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TESIS DOCTORAL

Estudio epidemiológico de enfermedades zoonósicas desde una perspectiva *One Health*

Epidemiological study of zoonotic diseases from a *One Health* approach

Autor: **David Cano Terriza**
Directores: **Ignacio García Bocanegra / María de los Ángeles Risalde Moya**

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TITULO: *ESTUDIO EPIDEMIOLÓGICO DE ENFERMEDADES ZOONÓSICAS DESDE UNA PERSPECTIVA ONE HEALTH*

AUTOR: *David Cano Terriza*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

https://www.uco.es/ucopress/index.php/es/
ucopress@uco.es

TÍTULO DE LA TESIS: Estudio epidemiológico de enfermedades zoonósicas desde una perspectiva *One Health*

DOCTORANDO/A: D. David Cano Terriza

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

La Tesis Doctoral titulada *Estudio epidemiológico de enfermedades zoonósicas desde una perspectiva One Health,* que ha realizado el doctorando D. David Cano Terriza, dio comienzo en el curso académico 2014/2015.

Tiene como objetivo general evaluar el papel de diferentes especies domésticas, peridomésticas y silvestres en la epidemiología de enfermedades zoonósicas de importancia médica y sanitaria.

Para la consecución de este objetivo general, se han planteado los siguientes objetivos específicos: 1) Determinar la prevalencia, distribución espacial y factores de riesgo asociados a la infección por micobacterias del Complejo *Mycobacterium tuberculosis* en explotaciones porcinas manejadas bajo sistemas de producción extensivos. 2) Evaluar la eficacia de la correcta gestión de los residuos de caza como medida de control de la tuberculosis en ungulados silvestres. 3) Determinar la prevalencia y factores de riesgo asociados a la infección por patógenos zoonósicos (flavivirus y *Toxoplasma gondii*) en

especies domésticas (perro) y peridomésticas (palomas y animales de zoológico). 4) Establecer la distribución espacio-temporal y factores implicados en los brotes de virus de West Nile en caballos en España.

Parte de los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral han sido publicados en seis revistas indexadas en el JCR:

- **Cano-Terriza D**, Guerra R, Lecollinet S, Cerdà-Cuéllar M, Cabezón O, Almería S, García-Bocanegra I. (2015). Epidemiological survey of zoonotic pathogens in feral pigeons (*Columba livia* var. *domestica*) and sympatric zoo species in Southern Spain. Comp Immunol Microbiol Infect Dis., 43:22-7.

- **Cano-Terriza D**, Puig-Ribas M, Jiménez-Ruiz S, Cabezón O, Almería S, Galán-Relaño A, Dubey JP, García-Bocanegra I (2016). Risk factors of *Toxoplasma gondii* infection in hunting, pet and watchdogs from Southern Spain and Northern Africa. Parasitol Int., 65:363-66.

- **Cano-Terriza D**, Guerra R, Mozos E, Rodríguez-Sánchez B, Borge C, García-Bocanegra I, Fatal *Acinetobacter baumannii* infection in the critically endangered European mink (*Mustela lutreola*) (2017). J Zoo Wildl Med., 48:220–223.

- García-Bocanegra I, Belkhiria J, Napp S, **Cano-Terriza D**, Jiménez-Ruiz S, Martínez-López B (2018). Epidemiology and spatio-temporal analysis of West Nile virus in horses in Spain between 2010 and 2016. Transbound Emerg Dis., 65:567-577.

- García-Bocanegra I, Jurado-Tarifa E, **Cano-Terriza D**, Martínez R, Pérez-Marín JE, Lecollinet S (2018). Exposure to West Nile virus and tick-borne encephalitis virus in dogs in Spain. Transbound Emerg Dis., 65:765-772.

- **Cano-Terriza D**, Risalde MA, Jiménez-Ruiz S, Vicente J, Isla J, Paniagua J, Moreno I, Gortázar C, Infantes-Lorenzo JA, I García-Bocanegra (2018). Management of hunting waste as control measure for tuberculosis in wild ungulates in south-central Spain. Transbound Emerg Dis. Doi: 10.1111/tbed.12857.

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

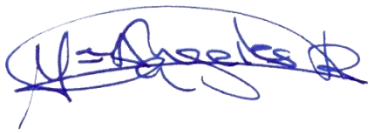
- Cano-Terriza D, Risalde MA, Rodríguez-Hernández P, Napp S, Fernández-Morente M, Moreno I, Bezos J, Fernández-Molera V, Sáez JL, García-Bocanegra I. Epidemiological surveillance of *Mycobacterium tuberculosis* complex in extensively raised pigs in the south of Spain. Prev Vet Med., En revisión.

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, 30 de mayo de 2018

Firma de los directores



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Fdo.: Mª de los Ángeles Risalde Moya

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RESUMEN

El concepto *One Health* (una sola salud) busca promover, mejorar y defender la salud y el bienestar de todas las especies mediante la cooperación y colaboración entre profesionales de Salud Pública, Sanidad Animal y Salud Ambiental. Dicho concepto está experimentando un resurgir importante en las últimas décadas debido al creciente interés sobre las relaciones epidemiológicas de las enfermedades compartidas entre el ser humano, los animales domésticos, la fauna silvestre y el medioambiente, y a la evidencia de que la lucha frente a estas enfermedades ha de abordarse desde una perspectiva holística. El objetivo general de la presente Tesis Doctoral es evaluar el papel de diferentes especies domésticas, peridomésticas y silvestres en la epidemiología de enfermedades zoonósicas bacterianas, víricas y parasitarias desde una perspectiva *One Health*.

En el primer estudio (Capítulo 1.1) se estableció la seroprevalencia, factores de riesgo, distribución espacial y espoligotipos del complejo *Mycobacterium tuberculosis* (CMT) circulantes en cerdos criados en extensivo en Andalucía, para evaluar su papel en el manteniendo y la transmisión de la tuberculosis (TB). La seroprevalencia individual de CMT fue del 2,3% (82/3.622) y la prevalencia de explotación del 24,8% (32/129). El censo porcino y la presencia de explotaciones caprinas colindantes fueron factores de riesgo asociados con la seropositividad de CMT en las granjas porcinas. Se identificaron dos clústeres espaciales estadísticamente significativos (P<0,001) y se aislaron un total de 25 espoligotipos de CMT diferentes en cerdos criados en extensivo en la zona de estudio, compartidos con otras especies domésticas y silvestres. La baja seroprevalencia individual encontrada sugiere que los cerdos ibéricos podrían actuar como hospedadores accidentales del CMT. Sin embargo, la alta prevalencia de rebaño, la identificación de clústeres espaciales y la detección de espoligotipos previamente aislados en diferentes especies, indican la necesidad de implementar programas de control en las explotaciones de cerdo manejadas bajo sistemas de producción extensivos en España.

El objetivo del Capítulo 1.2 fue evaluar la utilidad de la correcta eliminación de los residuos de caza mayor en áreas de alta prevalencia de TB como medida de control del CMT en jabalí (*Sus scrofa*) y ciervo (*Cervus elaphus*) (considerados reservorios naturales del CMT en España), durante las temporadas de caza 2008/2009 a 2016/2017. Los resultados evidenciaron que la eliminación adecuada de los subproductos de caza contribuyó a reducir la seroprevalencia del CMT en jabalí en un 25%. La correcta eliminación de los residuos de la caza mayor ha logrado reducir la seroprevalencia de tuberculosis en jabalí (*Sus scrofa*) hasta en un 25%, siendo por tanto una medida eficaz para control de tuberculosis en esta especie. Estos resultados son de especial relevancia en el contexto actual de enfermedades emergentes y reemergentes como la TB y la peste porcina africana en Europa.

El objetivo del Capítulo 2.1 fue determinar la prevalencia de agentes zoonósicos (flavivirus, virus de la influenza aviar (VIA), *Salmonella* spp. y *Toxoplasma gondii*) en palomas y especies simpátricas del parque zoológico de Córdoba (sur de España) entre 2013 y 2014. En este estudio, se detectaron anticuerpos frente a flavivirus en 7,8% de 142 palomas y en el 8,2% de los 49 animales de zoológico analizados. Aunque no se encontró seropositividad frente a VIA ni infección por *Salmonella* spp. en palomas, el 17,9% (5/28) y el 6,8% (3/44) de los animales de zoológico examinados mostraron resultados positivos a VIA y *Salmonella* spp., respectivamente. Además, se detectaron anticuerpos frente a *T. gondii* en el 9,2% de las 142 palomas y en el 26,9% de los 108 animales de zoológico testados. Estos resultados sugieren que las palomas y los animales de zoológico podrían ser empleados como especies centinela en la vigilancia de patógenos zoonósicos en zonas urbanas.

En el Capítulo 2.2 se describe el primer caso mortal de infección por *Acinetobacter baumannii* en visón europeo (*Mustela lutreola*), una especie catalogada como en peligro crítico de extinción. La bacteria se aisló en cultivo de pulmón y riñón, se identificó mediante una batería de pruebas bioquímicas (API 20NE) y se confirmó mediante desorción/ionización láser asistida por matriz (MALDI-TOF). Además, la cepa aislada

presentó resistencia a un total de 19 antibióticos. La confirmación de este caso no sólo es de interés para la conservación, sino también para la Salud Pública, dado que *A. baumannii* es uno de los patógenos más importantes implicados en las infecciones nosocomiales en humanos.

El Capítulo 3 se centra en el análisis de la distribución espacio-temporal y los factores de riesgo implicados en los brotes del virus de West Nile (VWN) en caballos de España entre 2010 y 2016. Los resultados obtenidos confirmaron que el VWN ha circulado de forma endémica en España desde el año 2010. Sin embargo, su distribución no es homogénea, estando la mayoría de los brotes (92,7%) concentrados en el suroeste del país, donde se han identificaron clústeres espaciales significativos en años no consecutivos. La temperatura media anual (49,5%), la presencia de *Culex pipiens* (19,5%), la precipitación media anual (16,1%) y la distancia a los humedales Ramsar (14,9%), fueron las variables más asociadas con la presencia de brotes de VWN en España. Este estudio proporcionó una información valiosa para el desarrollo de programas de vigilancia basados en riesgo, orientados a una mejor prevención y control del VWN en España.

En el Capítulo 4.1 se evalúa la seroprevalencia y los factores riesgo asociados a flavivirus antigénicamente relacionados (VWN, virus de Usutu y virus de la encefalitis transmitida por garrapatas (ETG)) en perros de España. La prevalencia global de anticuerpos frente a flavivirus fue del 4,8% (39/815), y se observó una seropositividad significativamente mayor en los perros de caza en comparación con los perros de compañía. Este es el primer estudio seroepidemiológico de VWN y ETG en perros en España, así como la primera descripción de circulación de ETG en el país. Estos resultados sugieren que la vigilancia serológica en perros podría ser una herramienta complementaria para monitorizar la actividad de los flavivirus emergentes en España.

El objetivo del Capítulo 4.2 fue determinar la seroprevalencia y los factores de riesgo asociados a la infección por *T. gondii* en perros de Andalucía y Ceuta. Se detectaron anticuerpos frente a *T. gondii* en el 30,6% (235/769) de los perros analizados. La edad

(perros adultos), la actividad (perros de caza) y el tamaño (perros grandes y medianos) fueron los principales factores de riesgo asociados a la infección por *T. gondii*. Los resultados indican que *T. gondii* está muy extendido en perros de la España peninsular y de Ceuta, lo que podría tener importantes implicaciones para la Salud Pública.

En esta Tesis Doctoral se aportan nuevos conocimientos sobre la implicación de las especies domésticas y silvestres en la epidemiología de diferentes enfermedades zoonósicas de importancia médica y sanitaria. Los resultados obtenidos permitirán mejorar la vigilancia epidemiológica de enfermedades zoonósicas compartidas entre especies animales que habitan en el entorno urbano, periurbano y en el medio natural.

SUMMARY

The *One Health* concept (one unique health) aims to promote, improve and defend the health and wellness status of all species through cooperation and collaboration among professionals from Public, Animal and Environmental Health. This concept is undergoing an important rebirth in recent decades due to the growing interest about the epidemiological relationships of diseases shared among humans, domestic animals, wildlife and the environment, and the evidence that the control against these diseases must be established from a holistic perspective. The general objective of this Doctoral Thesis is to assess the role of different domestic, peridomestic and wild species in the epidemiology of bacterial, viral and parasitic zoonotic diseases under a *One Health* approach.

In the first study (Chapter 1.1), we aimed to establish the seroprevalence, risk factors, spatial distribution and spoligotypes of *Mycobacterium tuberculosis* complex **(**MTC) circulating in pigs extensively farmed in Andalusia, in order to assess their role in the maintenance and transmission of tuberculosis (TB). The individual seroprevalence of MTC was 2.3% (82/3,622) and the herd prevalence was 24.8% (32/129). Pig census and the presence of neighboring goat flocks were risk factors associated with MTC seropositivity in farms. Two statistically significant spatial clusters ($P<0.001$) were identified and a total of 25 different MTC spoligotypes shared with other domestic and wild species were isolated in pigs bred extensively in the study area. The low individual seroprevalence found suggests that Iberian pigs could act as spillover hosts of MTC. However, the high herd prevalence, as well as the identification of significant spatial clusters, indicate the need to implement control programs in pig farms managed under extensive production systems in Spain.

The aim of the Chapter 1.2 was to assess the usefulness of the proper disposal of hunting waste (big game) in high TB prevalence areas as MTC control measure in wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) (which are considered natural MTC reservoirs in Spain), from 2008/2009 to 2016/2017 hunting seasons. The results evidenced that the proper disposal of hunting waste contributed to achieve a 25% reduction in MTC

seroprevalence in wild boar. This measure together with other wildlife disease control tools, should be implemented and maintained over time in integrated disease control strategies.

The objective of the Chapter 2.1 was to determine the prevalence of pathogenic zoonotic agents (flaviviruses, avian influenza virus (AIV), *Salmonella* spp. and *Toxoplasma gondii*) in feral pigeons and sympatric zoo animals from Córdoba (southern Spain) between 2013 and 2014. Antibodies against flaviviruses were detected in 7.8% out of 142 pigeons, and 8.2% out of 49 zoo animals tested. Even though seropositivity to AIV and *Salmonella* spp. was not detected in pigeons, 17.9% (5/28) and 6.8% (3/44) of the zoo animals tested, showed positive results to AIV and *Salmonella* spp, respectively. Moreover, antibodies against *Toxoplasma gondii* were found in 9.2% out of 142 pigeons and 26.9% out of 108 zoo animals tested. These results suggest that pigeons and sympatric zoo animals may be included as sentinel species for monitoring zoonotic pathogens in urban areas.

In Chapter 2.2, the first fatal case of *Acinetobacter baumannii* infection is reported in the critically endangered European mink (*Mustela lutreola*). This pathogen was isolated from lung and kidney culture, then identified through a battery of biochemical tests (API 20NE) and finally confirmed using desorption/ionization laser assisted by matrix (MALDI-TOF). The isolated strain showed resistance against 19 different antibiotics. Confirmation of this case is not only of conservation interest but also of public health concern given that *A. baumannii* is one of the most important pathogens implicated in nosocomial infections in humans.

Chapter 3 analyzed the spatio-temporal distribution and risk factors involved in the West Nile virus (WNV) outbreaks in horses in Spain between 2010 and 2016. WNV has circulated endemically in Spain since 2010. However, its distribution is not homogeneous, since most of the WNV outbreaks in Spain (92.7%) were concentrated in the southwestern part of the country, where significant clusters were detected in non-consecutive years. Mean annual temperature (49.5%), presence of *Culex pipiens* (19.5%), mean annual precipitation (16.1%) and distance to Ramsar wetlands (14.9%) were the environmental

variables most associated with WNV outbreaks occurrence in Spain. This study provided valuable information for the development of surveillance programs based on risk factors and aimed at improving prevention and control of WNV in Spain.

In Chapter 4.1, the seroprevalence and risk factors associated with antigenically related flaviviruses, (WNV, Usutu virus (USUV) and tick-borne encephalitis virus (TBEV)), were assessed in dogs in Spain. The overall prevalence of antibodies against flaviviruses was 4.8% (39/815) and a significantly higher seropositivity was observed in hunting dogs compared to pet dogs. This is the first seroepidemiological survey of WNV and TBEV in dogs in Spain, as well as the first report of TBEV circulation in this country. These results suggest that serosurveillance in dogs could be a complementary tool for monitoring the activity of emerging flaviviruses in Spain.

The aim of the Chapter 4.2 was to determine the seroprevalence and risk factors associated with *T. gondii* infection in dogs from Andalusia and Ceuta in Spain. Antibodies to *T. gondii* were detected in 30.6% (235/769) of the dogs analysed. The main risk factors for *T. gondii* infection were age (higher seroprevalence in older dogs), activity (hunting dogs) and size (higher seroprevalence in larger and medium dogs). The results indicate that *T. gondii* is widespread in dogs in mainland Spain and Ceuta, which might have important implications for Public health.

This Doctoral Thesis contributes to throw new knowledge about the role of domestic and wild species in the epidemiology of different zoonotic diseases of medical and health concern. The results obtained will allow to improve the epidemiological surveillance of zoonotic diseases shared among animal species from urban, periurban and natural habitats.

INTRODUCCIÓN

1. Concepto *One Health*

El concepto *One Health* (“Una Sola Salud”) hace referencia a una estrategia mundial encaminada a favorecer la comunicación y la colaboración interdisciplinar entre profesionales de la Salud Pública, la Sanidad Animal y la Salud Ambiental, entendiendo que la salud de las personas, de los animales y del medio ambiente están estrechamente interconectadas entre sí.

En el siglo XIX el científico alemán Rudolf Vircchow (1821-1902), considerado el padre de la medicina comparativa, la biología celular y la patología veterinaria, puso de manifiesto el estrecho vínculo entre las enfermedades de los seres humanos y los animales, instaurando el término "zoonosis". Vircchow indicó la importancia en la colaboración de la medicina veterinaria y la medicina humana, afirmando que no había más que una sola medicina (Schultz, 2008). Su afirmación "entre la medicina animal y la humana no hay líneas divisorias, ni debería haberlas", sigue siendo un referente a día de hoy (Kahn et al., 2007).

Sin embargo, fue el veterinario Calvin W. Schwabe (1927-2006), considerado el fundador de la epidemiología veterinaria y una autoridad global en el área de las zoonosis, quien acuñó el término *One Medicine* (*“una sola medicina”)* en el siglo XX (Monath et al., 2010). Schwabe reconoció que no hay diferencia de paradigma entre la medicina humana y la veterinaria y que ambas disciplinas pueden contribuir al desarrollo de la otra (Monath et al., 2010; Zinsstag et al., 2011). Así, desde 1922, el lema que figura en el escudo de la profesión veterinaria en España: “*Hygia pecoris, salus populi*” (la higiene del ganado, la salud del pueblo), ya hace referencia a este concepto, evidenciándose la estrecha relación entre la salud de personas y la de los animales.

El término *One Medicine* en algunos aspectos puede tener una connotación más clínica, por ello, teniendo en cuenta un enfoque más amplio, el concepto original de *One Medicine,* utilizado sobre todo a finales del siglo XX, ha sido gradualmente sustituido por *One Health* (“Una Sola Salud”) (Zinsstag et al., 2011). En consecuencia, el concepto actual busca potenciar el abordaje multidisciplinar de los riesgos sanitarios en la interfaz humano-

animal-ecosistema, integrando factores genéticos, biológicos, sociales, económicos, políticos, ecológicos, medioambientales, así como sus interacciones (Hudson, 2002; Wilcox y Gubler, 2005) (Figura 1).

En los últimos años, dicho concepto ha experimentado un auge importante debido al creciente interés sobre las relaciones epidemiológicas de los agentes infectocontagiosos entre humanos, animales domésticos, la fauna silvestre y el medioambiente en el que habitan (Kahn et al., 2007; Kaplan et al., 2009; Zinsstag et al., 2011).

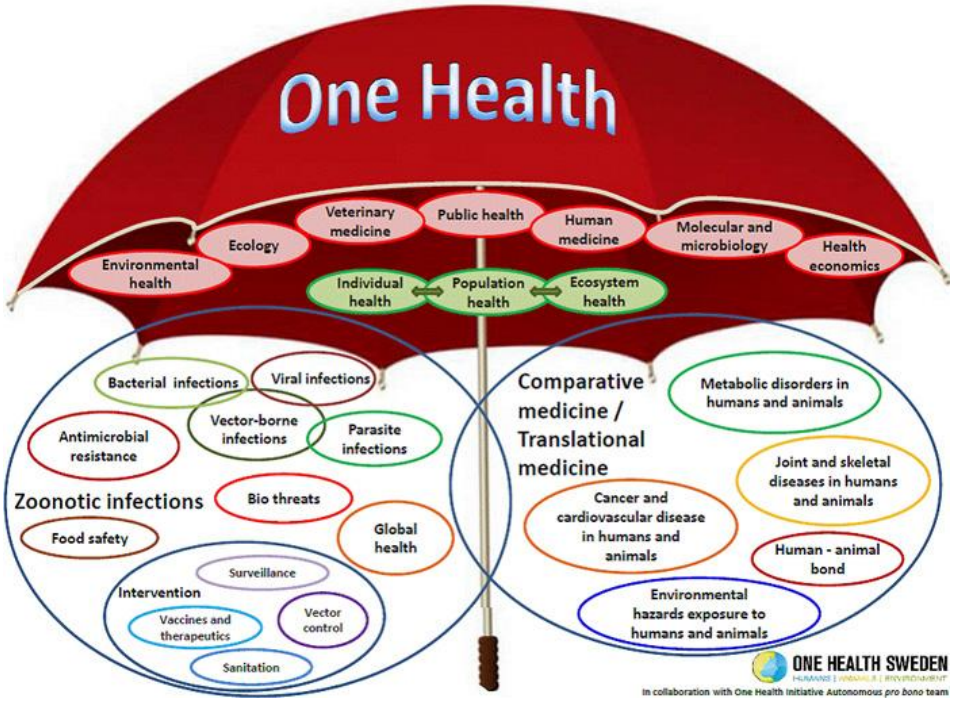

Figura 1. Representación gráfica del concepto *One Health,* desarrollado por las redes *One Health Sweden* y *One Health Initiative* (Gibbs, 2014).

2. La importancia de las zoonosis

Existe un elevado número de enfermedades animales que pueden transmitirse al hombre, y que han sido denominadas tradicionalmente como zoonosis desde que Virchow usó este término por primera vez (Schultz, 2008). Durante un tiempo dichas enfermedades fueron llamadas “antropozoonosis”, mientras que las enfermedades transmisibles de los humanos a los animales se denominaban “zooantroponosis”. Desafortunadamente, estos términos se usaron en sentido inverso o de forma indiscriminada en muchas ocasiones, y por tanto, se decidió abandonarlos y se recomendó usar el término "zoonosis" en el contexto en el que lo usamos hoy en día (Hubalek, 2003). No obstante, desde un punto de vista estrictamente etimológico, el término zoonosis (del griego "*zoon*" = animal y “*nosos*” = enfermedad) cuyo significado es “enfermedad de los animales” (sin hacer referencia a la transmisión a la especie humana), sería incorrecto. Por ello, el término se ha intentado sustituir sin éxito hasta el punto de que el Comité Mixto de la OMS y la FAO de Expertos Mundiales de Zoonosis recomendaron su utilización, definiendo zoonosis como “aquellas enfermedades o infecciones que se transmiten de forma natural entre los vertebrados y el hombre y viceversa”.

Son varios criterios los que se han usado para clasificar las zoonosis con el objetivo de facilitar su estudio y plantear su lucha. Así, por ejemplo, las zoonosis se pueden clasificar según las vías de transmisión a los humanos, en zoonosis por contacto directo, indirecto, alimentarias o transmitidas por vectores (CDC, 2018a). Otro criterio clasifica las zoonosis en distintas categorías epidemiológicas según el ecosistema en el que circulan, existiendo así zoonosis con un ciclo fundamentalmente urbano y periurbano, donde la fuente de infección son los animales domésticos y peridomésticos, mientras que otras, poseen un ciclo fundamentalmente selvático, ya que se suelen dar en el medio natural mediante el contacto con fauna silvestre. Sin embargo, al igual que con cualquier clasificación, agrupar las enfermedades en categorías epidemiológicas de acuerdo con la fuente de infección tiene como inconveniente que las complejas relaciones que se dan entre la fauna silvestre, el

ganado doméstico, los animales de compañía y el ser humano en cada contexto epidemiológico, propician que algunas zoonosis circulen tanto en ciclos urbanos como silvestres (Hubalek, 2003).

La lista de enfermedades que se agrupan dentro de las zoonosis es muy extensa, así, hoy en día, el 60% de los patógenos emergentes que afectan al ser humano son zoonósicos (Jones et al., 2008), y, de hecho, frecuentemente se registran nuevas relaciones patológicas entre animales y humanos. Aunque el conocimiento y la aparición de nuevas zoonosis están en parte vinculados al desarrollo y al perfeccionamiento de las herramientas de diagnóstico, las principales causas de su aparición están directamente asociadas a factores relacionados con la actividad humana. Así, factores como el creciente desarrollo del ecoturismo, los cambios en las prácticas agrícolas, la globalización del comercio, la invasión del hábitat natural de la fauna salvaje o el calentamiento global, están directamente relacionados con la emergencia de nuevas zoonosis (Chomel et al., 2007; Brault, 2009). Todos ellos se combinan para crear una situación propicia para la aparición de patógenos zoonósicos en la interfaz humano-animal y facilitan la rápida difusión de los agentes infectocontagiosos en nuevos escenarios epidemiológicos (Daszak et al., 2000).

3. Epidemiología de las enfermedades compartidas

La capacidad de una especie para actuar como reservorio de una enfermedad no sólo dependerá de ella misma y del patógeno, sino también de distintas circunstancias ambientales como el hábitat, los mecanismos de transmisión del agente, la densidad poblacional de la especie o las interacciones específicas con el resto de especies hospedadoras. En consecuencia, una misma especie animal puede actuar como hospedador accidental o verdadero reservorio según el contexto epidemiológico (Nugent, 2011).

Actualmente, tanto las especies animales silvestres como las domésticas se consideran verdaderos reservorios de un elevado número de enfermedades zoonósicas de

etiología bacteriana, vírica y parasitaria, jugando un importante papel en el mantenimiento, la transmisión y la dispersión de estos patógenos (OIE, 2018). Las diferentes relaciones epidemiológicas que se dan entre estas especies y el ser humano en cada contexto epidemiológico, propician la creación de una compleja red de conexiones que favorece el intercambio de patógenos (Wilcox y Gubler, 2005). Es en esta red de conexiones donde se establecen los diferentes nichos de interacción, con ciclos urbanos, periurbanos y silvestres entre los animales y el hombre que favorecen la adaptación de unas especies a otras, incrementando, consecuentemente, el riesgo de transmisión interespecífica de patógenos entre especies simpátricas (Siembieda et al., 2011).

Dentro de los ciclos urbanos, los animales de compañía son los que están más próximos a los humanos y, en ocasiones, las enfermedades zoonósicas transmitidas a través de los mismos pueden llegar a causar graves problemas de salud (Tabla 1) (Monath et al., 2010). En la última década, el número de mascotas que viven en estrecho contacto con las personas se ha incrementado exponencialmente, observándose que, en España, hasta el 40% de los hogares posee al menos un animal de compañía, destacando el perro por encima del resto (AMVAC, 2018). Adicionalmente, en los últimos tiempos la posesión de mascotas exóticas es cada vez más frecuente, lo que supone un mayor riesgo de exposición frente a nuevos agentes zoonósicos (Cleaveland et al., 2001).

Tabla 1. Principales zoonosis transmitidas por animales de compañía. Adaptado de Monath et al. (2010).

Zoonosis	Agente patógeno	Animal implicado en la transmisión
Bacteriano		
Enfermedad del arañazo del gato	*Bartonella henselae*	Gatos
Enfermedad de Lyme	*Borrelia burgdorferi*	Perros (vía garrapatas)
Campilobacteriosis	*Campylobacter*	Perros y gatos
Ornitosis	*Chlamydia psittaci*	Aves
Tularemia	*Francisella tularensis*	Roedores y lagomorfos
Leptospirosis	*Leptospira*	Perros, roedores
Salmonelosis	*Salmonella*	Reptiles, aves…
Fiebre por mordedura de rata	*Spirillum* sp.; *Streptobacillus* sp.	Roedores
Fúngico		
Tiñas	*Microsporum* spp.; *Trichophyton* sp.	Perros, gatos…
Criptococosis	*Cryptococcus neoformans*	Aves (palomas)
Vírico		
Viruela del mono	Monkeypox virus	Roedores
Rabia	Virus de la rabia	Perros, gatos …
Meningitis aseptica	Virus Coriomeningitis linfocítica	Roedores
Parasitario		
Larva migrans cutánea	*Ancylostoma* spp.	Perros, gatos y su ambiente
Criptosporidiosis	*Cryptosporidium*	Perros, gatos
Dipilidiasis	*Dipylidium caninum*	Perros y gatos (vía pulgas)
Dirofilariasis	*Dirofilaria immitis*	Perros (vía mosquitos)
Hidatidosis	*Echinoccocus granulosus*	Perros
Leishmaniasis	*Leishmania*	Perros, conejos (mosquitos)
Sarna sarcóptica	*Sarcoptes scabiei*	Perros, gatos, roedores…
Toxocariosis	*Toxocara* spp	Perros, gatos
Toxoplasmosis	*Toxoplasma gondii*	Gatos

Las especies peridomésticas y las especies silvestres mantenidas en cautividad en los zoológicos también pueden participar en el ciclo epidemiológico de múltiples patógenos en un entorno urbano o periurbano. De hecho, se han detectado más de 174 enfermedades

infectocontagiosas que potencialmente pueden aparecer en parques zoológicos, muchas de las cuales son zoonosis (AAVZ, 2018). En este sentido, se han descrito diversos brotes en humanos por patógenos como *Mycobacterium tuberculosis (M. tuberculosis)*, *M. bovis* o *Salmonella* spp. asociados a animales silvestres mantenidos en cautividad (Stetter et al., 1995, Friedman et al., 1998; Michalak et al., 1998; Chomel et al., 2007).

Anualmente, la Autoridad Europea de Seguridad Alimentaria (EFSA), de acuerdo con la información recibida por los Estados Miembros, elabora un informe con todos los datos de zoonosis y agentes zoonósicos (Figura 2). Este informe pone de manifiesto la importancia de las zoonosis de tipo alimentario transmitidas por el ganado doméstico, siendo la campilobacteriosis y la salmonelosis las enfermedades más frecuentemente reportadas en los países de la Unión Europea (UE).

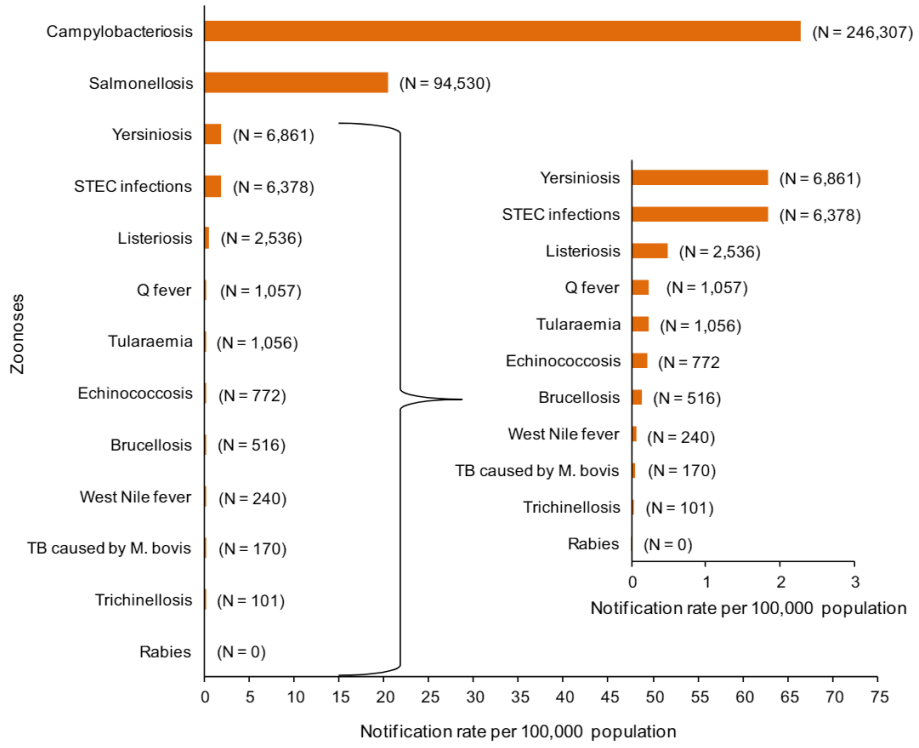

Figura 2. Notificación de zoonosis confirmadas en la UE, 2016 (EFSA, 2017).

En cuanto al interés por las enfermedades compartidas con la fauna silvestre, tradicionalmente sólo se han tenido en cuenta cuando éstas podían suponer una amenaza para la ganadería y/o la Salud Pública (Daszak et al., 2000; Fischer y Gerhold, 2002). Sin embargo, actualmente se reconoce que la transmisión de patógenos puede ser bidireccional entre animales domésticos y silvestres o entre humanos y animales, dependiendo de las diferentes condiciones epidemiológicas (Bengis et al., 2002; Nugent, 2011). Además, los agentes infectocontagiosos cuyos reservorios naturales son las especies silvestres no sólo son de importancia para la Salud Pública o la producción ganadera, sino que también pueden tener un importante impacto sobre sectores económicos como el cinegético (Barasona et al., 2016) o en la conservación de especies amenazadas (Briones et al., 2000; Masot et al., 2016).

Por lo tanto, el control de las enfermedades zoonósicas compartidas requiere el establecimiento de estrategias apropiadas que reduzcan la transmisión de patógenos entre seres humanos, animales domésticos y especies silvestres, actuando sobre todos los actores que contribuyen a su mantenimiento. Así, la instauración de programas de vigilancia realizados por equipos multidisciplinares, especialmente en los escenarios de mayor interacción, se hace fundamental para comprender la Sanidad Animal desde una perspectiva *One Health* y abordar con eficacia el control de dichas enfermedades compartidas (Zinsstag et al., 2011). En este contexto, la legislación nacional, en concreto el Real Decreto 1940/2004 sobre la vigilancia de zoonosis y los agentes zoonóticos, proponen una serie de enfermedades zoonósicas (la tuberculosis (TB) provocada por *M. bovis,* la salmonelosis o la triquinosis) que deben ser objeto de vigilancia (siempre), mientras que establecen una lista de zoonosis y agentes zoonósicos que deben ser objeto de vigilancia en función de la situación epidemiológica (la toxoplasmosis, la leptospirosis o las virosis transmitidas por artrópodos vectores, entre otras).

