Instituto de Investigación en Recursos Cinegéticos
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Paratuberculosis en España:

Importancia de los reservorios silvestres

Tania Carta

Tesis Doctorales

Los abajos firmantes, como directores de esta tesis, hacemos constar que la tesis titulada “Paratuberculosis en España: Importancia de los reservorios silvestres”, y realizada por **Tania Carta**, licenciada en veterinaria, reùne los requisitos necesarios para su defensa y aprobaciòn, y por tanto, para optar al grado de Doctor.

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A mia madre

Se dovessi scegliere tra il tuo amore e la mia vita, sceglierei il tuo amore, perché è la mia vita (Jim Morrison)

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Os quiero!!!!

LISTA DE ABREVIATURAS

AFB: Acid fast bacilli

b-PPD: Bovine purified protein derivative

DNA: Deoxyribonucleic acid

CFU: Colony forming unit

DMC: Digital Microfluidic Chip

DPP: Dual path platform

ELISA: Enzyme Linked Immunosorbent Assay

HE: Hematoxylin eosin

IS: Insertion sequence

LFA: Leukocyte function antigen

LN: Lymph node

LT: Linfocito T

LTh: LT helper

MAC: *Mycobacterium avium* complex

MAP: *Mycobacterium avium* subsp. *paratuberculosis*

NK: Natural killer

OD: Optical density

PCR: Polimerase Chain Reaction

PCR RT: Real time-PCR

PFGE: Pulsed Field Gel Electrophoresis

PPA: Protoplasmatic paratuberculosis antigen

RDA: Representational Difference Analysis

REA: Restriction Endonuclease Analysis

RFLP: Restriction Fragment Length Polymorphism

RT: Room temperature

RT-PCR: Reverse transcription polymerase chain reaction

TB: Tuberculosis

TNF: Tumor necrosis factor

ZN: Ziehl Neelsen

Ìndice general

ESTRUCTURA DE LA TESIS

Tras la introducción general y los objetivos, esta tesis se estructura en cinco capítulos. El primero de ellos es una revisión bibliográfica sobre paratuberculosis en fauna silvestre, que aborda el estado actual de conocimientos sobre su posible implicación en el mantenimiento de MAP, el efecto de MAP sobre sus poblaciones y sus consecuencias sobre la vigilancia sanitaria de otras micobacteriosis, particularmente la tuberculosis. Los siguientes tres capítulos describen el papel del conejo (*Oryctolagus cuniculus*) como posible reservorio local de MAP en la Península Ibérica; la utilidad de los cánidos ibéricos (lobo, *Canis lupus*; y zorro, *Vulpes vulpes*) como posibles centinelas de la circulación de MAP; y el papel del ciervo (*Cervus elaphus*) como reservorio de MAP en la península ibérica. El quinto y último capítulo describe la expresión de genes relacionados con la inmunidad, en respuesta a la infección de rumiantes por patógenos intracelulares de los géneros *Anaplasma* y *Mycobacterium*. Finalmente, la tesis se completa con una breve síntesis y las conclusiones.

PLANTEAMIENTO DEL TRABAJO DE LA TESIS

Participación en proyectos

Participación en proyectos

Título del proyecto: Paratuberculosis en España: Importancia de los reservorios silvestres. AGL2008-03875

Investigador principal: Christian Gortázar

Entidad financiadora: Plan Nacional I+D, MCINN

Entidades participantes: Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCM)

Duración: desde 2009, hasta 2011

Título del proyecto: Epidemiología molecular, epidemiología de campo y factores de riesgo biogeográficos asociados a la interacción sanitaria entre ungulados domésticos y silvestres FAU2008-00004-C03-02

Entidad financiadora: INIA-Plan Nacional

Entidades participantes: IREC (CSIC-UCLM-JCCM), UCM

Duración: desde 2009, hasta 2011

Investigador responsable: Joaquín Vicente Baños (IP y coordinador del proyecto)

Número de Investigadores participantes: 6

Gracias a la participación en estos proyectos, hemos tenido la posibilidad de estudiar en profundidad la problemática de la paratuberculosis en la fauna silvestre en relación a las características del territorio, a la epidemiología y al diagnóstico.

En España la paratuberculosis es una enfermedad importante en el ganado ovino, caprino y bovino; algunos mamíferos silvestres, particularmente cérvidos y lagomorfos, podrían participar activamente en la epidemiología de esta micobacteriosis, complicando su control en las especies de producción.

Mediante la combinación de herramientas de ecología, patología y genética, esta tesis pretende aportar información básica y aplicada para contribuir al conocimiento de la paratuberculosis en España.

INTRODUCCIÓN

Antecedentes y estado actual de los conocimientos

La paratuberculosis (enfermedad de Johne) es una enfermedad infecciosa crónica de los rumiantes y de otros mamíferos, causada por *Mycobacterium avium paratuberculosis* (MAP). Se trata de una de las enfermedades más importantes de los rumiantes domésticos. Su distribución es mundial y causa pérdidas económicas severas en la producción bovina (Hasanova and Pavlik, 2006), ovina y caprina (Dhand et al., 2007), y en la producción de ciervo en granjas y explotaciones en semilibertad (de Lisle et al., 2003). La ruta de transmisión típica es fecal oral. Aunque esta enfermedad se conoce desde hace mas de cien años, aún no tiene tratamiento y su control es complejo (Baumgartner et al., 2005). La emergencia o re-emergencia de esta enfermedad ha sido reportada repetidamente tanto en bovino (p. ej. Baumgartner et al., 2005) como en granjas de ciervo (de Lisle et al., 2003). En consecuencia, varios países están empezando a tomar medidas para el control de la paratuberculosis en los rumiantes domésticos (Baumgartner et al., 2005, McKenna et al., 2006). Recientemente, MAP ha sido objeto de una atención aún mayor porque existe una base científica creciente que sugiere que la infección de personas con esta bacteria puede causar algunos, quizá todos, los casos de la enfermedad de Crohn (Naser et al., 2004, Uzoigwe et al., 2007).

