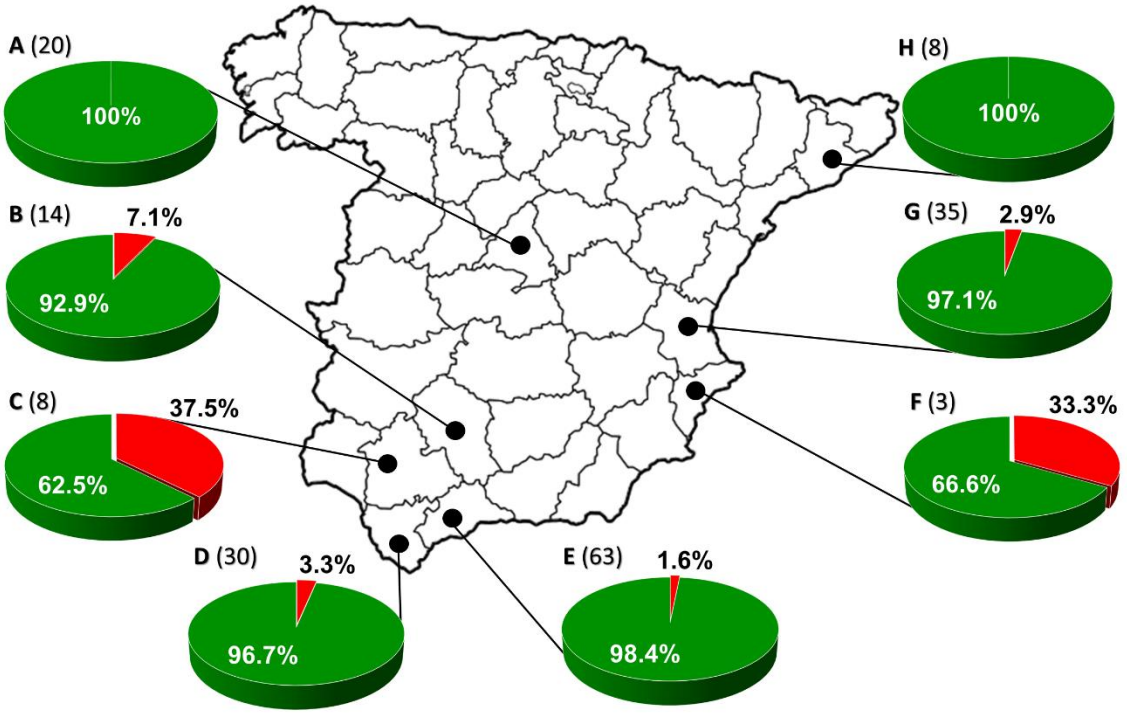

Figure 1. Seropositivity to hepatitis E virus in nonhuman primates (NHPs) in eight zoos in Spain. Pie charts indicate the frequencies of seropositive (red) and seronegative (green) animals. The total number of NHPs analyzed in each zoo (A-H) is depicted in parenthesis.

Table 2. Explanatory variables included in the bivariate analysis of seroprevalence of hepatitis E virus in captive nonhuman primates in zoos in Spain.

Variable	Categories	No. Positives/No. analyzed (%)	*P*-value
Age	Adult	6/102 (5.9%)	0.144
	Young	0/38 (0.0%)	
Sex	Female	6/85 (7.1%)	0.057
	Male	1/86 (1.2%)	
Hominidae family	Yes	4/41 (9.8%)	0.079
	No	4/140 (2.9%)	
Parvorder	New World monkey	0/14 (0.0%)	0.598
	Old World monkey	3/76 (3.9%)	

Discussion

Our results confirm that NHPs are naturally exposed to HEV in zoos in Spain. Five of the 33 species tested presented at least one animal with anti-HEV antibodies. The seropositivity detected in our study (4.4%) was in accordance with that found in NHPs in zoos in Italy (4.6%; 4/86) and Germany (3.9%; 10/259) (Melegari et al., 2018; Spahr et al., 2018b). In contrast, previous studies failed to detect anti-HEV antibodies in NHPs in zoos from China (0/2) and Korea (0/1) (Zhang et al., 2008; Song et al., 2013). Differences between studies should be carefully interpreted given the diversity of species analyzed and the number of animals tested. To the best of the author's knowledge, this is the first report of anti-HEV antibodies in Barbary macaques, black-and-white ruffed lemurs and mona monkeys, which increases the number of species susceptible to this virus. The seropositivity observed in Barbary macaques (22.2%) is within the values (range 3.6-36.7%) found previously in other NHP species of the *Macaca* genus in Asia (Arankalle, Goverdhan, & Banerjee, 1994; Hirano et al., 2003; Huang et al., 2011). Anti-HEV seropositivity was 6.8 times higher in primates of the Hominidae family than

in other NHP families. Higher genetic susceptibility to HEV infection, differences in behavior or interactions with other species are possible factors associated with the higher seropositivity detected in hominids. Additionally, since there is a direct relationship between volume of food ingested and size of animal, the probability of HEV infection could also be proportionally higher. Ingestion of contaminated water or meat products is considered the main route of HEV transmission in humans (Pavio et al., 2017) and probably also in NHPs (Spahr et al., 2018b). However, since the diet of these species is mainly herbivore and the virus has also been detected in fruit and vegetables (Kokkinos et al., 2012; Terio et al., 2017), they should be considered possible sources of infection.

None of the NHPs were positive for active infection, which indicates absence of genotypes 1-4 of HEV in sera at the time of sampling. The duration of HEV in serum ranged between one and eleven weeks in experimentally infected NHPs and seroconversion generally occurs three to five weeks after infection (Tsarev et al., 1993, 1994). Even though the specificity of the ELISA used in the present study has previously been shown to be high in both human and NHPs (Li et al., 2005; Liu et al., 2013; Avellon, Morago, Garcia-Galera del Carmen, Munoz, & Echevarría, 2015), cross reactions with other hepeviruses cannot be ruled out. In this context, divergent strains of HEV have previously been detected in chimpanzees in China (Zhou, Li, & Yang, 2014). Additional studies are required to assess the circulation of other hepeviruses in NHPs in the study area.

Anti-HEV antibodies were found twice in one NHP sampled after a time interval of seven months, which may be associated to a long-lasting humoral immune response. The persistence of anti-HEV antibodies in experimentally infected NHPs varies between less than 100 days and more than 7 years (Li, Zhuang, Kolivas, Locarnini, & Anderson, 1994; Arankalle

et al., 1999). Alternatively, the persistence of seropositivity found in this animal could be related with repeated exposure to the virus. In this context, the seroconversion detected in one chimpanzee confirms HEV circulation in zoo C between 2015 and 2016. Even though biosecurity measures are implemented in the facilities housing NHPs in the zoos sampled in the present study, the transmission of HEV by direct or indirect contact with other NHPs, humans, or other sympatric species, such as wild rats and cats, cannot be ruled out (Zhang et al., 2008; Huang et al., 2011; Spahr et al., 2018b). In this context, natural infection and transmission of HEV among NHPs housed together in the same facilities have been evidenced previously (Yamamoto et al., 2012).

In conclusion, the seropositivity found in 15.2% of the NHP species sampled indicates the susceptibility of these species to natural exposure of HEV or other related hepevirus. HEV infection in NHPs in zoos in Spain could be of public health and conservation concern. Control measures should be implemented to prevent transmission of this pathogen between NHPs and other sympatric species, including humans. Further studies are required to elucidate the role of these species in the epidemiology of HEV and to identify the sources of infection in NHPs housed in zoos.

Supplementary material. Analysis of 33 nonhuman primate species sampled in zoo parks in Spain for hepatitis E virus-specific antibodies.

SPECIES	HEV ELISA
	No. Positives/No. analyzed (%)
Baboons	0/11 (0.0)
Olive baboon (*Papio hamadryas)*	0/2 (0.0)
Sacred baboon (*Papio anubis*)	0/8 (0.0)
Yellow baboon (*Papio cynocephalus*)	0/1 (0.0)
Capuchin monkeys	
Brown capuchin monkey (*Sapajus apella*)	0/7 (0.0)

Cercopithecus monkeys	1/14 (7.1)
De Brazza's monkey (*Cercopithecus neglectus*)	0/10 (0.0)
Mona monkey (*Cercopithecus mona*)	1/2 (50.0)
Hamlyn's monkey (*Cercopithecus hamlyni*)	0/2 (0.0)
Chimpanzees	4/25 (16.0)
Common chimpanzee (*Pan troglodytes*)	3/17 (17.6)
Western chimpanzee (*Pan troglodytes verus*)	1/8 (12.5)
Colobus	
Colobus monkey *(Colobus guereza kikuyuensis*)	0/10 (0.0)
Gibbons	0/7 (0.0)
Lar gibbon (*Hylobates lar*)	0/3 (0.0)
Mueller's gibbon (*Hylobates muelleri*)	0/1 (0.0)
Yellow-cheeked crested gibbon (*Nomascus gabriellae*)	0/3 (0.0)
Gorillas	
Western lowland gorilla (*Gorilla gorilla gorilla*)	0/5 (0.0)
Lemurs	1/41 (2.4)
Black and white ruffed lemur (*Varecia variegata*)	1/7 (14.3)
Black lemur (*Eulemur macaco*)	0/4 (0.0)
Brown lemur (*Eulemur fulvus*)	0/2 (0.0)
Mongoose lemur (*Eulemur mongoz*)	0/1 (0.0)
Red-bellied lemur (*Eulemur rubriventer*)	0/4 (0.0)
Red ruffed lemur (*Varecia rubra*)	0/2 (0.0)
Ring-tailed lemur (*Lemur catta*)	0/20 (0.0)
White-fronted lemur (*Eulemur fulvus albifrons*)	0/1 (0.0)
Loris	
Pygmy slow loris (*Nycticebus pygmaeus*)	0/2 (0.0)
Macaques	2/10 (20)
Barbary macaque (*Macaca sylvanus*)	2/9 (22.2)
Japanese macaque (*Macaca fuscata*)	0/1 (0.0)
Mandrels	0/9 (0.0)
Drill (*Mandrillus leucophaeus*)	0/2 (0.0)
Mandrill (*Mandrillus sphinx*)	0/7 (0.0)
Mangabeys	
Sooty mangabey (*Cercocebus atys*)	0/5 (0.0)
Orangutans	
Bornean orangutan (*Pongo pygmaeus*)	0/11 (0.0)
Talapoins	
Northern talapoin monkey (*Miopithecus ogouensis*)	0/16 (0.0)
Titis	0/7 (0.0)
Golden-handed tamarin (*Saguinus midas*)	0/1 (0.0)

Golden lion tamarin (*Leontopithecus rosalia*)	0/1 (0.0)
Marmoset monkey (*Callithrix jacchus*)	0/5 (0.0)

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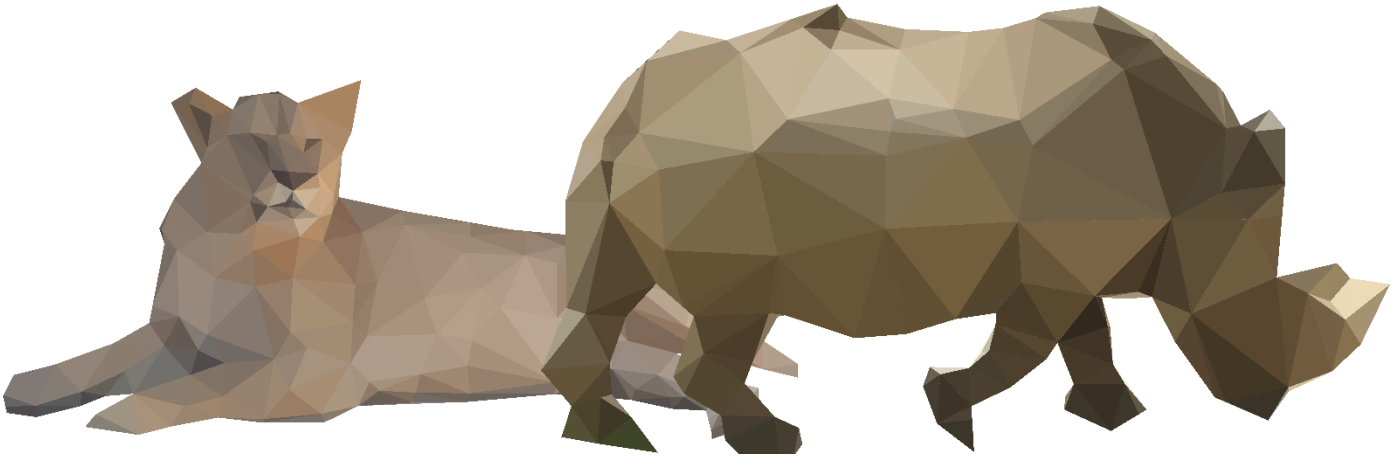
Chapter 2.5.

Monitoring of hepatitis E virus in zoo animals from Spain, 2007-2021



Caballero-Gómez, J., García Bocanegra, I., Cano-Terriza, D., Beato-Benítez, A., Ulrich, RG., Martínez, J., Guerra, R., Martínez-Valverde, R., Martínez-Nevado, E., Quevedo-Muñoz, MÁ., Sierra-Arqueros, C., Planas, J., de Castro-García, N., Rivero, A., Rivero-Juárez, A. **Monitoring of hepatitis E virus in zoo animals from Spain, 2007-2021.** *Transboundary and Emerging Diseases. Under review*.

Abstract

Hepatitis E virus (HEV, family *Hepeviridae*) is an important emerging and zoonotic pathogen. In recent decades, the number of human cases of zoonotic hepatitis E has increased considerably in industrialized countries and HEV has been detected in an expanding range of mammal species. Although domestic pigs and wild boar are considered the main reservoirs of zoonotic HEV genotypes, the role of other susceptible animals in the epidemiology of HEV is still poorly understood. A large-scale, long-term study was carried out (1) to assess HEV exposure in captive zoo animals in Spain and (2) to determine the dynamics of seropositivity in individuals that were sampled longitudinally during the study period. Between 2007 and 2021, serum samples from 425 zoo animals belonging to 109 animal species (including artiodactyls, carnivores, perissodactyls, proboscideans and rodents) were collected from 11 different zoological parks in Spain. Forty-six of these animals at seven of these zoos were also longitudinally sampled. Anti-HEV antibodies were detected in 36 (8.5%; 95%CI: 5.8-11.1) of 425 sampled zoo animals. Specific antibodies against HEV-3 and HEV-C1 antigens were confirmed in ELISA-positive animals using western blot assay. Two of 46 longitudinally surveyed animals seroconverted during the study period. Seropositivity was significantly higher in carnivores and perissodactyls than in artiodactyls, and also during the period 2012-2016 compared with 2007-2011. HEV RNA was not detected in any of the 262 animals that could be tested by RT-PCR. To the best of the author's knowledge, this is the first large-scale, long-term surveillance on HEV in different orders of zoo mammals. Our results indicate exposure to HEV-3 and HEV-C1 in zoo animals in Spain and confirm a widespread but not homogeneous spatiotemporal circulation of HEV in captive species in this country. Further studies are required to determine the role of zoo species, particularly carnivores and perissodactyls, in the epidemiology of HEV and to clarify the origins of infection in zoological parks.

Keywords: *Hepatitis E, emerging, zoonoses, zoological parks, epidemiology*.

Introduction

Hepatitis E virus (HEV) (family *Hepeviridae*; genus *Orthohepevirus*) is the most common cause of acute viral hepatitis in humans worldwide, with at least 20 million infections annually (Nimgaonkar et al., 2017). The virus has a positive-sense single-stranded RNA genome, which is divided into at least three open reading frames (ORF), named ORF1, ORF2 and ORF3. Four species (*Orthohepevirus A-D*) have been recognized so far, although *Orthohepevirus A* is the most important in terms of public health concern. Eight different genotypes (HEV-1 to HEV-8) of this species have been confirmed, of which HEV-3 to HEV-8 infect animals, with HEV-3 being the only one with global distribution and the most prevalent among animals (Izopet et al., 2019; Mulder et al., 2019). In industrialized countries, human cases of HEV-3 have increased sharply during the last decade (Aspinall et al., 2017), mainly associated with the consumption of animal products but also with the contact with infected species. Indeed, animal handlers, such as veterinarians, farmers, and forestry workers have been shown to be at increased risk for HEV infection (Mrzljak et al., 2021). While domestic pigs and wild boar are the main reservoirs of HEV-3, this genotype has the widest host range (Meng, 2016). HEV-3 has been detected in other artiodactyls and in different lagomorph, non-human primate, perissodactyl, rodent and carnivore species (Spahr et al., 2017a). In addition, rodents and wild carnivores have traditionally been considered the only reservoirs of the HEV-C1 and HEV-C2 genotypes of *Orthohepevirus C* species, respectively (Wang et al., 2020). However, several mammal species susceptible to this orthohepevirus have also been reported in the last few years, with HEV-C1 infections being confirmed in the Syrian brown bear (*Ursus arctos syriacus*) and also human beings (Spahr et al., 2017b; Sridhar et al., 2018). Exposure to

this emerging genotype has also recently been confirmed in dogs and cats (Caballero-Gómez et al., 2021).

Due to the high diversity of animal species and routine veterinary control, zoo animals are considered useful species for obtaining insights into the epidemiology and distribution of emerging zoonotic pathogens (Caballero-Gómez et al., 2020; Robinette et al., 2017). However only a very few studies have assessed HEV circulation in zoo animals worldwide to date, most of which have focused on certain animal species and/or analyzed a limited number of zoos or animals (Table 1). The main aims of the present large-scale study were (1) to assess HEV presence and circulation in captive zoo animals in Spain, and (2) to determine the dynamics of seropositivity in individuals that were sampled longitudinally during the study period.