4. Tuberculosis como modelo de enfermedad bacteriana compartida

Un ejemplo relevante del papel que juegan las especies domésticas y silvestres en la transmisión de enfermedades zoonósicas compartidas es la TB. Esta enfermedad infecciosa crónica causada por micobacterias del Complejo *Mycobacterium tuberculosis* (CMT), afecta a una gran variedad de mamíferos, incluido el hombre (Koch, 1882). Dentro de las especies de micobacterias del CMT con mayor importancia en Sanidad Animal y Salud Pública destacan *M. tuberculosis, M. bovis* y *M. caprae*, cuyos principales reservorios son el ser humano, el bovino y el ganado caprino, respectivamente.

Además de las micobacterias del CMT, otras micobacterias patógenas de interés en medicina humana y veterinaria se incluyen dentro del complejo *Mycobacterium avium*. No obstante, otras especies no incluidas en estos complejos también podrían tener repercusiones clínicas en animales y humanos (Tabla 2).

Tabla 2. Principales especies de micobacterias patógenas.

Complejo *M. tuberculosis*		**Complejo *M. avium***	
M. tuberculosis	*M. pinnipedii*	*M. a. avium*	*M. chimaera*
M. bovis	*M. canetti*	*M. a. silvaticum*	*M. columbiense*
M. bovis BCG	*M. orygis*	*M. a. paratuberculosis*	*M. marseillense*
M. africanum	*M. caprae*	*M. a. hominissuis*	*M. bouchedurhonense*
M. microti		*M. intracellulare*	*M. timonense*
		M. arosiense	
Micobacterias difícilmente cultivables patógenas		**Micobacterias atípicas de importancia zoonósica**	
M. leprae		*M. kansasii*	*M. marinum*
M. lepraemurium		*M. fortuitum*	*M. chelonae*

La TB es una zoonosis con especial importancia en países en vías de desarrollo en los que no se llevan a cabo procesos de pasteurización de la leche, principal fuente de infección en el hombre. En España, el número de casos de TB en humanos asociados a la infección por *M. bovis* se sitúa por encima de los 30 anuales (ISCIII, 2015). Además, la TB está considerada como una zoonosis profesional, asociada a la manipulación de animales o productos de origen animal infectados. Por otro lado, aunque *M. tuberculosis* afecta principalmente a humanos y primates, en España se han descrito casos de infecciones de *M. tuberculosis* en bovino por contacto directo con personas infectadas (Romero et al., 2011).

En Europa, la TB bovina (TBb) está incluida dentro de la lista de enfermedades de declaración obligatoria y representa uno de los principales retos en Sanidad Animal. Actualmente, la situación de la TBb es muy heterogénea. Gracias a las campañas de erradicación aplicadas desde hace varias décadas, se ha logrado que 18 países miembros de la Unión Europea sean considerados como oficialmente libres de TBb. Sin embargo, a pesar de los esfuerzos dirigidos al control y erradicación de esta enfermedad, en algunos países como España, la TBb sigue siendo endémica (EFSA, 2015; MAPAMA, 2018) (Figura 2).

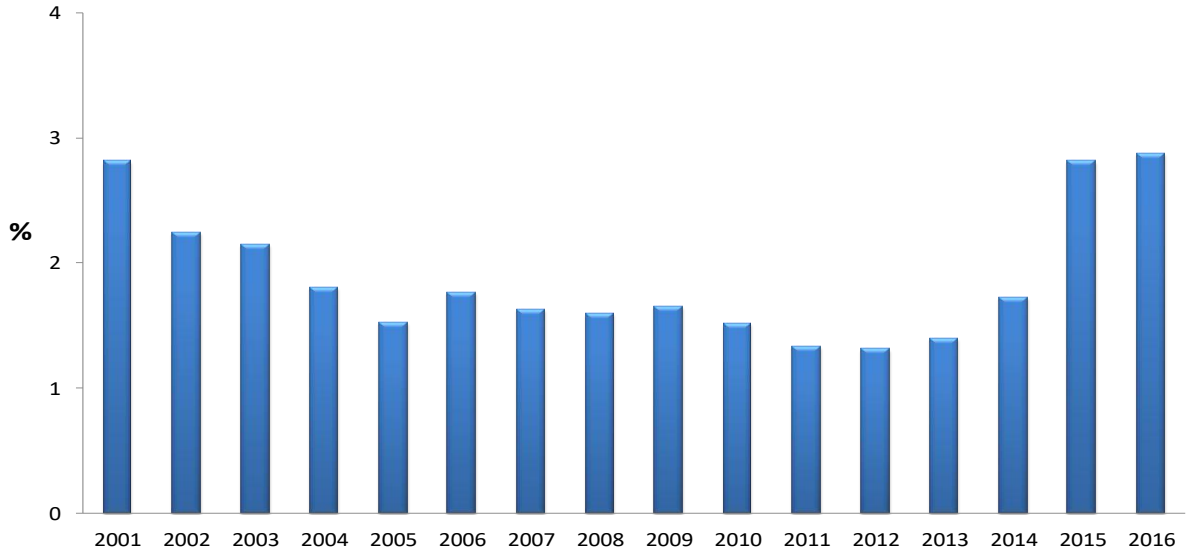

Figura 2. Prevalencia de TB en rebaños bovinos en España (2001-2016). Datos obtenidos de MAPAMA, (2018).

La TBb posee un marcado carácter multihospedador, y la infección por micobacterias del CMT se ha detectado en diferentes especies domésticas y silvestres (Gortázar et al., 2012; Ribeiro et al., 2017). Aunque el ganado bovino es el principal reservorio de la TBb, estudios previos realizados en España han demostrado el papel de la cabra como reservorio del CMT (Napp et al., 2013), compartiendo espoligotipos de *M. bovis* con el ganado vacuno y con especies silvestres como el jabalí (*Sus scrofa*) y el ciervo rojo (*Cervus elaphus*) (Rodríguez et al., 2010), considerados como los principales reservorios silvestres en la España mediterránea (PATUBES, 2017). Así mismo, también se ha demostrado que las ovejas, bajo condiciones específicas, también podrían actuar como reservorios de CMT (Muñoz-Mendoza et al., 2012).

En el caso del cerdo doméstico, tradicionalmente se ha considerado poco relevante en la epidemiología de la TB debido a los sistemas de crianza intensivos. Sin embargo, la situación podría ser diferente en el caso de los cerdos criados en sistemas de producción extensivos, particularmente en ecosistemas mediterráneos donde comparten recursos naturales con reservorios de TB domésticos y silvestres (Di Marco et al., 2012).

Dado el carácter multihospedador de la TB, la identificación de nuevos reservorios domésticos implicados en la epidemiología de la enfermedad, así como la implementación y evaluación de medidas de control de esta enfermedad en los reservorios silvestres, son líneas prioritarias para establecer programas de lucha eficaces.

5. Arbovirosis como modelo de enfermedades víricas compartidas

Otro grupo de enfermedades de especial relevancia dentro del contexto *One Health* son las arbovirosis. Dentro de este tipo de enfermedades se incluyen aquellas producidas por virus ARN mantenidos principalmente en la naturaleza mediante la transmisión biológica de vertebrado a vertebrado por la intermediación de artrópodos hematófagos.

Actualmente existen más de 300 arbovirus, muchos de los cuales son considerados patógenos emergentes o re-emergentes. Dentro de los arbovirus, los virus incluidos en el género *Flavivirus* (familia *Flaviviridae*) destacan por su carácter emergente y porque la mayoría de las 53 especies descritas son patógenas para el ser humano (Beck et al., 2013). Aunque algunos arbovirus son específicos de animales (virus de la lengua azul, virus de la peste equina, virus de Schmallenerg, etc.), la mayoría son patógenos zoonósicos, pudiendo infectar al hombre y a los animales simultáneamente.

De acuerdo con el tipo de vector artrópodo implicado en la transmisión, los flavivirus se clasifican en tres grupos: los flavivirus transmitidos por mosquitos, los transmitidos por garrapatas y los de vector desconocido (Beck et al., 2013) (Figura 3). Aquellos transmitidos por mosquitos pueden subdividirse, a su vez, según la especie de mosquito implicado en la transmisión (géneros *Culex* spp. o *Aedes* spp.). Así, los transmitidos por mosquitos del género *Culex* spp. poseen como principal reservorio a las aves silvestres, mientras que los transmitidos por *Aedes* spp. poseen como principales reservorios a los primates. Dentro de los flavivirus transmitidos por garrapatas, podemos encontrar por un lado al grupo de las encefalitis transmitidas por garrapatas, asociado principalmente a roedores, y por otro, a los virus que circulan entre aves marinas (Beck et al., 2013). Finalmente, el grupo de flavivirus de vector desconocido puede dividirse en dos subgrupos, aquellos aislados exclusivamente en murciélagos y los aislados en roedores (Blitvich et al., 2017).

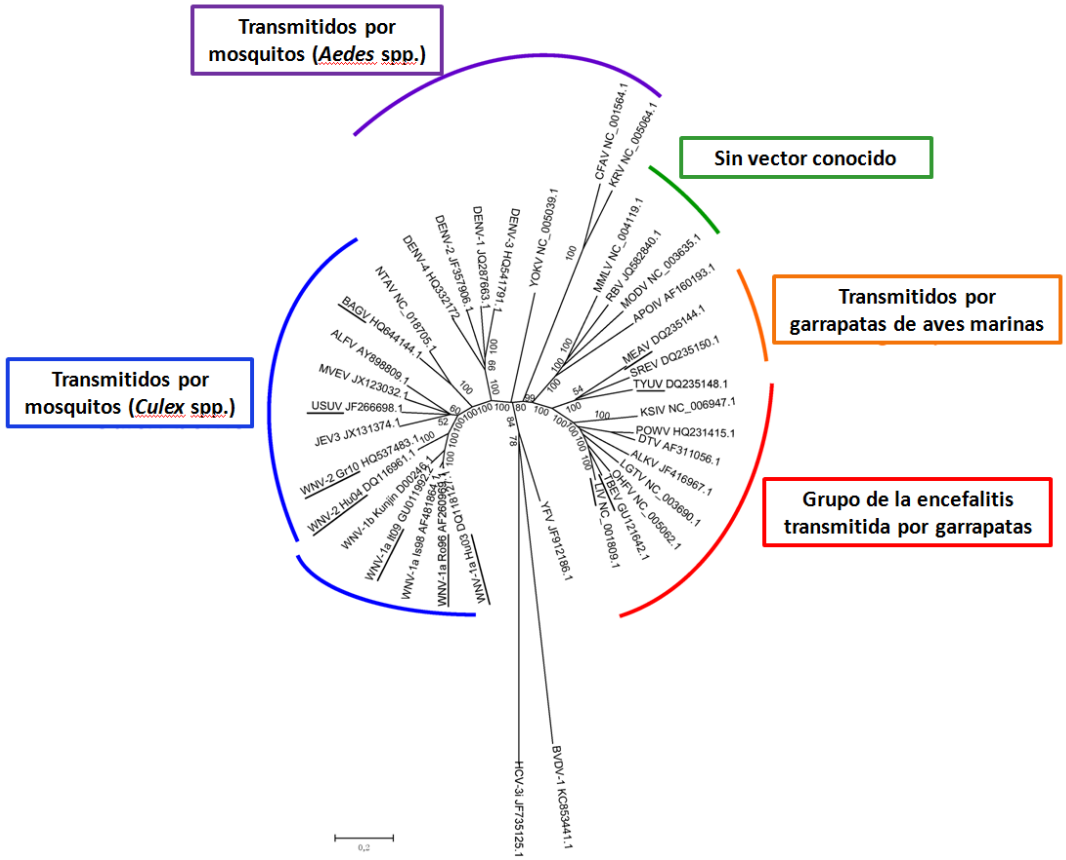

Figura 3. Relación genética de los flavivirus y sus vectores artrópodos. Adaptado de Beck et al., 2013.

En Europa se han detectado siete flavivirus en la última década, incluyendo el virus de West Nile (VWN), virus de la encefalitis transmitida por garrapatas (VETG), virus Usutu, virus del Dengue, virus de Bagaza, virus de la encefalomielitis ovina y virus de Meaban. Cinco de los siete (exceptuando el virus del Dengue y el VETG) ha circulado en España durante la última década (Beck et al., 2013; Arnal et al., 2014).

De todos los flavivirus transmitidos por mosquitos, el VWN es sin duda el flavivirus de mayor expansión geográfica y con mayor rango de hospedadores y vectores. Este flavivirus zoonósico está encuadrado en el serocomplejo de la Encefalitis Japonesa y puede infectar a numerosas especies de vertebrados. De forma habitual, este virus se mantiene en un ciclo selvático o rural, circulando entre aves silvestres y mosquitos ornitofílicos. Sin

embargo, en ocasiones puede pasar a un ciclo urbano o periurbano, transmitiéndose a hospedadores accidentales como los équidos o el hombre (CDC, 2018b).

En humanos, la infección asociada al VWN es asintomática en un 80% de los casos. No obstante, en el 20% restante se produce un cuadro clínico leve o moderado, que en menos del 1% de los casos produce sintomatología nerviosa que puede desembocar en un cuadro de meningoencefalitis de graves consecuencias. De igual forma, en caballos, menos del 20% de los infectados manifiestan clínica, caracterizada por fiebre y sintomatología nerviosa como ataxia, desorientación, hiperestesia, temblores musculares y fotofobia (CDC, 2018b).

En los últimos 5 años se han producido en humanos más de 1000 casos de infecciones por VWN (linajes 1 y 2) en Europa y la cuenca mediterránea (ECDC, 2017a). En España, los primeros brotes en humanos y en caballos se detectaron en el año 2010 (García-Bocanegra et al., 2011). En los años siguientes, el virus ha circulado endémicamente en el país, detectándose 5 brotes en humanos y 191 brotes en equinos (Figura 4), la mayoría de ellos en regiones del suroeste del país (López-Ruiz et al., 2018; RASVE, 2018). Además, se ha confirmado el contacto con VWN en otras especies domésticas y silvestres, así como en diferentes especies de mosquitos (Gaunt et al., 2015; García-Bocanegra et al., 2016).

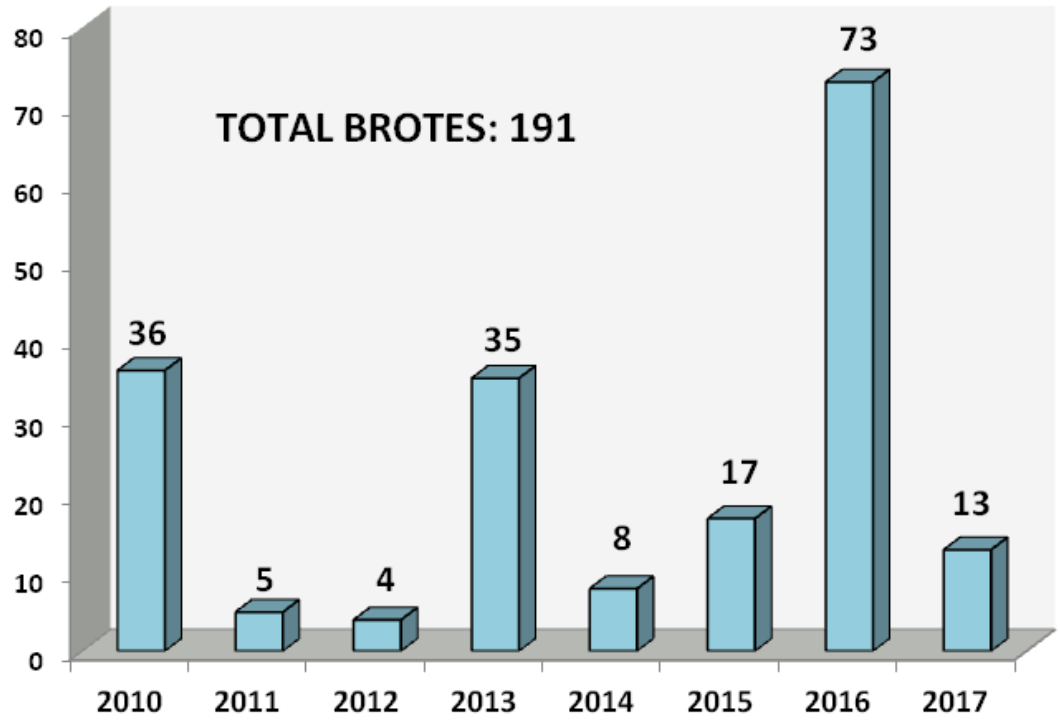

Figura 4. Distribución temporal del número de brotes de VWN en caballos detectados en España entre 2010 y 2017. Datos obtenidos de RASVE, 2018.

A pesar de la importancia médica y sanitaria de este y otros flavivirus, la información relacionada con la prevalencia, distribución espacio-temporal y factores de riesgo asociados a la transmisión en especies domésticas, peridomésticas y silvestres en España sigue siendo muy limitada. Por ello, su monitorización resulta de gran interés para detectar la circulación temprana en áreas de riesgo.

6. Toxoplasmosis como modelo de enfermedad parasitaria compartida

La toxoplasmosis es una zoonosis parasitaria provocada por el protozoo *Toxoplasma gondii (T. gondii),* parásito intracelular obligado que pertenece al phylum Apicomplexa. Dentro de las zoonosis de origen parasitario, la toxoplasmosis es la enfermedad de mayor

distribución mundial, estimándose que un tercio de la población mundial está infectada por *T. gondii* (Montoya y Liesenfeld 2004) (Figura 5).

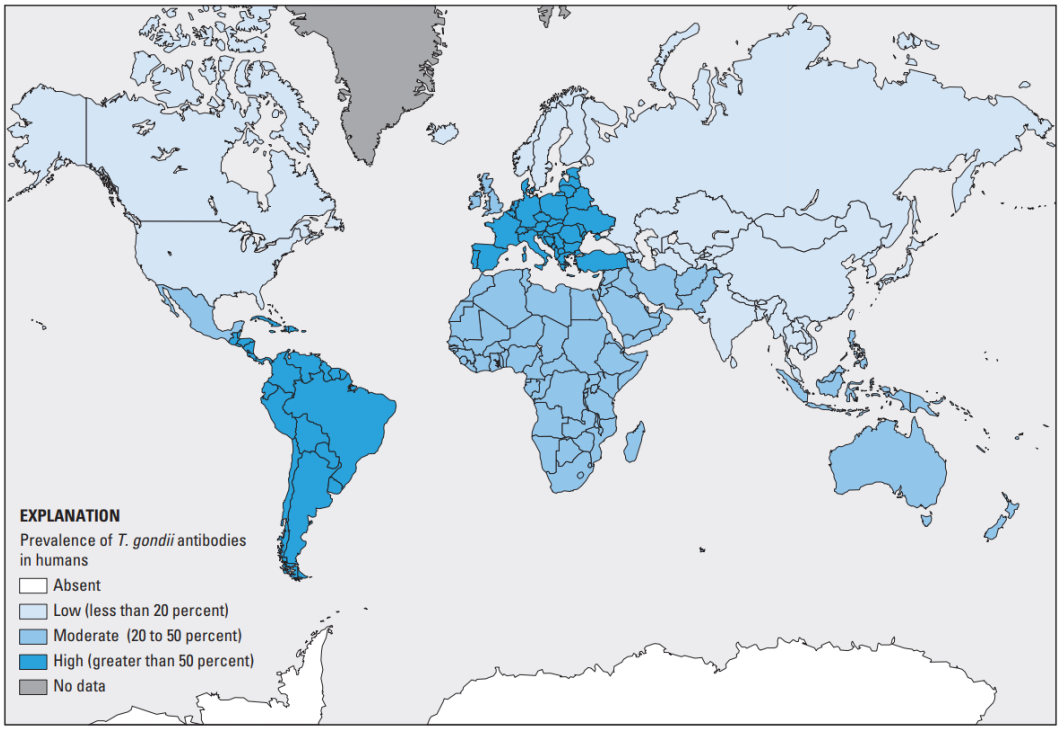

Figura 5. Seroprevalencia de *T. gondii* a nivel mundial (Hill y Dubey, 2014)

Los felinos, tanto domésticos como silvestres, son los hospedadores definitivos de *T. gondii*. Además, todas las especies de sangre caliente son susceptibles de infectarse por este protozoo mediante la ingestión de agua y alimentos contaminados o de forma congénita, pudiendo actuar como hospedadores intermediarios en su ciclo epidemiológico (Dubey, 2010) (Figura 6).

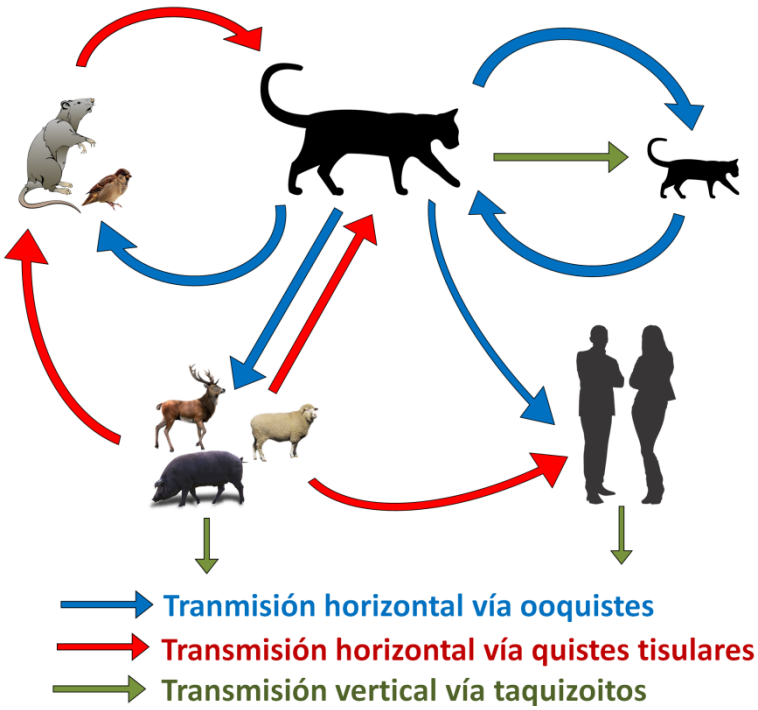

Figura 6. Principales rutas de transmisión de *T. gondii*.

Aunque la mayoría de las infecciones adquiridas en humanos son leves o asintomáticas, *T. gondii* puede causar mortalidad fetal. La infección congénita se produce cuando una mujer se infecta durante el embarazo o en mujeres inmunodeprimidas al reactivarse una infección adquirida previamente al embarazo (Hill y Dubey, 2014).

Por otro lado, la infección posnatal puede dar lugar a diversas formas clínicas localizadas o generalizadas. Así, pueden aparecer complicaciones oculares crónicas graves, así como linfadenopatía o encefalitis en personas inmunodeprimidas (Hill y Dubey, 2002). La toxoplasmosis está considerada como una enfermedad ocupacional en trabajadores de matadero, veterinarios o cazadores, los cuales pueden infectarse durante la evisceración y manipulación de las canales de los animales, siendo los sectores de la población que poseen un mayor riesgo de infección.

Aunque la toxoplasmosis es una de las zoonosis parasitarias más notificadas en seres humanos en la Unión Europea, el sistema de vigilancia se considera inadecuado, por lo que la incidencia de la enfermedad podría estar subestimada (EFSA, 2007) (Figura 7).

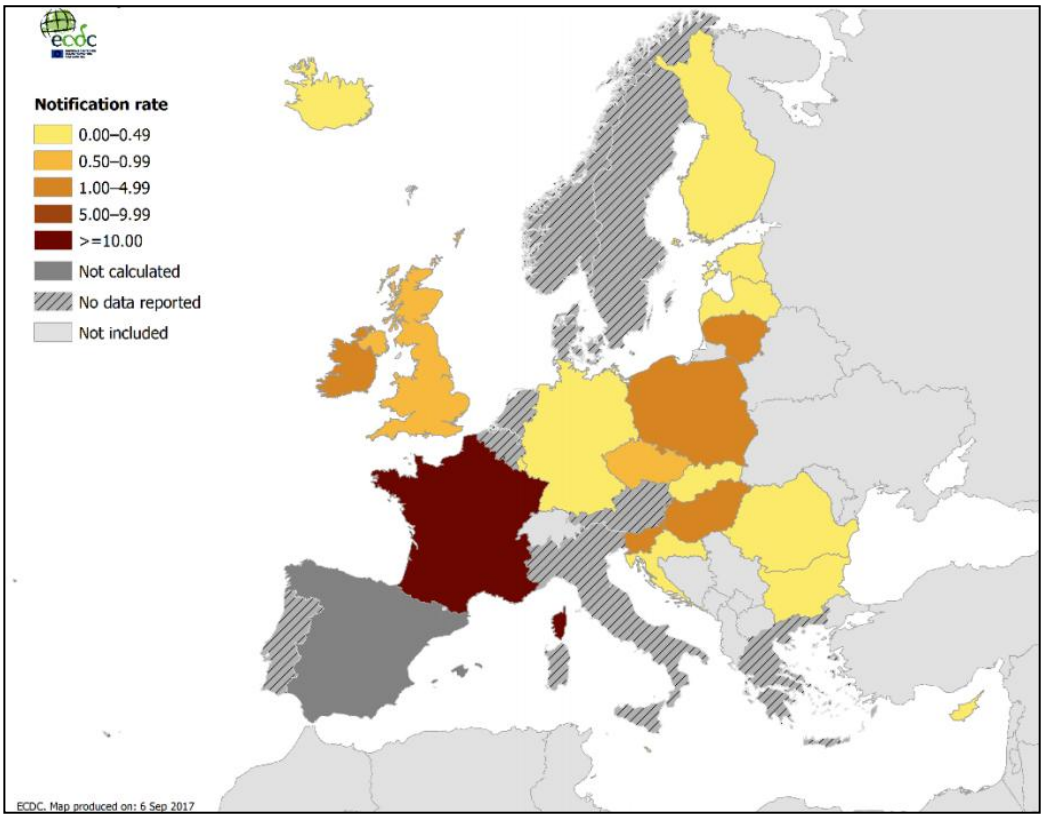

Figura 7. Ratio de casos confirmados de toxoplasmosis congénita en humanos (UE/EEE) por cada 100.000 habitantes (2015) (ECDC, 2017b).

La patogenicidad de *T. gondii* está determinada por la virulencia de la cepa y la susceptibilidad de la especie infectada (Dubey y Beattie, 1988). No obstante, en el ganado doméstico es capaz de producir cuadros clínicos severos, destacando el ovino como la especie donde es más frecuente la aparición de mortalidad perinatal y abortos asociados la infección por este protozoo. Otras especies de producción como el caprino o el porcino también suelen verse afectadas clínicamente, mientras que los equinos o el bovino parecen ser más resistentes a la enfermedad (Dubey y Beattie, 1988).

En fauna silvestre, los estudios serológicos indican que las infecciones son comunes en una amplia variedad de especies (Dubey, 2010). Además, destacan las formas clínicas y subclínicas causadas por el parásito en cérvidos, marsupiales, primates y mamíferos marinos, así como mortalidad en especies de marsupiales, monos del nuevo mundo o nutrias marinas (Cedillo-Peláez et al., 2011; Fernández-Aguilar et al., 2013; Hill y Dubey, 2014).

Los diferentes estudios a nivel mundial muestran que la infección por *T. gondii* en animales de compañía es frecuente. Así, además de los gatos, hospedadores definitivos del parásito, la infección en perros domésticos es habitual y ha demostrado ser un buen indicador de la presencia de *T. gondii* en ambientes urbanos (Cabezón et al., 2010; Dubey, 2010).

Por ello, dadas las importantes repercusiones que la toxoplasmosis clínica puede tener en la Salud pública y la Sanidad Animal, resulta de gran interés la instauración de programas de vigilancia en distintas especies animales, especialmente en aquellas que pueden ser usadas como centinelas en ambientes urbanos y periurbanos.

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OBJETIVOS/OBJECTIVES

OBJETIVOS

El **objetivo general** de la presente tesis es evaluar el papel de diferentes especies domésticas, peridomésticas y silvestres en la epidemiología de enfermedades zoonósicas desde una perspectiva *One Health*. Para la consecución de este objetivo general, se plantean los siguientes **objetivos específicos**:

1. Determinar el papel del cerdo criado en sistemas de producción extensivos en la epidemiología del complejo *Mycobacterium tuberculosis* (CMT) y evaluar la eficacia de la implementación de medidas de control frente a tuberculosis en ungulados silvestres en ecosistemas mediterráneos del sur de España.

Dicho objetivo ha sido abordado en el **Capítulo 1**, que incluye:

Capítulo 1.1. Cuyo objetivo es determinar la seroprevalencia, factores de riesgo, distribución espacial y espoligotipos circulantes de micobacterias del CMT en granjas de porcino Ibérico del sur de España, región con una elevada prevalencia de CMT en ganado bovino y en poblaciones de jabalí (*Sus scrofa*).

Cano-Terriza D, Risalde MA, Rodríguez-Hernández P, Napp S, Fernández-Morente M, Moreno I, Bezos J, Fernández-Molera V, Sáez JL, García-Bocanegra I. Epidemiological surveillance of Mycobacterium tuberculosis complex in extensively raised pigs in the south of Spain. Prev Vet Med., *En revisión.*

Capítulo 1.2. Cuyo objetivo es determinar la eficacia de la correcta eliminación de los residuos procedentes de la caza mayor como medida de control de la tuberculosis en jabalí y ciervo (*Cervus elaphus*) en el centro-sur de España.

Cano-Terriza D, Risalde MA, Jiménez-Ruiz S, Vicente J, Isla J, Paniagua J, Moreno I, Gortázar C, Infantes-Lorenzo JA, I García-Bocanegra (2018). Management of hunting waste as control measure for tuberculosis in wild ungulates in south-central Spain. Transbound Emerg Dis. Doi: 10.1111/tbed.12857.

2. Determinar la prevalencia de infección de patógenos zoonósicos de interés en especies peridomésticas (palomas) y animales de zoológico.

Dicho objetivo ha sido abordado en el **Capítulo 2**, que incluye:

Capítulo 2.1. Cuyo objetivo es establecer la prevalencia de flavivirus, virus influenza aviar, *Toxoplasma gondii* y *Salmonella* spp. en palomas domésticas (*Columba livia* var*. domestica*), así como evaluar el riesgo de transmisión a especies simpátricas del Parque Zoológico Municipal de Córdoba.

Cano-Terriza D, Guerra R, Lecollinet S, Cerdà-Cuéllar M, Cabezón O, Almería S, García-Bocanegra I. (2015). Epidemiological survey of zoonotic pathogens in feral pigeons (Columba livia var. domestica) and sympatric zoo species in Southern Spain. Comp Immunol Microbiol Infect Dis., 43:22-7.

Capítulo 2.2. Cuyo objetivo es describir el primer caso de mortalidad asociada a *Acinetobacter baumannii* en visón europeo (*Mustela lutreola*).

Cano-Terriza D, Guerra R, Mozos E, Rodríguez-Sánchez B, Borge C, García-Bocanegra I, Fatal Acinetobacter baumannii infection in the critically endangered European mink (Mustela lutreola) (2017). J Zoo Wildl Med., 48:220–223.

3. Realizar una descripción clínica y epidemiológica de los brotes del virus de West Nile (VWN) en caballos en España durante el período 2010-2016, establecer la distribución espacio-temporal del virus e identificar los factores implicados en la aparición de los brotes en España, empleando modelos de nicho ecológico basados en el concepto de máxima entropía. Dichos objetivos han sido abordados en el **Capítulo 3.**

García-Bocanegra I, Belkhiria J, Napp S, Cano-Terriza D, Jiménez-Ruiz S, Martínez-López B (2018). Epidemiology and spatio-temporal analysis of West Nile virus in horses in Spain between 2010 and 2016. Transbound Emerg Dis., 65:567-577.

4. Determinar la prevalencia y factores de riesgo asociados a la infección por patógenos zoonósicos en perro.

Dicho objetivo ha sido abordado en el **Capítulo 4**, que incluye:

Capítulo 4.1. Cuyo objetivo es determinar la prevalencia de anticuerpos frente a flavivirus antigénicamente relacionados (VWN, virus Usutu y virus de la encefalitis transmitida por garrapatas) en perros, en el suroeste de España.

García-Bocanegra I, Jurado-Tarifa E, Cano-Terriza D, Martínez R, Pérez-Marín JE, Lecollinet S (2018). Exposure to West Nile virus and tick-borne encephalitis virus in dogs in Spain. Transbound Emerg Dis., 65:765-772.

Capítulo 4.2. Cuyo objetivo es identificar los factores de riesgo asociados a la prevalencia de anticuerpos frente a *Toxoplasma gondii* en perros de caza, guarda y compañía en Andalucía (sur de España) y Ceuta (norte de África).

Cano-Terriza D, Puig-Ribas M, Jiménez-Ruiz S, Cabezón O, Almería S, Galán-Relaño A, Dubey JP, García-Bocanegra I (2016). Risk factors of Toxoplasma gondii infection in hunting, pet and watchdogs from Southern Spain and Northern Africa. Parasitol Int., 65:363-66.

OBJETIVES

The **main objective** of this thesis is to assess the role of different domestic, peridomestic and wild species in the epidemiology of several zoonotic diseases from a *One Health* approach. To achieve this global objective, the following **specific objectives** are addressed:

1. To determine the role of domestic pigs extensively farmed in the epidemiology of *Mycobacterium tuberculosis* complex (MTC) and to assess the usefulness of the proper disposal of hunting waste as tuberculosis control measure in wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*).

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

Chapter 1.1. To establish the seroprevalence, risk factors, spatial distribution and spoligotypes of MTC circulating in the Iberian pig farms in the south of Spain, a region where MTC is highly prevalent in both cattle and wild boar populations.

Cano-Terriza D, Risalde MA, Rodríguez-Hernández P, Napp S, Fernández-Morente M, Moreno I, Bezos J, Fernández-Molera V, Sáez JL, García-Bocanegra I. Epidemiological surveillance of Mycobacterium tuberculosis complex in extensively raised pigs in the south of Spain. Under review.

Chapter 1.2. To assess the usefulness of the proper disposal of big game hunting waste as control measure for tuberculosis in wild boar and red deer in south and central Spain.

Cano-Terriza D, Risalde MA, Jiménez-Ruiz S, Vicente J, Isla J, Paniagua J, Moreno I, Gortázar C, Infantes-Lorenzo JA, I García-Bocanegra (2018). Management of hunting waste as control measure for tuberculosis in wild ungulates in south-central Spain. Transbound Emerg Dis. Doi: 10.1111/tbed.12857.

2. To determine the prevalence of selected zoonotic pathogens in peridomestic species (pigeons) and zoo animals.

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

Chapter 2.1. To determine the prevalence of flaviviruses, avian influenza viruses, *Salmonella* spp. and *Toxoplasma gondii* in feral pigeons, and to assess the risk of transmission to sympatric zoological species in the Cordoba Municipal Zoo Park.

Cano-Terriza D, Guerra R, Lecollinet S, Cerdà-Cuéllar M, Cabezón O, Almería S, García-Bocanegra I. (2015). Epidemiological survey of zoonotic pathogens in feral pigeons (Columba livia var. domestica) and sympatric zoo species in Southern Spain. Comparative Immunology, Microbiology & Infectious Diseases, 43:22-7.