Material and methods

Sampling

A total of 425 zoo animals belonging to 109 species were sampled at 11 different zoological parks (A-J) in Spain between 2007 and 2021 (Table S1). Samples were obtained from serum banks or from animals subjected to health programs, surgical interventions, or medical check-ups during the study period. Serum samples were stored frozen at -20°C until shipment to the Animal Health Department at the University of Cordoba (Spain) for laboratory analysis. Whenever possible, epidemiological information, including species, age, zoo provenance and sampling date, was gathered from each animal, whenever possible. Longitudinal samples (between two and six samplings per animal) were also retrospectively gathered from 46 of the 425 analyzed animals at seven of the sampled zoos. The median (interquartile range Q1-Q3) of the period between consecutive samplings during follow-up was

36 months (12-60).

Laboratory analyses

The presence of antibodies against HEV was determined using a commercial double-antigen multispecies sandwich HEV ELISA 4.0v (MP Diagnostics, Illkirch, France), following the manufacturer's instructions. The plates of this ELISA are coated with the recombinant ET2.1 protein, which is highly conserved in HEV genotypes (Hu et al., 2008) and can detect the presence of total antibodies in sera from a wide range of mammals. This ELISA has previously been used in humans, artiodactyls and carnivores and has been reported to be highly sensitive and specific in detecting anti-HEV antibodies (Carpentier et al., 2012; Kukielka et al., 2015; Caballero-Gómez et al., 2021).

Whenever possible, samples from seropositive zoo animals were further investigated by western blot (WB) analysis to confirm exposure to *Orthohepevirus* A and/or C strains, including HEV-3 and HEV-C1 genotypes in zoo species. Carboxy-terminal segments of the capsid proteins of HEV-3 and rat HEV (HEV-C1) 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., 2011; Lundkvist et al., 2002) (for more details see Caballero Gómez et al. (2022)). Seropositivity was confirmed by WB when blotted bands matching either HEV-3 and/or HEV-C1 antigens were observed. The presence of specific antibodies against HEV-3 or HEV-C1 was considered when serum samples reacted against only one of these genotypes, otherwise the result was considered as indeterminate.

Whenever possible, RNA was extracted from 400µl pools of serum using the QIAamp

MinElute Virus Spin Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Each pool contained sera from four different individuals (100µl of each sample). The purified RNA was eluted in a total volume of 50µl. For detection of *Orthohepevirus A* RNA, real-time RT-PCR (CFX Connect Real Time PCR System) targeting a 70-nucleotide sequence of ORF3 was performed, using 25µL of RNA template and the QIAGEN One-Step RT-PCR kit, as previously described (Frias et al., 2021). The detection limit was set at 21.9 (95% confidence interval (95%CI): 17.4-34.3). A nested broad-spectrum RT-PCR, that amplifies a conserved 280-nucleotide segment of ORF1 (RdRp-coding sequence), was also carried out according, in accordance with Johne *et al*. (2010). This assay was designed and validated to detect strains belonging to *Orthohepevirus A, B* and *C*. The first round was performed using the QIAGEN One-Step RT-PCR kit and the nested PCR was carried out with a premixed 2X solution Taq DNA polymerase, dNTPs and reaction buffer kit (Promega). The second PCR products were examined on 1.5% agarose gel stained with RedSafe™ Nucleic Acid Staining solution.

Statistical analyses

Associations between prevalence of anti-HEV antibodies and HEV RNA and explanatory variables (age, sex, order, zoo provenance and sampling period [2007-2011, 2012-2016 and 2017-2021]) were analysed using the Pearson's chi-square or Fisher's exact tests. Variables with $p < 0.05$ in bivariate analysis were included for further analysis. Collinearity between pairs of variables was assessed by Spearman's Rho test. Finally, a multivariable analysis was carried out using a generalized estimating equations (GEE) model. The number of seropositive animals was assumed to follow a binomial distribution and zoo provenance was used as the subject variable. Given the absence of seropositivity in individuals of the orders

Proboscidea and *Rodentia*, these species were excluded from the multivariable analysis. Forward selection of variables was used, starting with the variable with the lowest *p*-value in bivariate analysis. 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).

Results and discussion

To the best of the author's knowledge, this is the first large-scale surveillance conducted on HEV and other *Orthohepevirus* in different orders of zoo mammals. A total of 36 (8.5%; 95%CI: 5.5-11.1) of the 425 sampled zoo animals showed anti-HEV antibodies using ELISA (Tables 1 and 2; Supplementary material, Table S1). Seropositivity was confirmed in 14 of the 19 ELISA-positive animals that could be analyzed by WB (Supplementary Material, Table S1). Of them, the presence of specific IgG antibodies against HEV-3 and HEV-C1 was detected in four and three animals, respectively, whereas seven individuals reacted against both HEV-3 and HEV-C1 antigens. These findings indicate circulation of both genotypes and related *Orthohepevirus* in zoo animals in Spain.

Table 1. Seroprevalence and RNA detection rate of hepatitis E virus in zoo animals worldwide.

Country	No. of captivity centres	Sampling period	Order(s) analyzed	No. Seropositives/ No. Analyzed (%)	No. RNA Positives/ No. Analyzed (%)	Reference
Germany	3 (most samples from 1)	2006-2016	Afrosoricida	0/1 (0.0)	NA*	Spahr et al., 2017b
			Artiodactyla	16/167 (9.6)	0/98 (0.0)	
			Carnivora	10/37 (27.0%)	3/37 (8.1)	
			Chiroptera	0/4 (0.0)	0/4 (0.0)	
			Diprodontia	0/2 (0.0)	0/1 (0.0)	
			Perissodactyla	2/24 (8.3)	0/20 (0.0)	
			Proboscidea	0/6 (0.0)	NA	
			Rodentia	0/2 (0.0)	0/3 (0.0)	
Germany	9	2015-2016	Primates	10/259 (3.9)	0/256 (0.0)	Spahr et al., 2017c
Italy	1	2001-2017	Primates	4/86 (4.7)	0/86 (0.0)	Melegari et al., 2018
Japan	1	2006	Artiodactyla	NA	7/23 (30.4)	Zhang et al., 2008
			Carnivora		2/3 (33.3)	
			Casuariiformes		0/1 (0.0)	
			Primates		0/2 (0.0)	
			Rodentia		0/1 (0.0)	
			Galliformes		1/2 (50.0)	
			Psittaciformes		0/1 (0.0)	
			Gruiformes		1/3 (33.3)	
			Struthioniformes		0/1 (0.0)	
Korea	1	2005-2010	Anseriformes	0/2 (0.0)	NA	Song et al., 2013
			Artiodactyla	38/168 (22.6)		
			Carnivora	1/14 (7.1)		
			Ciconiformes	0/1 (0.0)		
			Diprodontia	0/3 (0.0)		
			Falconiformes	0/2 (0.0)		
			Gruiformes	0/3 (0.0)		
			Perissodactyla	1/4 (25.0)		
			Primates	0/1 (0.0)		
			Rodentia	0/2 (0.0)		
			Struthioniformes	0/1 (0.0)		
Spain	8	2002-2018	Primates	8/181 (4.4)	0/181 (0.0)	Caballero-Gómez et al., 2019
Spain	11	2001-2021	Artiodactyla	6/195 (3.1)	0/262 (0.0)	*Present study*
			Carnivora	25/171 (14.6)		
			Perissodactyla	5/28 (17.9)		
			Proboscidea	0/14 (0.0)		
			Rodentia	0/17 (0.0)		
Thailand	1	2009	*Artiodactyla*	NA	0/19 (0.0)	Wiratsudakul et al., 2012

*Not analyzed

Table 2. Seropositivity to hepatitis E virus in zoo animals in Spain and results of the bivariate analyses.

Variable	Categories	No. Positives /No. analyzed (%)*	*P*
Order	Artiodactyla	6/195 (3.1)	<0.001
	Carnivora	25/171 (14.6)	
	Perissodactyla	5/28 (17.9)	
	Proboscidea	0/14 (0.0)	
	Rodentia	0/17 (0.0)	
Age	Young	2/50 (4.0)	0.143
	Adult	18/179 (10.1)	
Gender	Male	16/141 (11.3)	0.119
	Female	12/174 (6.9)	
Zoo	A	0/5 (0.0)	0.051
	B	10/59 (16.9)	
	C	3/40 (7.5)	
	D	2/27 (7.4)	
	E	11/66 (16.7)	
	F	1/36 (2.8)	
	G	4/68 (5.9)	
	H	0/20 (0.0)	
	I	0/5 (0.0)	
	J	0/16 (0.0)	
	K	5/83 (6.0)	
Sampling period	2007-2011	5/88 (5.7)	0.006
	2012-2016	18/119 (15.1)	
	2017-2021	10/188 (5.3)	

*Missing values omitted

The overall seroprevalence of HEV detected in the present study (8.5%) is of the same magnitude as that observed in zoo animals in Germany, where 11.5% of 244 individuals tested presented anti-HEV antibodies (Spahr et al., 2017b), but lower than the seropositivity found in captive animals in Korea (19.9%; 40/201) (Song et al., 2013). Comparisons between studies should be made with caution given the differences in the numbers of animals and species analyzed, study design and diagnostic assays employed. Nevertheless, our result confirms that animals in zoo parks are naturally exposed to this virus in Spain, which could be important for

public health since zoonotic HEV transmission through direct or indirect contact with infected captive zoo animals could occur, as previously suggested for this virus and other zoonotic pathogens transmitted by the oral-fecal route (Zhang et al., 2008; Köster et al., 2021).

Seropositivity was detected in 23 (21.1%) of the 109 species analyzed (Supplementary material, Table S1). We report for the first time HEV exposure in thirteen carnivore species, four artiodactyla and two perissodactyla. Of these, the presence of specific antibodies against HEV-3 was confirmed in the serval (*Leptailurus serval*) and the sun bear (*Helarctos malayanus*), and specific anti-HEV-C1 antibodies were detected in the Iberian wolf (*Canis lupus signatus*), white rhinoceros (*Ceratotherium simum*) and South American sea lion (*Otaria byronia*) (Supplementary material, Table S1). Previous studies have reported exposure to HEV-C1 in humans, captive Syrian brown bears, pet dogs and stray cats (Caballero-Gómez et al., 2021; Spahr et al., 2017b; Sridhar et al., 2018). Our results increase the range of species susceptible to HEV-3 and HEV-C1. Further studies are warranted to assess the role of these species in the epidemiology of HEV.

Seropositive animals were detected in orders Perissodactyla (17.9%; 5/28), Carnivora (14.6%; 25/171) and Artiodactyla (3.0%; 6/195), but not in orders Proboscidea (0/14) and Rodentia (0/17) (Table 2). Multivariate regression analyses identified the "order" as a potential risk factor associated with HEV exposure in zoo animals in Spain. Seropositivity was significantly higher in perissodactyls (5/28; OR= 6.4; P=0.006; 95%CI: 1.7-24.0) and carnivores (25/171; OR=6.2; P<0.001; 95%CI: 2.2-16.9) than in artiodactyls (6/195) (Table 3). It should be noted that anti-HEV antibodies were only found in three of the eight perissodactyl species tested (Malayan tapir (*Tapirus indicus*), Przewaslki's horse (*Equus caballus przewalskii*) and white rhinoceros). Our results could be related to an increased

genetic susceptibility to HEV infection of these three perissodactyl species, although further studies are needed to support this hypothesis. On the other hand, the differences observed between captive carnivores and artiodactyls could be associated with diet. The diet of captive artiodactyl species is mainly based on commercial feed for equines/ruminants, fruits and vegetables, whereas carnivores in the sampled zoos are fed with meat products from different animal species, some of which, including farmed rabbits, equines and rodents, are potential reservoirs of HEV (Wang et al., 2018; García-Bocanegra et al., 2019; Lack et al., 2012). Although fruits and vegetables have been recognized as potential sources of HEV, the consumption of products derived from infected animals is considered the main transmission route of HEV in humans and probably also in other mammals (EFSA, 2017; Spahr et al., 2017a). However, to date, cross-species foodborne transmission has only been confirmed from rabbits (Izopet et al., 2012). In any case, additional studies are needed to clarify the sources of HEV transmission in animals kept in captivity in zoo parks.

Table 3. Generalized estimating equation analysis of the potential risk factors associated with hepatitis E virus seropositivity in zoo animals in Spain.

Variable	Categories	*P*	OR (95%CI)
Order	Artiodactyla	[a]	[a]
	Carnivora	<0.001*	6.2 (2.2-16.9)
	Perissodactyla	0.006*	6.4 (1.7-24.0)
Sampling period	2007-2011	[a]	[a]
	2012-2016	0.022*	2.9 (1.2-7.3)
	2017-2021	0.931	1.0 (0.4-2.4)

[a]Reference category; * *P*-value < 0.05

Seropositive individuals were detected at seven of the 11 zoos tested, with within-zoo seropositivity ranging between 2.8% and 16.9% (Figure 1). Seropositivity was also found every year between 2008 and 2021, except for 2013. Significantly higher seroprevalence was

detected during 2012-2016 (18/119; OR= 2.9; *P*=0.022; 95%CI: 1.2-7.3) compared to 2007-2011 (5/88) (Table 3). These results indicate an endemic but not homogeneous spatiotemporal circulation of HEV in zoo animals in Spain.

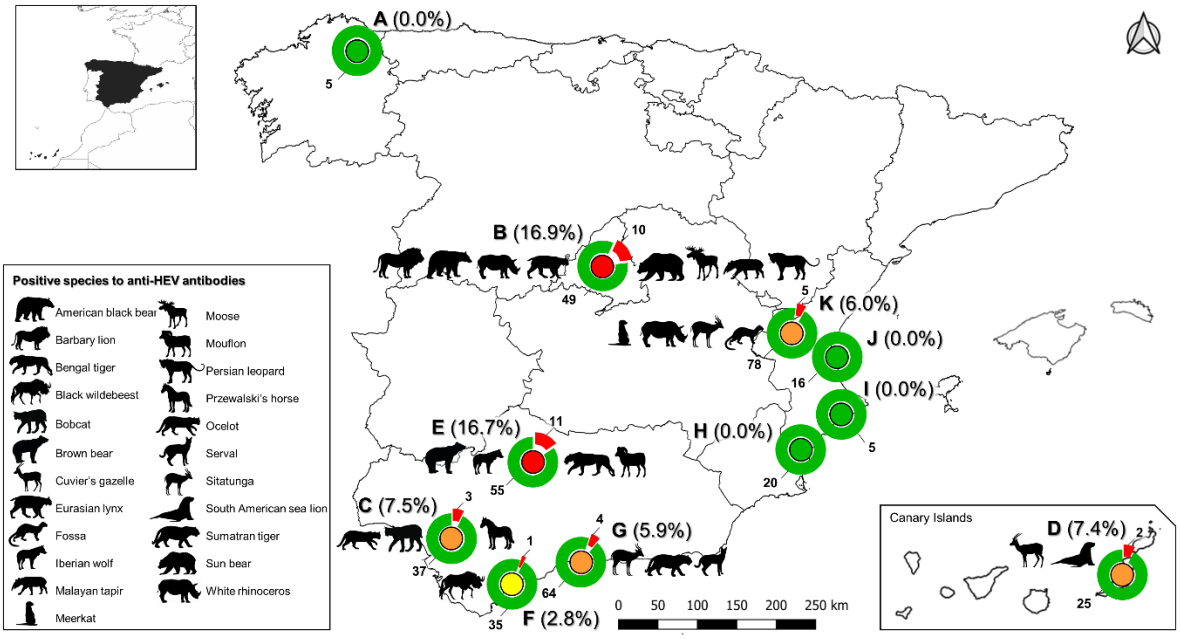


Figure 1. Distribution of the zoos (A-K) sampled in Spain. The number of positive (red) and negative (green) animals tested by ELISA at each zoo park is represented in a pie chart. Colored dots indicate the frequency of seropositivity (yellow: lesser than 5.0%; orange: between 5.1% and 10.0%; red: between 10.1% and 25.0%; green: absence).

Of the 46 animals sampled longitudinally, 44 were seronegative at all samplings. One fossa from zoo K showed seropositivity in both March and November 2009 (Table 4), which could be related to the life-long persistence of anti-HEV antibodies, as has been previously suggested for other animal species (Caballero-Gómez et al., 2019a), although repeated exposure to HEV cannot be ruled out. Interestingly, a Malayan tapir from zoo B seroconverted between 2014 and 2019, and an ocelot from zoo C that tested seronegative in 2008 showed

seropositivity in 2019. These results, coupled with the presence of anti-HEV antibodies in a yearling Iberian wolf in zoo E in 2014, suggest HEV circulation in zoo animals from these zoos during the study period. Previously detected seroconversion in a common chimpanzee from zoo E between 2015 and 2016 supports this hypothesis (Caballero-Gómez et al., 2019b).

Table 4. Antibodies against hepatitis E virus in longitudinally sampled zoo animals. Colored dots indicate information on antibodies to HEV (red: positive; green: negative). When two samplings were carried out in the same year, the sampled months are indicated in superscript.

Species	Zoo	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Malayan tapir	B								●					●
Ocelot	C		●											●
Fosa	K			●Mar; ●Nov										
Harbor seal	A								●				●	
Harbor seal	A									●			●	
Harbor seal	A									●			●	
Sun bear	B											●		
Giant panda	B											●		●
Giant panda	B											●		●
Bengal tiger	B										●			●
Black wildebeest	B										●			
Malayan tapir	B											●		●
Dama gazelle	C			●				●						
Przewaslki's horse	C			●					●					
Cape mountain zebra	C			●					●					
Asiatic elephant	E												●	●
Iberian wolf	E											●Nov; ●Dec		
Iberian wolf	E											●	●	
Iberian wolf	E										●	●		
Jaguar	E						●					●		
Binturong	G									●				
Malayan tapir	G		●		●		●		●		●	●		
Malayan tapir	G			●		●	●			●				
Malayan tapir	G								●	●	●			
Philippine spotted deer	G						●					●	●	
Philippine spotted deer	G										●	●		
Sri Lankan leopard	G									●	●			
South American coati	G						●							●
Spotted hyaena	K	●				●								
Spotted hyaena	K				●Jun; ●Oct									
Lion	K	●			●									
Lion	K			●	●									
Sri Lankan leopard	K				●Sep; ●Dec									
Common eland	K		●		●	●								
Bongo	K							●		●				
Blesbok	K					●	●							

Information about HEV viremia in animal species is still very scare. In non-human primates, rabbits and swine, the duration of HEV in sera has been shown to be limited, usually

between one and four weeks (Tsarev et al., 1994; Ma et al., 2010; Meester et al., 2021). Previous studies have confirmed active HEV infection in sera from different animal species kept in captivity (Spahr et al., 2017b; Yamamoto et al., 2012). Spahr et al. (2017b) detected closely related rat HEV (HEV-C1) RNA sequences in a captive Syrian brown bear and Norway rats (pests) from the same zoo in Germany, suggesting that infection in the bear originated from the rodents. In addition, cross-species transmission of HEV-4 was suspected in a zoo-like location involving different avian and mammal species in China (Zhang et al., 2008) and HEV-3 transmission was confirmed among captive macaques in Japan (Yamamoto et al., 2012). In our study, HEV RNA was not found in serum samples of the 262 animals tested, which indicates the absence of active infection in those individuals at the sampling point.

In conclusion, the results obtained in the present study indicate widespread but not homogeneous spatiotemporal circulation of HEV (and other orthohepeviruses) in zoo animals in Spain. Although active HEV infection was not detected, the frequency and dynamics of seropositivity provide evidence that animal zoo species are naturally exposed to this emerging virus, which could be of public health concern. Further studies are required to determine the role of these species in the epidemiology of HEV and to clarify the sources of infection in animals housed in zoo parks.

Supplementary material

Table S1. Antibodies against hepatitis E virus in zoo species sampled in zoo parks in Spain.

Species	Provenance zoo(s)	No. Positives by ELISA /No. analyzed (%)	Western Blot results		
			HEV-3	HEV-C1	Indeterminate**
Order Artiodactyla					
Angolan giraffe (*Giraffa camelopardalis angolensis*)	F	0/1 (0.0)			
Aoudad (*Ammotragus lervia*)	B, C, E, F	0/18 (0.0)			
Ankole-Watusi cattle (*Bos taurus*)	H	0/3 (0.0)			
Bactrian camel (*Camelus bactrianus*)	B	0/1 (0.0)			
Banteng (*Bos javanicus*)	G	0/2 (0.0)			
Black wildebeest (*Connochaetes gnou*)*	B, E, F	1/4 (25.0)			1
Blackbuck (*Antilope cervicapra*)	B	0/1 (0.0)			
Blesbok (*Damaliscus pygargus*)	K	0/7 (0.0)			
Blue duiker (*Philantomba monticola*)	G, K	0/3 (0.0)			
Bongo (*Tragelaphus euryceros*)	K	0/4 (0.0)			
Chinese muntjac (*Muntiacus reevesi*)	G	0/1 (0.0)			
Collared peccary (*Pecari tajacu*)	E, G	0/9 (0.0)			
Common eland (*Taurotragus oryx*)	D, F, K	0/6 (0.0)			
Cuvier's Gazelle (*Gazella cuvieri*)*	D	1/4 (25.0)			
Dama gazelle (*Nanger dama*)	B, C, F	0/6 (0.0)			
Dorcas gazelle (*Gazella dorcas*)	B, C	0/6 (0.0)			
Dromedary (*Camelus dromedarius*)	B, C, D, F, H, K	0/15 (0.0)			
European bison (*Bison bonasus*)	B, C, F	0/7 (0.0)			
Forest buffalo (*Syncerus caffer nanus*)	B, F	0/4 (0.0)			
Gemsbok (*Oryx gazella*)	D	0/3 (0.0)			
Giraffe (*Giraffa camelopardalis*)	D	0/3 (0.0)			
Guanaco (*Lama guanicoe*)	F	0/4 (0.0)			
Hairy babirusa (*Babyrousa babyrussa*)	G	0/1 (0.0)			
Impala (*Aepyceros melampus*)	F, K	0/8 (0.0)			
Kirk's Dik-diks (*Madoqua kirkii*)	I	0/1 (0.0)			
Lesser mouse-deer (*Tragulus javanicus*)	G	0/1 (0.0)			
Llama (*Lama glama*)	D, E	0/2 (0.0)			

Lowland anoa (*Bubalus depressicornis*)	F	0/1 (0.0)			
Moose (*Alces alces*)	B	0/1 (100.0)			
Mouflon (*Ovis aries musimon*)*	E	0/2 (50.0)	1		
Nilgai (*Boselaphus tragocamelus*)	B, H	0/2 (0.0)			
Philippine spotted deer (*Rusa alfredi*)	G	0/6 (0.0)			
Red deer (*Cervus elaphus hispanicus*)	B, E	0/9 (0.0)			
Red lechwe (*Kobus leche leche*)	D, F	0/2 (0.0)			
Red river hog (*Potamochoerus porcus pictus*)	G, K	0/7 (0.0)			
Reeves' muntjac (*Muntiacus reevesi*)	B	0/2 (0.0)			
Rothschild's giraffe (*Giraffa camelopardalis rothschieldii*)	K	0/1 (0.0)			
Scimitar-horned oryx (*Oryx dammah*)	H	0/1 (0.0)			
Sitatunga (*Tragelaphus spekii*)*	B, G, K	2/12 (16.7)			
Spanish ibex (*Capra pyrenaica*)	E	0/6 (0.0)			
Takin (*Budorcas taxicolor*)	B	0/1 (0.0)			
Thomson's gazelle (*Eudorcas thomsonii*)	K	0/10 (0.0)			
Vicuña (*Vicugna vicugna*)	C	0/3 (0.0)			
Waterbuck (*Kobus ellipsiprymnus defassa*)	F	0/1 (0.0)			
Wild boar (*Sus scrofa*)	B	0/1 (0.0)			
Wild yak (*Bos mutus*)	H	0/2 (0.0)			
TOTAL		6/195 (3.1)	1	0	1
Order Carnivora					
American black bear (*Ursus americanus*)*	B	1/1 (100.0)			1
Asiatic black bear (*Ursus thibetanus*)	B	0/1 (0.0)			
Asian small-clawed otter (*Aonyx cinereus*)	G	0/5 (0.0)			
Asiatic lion (*Panthera leo persica*)	B, C	0/3 (0.0)			
Atlantic walrus (*Odobenus rosmarus*)	J	0/3 (0.0)			
Banded mongoose (*Mungos mungo*)	K	0/1 (0.0)			
Barbary lion (*Panthera leo leo*)*	B	1/2 (50.0)			
Bengal tiger (*Panthera tigris tigris*)*	B, E, G, H	2/8 (25.0)			
Binturong (*Arctictis binturong*)	B, G	0/3 (0.0)			
Black-backed jackals (*Canis mesomelas*)	K	0/1 (0.0)			
Bobcat (*Lynx rufus*)*	C	1/4 (25.0)			
Brown bear (*Ursus arctos*)	B, E	2/5 (40.0)			2
California sea lion (*Zalophus californianus*)	I, J	0/3 (0.0)			
Cheetah (*Acinonyx jubatus*)	D, F	0/2 (0.0)			
Common genet (*Genetta genetta*)	G	0/1 (0.0)			
Dohle (*Cuon alpinus*)	G	0/3 (0.0)			

Dwarf mongoose (*Helogale parvula*)	K	0/1 (0.0)			
Egyptian mongoose (*Herpestes ichneumon*)	G	0/1 (0.0)			
Eurasian lynx (*Lynx lynx*)*	B, D, E, H	1/7 (14.3)			1
European mink (*Mustela lutreola*)	C, E	0/3 (0.0)			
European otter (*Lutra lutra*)	C, E	0/4 (0.0)			
Fossa (*Cryptoprocta ferox*)*	K	2/2 (100.0)			
Giant panda (*Ailuropoda melanoleuca*)	B	0/3 (0.0)			
Grey seal (*Halichoerus grypus*)	I	0/1 (0.0)			
Harbor seal (*Phoca vitulina*)	A, I, J	0/10 (0.0)			
Iberian lynx (*Lynx pardinus*)	F	0/1 (0.0)			
Iberian wolf (*Canis lupus signatus*)*	C, E	6/12 (50.0)		1	
Jaguar (*Panthera onca*)	E	0/1 (0.0)			
Katanga lion (*Panthera leo bleyenberghi*)	K	0/3 (0.0)			
Kinkaju (*Potos flavus*)	G	0/1 (0.0)			
Lion (*Panthera leo*)	E, H, K	0/7 (0.0)			
Meerkat (*Suricata suricatta*)*	C, D, G, K	1/7 (14.3)			
Ocelot (*Leopardus pardalis*)*	C	1/1 (100.0)			
Persian leopard (*Panthera pardus saxicolor*)	B	2/2 (100.0)	1		
Raccoon (*Procyon lotor*)	B, G	0/3 (0.0)			
Red fox (*Vulpes vulpes*)	G	0/1 (0.0)			
Red panda (*Ailurus fulgens fulgens*)	B	0/2 (0.0)			
Serval (*Leptailurus serval*)*	G	2/5 (40.0)	1		
South African fur seals (*Arctocephalus pusillus*)	H	0/2 (0.0)			
South American coati (*Nasua nasua*)	G	0/1 (0.0)			
South American sea lion (*Otaria byronia*)*	B, D, K, I	1/18 (5.6)		1	
Spotted hyaena (*Crocuta crocuta*)	F, K	0/7 (0.0)			
Spotted-necked otter (*Hydrictis maculicollis*)	K	0/2 (0.0)			
Sri Lankan leopard (*Panthera pardus kotiya*)	G, K	0/9 (0.0)			
Striped skunk (*Mephitis mephitis*)	G	0/1 (0.0)			
Sumatran tiger (*Panthera tigris sumatrae*)*	G	1/5 (20.0)			
Sun bear (*Helarctos malayanus*)*	B	1/2 (50.0)	1		
TOTAL		25/171 (14.6)	3	2	4
Order Perissodactyla					
Cape mountain zebra (*Equus zebra*)	C	0/3 (0.0)			
Common zebra (*Equus quagga*)	C, D, H, K	0/7 (0.0)			

Indian rhinoceros (*Rhinoceros unicornis*)	B	0/1 (0.0)			
Lowland tapir (*Tapirus terrestris*)	C	0/2 (0.0)			
Malayan tapir (*Tapirus indicus*)*	B, G	1/6 (16.7)			1
Przewaslki's horse (*Equus caballus przewalskii*)	C	1/3 (33.3)			1
Somali wild ass (*Equus africanus somalicus*)	F	0/1 (0.0)			
White rhinoceros (*Ceratotherium simum*)*	B, K	3/5 (60.0)		1	
TOTAL		5/28 (17.9)	0	1	2
Order Proboscidea					
African elephant (*Loxodonta africana*)	K	0/6 (0.0)			
Asian elephant (*Elephas maximus*)	B, E, F	0/8 (0.0)			
TOTAL		0/14 (0.0)	0	0	0
Order Rodentia					
Cape porcupines (*Hystrix africaeaustralis*)	G	0/3 (0.0)			
Capybara (*Hydrochoerus hydrochaeris*)	C, E	0/3 (0.0)			
Coypu (*Myocastor coypus*)	K	0/1 (0.0)			
Crested porcupine (*Hystrix cristata*)	E	0/3 (0.0)			
Mara (*Dolichotis patagonum*)	E	0/5 (0.0)			
Woodchuck (*Marmota monax*)	D	0/2 (0.0)			
TOTAL		0/17 (0.0)	0	0	0
TOTAL ZOO ANIMALS		**36/425 (8.5)**	**4**	**3**	**7**

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Chapter 2.6.

Hepatitis E virus circulation in free-ranging and captive cetaceans in Spain, 2011-2022

Caballero-Gómez, J., Rivero-Juárez, A., Beato-Benítez, A., Fernández-Maldonado, C., Domingo, M., García-Párraga, D., Sierra, E., Segura-Göthlin, S., Martínez-Nevado, E., Sierra, C., Canales, R., García-Bocanegra, I. **Hepatitis E virus circulation in free-ranging and captive cetaceans in Spain, 2011-2022**. *Transboundary and Emerging Diseases*. *Under review.*

Abstract

Hepatitis E virus is an emerging zoonotic pathogen of worldwide distribution that has been detected in an increasing range of terrestrial and even aquatic animal species over the last decade. Information about the susceptibility of cetaceans to this emerging virus is still very scare. The aims of this study were: (1) to determine the seroprevalence and prevalence of HEV in both free-ranging and captive cetaceans in Spain; and (2) to assess the dynamics of seropositivity in captive individuals sampled longitudinally during the study period. Between 2011 and 2022, serum and/or liver samples from 305 cetaceans, including 241 free-ranging and 64 captive animals, were collected across the Spanish coastline and in six different zoological parks of this country. In addition, 30 of the 64 cetaceans kept in captivity were longitudinally surveyed during the study period. Sixty-eight (50.0%; 95% CI: 41.6-58.4) of the 136 cetaceans tested by ELISA showed anti-HEV antibodies. Seropositivity was detected in six of the nine species serologically analyzed. Significantly higher seroprevalence was found in free-ranging (61.1%) than in captive (37.5%) animals. Within the free-ranging population, the multivariate analyses identified “age” (adult) as a risk factor potentially associated with HEV exposure in cetaceans. In the longitudinal survey, seroconversions were detected in two out 64 bottlenose dolphins (*Tursiops truncatus*) during the study period. None (0.0%; 95% CI: 0.0-1.2) of the 303 analyzed cetaceans were positive for active HEV infection by PCR. To the best of the authors’ knowledge, this is the first study to assess HEV circulation in cetaceans, the first report of exposure to the virus being in Atlantic spotted (*Stenella frontalis*), common (*Delphinus delphis*), Risso (*Grampus griseus*) and striped (*Stenella coeruleoalba*) dolphins as well as in Cuvier's Beaked (*Ziphius cavirostris*) and killer (*Orcinus orca*) whales. Our results point high HEV exposure in cetaceans in Spain, indicating widespread circulation of this virus

in both free-ranging and captive populations over the last decade. Further studies are required to assess the role of cetaceans in the epidemiology of the virus and to identify the sources of HEV transmission in these species.

Keywords: *Hepatitis E, Orthohepevirus, emerging, cetaceans, aquatic*.

Introduction

Hepatitis E virus (HEV; family *Hepeviridae*; species *Orthohepevirus A*) is the leading cause of acute viral hepatitis in humans with more than 20 million cases annually worldwide (WHO, 2021). Eight different genotypes (HEV-1 to HEV-8) have been recognized so far, HEV-1 to HEV-4 being the most important in terms of public health concern. HEV-1 and HEV-2 are restricted to human beings whereas HEV-3 and HEV-4 are zoonotic (Kamar et al., 2017). Of them, HEV-3 is the genotype with the widest distribution, including Europe where the number of autochthonous hepatitis E (HE) cases have sharply increased during the past decade (Aspinall et al., 2017). The main reservoirs of HEV-3 are domestic pigs and wild boar, but a wide range of other mammals has been shown to be susceptible to this emerging genotype (Kenney, 2019; Wang & Meng, 2020).

The virus is mainly shed in the faeces of infected species, which may lead to viral contamination of the environment (Fenaux et al., 2019). In this regard, a wide variety of water sources has tested positive to HEV, including those from tap, river and sea water, and a high homology with human and/or animal HEV-3-related strains have been confirmed in these samples (Iaconelli et al., 2015; La Rosa et al., 2017; Wang et al., 2020). Of note, similar findings have also been detected in echinoderms and several species of bivalve shellfish from coastal waters (Crossan et al., 2012; Rivadulla et al., 2019; Santos-Ferreira et al., 2020; Gao et al., 2015), which indicate HEV circulation in marine environments.

The marine ecosystems from Atlantic Ocean and Mediterranean Sea harbor a large biodiversity, which include a wide range of cetacean species (Coll et al., 2010; Danovaro et al., 2010; MITECO, 2020). However, over the last decades cetacean populations have been threatened by different biotic and abiotic factors, such as industrial fishing, chemical pollution,

ocean warming, and also infectious diseases (Bearzi & Reeves, 2021; Coll et al., 2010; Bossart & Duignan, 2018). With respect to this last factor, different emerging pathogens, including cetacean morbillivirus, have caused important mortalities in many cetacean species, thus provoking a great impact on their populations (Van Bressem et al., 2012, 2014). In addition, for some of those agents, terrestrial mammals act as their natural reservoirs (Dubey, 2010). The circulation of shared pathogens, such as *Brucella sp.*, *Toxoplasma gondii*, *Erysipelothrix rhusiopathiae* and *Lacazia loboi*, among others, in both terrestrial and marine ecosystems (Miller et al., 2008; Van Bressem, 2012; Paniz-Mondolfi et al., 2012), provides evidence of common epidemiological cycles among land and aquatic animals. However, the susceptibility of cetaceans to other shared and emergent pathogens, such as HEV, and their possible role in the epidemiology of this virus is still unknown (Montalvo-Villalba et al., 2021). The aims of the present large-scale were therefore: (1) to determine the seroprevalence and prevalence of HEV in both free-ranging and captive cetacean populations in Spain and (2) to assess the dynamics of seropositivity in individuals that were sampled longitudinally during the study period.

Material and methods

Population and sampling

Between 2011 and 2022, 305 cetaceans belonging to 13 different species were sampled in Spain (Figure 1; Table 1). A total of 241 (79.0%) were free-ranging animals found dead across the coastline of the Atlantic Ocean and the Mediterranean Sea of this country. In addition, 64 (21.0%) were cetaceans kept in captivity in six different zoological parks (A-F) from Spain, the country of the European Union (EU) with the highest number of cetacean zoo parks (EU Dolphinaria report, 2014).

Blood samples were collected from 146 cetaceans (Figure 1), including 82 free-ranging and 64 captive individuals. Thirty of these 64 animals kept in captivity were longitudinally surveyed during the study period. During follow-up, the median (Q1-Q3) interval between consecutive samplings was 35 months (22-118.5). Sera were obtained by blood centrifugation at 400x *g* for 15 min. In addition, liver was gathered from 235 of the free-ranging sampled animals (Figure 1). Samples were stored at -20ºC until laboratory analyses. Epidemiological information, including species, age, gender, habitat status (free-ranging *vs* captivity), origin (free-ranging areas [Atlantic Ocean *vs* Mediterranean Sea] and zoological parks), sampling date and georeferenced location, was gathered from each animal, whenever possible.