Chapter 2.2. To report the first case of fatal *Acinetobacter baumannii* infection in the critically endangered European mink (*Mustela lutreola*).

Cano-Terriza D, Guerra R, Mozos E, Rodríguez-Sánchez B, Borge C, García-Bocanegra I, Fatal Acinetobacter baumannii infection in the critically endangered European mink (Mustela lutreola) (2017). J Zoo Wildl Med., 48:220–223.

3. **To describe the main epidemiological and clinical findings of the West Nile virus (WNV) outbreaks in horses during the period 2010–2016 in Spain, to assess the spatio-temporal distribution of WNV and to identify the drivers of WNV occurrence in Spain using a presence-only maximum entropy ecological niche model.**

This objective has been addressed in **Chapter 3.**

García-Bocanegra I, Belkhiria J, Napp S, Cano-Terriza D, Jiménez-Ruiz S, Martínez-López B (2018). Epidemiology and spatio-temporal analysis of West Nile virus in horses in Spain between 2010 and 2016. Transbound Emerg Dis., 65:567-577.

4. To determine the prevalence and risk factors associated with the infection of selected zoonotic pathogens in dogs.

This objective has been addressed in **Chapter 4,** which includes:

Chapter 4.1. To determine the prevalence of antibodies against antigenically related zoonotic flaviviruses, particularly WNV, Usutu virus and tick-borne encephalitis virus, in dogs in the south-western regions of Spain.

García-Bocanegra I, Jurado-Tarifa E, Cano-Terriza D, Martínez R, Pérez-Marín JE, Lecollinet S (2018). Exposure to West Nile virus and tick-borne encephalitis virus in dogs in Spain. Transbound Emerg Dis., 65:765-772.

Chapter 4.2. To determine the risk factors affecting the prevalence of antibodies against *Toxoplasma gondii* in hunting, pet and watchdogs in Andalusia (Southern Spain) and Ceuta (Northern Africa).

Cano-Terriza D, Puig-Ribas M, Jiménez-Ruiz S, Cabezón O, Almería S, Galán-Relaño A, Dubey JP, García-Bocanegra I (2016). Risk factors of Toxoplasma gondii infection in hunting, pet and watchdogs from Southern Spain and Northern Africa. Parasitol Int., 65:363-66.

CAPÍTULO 1

Papel del cerdo criado en extensivo en la epidemiología del complejo *Mycobacterium tuberculosis* y evaluación de medidas de control frente a tuberculosis en ungulados silvestres

CAPÍTULO 1.1

Vigilancia epidemiológica del complejo *Mycobacterium tuberculosis* en cerdos criados en sistemas de producción extensivos en el sur de España

Cano-Terriza D, Risalde MA, Rodríguez-Hernández P, Napp S, Fernández-Morente M, Moreno I, Bezos J, Fernández-Molera V, Sáez JL, García-Bocanegra I. **Epidemiological surveillance of *Mycobacterium tuberculosis* complex in extensively raised pigs in the south of Spain.** Preventive Veterinary Medicine. *En revisión*.

Resumen

Se considera que el papel de los cerdos domésticos en la epidemiología del complejo *Mycobacterium tuberculosis* (CMT) es limitado debido a las características de los sistemas de producción intensiva. Sin embargo, en el suroeste de España, los cerdos ibéricos suelen criarse bajo sistemas de producción extensivos, compartiendo hábitat con otras especies domésticas y silvestres, algunas de las cuales pueden actuar como reservorios de CMT. El objetivo del presente trabajo es establecer la seroprevalencia, factores de riesgo, distribución espacial y espoligotipos de CMT que circulan en cerdos en extensivo en Andalucía (sur de España), una región donde la tuberculosis tiene una alta prevalencia en bovino y jabalí. Para ello, se analizaron muestras de suero de 3.622 cerdos ibéricos criados en extensivo en 129 granjas aleatoriamente seleccionadas para detectar anticuerpos frente a CMT utilizando un P22-ELISA indirecto. Se detectaron anticuerpos frente a CMT en 82 cerdos (2,3%; IC95%: 1,78-2,75%). Se encontró una seropositividad significativamente superior en reproductores (3,7%) comparado con los cerdos de cebo (1,7%) (P=0,0001). La seroprevalencia de granja fue del 24,8% (IC95%: 17,4-32,3%). Se identificaron dos factores de riesgo asociados con la seropositividad por CMT en las explotaciones de cerdo en extensivo: el censo de cerdos (mayor seroprevalencia en granjas con mayor censo) (OR=1,001; IC95%: 1,000-1,002) y la presencia de explotaciones caprinas colindantes (OR=7,345; IC95%: 1,464-36,848). En el noroeste de Andalucía se identificaron dos clústeres espaciales estadísticamente significativos (P<0,001). Además, se aislaron un total de 25 espoligotipos diferentes de CMT en cerdos en extensivo en el área de estudio. La baja seroprevalencia individual encontrada sugiere que los cerdos ibéricos podrían actuar como hospedadores accidentales de CMT. Sin embargo, la alta seroprevalencia de explotación, así como la identificación de clústeres espaciales, indica una amplia, aunque no homogénea, circulación de CMT en las explotaciones porcinas en extensivo de Andalucía. Por ello, se deberían implementar programas de vigilancia basados en riesgo y programas de control en estas explotaciones porcinas en España.

Abstract

The role of domestic pigs in the epidemiology of *Mycobacterium tuberculosis* complex (MTC) is considered to be limited due to the characteristics of the intensive production systems. However, in southwestern Spain, Iberian pigs are usually raised under extensive management systems, sharing habitat with other domestic and wild species, some of which may act as reservoirs of MTC. Here, we aimed to establish the seroprevalence, risk factors, spatial distribution and spoligotypes of MTC circulating in pigs extensively farmed in Andalusia (southern Spain), a region where tuberculosis is highly prevalent in both cattle and wild boar populations. Serum samples from 3,622 extensively raised Iberian pigs from 129 randomly selected farms were tested for antibodies against MTC using an indirect P22-ELISA. Antibodies to MTC were detected in 82 pigs (2.3%; 95%CI: 1.78-2.75%). Significantly higher seropositivity was found in sows (3.7%) compared with fattening pigs (1.7%) (P=0.0001). The herd prevalence was 24.8% (95%CI: 17.4-32.3%). Two risk factors were associated with MTC seropositivity in farms: pig census (higher seroprevalence in larger farms) (OR=1.001; 95%CI: 1.000–1.002), and the presence of neighboring goat flocks (OR=7.345; 95%CI: 1.464-36.848). Two statistically significant spatial clusters (P<0.001) were identified in the north-west of Andalusia. A total of 25 different MTC spoligotypes were isolated in pigs bred extensively in the study area. The low individual seroprevalence found in the present study suggests that Iberian pigs could act as spillover hosts of MTC. On the other hand, the high herd prevalence, as well as the identification of significant spatial clusters, indicates a widespread, but not homogenous MTC circulation among extensively managed pig farms in Andalusia. Risk-based surveillance and control programs should be implemented in those farms in Spain.

Keywords: Tuberculosis, Iberian pigs, reservoir, risk factors, spatial analysis, spoligotyes.

1. Introduction

Tuberculosis (TB) is an important zoonosis caused by members of the *Mycobacterium tuberculosis* complex (MTC), which can infect a broad range of domestic and wild mammals worldwide (Bailey et al., 2013). Even though bovine TB (bTB) has been subject to compulsory eradication programs in the European Union (EU) for many years, and in fact 18 EU member countries have managed to get an officially bTB-free status, in several others the disease is still endemic, and prevalence levels have remained constant or even increased in recent years (EFSA, 2017).

In Spain, despite the efforts involved in the eradication of the disease, a high bTB prevalence is still reported in the southwestern regions (MAPAMA, 2018a). In these endemic regions, cattle are usually bred under extensive production systems, sharing habitat with other domestic and wild MTC hosts, which may act as potential reservoirs. In this multi-host context, it is essential to identify and characterize the role of the different species in the transmission of MTC. However, the ability of mammal species to act as spillover or true MTC maintenance hosts depends on the specific epidemiological scenario (Nugent et al., 2015). For instance, the Eurasian wild boar (*Sus scrofa*) acts probably as a spillover MTC host in the Atlantic habitats of the north of the Iberian Peninsula (Muñoz-Mendoza et al., 2013), while it is the most important wildlife in the Iberian Mediterranean ecosystems (Naranjo et al., 2008; Gortázar et al., 2012). Similarly, even though the role of the domestic pigs in the transmission of MTC has been traditionally considered limited, studies have shown that free-ranging domestic pigs may act as a true MTC reservoirs in Mediterranean ecosystems (Di-Marco et al., 2012).

Spain has the largest pig population in Europe, and it is the fourth largest pork producer in the world behind China, the United States and Germany. Pig production is an important driver of the Spanish economy, accounting for around 36.4% of the total animal husbandry production (MAPAMA, 2018b). It is noteworthy that Iberian pigs extensively farmed represent around 10% of the national pig farming in Spain, and the southwestern

regions concentrate approximately the 80% of the Spanish Iberian pig farms. TB-like lesions (TBLL) are an important cause of condemnation in Iberian pigs at slaughterhouses, resulting in significant economic losses for this type of production (Cardoso-Toset et al., 2015). Because epidemiological information on MTC in domestic pigs is still very scarce, we aimed to establish the seroprevalence, risk factors, spatial distribution and spoligotypes of MTC circulating in the Iberian pig farms in the south of Spain, a region where MTC is highly prevalent in both cattle and wild boar populations.

2. Materials and methods

2.1 Study design and sample collection

Between 2015 and 2017, a cross-sectional survey was carried out to assess the prevalence of antibodies against MTC in 129 pig farms managed under extensive production systems in Andalusia, the Spanish region with the highest herd prevalence of bTB (MAPAMA, 2018a). The sample size was calculated assuming a herd prevalence of 50%, with a 95% confidence interval and an accepted error of 9%. The number of farms sampled in each province was proportional to the census of Iberian pigs in that province. Within each province, pig farms were randomly selected. The number of pigs collected at each farm (30 whenever possible) was chosen to ensure a 95% probability of detecting at least one positive animal for an assumed minimum within-herd prevalence of 10%.

A total of 3,622 blood samples were collected in the selected farms using the orbital sinus puncture method. Samples were kept at 4°C, centrifuged at 400g during 10 min for serum separation, and stored at -20ºC until analysis. Epidemiological information related to the animals sampled, management and production parameters, as well as environmental data and biosecurity measures implemented in the farms, was gathered by direct interview with farmers.

2.2 Serological analysis

Serum samples were analyzed by indirect ELISA to detect antibodies against MTC using as antigen an immunopurified subcomplex protein from bovine tuberculin purified protein derivative (bPPD) (CZ Veterinaria SA, Porriño, Spain), named P22 (Infantes-Lorenzo et al., 2017). The protocol used has been described (patent EP16382579, "Methods and compositions for tuberculosis diagnosis") and previously detailed (Cano-Terriza et al., 2018). The estimated sensitivity and specificity of this ELISA in swine were 84.1% and 98.4%, respectively (Thomas et al., unpublished data).

2.3 Statistical analyses

The individual and herd seroprevalences were calculated from the ratio of results positive to MTC to the total number of pigs and farms examined, respectively, using exact binomial confidence intervals (95%CI) (Martin et al., 1987). A farm was considered as positive if at least one seropositive pig was detected.

Information gathered in the farm questionnaires was used to evaluate risk factors associated with swine TB. A bivariate chi-square and Fisher's exact test were performed to obtain the statistical significance of the explanatory variables in the risk of a farm being MTC positive. Variables with a *P*-value <0.15 in the bivariate analysis were selected as potential risk factors. Then, in order to detect collinearity problems, the spearman's rho test between pairs of variables was computed. When collinearity between variables was detected, only the variable more clearly linked to MTC based on biological relevance was retained. Finally, the effect of the exploratory variables selected on positivity to MTC was investigated using a logistic regression model. The model was re-run until all remaining variables presented statistically significant values ($P<0.05$). The fit of the models was assessed using a goodness-of-fit test. SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analyses.

2.4 Spatial analysis

A spatial cluster analysis was carried out using a Bernoulli model to detect the presence of areas with significant aggregation of MTC positive pig farms (Kulldorff et al., 2006). The maximum spatial window was set up at 50% of the study region surface. The number of Monte Carlo simulations was set at 999 for the cluster scan statistic. Analyses were run using SaTScan^{TM} v9.5. Clusters were considered to be significant at $P<0.05$.

2.5 Microbiological analysis

Throughout the study period, tissue samples from Iberian pigs showing TBLL during routine inspections at slaughterhouse were collected in the study area by government veterinarians and submitted to the official laboratory for microbiological and molecular analysis. Samples were cultured in Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media (Dismalab, Madrid, Spain) after decontamination with a final concentration of 0.37% hexadecylpyridinium chloride (Corner and Trajstman, 1988). Positive cultures were identified as members of the MTC by PCR of *Mycobacterium* genus-specific 16S rRNA fragment and MPB70 sequences (Wilton and Cousins, 1992). Subsequently, isolates were spoligotyped using the standardized membrane with 43 spacers as previously described (Kamerbeek et al., 1997). All profiles were included in Spanish Database of Animal Mycobacteriosis, mycoDB.es database (Rodríguez-Campos et al., 2012).

3. Results

The individual seroprevalence of MTC was 2.3% (82/3,622; 95%CI: 1.8-2.8%). Seropositivity was significantly higher in sows (3.7% of 1,044) than in fattening pigs (1.7% of 2,329) (P=0.0001). Significantly higher seroprevalence was also found in fattening pigs from farms with seropositive sows (4/30; 13.3%) compared with fattening pigs sampled in farms where seropositivity was not detected in sows (6/374; 1.6%) ($P<0.001$).

The herd prevalence was 24.8% (32/129; 95%CI: 17.4-32.3%). In positive farms, the within-herd prevalence ranged from 2.8% to 33.3% (median 6.7%; mean 8.9%). At least one positive farm was detected in 41% (25/61) of the analyzed municipalities. The logistic regression model showed that the main risk factors associated with MTC seropositivity in pig farms were: the census (higher seroprevalence in larger farms) (OR=1.001; 95%CI: 1.000–1.002) and the presence of neighboring goat flocks (OR=7.345; 95%CI: 1.464-36.848).

The Bernoulli model identified two statistically significant spatial clusters (*P*<0.001) in Andalusia (Figure 1). The first cluster (34.93 km radius), included 11 pig farms and was located in northern Andalusia. The second cluster (9.85 km radius), included two farms and was located in western Andalusia.

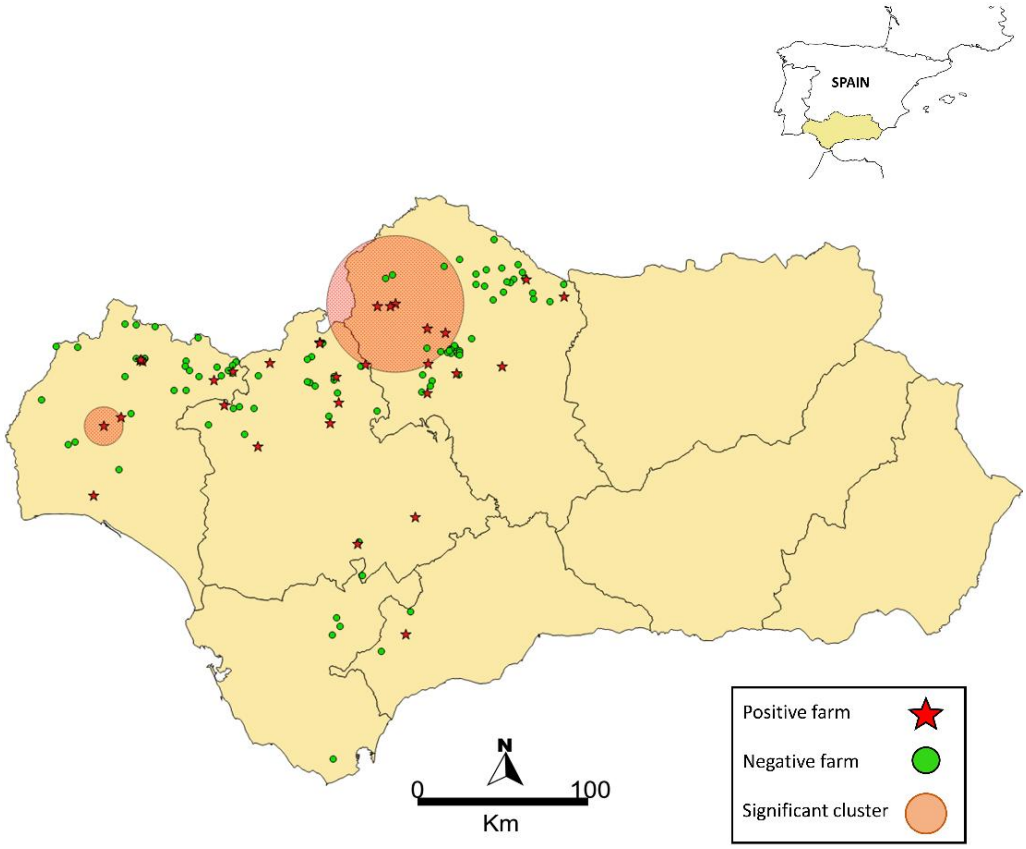

Figure 1. Map of Andalusia (southern Spain) showing the distribution of the sampled pig farms. Red stars and green dots indicate positive and negative farms, respectively. Dots in light red represents the two significant spatial clusters (*P*<0.001).

A total of 189 TBLL were positive for MTC by culture. Of them, 175 (92.6%) were identified as *Mycobacterium bovis*, and the remaining 14 (7.4%) as *Mycobacterium caprae*. Twenty-five different spoligotypes were identified. To avoid sampling bias, only one spoligotype per herd and year was taken into account to determine the frequency of spoligotypes. The most frequently detected spoligotypes was SB0121 (26.6%), followed by SB0134 (20.7%), SB0295 / SB1190 (10.1%), SB0157 (5.3%) and SB0265 / SB1174 (4.1%) (Figure 2).

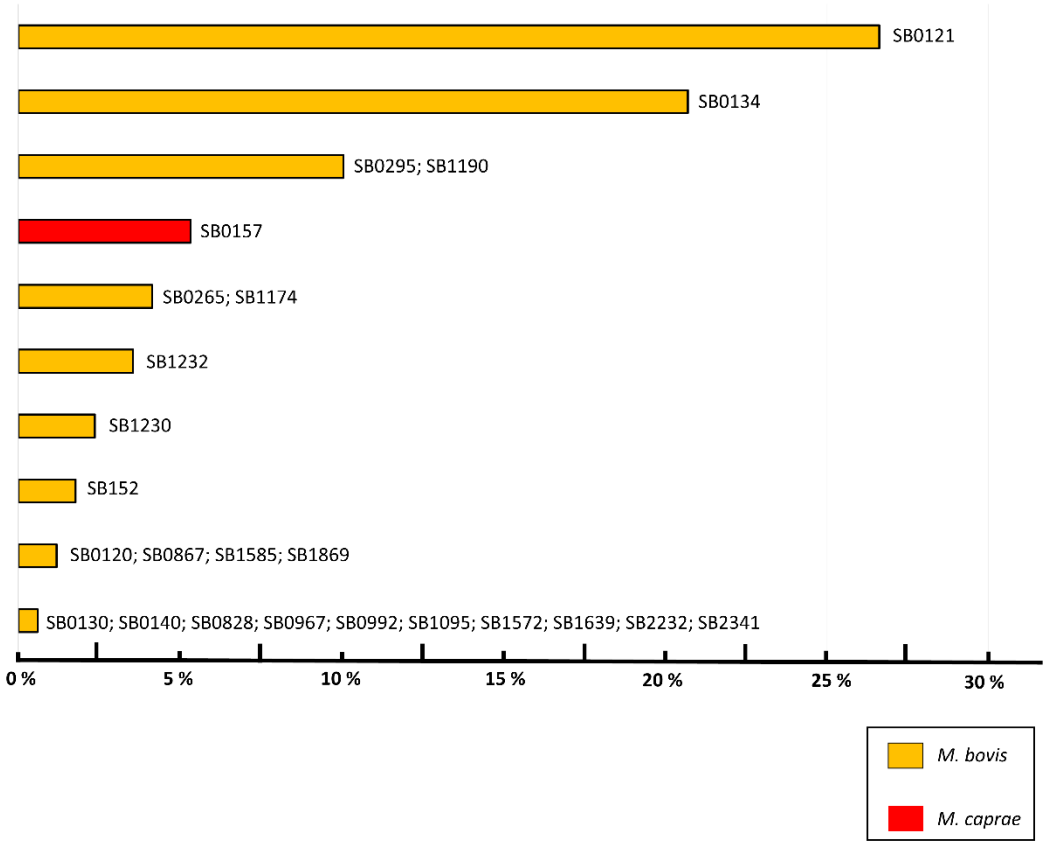

Figure 2. Spoligotypes of MTC isolated in Iberian pigs in Andalusia (southern Spain) during the period 2015–2017.

4. Discussion

To the author's knowledge, this is the first cross-sectional serological survey carried out on TB in domestic pigs. The individual seroprevalence observed in Iberian pigs (2.3%) confirms the susceptibility of this species to MTC infection. The results obtained in this breed are in accordance with that described by Di Marco et al. (2012) in extensively raised Sicilian black pigs. These authors, based on the location and characteristics of the TBLL, and the genetic profiles of the isolates, concluded that the Sicilian black pig could act as a true MTC reservoir. In contrast, although the extensively farmed domestic pig has been suggested to be a useful sentinel species for animal TB monitoring, it has not been considered relevant in the transmission of MTC in epidemiological scenarios other than in Mediterranean ecosystems (Nugent et al., 2015; Bailey et al., 2013).

Significantly higher seropositivity was found in sows compared to fattening pigs, which is in agreement with that observed in Sicilian black pigs (Di-Marco et al., 2012) and wild boars (Matos et al., 2014; Cano-Terriza et al., 2018). The results reflect a more likely exposure to MTC in sows as a consequence of their longer productive life, as well as the lifelong persistence of antibodies. Interestingly, significantly higher prevalences were found in fattening pigs from farms with presence of seropositive sows. This finding suggests possible transmission within the farm at an early age, either from their dams or by contact with infected piglets. Similarly, seropositivity detected in fattening pigs from farms with absence of MTC infection in sows, may indicate that these animals were infected during the fattening period, in which pigs are reared under free-ranging conditions, increasing the risk of infection by direct or indirect contact with other sympatric MTC reservoirs.

The herd seroprevalence (24.8%) as well as the high positivity at municipality level (41.0%), indicated widespread distribution of MTC in the extensively managed pig farms in southern Spain. However, the spatial distribution was not homogeneous across the studied area. The spatial analysis showed a significantly higher positivity in the northwestern part of

Andalusia, with the presence of two statistically significant clusters (Figure 1). Interestingly, both clusters were located in areas with high prevalence of MTC in both cattle herds and wildlife (García-Bocanegra et al., 2012; MAPAMA, 2018a). Likewise, the individual and herd prevalence detected in Iberian pigs in the present study (2.3% and 24.8%, respectively) is consistent with the individual and herd bTB prevalence found in cattle (2.1% and 17.1%, respectively) in the same region (MAPAMA, 2018a). However, because cattle are subjected to an intensive bTB eradication program, with strict scheduled test and slaughter program, this agreement should be carefully interpreted.

The logistic regression model identified the pig census as a risk factor associated with herd positivity to MTC. These results could be associated with the density-dependent nature of TB. Accordingly, Vicente et al. (2013) reported a significantly higher MTC prevalence in wild ungulates from areas with high population densities and intensive hunting management. In addition, an increasing herd size has also been associated with a higher risk of bTB herd breakdowns in cattle (Ramírez-Villaescusa et al., 2010). The risk of TB infection was 7.4 times higher in Iberian pig herds in close contact with goat flocks. These results are consistent with those reported by Napp et al. (2013), who evidenced that MTC positive goat flocks may pose a source of MTC transmission to neighboring cattle herds. Moreover, the high frequency of *M. caprae* detected in the present study (Figure 2), seem to be in accordance with this finding. In fact, in Spain, the national bTB eradication program includes the surveillance of goats in mixed herds and when there is a potential epidemiological link to an outbreak in cattle (MAPAMA, 2018a). This is related not only to the importance of the goat production sector in Spain (the second largest goat population in the EU), but also to the high susceptibility of this species to MTC infection (Pérez de Val et al., 2011), and its potential role in TB transmission. In Spain, only a few regions have either voluntary or compulsory TB eradication programs in goats. That means that most goat flocks are not subjected to routine MTC diagnostic tests, and therefore the prevalence and

spatial distribution of MTC in goat flocks in Spain is unknown. Given the role of this species as reservoir of MTC, further studies are needed.

A total of 25 different spoligotypes of MTC were identified in Iberian pigs extensively raised in the study area between 2015 and 2017. All of them have been isolated previously from other domestic and wild species in Spain (Parra et al., 2005; Rodríguez et al., 2010; García-Jiménez et al., 2016). The *M. bovis* SB0121 spoligotype, the most frequently isolated in our study, is also the most commonly found in both cattle and wild boars in Spain (Rodríguez et al., 2010; García-Jiménez et al., 2016). Fourteen out of the 189 isolates identified corresponded to SB0157, which is also the most prevalent spoligotype of *M. caprae* isolated in livestock and wildlife species in Spain (Rodríguez et al., 2011). The presence in pigs of spoligotypes shared with domestic and wild species suggest a complex inter-species transmission cycle in the study area.

In conclusion, the low individual MTC seroprevalence detected in extensively raised Iberian pigs in the present study suggests that this species could be considered as spillover hosts and not true TB reservoirs in Spanish Mediterranean ecosystems. Contact not only with infected pigs but also with other domestic or wild MTC reservoirs are potential sources of MTC infection in Iberian pigs. The high MTC herd prevalence, as well as the identification of significant spatial clusters and spoligotypes shared with other domestic and wild species, evidence the need to survey the pig farms extensively managed in Spain, focusing on the risk areas identified.

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Conflict of interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the manuscript.

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CAPÍTULO 1.2

Gestión de los subproductos de la caza como medida de control de la tuberculosis en ungulados silvestres en el centro-sur de España

Cano-Terriza D, Risalde MA, Jiménez-Ruiz S, Vicente J, Isla J, Paniagua J, Moreno I, Gortázar C, Infantes-Lorenzo JA, I García-Bocanegra (2018). **Management of hunting waste as control measure for tuberculosis in wild ungulates in south-central Spain.** Transboundary and Emerging Diseases. Doi: 10.1111/tbed.12857.

Resumen

En las últimas décadas, el cambio en los usos del suelo y el manejo intensivo de los ungulados silvestres para la caza han provocado un aumento de sus poblaciones en el centro-sur de España. Esto implica una mayor generación de residuos de caza, lo que puede favorecer la transmisión de enfermedades infecciosas como la tuberculosis (TB). El objetivo de este estudio fue evaluar la utilidad de la correcta eliminación de los residuos de caza mayor como medida de control de la TB en jabalí (*Sus scrofa*) y ciervo (*Cervus elaphus*) durante las temporadas de caza 2008/2009-2016/2017. Para ello, se obtuvieron muestras de sangre de 664 jabalíes y 934 ciervos en 14 fincas cinegéticas de dos provincias andaluzas (Área 1), en las que se llevó a cabo la correcta eliminación de los residuos cinegéticos desde la temporada de caza 2012/2013. Además, se utilizaron como controles seis fincas cinegéticas de la provincia de Ciudad Real, en Castilla-La Mancha (Área 2), región colindante en la que no se aplicó esta medida de gestión durante el periodo de estudio. En el Área 2 se tomaron muestras de sangre en 277 jabalíes y 427 ciervos. La seroprevalencia detectada de complejo *Mycobacterium tuberculosis* (CMT) en jabalíes del Área 1, fue significativamente mayor antes de la correcta eliminación de los subproductos de la caza (82,8%; 2008/2009-2012/2013) en comparación con el segundo período (61,8%; 2013/2014-2016/2017) (P<0,001), después de que se estableciera esta medida de control. Por el contrario, no se observaron diferencias significativas entre períodos en jabalíes (41,3% frente a 44,8%; P=0,33) y ciervos (14,9% frente a 11,6%; P=0,19) del Área 2, ni en ciervos del Área 1 (10,8% frente a 10,5%; P=0,48). La correcta eliminación de los residuos de la caza contribuyó a reducir la seroprevalencia del CMT en jabalíes en un 25%. Estos resultados son de especial relevancia, fundamentalmente en el jabalí y en el contexto actual de enfermedades emergentes y reemergentes como la TB y la peste porcina africana en Europa. Se necesitan más estudios para evaluar el efecto de esta medida en el estatus sanitario del ganado y otras especies silvestres.

Abstract

In recent decades, habitat change and the intensive management of wild ungulates for hunting have led to an increase in their populations in south-central Spain. This implies a higher generation of hunting waste, which can favour the transmission of infectious diseases, including tuberculosis (TB). The aim of this study was to assess the usefulness of the proper disposal of hunting waste as TB control measure in wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) during the 2008/2009 to 2016/2017 hunting seasons. Blood samples from 664 wild boar and 934 red deer were obtained in 14 game estates in two provinces in Andalusia (Area 1), where the disposal of hunting waste was implemented since the 2012/2013 hunting season. Besides, six game estates in the province of Ciudad Real, in Castilla-La Mancha (Area 2), an adjacent region where this management measure was not implemented during the studied period, were used as controls, sampling 277 wild boar and 427 red deer sera. The *Mycobacterium tuberculosis* complex (MTC), seroprevalence detected in wild boar from Area 1, was significantly higher before the disposal of big game hunting by-products (82.8%; 2008/2009–2012/2013) compared to the second period (61.8%; 2013/2014–2016/2017) (P<0.001), after this control measure became established. By contrast, no significant differences between periods were found in wild boar (41.3% versus 44.8%; P=0.33) and red deer (14.9% versus 11.6%; P=0.19) from Area 2 as well as in red deer (10.8% versus 10.5%; P=0.48) from Area 1. The proper disposal of hunting waste contributed to achieve a 25% reduction in MTC seroprevalence in wild boar. These results are of particular relevance regarding wild boar in the current context of re-emerging and emerging diseases such as TB and African Swine Fever in Europe. Further studies are needed to assess the effect of this measure on the health status of livestock and other wildlife species.

Keywords: Animal tuberculosis, big game waste management, *Mycobacterium tuberculosis* complex, red deer, wild boar

1. Introduction

Tuberculosis (TB) is a chronic infectious disease, caused by members of the *Mycobacterium tuberculosis* complex (MTC), which infects a wide range of hosts including domestic animals, wildlife and humans. Because of its zoonotic potential and its high economic impact on livestock production due to movement restrictions and the costs of testing and culling, bovine TB (bTB) is subjected to eradication programmes in the European Union (OIE, 2017). Spain is the third country with the highest bTB herd prevalence in the European Union, behind the United Kingdom and the Republic of Ireland (EFSA, 2015). Even though the Spanish eradication programme has reduced the bTB herd prevalence from 11.1% in 1986 to 2.9% by the end of 2016, the prevalence levels have remained stable or even increased in the last few years (MAPAMA, 2017). In 2016, Andalusia was the Spanish region with highest bTB herd prevalence (17.10%), followed by Extremadura (12.96%) and Castilla-La Mancha (CLM) (7.84%) (MAPAMA, 2017). These regions are located in south-central Spain (SCS), where beef cattle are usually bred in extensive production systems, sharing habitats with wildlife (Carrasco-García et al., 2016). In SCS, TB is highly prevalent in some big game species such as Eurasian wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*), which are considered natural MTC reservoirs (Gortázar et al., 2012).

During the last decades, habitat changes and supplementary feeding have contributed to allow SCS wild ungulates reaching densities above the natural carrying capacity (Acevedo et al., 2007, 2008). The rise of the wild ungulate harvest during the hunting season contributes to generate large amounts of hunting by-products not intended for human consumption, which include carcasses, viscera, limbs or heads when not used as trophies. It has been estimated that in big game areas of CLM, there is a production of 2.3 (approximately 60 kg) and 0.7 (approximately 10 kg) individual gut piles per km2 and year, for red deer and wild boar, respectively. The generation of deer remains may reach up to 400 kg per km2 and hunting season in some high-density hunting estates (Vicente et al., 2011). It is obvious that abundance of carrion can favour the spread of a broad range of

pathogens (Jennelle et al., 2009; Carrasco-García et al., 2018; Ruiz-Fons, 2017). This is of particular relevance regarding wild boar in the current context of re-emerging TB (Gortázar et al., 2012) and emerging African Swine Fever (ASF; Depner et al., 2017) in Europe.

In May 2012, the Regional Government of Andalusia regulated the management of big game hunting by-products not intended for human consumption, including their compulsory collection, transport and disposal by incinerating in processing plants or using selective avian scavenging feeding points (CAP, 2012). However, the effectiveness of this control measure has not yet been evaluated. The aim of this study was to assess the usefulness of the proper disposal of big game hunting waste as control measure for TB in wild boar and red deer in SCS under contrasting waste management regimes.

2. Materials and methods

2.1 Study design

A long-term survey study was carried out to assess the prevalence of MTC-specific antibodies in wild ungulates in two areas in SCS. Area 1 included 14 game estates (32,115 ha), where the proper disposal of hunting waste was verified by the authors of this study, located in the provinces of Cordoba and Seville (Andalusia region). Area 2 included six game estates (7,536 ha) located in the province of Ciudad Real (CLM region) (Figure 1), where this control measure was not implemented during the studied period. Blood samples from 664 wild boar and 934 red deer were collected in Area 1, and a total of 277 wild boar and 427 red deer were sampled in Area 2. Although a total of 20 game estates were included in the study, in one game estate in Area 2, only wild boar was sampled. In the same way, in one game estate in Area 2, only samples from red deer were collected (Table S1).

The game estates sampled in the study areas had both geographically proximity and similar climatic and environmental conditions. In Andalusia, the disposal of the big game hunting byproducts was properly regulated since May 2012 (CAP, 2012). Even though the

management of the big game hunting by-products started before the 2012/2013 hunting season (October to February), its effects in the TB prevalence would not be theoretically detected in the wild ungulate populations until the following 2013/2014 hunting season. Therefore, two periods were defined: the first period included from 2008/2009 to 2012/2013 hunting seasons and the second one from 2013/2014 to 2016/2017 hunting seasons. All game estates were sampled at least once in both study periods.

2.2 Sample and data collection

Blood samples were taken by endocranial venous sinus puncture (Arenas-Montes et al., 2013; Jiménez-Ruiz et al., 2016). Samples were collected into sterile tubes without anticoagulant and centrifuged at 400 g for 10 min. Serum samples were stored at -20°C until analysis. Data on gender and age were recorded for each animal. Age was estimated according to dentition patterns (yearlings, subadults, adults) (Sáenz de Buruaga et al., 2001). Epidemiological data related to the management of by-products and presence of perimeter fence were also gathered during the sampling through personal interviews with the managers and gamekeepers of each game estate.

2.3 Serological analysis

Serum samples were tested to determine the presence of MTC-specific antibodies using an in-house indirect enzyme-linked assay (P22-ELISA), with P22 as antigen, an immunopurified subcomplex of bovine-purified protein derivative (bPPD) (Infantes-Lorenzo et al., 2017), as well as horseradish peroxidase (HRP)-conjugated goat antipig IgG antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) for wild boar and a HRP-conjugated protein G (Sigma-Aldrich Química SA, Madrid, Spain) for red deer as conjugates, following the protocols previously described (patent EP16382579, “Methods and compositions for tuberculosis diagnosis”). Briefly, after coating the plates overnight at 4°C with 10 µg/ml of antigen in phosphate-buffered saline (PBS) solution, plates were washed with PBS solution containing 0.05% Tween-20 (PBST) and blocked at room temperature with blocking solution

(skim milk in PBS) for 1 hr. Sera were diluted 1:100, v/v, for wild boar, and 1:10, v/v, for red deer, in blocking solution and were added into wells. After an incubation period (1 and 1.5 hr for wild boar and red deer, respectively) at 37°C, the plates were washed three times with PBST and the conjugates were added (1:20,000 and 1:1,500, v/v, in PBS, for wild boar and red deer, respectively) and incubated at room temperature for 1 hr. After three washes, substrate solution (Fast OPD, Sigma, Barcelona, Spain) was added. The reaction was stopped after 20 min with H_2SO_4 3 N, and the optical density (OD) was measured in a spectrophotometer at 450 nm. Sample results were expressed as an ELISA percentage (E%) that was calculated as: [sample E% = (mean sample OD/2 X mean of negative control OD) X 100]. Cut-off values were defined as the ratio of the mean sample OD to the double of mean OD of the negative control. Serum samples with E% values greater than 100 were considered positive. In wild boar, P22-ELISA has a sensitivity (Se) of 82.3% and a specificity (Sp) of 92.6%, whereas in red deer, the Se and Sp reached 70.1% and 99.0%, respectively (Thomas et al., 2017). Positive control sera to MTC-specific antibodies were obtained from wild boar and red deer previously confirmed as TB-positive based on the TB-compatible lesions and *M. bovis* positive cultures, while negative controls for each species were obtained from TB-free wild boar and red deer previously confirmed as *M. bovis* negative by culture and without presence of TB-compatible lesions, from TB-free areas. Positive and negative controls were tested in quadruplicate in every plate.

2.4 Statistical analyses

Prevalence of MTC-specific antibodies was estimated from the ratio of positive to the total number of samples tested, with the exact binomial confidence intervals of 95% (CI95%) (Martin et al., 1987). Statistically significant differences between the seroprevalence to MTC and independent variables (periods, species, gender, age and perimeter fence) were assessed by means of a Pearson's chi-square test or Fisher's exact, when there were fewer than six observations per category. Differences were considered

statistically significant when $P<0.05$. The statistical analyses were performed using SPSS v22.0 software (Statistical Package for Social Sciences (SPSS) Inc., Chicago, IL, USA).

2.5 Ethics statement

This study did not involve purposeful killing of animals. Samples were collected from dead animals legally hunted during the hunting seasons. No ethical approval was necessary.

3. Results

The overall seroprevalence to MTC was significantly higher in wild boar (59.9%; 564/941, CI95%: 56.8%–63.1%) compared to red deer (11.5%; 156/1361, CI95%: 9.8%–13.2%) ($P<0.001$). Seropositivity to MTC in wild boar and red deer was detected in 100% (19/19) and 94.7% (18/19) of the sampled game estates, respectively (Figure 1). Adult (324/463; 70.0%) and subadult (121/189; 64.0%) wild boar had significantly higher seroprevalence ($P<0.001$) compared to yearlings (106/261; 40.6%). The prevalence of MTC-specific antibodies in adult red deer (99/674; 14.7%) was also significantly higher than in subadults (21/328; 6.4%) and yearlings (15/171; 8.8%) ($P<0.001$). Significantly higher seropositivity was found in both wild boar ($P=0.016$) and red deer ($P=0.025$) from fenced game estates compared to those sampled in open game estates. Statistically significant differences between sexes were not found.

In Area 1, seropositivity to MTC was detected in 82.8% of the wild boar and 10.8% of the red deer in the first period, before the improved disposal of big game hunting by-products was implemented (2008/2009–2012/2013 hunting seasons) (Table 1 and Figure 1). In the second period (2013/2014–2016/2017 hunting seasons), the prevalence of antibodies decreased significantly in wild boar (61.8%; $P<0.001$ (25.4% reduction in MTC seroprevalence) but not in red deer (10.5%; $P=0.48$). Statistically significant differences between periods were not found in both wild boar (41.3% versus 44.8%; $P=0.33$) and red deer (14.9% versus 11.6%; $P=0.19$) in Area 2. In wild boar, similar results were found when

only open game estates were selected (82.9% versus 59.6%; *P*<0.001). In fenced game estates, a marked downward trend between the first and the second study periods was observed in this species (82.8% versus 69.3%), but differences between periods were not statistically significant (*P*=0.111) (Table S1).

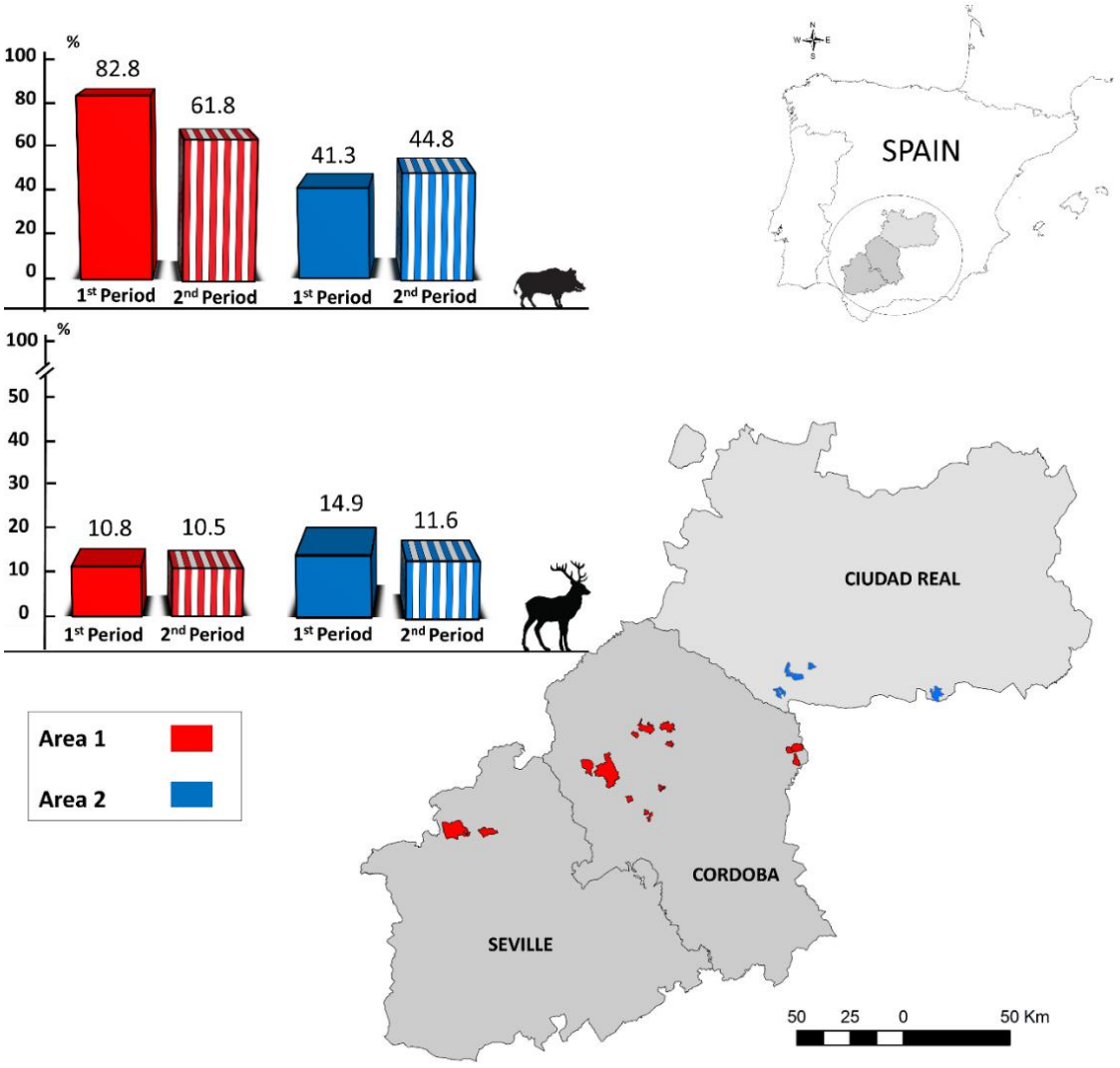

Figure 1. Map of south-central Spain showing the location of the game estates sampled in Area 1 (red) and Area 2 (blue). Bars indicate seroprevalence in wild boar and red deer in the first (solid pattern) and second (striped pattern) study period.

Table 1. Distribution of seroprevalence to MTC by sampled areas, species and study periods

Area	Species	First period (2008/2009 to 2012/2113)	Second period (2013/2014 to 2016/2017)	*P*-value
		positives/tested (%; 95% CI)	positives/tested (%; 95% CI)	
Area 1	Wild boar	140/169 (82.8; 77.2-88.5)	306/495 (61.8; 57.5-66.1)	<0.001
	Red deer	33/305 (10.8; 7.3-14.3)	66/629 (10.5; 8.1-12.9)	0.480
Area 2	Wild boar	71/172 (41.3; 33.9-48.6)	47/105 (44.8; 35.3-54.3)	0.328
	Red deer	34/228 (14.9; 10.3-19.5)	23/199 (11.6; 7.1-16.0)	0.191

4 Discussion

The multihost complexity in the epidemiology of TB requires combined and synergistic strategies for its control, acting on all maintenance hosts and including specific measures in wildlife (PATUBES, 2017). Our results showed that the MTC seroprevalence in wild boar decreased by 25.4% after the adequate management of big game hunting by-products. By contrast, no differences between periods were found in wild boar from Area 2. Even though further studies are required to assess the usefulness of the proper hunting waste management in other regions with lower TB prevalence, our results evidenced that this control measure can be an important aspect to consider in wildlife management programmes in order to control TB in wildlife populations and at the wildlife/livestock interface.

TB control requires an integral approach where the vaccination is also a suitable tool for the control of TB in wild boar, with an up to 66% of reduction in TB-compatible lesion prevalence in 4 years (Díez-Delgado et al., 2017). Similarly, Boadella et al. (2012) established that culling of at least 50% of the estimated wild boar population allowed a reduction in TB prevalence by 21%–48% in 2 to 3 years. Wild boar culling, as well as other management

practices such as segregating wild ungulates and livestock, has proven to be effective to decrease TB prevalence not only in wild boar, but also in sympatric wild and domestic species (Boadella et al., 2012; Barasona, et al., 2013; García-Jiménez et al., 2013). In this context, the proper disposal of hunting by-products could have an indirect positive effect on the health status of livestock, particularly in areas with high wildlife–livestock interactions.

The disposal of big game by-products at avian scavenging feeding points is an essential resource for the maintenance of the avian scavenging community (Blázquez and Sánchez-Zapata, 2009). In SCS, avian scavenging species comprise most of European vultures, including the endangered Egyptian vulture (*Neophron percnopterus*). Even though avian species are not usually susceptible to mammal infectious diseases, consumption of infected tissues by facultative mammal scavengers, such as wild boar, is regarded as a key factor in the spread of infectious diseases, including TB (Carrasco-García et al., 2018). In this context, differences between wild ungulates observed in the present study can be related to the scavenging behaviour of wild boar. Therefore, avian scavenging feeding points must have appropriate temporal or permanent physical barriers to avoid the entry of mammalian scavengers (Moreno-Opo et al., 2012). Additionally, the high fertility rate and the faster population turnover in wild boar are also possible factors related with the differences found between periods in this species, in which the effect of the adequate disposal of big game waste was firstly reflected. It will probably take longer time periods and larger sample sizes to detect such changes in red deer, too.

ELISA test is suitable diagnosis method for surveillance in wild ungulate populations (Boadella et al., 2011). The Se and Sp described for the P22-ELISA used in our study are within the accuracy values previously reported in wild boar (Se: 72.6%–100%; Sp: 89%–100%) and red deer (Se: 45.7%–86.7%; Sp: 52.0%–100%) (Griffin et al., 1991; Griffin, et al., 1994; Aurtenetxe et al., 2008; Boadella et al., 2011; García-Bocanegra et al., 2012; Wadhwa et al., 2013). The Se of the P22-ELISA (82.3% and 70.1% in wild boar and red deer, respectively) suggests that the seroprevalence found in the study areas may be

underestimated. The prevalence of TB in big game species, particularly wild boar, in SCS is among the highest ones cited in the scientific literature, ranging from 50% to 100% (Gortázar et al., 2012; Carrasco-García et al., 2016). The high individual (59.9%) and game estate (100%) seroprevalence detected in wild boar in the present study confirms a widespread contact of wild boar to MTC in SCS. The results agree with those previously reported in this region (García-Bocanegra et al., 2012; Vicente et al., 2013) and confirm the key role of wild boar as the main wild reservoir of MTC in Mediterranean ecosystems of the Iberian Peninsula (Naranjo et al., 2008). The significantly higher seroprevalence observed in adults reflects a greater probability of exposure to the mycobacteria and the long persistence of MTC antibodies and suggests horizontal infection as the main route of MTC transmission. The results are in agreement with other studies (García-Bocanegra et al., 2012; Vicente et al., 2006). In contrast, other authors found a higher prevalence of MTC in juvenile wild boar, possibly as a result of recent expansion of the infection (Pérez de Val et al., 2017). Statistically significant differences between sexes were not found, which is consistent with previous reports (Martín-Hernando et al., 2007; García-Bocanegra et al., 2012; Pérez de Val et al., 2017). Conversely, a significantly higher TB prevalence was detected in deer males by gross TB-lesion pattern (O'Brien et al., 2000; Vicente et al., 2006). The higher seropositivity in fenced game estates had also been described in SCS (Vicente et al., 2013). These findings may be related to the higher population densities, intensive management measures, inbreeding and spatial aggregation in fenced game estates, which increase the risk of infectious disease transmission.

In conclusion, our results indicate that a proper disposal of hunting waste can be a complementary control strategy for the control of TB in wild boar, at least in Mediterranean ecosystems. This management tool may be extensive to the prevention and control of other wild boar and pig diseases, such as Aujeszky's disease, ASF, swine brucellosis or trichinellosis. The proper management of hunting waste, together with other wildlife disease control tools, should be implemented and maintained along the time in integrated

disease control strategies. Further studies are required to assess the effect of the disposal of big game waste on the prevalence of TB in livestock and other wildlife species.

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Conflict of interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the manuscript.

Supplementary File 1. Table S1. Prevalence of MTC-specific antibodies in wild ungulate species by game estate in the two study periods.

		RED DEER				WILD BOAR			
		First period		Second period		First period		Second period	
Game estate	Perimeter	Positives/tested (%)	Times sampled	Positives/tested (%)	Times sampled	Positives/tested (%)	Times sampled	Positives/tested (%)	Times sampled
AREA 1									
1	Open	13/82 (15.9)	3	17/122 (13.9)	4	24/25 (96.0)	3	44/57 (77.2)	4
2	Open	6/63 (9.5)	2	5/39 (12.8)	2	4/4 (100.0)	1	12/29 (41.4)	1
3	Open	2/5 (40.0)	1	0/1 (0.0)	1	3/5 (60.0)	1	4/5 (80.0)	1
4	Open	2/26 (7.7)	2	0/57 (0.0)	3	6/11 (54.5)	2	12/27 (44.4)	3
5	Open	0/6 (0.0)	1	3/60 (5.0)	4	1/5 (20.0)	1	9/44 (20.5)	4
6	Open	0/15 (0.0)	1	1/79 (1.3)	4	-	-	26/49 (53.1)	4
7	Open	1/14 (7.1)	2	6/59 (10.2)	3	3/3 (100.0)	1	38/59 (64.4)	3
8	Open	7/49 (14.3)	3	9/90 (10.0)	4	75/87 (86.2)	3	82/111 (73.9)	4
Total	**N=8**	31/260 (11.9)	15	41/507 (8.1)	25	116/140 (82.9)	12	227/381 (59.6)	24
9	Fenced	1/5 (20.0)	1	4/26 (15.4)	3	8/9 (88.9)	1	50/68 (73.5)	3
10	Fenced	0/9 (0.0)	1	1/9 (11.1)	1	0/1 (0.0)	1	1/3 (33.3)	1
11	Fenced	0/4 (0.0)	1	0/8 (0.0)	8	6/6 (100.0)	1	2/6 (33.3)	2
12	Fenced	0/6 (0.0)	1	1/4 (25.0)	1	2/4 (50.0)	1	13/16 (81.3)	1
13	Fenced	1/8 (12.5)	1	11/58 (19.0)	3	-	-	13/20 (65.0)	3
14	Fenced	0/13 (0.0)	1	8/17 (47.1)	1	8/9 (88.9)	1	0/1 (0.0)	1
Total	**N=6**	2/45 (4.4)	6	25/122 (20.5)	17	24/29 (82.8)	5	79/114 (69.3)	11
Total (Area 1)	**N=14**	**33/305 (10.8)**	**21**	**66/629 (10.5)**	**42**	**140/169 (82.8)**	**17**	**306/495 (61.8)**	**35**
AREA 2									
15	Open	-	-	-	-	16/75 (21.3)	4	6/39 (15.4)	4
16	Open	4/25 (16.0)	2	6/27 (22.2)	1	-	-	-	-
Total	**N=2**	4/25 (16.0)	2	6/27 (22.2)	1	16/75 (21.3)	4	6/39 (15.4)	4
17	Fenced	0/6 (0.0)	1	7/26 (26.9)	2	12/18 (66.7)	2	0/1 (0.0)	1
18	Fenced	5/26 (19.2)	2	1/45 (2.2)	2	10/15 (66.7)	1	13/23 (56.5)	2
19	Fenced	2/32 (6.3)	3	1/4 (25.0)	1	18/36 (50.0)	3	1/1 100.0)	1
20	Fenced	23/139 (16.5)	5	8/97 (8.2)	4	15/28 (53.6)	4	27/41 (65.9)	4
Total	**N=4**	30/203 (14.8)	11	17/172 (9.9)	8	55/97 (56.7)	10	41/66 (62.1)	8
Total (Area 2)	**N=6**	**34/228 (14.9)**	**13**	**23/199 (11.6)**	**9**	**71/172 (41.3)**	**14**	**47/105 (44.8)**	**12**

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CAPÍTULO 2

Prevalencia de infección de patógenos zoonósicos de interés en especies peridomésticas y animales de zoológico

CAPÍTULO 2.1

Estudio epidemiológico de patógenos zoonósicos en paloma doméstica (*Columba livia* var. *domestica*) y especies simpátricas de zoológico en el sur de España

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Resumen

En el presente trabajo se realizó un estudio transversal entre 2013 y 2014 para determinar la prevalencia de diversos agentes zoonósicos (flavivirus, virus influenza aviar, *Salmonella* spp. y *Toxoplasma gondii*) en paloma doméstica y animales simpátricos del Parque zoológico de Córdoba (sur de España). Se detectaron anticuerpos frente a flavivirus en el 7,8% de las 142 palomas (IC95%: 3,7-11,8) y en el 8,2% de los 49 (IC95%: 0,9-15,4) animales de zoológico analizados. Se confirmó la presencia de anticuerpos específicos frente a virus de West Nile y virus Usutu, tanto en palomas como en aves de zoológico. Aunque no se detectó seropositividad a virus influenza aviar en ninguna de las palomas analizadas, el 17,9% de las 28 aves de zoológico analizadas mostraron resultados positivos. No se aisló *Salmonella* spp. en ninguna de las 152 muestras fecales procedentes de palomas, mientras que el 6,8 % de los 44 animales de zoológico analizados fueron positivos. Se encontraron anticuerpos frente a *T. gondii* en el 9,2% de las 142 (IC95%: 4,8-13,6) palomas y en el 26,9% de los 108 (IC95%: 19,57-34,14) animales de zoológico analizados. Este es el primer estudio sobre flavivirus y *T. gondii* en palomas urbanas y especies de zoológico en España. Los anticuerpos frente al virus West Nile y el virus Usutu detectados en palomas no migratorias y animales de zoológico en cautividad indican la circulación local de estos patógenos emergentes en la zona de estudio. Se encontró una elevada dispersión de *T. gondii* entre las especies analizadas. Este hallazgo podría ser importante para la salud pública y la conservación de especies en peligro de extinción presentes en los parques zoológicos. Las palomas y los animales de zoológico podrían incluirse como especies centinelas para la vigilancia de patógenos zoonóticos en zonas urbanas.

Abstract

A cross-sectional study was carried out to determine the prevalence of pathogenic zoonotic agents (flaviviruses, avian influenza viruses (AIVs), *Salmonella* spp. and *Toxoplasma gondii*) in feral pigeons and sympatric zoo animals from Córdoba (Southern Spain) between 2013 and 2014. Antibodies against flaviviruses were detected in 7.8% out of 142 ($CI_{95\%}$: 3.7-11.8) pigeons, and 8.2% of 49 ($CI_{95\%}$: 0.9-15.4) of zoo animals tested. Antibodies with specificity against West Nile virus (WNV) and Usutu virus (USUV) were confirmed both in pigeons and in zoo birds. Even though seropositivity to AIVs was not detected in any of the analyzed pigeons, 17.9% of 28 zoo birds tested showed positive results. *Salmonella* spp. was not isolated in any of 152 faecal samples collected from pigeons, while 6.8% of 44 zoo animals were positive. Antibodies against *T. gondii* were found in 9.2% of 142 ($CI_{95\%}$: 4.8-13.6) feral pigeons and 26.9% of 108 ($CI_{95\%}$: 19.57-34.14) zoo animals. This is the first study on flaviviruses and *T. gondii* in feral pigeons and captive zoo species in Spain. Antibodies against WNV and USUV detected in non-migratory pigeons and captive zoo animals indicate local circulation of these emerging pathogens in the study area. *T. gondii* was widespread in species analyzed. This finding could be of importance for Public Health and Conservation of endangered species present in zoo parks. Pigeons and zoo animals may be included as sentinel species for monitoring zoonotic pathogens in urban areas.

Keywords: Avian influenza viruses; Captive zoo animals; Feral pigeons; Flaviviruses; *Salmonella* spp.; *Toxoplasma gondii*.

1. Introduction

Feral pigeon (*Columba livia* var. *domestica; Columbidae* family) is one of the bird species most frequently found in urban and peri-urban areas. During the last decades, their populations have increased exponentially in different countries, reaching densities higher than 2000 birds/km^2 in many European cities (Sacchi et al., 2002; Senar et al., 2009). Favorable environmental conditions and abundant food, which imply loosing the breeding season, and the absence of predators are the main factors implicated in the high increase of their populations (Amoruso et al., 2014). Feral pigeon is considered a pest species in many urban areas due to the destruction architectonic and urban heritage, damage to agriculture and risk of disease transmission to other sympatric species, including humans. In this sense, control measures including surgical or chemical sterilization, removal of eggs, physical repellents, use of birds of prey or capturing and controlled elimination are frequently implemented in urban areas (Senar et al., 2009; Albonetti et al., 2015).

Zoological parks are a very favorable habitat for pigeons, where they are found in higher densities than in urban areas. High populations imply significant economic losses associated to the consumption of food intended for captive zoo animals. Furthermore, the risk of disease transmission from pigeons to zoo sympatric species can be of concern for Animal Health and Conservation. In this sense, the importance of zoos for surveillance of zoonotic and emerging diseases has been previously suggested (Mcnamara, 2007).

Even though feral pigeons are considered reservoirs of zoonotic diseases including Newcastle disease, histoplasmosis, ornithosis, salmonellosis, or cryptococcosis (Dovc et al., 2004; De-Sousa et al., 2010), information about zoonotic diseases in this species in Spain is yet very limited. High prevalence of *Chlamydophila psittaci* infection was found in central (Madrid: 52.6%) and southwest (Murcia: 35.9%) areas (Salinas et al., 1993; Vázquez et al., 2010). Prevalence of *Campylobacter* spp. infection ranged between 1.1% and 69.1% in Barcelona and Madrid, respectively (Casanovas et al., 1995, Vázquez et al., 2010). Also, 1.5% of *Salmonella* spp. and 0.25% of *Yersinia intermedia* infections was detected in pigeons from

Barcelona city (Casanovas et al., 1995). In addition, 70.3% of pigeons analyzed in Alicante (southwest Spain) showed *Cryptococcus neoformans* infection (Colom-Valiente et al., 1993).

The aim of this study was to determine the prevalence of selected zoonotic pathogens (flaviviruses, avian influenza viruses (AIVs), *Salmonella* spp. and *T. gondii*) in feral pigeons, and to assess the risk of transmission to sympatric zoological species in the Córdoba Municipal Zoo Park (CMZP, Southern Spain).

2. Materials and Methods

2.1 Study design and sampling

A total of 152 feral pigeons were captured in the CMZP (Southern Spain) between November 2013 and May 2014. Captures were performed within the course of a pest control program undertaken by the local authorities following European guidelines (UNE 171210: 2008). Animals were handled and sampled according to regulations of the Regional Government of Andalusia.

Blood samples were taken from the brachial vein, after those animals were humanely euthanized using pentobarbital sodium (DOLETHAL®) (0.5 ml/kg). Samples were placed into sterile tubes without anticoagulant and centrifuged at 3000 rpm for 10 min. Sera were stored at -20ºC until analysis. All individuals were necropsied and inspected for the presence of macroscopic lesions. Pigeons were classified as juveniles (< 8 months) and adult (> 8 months) according to previously established criteria (Gibbs et al., 2010). Sera from 108 zoo animals belonging to 34 species (Table 1), which share habitat with the sampled pigeons, were also collected. In addition, digestive content (2-3 gr) from all pigeons and 44 fresh faecal samples from 35 different zoo species were collected for *Salmonella* spp. isolation.

2.2 Laboratory analysis

Sera from 142 pigeons and 49 captive zoo animals were tested for antibodies against one epitope of the preMembrane-Envelope (prM-E) protein common to flaviviruses of the Japanese Encephalitis antigenic complex (JEV) using a commercial blocking ELISA (bELISA 10.WNV.K3 INGEZIM West Nile COMPAC®, Ingenasa, Madrid, Spain). ELISA-positive sera were confirmed by virus neutralization test (VNT) for the detection of specific neutralizing antibodies against West Nile virus (WNV; Is98 strain), Usutu virus (USUV; It12 strain) and Meaban virus (MBV; Brest ART707 strain). VNTs were performed as previously described (Arnal et al., 2014; Chaintoutis et al., 2014). Samples that showed neutralization and absence of cytopathic effect at dilutions ≥ 10 for WNV and USUV and ≥ 20 for MBV, were considered positive. Interpretation of results was based on comparison of VNT titers obtained in parallel against the 3 flaviviruses. The neutralizing immune response observed was considered specific when VNT titers for a given virus was ≥ 4-fold higher than titers obtained for the other viruses. Samples showing VNT titers differences ≤ 2-fold between the viruses examined were considered positive for flavivirus but not conclusive for any specific virus.

The presence of antibodies against the nucleoprotein of AIVs type A was determined using a commercial (bELISA) (1.0.FLU.K3 INGEZIM INFLUENZA A®, Ingenasa, Madrid, Spain) according to the manufacturer´s recommendations. A total of 148 pigeons and 28 zoo birds were analyzed.

Faecal droppings and faecal samples were processed as described in Annex D of the Spanish standard UNE-EN ISO 6579:2002 for *Salmonella* isolation. *Salmonell*a-presumptive colonies were tested with the Mucap (Biolife, Milano, Italy) and indole tests. *Salmonella* serotyping was carried out according to the Kauffman-White scheme (Popoff et al., 2001) by the Agri-food laboratory of Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural (Generalitat de Catalunya, Spain).

Sera from 142 feral pigeons and 108 zoo animals, including samples from 28 carnivores, 43 ungulates, 35 birds and 2 monkeys were examined to detect antibodies against *T. gondii* using the modified agglutination test (MAT) as previously described (Dubey and Desmonts, 1987). Serum was tested at 1:25, 1:50, 1:100 and 1:500 dilutions and both positive and negative controls were included in all tests. Sera with titers ≥ 1:25 were considered positive.

Table 1. Prevalence of selected zoonotic pathogens in feral pigeons (*Columba livia* var. *domestica*) and sympatric zoological species in Córdoba, Southern Spain.

SPECIES	Flaviviruses	Influenza	*Salmonella* spp.	*Toxoplasma gondii*
ORDER COLUMBIFORMES				
Feral pigeon (*Columba livia* var. *domestica*)	11/142 (7.8 %)	0/148	0/152	13/142 (9.2%)
ORDER ARTIODACTYLA				
Aoudad (*Ammotragus lervia*)	0/3	-	0/1	2/10 (20%)
Red deer (*Cervus elaphus*)	0/7	-	-	3/15 (20%)
Fallow deer (*Dama dama*)	0/1	-	-	1/1(100%)
Mouflon (*Ovis musimon*)	0/5	-	-	4/6 (66.7%)
Collared Peccary (*Pecari tajacu*)	0/3	-	0/1	2/3 (66.7%)
Black Wildebeest (*Connochaetes gnou*)	1/1 (100 %)	-	0/1	0/1
Roe deer (*Capreolus capreolus*)	-	-	0/1	-
Wild boar (*Sus scrofa*)	-	-	1/1 (100 %)	-
Llama (*Lama glama*)	-	-	0/1	-
Sheep (*Ovis aries*)	-	-	-	1/3 (33.3%)
Goat (*Capra hircus*)	-	-	-	0/3
TOTAL	**1/20 (5%)**	**-**	**1/6 (16.7%)**	**13/42 (31%)**
ORDER PERISSODACTYLA				
Plains zebra (*Equus burchellii*)	0/1	-	-	0/1
Donkey (*Equus africanus asinus*)	-	-	0/3	-
Lowland Tapir (*Tapirus terrestris*)	-	-	0/1	-
TOTAL	**0/1**	**-**	**0/4**	**0/1**
ORDER ANSERIFORMES				
Rosy-billed Pochard (*Netta peposaca*)	0/1	1/1 (100 %)	-	0/1
Black-bellied whistling duck (*Dendrocygna autummalis*)	0/3	1/3 (33.3 %)	-	0/3
Mallard (*Anas platyrhynchos*)	0/4	1/4 (25 %)	-	1/4 (25 %)
Bar-headed goose (*Anser indicus*)	0/3	0/3	-	1/11 (9.1%)
Emperor goose (*Chen canagica*)	1/2 (50%)	1/2 (50 %)	-	1/2 (50%)
TOTAL	**1/13 (7.7%)**	**4/13 (30.8%)**	**-**	**3/21 (14.3%)**
ORDER ACCIPITRIFORMES				
Griffon vulture (*Gyps fulvus*)	0/1	0/1	0/1	0/1
Booted eagle (*Hieraaetus pennatus*)	0/1	0/1	0/1	1/1 (100%)
Short-toed snake eagle (*Circaetus gallicus*)	-	-	0/1	-
Common buzzard (*Buteo buteo*)	-	-	0/1	-
TOTAL	**0/2**	**0/2**	**0/4**	**1/2 (50%)**
ORDER GALLIFORMES				
Indian peafowl (*Pavo cristatus*)	0/1	0/1	0/1	0/1
ORDER STRUTHIONIFORMES				
Greater rhea (*Rhea americana*)	0/2	0/2	0/1	0/2
Emu (*Dromaius novaehollandiae*)	0/4	0/4	0/1	0/4
Ostrich (*Struthio camelus*)	1/2 (50 %)	0/2	1/2	0/2
TOTAL	**1/8 (12.5%)**	**0/8**	**1/4 (25%)**	**0/8**
ORDER CICONIIFORMES				
White stork (Ciconia ciconia)	1/2 (50 %)	0/2	-	0/1
ORDER PELECANIFORMES				
African sacred ibis (*Threskiornis aethiopicus*)	0/2	1/2 (50 %)	-	0/1
ORDER PSITTACIFORMES				
Muir's corella (*Cacatua pastinator*)	-	-	-	0/1

ORDER CARNIVORA				
Lion (*Panthera leo)*	-	-	0/1	2/2 (100%)
Jaguar (*Panthera onca*)	-	-	0/2	1/1 (100%)
Bengal tiger (*Panthera tigris tigris*)	-	-	-	3/3 (100%)
Eurasian lynx (*Lynx lynx*)	-	-	-	0/7
Brown bear (*Ursus arctos pyrenaicus*)	-	-	0/1	3/3 (100%)
European otter (*Lutra lutra*)	-	-	0/1	0/1
Iberian wolf (*Canis lupus signatus*)	-	-	-	1/8 (12.5%)
European mink (*Mustela lutreola*)	-	-	-	2/3 (66.6%)
TOTAL	**-**	**-**	**0/5**	**12/28 (42.9%)**
ORDER PRIMATES				
Northern white-cheeked gibbon (*Hylobates leucogenys*)	-	-	0/2	-
Brown lemur (*Eulemur fulvus*)	-	-	0/1	-
Black-and-white ruffed lemur (*Varecia variegata*)	-	-	0/1	-
Ring-tailed lemur (*Lemur catta*)	-	-	0/1	-
Barbary macaque (*Macaca sylvanus*)	-	-	0/2	0/1
White-collared Mangabey (*Cercocebus atys ssp. lunulatus*)	-	-	0/2	-
De Brazza's Monkey (*Cercopithecus neglectus*)	-	-	0/2	-
White-tufted-ear Marmoset (*Callithrix jacchus*)	-	-	0/1	0/1
Western Pygmy Marmoset (*Callithrix pygmaea*)	-	-	0/1	-
South American Squirrel Monkey (*Saimiri sciureus*)	-	-	0/1	-
TOTAL	**-**	**-**	**0/14**	**0/2**
ORDER RODENTIA				
Patagonian Cavy (*Dolichotis patagonum*)	-	-	0/1	-
Capybara (*Hydrochoerus hydrochaeris*)	-	-	0/1	-
TOTAL	**-**	**-**	**0/2**	**-**
ORDER SQUAMATA				
Corn snake (*Elaphe guttata*)	-	-	0/1	-
Milk snake (*Lampropeltis triangulum*)	-	-	1/1 (100 %)	-
TOTAL	**-**	**-**	**1/2 (50 %)**	**-**
ORDER TESTUDINES				
African spurred tortoise (*Geochelone sulcata*)	-	-	0/2	-
TOTAL IN CAPTIVE ANIMALS	**4/49 (8.2%)**	**5/28 (17.9 %)**	**3/44 (6.8%)**	**29/108 (26.9%)**

2.3 Statistical analysis

The prevalence of the different pathogens was estimated from the ratio of positive to the total number of samples tested, with the exact binomial confidence intervals of 95% ($CI_{95\%}$). Correlation between estimated prevalences and independent variables (species, sex and age) were analyzed by means of a Pearson's chi-square test or, when there were less than six observations per category, by the Fisher´s exact test. The differences between variables were analyzed by Tukey tests. Values with $P < 0.05$ were considered as statistically significant. Statistical analyses were performed using SPSS 22.0 (Statistical Package for Social Sciences (SPSS) Inc., Chicago, IL, USA).