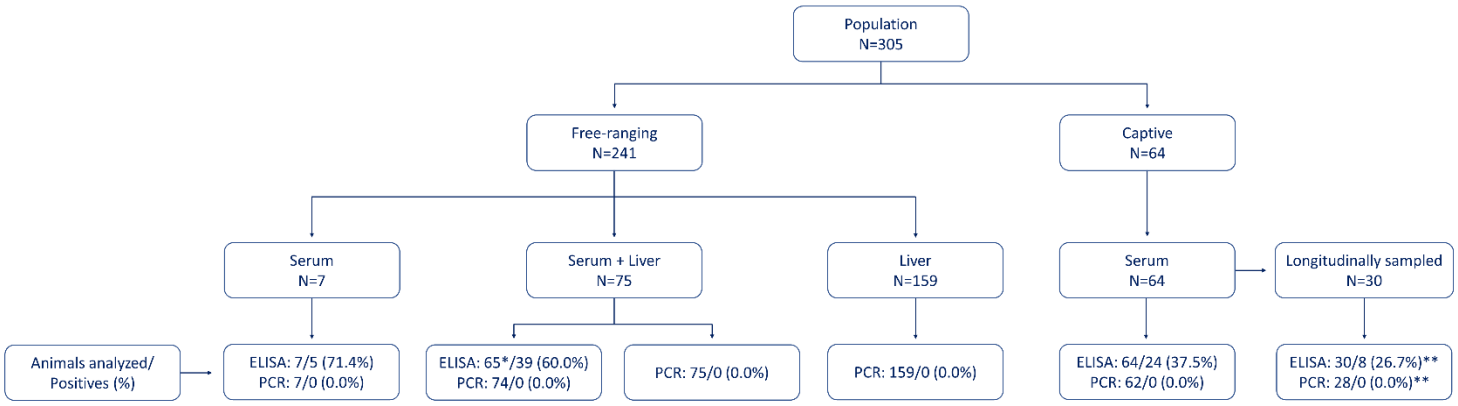

Figure 1. Description of the study population, number of cetaceans and type of samples analyzed by ELISA and PCR and results obtained in each assay. *Ten out of 75 serum samples were discarded for serological analyses due to hemolysis. **97 and 78 samples were analyzed by ELISA and PCR respectively.

Laboratory analyses

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

from a wide range of animal species.

RNA from serum and liver samples was extracted using the QIAmp MinElute Virus Spin and Rneasy Mini kits (QIAgen, Hilden, Germany), respectively, whenever possible (Figure 1). Liver samples were extracted individually whereas RNA from serum samples were obtained using pools of four samples (total volume: 400µl). The presence of HEV RNA was determined using two RT-PCR assays in parallel. A real-time RT-PCR (CFX Connect Real Time PCR System) that detects all the genotypes of HEV-A was performed using 25µl of RNA template and the QIAGEN One-Step RT-PCR kit as previously described (Frias et al., 2021). The detection limit was set up at 21.9 IU/mL (95% Confidence Interval (95% CI): 17.4-34.3). In addition, a nested broad-spectrum RT-PCR (Fisher Scientific Applied Biosystems SimpliAmp™), which is capable of detecting HEV-A, HEV-B, HEV-C and HEV-D, was carried out using the QIAGEN One-Step RT-PCR kit for the first round of RT-PCR and the premixed 2X solution containing Taq DNA Polymerase, dNTPs and Reaction Buffer (Promega, Madison, WI, USA) for the second round (Johne et al., 2010). The amplicons of the nested RT-PCR were examined on 1.5% agarose gels stained with RedSafe™ Nucleic Acid Staining solution (iNtRON Biotechnology, Seongnam, Korea).

Statistical analyses

Seroprevalence and prevalence were calculated by dividing the number seropositive and positive animals by the total of animals tested, using two-sided exact binomial 95% CI. Associations between presence of anti-HEV antibodies and the variable “habitat status” were analyzed using Pearson’s chi-square test. To avoid a possible collinearity bias, free-ranging and captive cetacean populations were tested separately. Associations between seroprevalence and explanatory variables (species, age and gender) were analyzed using the Pearson’s chi-

square or Fisher's exact tests, as appropriate. Variables with $p<0.05$ in the bivariate analysis were included for further analysis. Collinearity between pairs of variables was tested using the Spearman's Rho test. Finally, a generalized estimating equation model (GEE) was used to assess the effect of the variables previously selected in the bivariate analysis. "Origin" was included as a random factor and the number of seropositive animals was assumed to follow a binomial distribution. Variables with $p<0.05$ in the multivariate analyses were considered statistically significant. Statistical analyses were carried out using SPSS 25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA).

Results

A total of 68 (50.0%; 95% CI: 41.6-58.4) out of 136 cetaceans showed anti-HEV antibodies (Figures 1 and 2). The distribution of seroprevalence according to species, age and gender is shown in Table 1. Seropositivity was detected in six of the nine species serologically analyzed, with frequencies that ranged between 35.1% in bottlenose dolphin (*Tursiops truncatus*) to 100.0% in Atlantic spotted (*Stenella frontalis*) dolphin and Cuvier's Beaked whale (*Ziphius cavirostris*) (Table 1). None of the 212 serum (individual prevalence: 0/143; 0.0%; 95%CI: 0.0-2.6) and 234 liver (0.0%; 95%CI: 0.0-1.6) samples were positive for HEV RNA (Figure 2).

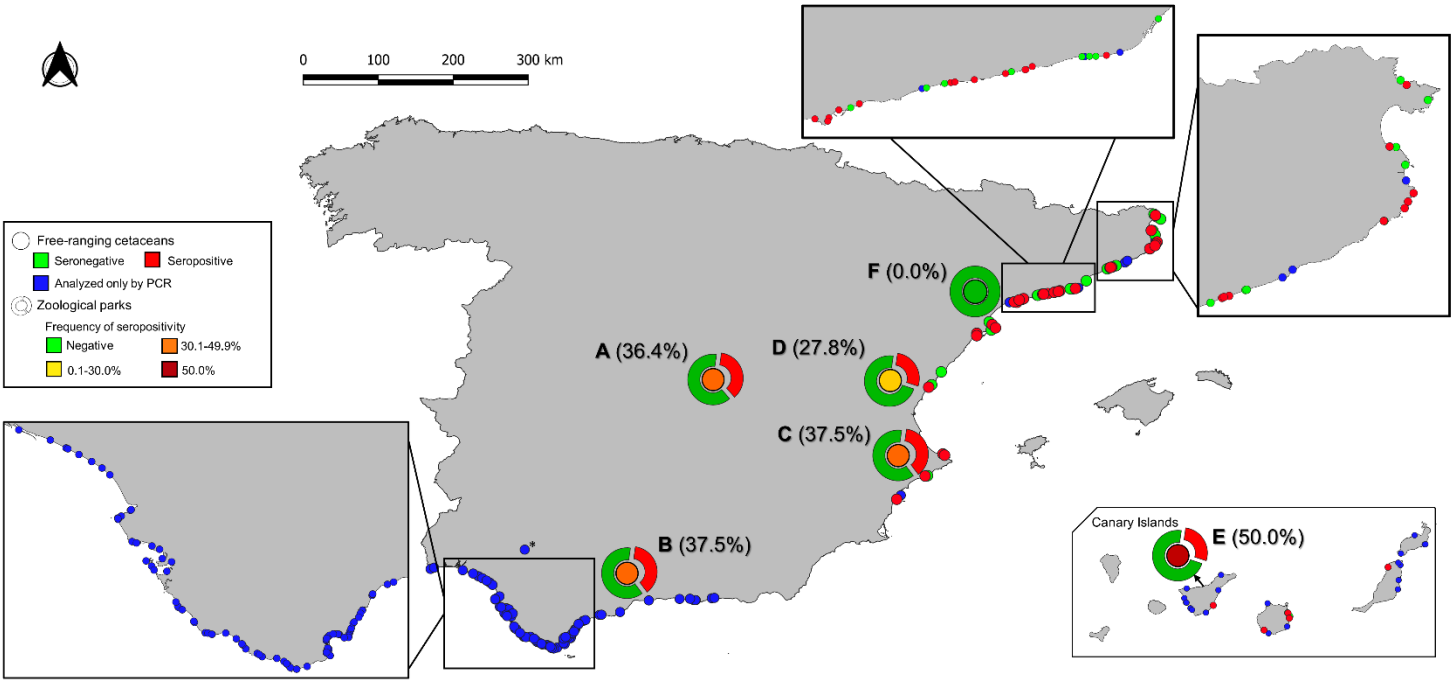

Figure 1. Spatial distribution of the cetaceans sampled in Spain. The frequency of seropositivity at each zoological park is shown in brackets. *Animal sampled in the Guadalquivir River.

Table 1. Distribution of HEV seroprevalence in free-ranging and captive cetacean populations and results of bivariate analysis.

		Free-ranging		Captivity	
Variable	**Categories**	**No. Positives/ No. Analyzed (%)***	***P*-value**	**No. Positives/ No. Analyzed (%)***	***P*-value**
Species**	Atlantic spotted dolphin	1/1 (100.0)	0.191	-	0.304
	Beluga	-		0/2 (0.0)	
	Bottlenose dolphin	0/2 (0.0)		20/55 (36.4)	
	Common dolphin	1/2 (50.0)		-	
	Cuvier's Beaked whale	1/1 (100.0)		-	
	Killer whale	-		4/7 (57.1)	
	Risso's dolphin	3/8 (37.5)		-	
	Southern long-finned pilot whale	0/1 (0.0)		-	

	Striped dolphin	38/57 (66.7)		-	
Age	Adult	33/45 (73.3)	0.006	20/47 (42.6)	0.220
	Young	11/27 (40.7)		3/12 (25.0)	
Gender	Female	25/39 (64.1)	0.373	12/33 (36.4)	0.593
	Male	19/33 (57.6)		11/30 (36.7)	

*Missing values excluded; **Samples from harbor porpoise and fin whale, minke and humpback whales were also included in the study but were only tested by PCR.

The seroprevalence was significantly higher in free-ranging (44/72; 61.1%; 95% CI: 49.9-72.4) than in captive (24/64; 37.5%; 95% CI: 25.6-49.4) animals (p=0.005; Relative risk=2.6; 95% CI: 1.3-5.2). In free-ranging cetaceans, the GEE model identified "age" as a risk factor potentially associated with HEV seropositivity. The seroprevalence was significantly higher in adult (33/45; 73.3%; p=0.006; OR: 4.0; CI95%: 1.4-11.0) compared with young animals (11/27; 40.7%). Of note, anti-HEV antibodies were detected in four yearling individuals (two striped (*Stenella coeruleoalba*), one Risso's (*Grampus griseus*) and one Atlantic spotted dolphins) in 2011, 2019 and 2021 from Mediterranean and Atlantic areas.

Although risk factors associated with HEV seropositivity were not found in captive cetaceans, seropositive animals were detected in five of the six sampled zoological parks, with within-zoo seroprevalence ranging between 27.8% in zoo D and 50.0% in zoo E (Table 2; Figure 2). Longitudinal samples were obtained in five of the six analyzed zoos. Twenty-one of the 30 longitudinally sampled animals remained seronegative, whereas six individuals showed seropositivity at all samplings during the study period (Table 3). In addition, two bottlenose dolphins from zoo D seroconverted in 2013 and 2017. Seroreversions were detected in two dolphins; one tested negative one and three years after the first sampling and the other animal, which was negative in 2009, was seropositive in two consecutive samplings in 2013 and 2017 and showed absence of anti-HEV antibodies in 2018 and 2020 (Table 3).

Table 2. Distribution of HEV seroprevalence in cetaceans in Spain by origin and results of bivariate analyses.

Variable	Categories	No. Positives /No. analyzed (%)	*P*
Free-ranging areas	Atlantic Ocean	6/8 (75.0)	0.327
	Mediterranean Sea	62/128 (48.4)	
Zoo	A	4/11 (36.4)	0.772
	B	3/8 (37.5)	
	C	3/8 (37.5)	
	D	5/18 (27.8)	
	E	9/18 (50.0)	
	F	0/1 (0.0)	

Table 3. Antibodies against hepatitis E virus in longitudinally sampled cetaceans. Colored dots indicate antibodies to hepatitis E virus (red: positive; green: negative). When two samplings were carried out in the same year, the abbreviated months are indicated in superscript.

Species	Zoo	Event	2009	2010	2013	2016	2017	2018	2019	2020	2021
Beluga	D	Seronegative at all samplings					●			●	
Bottlenose dolphin	A	Seronegative at all samplings					●		●		
Bottlenose dolphin	A	Seronegative at all samplings					●			●	
Bottlenose dolphin	A	Seropositive at all samplings					●		●	●	
Bottlenose dolphin	A	Seropositive at all samplings					●		●		
Bottlenose dolphin	A	Seronegative at all samplings				●Ap; ●Jun					
Bottlenose dolphin	A	Seronegative at all samplings					●		●		
Bottlenose dolphin	A	Seronegative at all samplings					●		●		
Bottlenose dolphin	A/B	Seronegative at all samplings					●			●	
Bottlenose dolphin	A/B	Seronegative at all samplings					●			●	
Bottlenose dolphin	C	Seronegative at all samplings							●	●	
Bottlenose dolphin	C	Seropositive at all samplings							●	●	
Bottlenose dolphin	C	Seronegative at all samplings							●		●
Bottlenose dolphin	C	Seronegative at all samplings							●		●
Bottlenose dolphin	D	Seroconversion			●Aug; ●Nov		●	●		●	
Bottlenose dolphin	D	Seropositive at all samplings					●	●		●	
Bottlenose dolphin	D	Seropositive at all samplings					●	●		●	
Bottlenose dolphin	D	Seroconversion & Seroreversion	●		●		●	●		●	
Bottlenose dolphin	D	Seroreversion					●	●		●	
Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
Bottlenose dolphin	D	Seronegative at all samplings					●	●			
Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
Bottlenose dolphin	D	Seronegative at all samplings	●		●		●	●		●	
Bottlenose dolphin	D	Seronegative at all samplings		●	●		●	●		●	
Bottlenose dolphin	E	Seropositive at all samplings									●Feb; ●Jun

Discussion

To the best of the authors' knowledge, this is the first study to assess HEV exposure in free-ranging cetaceans and the first large-scale and long-term surveillance to evaluate circulation of the virus in captive populations of these marine mammals. Our results show a high exposure to this emerging virus in both free-ranging and captive cetacean populations in coastline and zoo parks in Spain. Seropositivity was detected in six different cetacean species. The. The detection for first time of anti-HEV antibodies in Atlantic spotted, common (*Delphinus delphis*), Risso and striped dolphins as well as in Cuvier's Beaked and killer whales (*Orcinus orca*) increases the range of susceptible species to this virus.

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 it has also been suggested for dolphins (Montalvo-Villalba et al., 2017). The seropositive species detected in the present study have a wide variety of food resources, including fish and cephalopods, among others. Although the presence of HEV in these food resources has not been assessed yet, the virus has frequently been detected in other marine animals such as sea urchins (*Paracentrotus lividus*) and bivalve shellfish in different European areas, including coastline Spain (Crossan et al., 2012; Pol-Hofstad et al., 2014; Mesquita et al., 2016; Santos-Ferreira et al., 2020), which evidences the presence of HEV in sea environments. Of note, HEV has been shown to be highly resistant to even high concentrations of salt (up to 20%) (Wolff et al., 2020). Additionally, contaminated water has also been considered a potential source for zoonotic HEV (Fenaux et al., 2019) since its consumption from private well or a nearby river as well as of tap water have been suggested as risk factors for acquiring HEV infection in humans (Renou

et al., 2008; Mansuy et al., 2016). The detection of seropositivity or active HEV infection in captive cetaceans that all shared the same pools in the only study conducted in cetaceans to date supports this hypothesis (Montalvo-Villalba et al., 2017).

Significantly higher seroprevalence was found in adult free-ranging individuals compared with young animals, which reflects the likelihood of cumulative exposure to HEV in these species. An increasing trend can also be observed in older captive animals although statistically significant differences were not detected, probably due to the low number of young cetaceans sampled. Anti-HEV antibodies were found in four free-ranging yearlings individuals in 2011, 2019 and 2021, which could suggest endemic circulation of HEV in cetaceans living in Spanish coasts during the study period, although the presence of maternal antibodies in yearling mammals cannot be ruled out. In addition, free-ranging cetaceans had 2.5-times higher risk of being exposed to HEV than those kept in captivity. This finding could be explained by differences in feeding or longer exposure through environmental contamination. Human and swine related HEV-3 has been detected in sewage and slurry of Spain (Clemente-Casares et al., 2009) as well as in river or coastal waters from Italy (La Rosa et al., 2018; Iaconelli et al., 2020). The high census of some susceptible domestic and wildlife species (Bosch et al., 2012; MAPA, 2020) as well as the great urbanization of the coast and the insufficient control of urban wastewaters in some regions of the study area (EU Council Directive 91/271/CEE) (EC, 2020, 2022) are possible factors associated with the higher seropositivity detected in free-ranging cetaceans. By contrast, cetaceans in zoological parks live in large water tanks frequently decontaminated with chlorine, to which HEV has been observed to be sensitive (Fenaux et al., 2019). Of note, the high seroprevalence observed in captive (37.5%) cetaceans indicates that the virus also circulates in these more controlled environments. In this regard, although the

presence of HEV in water zoo tanks has not been assessed so far, it should be noted that the amount and contact time of chlorine and the presence of sewage, among others, affect the inactivation of HEV in water (Girones et al., 2014). Additional studies in both captive and free-ranging settings are needed to elucidate the differences in HEV seroprevalences.