3. Results

Antibodies against flavivirus of the JEV serocomplex were found in 11 of 142 pigeons (7.8%; $CI_{95\%}$: 3.7-11.8) tested by bELISA. No statistically significant differences were observed among sex or age. Six of the 11 positive samples were confirmed by VNT (Table 2). Specific antibodies against WNV were detected in three pigeons (3/142: 2.1%; $CI_{95\%}$: 0.0-4.3), while USUV infection was also confirmed in three individuals (3/142: 2.1%; $CI_{95\%}$: 0.0-4.3). No MBV-specific antibodies were detected in the pigeon sera tested.

Seroprevalence of flaviviruses in zoo animals was 8.2% (4/49). Presence of antibodies was found in ostrich (*Struthio camelos*), white stork (*Ciconia ciconia*), emperor goose (*Chen canagica*) and black wildebeest (*Connochaetes gnou*) (Table 2). Specific antibodies against WNV (1:40) and USUV (1:20) were confirmed in white stork and ostrich, respectively. Moreover, the seropositive emperor goose found positive in bELISA presented neutralizing antibodies against WNV (1:80), USUV (1:40) and MBV (1:20), which are indicative of at least two independent flavivirus infections (by a flavivirus belonging to the JEV serocomplex like WNV or USUV and tick-borne flavivirus like MBV).

Antibodies against AIV were not found in any of the 148 pigeons tested (0.0%; $CI_{95\%}$: 0.0-1.9). Seropositivity was observed in five out of 28 (17.9%) sera from captive zoo birds, including mallard (*Anas platyrhynchos*), emperor goose, African sacred ibis (*Threskiornis aethiopicus*), rosy-billed pochard (*Netta peposaca*) and black-bellied whistling duck (*Dendrocygna autummalis*) (Table 1).

Salmonella spp. was not isolated from any of the 152 (0.0%; 95% CI: 0.0-1.9) pigeons analyzed. In contrast, in three faecal samples from the 44 captive zoo animals tested (6.8%) positive isolation results were obtained (Table 1). In particular, *Salmonella* Panama was detected in wild boar (*Sus scrofa*), while *Salmonella* Apapa was isolated in milk snake (*Lampropeltis triangulum*). The *Salmonella* serovar isolated in ostrich could not be identified.

Antibodies against *T. gondii* were observed in 9.2% of 142 ($CI_{95\%}$: 4.8-13.6) feral pigeons. Titers of 1:25, 1:50, 1:100 and 1:500 were detected in three (23.1%), six (46.2%), three (23.1%) and one (7.7%) pigeons, of the 13 seropositive animals, respectively. Statistically significant differences between the seropositivity against *T. gondii* and sex or age were not found. The overall seropositivity to *T. gondii* in zoo animals was 26.9% (29/108). Antibodies were detected in 42.9% carnivore (12/28), 30.2% ungulate (13/43) and 11.4% bird (4/35) samples, but were not detected in two primates analyzed. Titers of 1:25 were found in nine (31.0%), 1:50 in three (10.3%), 1:100 in 10 (34.5%) and 1:500 in seven (24.1%) of the 29 seropositive animals. Statistically significant differences between seropositivity and sex, age or group of species (birds, carnivores, ungulates or primates) were not observed.

Table 2. Results of virus neutralization test for the detection of flaviviruses in feral pigeons and captive zoo animals.

Species, (no.)	VNT titers			Interpretation
Feral pigeon (*Columba livia*) (n=142)	WNV	USUV	MBV	
Feral pigeon (n= 1)	<10	10	<20	USUV
Feral pigeon (n= 1)	**20**	<10	<20	WNV
Feral pigeon (n= 1)	**80**	<10	<20	WNV
Feral pigeon (n= 1)	<10	**10**	<20	USUV
Feral pigeon (n= 1)	**80**	**10**	<20	WNV
Feral pigeon (n= 1)	<10	**20-40**	<20	USUV
Feral pigeon (n= 5)	<10	<10	<20	Undetermined Flavivirus
Total (%)	**3(2.1)**	**3(2.1)**	0	
Zoo animals (n=49)				
Emperor goose *(Chen canagica)* (n= 1)	**80**	**40**	**20**	Undetermined Flavivirus
White stork (*Ciconia ciconia*) (n= 1)	**40**	<10	NA[1]	WNV
Ostrich (*Rhea americana)* (n= 1)	<10	**20**	<20	USUV
Black wildebeest (*Connochaetes gnou*) (n= 1)	<10	<10	<20	Undetermined Flavivirus
Total (%)	**1(2.0)**	**1(2.0)**		

WNV, West Nile virus; USUV, Usutu virus; MV, Meaban virus; VNT, virus neutralization test;

NA[1]: Not analyzed, low volume.

4. Discussion

The results from the present study confirm local circulation of flavivirus, including WNV and USUV, in non-migratory feral pigeons in Córdoba city. Five out of 11 seropositive pigeons detected by bELISA were negative in USUV, WNV and MBV VNT. The results could be due to the higher sensitivity of the bELISA test, to the detection of different types of antibodies using bELISA and VNTs, or to the circulation of other antigenically related flaviviruses (Blitvich et al., 2003). Accordingly, circulation and mortality associated to Bagaza virus infections have been detected in wild bird species, including the genus *Columba,* in Spain (Gamino et al., 2012; García-Bocanegra et al., 2013). Similar rates of seropositivity against WNV and USUV were detected in pigeons and zoo birds, which indicates a homogeneous circulation of these flaviviruses in these species. To date, the WNV outbreaks reported in horses (n= 88) and in humans (n= 2) in Spain have only been detected in the southern region (RASVE, 2015a). Even though pigeons are included in the Spanish WNV Monitoring Program (RASVE, 2015b), to our knowledge, these are the first results in this species in Spain. Similar seroprevalences against flaviviruses were previously reported in both horses and wild birds in southern Spain (García-Bocanegra et al., 2011; López et al., 2011; García-Bocanegra et al., 2012), which suggests an endemic circulation of flaviviruses in this area. Higher seroprevalence (12.8%) to WNV was reported in game birds (partridges and pheasants) sampled in the same region where the highest number of outbreaks in horses in Spain was reported (Llorente et al., 2013). The seroprevalence to WNV in pigeons from other European countries ranges between 0 and 54% (Lena et al., 2006; Chaintoutis et al., 2014), and pigeons have been proposed as interesting sentinels of WNV transmission risk to humans (Chaintoutis et al., 2014). During the last few years, the number of USUV outbreaks reported in birds and humans has significantly increased in different European countries, including Spain (Weissenböck et al., 2002; Höfle et al., 2013; Santini et al., 2015). Seroprevalence obtained in pigeons in this study was in accordance with those previously reported in columbids and other wild bird species in Europe (Llorente et al., 2013; Chaintoutis et al., 2014).

Specific antibodies against WNV and USUV were confirmed in captive white stork and ostrich, respectively. Furthermore, one emperor goose showed specific neutralizing antibodies against the three flaviviruses tested using VNT. Although the higher titers against WNV detected in this species suggest infection with this flavivirus, contact with USUV and/or MBV cannot be ruled out. Interestingly, MBV circulation has been detected in gull populations in Spain (Arnal et al., 2014). Susceptibility to WNV infection in white stork and emperor goose has been previously confirmed (Malkinson et al., 2001; CDC, 2015). Seroprevalence to USUV detected in zoo birds in our study (3.6%) was in accordance with that found in other zoos in Europe (Buchebner ET AL., 2013). To the best of our knowledge, this is the first report of USUV infection in ostriches.

None of the pigeons tested showed antibodies against AIV. Although susceptibility to AIV infection in columbids has been previously demonstrated (Abolnik, 2014), our results are consistent with that reported in columbid species in Spain (Pérez-Ramírez et al., 2010) and other European countries (Lillehaug et al., 2005; Teske et al., 2013a). Experimental studies have showed that pigeons could be considered as AIV "dead end" host because the shedding levels are below the threshold of the minimal infective particles required to infect other species. Therefore, disseminating AIV in this species is more likely to occur via mechanical route (Abolnik, 2014). AIV seropositivity was detected in five captive bird species, four of them belonging to the order *Anseriformes*, which are the main reservoirs of AIV (Kim et al., 2009). To author's knowledge, this is the first report of antibodies against AIV in African sacred ibis.

The absence of *Salmonella* spp. isolation in pigeons suggests a limited role of feral pigeons in the transmission of this enteropathogen in the study area. These results are consistent with those previously reported in European countries including Spain (Casanovas et al., 1995; Teske et al., 2013b; Gargiulo et al., 2014). *Salmonella* spp. was isolated in 3 out of 44 samples from zoo animals, in particular, in wild boar, milk snake and in ostrich. Due the intermittent shedding of *Salmonella* spp. in feces; the prevalence found might have

been underestimated. The number of reports about *Salmonella* infections in humans associated to contact with reptiles has increased in the last years due to the use of these species as exotic pets (Geue and Löschner, 2002). Also, clinical infections have been confirmed in zoo animals and zoo visitors by direct or indirect contact with reptiles infected with *Salmonella* spp. (Friedman et al., 1998; Bauwens et al., 2006). The serotype (*Salmonella* Apapa) detected in milk snake in the present study has been previously associated with sepsis in human neonates due to direct or indirect contact with bearded dragons (Haase et al., 2011). *Salmonella* Panama was confirmed in one wild boar without clinical symptoms. Although this species is considered natural reservoir of *Salmonella* spp. in Spain (Navarro-González et al., 2012) and this serotype has previously been isolated in domestic pig (Kich et al., 2011), this is the first report of *Salmonella* Panama infection in wild boar.

Pigeons are a good model and indirectly indicate soil contamination by *T. gondii* oocysts, since they feed from the ground and they are highly susceptible to oral infection with oocysts (Waap et al., 2012). Importantly, an association of the consumption of pigeon meat with *T. gondii* infection has been reported (Alvarado-Esquivel et al., 2012). There have been many reports of *T. gondii* antibodies in feral pigeons, and the parasite has been isolated from this species (Dubey, 2010) but to our knowledge there have not been previous reports of seroprevalence of *T. gondii* in feral pigeons in Spain. Seroprevalence levels observed in the present study (less than 10%) are in agreement with most of the previously reported studies in pigeons worldwide (Dubey, 2010). The wide geographic distribution of *Columba livia,* the ecological association to human settlements and the sedentary behavior suggest the pigeon as good sentinel species for the presence of oocysts in the human environment. Additional studies are required in order to investigate the role of pigeons in the epidemiology of *T. gondii.*

Widespread *T. gondii* seroprevalence was observed in captive zoological species in CMZP. Sixteen of 33 (48.5%) zoological species analyzed had at least one seropositive

animal to *T. gondii* with 26.9% as average seroprevalence. This study is the first report of presence of *T. gondii* antibodies in European mink, a critically endangered species (UICN, 2015). High seroprevalence was observed in the felid species (46.2%), and importantly all the samples analyzed from lions, jaguars and Bengal tigers were positive. These results could be of Animal Health and Conservation concern in CMZP due to the fact that seropositive felids might shed oocysts and the high susceptibility to *T. gondii* infection of some zoo species such as Marsupials and New World monkeys (Juan-Sallés et al, 1998; Dubey, 2010; Fernández-Aguilaret al., 2013).

In the present study, antibodies against *T. gondii* were widespread in captive-ungulates, being observed in 6 of 9 species analyzed. In Spain, presence of such antibodies has been previously described in free-living wild ruminants (Gauss et al., 2006) and in wild boars (Gauss et al., 2005). Birds showed lower seroprevalence levels and antibodies were detected in only 4 of 14 species analyzed. The prevalence of antibodies against *T. gondii* in wild bird species in Spain has been highly heterogeneous, depending on their feeding behaviour and geographical location with higher levels in carnivore wild birds (Cabezón et al., 2011). Low number of carnivore birds was included in the present study, which could be one reason for the low prevalence observed. Another explanation could be low exposure to oocysts in the environment. In addition, some recent studies have suggested that lower sensitivity of serological tests, such as MAT, in birds may sub-estimate seropositivity levels in these species (Casartelli-Alves et al., 2014).

The results obtained in the present study suggest a limited role of pigeons in the transmission of AIVs and *Salmonella* spp. However, the seroprevalence against flaviviruses and *T. gondii* found in this species, as well the presence of flaviviruses, AIV, *Salmonella* spp. and *T. gondii* detected in captive zoo animals, indicate the need to monitor these species in the study area. Control measures, including monitoring, population control, vaccination programs and management measures should be maintained and/or implemented in order

to prevent transmission of these zoonotic agents between pigeons and sympatric species, included human.

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CAPÍTULO 2.2

Infección letal por *Acinetobacter baumannii* en visón europeo (*Mustela lutreola*)

Cano-Terriza D, Guerra R, Mozos E, Rodríguez-Sánchez B, Borge C, García-Bocanegra I (2017). **Fatal *Acinetobacter baumannii* infection in the critically endangered European mink (*Mustela lutreola*).** Journal of Zoo and Wildlife Medicine, 48:220–223.

Resumen

En el presente estudio se describe por primera vez un caso de infección letal por *Acinetobacter baumannii* (*A. baumannii)* en visón europeo (*Mustela lutreola*), una especie catalogada como en peligro crítico de extinción. El examen macroscópico reveló la presencia de una neumonía hemorrágica grave y difusa y congestión generalizada. Las principales lesiones microscópicas incluyeron neumonía fibrino-hemorrágica aguda y severa, asociada a la proliferación de cocobacilos, y congestión aguda-subaguda generalizada. El cultivo microbiológico y posterior identificación mediante desorción/ionización láser asistida por matriz (MALDI-TOF) confirmaron que el agente etiológico implicado en este caso fue *A. baumannii*. La cepa aislada presentó un patrón multirresistente a antibióticos. Nuestros resultados no sólo son de importancia desde el punto de vista de la conservación, sino también desde el punto de vista de la Salud Pública, dado que *A. baumannii* es uno de los patógenos más importantes implicados en las infecciones nosocomiales en humanos.

Abstract

In the present study we report the first case of fatal *Acinetobacter baumannii* infection in the critically endangered European mink (*Mustela lutreola*). Gross examination revealed a severe, diffuse haemorrhagic pneumonia and generalized congestion as main features. Microscopically, the main lesions were an acute, severe fibrinous-haemorrhagic pneumonia associated with proliferation of coccobacilli and generalized acute-subacute congestion. Cultures yielded *A. baumannii*; the species was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) and the strain presented a multidrug-resistant pattern. Our results are not only of conservation concern but also of public health concern given *A. baumannii* is one of the most important pathogens implicated in nosocomial infections in humans.

Keywords: European mink; Pneumonia; *Acinetobacter baumannii*; *Mustela lutreola,* Public health.

Brief communication

In October 2013, a 7-yr-old, male captive-bred European mink (*Mustela lutreola*) was shipped to the Cordoba Municipal Zoo Park (CMZP) (southern Spain) for captive breeding within the European *Ex situ* conservation program. In February 2014, the animal was admitted at the CMZP hospital with clinical signs of severe tachycardia (230-260 beats/min), tachypnea with increased breath sounds, depression, lethargy and low body temperature (36.5-37.8ºC). Radiographs demonstrated a widespread increase in radiodensity mostly in cranial and middle lobes of right lung and bronchoalveolar pattern of the apical regions (Figure 1). Plasma biochemistry values revealed a marked leucopenia (white blood cell count: $1.07x10^9$/L).

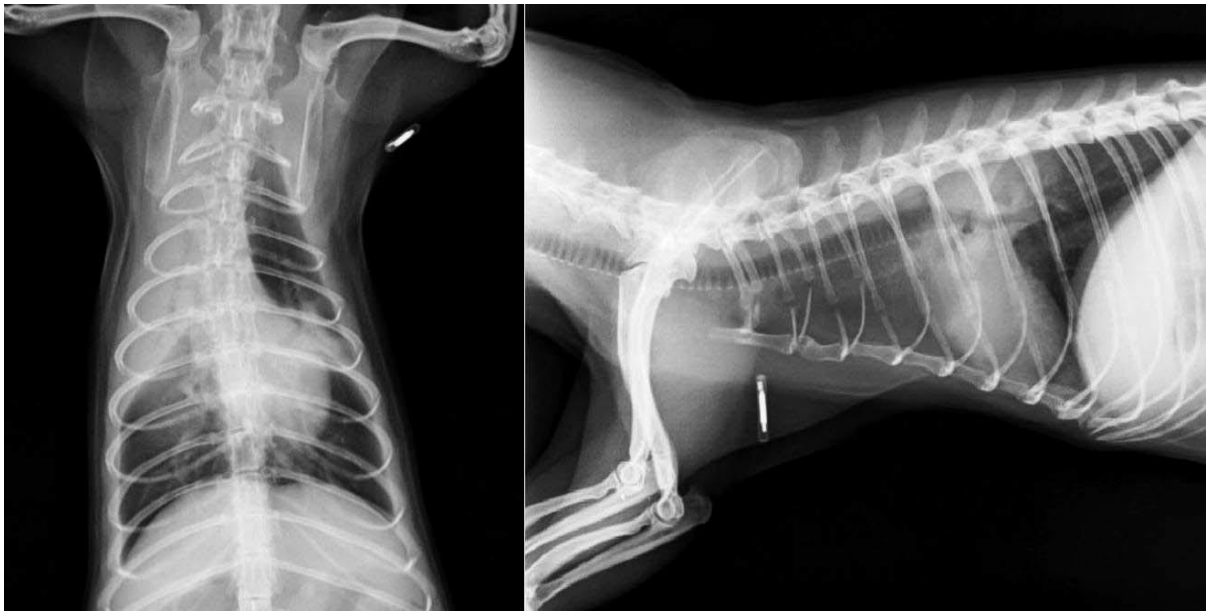

Figure 1. Ventrodorsal (left) and laterolateral (right) projections of the thoracic cavity by radiographic analysis.

The mink was treated with a combination of marbofloxacin (2 mg/kg body weight [BW]), amoxicillin-clavulanic acid (15 mg/kg BW), furosemide (4 mg/kg BW) and aminophylline (5 mg/kg BW). Intraosseous fluid therapy (lactated Ringer solution) and

oxygen were also administered. Despite treatment the mink died 5 hr after admission. At necropsy, the animal was in good body condition and weighed 1.16 kg. Gross examination revealed a severe, bilateral, acute haemorrhagic pneumonia, slight reddish pleural effusion, acute-subacute congestion of major organs, and moderate splenomegaly. No other abnormalities were observed.

Tissue samples from lung, heart, liver, spleen, kidney, urinary bladder, oesophagus, stomach, intestine, pancreas and adrenal cortex were fixed in 10% neutral buffered formalin, routinely processed, and stained with hematoxylin and eosin, periodic acid-Schiff, Frasser-Lendrum and Gram stain. Histopathologic examination of the lungs revealed severe generalized acute congestion, with the alveoli and bronchioles diffusely filled with oedema and moderate to abundant cellular exudates composed of neutrophils, macrophages, erythrocytes and detached pneumocytes. Numerous Gram-negative coccobacilli were observed admixed with the oedema, cells debris and proteinaceous material. Macrophages frequently contained intracytoplasmic coccobacilli (Figure 2a, b). In the spleen the red pulp showed acute congestion, few venous microthrombi in vessels and occasional megakaryocytes. In the myocardium, acute congestion, interfibrillar edema and extensive myofibril coagulation was observed. The liver presented centrilobulillar congestion and the kidneys showed congestion and multiple microhemorrhages in the corticomedullary junction. Acute congestion and microhemorrhages was found in adrenal cortex. Congestion was also observed in the esophagus, stomach, intestine and pancreas. A morphologic diagnosis of acute, severe fibrinous-haemorrhagic pneumonia with intralesional coccobacilli and acute septic shock was made.

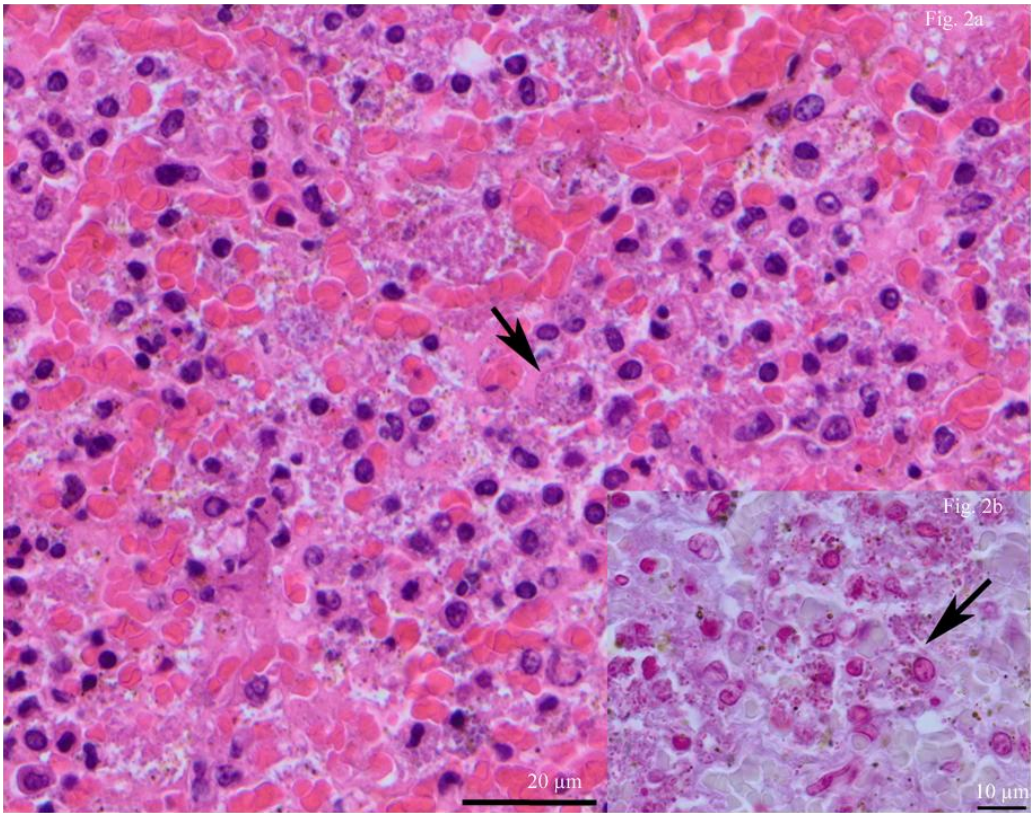

Figure 2. a. Lung. At high magnification the alveoli are filled with macrophages and neutrophils, as well as erythrocytes; numerous coccobacilli appear in the cytoplasm of large macrophages (arrow). The alveolar capillaries are congested. Hematoxylin and eosin, x40. **b.** Lung. Numerous gram-negative coccobacilli are present in the cytoplasm of macrophages in the alveolar lumina (arrow). Gram stain, x100.

The indirect immunofluorescent antibody test yielded negative results (titers < 1:20) for canine distemper virus. Thus, the acute character of clinical signs and the normal values of gamma globulin (11.7% of total protein) using protein electrophoresis, indicated absence of Aleutian disease infection (Best and Bloom, 2005). Lung and kidney samples were subjected to bacteriology analyses and cultured individually by using standard procedures. Samples were directly cultured on blood agar base supplemented with 5% sterile defibrinated sheep blood (Oxoid S.A., Spain). Pure colonies based on uniform colony

morphology were obtained after 24-48 h of aerobic incubation at 37ºC from both organs. Gram staining revealed gram-negative coccobacillus. Isolates were tested using API 20NE (API CS; BioMerieux, 69280, Marcy l'Etoile, France) according to the manufacturer's recommendations. Bacterial isolates were also analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) using a Microflex LT bench top mass spectrometer (Bruker Daltonics, 28359, Bremen, Germany) as described (Rodríguez-Sánchez et al., 2014). The isolates were identified as *Acinetobacter baumannii* with a score ≥ 2.0, which indicate that the identification was reliable at the species level according to the manufacturer's criteria. The ability of MALDI-TOF to differentiate among *Acinetobacter* species has been previously demonstrated (Toh et al., 2015). Besides, in our study, out of the ten results provided by MALDI-TOF MS for every analyzed sample, *Acinetobacter baumannii* was the only species detected.

Antimicrobial susceptibility of *A. baumanni* isolates was determined by the disc diffusion method (Bauer et al., 1966), using a panel of 27 antimicrobial agents. The diameter of the bacterial growth inhibition was measured on the basis of Clinical Laboratory Standards (Neo-Sensitabs potency according to CLSI 2006 and Veterinary Practise CLSI 2006). The bacterium was resistant to amoxicillin-clavulanic acid, ampicillin, cefazolin, cefotaxime, cefoxitin, ciprofloxacin, chloramphenicol, enrofloxacin, florfenicol, fosfomycin, lincomycin, metronidazole, neomycin, oxytetracycline, penicillin G, streptomycin, tetracycline, trimethoprim-sulfamethoxazole and vancomycin. Sensitivity was found for amikacin, cefepime, ceftazidime, co-trimoxazole, gentamicin, imipenem, piperacillin-tazobactam and tobramycin.

The results obtained in the present study indicate that the European mink died by an acute septic infection caused by *A. baumannii*. Even though fatal pneumonia associated with *A. baumannii* has been reported recently in American minks (*Neovison vison*) (Molenaar and Van Engelen, 2015) to the authors' knowledge, this is the first report on acute mortality by *A. baumannii* infection in the critically endangered European mink.

Histopathological lesions observed in lung were in accordance with those the previously reported in American minks infected with *A. baumannii* (Molenaar and Van Engelen, 2015).

European mink is a semi-aquatic mustelid species native to Europe. Their populations have been reduced more than 90% in the last 50 years and currently, only isolated populations are present in Northern Spain, Western France and Eastern Europe. Considered as critically endangered (IUCN, 2015), the progressive disappearance of the European mink has been mainly attributed to anthropogenic factors. Overexploitation, habitat destruction, watercourse quality loss, illegal hunting and trapping, introduction of the alien American mink, hybridization with European polecat (*Mustela putorius*) and diseases have been important factors in the decrease of their populations (Maran and Robinson, 1996) To save this species from extinction, *ex situ* and *in situ* conservation projects, which include habitat preservation, mink population monitoring and captive breeding, have been initiated to maintain the genetic variability and produce individuals for future reintroduction efforts.

Acinetobacter baumannii is considered as one of the most important pathogens implicated in nosocomial infections in human hospitals (Eveillard et al., 2013). In particular this bacterium affects immunocompromised patients causing local or generalized septic processes. The main clinical symptoms associated with *A. baumannii* infection in humans include pneumonia, respiratory, urinary and bloodstream infection. *Acinetobacter baumannii* infection has been also reported in animal species mostly from intensive care units (ICU) (Francey et al., 2000; Endimiani et al., 2011; Smet et al., 2012; Eveillard et al., 2013). Moreover, *A. baumannii* has been also isolated in both human body and head lice and the role of fleas as competent vectors for *A. baumannii* in American mink has been also suggested (La Scola and Raoult, 2004; Bouvresse et al., 2011; Molenaar and Van Engelen, 2015). Because ectoparasites (including ticks, fleas and lice) were not detected in the present case, this hypothesis could not be tested.

Acinetobacter baumannii is able to express a variety of mechanisms that make it frequently multidrug-resistant (Towner, 2009). The strain isolates in the European mink presented a multidrug-resistant pattern being resistant to 19 out of the 27 antibiotics tested. The results are in accordance with those found in human and other species including the American mink (Towner, 2009; Molenar and Van Engelen, 2015). Besides implications for the conservation of endangered species, *A. baumannii* is of public health concern due to its zoonotic potential. Additional studies are needed to determine the role of the European mink in the epidemiology of *A. baumannii* and its implication in the transmission to other species with which they share habitat.

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CAPÍTULO 3

Estudio epidemiológico y espacio-temporal del virus West Nile en caballos en España entre 2010 y 2016

García-Bocanegra I, Belkhiria J, Napp S, **Cano-Terriza D**, Jiménez-Ruiz S, Martínez-López B (2018). **Epidemiology and spatio-temporal analysis of West Nile virus in horses in Spain between 2010 and 2016.** Transboundary and Emerging Diseases, 65:567-577.

Resumen

Durante la última década, los brotes del virus de West Nile (VWN) han aumentado drásticamente tanto en caballos como en humanos en Europa. Los objetivos de este estudio fueron evaluar las principales características y distribución espacio-temporal de los brotes de VWN en caballos en España entre 2010 y 2016 con el fin de identificar las principales variables ambientales asociadas al VWN y generar mapas de presencia del virus que sirvan como estrategias de vigilancia basadas en el riesgo en este país. Entre agosto de 2010 y noviembre de 2016, se investigaron un total de 403 casos sospechosos en caballos, de los cuales, 177 (43,9%) fueron confirmados mediante técnicas laboratoriales. Los valores medios de las tasas de morbilidad, mortalidad y letalidad fueron respectivamente 7,5%, 1,6% y 21,2%. Los síntomas clínicos más comunes fueron: cansancio/apatía, decúbito, temblor muscular, ataxia, descoordinación e hiperestesia. Los brotes confirmados de VWN durante los últimos siete años, así como la detección del linaje 1 en 2010, 2012, 2013, 2015 y 2016, sugieren una circulación endémica del virus en España. La distribución espacio-temporal de los brotes de VWN no fue homogénea en este país, ya que la mayoría de ellos (92,7%) se concentraron en la parte occidental de Andalucía (sur de España) y se detectaron clústeres espaciales significativos en esta región en dos años no consecutivos. Estos hallazgos estuvieron apoyados con los resultados obtenidos en el modelo de permutación estadística espacio-temporal. Además, se realizó un modelo de nicho ecológico basado en máxima entropía (MaxEnt) para generar un mapa de probabilidad de presencia de VWN en Andalucía. Los principales factores ambientales seleccionados por el modelo fueron: temperatura media anual (49,5% de contribución), presencia de *Culex pipiens* (19,5% de contribución), precipitación media anual (16,1% de contribución) y distancia a humedales Ramsar (14,9% de contribución). Nuestros resultados constituyen un paso importante para entender la emergencia y propagación del VWN en España y proporcionarán una valiosa información para el desarrollo de programas de vigilancia más efectivos basados en el riesgo y programas de control para mejorar la protección de las poblaciones de caballos y humanos en las áreas endémicas del virus.

Abstract

During the last decade, West Nile virus (WNV) outbreaks have increased sharply in both horses and human in Europe. The aims of this study were to evaluate characteristics and spatio-temporal distribution of WNV outbreaks in horses in Spain between 2010 and 2016 in order to identify the environmental variables most associated with WNV occurrence and to generate high-resolution WNV suitability maps to inform risk-based surveillance strategies in this country. Between August 2010 and November 2016, a total of 403 WNV suspected cases were investigated, of which 177 (43.9%) were laboratory confirmed. Mean values of morbidity, mortality and case fatality rates were respectively 7.5%, 1.6% and 21.2%, respectively. The most common clinical symptoms were: tiredness/apathy, recumbency, muscular tremor, ataxia, incoordination and hyperesthesia. The outbreaks confirmed during the last seven years, with detection of WNV RNA lineage 1 in 2010, 2012, 2013, 2015, 2016, suggest an endemic circulation of the virus in Spain. The spatio-temporal distribution of WNV outbreaks in Spain was not homogeneous, as most of them (92.7%) were concentrated in western part of Andalusia (southern Spain) and significant clusters were detected in this region in two non-consecutive years. These findings were supported by the results of the space-time scan statistics permutation model. A presence-only MaxEnt ecological niche model was used to generate a suitability map for WNV occurrence in Andalusia. The most important predictors selected by the ENM were: mean annual temperature (49.5% contribution), presence of *Culex pipiens* (19.5% contribution), mean annual precipitation (16.1% contribution) and distance to Ramsar wetlands (14.9% contribution). Our results constitute an important step for understanding WNV emergence and spread in Spain and will provide valuable information for the development of more cost-effective surveillance and control programs and improve the protection of horse and human populations in WNV endemic areas.

Keywords: West Nile Disease, Emerging disease, Maximum Enthropy, MaxEnt, SaTScan; Risk-based surveillance

1. Introduction

West Nile disease (WND) is a re-emerging zoonotic disease in Europe and neighboring countries. The causative agent, West Nile Virus (WNV), is a positive, single-stranded, enveloped RNA virus classified within the Japanese encephalitis virus serogroup, in the genus *Flavivirus* (family Flaviviridae). To date, seven (or nine according to some authors) different WNV lineages have been identified, but only lineage 1 and 2 have been associated with human and horse cases (Rizzoli et al., 2015).

The virus is mainly transmitted by ornithophilic mosquitoes of the *Culex pipiens* complex and their hybrids in an enzootic life-cycle in which certain birds act as natural reservoir hosts, amplifying the virus. Although mammals are susceptible to infection, most are considered dead-end or incidental hosts, as the viraemia is too low to infect competent vectors. Humans can also become infected by blood transfusion, organ transplantation, intrauterine transmission, handling of infected carcasses and breast feeding (CDC, 2017). In general, WNV infection in humans and horses is asymptomatic or associated with influenza-like illness; however, in some cases (< 1%) the infection can lead to severe neurological symptoms and mortality (Hayes et al., 2005).

Compared with the rapid and widespread distribution of WNV in the United States after its introduction in 1999 (CDC, 2017), in Europe, WNV was initially considered to have minor health effects, with sporadic cases in human and horses. However, the number of notified WNV outbreaks caused by both lineage 1 and lineage 2 **has** significantly increased in Mediterranean Basin during the last decade, which has raised concerns in relation to both public and animal health (Hernández-Triana et al., 2014; Benjelloun et al., 2016).

Before the 2010 WNV epidemic in Spain, antibodies had already been detected in humans, horses and wild bird species, but only sporadic clinical cases were reported in humans and raptors in Spain (Kaptoul et al., 2007; Jiménez-Clavero et al., 2008; García-Bocanegra et al., 2011a). In late summer 2010, the first WNV horse outbreak was reported in southern Spain (Andalusia). Throughout that year, 35 further cases were reported in

horses, and also 2 human cases were confirmed in the region (García-Bocanegra et al., 2011b). Since then, WNV outbreaks in horses have been reported every year. The goals of this study were: (1) to describe the main epidemiological and clinical findings of the WNV outbreaks in horses during the period 2010-2016 in Spain, (2) to assess the spatio-temporal distribution of WNV, and (3) to identifying the drivers of WNV occurrence in the endemic areas in Spain using a presence only maximum entropy ecological niche model.