The frequency of seropositivity observed in zoo animals (33.3%) is consistent with the study conducted by Montalvo-Villalba et al. (2017) in a zoological park from Cuba (Montalvo-Villalba et al., 2017). These authors found that ten out of the 31 (32.3%) tested bottlenose dolphins had anti-HEV antibodies. In the present study, high seroprevalences were detected in the five seropositive zoos, which denote widespread circulation of HEV in captive cetaceans in zoos in Spain. In addition, the two seroconversions detected in captive bottlenose dolphins support the hypothesis of HEV circulation in zoos during the study period. On the other hand, four of the longitudinally surveyed cetaceans remained seropositive at all samplings. This could be associated with the lifelong persistence of anti-HEV antibodies in cetaceans, which has previously been denoted in other mammal species (Caballero-Gómez et al., 2019) and is supported by the significantly higher seroprevalence detected in older free-ranging cetaceans. However, the information about the persistence of anti-HEV antibodies in these species is unknown and studies conducted to date on human and non-human primates report a wide range between two and more than 14 years post-infection (Arankalle et al.,1999; Li et al., 1994; Krain et al., 2014). Thus, possible loss of antibodies and re-exposure in some of the persistently seropositive cetaceans during the study period cannot be ruled out. In connection with this, seroreversions were observed in two bottlenose dolphins one and five years after the first seropositive sampling was detected.

None of the serum and liver samples tested positive for *Orthohepevirus* species RNA,

which evidence absence of active infection in these individuals at the sampling point. In previous studies, Montalvo-Villalba et al. (2017) found HEV RNA in five (16.1%) of the 31 zoo dolphins, confirming infection by the zoonotic HEV-3 genotype in serum of two animals. Molecular results obtained in the present study suggest that cetaceans could play a limited role in the transmission of HEV, as previously suggested (Montalvo-Villalba et al., 2017). Nevertheless, given the high frequency of seropositivity observed, the close contact between cetaceans and humans in certain epidemiological contexts, such as zoos, and taking into account that HEV is mainly shed in the faeces and that it was not possible to assess HEV excretion in stool from these animals, further studies should be carried out to assess the risk of HEV transmission from cetaceans to other mammals, including humans.

In conclusion, the seropositivity found in the present study indicates widespread circulation of HEV in both free-ranging and captive cetacean populations of southwestern Europe. However, the absence of active infection suggests that these species play a limited role in the epidemiology of HEV. Additional studies are warranted to elucidate the sources and differences of HEV infection in free-ranging and captive cetacean populations.

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SYNTHESIS

During the last decades, the number of zoonotic hepatitis E (HE) cases in humans in industrialized countries has drastically increased. At the same time, the host range of the virus (HEV) has considerably expanded, being not only Suidae, which are considered the main reservoirs of HEV, but also other animal species recognized as susceptible to the virus. However, the information about their role in the epidemiology of HEV and the risk of zoonotic transmission from these species is still very limited. The present doctoral thesis evaluates the role of domestic animals, including pet and livestock, and both free-ranging and captive wildlife species in the epidemiology of HEV in Spain. This synthesis assembles the most relevant results derived from the research carried out, highlighting the risk factors associated with HEV exposure and the potential implications for public health.

Dogs and cats are the most common companion animal species in the world. In Europe, there are more than 195 million cats and dogs and approximately 25% of households own either of these two species. The risk of transmission of different zoonotic pathogens from cats and dogs has already been evidenced. This threat increases taking into account the growing number of stray animal populations without veterinary check-ups, particularly feral cats, observed in different countries, including Spain. Contact with infected animals has shown to be one of the main transmission routes for zoonotic genotypes of HEV. Exposure to the virus has already been confirmed in dogs and cats worldwide, zoonotic transmissions from both species being already suggested. However, the information about HEV infection in cats and dogs is still very limited. Therefore, in **Chapter 1.1**, we assessed HEV circulation, including HEV-A, HEV-B and HEV-C, in sympatric urban cats and dogs in Spain. Our results confirmed that both species are natural but not equally exposed to the virus and suggest circulation of

HEV-3 and HEV-C1 in urban cats and dogs in the province of Cordoba (southern Spain). Taking into consideration that these species may be exposed to HEV through the same source of contamination as humans, cats and dogs could be potential sentinels of environmental circulation of orthohepeviruses in urban and periurban areas. However, the absence of active infection denotes that these pet species may play a limited role in the epidemiology of HEV in southern Spain.

Other domestic animals that are in close contact with human beings are equines. These species are among the most important animals in human history given their utility in work, as a mean of transport, farming, leisure and also as a common food source. In this regard, besides the direct or indirect contact with infected animals, the consumption of animal products is also considered one of the major transmission routes of zoonotic HEV genotypes. Despite this, only two studies have assessed so far HEV circulation in equines, one conducted in Africa and the other in Asia. Andalusia is the region with the largest number of both equines and herds in Spain, with more than 220 thousand individuals and 74 thousand herds, respectively. In the **Chapter 1.2**, we determined the prevalence of HEV in equine species in Andalusia (southern Spain), being 0.4% in horses, 1.2% in donkeys and 3.6% in mules. These results, together with the detection of human related HEV strains in one horse confirm the susceptibility of equine species to HEV and suggest a potential risk of zoonotic transmission, although these species seem to play an epidemiological role as spillover hosts rather than true reservoirs.

Besides equines, HEV infection has also been confirmed not only in the main domestic animal reservoir of the virus, the domestic pig, but also in other food production animal species, such as ruminants. In addition, the high homology between viral strains obtained from domestic ruminants and human beings as well as the detection of the virus in derived products

from these species have raised concern about the risk of zoonotic transmission. Spain, with more than 15.4 and 2.6 million sheep and goats respectively, is the country with the largest census of small domestic ruminants in the European Union (EU) but the prevalence, spatial distribution and risk factors associated with HEV exposure in sheep and goats from this country is unknown. Thus, the **Chapter 1.3** provides evidence of widespread but unequal HEV exposure in small ruminant populations from southern Spain. In addition, the species and the number of animals within the farm was identified as risk factors associated with exposure to HEV, which add information about potential control measures to limit the transmission of this zoonotic virus in small ruminant populations.

A widespread distribution of HEV has not only been described in domestic animals but also in wildlife, such as wild boar and in a lesser extent deer. However, insights into the epidemiology of HEV in wildlife originate mostly from these population species and also from hunting estates, which particularly in southwestern Spain are frequently intensively managed and shares natural resources with extensively raised domestic pigs. Therefore, the circulation of this emerging virus in other areas with limited human activity and absence of pig industry, such as national parks is still poorly understood. In **Chapter 2.1**, the widespread distribution of HEV in wild boar populations in the Doñana National Park and the detection of an emergent and zoonotic strain of HEV (HEV-3r, currently renamed as HEV-3m) highlight the importance of wild boar in the epidemiology of HEV in these scenarios and confirm the risk of zoonotic transmission from this species.

Whereas wild boar is the most abundant big game harvested species in Spain, wild lagomorphs are the most important small game animals in terms of abundance, and hunting and conservation interests. In this country, about 5.3 million wild rabbits and 890 thousand

hares are harvested annually and are commonly consumed without sanitary inspection, since they are not usually intended for retail sale. In addition, wild rabbit and Iberian hare are keystone species in the Iberian Mediterranean ecosystems, being the staple prey for a large number of predators, including endangered species such as the Iberian lynx (*Lynx pardinus*) and the Spanish imperial eagle (*Aquila adalberti*). Despite this, there is very little information on the role of wild lagomorphs in the epidemiology of HEV in Europe. In **Chapter 2.2**, we described the absence of active circulation in both wild rabbit and Iberian hare populations from southern Spain, thus indicating a limited risk of transmission to other sympatric mammals. With this respect, the Iberian lynx is one of the most endangered felids in the world and one of the most endangered carnivores in Europe. This species, as commented above, is a trophic specialist that depends mainly on wild rabbits. In addition, free-ranging populations of the Iberian lynx shares habitat and natural resources with other domestic and wildlife susceptible species to HEV in the Mediterranean ecosystems, where endemic circulation of the virus has been confirmed. Despite this and the surveillance health programs conducted in free-ranging and captive populations, their susceptibility to HEV has not been evaluated to date. For these reasons, **Chapter 2.3** aimed to assess HEV circulation in both free-living and captive Iberian lynx populations, to determine potential risk factors associated to HEV exposure in this species and to evaluate the dynamics of seropositivity in longitudinally sampled animals during the study period. The high frequency of seropositivity, particularly in captive Iberian lynxes the detection of zoonotic genotypes, and the seroconversions observed along the study period indicate a widespread but not homogeneous spatiotemporal circulation of HEV in the Iberian lynx populations and evidence HEV infection in this endangered species, thus suggesting a possible role for this species as potential spillover hosts of the virus.

Interestingly, the high HEV circulation in Iberian lynx maintained in zoo-like settings with absence of contact with reservoir species, such as domestic pigs and wild boar, denote transmission of the virus through feed and raises concern about exposure to HEV in other wildlife species housed in similar epidemiological contexts, such as zoological parks. In this regard, zoo animals have been recognized to be involved in the epidemiological cycles of different pathogens of public health concern. In addition, zoos house a large diversity of animal species which are routinely veterinary controlled, being therefore considered useful settings to obtain insights into the epidemiology and distribution of emerging zoonotic pathogens with wide host range, such as HEV. However, the information about the epidemiology of this virus in zoo species is very scarce and originate mostly from certain animal species and/or analyzed a limited number of zoos or animals. One of the main orders of interest to conduct surveys on zoonotic infectious diseases are non-human primates (NHPs) due to their close genetic and physiological similarities with human beings. The results obtained in **Chapter 2.4** confirm susceptibility of NHPs to natural exposure to HEV and denote circulation of the virus in zoological parks in Spain. These findings were also consistent with the first large-scale surveillance conducted on HEV in different orders of zoo mammals, presented in **Chapter 2.5**. This study provides evidence of exposure to HEV-3 and/or HEV-C1 in artiodactyl, carnivore and perissodactyl species and indicate a widespread but not homogeneous spatiotemporal circulation of HEV in zoo animals in Spain. **Chapters 2.4** and **2.5** have increased the host range of HEV, since exposure to the virus was described for the first time in different non-human primate, carnivore, artiodactyl and perissodactyl species, and suggest differences in the susceptibility of zoo species to HEV exposure, being higher in hominids than in other NHP families and in carnivores and perissodactyls than in artiodactyl species.

Cetaceans are other important animal order often kept in captivity in zoo parks worldwide. In addition, both free-ranging and captive populations have been recognized as useful sentinel species to monitor zoonotic emerging infectious diseases in marine-terrestrial interface ecosystems wherein humans are integrated. HEV is mainly shed in the faeces of infected mammals, thus facilitating viral contamination of the environment, including water resources such as tap, river and sea water. However, there is very little information so far about the susceptibility of cetacean species to HEV infection and their possible role in the epidemiology of the virus. In **Chapter 2.6**, we describe the first study to assess *Orthohepevirus* exposure in free-ranging cetaceans and the first large-scale and long-term surveillance to evaluate circulation of the virus in captive populations of these marine mammals. Serological results found in that study confirmed endemic but not equal HEV circulation in both free-ranging and captive cetacean populations of southwestern Europe. The high seroprevalence detected in these species (49.3%), particularly in those free-ranging (61.1%), together with their long-life spans, long-term coastal residences (of free-ranging animals) and feed at a high trophic level may indicate that cetaceans could be useful sentinel species to monitor HEV activity in aquatic ecosystems. However, the absence of active infection suggests that these marine mammal species play a limited role in the transmission of HEV.

The results obtained in the present Doctoral Thesis gives insights into the epidemiological role of unknown and already known susceptible species to HEV that will allow to understand better the epidemiology of this emerging virus and to prioritize HEV monitoring on certain species. HEV circulation has been confirmed in different epidemiological contexts, including households (**Chapter 1.1**), farms (**Chapter 1.3**), Mediterranean ecosystems (**Chapters 1.2, 2.1, and 2.3**), zoos (**Chapters 2.3, 2.4, 2.5 and 2.6**)

and sea (**Chapter 2.6**). The identification of novel hosts that could be implicated in the epidemiology of HEV (**Chapter 2.3, 2.4, 2.5 and 2.6**), the detection of emerging subtypes in both known (**Chapter 2.5**) or unknown (**Chapters 1.1** and **2.1**) susceptible species and the identification of risk factors associated with HEV exposure in both domestic (**Chapters 1.1** and **1.2**) and wildlife (**Chapters 2.1**, **2.3**, **2.4**, **2.5** and **2.6**) species, or potential sentinel species (**Chapter 1.1** and **2.6**) should be taken into account for epidemiological monitoring of HEV.

Finally, the research carried out in this Doctoral Thesis opens the door to many future research directions. Given that the viraemia in animal reservoirs seems to be limited, the evaluation of the presence of anti-HEV antibodies, that persist longer, would allow to get a more global information about HEV exposure in those animal species that were not analyzed by ELISA, such as horses, donkeys, mules and wild rabbits. Antibodies against HEV-C1 was confirmed in dogs, cats and different zoo species, as well as suggested in Iberian lynx. Taking into consideration that rodents are the main reservoir species of this zoonotic subtype and that the information about HEV-C1 circulation in rodents in Spain is still very limited, molecular and serological surveys on this population should be conducted. On the other hand, the high frequency of seropositivity to HEV found in captive Iberian lynx, which are mainly fed with farmed rabbits, point the need to carry out a surveillance program of this emerging virus in these lagomorphs in Spain. Additionally, a high seroprevalence was observed in cetaceans kept in captivity but it was even higher in free-ranging populations. The assessment of HEV circulation in marine environments, including samples from sea water and aquatic animal species, would provide valuable information in the epidemiology of the virus.

CONCLUSIONS

1. Exposure to HEV was confirmed in different companion and food production domestic species, including horses, donkeys, mules, goats, sheep, cats and dogs. The absence or limited active infection detected in these animals suggests that these species might not have a relevant role in the epidemiology of HEV in southern Spain.

2. Antibodies against HEV-3 and/or HEV-C1 found in cats and dogs point circulation of both genotypes in these species in southern Spain. Taking into account that these pets may be exposed to HEV through the same source of contamination as humans, cats and dogs could be potential sentinel species of environmental circulation of hepeviruses in urban and periurban areas.

3. The seropositivity found in small ruminants provides evidence of widespread but unequal HEV exposure in these species in southern Spain. Goats, and big (≥538 animals) and small (≤348 animals) flocks of small ruminants presented higher risk to be exposed to HEV. Surveillance on this species and in small ruminant farms with these flock sizes would better and more efficiently characterize the risk of zoonotic transmission of this emerging virus from small ruminants.

4. The prevalence of antibodies against HEV in wild boar from the Doñana National Park highlighted a high exposure, widespread distribution and endemic circulation of the virus in this protected area. This finding, together with the detection of the emergent HEV-3m strain in this species, point the importance of wild boar in the epidemiological cycle of this virus and their role in the maintenance of HEV even in the absence of domestic pigs.

5. Active HEV circulation was not detected in European wild rabbit and Iberian hare in southern Spain, which suggest a limited risk of transmission of HEV from these wild lagomorph species to other mammals in this country. By contrast, serological and molecular results provide evidence of HEV-3 infection in both free-ranging and captive populations of the Iberian lynx, a trophic specialist that depends mainly on wild rabbits, and suggest a possible role for this endangered felid species as spillover host of this virus in Iberian Mediterranean ecosystems. We also confirmed a widespread but not homogeneous circulation of HEV in Iberian lynx populations, being particularly high in those kept in captivity.

7. The seropositivity found in the zoo species sampled confirms the susceptibility of these animals to natural exposure of HEV, which could be of public health and conservation concerns. In addition, the results increase the range of species susceptible to HEV. The detection of higher seroprevalence in hominids compared with other non-human primate families and in perissodactyl and carnivore species compared with artiodactyls suggest differences in HEV exposure among zoo animals. The identification of specific antibodies against HEV-3 and HEV-C1 in different zoo species highlights circulation of two different emerging and zoonotic genotypes of HEV in urban and periurban areas, which could be of importance for public health.

8. The high prevalences of antibodies found against HEV in free-ranging and captive cetaceans point a high but not equal exposure as well as widespread distribution and endemic circulation of this virus in both populations over the last decade. Seropositivity to HEV was reported for the first time in five cetacean species, which increases the host range of this virus in aquatic ecosystems.

APPENDIX

Appendix I. Scientific communications derived from this doctoral thesis in international or national meetings.

Javier Caballero Gómez is the first author or co-author of three and 13 scientific communications related with the epidemiology of HEV presented in international and national meetings, respectively.

I.A. International meetings

Title: Hepatitis E virus in sympatric cattle and wild ungulates in the Doñana National Park, Spain.
Congress: Hepatitis E Workshop: Paradigm of a food-borne zoonotic emerging disease in Europe
Place: Madrid, Spain.
Date: 4-5th June 2018.
Organizing institution: VISAVET centre of Complutense University of Madrid.
Type of communication: Poster.
Authors: Caballero, J., García-Bocanegra, I., Jiménez-Ruiz, S., Vicente, J., Rivero, A., Risalde, M. A., Barasona, J. A., López-López, P., Rivero-Juárez, A.

Title: Subtipo emergente del virus de la hepatitis E en España.
Congress: 37èmes rencontres du Groupe d'Etudes sur l'Eco-pathologie de la Faune Sauvage de Montagne (GEEFSM).
Place: Etrouble, Italy.
Date: 13-16th June 2019.
Organizing institution: GEEFSM.
Type of communication: Oral.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Jiménez-Ruiz, S., López-López, P., Risalde, M. A., Cano-Terriza, D., Frias, M., Barasona, J. A., Rivero, A., García-Bocanegra, I.

Title: Estudio epidemiológico del virus de la hepatitis E en lince ibérico (*Lynx pardinus*).*
Congress: 38èmes Rencontres du GEEFSM.
Place: Lanslebourg, France.
Date: 7-10th October 2021.
Organizing institution: GEEFSM.
Type of communication: Oral.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Zorrilla, I., Alcaide-Martín, E., López, G., Nájera, F., Salcedo, J., Paniagua, J., García-Bocanegra, I.
*Awarded communication

I.B. National meetings

Title: Prevalencia de infección por virus de la Hepatitis E en el ganado bovino y ungulados silvestres en el Parque Nacional de Doñana

Congress: XXII Congreso Internacional de la Asociación Nacional de Especialistas en Medicina Bovina de España (ANEMBE).
Place: Pamplona, Spain.
Date: 28-30th June 2017.
Organizing institution: ANEMBE.
Type of communication: Poster.
Authors: Jiménez-Ruiz, S., Rivero-Juárez, A., Vicente, J., Rivero, A., Risalde, M. A., Cano-Terriza, D., Caballero, J., Barasona, J. A., García-Bocanegra, I.

Title: Infección por el virus de la Hepatitis E en equinos en España
Congress: III Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC).
Place: Seville, Spain.
Date: 28-30th September 2017.
Organizing institution: SEIMC.
Type of communication: Poster.
Authors: Jiménez Ruiz, S., García Bocanegra, I., Rivero Juárez, A., Caballero, J., Cano Terriza, D., Frías, M., Risalde, M. A., López-López, P., Zafra, I., Isla, J., Marmesat, B., Ruiz-Torres, L., Gómez Villamandos, J. C., Rivero, A.

Title: Circulación del virus de la hepatitis E en bovinos y ungulados silvestres en el Parque Nacional de Doñana
Congress: II Congreso de Investigadores Nóveles.
Place: Cordoba, Spain.
Date: 15th November 2017.
Organizing institution: University of Cordoba.
Type of communication: Poster.
Authors: Caballero, J., García-Bocanegra, I., Rivero-Juárez, A.

Title: Situación epidemiológica del virus de la hepatitis E en ungulados domésticos y silvestres en el Parque Nacional de Doñana
Congress: II Congreso de Veterinaria y Ciencia y Tecnología de los Alimentos.
Place: Cordoba, Spain.
Date: 9th February 2018.
Organizing institution: Faculty of Veterinary Medicine.
Type of communication: Oral.
Authors: Caballero, J., Jiménez-Ruiz, S., García-Bocanegra, I., Vicente, J., Rivero, A., Risalde, M. A., Barasona, J. A., Rivero-Juárez, A.

Title: Hepatitis E virus in captive nonhuman primates in zoos in Spain.
Congress: X Young Investigators Meeting of the Maimonides Research Institute of Cordoba (IMIBIC)
Place: Cordoba, Spain.
Date: 19-20th May 2019.
Organizing institution: IMIBIC.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Cano-Terriza, D., Risalde, M. A., Lopez-Lopez, P., Frias, M., Jiménez-Ruiz, S., Rivero, A., García-Bocanegra, I.

Title: Hepatitis E en conejo silvestre (*Oryctolagus cuniculus*) y liebre ibérica (*Lepus granatensis*): ¿son una fuente de infección zoonósica en el sur de España?
Congress: V Congreso Nacional de GEHEP de la SEIMC
Place: Caceres, Spain.
Date: 26-28th September 2019.
Organizing institution: SEIMC.
Type of communication: Oral.
Authors: Caballero-Gómez, J., García-Bocanegra, I., Gómez-Guillamón, F., Camacho-Sillero, L., Zorrilla, I., López-López, P., Frias, M., Zafra, I., Ruiz-Rubio, C., Rivero-Juárez, A.

Title: Subtipo 3r del virus de la hepatitis E en jabalí: un riesgo emergente ¿de importancia en Salud Pública?
Congress: V Congreso Nacional de GEHEP de la SEIMC.
Place: Caceres, Spain.
Date: 26-28th September 2019.
Organizing institution: SEIMC.
Type of communication: Poster.
Authors: Caballero-Gómez, J., López-López, P., Jiménez-Ruiz, S., Vicente, J., Risalde, M. A., Cano-Terriza, D., Frías, M., Barasona, J. A., Zafra, I., Rivero, A., Rivero-Juárez, A., Diaz-Cao, J. M., Ruiz-Torres, L., García-Bocanegra, I.

Title: Identificación de un subtipo emergente del virus de la Hepatitis E en el Parque Nacional de Doñana.
Congress: XXI Congreso de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI).
Place: Seville, Spain.
Date: 21-23rd November 2019.
Organizing institution: SAEI.
Type of communication: Poster.
Authors: Caballero-Gómez, J., López-López, P., Rivero-Juárez, A., Jiménez-Ruiz, S., Vicente, J., Risalde, M. A., Frías, M., Cano-Terriza, D., Zafra-Soto, I., Ruiz-Torres, L., Barasona, J.A., Rivero, A., García-Bocanegra, I.

Title: Hepatitis E en conejo silvestre y liebre ibérica: ¿son una fuente de infección zoonósica en Andalucía?
Congress: VIII Congreso científico de investigadores en formación de la Universidad de Córdoba.
Seville, Spain.
Date: 21-23rd November 2019.
Organizing institution: SAEI.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Gómez-Guillamón, F., Camacho-Sillero, L., Zorrilla, I., López-López, P., Frías, M., Ruiz-Torres, L., Zafra-Soto, I., García-Bocanegra, I.

Title: Hepatitis E en conejo silvestre (*Oryctolagus cuniculus*) y liebre ibérica (*Lepus granatensis*): ¿son una fuente de infección zoonósica en el sur de España?
Congress: VIII Congreso científico de investigadores en formación de la Universidad de Córdoba.

Place: Cordoba, Spain.
Date: 18-19th February 2020.
Organizing institution: University of Cordoba.
Type of communication: Oral.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., García-Bocanegra, I.

Title: Virus de la Hepatitis E en ovino y caprino en el sur de España
Congress: VI Congreso Nacional de GEHEP de la SEIMC.
Place: Granada, Spain.
Date: 21-23rd September 2021.
Organizing institution: SEIMC.
Type of communication: Oral.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Jiménez-Martín, D., Cano-Terriza, D., Risalde, MA., Casares-Jiménez, M., García-Bocanegra, I.

Title: Circulación del virus de la Hepatitis E en gatos y perros en la provincia de Córdoba
Congress: VI Congreso Nacional de GEHEP de la SEIMC.
Place: Granada, Spain.
Date: 21-23rd September 2021.
Organizing institution: SEIMC.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Jurado-Tarifa, E., Jiménez-Martín, D., Jiménez-Ruiz, E., Castro-Scholten, S., López-López, P., García-Bocanegra, I.

Title: Virus de la Hepatitis E en ovino y caprino en el sur de España
Congress: III Congreso Andaluz de Salud Pública.
Place: Cordoba, Spain.
Date: 23-24th September 2021.
Organizing institution: Consejo Andaluz de Colegios Oficiales Veterinarios.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Jiménez-Martín, D., Cano-Terriza, D., Risalde, MA., Casares-Jiménez, M., García-Bocanegra, I.

Appendix II. Other scientific publications derived from the research activity during the PhD.

Javier Caballero Gómez is the first or co-first author of five scientific articles related with the epidemiology of emerging infectious diseases. In addition, the PhD candidate is co-author of 26 scientific articles and five book chapters, the vast majority of those dealing with the epidemiology, diagnosis or control of infectious diseases of public and animal health concerns. Javier Caballero Gómez is also the first author or co-author of 13 and 26 scientific communications presented in international and national meetings, respectively, as well as author of two dissemination articles.

II.A. Research articles

Authors: Caballero-Gómez, J., Cano Terriza, D., Pujols, J., Martínez-Nevado, E., Carbonell, M. D., Guerra, R., Recuero, J., Soriano, P., Barbero, J., García-Bocanegra, I.
Title: Monitoring of bluetongue virus in zoo animals in Spain, 2007–2019.
Journal: Transboundary and Emerging Diseases (2021). Doi: 10.1111/tbed.14147

Authors: Caballero-Gómez, J., García-Bocanegra, I., Navarro, N., Guerra, R., Martínez-Nevado, E., Soriano, P., Cano-Terriza, D.
Title: Zoo animals as sentinels for Schmallenberg virus monitoring in Spain.
Journal: Veterinary Microbiology (2021), *252*, 108927. Doi: 10.1016/j.vetmic.2020.108927.

Authors: Caballero-Gómez, J., Cano-Terriza, D., Lecollinet, S., Carbonell, M. D., Martínez-Valverde, R., Martínez-Nevado, E., García-Párraga, D., Lowenski, S., García-Bocanegra, I.
Title: Evidence of exposure to zoonotic flaviviruses in zoo mammals in Spain and their potential role as sentinel species.
Journal: Veterinary Microbiology (2020), *247*,108763 Doi: 10.1016/j.vetmic.2020.108763

Authors: López-López, P., Frias, M., Camacho, A., Machuca, I., Caballero-Gómez, J., Risalde, M. A., García-Bocanegra, I., Pérez-Valero, I., Gómez-Villamandos, JC., Rivero, A.
Title: Seroreversion of IgG anti-HEV in HIV cirrhotic patients: A long-term multi-sampling longitudinal study.
Journal: Transboundary and Emerging Diseases (2022). Doi: 10.1111/tbed.14486.

Authors: Dashti, A., Rivero-Juárez, A., Santín, M., George, NS., Koster, PC., López-López, P., Risalde, MA., García-Bocanegra, I., Gómez-Villamandos, JC., Caballero-Gómez, J., Frías, M., Bailo, B., Ortega, S., Salimo-Muadica, A., Calero-Bernal, R., González-Barrio, D., Rivero, A., Briz, V., Carmena, D.
Title: Diarrhoea-causing enteric protist species in intensively and extensively raised pigs (*Sus scrofa domesticus*) in Southern Spain. Part I: Prevalence and genetic diversity.
Journal: Transboundary and Emerging Diseases (2021). Doi: 10.1111/tbed.14388.

Authors: Rivero-Juárez, A., Dashti, A., Santín, M., Köster, P. C., López-López, P., Risalde, MA., García-Bocanegra, I., Gómez-Villamandos, JC., Caballero-Gómez, J., Frías, M., Bailo, B., Ortega, S., Salimo-Muadica, A., Calero-Bernal, R., González-Barrio, D., Rivero, A., Briz, V., Carmena, D.
Title: Diarrhoea-causing enteric protist species in intensively and extensively raised pigs (*Sus scrofa domesticus*) in Southern Spain. Part II: Association with Hepatitis E virus susceptibility.
Journal: Transboundary and Emerging Diseases (2021). Doi: 10.1111/tbed.14408.

Authors: Casares-Jiménez, M., López-López, P., Caballero-Gómez, J., Frías, M., Perez-Hernando, B., Oluremi, A. S., Risalde, MA., Ruiz-Cáceres, I., Oladele Opaleye, O., García-Bocanegra, I., Rivero-Juárez, A., Rivero, A.
Title: Global molecular diversity of Hepatitis E virus in wild boar and domestic pig.
Journal: One Health (2021), *13*, 100304. Doi: 10.1016/j.onehlt.2021.100304.

Authors: Rivero-Juárez, A., Risalde, MA., Gortázar, C., López-López, P., Barasona, JA., Frias, M., Caballero-Gómez, J., de la Fuente, J., Rivero, A.
Title: Detection of Hepatitis E Virus in *Hyalomma lusitanicum* Ticks Feeding on Wild Boars.
Journal: Frontiers in Microbiology (2021), *12*, 1827. Doi: 10.3389/fmicb.2021.692147.

Authors: Barroso, P., Risalde, M. A., García-Bocanegra, I., Acevedo, P., Barasona, J. Á., Caballero-Gómez, J., Jiménez-Ruiz, S., Rivero-Juárez, A., Montoro, V., Vicente, J.
Title: Long-Term Determinants of the Seroprevalence of the Hepatitis E Virus in Wild Boar (*Sus scrofa*).
Journal: Animals (2021), *21*, 1805. Doi: 10.3390/ani11061805.

Authors: Arenas, A., Borge, C., Carbonero, A., Garcia-Bocanegra, I., Cano-Terriza, D., Caballero, J., Arenas-Montes, A.
Title: Bovine Coronavirus Immune Milk Against COVID-19.
Journal: Frontiers in Immunology (2021), *12*, 843. Doi: 10.3389/fimmu.2021.637152.

Authors: Díaz-Cao, J. M., Adaszek, Ł., Dzięgiel, B., Paniagua, J., Caballero-Gómez, J., Winiarczyk, S., Cano-Terriza, D., García-Bocanegra, I.
Title: Prevalence of selected tick-borne pathogens in wild ungulates and ticks in southern Spain.
Journal: Transboundary and Emerging Diseases (2021). Doi: 10.1111/tbed.14065.

Authors: Nimgaonkar, I., Archer, N. F., Becher, I., Shahrad, M., LeDesma, R. A., Mateus, A., Caballero-Gómez, J., Berneshawi, AR., Ding, Q., Douam, F., Gaska, JM., Savitski, MM., Kim, H., Ploss, A.
Title: Isocotoin suppresses hepatitis E virus replication through inhibition of heat shock protein 90.
Journal: Antiviral Research (2021), *185*, 104997. Doi: 10.1016/j.antiviral.2020.104997.

Authors: Martínez-Padilla, A.†, Caballero-Gómez, J.†, Magnet, Á., Gómez-Guillamón, F., Izquierdo, F., Camacho-Sillero, L., Jiménez-Ruiz, S., del Águila, C., García-Bocanegra, I.
Title: Zoonotic Microsporidia in Wild Lagomorphs in Southern Spain.
Journal: Animals (2021), *10*, 2218. Doi: 10.3390/ani10122218.
†Equally contributed

Authors: Frías, M., López-López, P., Zafra, I., Caballero-Gómez, J., Machuca, I., Camacho, Á., Risalde, MA., Rivero-Juárez, A., Rivero, A.
Title: Development and Clinical Validation of a Pangenotypic PCR-Based Assay for the Detection and Quantification of Hepatitis E Virus (*Orthohepevirus A* Genus).
Journal: Journal of Clinical Microbiology (2021), *59*, e02075-20. Doi: 10.1128/JCM.02075-20.

Authors: Rivero-Juárez, A., Frías, M., López-López, P., Berenguer, J., García, F., Macias, J., Alcaraz, B., Castro-Iglesias, A., Caballero-Gómez, J., Rivero, A.

Title: Hepatitis E 3ra Genotype Infection in People Living With HIV in Spain.
Journal: Frontiers in Microbiology (2021), 2189. Doi: 10.3389/fmicb.2020.564486.

Authors: García-Bocanegra, I., Camacho-Sillero, L.†, Caballero-Gómez, J.†, Agüero, M., Gómez-Guillamón, F., Ruiz-Casas, JM., Díaz-Cao, JM., García, E., Ruano, MJ., de la Haza, R.
Title: Monitoring of emerging myxoma virus epidemics in Iberian hares (*Lepus granatensis*) in Spain, 2018-2020.
Journal: Transboundary and Emerging Diseases (2021), *68*, 1275-1282. Doi: 10.1111/tbed.13781.
†Equally contributed

Authors: Risalde, M. A., Rivero-Juárez, A., Frías, M., Olivas, I., López-López, P., García-Bocanegra, I., Brieva, T., Caballero-Gómez, J., Camacho, A., Fernández-Molera, V., Gómez-Villamandos, JC., Rivero, A.
Title: Evaluation of a non-invasive screening approach to determine hepatitis E virus status of pig farms.
Journal: Veterinary Record (2021), *187*, 272. Doi: 10.1136/vr.105840.

Authors: Gómez-Guillamón, F.†, Caballero-Gómez, J.†, Agüero, M., Camacho-Sillero, L., Risalde, M. A., Zorrilla, I., Villalba, R., Rivero-Juárez, A., García-Bocanegra, I.
Title: Re-emergence of bluetongue virus serotype 4 in Iberian ibex (*Capra pyrenaica*) and sympatric livestock in Spain, 2018-2019.
Journal: Transboundary and Emerging Diseases (2021), *68*, 458-466. Doi: 10.1111/tbed.13696.
†Equally contributed

Authors: Dashti, A., Rivero-Juárez, A., Santín, M., López-López, P., Caballero-Gómez, J., Frías-Casas, M., Koster, PC., Bailo, B., Calero-Bernal, Briz, V., Carmena, D.
Title: *Enterocytozoon bieneusi* (Microsporidia): Identification of novel genotypes and evidence of transmission between sympatric wild boars (*Sus scrofa ferus*) and Iberian pigs (*Sus scrofa domesticus*) in Southern Spain.
Journal: Transboundary and Emerging Diseases (2020), *67*, 2869-2880. Doi: 10.1111/tbed.13658.

Authors: Cano-Terriza, D., Jiménez-Martín, D., Jiménez-Ruiz, S., Paniagua, J., Caballero-Gómez, J., Guerra, R., Franco, JJ., García-Bocanegra, I.
Title: Serosurvey of Peste des Petits Ruminants in southern Spain.
Journal: Transboundary and Emerging Diseases (2020), *67*, 3033-3037. Doi: 10.1111/tbed.13602.

Authors: Díaz-Cao, J. M., Lorca-Oró, C., Pujols, J., Cano-Terriza, D., de los Ángeles Risalde, M., Jiménez-Ruiz, S., Caballero-Gómez, J., García-Bocanegra, I.
Title: Evaluation of two enzyme-linked immunosorbent assays for diagnosis of bluetongue virus in wild ruminants.
Journal: Comparative Immunology, Microbiology and Infectious Diseases (2020), *70*, 101461. Doi: 10.1016/j.cimid.2020.101461.

Authors: Cano-Terriza, D., Almería, S., Caballero-Gómez, J., Jiménez-Martín, D., Castro-Scholten, S., Dubey, J. P., García-Bocanegra, I.
Title: Exposure to *Toxoplasma gondii* in zoo animals in Spain.
Journal: Preventive Veterinary Medicine (2020), *176*, 104930. Doi: 10.1016/j.prevetmed.2020.104930.

Authors: Rivero-Juárez, A., Vallejo, N., López-López, P., Díaz-Mareque, A. I., Frías, M., Vallejo, A., Caballero-Gómez, J., Rodríguez-Velasco, M., Molina, E., Aguilera, A.
Title: Ribavirin as a first treatment approach for hepatitis e virus infection in transplant recipient patients.
Journal: Microorganisms (2019), *8*, 51. Doi: 10.3390/microorganisms8010051.