2. Materials and methods

2.1 Descriptive analysis

After the confirmation of the first WNV outbreak in a horse herd on the 31st of August 2010, a passive surveillance system which included horses, humans, wild birds, and mosquitoes was launched by the veterinary and health authorities in Spain. All the herds in which horses with clinical symptoms compatible with WND were observed, were investigated by veterinary officers. Blood samples were obtained from suspected horses by puncture of the jugular vein. Brain and cerebrospinal fluid samples were also collected from dead or euthanized animals.

Serum samples from all investigated horses were tested to detect IgM antibodies against WNV using a commercial competitive ELISA (cELISA; IDEXX IgM WNV Ab, IDEXX Lab). Furthermore, 248 of the 403 (61.5%) investigated herds (including 115 IgM-positive herds and 133 IgM-negative herds) were randomly selected and analyzed by a commercial blocking ELISA (bELISA; Ingezim West Nile compac R.10.WNV.K3, Ingenasa Lab), which detects IgG antibodies against one epitope of the prM-E protein of the flaviviruses of the Japanese Encephalitis antigenic group. Both ELISAs were performed according to the manufacturer's recommendations. Ninety-four of 177 (53.1%) of the IgM-positive herds, as well as 31 of 133 (23.3%) of the IgM-negative herds, were selected, using a convenience sampling, and analyzed also by virus serum-neutralization test (VNT) against WNV (strain

Eg101) according to the OIE guidelines (Table 1). Blood and cerebrospinal fluid samples (CFS) were analyzed for detection of WNV lineage 1 and 2 by real time RT-PCR as previously described (Del Amo et al., 2013). All laboratory tests were performed at the National WNV Reference Laboratory in Algete (Madrid, Spain).

Table 1. Results of the laboratory analyses for West Nile virus (WNV) in horse herds between August 2010 and November 2016, in southern Spain.

Laboratory diagnostics	% Confirmed herds (positive/tested)	% Non-confirmed herds (positive/tested)	% Investigated herds (positive/tested)
IgM antibodies	100 (177/177)	0.0 (0/226)	43.9 (177/403)
IgG antibodies	93.0 (107/115)	30.8 (41/133)	59.7 (148/248)
Neutralizing	53.2 (50/94)	45.2 (14/31)	51.2 (64/125)
WNV RNA	7.9 (14/177)	0.0 (0/155)	4.2 (14/332)

An outbreak was defined as a herd with at least one confirmed case. A case was defined as a horse with clinical symptoms that were compatible with WND and confirmed by the National Reference Laboratory as positive by detection of IgM antibodies to WNV or RT-PCR positivity. The WNV-specific morbidity was expressed as the number of WND cases in the investigated horse herds divided by the total number of horses in these affected herds. The WNV-specific mortality was calculated as the number of deaths due to WNV infection divided by the total number of horses in the confirmed herds. The WNV-specific case fatality was expressed as the proportion cases that died of WNV infection by the total number of WND cases.

Epidemiological data were collected during clinical inspections using a standardized questionnaire in Andalusia, the region where the majority of outbreaks (92.7%) were reported. Information on the characteristics of the herds and affected animals was also recorded in this region for the descriptive analysis.

2.2 Spatio-temporal cluster analysis

A spatio-temporal analysis was carried out in Andalusia. Geolocations (UTM coordinates) of all investigated horse herds were provided by the Regional Government of Andalusia and the Spanish Ministry of Agriculture Food and Environment. Data from August 2010 to November 2016 were analyzed using a space-time scan statistic, with a space-time permutation model (Kulldorff et al., 2005), to detect the presence of areas and time periods with significant aggregation of WNV outbreaks in horse herds. Space-time permutation model, similarly to the other more commonly used Bernoulli- and Poisson- based models, creates thousands of overlapping cylinders over the study area and compare the observed number of cases within the cylinder (i.e., at particular space -based of the cylinder- and particular time -height of the cylinder-) to the "expected" number of cases in that cylinder. The main difference of the permutation model is that the expected is calculated using only the cases as described by Kulldorff et al, 2005. The maximum spatial and temporal window were set up to be 50% of the study region surface and 15% of the study period (one year), respectively. The number of Monte Carlo simulations was set to 999 for the cluster scan statistic. Analyses were run using SaTScan^{TM} v9.4.4. Clusters were considered to be significant at $P < 0.05$.

2.3 Ecological Niche Modeling

Risk areas for WNV outbreaks occurrence in Andalusia were detected using the presence-only maximum entropy ecological niche model (MaxEnt) (Phillips et al., 2006). The model was performed with the MaxEnt program version 3.3.3 via the “dismo” package in R Studio version 1.0.44 (Hijmans et al., 2016). Briefly, the maximum entropy ecological niche model looks at the association between the presence data and several environmental predictors known to be related to the disease in order to characterize the most important environmental requirements for the disease agent to be present and estimate a suitability probability in sampled and non-sampled geographic areas.

In order to determine the suitability area for WNV occurrence in Andalusia, the MaxEnt model used the WNV outbreaks locations in the region between 2010 and 2016 as presence data and 10.000 randomly chosen background points from Andalucía as "Pseudo-Absence" data. Potential predictors for the MaxEnt model consisted of a set of 14 climatic, environmental and demographic factors that were previously described as important for WNV presence. The model was calibrated with a default convergence threshold, a regularization of 1 and a number of iterations of 1,000. In addition, a logistic model was used to ensure that predictions gave estimates between 0 and 1 for the spatial suitability per map cell. Climatic predictors were provided by the Regional Government of Andalusia (CMAOT, 2016). They included mean annual temperature (ºC), mean maximum annual temperature (ºC), mean minimum annual temperature (ºC), mean annual rainfall (mm) and average number of rainy days per year (days). Environmental/demographic variables included altitude (m), evotranspiration defined as theoretical water requirements by the vegetation cover (mm), type of soil, distance to wetlands of national importance for water birds (Ramsar wetlands) (Km) (ICWII, 2017), land cover, presence of *Culex pipiens*, presence of *Culex theilery,* horse herd density and human population density. Human and horse densities (at municipality level), altitude, evotranspiration, type of soil, distance to Ramsar wetlands and land cover were provided by the Regional Government of Andalusia (CMAOT, 2016). The land cover was obtained from the Land Cover Change 2006-2012 (http://land.copernicus.eu/pan-european/corine-land-cover/lcc-2006-2012/view). Raster maps of presence/absence of *C. pipiens* and *C. theileri* were generated based on the criteria defined by Tran et al. (2013). In brief, they evaluated whether different mosquito species were present in each CORINE (Coordination of Information on the Environment, http://www.eea.europa.eu) land cover class based on a literature review and the opinion of expert entomologists. Therefore, presence or absence of *C. pipiens* and *C. theileri* (dichotomous variable) throughout Andalusia was defined as based on the corresponding CORINE land cover class at each location. The 2006 CORINE land cover map was obtained from the European Environment Agency website (http://www.eea.europa.eu). All

predictors were rescaled in rasters format with 100m × 100m spatial resolution, the same extent and the common UTM 30N projection. Correlation between predictors was assessed using pairwise Spearman's rank correlation coefficient (*rho*). When the correlation between two variables was 0.5 or higher, only the variable more biologically linked to WNV occurrence was included in the model. A first "full" MaxEnt model was fit with the 14 potential predictors. Predictors that contributed to 5% or more to the first model were selected to be run in a final "reduced" model. The final model was evaluated after partitioning the presence data into a training and a testing using the A k-fold method (Jung and Hu, 2015). A total of 80% of the WNV outbreaks locations were randomly selected for model building, whereas the remaining 20% locations were set aside for external validation. Model performance was assessed using the area under the curve (AUC) of the receiver operating characteristics curve (ROC) using the "dismo" package in R. Since AUC has been shown to be influenced by spatial sorting bias, the calibrated AUC (AUCc) was also used as suggested by Hijmans et al. (2012). The AUCc provides a more accurate estimate of the real performance of a model.

The Jackknife training gain test and percent contribution were used to estimate the contribution of each predictor in the final model. Predictors with the highest training gains or those that reduced the training gain the most when left out of the model, were considered the most valuable variables to the model. The final model was then used to generate the corresponding suitability map for WNV occurrence in Andalusia and a partial plot for the contribution of each predictors in the model were generated.

Non-confirmed herds were also overlaid over the WNV suitability map in order to assess how many of them where located in high risk areas. Specifically, WNV suspected cases were classified into two categories ("high risk" or "low risk") based on the median value of the outbreaks. Map was created using ArcMap version 10.3 (ESRI, Environmental Systems Resource Institute, www.esri.com).

3. Results

3.1 Descriptive analysis

Between August 2010 and November 2016, 403 suspected horse herds were investigated, 177 (43.9%) were confirmed as WNV outbreaks (presence of both clinical symptoms and IgM antibodies or RT-PCR positivity to WNV in at least on horse) (Table 1, Figure 1). Within the WNV positive herds, 236 (8.5%) of the 2,779 horses were considered clinically suspected, of which 215 were confirmed as cases, resulting in a mean WNV-specific morbidity of 7.7%. The mean age of the cases was 7 years (ranging between one and 22 years), and the census of the infected herds varied from 1 to 325 horses (median=4).

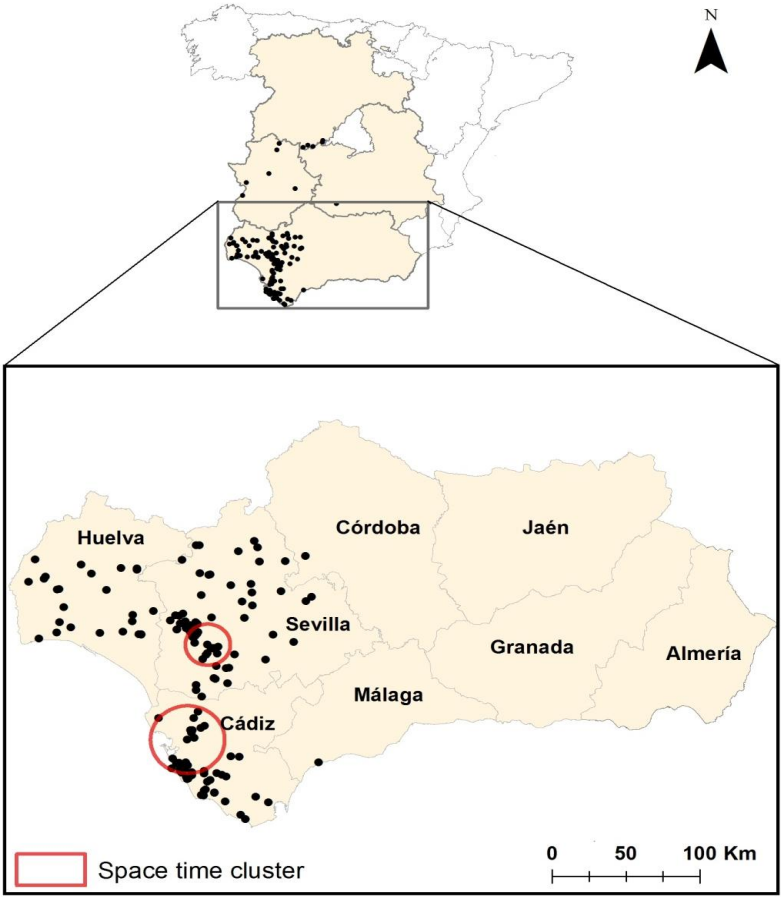

Figure 1. Spatial distribution of the 177 confirmed WNV outbreaks in Spain between 2010 and 2016. Black dots indicate outbreaks in horse herds.

Vaccination programs were not implemented in any of the confirmed or non-confirmed herds and movement of animals were not performed in these herds one month before the outbreak was reported. In those WNV positive herds, mean WNV-specific mortality and WNV-specific case fatality rates were 1.6% and 21.2%, respectively. The most common clinical symptoms detected in the positive herds were: tiredness/apathy (74.2%), recumbency (54.8%), muscular tremor (51.6%), ataxia (48.4%), incoordination (48.4%) and hyperesthesia (45.2%). Fever (32%), anorexia (23%) and convulsion (23%) were also frequently observed (Figure 2).

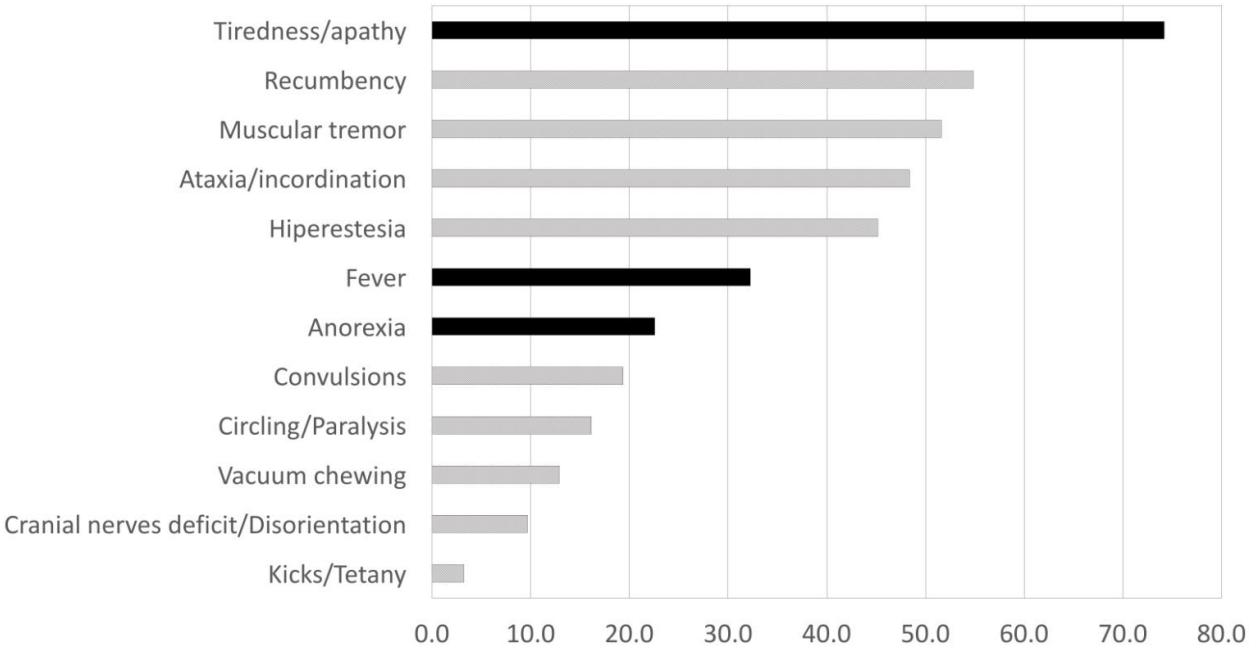

Figure 2. Frequency in which different clinical symptoms associated to WNV infection were observed in herds affected by WND in Spain (2010-2016). Black and grated bars indicate general and nervous symptoms, respectively.

Presence of IgG antibodies was confirmed in 107 (93.0%) of the 115 WNV confirmed (IgM-positive) herds tested (Table 1). WNV neutralizing antibodies were observed in 50 (53.2%) of the 94 confirmed herds that could be tested using VNT, with titers of 1:10 in 20% of them, 1:20 in 30%, 1:40 in 18%, 1:80 in 22% and 1:160 in 10%. Ten samples (10.6%) could not be analyzed due to cytopathic effect. Besides, IgG antibodies was detected in 41 of the 133 (30.8%) non-confirmed herds analyzed. WNV neutralizing antibodies were detected in 14 out of 31 (45.2%) non-confirmed (IgM-negative) herds analyzed by VNT, with titers of 1:20, 1:40, 1:80, 1:240 and 1:640 in two, five, four, two and one horse herds, respectively.

WNV RNA was found in 14 (4.2%) out of 332 horse herds. Within the positive herds, WNV RNA was detected in 5 (2.2%) of the 229 blood samples of the analyzed horses, and in 11 (61.1%) of the 18 CFS from dead or euthanized animals. Two horses were WNV RNA positive in both blood and CFS. WNV RNA was detected in 2010, 2012, 2013, 2015 and 2016. All RT-PCR positive samples were confirmed as WNV lineage 1.

The first outbreak was reported on the 31th of August 2010 in a horse herd in Cádiz province. Since then, outbreaks have been reported every year during the studied period (Figure 3). The total number of outbreaks per year was: 36 (20.3%) in 2010, 6 (3.4%) in 2011, 3 (1.7%) in 2012, 35 (19.8%) in 2013, 8 (4.5%) in 2014, 17 (9.6%) in 2015 and 72 (40.7%) in 2016. There was a clear seasonal pattern in the outbreak temporal distribution. Outbreaks were concentrated between the months of July and January, and peaked in September (Figure 3). The last three outbreaks were reported on the 11th of November 2016, two in Badajoz province (Extremadura) and one in Avila province (Castile and Leon). A total of 82 municipalities located in four different regions have been affected by the WNV outbreaks that occurred in Spain between 2010 and 2016. Most of the outbreaks were located in Andalusia (92.7%), followed by Extremadura (3.9%), Castile and Leon (2.8%) and Castile La Mancha (0.7%). One herd reported outbreaks in three consecutive years (2014-2016), and other three herds reported outbreaks two different years (2010-2012, 2014-2015 and 2015-2016). The remaining 173 horse herds affected reported a single outbreak.

3.2 Spatio-temporal analysis

The space-time permutation model identified two statistically significant clusters ($P < 0.001$) centered in the west part of Andalusia (Table 2 and Figure 1). The most likely cluster included 36 outbreaks and was located in south-western Andalusia (Cádiz province) in September 2010. Another cluster, with 34 outbreaks, emerged in August 2016 in central western Andalusia (Seville province).

Table 2. Results of the space–time permutation model for West Nile virus outbreaks in horses between August 2010 and November 2016, in Andalusia, southern Spain

Cluster	Radius (km)	Cluster time frame	No. of observed cases	No. of expected cases	Observed/ expected	*P*-value
1	25.00	31 August to 29	36	8.45	4.26	<0.001
2	15.17	4 August to 7	34	7.62	4.46	<0.001

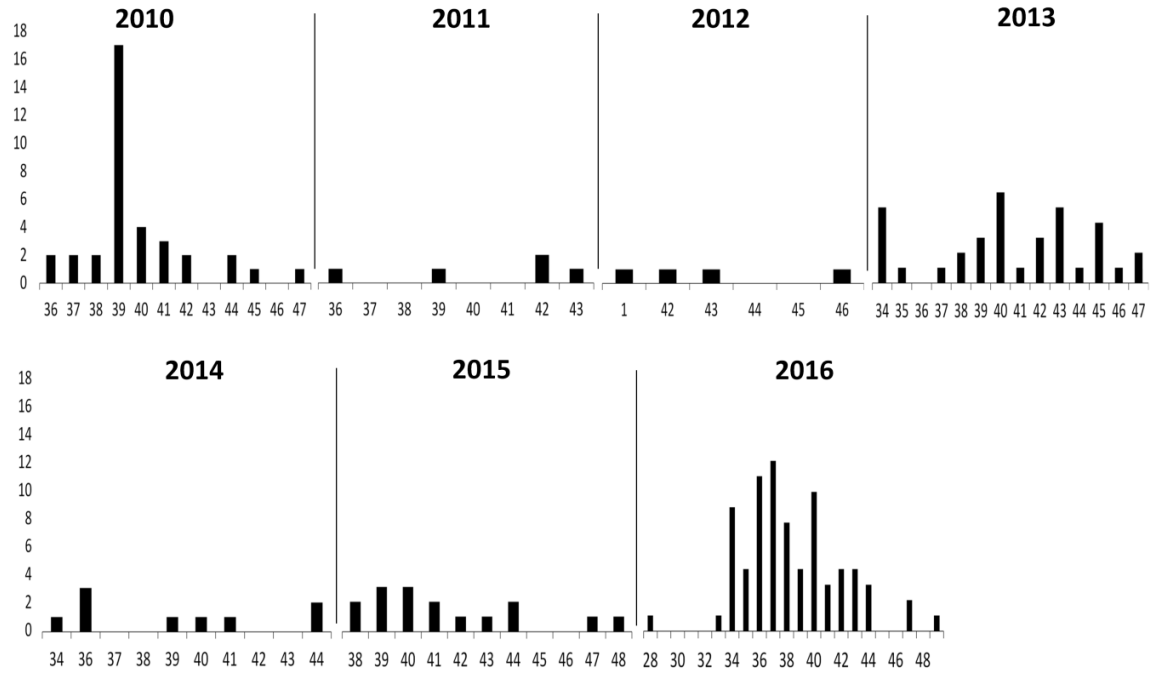

Figure 3. Temporal evolution (in weeks) of WNV outbreaks in Spain during the period 2010-2016.

3.3 MaxEnt modeling

Mean annual temperature (49.5% contribution), presence of *Culex pipiens* (19.5% contribution), mean annual rainfall (16.1% contribution) and distance to Ramsar wetlands (14.9% contribution) were identified as the most important predictors for WNV occurrence in Andalusia (Figure 4). The AUC of the final MaxEnt model was 0.918 and the AUCc values 0.914. Spearman correlation showed very low correlation between the selected predictors (see Supplementary Figure 1). Results of Jackknife and partial plots of the variables in the final model are shown in Supplementary Figure 2 and Supplementary Figure 3. A total of 85 of the 226 (37.6%) non-confirmed herds were identified in "high risk" areas (Figure 4).

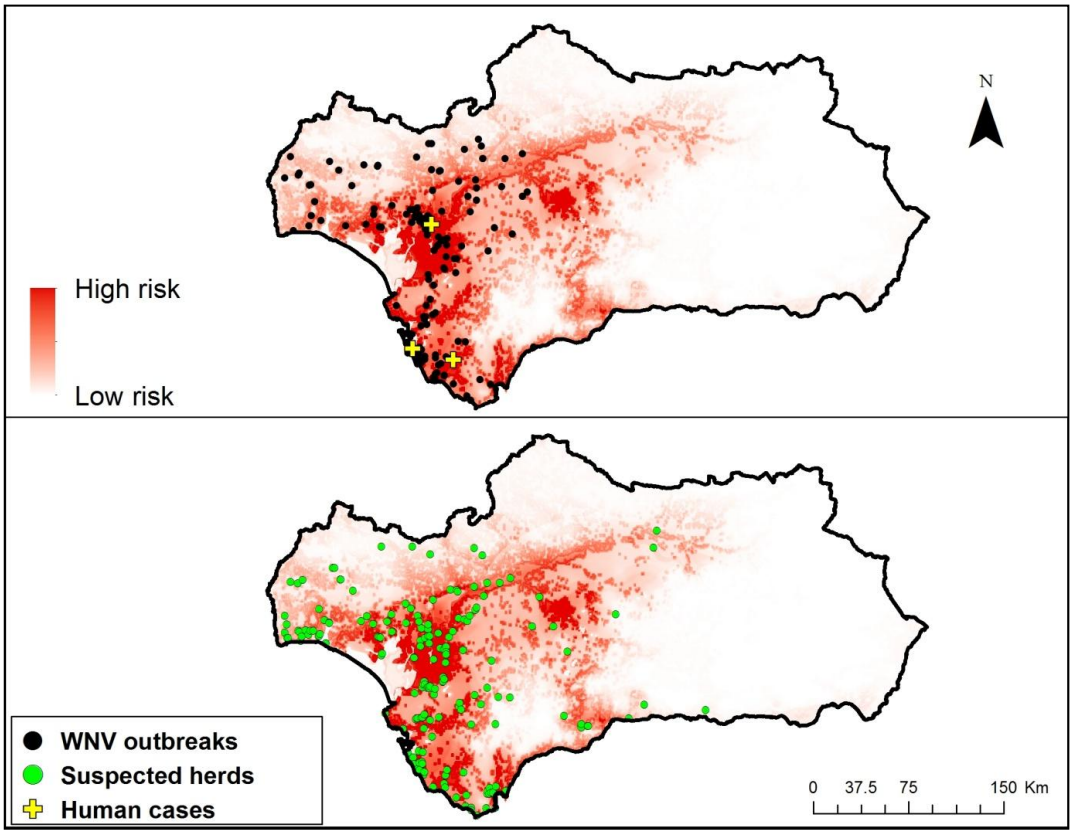

Figure 4. Map of Andalusia (southern Spain) showing high risk areas for WNV occurrence. Color gradient represents the WNV occurrence risk. Black and green dots indicate the confirmed and non-confirmed WNV outbreaks in horses, respectively. Yellow crosses indicate WNV outbreaks in human.

4. Discussion

4.1 Descriptive analysis

Even though antibodies to WNV had been previously detected in different species in Spain, clinical cases in horses or humans were not reported until 2010 (García-Bocanegra et al., 2011b). Since 2010, a total of 403 suspected WNV outbreaks were investigated, and almost half of them (43.9%) were confirmed as outbreaks. The occurrence of WNV **outbreaks** over Spain was not homogeneous, as it was higher in the western part of Andalusia (Figure 1). However, the temporal evolution confirms important changes in the spread of WNV in the last decade in Spain, in agreement with those previously reported in other European countries (ECDC, 2016).

Clinical signs observed in WNV infected horses included both general and nervous signs, being similar to those previously reported (Murgue et al., 2001; Kutasi et al., 2011; Porter et al., 2011; van Galen et al., 2013; Bouzalas et al., 2016). The WNV-specific case fatality found in Spain (21%) was in accordance the values reported in Italy in 2011 (25%) (Cantile et al., 2000; Autorino et al., 2002), but was lower than that reported in other countries such as Hungary in 2008 (29%), Greece in 2010 (30%), France in 2000 (45%) and Morocco in 2003 (56%) (Murgue et al., 2001; Schuffenecker et al., 2005; Kutasi et al., 2011; van Galen et al., 2013; Bouzalas et al., 2016). Discrepancies in fatality rate among countries may be explained by differences in the viral strains involved or individual factors (Rios et al., 2010; Porter et al., 2011). In this respect, cross-immunity associated to previous exposure to other flaviviruses may also influence the clinical presentation of the disease (Tesh et al., 2002; Rodríguez et al., 2010). Several flaviviruses, including Usutu virus, Bagaza virus and Meaban virus have circulated in the study area in the last decade (García-Bocanegra et al., 2012a, Jurado-Tarifa et al., 2016). Consequently, laboratory analyses are required to confirm or exclude WNV infection.

Given the low viral load and short viremia in horses and humans, the voluntary vaccination in horses as well as the late appearance of clinical symptoms, frequently when the viremia phase is over, confirmation of WNV outbreaks is based on both presence of clinical symptoms and the detection of early IgM antibodies against WNV. Based on that criteria, 177 (43.9%) of the 403 suspected herds were confirmed as WNV outbreaks. VNT positivity was only confirmed in 53.2% of the total confirmed herds. Additionally, two cases were negative by VNT but positive using RT-PCR. Although VNT is recommended by OIE as the gold standard method for WNV diagnosis particularly in areas with circulation of other flaviviruses, our results indicate that the combination of clinical symptoms and detection of IgM antibodies against WNV, may be a good criterion to confirm a case in specific epidemiological scenarios.

A high percentage of WND cases (93.0%; 107 of the 115 WNV positive herds) presented both IgM and IgG antibodies. Experimental WNV infections indicate that IgM antibodies can be found in serum around 7-10 days post-infection (dpi) until 1-3 months, while IgG-specific antibodies can be detected for several years after infection (Ostlund et al., 2001; Durand et al., 2002; Castillo-Olivares and Wood, 2004; Bouzalas et al., 2016). WNV RNA was only detected in blood in 2.2% of the total analyzed animals from positive herds, which is consistent with the short viremia reported in this species (Bunning et al., 2002). The presence of WNV RNA in 11 of the 18 (61.1%) CFS confirms the higher persistence of the virus in central nervous system as well as the usefulness of using this fluid for RNA WNV detection (Kleiboeker et al., 2004). Although both WNV lineages 1 and 2 have been reported in Europe and the Mediterranean Basin (Calistri et al., 2010; Papa et al., 2011), all RT-PCR positive samples were confirmed as WNV lineage 1. This coincides with previous studies that reported only WNV lineage 1 in birds, horses and mosquitoes in Spain (Jiménez-Clavero et al., 2008; García-Bocanegra et al., 2011b; Vázquez et al., 2011). However, given the active circulation of WNV lineage 2 in other Mediterranean countries during the last few years (Hernández-Triana et al., 2014), its introduction and spread in Spain cannot be ruled out (Fros et al., 2015).

The highest risk period for WNV outbreaks occurrence in Spain ranges between mid-August and mid-November, concentrating 94.4% of the total horse outbreaks. This temporal distribution of WNV outbreaks is consistent with the findings in other Mediterranean countries (Murgue et al., 2001; Autorino et al., 2002; Porter et al., 2011; Kutasi et al., 2011) and USA states with Mediterranean-like climate (CDC, 2017). The outbreaks confirmed consecutively during the last seven years as well as the detection of WNV RNA lineage 1 in 2010, 2012, 2013, 2015 and 2016, suggest an endemic circulation of WNV in Spain. Annual reintroduction of the virus through transportation of migratory infected birds, infected vectors putatively from Africa or overwintering, may explain pathways for the endemic circulation observed in Spain in the last years. Further phylogenetic analyses are needed to elucidate the origin and evolution of the viruses circulating in Spain.

4.2 Spatio-temporal distribution

Andalusia is the region of Spain with the largest populations of horses with about 219,198 animals, and the density ranges between 3.7 and 1.2 horses/km^2 in the western and eastern regions, respectively (MAPAMA, 2016). The provinces of Seville and Cadiz, where the two clusters were detected, were also consistently associated with the detection of the three human cases reported in Spain to date (Figure 3). The strategic location within important wild birds migratory flyways, the high number of wetlands, environmental conditions, higher density of competent vectors and the high density of horses, are possible factors implicated in the higher spread of WNV in this area.

The immunity of the horse population to WNV is directly related with the natural exposure and the vaccination. Seroprevalence against WNV in horses in Andalusia after the first WNV epidemic was 7.1% (36/510), being significantly higher in areas where the outbreaks were reported (García-Bocanegra et al., 2012). Further studies are required to assess the evolution of the immunity in the horse populations after seven years of WNV circulation in this region. Even though vaccination is known to be an effective measure for WNV prevention and control, because of the cost of vaccines, its application is voluntary

and commonly restricted to regions where outbreaks were previously detected. The secondary cluster was identified in the region of Andalusia with highest horses and human population density, which highlights the high risk of WNV potential infection in humans. In fact, confirmed WNV outbreaks in horses is considered an early indicator of the risk of exposure to humans (Saegerman et al., 2016).

4.3 MaxEnt modeling

We have shown that MaxEnt modeling can be successfully applied to emerging diseases, in which climatic and environmental characteristics play an essential role. The presented presence-only model showed high accuracy capturing risk areas for WNV outbreaks (AUCc=0.914). The WNV risk map accurately identifies areas at high risk for WNV outbreaks in both humans and animals and thus provides a useful tool to design more accurate and cost-effective surveillance and control programs. Our results were in general consistence with those previously obtained using Mahalanobis distance analysis (Conte et al., 2015). Similar risk areas were also identified by Sánchez-Gómez et al. (2017) using a logistic regression-based spatial model with a limited number of predictors. The suitability areas obtained in our study provide a much higher spatio-temporal resolution than previous studies, allowing to refine the implementation of target interventions. Additionally, the MaxEnt model also highlighted new high risk areas for WNV occurrence (Figure 4), which provides valuable information to guide surveillance strategies for early detection of new cases in currently free areas. Interestingly, the MaxEnt model identified WNV risk areas in central regions in Andalusia where, although outbreaks have not been reported yet, seropositivity to WNV has been detected in different species including horses, birds and wild ruminants (García-Bocanegra et al., 2012b, García-Bocanegra et al., 2016; Jurado-Tarifa et al., 2016). Moreover, a high percentage (37.6%; 85/226) of non-confirmed herds were located in high suitability areas. This finding, together with the high percentage of non-confirmed herds positive to IgG antibodies (30.8%; 41/133), most of them (45.2%; 14/31) also confirmed by VNT, indicate that the number of WNV outbreaks confirmed in horses in

Spain may be underestimated. The notification of WND clinically suspected horses to the official veterinary services, which usually occurs at late stages of the disease, as well the relatively short duration of WNV-specific IgM antibodies, are possible factors that can hamper the confirmation of clinically suspected cases.

The final presence-only model mostly relied on climatic and environmental factors to determine the WNV suitability in Spain between 2010 and 2016. Specifically, mean annual temperature and mean annual rainfall which were the most important climatic predictors for WNV occurrence. Areas with mean annual temperature higher than 18º C were found most risk for WNV (Supplementary Figure 2). Different studies have reported the influence of temperature on the risk of WNV transmission (Ben Hassine et al., 2014; Paz, 2015). Increased temperature accelerates both WNV amplification and transmission through the acceleration of the development of competent vectors, increasing the biting behavior, increasing the reproductive rate, increasing the duration of the breeding season, and by reducing the extrinsic incubation period (Chevalier et al., 2014; Paz, 2015). Moreover, areas with annual precipitation between 550 and 1100 mm were found most risk for the presence of WNV (Supplementary Figure 2). These are consistent with the notion that regions with wet and warm climatic conditions are risk habitats for WNV transmission (Ozdenerol et al., 2013; Mughini-Gras et al., 2014; Paz, 2015). As the impact of rainfall in WNV transmission is not as straightforward as temperature, the influence of rainfall on WNV transmission remains controversial. Even though previous studies demonstrated that the rainfall is positively correlated with both the presence of mosquitoes and WNV outbreaks in humans (Papa et al., 2010; Bisanzio et al., 2011; Hartley et al., 2012; Chevalier et al., 2014), heavy precipitation may have a negative effect on their abundance through the dilution of nutrients and the flushing of the breeding sites (Koenraadt et al., 2008). Furthermore, drought may concentrate resources for both avian reservoirs and mosquitoes, leading to more likely contact between both groups of species.

Areas close to Ramsar wetland (< 0.5 km) were also found to be suitable for WNV occurrence (Supplementary Figure 2). This finding is consistent with previous observations (Rodríguez-Prieto et al., 2012; Valiakos et al., 2014; Bargaoui et al., 2015; Sánchez-Gómez et al., 2017). WNV transmission depends on the co-occurrence in space and time of both virus, competent vectors and susceptible bird hosts. Wetlands provide suitability habitats for mosquito larva presence (Bian et al., 2006). Moreover, Ramsar wetlands present a particularly high diversity and abundance of wild bird species, increasing the risk of WNV transmission to both natural reservoirs and dead-end hosts close to these areas (Valiakos et al., 2014).