Authors: López-López, P., Rivero-Juárez, A., Frías, M., Machuca, I., Caballero-Gómez, J., Olivas, I., Camacho, A., Risalde, MA., García-Bocanegra, I., Rivero, A.
Title: Mutations in the Progesterone Receptor (PROGINS) May Reduce the Symptoms of Acute Hepatitis E and Protect Against Infection.
Journal: Frontiers in Microbiology (2019), 2617. Doi: 10.3389/fmicb.2019.02617.

Authors: Cano-Terriza, D., Arenas, A., Borge, C., Carbonero, A., Paniagua, J., Risalde, M. A., Caballero-Gómez, J., Jiménez-Ruiz, S., Díaz, J. M., Martínez-Padilla, A., García-Bocanegra, I.
Title: Gamificación como apoyo a la docencia en el Grado en Veterinaria.
Journal: Revista de Docencia Veterinaria (2019), *3*, 111-112.

Authors: García-Bocanegra, I., Camacho-Sillero, L., Risalde, M. A., Dalton, K. P., Caballero-Gómez, J., Agüero, M., Zorrilla, I., Gómez-Guillamón, F.
Title: First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*).
Journal: Transboundary and Emerging Diseases (2019), *66*, 2204-2208. Doi: 10.1111/tbed.13289.

Authors: Camacho-Sillero, L., Caballero-Gómez, J., Gómez-Guillamón, F., Martínez-Padilla, A., Agüero, M., San Miguel, E., Zorrilla, I., Rayas, E., Talavera, V., García-Bocanegra, I.
Title: Monitoring of the novel rabbit haemorrhagic disease virus type 2 (GI.2) epidemic in European wild rabbits (*Oryctolagus cuniculus*) in southern Spain, 2013-2017.
Journal: Veterinary Microbiology (2019), *237*, 108361. Doi: 10.1016/j.vetmic.2019.07.013.

Authors: Jiménez-Ruiz, S., Paniagua, J., Isla, J., Martínez-Padilla, A. B., Risalde, MA., Caballero-Gómez, J., Cano-Terriza, D., Pujols, J., Arenas, A., García-Bocanegra, I.
Title: Description of the first Schmallenberg disease outbreak in Spain and subsequent virus spreading in domestic ruminants.
Journal: Comparative Immunology, Microbiology and Infectious Diseases (2019), *65*, 189-193. Doi: 10.1016/j.cimid.2019.06.002.

Authors: Cano-Terriza, D., Almería, S., Caballero-Gómez, J., Díaz-Cao, JM., Jiménez-Ruiz, S., Dubey, JP., García-Bocanegra, I.
Title: Serological survey of *Toxoplasma gondii* in captive nonhuman primates in zoos in Spain.
Journal: Comparative Immunology, Microbiology and Infectious Diseases (2019), *65*, 54-57. Doi: 10.1016/j.cimid.2019.04.002.

Authors: López-López, P., de los Angeles Risalde, M., Frias, M., García-Bocanegra, I., Brieva, T., Caballero-Gómez, J., Camacho, A., Fernández-Molera, V., Machuca, I., Gómez-Villamandos, JC., Rivero, A., Rivero-Juárez, A.
Title: Risk factors associated with hepatitis E virus in pigs from different production systems.
Journal: Veterinary Microbiology (2018), *224*, 88-92. Doi: 10.1016/j.vetmic.2018.08.020.

Authors: Romeo, C., Lecollinet, S., Caballero, J., Isla, J., Luzzago, C., Ferrari, N., García-Bocanegra, I.
Title: Are tree squirrels involved in the circulation of flaviviruses in Italy?
Journal: Transboundary and Emerging Diseases (2018), *65*, 1372-1376. Doi: 10.1111/tbed.12874.

II.B. Book chapters

Authors: Javier Caballero Gómez, José Manuel Díaz Cao, Ana Belén Martínez Padilla, Julio Isla Rodríguez de Tembleque.
Title: Leucosis bovina.
Book: Enfermedades infectocontagiosas en rumiantes: Manuales clínicos de Veterinaria. ISBN:9788491133537.
Publisher: ELSEVIER.
Year: 2019.

Authors: Javier Caballero Gómez, Julio Isla Rodríguez de Tembleque, Estefanía Jurado Tarifa, Ignacio García Bocanegra.
Title: Pedero.
Book: Enfermedades infectocontagiosas en rumiantes: Manuales clínicos de Veterinaria. ISBN:9788491133537.
Publisher: ELSEVIER.
Year: 2019.

Authors: Ignacio García Bocanegra, Javier Caballero Gómez, Antonio Rivero Juárez, Saúl Jiménez Ruiz.
Title: Lengua Azul.
Book: Enfermedades infectocontagiosas en rumiantes: Manuales clínicos de Veterinaria. ISBN:9788491133537.
Publisher: ELSEVIER.
Year: 2019.

Authors: Estefanía Jurado Tarifa, Saúl Jiménez Ruiz, Javier Caballero Gómez, Ignacio García Bocanegra.
Title: Fiebre del Valle del Rift.
Book: Enfermedades infectocontagiosas en rumiantes: Manuales clínicos de Veterinaria. ISBN:9788491133537.
Publisher: ELSEVIER.
Year: 2019.

Authors: Ana Belén Martínez Padilla, Alfonso Carbonero Martínez, Javier Caballero Gómez, David Cano Terriza.

Title: Clostridiosis histotóxicas y neurotóxicas.
Book: Enfermedades infectocontagiosas en rumiantes: Manuales clínicos de Veterinaria. ISBN:9788491133537.
Publisher: ELSEVIER.
Year: 2019.

II.C. Congress communications

II.C.1. International meetings

Title: Epidemiología y análisis espacio-temporal del virus de West Nile en caballos en España entre 2010 y 2016
Congress: III Congreso de la Sociedad Iberoamericana de Epidemiología Veterinaria y Medicina Preventiva (SIEVMP).
Place: Valdivia, Chile.
Date: 17-19th October 2017.
Organizing institution: SIEVMP.
Type of communication: Poster.
Authors: García-Bocanegra, I., Belkhiria, J., Napp, S., Cano-Terriza, D., Jurado-Tarifa, E., Jiménez-Ruiz, S., Caballero, J., Martínez-López, B.

Title: Acquisition of professional skills in Veterinary Medicine degree by using ICT: camera trapping techniques in wildlife
Congress: I Congreso Internacional Virtual de Innovación Docente Universitaria "We teach & We Learn".
Place: Cordoba, Spain.
Date: 20-21st June 2018.
Organizing institution: University of Cordoba.
Type of communication: Poster.
Authors: Paniagua-Risueño, J., Cano-Terriza, D., Risalde, M. A., Jiménez-Ruiz, S., Caballero, J., Carbonero, A., Martínez-Padilla, A. B., Borge, C., Arenas, A., García-Bocanegra, I.

Title: Monitoring of the Rabbit Hemorrhagic Disease virus 2 (RHDV2) epidemics in European wild rabbit (*Oryctolagus cuniculus*) in Andalusia (Spain), 2013-2017
Congress: 13rd Conference of European Wildlife Disease Association (EWDA).
Place: Larissa, Greece.
Date: 27-31st August 2018.
Organizing institution: EWDA.
Type of communication: Poster.
Authors: Camacho-Sillero, L., Gómez-Guillamón, F., Caballero, J., Martínez-Padilla, A., San Miguel Ibáñez, E., Sánchez Sánchez, A., Agüero García, M., Rocha Roso, A., Rayas, E., Talavera, V., Zorrilla, I., García-Bocanegra, I.

Title: Evaluación de la gestión de subproductos procedentes de actividades cinegéticas de caza mayor en muladares de Sierra Morena (sur de España).*
Congress: 36èmes rencontres du GEEFSM.
Place: Orlu, France.
Date: 14-16th September 2018.
Organizing institution: GEEFSM.

Type of communication: Oral.
Authors: Jiménez-Ruiz, S., García-Bocanegra, I., Díaz, J. M., Cano-Terriza, D., Isla, J., Rodríguez-Hernández, P., Caballero-Gómez, J., Romero-Herrera, P., Arenas, A., Paniagua, J. *Awarded communication

Title: Circulación del virus de la hepatitis E y papel regulador de los microARNs en jabalíes infectados de forma natural en el sur de España
Congress: 36èmes rencontres du GEEFSM.
Place: Orlu, France.
Date: 14-16th September 2018.
Organizing institution: GEEFSM.
Type of communication: Oral.
Authors: Caballero-Gómez, J., Garrido, J. J., Risalde, M. A., Zaldívar-López, S., Martínez-Padilla, A., López-López, P., Cano-Terriza, D., Jiménez-Ruiz, S., Zafra, I., García-Bocanegra, I.

Title: Prevalence of Hepatitis E virus infection in wild boar in Mediterranean ecosystems. Can microRNAs act as biomarkers of chronic infections?
Congress: IX Reunión de Ungulados Silvestres Ibéricos (RUSI).
Place: Capileira, Spain.
Date: 4-6th October 2018.
Organizing institution: RUSI.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Garrido, J. J., Risalde, M. A., Zaldívar-López, S., Martínez-Padilla, A., López-López, P., Cano-Terriza, D., Jiménez-Ruiz, S., Zafra, I., García-Bocanegra, I.

Title: Evaluation of the management of hunting waste in selective avian scavenging feeding points in Sierra Morena (southern Spain)
Congress: IX RUSI.
Place: Capileira, Spain.
Date: 4-6th October 2018.
Organizing institution: RUSI.
Type of communication: Poster.
Authors: Jiménez-Ruiz, S., García-Bocanegra, I., Díaz, J. M., Cano-Terriza, D., Isla, J., Rodríguez-Hernández, P., Caballero-Gómez, J., Romero-Herrera, P., Arenas, A., Paniagua, J.

Title: Estudio epidemiológico de *Toxoplasma gondii* en rumiantes domésticos en extensivo y ungulados silvestres simpátricos en el sur de España
Congress: I Congreso Hispano-Luso de Ganadería Extensiva.
Place: Seville, Spain.
Date: 8-9th November 2018.
Organizing institution: Cooperativas Agro-alimentarias de Andalucía and the Federación de Agrupaciones de Defensa Sanitaria Ganadera.
Type of communication: Poster.
Authors: Cano-Terriza, D., Almería, S., Cabezón, O., Paniagua, J., Jiménez-Ruiz, S., Caballero-Gómez, J., Arenas-Montes, A., Dubey, J. P., García-Bocanegra, I.

Title: Serosurvey of Peste des petits ruminants in Andalusia (Spain).*****

Congress: X RUSI.
Place: Lousa, Portugal.
Date: 18-19th October 2019.
Organizing institution: RUSI.
Type of communication: Poster.
Authors: Jiménez-Martín, D., Cano-Terriza, D., Morales, F., Jiménez-Ruiz, S., Paniagua, J., Caballero-Gómez, J., Guerra, R., Franco JJ., García-Bocanegra, I.
*Awarded communication.

Title: Investigating the enteric coinfection of Hepatitis E virus with *Giardia duodenalis*, *Blastocystis* sp., and *Balantioides coli* in intensively and extensively raised pigs (*Sus scrofa domesticus*) in Southern Spain
Congress: 3rd International Blastocystis conference.
Place: Virtual.
Date: 2-4th June 2021.
Organizing institution: University of Kent.
Type of communication: Oral.
Authors: Dashti, A., Rivero-Juárez, A., Köster, P., López-López, P., Risalde, MÁ., García-Bocanegra, I., Gómez-Villamandos, JC., Caballero-Gómez, J., Frías, M., Bailo, B., Ortega, S., Muadica, AS., Calero-Bernal, R., González-Barrio, D., Rivero, A., Briz, V., Carmena, D.

Title: Are zoo mammals potential sentinels for zoonotic flaviviruses monitoring?
Congress: 69th annual Wildlife Disease Association (WDA) and 14th biennial European Wildlife Disease Association (EWDA).
Place: Cuenca, Spain.
Date: 31-2nd August-September 2021.
Organizing institution: WDA/EWDA.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Cano-Terriza, D., Lecollinet, S., Carbonell, MD., Martínez-Valverde, R., Martínez-Nevado, E., García-Párraga, D., Lowenski, S., Beato, A., Barbero, J., García-Bocanegra, I.

Title: Zoo animals as sentinels for bluetongue virus monitoring in Spain.
Congress: 69th annual Wildlife Disease Association (WDA) and 14th biennial European Wildlife Disease Association (EWDA).
Place: Cuenca, Spain.
Date: 31-2nd August-September 2021.
Organizing institution: WDA/EWDA.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Cano-Terriza, D., Pujols, J., Martínez-Nevado, E., Carbonell, MD., Guerra, R., Recuero, J., Soriano, P., de Castro, N., Castro-Scholten, S., Barbero, J., García-Bocanegra, I.

Title: Monitoring of Schmallenberg virus in zoo animals in Spain, 2002-2019.
Congress: 69th annual Wildlife Disease Association (WDA) and 14th biennial European Wildlife Disease Association (EWDA).
Place: Cuenca, Spain.
Date: 31-2nd August-September 2021.

Organizing institution: WDA/EWDA.
Type of communication: Poster.
Authors: Jiménez-Martín, D., Caballero-Gómez, J., García-Bocanegra, I., Navarro, N., Gerique, C., Martínez-Nevado, E., Soriano, P., Castro-Scholten S., Beato, A., Cano-Terriza, D.

II.C.2. National meetings

Title: Persistencia del virus de la hepatitis E en el hígado de jabalíes no virémicos infectados de forma natural
Congress: III Congreso Nacional de GEHEP de la SEIMC.
Place: Seville, Spain.
Date: 28-30th September 2017.
Organizing institution: SEIMC.
Type of communication: Poster.
Authors: Caballero, J., Risalde, M. A., Rivero Juárez, A., Romero Palomo, F., Frias, M., López López, P., Cano Terriza, D., García Bocanegra, I., Jiménez Ruiz, S., Zafra, I., Isla, J., Camacho, Á., Machuca, I., Brieva, T., Gómez Villamandos, JC., Rivero, A.

Title: Método no invasivo para la detección del virus de la hepatitis E en explotaciones de porcino intensivo.
Congress: XXII Simposio Asociación de Veterinarios Especialistas en Diagnóstico de Laboratorio (AVEDILA).
Place: Valladolid, Spain.
Date: 16-17h November 2017.
Organizing institution: AVEDILA.
Type of communication: Poster.
Authors: Risalde, MA., Rivero-Juárez, A., Frías, M., López-López, P., Zafra, I., Jiménez-Ruiz, S., García-Bocanegra, I., Cano-Terriza, D., Caballero, J., Machuca, I., Ruiz-Torres, L., Camacho, A., Brieva, T., Gómez-Villamandos, JC., Rivero, A.

Title: Control de la tuberculosis en gamo (*Dama dama*) mediante la correcta gestión de subproductos procedentes de la caza mayor.
Congress: II Congreso de Veterinaria y Ciencia y Tecnología de los Alimentos.
Place: Cordoba, Spain.
Date: 9th September 2018.
Organizing institution: Faculty of Veterinary Medicine.
Type of communication: Poster.
Authors: Cano-Terriza, D., Risalde, M. A., Isla, J., Jiménez-Ruiz, S., Paniagua, J., Rodríguez, P., Caballero, J., García-Bocanegra, I.

Title: Prevalencia de infección por *Encephalitozoon cuniculi* en conejo silvestre (*Oryctolagus cuniculus*) en Andalucía
Congress: II Congreso de Veterinaria y Ciencia y Tecnología de los Alimentos.
Place: Cordoba, Spain.
Date: 9th September 2018.
Organizing institution: Faculty of Veterinary Medicine.
Type of communication: Poster.

Authors: Martínez-Padilla, A., Caballero, J., Gómez-Guillamón, F., Camacho, L., Jiménez-Ruiz, S., Isla, J., García-Bocanegra, I.

Title: Estudio epidemiológico del Complejo *Mycobacterium tuberculosis* en suidos domésticos y silvestres en Andalucía*
Congress: II Congreso Nacional de Sanidad Animal.
Place: Cordoba, Spain.
Date: 17-18th October 2018.
Organizing institution: Consejo General de Colegios Veterinarios.
Type of communication: Oral.
Authors: Cano-Terriza, D., Risalde, M. A., Jiménez-Ruiz, S., Paniagua, J., Romero, B., Fernández-Morente, M., Moreno, I., Isla, J., Caballero-Gómez, J., Fernández-Molera, V., Arenas, A, Sáez, J. L., García-Bocanegra, I.
*Awarded communication

Title: Evaluación de la imagen térmica infrarroja para el diagnóstico de la tuberculosis bovina mediante intradermotuberculinización
Congress: II Congreso Nacional de Sanidad Animal.
Place: Cordoba, Spain.
Date: 17-18th October 2018.
Organizing institution: Consejo General de Colegios Veterinarios.
Type of communication: Poster.
Authors: Arenas, A., Borge, C., Carbonero, A., Jiménez-Ruiz, S., Caballero, J., Rodríguez, P., García-Bocanegra, I., Perea, MA., Arenas-Montes, A.

Title: Primer brote de Enfermedad de Schmallenberg en España: dispersión del virus y factores de riesgo asociados
Congress: II Congreso Nacional de Sanidad Animal.
Place: Cordoba, Spain.
Date: 17-18th October 2018.
Organizing institution: Consejo General de Colegios Veterinarios.
Type of communication: Poster.
Authors: Jiménez-Ruiz, S., Isla, J., Paniagua, J., Martínez-Padilla, A., Risalde, MA., Caballero-Gómez, J., Cano-Terriza, D., Pujols, J., Arenas, A., García-Bocanegra, I.