The final model included the presence of *C. pipiens* as the second most relevant predictor of WNV occurrence. This result is consistent with other studies that identified this mosquito species as the primary vector of WNV in Europe (Hubálek and Halouzka, 1999; Calistri et al., 2010; Chevalier et al., 2014). Although data on the distribution of *Culex* species are still limited in Spain, previous studies have shown that *C. pipiens, C. perexiguus, C. theileri* and *C. modestus* are the main *Culex* species detected in western Andalusia (Aranda et al., 2009, Vázquez et al., 2011, García-Bocanegra et al., 2012b). The absence of *C. theileri* in the final model could indicate a less relevant role of this vector species in WNV transmission in the study area. Future studies should be conducted considering other competent vector species (eg. *C. perexiguus* and *C. modestus*) as well as other environmental and climatic variables for which data was not available in this study (e.g., relative humidity, seasonality) to assess the implication of these variables in the WNV occurrence in Spain.

5. Conclusions

The results obtained in this study contribute to a better understanding of WNV transmission in Spain. The outbreaks confirmed consecutively during the last seven years, as well as the detection of WNV RNA lineage 1 in five different periods, suggest an endemic circulation of the virus in the south part of the country. The spatio-temporal distribution of

WNV outbreaks in Spain was not homogeneous, as significant clusters were detected in western Andalusia in two non-consecutive years. Additionally, the highest number of outbreaks reported in 2016 compared to the previous years and the expansion to northern areas outside Andalusia province, suggests an active circulation and expansive trend. Therefore, there is a potential risk of WNV spread to previously unaffected areas within the Iberian Peninsula in the following years. The identification of WNV risk areas and spatio-temporal clusters in this study can serve to inform risk-based, more cost-effective strategies towards better prevention and control of WND in Spain. In order to better prevent future WNV cases in the horses and humans, specific measures, including vaccination programs, risk-based surveillance and outreach and communication of horse owners and the general public should be implemented in the identified areas.

Conflict of interest statement

None of the authors of this study has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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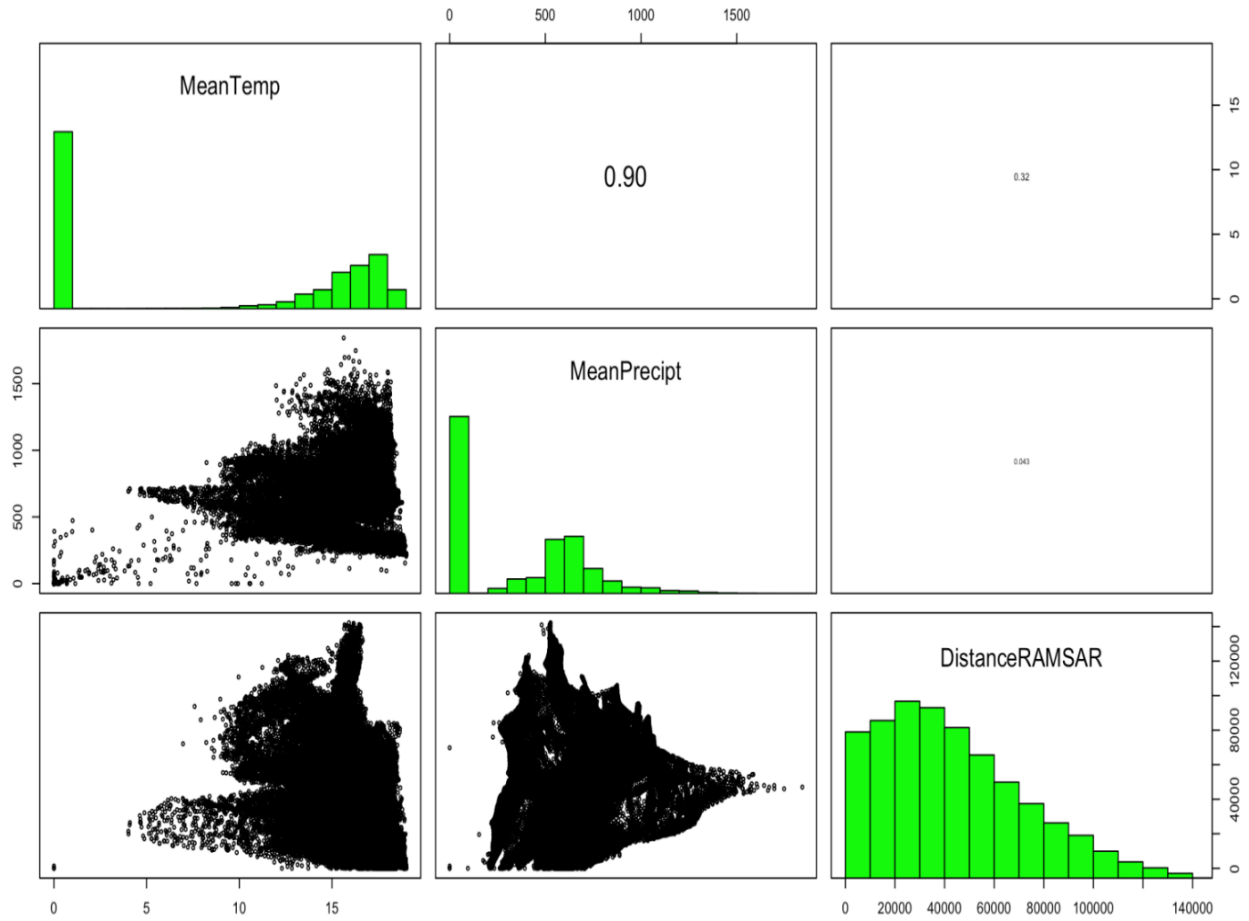
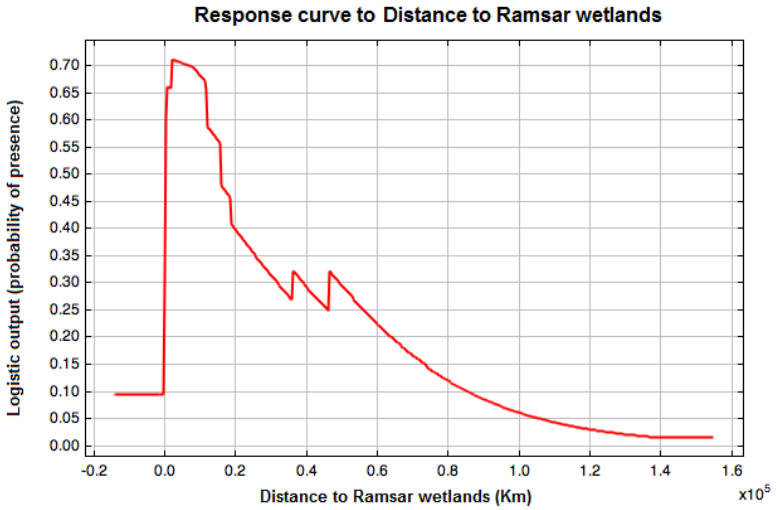
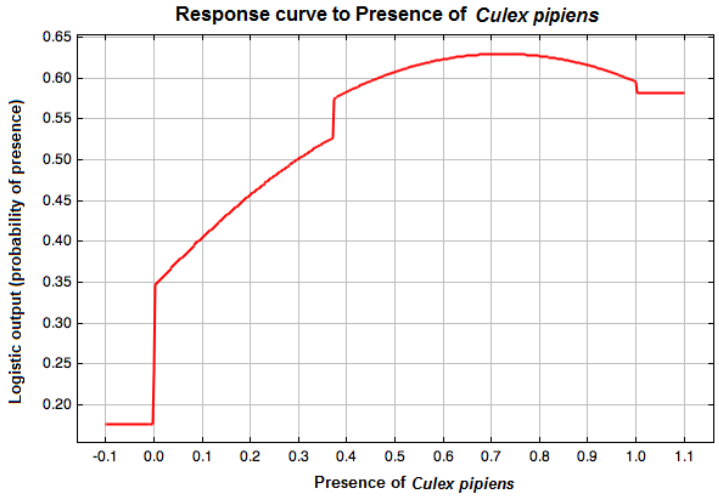
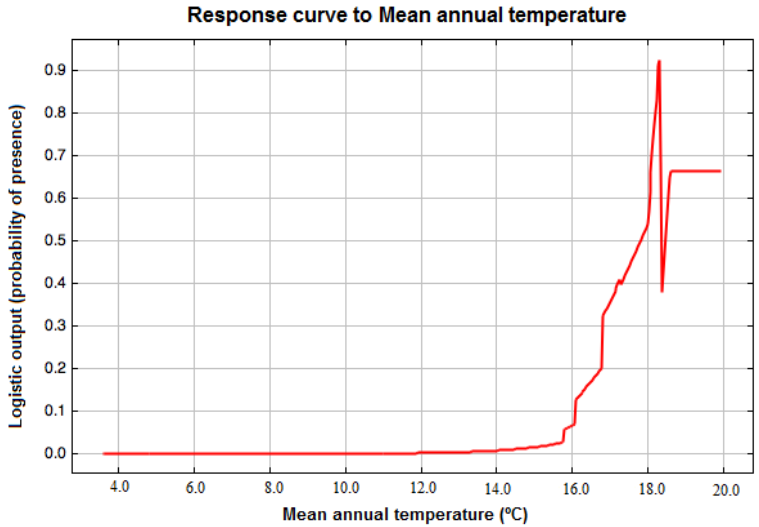


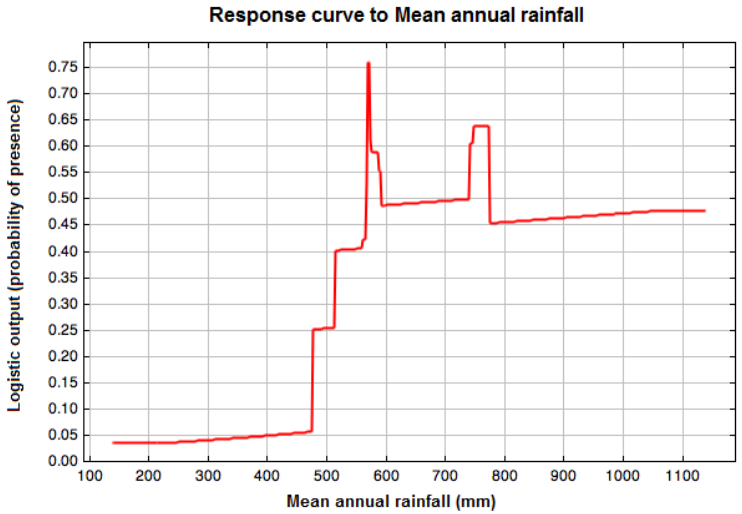
Supplementary Figure 1. Spearman correlation plots for the predictors variables included in the final MaxEnt model.

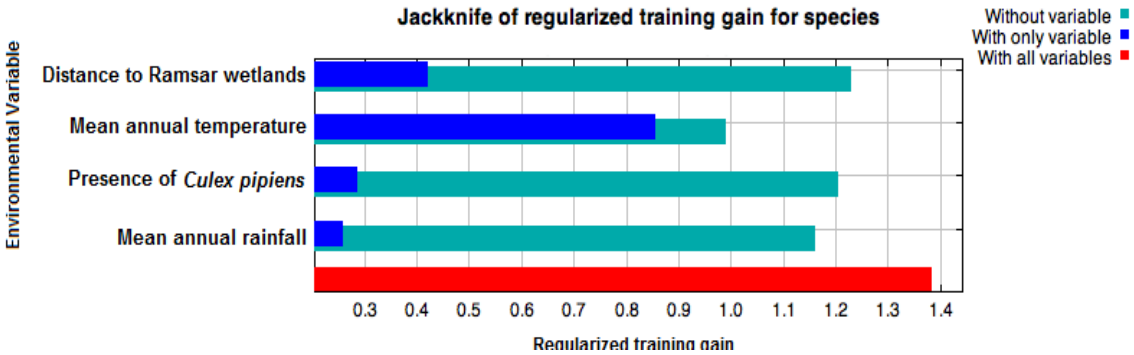
Supplementary Figure 2. Response curve for the predictors included in the final MaxEnt model.











Supplementary Figure 3. Jackknife of Regularized Training Gain of the predictors variables included in the final MaxEnt model. The red bar shows the overall training gain with all the included predictors. The blue bar represents the training gain when using each predictor in isolation. The clear blue bar represents the training gain when the predictor is excluded from the model.

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CAPÍTULO 4

Prevalencia y factores de riesgo asociados a la infección por patógenos zoonósicos en perro

CAPÍTULO 4.1

Exposición al virus de West Nile y al virus de la encefalitis transmitida por garrapatas en perros en España

García-Bocanegra I, Jurado-Tarifa E, **Cano-Terriza D**, Martínez R, Pérez-Marín JE, Lecollinet S (2018). **Exposure to West Nile virus and tick-borne encephalitis virus in dogs in Spain.** Transboundary and Emerging Diseases, 65:765-772.

Resumen

En la última década se ha confirmado la circulación de diversos flavivirus zoonósicos emergentes (género *Flavivirus*, familia *Flaviviridae*) en diversas regiones del mundo, lo que representa un reto para la salud humana y animal. En el presente trabajo, se llevó a cabo un estudio seroepidemiológico en perros en España para evaluar la exposición y los factores riesgo asociados a flavivirus antigénicamente relacionados, en particular, virus de West Nile (VWN), virus Usutu (VU) y virus de la encefalitis transmitida por garrapatas (VETG). Se detectaron anticuerpos frente a flavivirus en 39 de los 815 (4,8%; IC95%: 3,3-6,3) perros analizados mediante ELISA. Se observó una seropositividad significativamente mayor en los perros de caza en comparación con los perros de compañía. Además, los análisis mediante test de seroneutralización vírica confirmaron la presencia de anticuerpos específicos frente a VWN y VETG en 11 y 14 perros seropositivos a ELISA, respectivamente. Este es el primer estudio seroepidemiológico de VWN y VETG en perros en España y la primera descripción de circulación del VETG en este país. La seropositividad obtenida indica una amplia, aunque no homogénea, distribución del VWN y el VETG en perros en España. Los perros seropositivos a VWN detectados en 2013 y 2015 coincidieron con las mismas áreas de Andalucía en las que se notificó el mayor número de brotes de caballos y humanos. Además, se han encontrado anticuerpos frente a VETG en perros procedentes de dos grupos y regiones diferentes de España. La serovigilancia en perros podría ser una herramienta complementaria para monitorizar la actividad de flavivirus emergentes en España.

Abstract

In the past decade, the spread of emerging zoonotic flaviviruses (genus Flavivirus, family Flaviviridae) has been reported in many regions worldwide, representing a threat to both human and animal health. A serosurvey was carried out to assess exposure and risk factors associated with antigenically related flaviviruses, particularly West Nile virus (WNV), Usutu virus (USUV) and tick-borne encephalitis virus (TBEV), in dogs in Spain. Flavivirus antibodies were detected in 39 of 815 dogs (4.8%; 95% CI: 3.3–6.3) by bELISA. Significantly higher seropositivity was observed in hunting dogs compared to pet dogs. Virus neutralization tests confirmed WNV-specific and TBEV-specific antibodies in 11 and 14 bELISA-positive dogs, respectively. This is the first serosurvey of WNV and TBEV in dogs in Spain and the first report of TBEV circulation in this country. The seropositivity obtained indicates widespread, but not homogeneous, distribution of WNV and TBEV in dogs in Spain. In 2013 and 2015, WNV-seropositive dogs were detected in those areas of Andalusia where the highest number of WNV outbreaks were reported in both horses and humans. Antibodies against TBEV have been found in dogs sampled in two different periods and regions in Spain. Serosurveillance in dogs could be a complementary way of monitoring the activity of emerging flaviviruses in Spain.

Keywords: Dogs, emerging disease, flavivirus, risk factors, tick-borne encephalitis virus, Usutu virus, West Nile virus

1. Introduction

During the past decade, the number of outbreaks of emerging flaviviruses (genus *Flavivirus*, family *Flaviviridae*) has increased sharply in Europe, representing a threat to both human and animal health. In nature, these viruses are maintained in an enzootic life cycle involving ornithophilic mosquitoes or ticks as competent vectors, and birds and small rodents as main reservoir hosts. Although mammals are susceptible to flavivirus infections, most are considered dead-end or incidental hosts, as they produce viremia levels of insufficient magnitude and duration to infect competent vectors.

Dogs do not appear to play a major role in the epidemiology of flaviviruses. In experimental studies of dogs infected with West Nile virus (WNV), viremia of low magnitude and short duration was detected, but the animals failed to develop clinical signs of the disease (Austgen et al., 2004). Nonetheless, clinical signs of disease, including encephalitis, polyarthritis and myocarditis, have been reported in naturally occurring WNV infection in dogs (Lichtensteiger et al., 2003; Cannon et al., 2006). Serosurveys carried out in this species have detected WNV, Usutu virus (USUV) and tick-borne encephalitis virus (TBEV) exposure in many regions worldwide (Table 1). WNV seroconversion was detected in juvenile dogs in Houston (USA) six weeks before the first case was reported in humans (Resnick et al., 2008). Previous studies have also shown that dogs are common feeding hosts of *Culex* mosquitoes and *Ixodes* ticks (Molaei et al., 2007; Muñoz et al., 2011, Estrada-Peña et al., 2017). Because of the close association between dogs and humans, as well as their convenience for sampling purposes, this species has been proposed as a suitable sentinel for monitoring WNV (Resnick et al., 2008; Durand et al., 2016). However, information pertaining to dogs as amplifying hosts for flaviviruses other than WNV is still very limited.

Spain is considered to be a country at risk for the introduction of flaviviruses. Indeed, five of the six main *Culex*-borne and tick-borne flaviviruses found in Europe, including WNV, USUV, Meaban virus (MBV), Louping ill virus (LIV) and Bagaza virus (BGV) have been detected in this country in the last few years (Busquets et al., 2008; Jiménez-Clavero et al.,

2008; Agüero et al., 2011; Balseiro et al., 2012; García-Bocanegra et al., 2016). Between 2010 and 2016, a total of 177 WNV outbreaks in horses and three clinical cases in humans have been reported in Spain (García-Bocanegra et al., 2017). Antibodies against WNV have also been frequently detected in both bird and mammal species (Boadella et al., 2012; García-Bocanegra et al., 2012a; Gutiérrez-Guzmán et al., 2012; Jurado-Tarifa et al., 2016), and clinical disease and mortality associated to WNV infection was evidenced in wild birds in this country (Jiménez-Clavero et al., 2008). Although TBEV infection has not yet been confirmed on the Iberian Peninsula, the risk of its introduction into mainland Spain should be considered high as a result of the emerging spread of TBEV virus to neighboring European countries, such as France and Italy (Herpe et al., 2007; Rezza et al., 2015). Despite an estimated census of 7.4 million domestic dogs (MAPAMA, 2015), survey studies to detect flavivirus circulation have not been carried out in the Spanish canine population. The objective of this study was to determine the prevalence of antibodies against antigenically-related zoonotic flaviviruses, particularly WNV, USUV and TBEV, in dogs in the southwestern regions of Spain. This area is considered to present the highest risk of flavivirus circulation due to the strategic location within important migratory bird flyways, the high number of wetlands, environmental conditions and the high density of competent vectors (García-Bocanegra et al., 2017).

2. Materials and methods

2.1 Study design and sampling

A cross-sectional study was designed to analyze the seroprevalence of antigenically-related flaviviruses among dogs in Andalusia (southern Spain) and Ceuta (North Africa) (Figure 1). In consideration of the large population of dogs in Andalusia (n> 10,000), an estimated prevalence of 50% (which provides the highest sample size in studies with unknown prevalence), the desired precision was set at ±5% and confidence level at 95% (95% CI), resulting in 385 animals to be sampled. A total of 419 dogs (409 from Andalusia,

10 from Ceuta) were finally selected and the sampling was stratified by provinces according to the proportion of dogs in each province. Blood samples were collected from dogs admitted into veterinary clinics located in 133 municipalities between May 2013 and June 2015. A retrospective analysis of sera collected from 396 hunting dogs in 32 municipalities in Extremadura (southwestern Spain) in 2003 was also performed. Dogs from Extremadura were selected using a convenience sampling by municipality. A total of 36 hunting dog group were sampled in this region. Within each hunting dog group, 11 animals were randomly selected. All samples were obtained by puncture of the cephalic or jugular veins using a sterile collection system. Serum was separated by centrifugation at 400 *g* for 15 minutes and stored at -20ºC until analysis. Data on location, year, gender, age (<12, 12-24, 25-36 and >36 months), breed (pure or crossbred), activity (pet or hunting) and size (height at the withers: small (<40 cm), medium (41-60 cm) and large (>60 cm)) were recorded for each animal (Table 2). The geographical distribution of the sampled municipalities is represented in Figure 1.

2.2 Serological assay

Serum samples were screened for flavivirus antibodies using a commercial blocking enzyme-linked immunosorbent assay (bELISA 10.WNV.K3 INGEZIM West Nile COMPAC®, Ingenasa, Madrid, Spain), carried out in accordance with the manufacturer's instructions. bELISA detects antibodies targeting epitopes on domain III of the envelope protein common to antigenically-related flaviviruses. bELISA-positive results were indicative of the presence of immunoglobulins against flaviviruses. Seropositive sera were then confirmed by virus neutralization test (VNT) for the detection of specific neutralizing antibodies against WNV (lineage 1, Is98 strain, Genbank accession number AF481864.1), USUV (It12 strain, Genbank accession number KF055446.1) and TBEV (Hypr strain, Genbank accession number U39292). VNT analyses were performed at the European Union Reference Laboratory for equine diseases (ANSES, Maisons-Alfort), as previously described (OIE, 2013). Titers were expressed as the reciprocal of the highest serum dilution most protective of the Vero cells. Samples

showing neutralization titers (absence of cytopathic effects) at dilutions ≥ 10 (WNV, USUV) and ≥ 20 (TBEV) were considered positive. Interpretation of results was based on comparison of VNT titers obtained in parallel against all three flaviviruses. The neutralizing immune response observed was considered specific when VNT titers against one virus increased ≥4-fold compared with the titers against the other two. Samples showing ≤ 2-fold differences between VNT titers for the viruses examined were considered positive for flavivirus but not conclusive for a specific virus. Positive and negative control sera were included for both bELISA and VNT analyses.

2.3 Statistical analysis

Possible associations between seropositivity and explanatory variables (region, gender, age, activity, size and breed) were assessed using multivariate analysis. Due to low seropositivity against WNV, USUV and TBEV, statistical analysis of overall flavivirus seroprevalence was performed. Pearson's chi-square test, or Fisher's exact test when there were fewer than six observations per category, was applied to test the independence of explanatory variables according to outcome. Factors showing a *P*-value of <0.20 were further scrutinized for associations, using Cramer's V coefficient between pairs of variables to prevent collinearity. Variables with coefficients greater than 0.60 were considered to be correlated and were not included together in the same model. When collinear variables were detected, only the variable with the a priori stronger biological association with flavivirus seropositivity was retained. The third step involved a generalized estimating equation (GEE) model (Hanley et al., 2003). The number of seropositive animals was assumed to follow a binomial distribution and "municipality" was included as a random effect. A Poisson distribution and logit link function were used. Biologically-plausible confounding factors were assessed using the Mantel-Haenszel method and confounding was considered to be potentially significant if the odds ratios (ORs) shifted appreciably. Variables that altered coefficients of independent variables of interest by 30% or more when removed from the model were classified as confounding factors. The model was re-

run until all remaining variables presented statistically significant values (likelihood ratio and Wald tests, $P < 0.05$). Values with $P < 0.05$ were considered statistically significant. Statistical analyses were performed using SPSS 22.0 (Statistical Package for Social Sciences (SPSS), IBM Corp., Armonk, NY, USA).

3. Results

Antibodies to antigenically-related flaviviruses were detected in 39 of 815 dogs (4.8%; 95% CI: 3.3-6.3) using bELISA. The distribution of seroprevalence according to the various explanatory variables is shown in Table 2. Twenty six of the 165 (15.8%) sampling sites analysed presented at least one seropositive dog. Flavivirus seropositivity was found in Andalusia (3.9%; 16/409) and Extremadura (5.8%; 23/396) but not in Ceuta (0/10) (Figure 1). Statistically significant differences in seropositivity between Andalusia and Extremadura were not found ($P = 0.112$). Since most of the samples from hunting dogs (90%; 396/440) were collected in Extremadura, the explanatory variable "region" was excluded from multivariate analysis due to collinearity with "hunter activity". The GEE model showed that "hunter activity" was a potential risk factor associated with flavivirus exposure. Significantly higher seroprevalence was found in hunting dogs (6.6%; Odds ratio (OR)= 5.1, $P = 0.004$, 95%CI= 1.67–15.03) compared to pet dogs (2.7%). Similar results were found in the GEE model when only dogs from Andalusia were selected (hunting dogs = 13.6%, 6/44; OR= 6.0, $P = 0.006$, 95%CI= 1.67 – 21.59 *vs* pet dogs = 2.7, 10/375).

A total of 12 sera positive by bELISA showed negative results against the three flaviviruses tested by VNT. Specific neutralizing antibodies against WNV and TBEV were confirmed in 11 (28.2%) and 14 (35.7%), respectively, of the 39 bELISA-positive dogs (Table 1). In addition, the simultaneous occurrence of antibodies against WNV+USUV and WNV+USUV+TBEV was detected in two animals. With respect to possible co-occurrences, the frequency of seropositive dogs ranged between 1.3 and 1.6% for WNV, 0.0 and 0.3% for USUV, and 1.7 and 1.8% for TBEV. Specific diagnosis of WNV and TBEV infections was

confirmed in 10 (6.1%) and 12 (7.3%) of the 165 municipalities sampled, respectively. WNV seropositive dogs were detected in Extremadura in 2003, and in dogs sampled in western Andalusia in 2013 and 2015. TBEV circulation was found in Extremadura in 2003 and in Andalusia in 2013 and 2014.

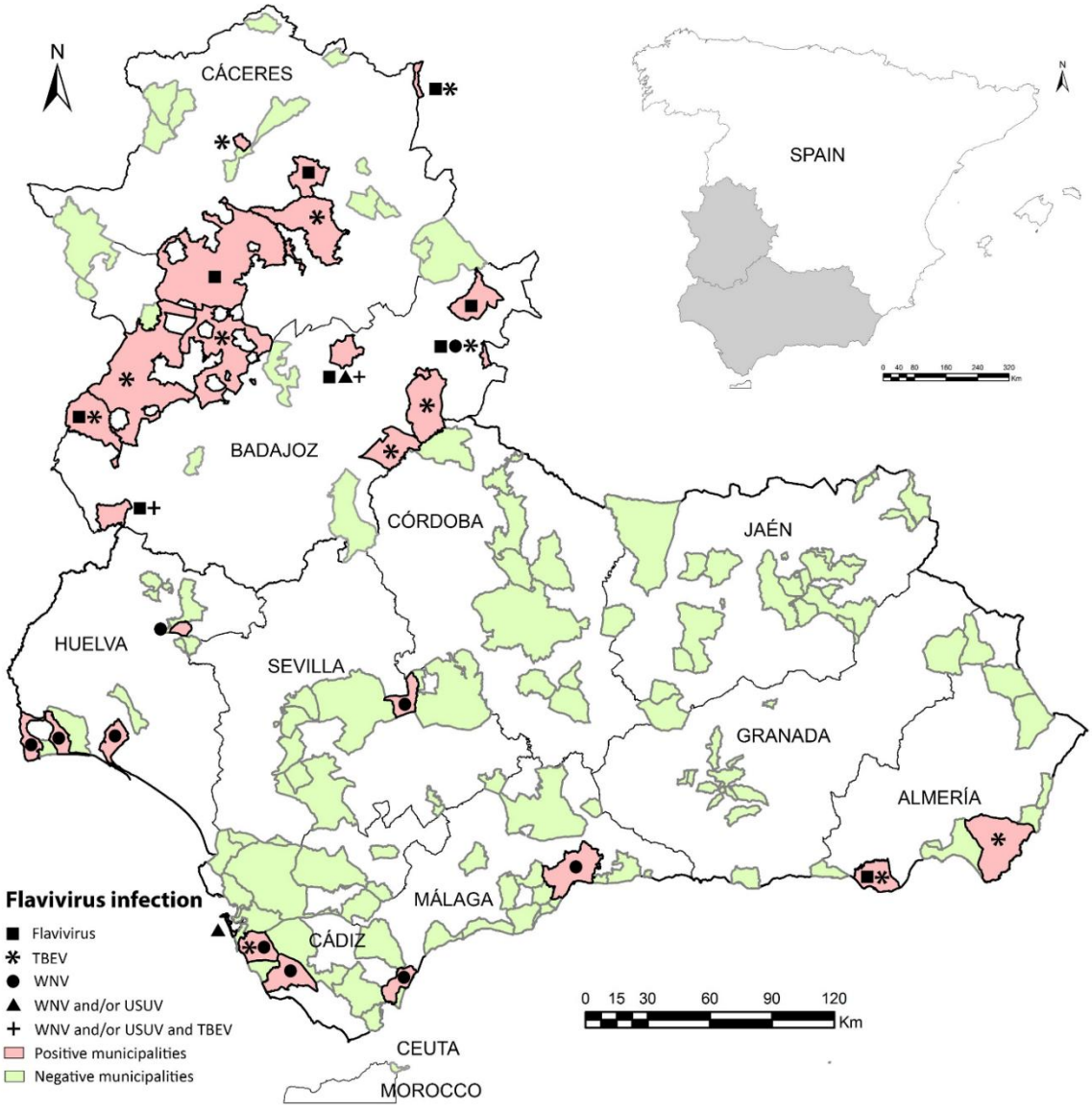

Figure 1. Map of Andalusia (southern Spain), Extremadura (southwestern Spain) and Ceuta (North Africa), showing the locations of dogs sampled. Black and white dots indicate seropositive and seronegative animals, respectively.

Table 1. Prevalence of antigenically-related flavivirus (WNV, USUV and TBEV) antibodies in dogs. *ELISA: Enzyme-linked immunosorbent assay, RFFIT: Rapid fluorescent focus inhibition confirmation test, WBT: Western-blot test, PRNT: Plaque reduction neutralization test, HI: Hemagglutination inhibition test, VNT: virus neutralization test.

Country	Test*	N. Tested	%	% WNV	% USUV	% TBEV	Reference
Belgium	ELISA/RFFIT	880		0.0		0.1	Roelandt et al., 2011
Canada	ELISA	143		28.0			Gaunt et al., 2015
Chad	ELISA/WBT	55	32.7	30.9			Davoust et al., 2014
China	ELISA/PRNT	367	5.7	4.6			Lan et al., 2011
Czech Republic	HI	151				3.3	Klimes et al., 2001
Democratic Republic of the Congo	ELISA/WBT	24	16.7	12.5			Davoust et al., 2014
Denmark	ELISA/VNT	125	30.0			4.8	Lindhe et al., 2009
Djibouti	ELISA/WBT	47	17.0	12.8			Davoust et al., 2014
Finland	ELISA	148				6.7	Levanov et al., 2016
France	ELISA	71		8.4	0.00		Marquart et al., 2017
France	ELISA/WBT	104	12.5	6.7			Davoust et al., 2014
Gabon	ELISA/WBT	250	0.0	0.0			Davoust et al., 2014
Germany	ELISA/VNT	331				2.1	Balling et al., 2015
Italy	ELISA	36		55.5			Busani et al., 2011
Ivory coast	ELISA/WBT	137	5.1	2.2			Davoust et al., 2014
Morocco	ELISA/PRNT	231	62.0	80.0	12.00		Durand et al., 2016
Norway	ELISA	317				16.4	Cçangó 2004
Puerto Rico	ELISA/PRNT	269		8.5			Phoutrides et al., 2011
Senegal	ELISA/WBT	11	27.3				Davoust et al., 2016
Senegal	ELISA/WBT	141	8.5	4.9			Davoust et al., 2014
Spain	ELISA/VNT	810	4.8	1.3-1.6	0.0-0.3	1.7-1.8	Present study
The United States	PRNT	442		26.0			Kile et al., 2005
The United States		189		5.3			Komar et al., 2001
The United States	PRNT	414		55.9			Levy 2011
The United States	HI	154		56.5			Lillibridge et al., 2004
The United States	ELISA	246	18.0				Resnick et al., 2008
Turkey	ELISA/VNT	114		37.7			Okzul et al., 2006

Table 2. Seroprevalence of antigenically-related flaviviruses in dogs from southwestern Spain and North Africa. [a]Missing values omitted.

Variable	Exposure	% flaviviruses	Number/overall [a]	*P*-value
Region	Andalusia	3.9	16/409	0.122
	Extremadura	5.8	23/396	
	Ceuta	0.0	0/10	
Sex	Male	5.7	28/492	0.090
	Female	3.4	11/323	
Age	<12	4.9	4/81	0.363
	12-24	2.2	3/134	
	25-36	6.9	9/131	
	>36	5.3	22/417	
Size	Small	3.9	3/76	0.873
	Medium	5.3	14/263	
	Large	5.4	17/316	
Activity	Hunting dog	6.6	29/440	**0.009**
	Pet dog	2.7	10/375	
Breed	Pure-breed	4.0	22/545	0.451
	Mixed-breed	5.3	17/321	
Year	2003	5.8	23/396	0.087
	2013	3.0	11/367	
	2014	11.5	3/26	
	2015	7.7	2/26	

4. Discussion

In order to identify the possible establishment of an urban cycle for flaviviruses, several serosurvey studies have been conducted in dogs worldwide (Table 1). To the best of author's knowledge, this is the first report on exposure to flaviviruses in dogs in Spain. The overall flavivirus seroprevalence detected in this species (4.8%) is consistent with that observed in clinically healthy horses (7.1%) and wild ruminants (ranging between 1.4% and 4%) (Boadella et al., 2012; García-Bocanegra et al., 2012; García-Bocanegra et al., 2016), but slightly lower than in domestic birds (13.0%), wild boar (12.6%) and red foxes (20.4%) in this country (Gutiérrez-Guzmán et al., 2012; Jurado-Tarifa et al., 2016). Our results were also

within the values previously found in both resident and migratory wild birds in Spain (García-Bocanegra et al., 2011; López et al., 2011; Cano-Terriza et al., 2015; Alba et al., 2014; Ferraguti et al., 2016). Although differences in seroprevalence were observed among regions, they were probably related to certain bias in the sampling, since most of the hunting dogs were collected in Extremadura in 2003. The absence of seropositivity in the province of Ceuta, in North Africa, may also be due to sampling bias, as only ten animals were tested in this region. However, since WNV and USUV infections have been reported in neighboring Morocco in horses, birds, dogs and humans during the last decade (El Rhaffouli et al., 2012; Benjelloun et al., 2016; Durand et al., 2016), the risk of flavivirus circulation in this region should be considered high.

Significantly higher seropositivity was found in hunting dogs compared to pet dogs. In Spain, there are around 3,000 hunting dog groups for large game purposes, with an estimated census population of 45,000 hunting dogs (FEDENCA, 2012). Our results indicate that this group of canids could be used as a suitable sentinel species for monitoring flavivirus activity. Big game hunting is an activity that is carried out in different environments, such as wetlands, forests and agricultural fields, that support the development of competent vectors for flaviviruses and can therefore increase the risk of canine vector-borne disease transmission. Additionally, seroconversion by ingestion of infected prey, which may be more frequent in hunting dogs, has also been suggested as a source of WNV infection in carnivore species (Austgen et al., 2004). Previous studies have shown that dogs that spend longer periods of time outdoors are at significantly higher risk for exposure to WNV (Kile et al., 2005; Gaunt et al., 2015).