Title: Prevalencia de infección por el virus de la Hepatitis E y expresión diferencial de microARNs en jabalíes en el sur de España**.***
Congress: III Congreso Científico de Investigadores Nóveles.
Place: Cordoba, Spain.
Date: 19th November 2018.
Organizing institution: University of Cordoba.
Type of communication: Oral.
Authors: Caballero-Gómez, J., Garrido, J. J., Risalde, M. A., Rivero-Juárez, A., Zaldívar-López, S., Martínez-Padilla, A., López-López, P., Cano-Terriza, D., Jiménez-Ruiz, S., Frias, M., Zafra, I., García-Bocanegra, I.
*Awarded communication

Title: Evaluación de dos técnicas ELISA comerciales para el diagnóstico de lengua azul en rumiantes silvestres

Congress: I Congreso Ibérico de Ciencia Aplicada a los Recursos Cinegéticos (CICARC).
Place: Ciudad Real, Spain.
Date: 1-4th July 2019.
Organizing institution: Instituto de Investigación en Recursos Cinegéticos (IREC).
Type of communication: Poster.
Authors: Díaz-Cao, J. M., Lorca-Oró, C., Pujols, J., Cano-Terriza, D., Risalde, M. A., Jiménez-Ruiz, S., Caballero-Gómez, J., García-Bocanegra, I.

Title: Mortalidad asociada al serotipo 4 del virus de la lengua azul en poblaciones de cabra montés (*Capra pyrenaica hispanica*) en Andalucía.
Congress: I Congreso Ibérico de Ciencia Aplicada a los Recursos Cinegéticos (CICARC).
Place: Ciudad Real, Spain.
Date: 1-4th July 2019.
Organizing institution: Instituto de Investigación en Recursos Cinegéticos (IREC).
Type of communication: Oral.
Authors: Gómez-Guillamón, F., Camacho-Sillero, L., Risalde, M. A., Díaz-Cao, J. M., Caballero-Gómez, J., Agüero, M., González, M. A., Zorrilla, I., Talavera, V., García-Bocanegra, I.

Title: Gamificación como apoyo a la docencia en el Grado en Veterinaria.
Congress: V Congreso VetDoc de Docencia en Veterinaria.
Place: Madrid, Spain.
Date: 8-9th July 2019.
Organizing institution: Asociación Española de Veterinarios Docentes.
Type of communication: Oral.
Authors: Cano-Terriza, D., Arenas, A., Borge, C., Carbonero, A., Paniagua, J., Risalde, M. A., Caballero-Gómez, J., Jiménez-Ruiz, S., Díaz, J. M., Martínez-Padilla, A., García-Bocanegra, I.

Title: Prevalence of *Leishmania infantum* in wild animals from southern Spain**.**
Congress: XXI Congreso Sociedad Española de Parasitología (SOCEPA)
Place: Pontevedra, Spain.
Date: 3-5th July 2019.
Organizing institution: SOCEPA.
Type of communication: Poster.
Authors: Ortuño-Gil, M., Resa-Collados, M., García-Bocanegra, I., Camacho-Sillero, L., Jiménez-Ruiz, S., Caballero-Gómez, J., Cano-Terriza, D., Berriatua, E.

Title: Estudio serológico de Anemia Infecciosa Equina en équidos en Europa
Congress: XXIV Simposium AVEDILA.
Place: Pamplona, Spain.
Date: 7-8th November 2019.
Organizing institution: AVEDILA.
Type of communication: Poster.
Authors: Franco, JJ., Cano-Terriza, D., Cunilleras, J., Alguacil, E., García, J., Caballero-Gómez, J., Jiménez-Martín, D., Sanz, A., García-Bocanegra, I.

Title: La cinética viral del VHC en células mononucleares de sangre periférica durante el tratamiento no es un factor de predicción de recidivas

Congress: XXI Congreso de la SAEI.
Place: Seville, Spain.
Date: 21-23rd November 2019.
Organizing institution: SAEI.
Type of communication: Poster.
Authors: Frías, M., Rivero-Juárez, A., De Salazar, A., López-López, P., Fuentes, A., Machuca, I., Ruiz-Cáceres, I., Camacho, A., Caballero-Gómez, J., García, F., Rivero, A.

Title: Impacto del inhibidor de la proteasa NS3/4A en la homeostasis lipídica de pacientes VIH/VHC co-infectados
Congress: XXI Congreso de la SAEI.
Place: Seville, Spain.
Date: 21-23rd November 2019.
Organizing institution: SAEI.
Type of communication: Poster.
Authors: Frías, M., Rivero-Juárez, A., Camacho, A., Ruiz-Cáceres, I., Caballero-Gómez, J., Machuca, I., López-López, Rivero, A.

Title: Los pacientes VIH infectados no tienen mayor riesgo de infección por el virus de la hepatitis E: resultados de un metaanálisis
Congress: XXI Congreso de la SAEI.
Place: Seville, Spain.
Date: 21-23rd November 2019.
Organizing institution: SAEI.
Type of communication: Poster.
Authors: López-López, P., Frías, M., Camacho, A., Zafra-Soto, I., Ruiz-Torres, L., Ruiz-Cáceres, I., Muñoz-Moreno, V., Caballero-Gómez, J., Milla-Serrano, L., Rivero-Juárez, A., Rivero, A.

Title: Zoo animals as potential sentinels for zoonotic flaviviruses monitoring.
Congress: XI Young Investigators Meeting of the IMIBIC.
Place: Cordoba, Spain.
Date: 29-30th October 2020.
Organizing institution: IMIBIC.
Type of communication: Oral.
Authors: Caballero-Gómez, J., Cano-Terriza, D., Lecollinet, S., Guerra, R., Carbonell MD., Martínez-Velarde, R., Martínez-Nevado, E., García-Párraga, D., Lowenski, S., Rivero-Juárez, A., Frias, M., López-López, P., García-Bocanegra, I.

Title: Protist enteroparasite in wild boar (*Sus scrofa ferus*) and Iberian pig (*Sus scrofa domesticus*) in Southern Spain: A protective effect on Hepatitis E acquisition?
Congress: XI Young Investigators Meeting of the IMIBIC.
Place: Cordoba, Spain.
Date: 29-30th October 2020.
Organizing institution: IMIBIC.
Type of communication: Poster.
Authors: López-López, P., Frías, M., Risalde, MA., Caballero-Gómez, J., García-Bocanegra, I., Machuca, I., Ruiz-Cáceres, I., Zafra-Soto, I., Rivero, A., Rivero-Juárez, A.

Title: *Enterocytozoon bieneusi* in Iberian pigs and wild boar in Mediterranean ecosystems from Spain: are they a potential source of zoonotic infection?
Congress: XI Young Investigators Meeting of the IMIBIC.
Place: Cordoba, Spain.
Date: 29-30th October 2020.
Organizing institution: IMIBIC.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Dahsti, A., Rivero-Juárez, A., Santín, M., López-López, P., Ruiz-Cáceres, I., Frías, M., Koster, PC., Bailo, B., Calero-Bernal, R., Briz, V., Carmena, D.

Title: Hepatitis E virus in people living with HIV: first report of HEV-3ra infection in Spain
Congress: XI Young Investigators Meeting of the IMIBIC.
Place: Cordoba, Spain.
Date: 29-30th October 2020.
Organizing institution: IMIBIC.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Frías, M., López-López, P., Ruiz-Cáceresm I., Berenguer, J., García, F., Macías, J., Alcaraz, B., Castro-Iglesias, A., Rivero, A.

Title: Cinética de anticuerpos del VHE en pacientes VIH cirróticos
Congress: VI Congreso Nacional de GEHEP de la SEIMC.
Place: Granada, Spain.
Date: 21-23rd September 2021.
Organizing institution: SEIMC.
Type of communication: Oral.
Authors: López-López, P., Frías, M., García-Delgado, G., Machuca, I., Caballero-Gómez, J., Casares-Delgado, M., Risalde, MÁ., Ruiz-Cáceres, I., García-Bocanegra, I., Zafra-Soto, I., Pérez-Valero, I., Ruiz-Torres, L., Gómez-Villamandos, JC., Camacho, A., Rivero-Juárez, A., Rivero, A.

Title: Prevalencia del virus de la Hepatitis E en alimentos de origen porcino
Congress: VI Congreso Nacional de GEHEP de la SEIMC.
Place: Granada, Spain.
Date: 21-23rd September 2021.
Organizing institution: SEIMC.
Type of communication: Poster.
Authors: Casares-Jiménez, M., López-López, P., Caballero-Gómez, J., Martínez-Blasco, J., Martín-Gómez, A., Agulló-Ros, I., Frías, M., García-Bocanegra, I., Rivero, A., Gómez-Villamandos, JC., Risalde, MÁ., Rivero-Juárez, A.

Title: Detección del virus de la Hepatitits E en *Hyalomma lusitanicum* procedentes de jabalíes
Congress: VI Congreso Nacional de GEHEP de la SEIMC.
Place: Granada, Spain.
Date: 21-23rd September 2021.
Organizing institution: SEIMC.
Type of communication: Poster.
Authors: Caballero-Gómez, J., Rivero-Juárez, A., Risalde, MÁ., Gortázar, C., López-López, P., Barasona, J., Frías, M., de la Fuente, J., Rivero, A.

Title: Evaluación de la competencia vectorial del mosquito común (*Culex pipiens*) para la transmisión del virus de la hepatitis E
Congress: VI Congreso Nacional de GEHEP de la SEIMC.
Place: Granada, Spain.
Date: 21-23rd September 2021.
Organizing institution: SEIMC.
Type of communication: Poster.
Authors: Frias-Casas, M., Casades-Martí, L., López-López, P., Peralbo-Morena, Caballero-Gómez, J., Cuadrado-Matías, R., Rviero-Juárez, A., Ruiz-Fons, F.

Title: Desarrollo de una técnica de PCR con capacidad de detectar todas las especies de *Orthohepevirus*
Congress: VI Congreso Nacional de GEHEP de la SEIMC.
Place: Granada, Spain.
Date: 21-23rd September 2021.
Organizing institution: SEIMC.
Type of communication: Oral poster.
Authors: Zafra-Soto, I., García-Delgado, G., López-L, Caballero-Gómez, J., Casares-Jiménez, M., Ruiz-Torres, L., Camacho, A., Frias, M., Rivero, A., Rivero-Juárez, A.

Title: Diversidad molecular global del virus de la hepatitis E en jabalí y cerdo doméstico
Congress: VI Congreso Nacional de GEHEP de la SEIMC.
Place: Granada, Spain.
Date: 21-23rd September 2021.
Organizing institution: SEIMC.
Type of communication: Oral poster.
Authors: Casares-Jiménez, M., López-López, P., Caballero-Gómez, J., Frías, M., Pérez-Hernando, B., Risalde, MÁ., Ruiz-Cáceres, I., García-Bocanegra, I., Rivero, A., Rivero-Juárez, A.

D. Dissemination articles

Authors: Caballero-Gómez, J., Rivero-Juárez, A., Risalde, MA., Frías, M., Jiménez-Ruiz, S., López-López, P., Rivero, A., García-Bocanegra, I.
Title: Situación actual de la hepatitis E en el ganado porcino: una enfermedad emergente de interés en salud pública.
Journal: SUIS (August 2020).

Authors: Jiménez-Martín, D., Caballero-Gómez, J., García-Bocanegra, I., Cano-Terriza, D., Risalde, MA., López-López, P., Jiménez-Ruiz, S., Rivero, A., Rivero-Juárez, A.

Title: Situación actual de la hepatitis E en el ganado porcino: una enfermedad emergente de interés en salud pública.

Journal: Tierras (March 2022).

Appendix III. Projects funded in competitive public calls in which the doctoral student participates

Javier Caballero has participated in 13 and four research and teaching innovation projects, respectively.

III. A. Research projects

1. **Epidemiología de la tuberculosis bovina en animales domésticos en España: Implicación de reservorios domésticos y silvestres y evaluación de medidas de lucha**. REF: AGL2013-49159-C2-2-R. IP: Ignacio García Bocanegra/Sebastián Napp Avelli. Entidad financiadora: Ministerio de Economía y Competitividad. Periodo: 2014-2016. Presupuesto: 125.000€. **Investigador colaborador**.

2. **Impacto de la infección aguda por virus de hepatitis E en pacientes infectados por VIH**. REF: PI16/01297. IP: Antonio Rivero Juárez. Entidad financiadora: Ministerio de Ciencia e Innovación. Periodo: 2016-2018. Presupuesto: 92.565€. **Investigador colaborador**.

3. **Evaluación de protocolos de bioseguridad y de la gestión de ungulados en la transmisión de enfermedades compartidas**. REF: AGL2016-76358-R. IP: Pelayo Acevedo Lavandera/Joaquín Vicente Baños. Entidad financiadora: Ministerio de Economía y Competitividad. Periodo: 2017-2019. Prespuesto: 120.000€. **Investigador colaborador**.

4. **GO-TUBERCULOSIS: TbB nuevas medidas y técnicas de control de la tuberculosis bovina en Andalucía (subproyecto AGR-149)**. REF: GOP21-C0-16-0010. IP: Ignacio García Bocanegra/Antonio Arenas Casas. Entidad financiadora: Consejería de Agricultura, Pesca, y Desarrollo Rural. Junta de Andalucía. Periodo: 2018-2020. Presupuesto: 15.553,15€ (todo el proyecto: 264.550,21€). **Equipo de trabajo**.

5. **Diseño, implantación y evaluación de programas sanitarios para la mitigación del riesgo de transmisión de la tuberculosis en el ganado porcino extensivo**. REF: AA-17-0031-1. IP: Ignacio García-Bocanegra/María de los Ángeles Risalde Moya/David Cano Terriza. Período: 2018-2020. Presupuesto: 77.477,98€. **Investigador colaborador**.

6. **Improvement of preventive actions to emerging lagoviruses in the Mediterranean basin: development and optimization of methodologies for pathogen detection and control**. REF: PRIMA-S2-11-PCI2019-103698. IP: Carlos Rouco Zufiaurre. Entidad convocante: PRIMA-Partnership for Research and Innovacion in the Mediterranean Area. Entidad financiadora: Ministerio de Ciencia, Innovación y Universidades. Período: 2019-2022. Presupuesto: 902.400€ (subproyecto UCO: 95.000€). **Investigador colaborador.**

7. **Evaluación de medidas de lucha integradas para el control de la tuberculosis en el ganado porcino manejado en sistemas de producción extensivos**. IP: David Cano Terriza. Entidad financiadora: Universidad de Córdoba (Plan Propio Galileo 2020; Submodalidad 1.2 UCO-ACTIVA). Período: 2020-2021. Presupuesto: 12.000€. **Equipo de trabajo**.

8. **Trazabilidad epidemiológica y microbiológica de la infección por el virus de la hepatitis E: Estudio TrazHE**. REF: PI19/00864. IP: Antonio Rivero Juárez. Entidad financiadora:

Instituto de Salud Carlos III y Ministerio de Ciencia, Innovación y Universidades. Periodo: 2020-2022. Presupuesto: 34.974€. **Investigador colaborador**.

9. **Evaluación epidemiológica de reservorios domésticos y silvestres de *Leishmania spp*. en Andalucía.** REF: UCO-FEDER-1264967. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba/Consejería de Economía y Conocimiento de la Junta de Andalucía. Período: 2020-2022. Presupuesto: 34.974€. **Investigador colaborador**.

10. **Infectividad del virus de la Hepatitis E detectado en alimentos de origen porcino utilizando modelos animales y cultivos celulares (Estudio HepEfood).** REF: PI-0287-2019. IP: María de los Ángeles Risalde Moya. Entidad financiadora: Consejería de Salud y Familias de la Junta de Andalucía. Periodo: 2020-2023. Presupuesto: 65.009,96€. **Investigador colaborador**.

11. **Avances en el conocimiento de enfermedades en lagomorfos silvestres en ecosistemas mediterráneos de España desde una perspectiva de salud global (LagoHealth)**. REF: PID2019-111080RB-C21. IP: Ignacio García Bocanegra. Entidad financiadora: Ministerio de Ciencia e Innovación. Período: 2020-2023. Presupuesto: 181.500€. **Equipo de trabajo.**

12. **Evaluación de la infección por *Orthohepevirus C* como causa emergente de enfermedad de origen zoonótico.** REF: PI21/00793. IP: Antonio Rivero Román. Entidad financiadora: Instituto de Salud Carlos III. Período: 2021-2024. Presupuesto: 90.750€. **Investigador colaborador.**

13. **Diseño y evaluación de estrategias de lucha integradas para el control de enfermedades transmisibles en ganado porcino en sistemas silvopastorales en Andalucía.** REF: PYC20 RE-056 UCO. IP: Ignacio García Bocanegra/Inmaculada Luque Moreno/ Jaime Gómez Laguna. Entidad Financiadora: Consejería de Transformación Económica, Industria, Conocimiento y Universidades. Junta de Andalucía. Fondo Europeo de Desarrollo Regional Período: 2022-2023. Presupuesto: 260.000€. **Equipo de trabajo**.

III. B. Teaching Innovation Projects

1. **La gamificación en el aula: Uso de aplicaciones TICs como apoyo para la docencia en el Grado en Veterinaria**. REF: 2018-1-3015. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba. Período: 2018-2019. **Equipo de trabajo**.

2. **La gamificación en el aula 2.0: Evaluación de la percepción del alumnado del Grado en Veterinaria sobre la implementación de aplicaciones TICs como apoyo a la docencia**. REF: 2019-1-3007. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba. Período: 2019-2020. **Equipo de trabajo**.

3. **Uso del eBook como herramienta docente para el desarrollo de contenidos bilingües aplicados a asignaturas de Grado y Postgrado**. REF: 2020-1-3014. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba (Modalidad 1. Proyectos de Innovación Docente). Período: 2020-2021. **Equipo de trabajo.**

4. **Implementación de nuevas herramientas en la docencia práctica del Grado en Veterinaria y el Máster en Salud Pública Veterinaria: Uso de drones para la**

monitorización de la fauna silvestre y la evaluación de implicaciones sanitarias. REF: 2020-5-3005. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba (Modalidad 5. Ayudas para desarrollar y consolidar buenas prácticas innovadoras). Período: 2020-2021. **Equipo de trabajo**.

5. **La gamificación en ciencias de la salud: Implantación de aplicaciones para reforzar la docencia en títulos de Grado y Máster de ámbito Sanitario.** REF: 2021-2-3001. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba (Modalidad 2. Proyectos de innovación para formación en innovación docente). Período: 2021-2022. **Equipo de trabajo**.