WNV seropositivity was detected only in the western part of Andalusia and the southern part of Extremadura, which is consistent with the greatest number of outbreaks reported in horses and human in Spain. Since the first clinical case was detected in summer 2010, a total of 177 WNV outbreaks in horses and three cases in humans have been reported in southwestern regions (RASVE, 2017). The strategic location in major migratory

bird flyways, the many wetland areas, favorable environmental conditions and a higher density of competent vectors are possible factors implicated in the greater spread of WNV in this area. In this respect, it has been shown that *C. pipiens, C. perexiguus, C. theileri* and *C. modestus* are the main *Culex* species detected in the regions studied here (Aranda et al., 2009, Vázquez et al., 2011, García-Bocanegra et al., 2012b; Bravo-Barriga et al., 2017). In other European countries, such as France, seropositivity for WNV in dogs ranged between 6.7% and 8.4% (Davoust et al., 2014; Marquart et al., 2017). Seroprevalence in this species was higher in Turkey (37.7%) (Okzul et al., 2006) and Italy (55.5%) (Busani et al., 2011). Conversely, antibodies to WNV were not detected in canines in Belgium (Roelandt et al., 2011).

In Spain, USUV infections have been detected in resident and migratory birds, wild ruminants and mosquitoes in south-central Spain (Busquets et al., 2008; Höfle et al., 2013; Bakonyi et al., 2014; García-Bocanegra et al., 2016). USUV-specific neutralizing antibodies were not confirmed in dogs in the present study. The results are consistent with those previously reported in France (Marquart et al., 2017). Two animals were found to be seropositive for both WNV and USUV by VNT assay, and one of them was also positive for TBEV, with a ≤ 2-fold difference in titers. Although co-infections cannot be ruled out, because the co-circulation of flaviviruses was not confirmed in the dog populations analysed, the results are more likely to be related to cross-reactions among flaviviruses in VNT.

This is the first report of TBEV circulation in mainland Spain. Interestingly, seropositivity for TBEV was found in animals less than 12 months of age in Extremadura in 2003 and in Andalusia in 2014, which suggests that the virus was circulating in these regions during those periods. Neutralizing antibodies against TBEV were recently detected in a horse on the island of Mallorca (off the eastern coast of Spain) (Vanhomwegem et al., 2017). The seroprevalence detected in Spanish canines lies between the rates reported in other European countries, with levels ranging between 0.1% and 16.4% (Table 1).

Twelve serum samples were positive by bELISA but negative for WNV, USUV and TBEV by VNT. The results could be due to cross-reactivity with other antigenically-related flaviviruses. In this respect, MBV seropositivity in wild birds and ruminants, as well as mortality in wild game birds and ruminant species due to BGV and LIV infections, respectively, has been detected in south-central Spain in the last few years (Agüero et al., 2011; Balseiro et al., 2012; García-Bocanegra et al., 2016). Unfortunately, low volumes precluded analysis of every flavivirus evidenced in Spain. Because of cross-reactions between TBEV and LIV using rapid diagnostic tests and possibly VNT, a further entomological survey of *Ixodes* ticks is required to confirm TBEV circulation in Spain.

5. Conclusions

We confirm for the first time exposure to flaviviruses, particularly WNV and TBEV, in dogs in Spain. The presence of neutralizing antibodies against TBEV in different regions and at different times suggests that this virus may have circulated silently on the Iberian Peninsula in the past two decades. Our results indicate that dogs could be used as a suitable sentinel species for monitoring flavivirus activity.

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Conflict of interest statement

None of the authors has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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CAPÍTULO 4.2

Factores de riesgo asociados a la infección por *Toxoplasma gondii* en perros de caza, guarda y compañía en el sur de España y el norte de África.

Cano-Terriza D, Puig-Ribas M, Jiménez-Ruiz S, Cabezón O, S Almería, Galán-Relaño A, Dubey JP, García-Bocanegra I (2016). **Risk factors of *Toxoplasma gondii* infection in hunting, pet and watchdogs from Southern Spain and Northern Africa.** Parasitology International, 65:363-366.

Resumen

Los perros pueden actuar como reservorios de *Toxoplasma gondii* (*T. gondii)* para humanos y otros hospedadores. En este estudio se determinó la seroprevalencia y factores de riesgo asociados a la infección por *T. gondii* en perros de Andalucía (sur de España) y Ceuta (norte de África). Se detectaron anticuerpos frente a *T. gondii* en 235 de 769 perros (30,6%; IC95%: 27,3-33,8) mediante la prueba de aglutinación modificada (MAT, punto de corte 1:25), con títulos de 1:25 en 91, 1:50 en 43, 1:100 en 84 y ≥ 1:500 en 17 perros. Los principales factores de riesgo asociados con la infección por *T. gondii* fueron la edad (perros de mayor edad), la actividad (perros de caza) y el tamaño (perros grandes y medianos). Este es el primer estudio sobre infección por *T. gondii* en perros domésticos en España. Los resultados obtenidos indican que *T. gondii* está ampliamanete extendido en esta especie en la España peninsular y de Ceuta, lo que podría ser de relevancia para la Salud Pública.

Abstract

Dogs can act as reservoirs of *Toxoplasma gondii* infections for humans and other hosts. Here we determined seroprevalence and risk factors of *T. gondii* infection in dogs from Andalusia (Southern Spain) and Ceuta (Northern Africa). Antibodies to *T. gondii* were detected in 235 out of 769 dogs (30.6%; $CI_{95\%}$: 27.3-33.8) by the modified agglutination test (MAT, cut-off of 1:25) with titers of 1:25 in 91, 1:50 in 43, 1:100 in 84, and ≥ 1:500 in 17 dogs. The main risk factors associated with *T. gondii* infection in dogs were age (higher seroprevalence in older dogs), hunter activity and size (higher seroprevalence in larger and medium dogs). This is the first study on *T. gondii* infection in pet dogs from Spain and Ceuta. The results indicate that *T. gondii* is widespread in dogs in mainland Spain and Ceuta, which might have important implications for public health.

Keywords: *Toxoplasma gondii*, Dogs, Seroprevalence, Risk factors.

1. Introduction

Toxoplasma gondii is a zoonotic protozoan which can potentially infect all warm-blooded animals, including humans [1]. Felids are the only definitive hosts able to excrete oocysts in their feces. *T. gondii* infection in hosts occurs by ingestion of tissue cysts from undercooked or raw meat, consumption of contaminated food or drinks with sporulated oocysts, or congenitally (Dubey, 2010; Pereira et al., 2010).

Although only Felidae family are known to produce *T. gondii* oocysts, dogs can act as mechanical vectors in the transmission of *T. gondii* to humans. Rolling over cat feces can be a normal behavior in dogs increasing the risk of *T. gondii* transmission by direct contact (Frenkel et al., 1995). In addition, dogs can eat cat feces infected with *T. gondii* oocysts, and oocysts can be passed in dog feces unchanged and remain viable (Lindsay et al., 1997; Schares et al., 2005). Viable *T. gondii* has been isolated from tissues of dogs in many countries (Dubey, 2010; Dubey et al., 2016). Studies have shown that pet dog owners had significantly higher seroprevalence against *T. gondii* compared to people without pet dogs (reviewed in [Duan et al., 2012]). High risk of *T. gondii* infection has also been reported in children in contact with dogs (Frenkel et al., 1995; Etheredge et al., 2004). Additionally, consumption of undercooked or raw meat from dog as part of the diet in some countries may also be implicated in the direct transmission of *T. gondii* to humans (Yang et al., 2014; Zhang et al., 2015).

In Spain, antibodies in dogs have been previously reported but in small population studies (Millán et al., 2009; Cabezón et al., 2010). The aim of the present study was to determine the risk factors affecting the prevalence of antibodies against *T. gondii* in a high number of dogs from Andalusia (Southern Spain) and Ceuta (Northern Africa), including hunting, pet and watchdogs. To our knowledge, this is the first report of *T. gondii* seroprevalence in pet dogs from Spain.

2. Materials and Methods

2.1. Study design and sampling

A survey study was designed to analyse seroprevalence of *T. gondii* in dogs from Andalusia (Southern Spain; 36º N-38º 60´ N, 1º 75´ W-7º 25´ W) and Ceuta (Northern Africa; 35º 53´ 18´´ N, 5º 18´ 56´´ W) (Figure 1). A total of 769 blood samples were collected from dogs admitted into veterinary clinics located in 155 cities between May 2013 and June 2015. Blood samples were obtained by puncture from cephalic or jugular veins. Serum was separated by centrifugation at 400 *g* for 15 minutes, and was stored at -20ºC until analysis. Epidemiological information was provided by the veterinary clinics. Data on sex, age (<12, 12-24, 25-36 and >36 months), location, breed (pure or crossbred), activity (pet, hunting or watchdog) and size (height at the withers: small (<40 cm), medium (41-60 cm) and large (>60 cm)) were recorded for each animal.

2.2. Serological assay

Sera were tested to detect antibodies against *T. gondii* using the modified agglutination test (MAT) as previously described (Dubey and Desmonts, 1987). Serum was tested at 1:25, 1:50, 1:100 and 1:500 dilutions and both positive and negative controls were included in all tests. Samples with doubtful results were re-examined, and animals with titers ≥ 1:25 were considered positive. MAT has been used in many studies in dogs (de Souza et al., 2003; Dubey et al., 2007; Dubey et al., 2009; Millán et al., 2009; Cabezón et al., 2010; Wu et al., 2011).

2.3. Statistical analysis

The prevalence of antibodies against *T. gondii* was estimated from the ratio of positive to the total number of samples tested, with the exact binomial confidence intervals of 95% ($CI_{95\%}$) (Martin et al., 1987). Correlation between estimated prevalences and independent variables (region, gender, age, activity, size and bred) were analyzed by means

of a Pearson´s chi-square test and by Fisher´s exact test when there were less than six observations per category. The differences between categories were analyzed by Tukey´s test. Factors showing a *P-value* <0.15 were further scrutinized for associations using Spearman's rank correlation coefficient (*r*) to avoid colinearity problems. When colinearity ($P < 0.05$ and $r > 0.4$) occurred, only the variable more clearly linked to *T. gondii* seropositivity was retained. The third step involved a multiple logistic regression analysis (Hosmer and Lemeshow, 2000). The goodness of fit was assessed through a multivariate logistic regression using the Hosmer–Lemeshow goodness-of-fit test (Petrie and Watson, 2006). Biologically plausible confounding factors were assessed using Mantel-Haenszel analysis and confounding was considered to be potentially significant if odd ratios (ORs) shifted appreciably. Variables that altered the coefficients for the independent variables of interest by 30% or more when removed from the model were classified as confounding factors. The model was re-run until all remaining variables presented statistically significant values (likelihood-ratio Wald's test, $P < 0.05$), and a potential causal relationship with the response variable existed. Values with $P < 0.05$ were considered as statistically significant. Statistical analyses were performed using SPSS 20.0 (Statistical Package for Social Sciences (SPSS), IBM Corp., Armonk, NY, USA).

3. Results

Antibodies to *T. gondii* were detected in 235 out of 769 dogs (30.6%; $CI_{95\%}$: 27.3-33.8). Titers of 1:25, 1:50, 1:100 and ≥ 1:500 were found in 91 (38.7%), 43 (18.3%), 84 (35.7%) and 17 (7.2%) animals, respectively. Distribution of *T. gondii* seropositivity by sex, age, region, size, activity and breed is shown in Table 1. Significant differences among provinces were observed, with prevalences ranging from 24.6% in Cádiz (32/130) to 48.2% in Huelva (41/85) (Table 1). Significantly higher seroprevalence was found in the province of Huelva compared to Jaén (P= 0.028), Córdoba (P= 0.019) and Cádiz (P= 0.008). Since most of the samples from hunting dogs (39.81%; 43/108) were collected in the province of Huelva,

this region was excluded from the multivariate analysis due to collinearity with hunter activity. At least one seropositive animal was detected in 87 out of the 155 (56.13%) cities sampled (Data not shown). Statistically significant differences of seroprevalence among provinces were not detected when only pet dogs were selected. Statistically significant differences in seroprevalence to *T. gondii* were not observed between sexes and breeds (Table 1).

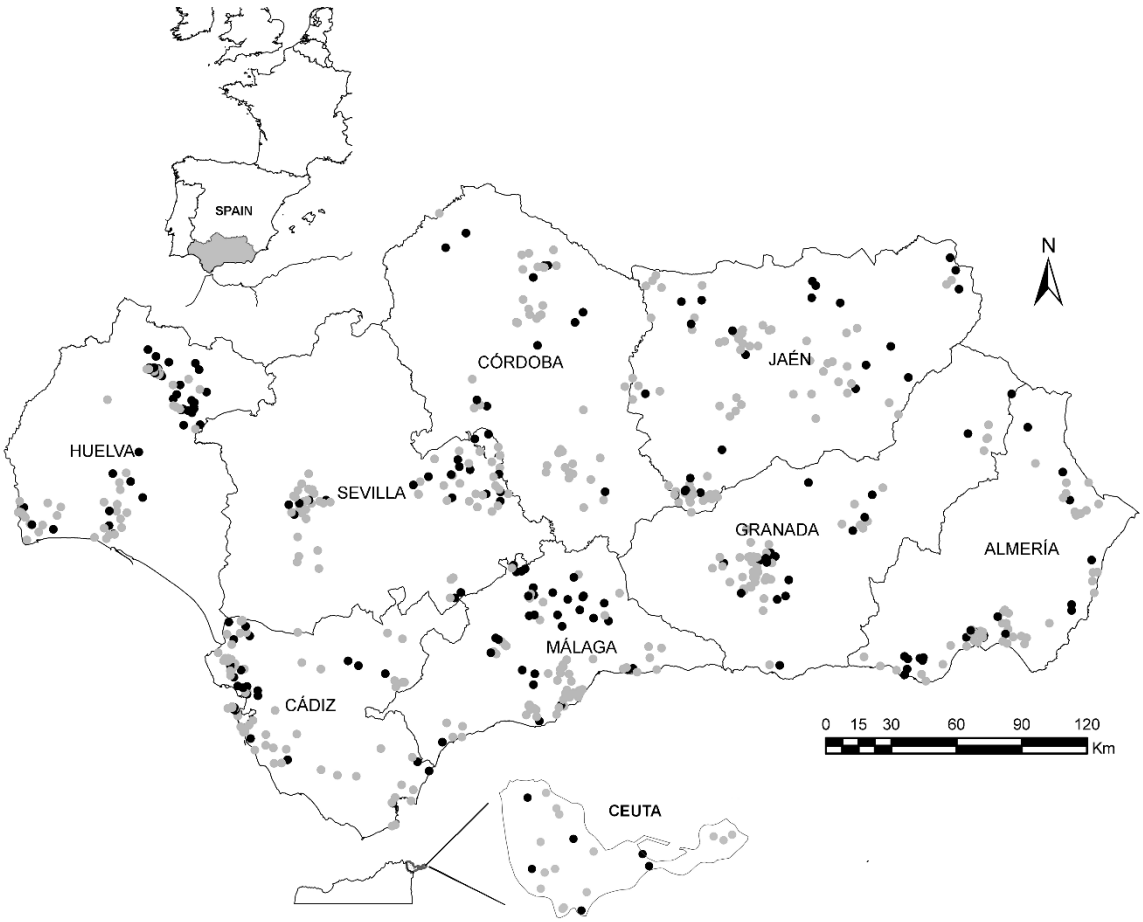

Figure 1. Map of Andalusia (Southern Spain) and Ceuta (Northern Africa), showing the location of dogs sampled. Black and grey dots indicate seropositive and seronegative animals, respectively.

The final multivariate analysis showed that the risk factors potentially associated with *T. gondii* infection were age, hunter activity and size of the dogs (Table 2). Seroprevalence was significantly higher in dogs older than 25 months when compared to younger animals (Table 1). Significantly higher seroprevalence was observed in hunting dogs (52.8%) compared to pet dogs (26.3%), and significantly higher seroprevalence was observed in large (35.2%) and medium (33.3%) dogs compared to small dogs (14.0%) (Table 2).

Table 1. Seroprevalence of *T. gondii* in dogs from Andalusia (Southern Spain) and Ceuta (Northern Africa).

Variable	Exposure levels	% MAT positive	Number/overall [a]	*P*-value
Region	Almería	34.1	28/82	0.012
	Cádiz	24.6	32/130	
	Córdoba	24.8	25/101	
	Granada	26.2	17/65	
	Huelva	48.2	41/85	
	Jaén	25.0	23/92	
	Málaga	33.9	37/109	
	Sevilla	31.4	27/86	
	Ceuta	26.3	5/19	
Sex	Male	32.9	94/286	0.059
	Female	26.7	83/311	
Age (months)	<12	12.5	4/32	0.005
	12-24	20.8	15/72	
	25-36	33.8	27/80	
	>36	33.4	138/413	
Size	Small	14.0	15/107	< 0.001
	Medium	33.3	71/213	
	Large	35.2	37/105	
Activity	Hunting dog	52.8	57/108	< 0.001
	Pet dog	26.3	160/609	
	Watchdog	44.4	8/18	
Breed	Pure-breed	29.0	114/393	0.364
	Mixed-breed	30.7	63/205	

[a] Missing values omitted.

Table 2. Logistic regression analysis of potential risk factors associated with *T. gondii* seropositivity in dogs from Andalusia (Southern Spain) and Ceuta (Northern Africa).

Variable	Categories	*β (S.E.)*	*P*-value	df	OR 95%CI
Age (months)	≤12	[a]	[a]		[a]
	13-24	0.01 (0.31)	0.979	1	0.99 (0.54-1.82)
	25-36	0.76 (0.36)	0.036	1	2.14 (1.05-4.36)
	>36	2.21 (0.98)	0.025	1	9.11 (1.32-62.65)
Size	Small	[a]	[a]		[a]
	Medium	1.07 (0.31)	0.001	1	2.91 (1.57-5.39)
	Large	1.06 (0.30)	<0.001	1	2.90 (1.61-5.23)
Activity	Pet	[a]	[a]		[a]
	Watchdog	1.63 (1.48)	0.271	1	5.09 (0.28-92.62)
	Hunting dog	0.73 (0.31)	0.019	1	2.08 (1.13-3.84)

[a] Reference category

4. Discussion

The results obtained in the present study indicate that *T. gondii* infection is widespread in dogs in Southern Spain and Ceuta (Northern Africa). Although some differences were observed among regions, they were probably related to certain bias in the sampling since most of the hunting dogs were collected in the same province (Huelva). In fact, statistically significant differences in seroprevalence among provinces were not detected in pet dogs. The mean seroprevalence of *T. gondii* detected in our study is similar to those reported in dogs in other European countries including Portugal (38.0%; 256/673) (Lopes et al., 2011), Greece (ranging between 21% and 34%) (reviewed in [Hamel et al., 2016]) and Austria (26%; 63/242) (Wanha et al., 2005). Previous studies in small number of dogs in Spain using the same technique showed higher seroprevalence levels in shepherding and watchdogs from Southern Spain (54.2%; 13/24) (Millán et al., 2009) and from kennel dogs in Majorca Island (Spain) (58.7%; 27/46) (Cabezón et al., 2010). However, differences

in seroprevalence among studies have to be compared carefully since type of dogs sampled, diagnosis methods and cut-off values used varied among studies.

Gender and breed were not risk factors associated with *T. gondii* infection, which is in agreement with previous reports (Lopes et al., 2011; Yoshikawa et al., 2015). In contrast, Raimundo et al. (2015) observed significantly higher seropositivity in mixed-breed than in pure-breed dogs. In our study, the prevalence of *T. gondii* antibodies was age-related, in accordance with several preceding studies [Cabezón et al., 2010; Dubey et al., 2009; Wu et al., 2011). Recently, Lopes et al. (2014) observed that for each year increase in age, the risk of a dog being found seropositive significantly increased by an OR of 1-18 ($CI_{95\%}$: 1.02-1.36). The higher seroprevalence in older animals reflects a cumulative likelihood for exposure to *T. gondii* and lifelong persistence of antibodies. The results suggest that horizontal transmission is the main route of infection in dogs.

To the best of our knowledge, this is the first study on *T. gondii* seroprevalence in pet dogs from Spain and Ceuta (Northern Africa). Significantly higher seropositivity was found in hunting dogs compared to pet dogs. Statistically significant differences between watchdogs (44.4%) and pet dogs were not observed, probably due to the low number of watchdogs sampled (n=18). In agreement with our study, several studies have also observed lower *T. gondii* seroprevalence in pet dogs (e.g. 15.5% of 103 dogs in Luanda, Angola (Lopes t al., 2014) and 21.6% in pet dogs in China (Duan et al., (2012)). De Souza et al. (2003) observed *T. gondii* seroprevalences of 31.6% in 610 urban stray dogs, 34.3% of 134 rural dogs, but only 5.2% of 500 urban owned dogs in Brazil. The lower occurrence of *T. gondii* in pet owned dogs is of interest indicating that only a small proportion of dogs in homes had been exposed to either oocysts or infected meat (de Souza et al., 2003), while the higher occurrence of *T. gondii* in hunting and watchdogs in the present study reflects more opportunities to ingest tissues of animals or oocysts from the environment. Similar seroprevalences to those observed in hunting dogs and watchdogs in the present study were also found in kennel, shepherding and watchdogs in previous studies in Spain (Millán

et al., 2009; Cabezón et al., 2010). These types of dogs are usually kept outdoors in rural areas, which often roam more freely with greater access to parasites than dogs kept indoors (Ahmad et al., 2014). Additionally, hunting dogs usually eat preys or parts of the preys during the hunting activity, which may also be a cause of infection by *T. gondii* as suggested for other related protozoa parasites (Godim et al., 2004). Survey studies carried out in wild rabbits (*Oryctolagus cuniculus*) and wild ungulates have shown *T. gondii* infections in these species commonly hunted by dogs in Spain (Almería et al., 2004; Gauss et al., 2005; Calero-Bernal et al., 2015).

The risk for being seropositive for *T. gondii* was 2.91 and 2.9 times higher in medium and large dogs than in small dogs, respectively. The volume of food ingested is directly related with the size of the animal, so the probability of *T. gondii* infection could be also proportionally increased. Further studies are required to assess the relationship between size and the risk of *T. gondii* infection.

The high seroprevalence found in the present study indicates that *T. gondii* is widespread in dogs in Southern Spain and Ceuta (Northern Africa), which might have important implications for public health. Samples from dogs, which are easily accessible, may be a useful tool to monitor the presence of *T. gondii* in the environment. Management measures such as feeding dogs with dry and processed food or cooked meat as well as avoiding contact with definitive or intermediate hosts may limit the infection by *T. gondii* in dogs (Dubey et al., 2009). In the same way, hygienic measures including hand washing after contact with dogs or their feces are recommended to reduce the transmission of *T. gondii* from dogs to humans (Lindsay et al., 1997).

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SÍNTESIS

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La presente Tesis Doctoral tiene como objetivo general evaluar el papel de especies animales domésticas, peridomésticas y silvestres en la epidemiología de diferentes enfermedades zoonósicas bacterianas, víricas y parasitarias en ecosistemas mediterráneos de España. Esta Tesis Doctoral se encuadra dentro del concepto *One Health,* incorporando nuevos conocimientos relacionados con los agentes zoonósicos, los factores de riesgo potencialmente asociados a su transmisión y la evaluación de las medidas de lucha frente a ellos. En esta síntesis se integran los resultados más destacables, para proporcionar una visión general de los hallazgos más relevantes.

El **Capítulo 1** se centra en el estudio de la tuberculosis (TB). Por un lado, se evaluó el potencial papel como reservorio del cerdo doméstico criado en extensivo (**Capítulo 1.1**) y por otro, la eficacia de una de las posibles medidas de control de la enfermedad en ungulados silvestres considerados reservorios de micobacterias del Complejo Mycobacterium tuberculosis (CMT) (**Capítulo 1.2**). En el **Capítulo 2** se tomó como base de estudio a diversas especies peridomésticas (paloma y especies simpátricas del zoológico de Córdoba) como potenciales reservorios de patógenos zoonósicos en zonas urbanas. Así, en el **Capítulo 2.1** se estableció la prevalencia de flavivirus zoonósicos, virus de la influenza aviar, *Salmonella* spp. y *Toxoplasma gondii (T. gondii*) en estas especies en el parque zoológico de Córdoba. Además, se incluyó el estudio de un caso de mortalidad de visón europeo (*Mustela lutreola*) mantenido en cautividad en este parque, debido a una infección por *Acinetobacter baumannii* (**Capítulo 2.2**). Por su parte, el **Capítulo 3** se centró en el análisis de la distribución espacio-temporal y los factores de riesgo asociados a los brotes de virus de West Nile (VWN) en caballos de España; mientras que en el **Capítulo 4** se evaluó la utilidad del perro como especie centinela frente a diferentes flavivirus zoonósicos (**Capítulo 4.1**) y frente a *T. gondii* (**Capítulo 4.2**), así como los factores de riesgo implicados en la transmisión de estos patógenos en esta especie animal.

En el **Capítulo 1.1** se observó una baja seroprevalencia individual (2,3%) frente a micobacterias del CMT en porcino criado en extensivo, lo que sugiere que los cerdos criados en estos sistemas productivos podrían actuar como hospedadores *spillover* más que como verdaderos reservorios del CMT. Por otro lado, la alta prevalencia de rebaño encontrada (24,8%), la detección de clústeres significativos, así como el aislamiento de 25 espoligotipos diferentes de CMT todos ellos compartidos con otras especies domésticas y silvestres, evidenciaron la necesidad de establecer programas de control en el cerdo cuando se maneja en sistemas extensivos. Por su parte, en el **Capítulo 1.2** se comprobó como la correcta eliminación de los residuos de caza mayor redujo la seroprevalencia de CMT en jabalí hasta en un 25%. Así, confirmándose la eficacia de la implementación de esta medida para el control de la TB en el principal reservorio silvestres en los ecosistemas del centro y sur de España.

En el **Capítulo 2.1** se confirmó la circulación local de flavivirus (VWN y virus Usutu) y *T. gondii* en palomas no migratorias y animales de zoológico en la ciudad de Córdoba. Estos resultados han puesto de manifiesto que tanto las palomas como los animales de zoológico podrían ser utilizados como especies centinela en la vigilancia de estos patógenos zoonósicos en zonas urbanas. Adicionalmente, en el **Capítulo 2.2** se evidenció que *A. baumannii* no sólo es un patógeno de importancia en Salud Pública, al ser considerado uno de los patógenos más frecuentes en las infecciones nosocomiales en humanos, sino que también puede tener importantes repercusiones en Sanidad Animal y para la conservación de especies en peligro crítico de extinción, categoría en la que se encuadra el visón europeo.

En el **Capítulo 3** se confirmó que el VWN ha circulado en caballos de forma endémica en desde la aparición del primer brote en 2010. También, se mostró como la mayoría de los brotes declarados en esta especie en España (92,7%) se concentran en la parte occidental de Andalucía, donde además, se detectaron clústeres significativos en diferentes periodos. No obstante, la expansión a otras regiones fuera de Andalucía en los últimos años, sugiere

una tendencia expansiva del virus. Por lo tanto, existe un potencial riesgo de propagación del virus a zonas previamente no afectadas dentro de la Península Ibérica. La temperatura media anual, la presencia de *Culex pipiens*, la precipitación media anual y la distancia a los humedales Ramsar, fueron las principales variables ambientales asociadas con la presencia del VWN. Este estudio ha permitido desarrollar mapas de riesgo de presencia del virus, proporcionando una valiosa información que puede ser aplicada a los programas de vigilancia vigentes, permitiendo así una mejor protección de las poblaciones de caballos y humanos en España.

En el **Capítulo 4.1** se evaluó la seroprevalencia y los factores riesgo asociados a flavivirus antigénicamente relacionados (VWN, virus de Usutu y virus de la encefalitis transmitida por garrapatas (ETG)) en perros de España. Se encontró una seroprevalencia global de anticuerpos frente a flavivirus del 4,8% y se observó una seropositividad significativamente mayor en los perros de caza en comparación con los perros de compañía. En este estudio se describe por primera vez la circulación de ETG en España y se demuestra que la vigilancia serológica en perros podría ser una herramienta complementaria para monitorizar la actividad de los flavivirus emergentes en el país.

En el **Capítulo 4.2** se determinó la seroprevalencia y los factores de riesgo asociados a la infección por *T. gondii* en perros de Andalucía y Ceuta. Se detectaron anticuerpos frente a *T. gondii* en el 30,6% de los perros analizados. La edad (perros adultos), la actividad (perros de caza) y el tamaño (perros grandes y medianos) fueron los principales factores de riesgo asociados a la infección por *T. gondii*. Los resultados indicaron que *T. gondii* está ampliamente diseminado en perros de la España peninsular y Ceuta, lo que podría tener importantes implicaciones para la Salud Pública.

En esta Tesis Doctoral se pone de manifiesto que el establecimiento de un adecuado programa de vigilancia, tanto en especies domésticas como silvestres, debe ser un paso previo a la decisión de intervenir o no y al establecimiento de estrategias capaces de reducir la transmisión de patógenos. La definición clara de los reservorios de las enfermedades en

cada contexto epidemiológico, los factores de riesgo asociados a su presentación y las potenciales especies que podrían ser usadas como centinelas, permiten el diseño de programas de vigilancia epidemiológica más eficientes y optimizados basados en el riesgo.

Los resultados obtenidos en la presente tesis aportan valiosos conocimientos que permiten mejorar la vigilancia epidemiológica de las especies animales que habitan en el entorno urbano, periurbano y en el medio natural, y que comparten enfermedades entre ellas y con el ser humano.

CONCLUSIONES

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PRIMERA: La seroprevalencia individual frente a micobacterias del complejo *Mycobacterium tuberculosis* (CMT) en cerdo doméstico (2,3%), sugiere que esta especie podría actuar como un hospedador accidental más que como un verdadero reservorio del CMT. Sin embargo, la alta prevalencia de rebaño encontrada (24,8%), la detección de clústeres espaciales significativos, así como el aislamiento de 25 espoligotipos diferentes de CMT previamente detectados en otras especies domésticas y silvestres, evidencian la necesidad de establecer programas de control en esta especie cuando se maneja en sistemas de producción extensivos. **(Capítulo 1.1. *Cano-Terriza D, Risalde MA, Rodríguez-Hernández P, Napp S, Fernández-Morente M, Moreno I, Bezos J, Fernández-Molera V, Sáez JL, García-Bocanegra I. Epidemiological surveillance of Mycobacterium tuberculosis complex in extensively raised pigs in the south of Spain. En revisión*).**

SEGUNDA: La correcta eliminación de los residuos de la caza mayor ha logrado reducir la seroprevalencia de tuberculosis en jabalí (*Sus scrofa*) hasta en un 25%, siendo por tanto una medida eficaz para control de tuberculosis en esta especie. **(Capítulo 1.2. *Cano-Terriza D, Risalde MA, Jiménez-Ruiz S, Vicente J, Isla J, Paniagua J, Moreno I, Gortázar C, Infantes-Lorenzo JA, I García-Bocanegra (2018). Management of hunting waste as control measure for tuberculosis in wild ungulates in south-central Spain. Transbound Emerg Dis. Doi: 10.1111/tbed.12857*).**

TERCERA: La prevalencia de anticuerpos frente a flavivirus (virus West Nile (VWN) y virus Usutu) y *Toxoplasma gondii (T. gondii)* detectada en palomas y animales de zoológico indican su utilidad como especies centinelas en la vigilancia de estos patógenos zoonósicos en zonas urbanas. **(Capítulo 2.1. *Cano-Terriza D, Guerra R, Lecollinet S, Cerdà-Cuéllar M, Cabezón O, Almería S, García-Bocanegra I. (2015). Epidemiological survey of zoonotic pathogens in feral pigeons (Columba livia var. domestica) and sympatric zoo species in Southern Spain. Comp Immunol Microbiol Infect Dis., 43:22-7.*)**

CUARTA: La mortalidad detectada en visón europeo (*Mustela lutreola*) confirma la susceptibilidad de esta especie en peligro crítico de extinción a la infección por la bacteria zoonósica *Acinetobacter Baumannii*. *(***Capítulo 2.2.** ***Cano-Terriza D, Guerra R, Mozos E, Rodríguez-Sánchez B, Borge C, García-Bocanegra I, Fatal Acinetobacter baumannii infection in the critically endangered European mink (Mustela lutreola) (2017). J Zoo Wildl Med., 48:220–223).***

QUINTA: El VWN ha circulado de forma endémica en España desde el año 2010. Sin embargo, su distribución no es homogénea, estando la mayoría de los brotes (92,7%) concentrados en el suroeste del país. La temperatura media anual (49,5%), la presencia de *Culex pipiens* (19,5%), la precipitación media anual (16,1%) y la distancia a los humedales Ramsar (14,9%), fueron las variables más asociadas con la presencia de brotes de VWN en España. **(Capítulo 3.** ***García-Bocanegra I, Belkhiria J, Napp S, Cano-Terriza D, Jiménez-Ruiz S, Martínez-López B (2018). Epidemiology and spatio-temporal analysis of West Nile virus in horses in Spain between 2010 and 2016. Transbound Emerg Dis., 65:567-577*****).**

SEXTA: Este es el primer estudio sobre VWN y virus de la encefalitis transmitida por garrapatas (VETG) en perros en España y la primera descripción de circulación de VETG en este país. La presencia de anticuerpos neutralizantes frente a VETG en diferentes regiones y periodos sugiere que este virus puede haber circulado de forma silenciosa durante las últimas dos décadas. Los resultados ponen de manifiesto la utilidad de los perros como especie centinela para monitorizar la actividad de flavivirus emergentes. **(Capítulo 4.1.** ***García-Bocanegra I, Jurado-Tarifa E, Cano-Terriza D, Martínez R, Pérez-Marín JE, Lecollinet S (2018). Exposure to West Nile virus and tick-borne encephalitis virus in dogs in Spain. Transbound Emerg Dis., 65:765-772*****).**

SÉPTIMA: *T. gondii* está ampliamente diseminado en perros de Andalucía y Ceuta, detectándose anticuerpos en el 30,6% de los animales analizados, lo que podría tener importantes implicaciones en Salud Pública. La edad (perros adultos), la actividad (perros de caza) y el tamaño (perros grandes y medianos) fueron los principales factores de riesgo

asociados a la infección por *T. gondii* en esta especie. **(Capítulo 4.2. *Cano-Terriza D, Puig-Ribas M, Jiménez-Ruiz S, Cabezón O, Almería S, Galán-Relaño A, Dubey JP, García-Bocanegra I (2016). Risk factors of Toxoplasma gondii infection in hunting, pet and watchdogs from Southern Spain and Northern Africa. Parasitol Int., 65:363-66*).**