En España, la paratuberculosis se considera ampliamente distribuida, y los estudios más recientes sitúan la prevalencia entre el 10 y el 86% de explotaciones, según la especie y la región. La distribución regional y de hospedadores de las diferentes cepas varía, según los autores, pero es evidente que las cepas de tipo II "bovino" están más extendidas y son capaces de infectar a una gran variedad de especies hospedadoras (de Juan et al., 2006, Sevilla, 2007). Además de las pérdidas económicas directas que esto conlleva para el sector ganadero, la existencia de paratuberculosis en explotaciones que son objeto de erradicación de tuberculosis supone un problema añadido a causa de las dificultades para el diagnóstico diferencial *in vivo* con la tuberculosis (Álvarez et al., 2008).

En España, se considera que la infección por MAP es endémica en una población local de gamos en la sierra del Sueve, en Asturias (Marco et al., 2002). MAP también ha sido ocasionalmente reportado en conejo, gamo (*Dama dama)*, y jabalí (*Sus scrofa)* en otras regiones (Álvarez et al., 2005; Fernández-de-Luco et al. 1995). Un estudio preliminar permitió detectar anticuerpos en los sueros del 30% de 852 ciervos Ibéricos (Reyes-García et al., 2008).

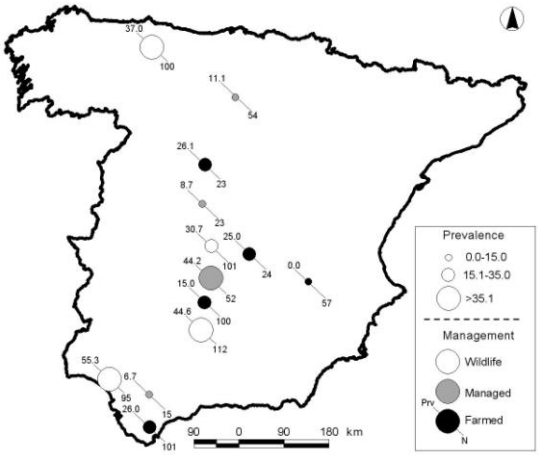

Figura 1.- Mapa de la prevalencia de paratuberculosis obtenido mediante ensayos serológicos (para detectar presencia de anticuerpos frente a MAP) en ciervos (*Cervus elaphus)* en España. Se representa la prevalencia (arriba izq.) y el tamaño de la muestra (abajo dcha.). El color indica el tipo de población (blanco silvestre, gris manejado/vallado, negro granja). Fuente: Reyes-García et al., 2008.

La existencia de hospedadores silvestres con elevadas prevalencias de contacto con MAP es indicativa del interés que tiene una prospección más amplia de la situación de la fauna silvestre española en relación con MAP.

Diagnóstico y tipado

El diagnóstico de las micobacteriosis es complejo. Clínicamente, la paratuberculosis es una enteritis crónica y se caracteriza, tanto en rumiantes domésticos como en el ciervo, por una pérdida de peso y condición general, frecuentemente acompañado de diarrea. En contraste con el ganado ovino y bovino, los brotes de paratuberculosis clínica ocurren con frecuencia en ciervos jóvenes, de entre 8 y 15 meses (Mackintosh et al, 2008). Histopatológicamente se trata de una enteritis granulomatosa y linfadenitis, particularmente a nivel de yeyuno, íleon y linfonodos mesentéricos asociados (Mackintosh et al., 2004; Balseiro et al., 2008). Además de la patología, en paratuberculosis existen pruebas serológicas (test ELISA, con sensibilidad limitada y especificidad variable, Gumber et al., 2006), cultivos (con limitaciones logísticas debidas al crecimiento muy lento de algunas cepas, Sevilla, 2007), y cada vez más técnicas basadas en PCR, ya que éstas están mostrando mayor sensibilidad que los cultivos (Kawaji et al., 2007).

Los ensayos immunoenzimáticos (ELISA) se encuentran entre las técnicas más comunemente empleadas, por ser flexibles, de bajo coste, y susceptibles de automatización. Aunque su sensibilidad sea limitada, su especificidad es considerada aceptable cuando la técnica se optimiza para situaciones concretas (Griffin et al., 2005). Existe, además, cierta asociación entre individuos ELISA positivos e individuos con mayor capacidad de excreción de micobacterias, que resulta de utilidad práctica en el control de los niveles de prevalencia a escala de explotación (Mackintosh et

al., 2007). El cultivo, considerado como prueba "gold standard", tiene dos inconvenientes logísticos importantes: primero, algunas cepas son de crecimiento muy lento, lo que dificulta y alarga los estudios; segundo, las cepas tienen requerimientos muy particulares, de forma que sólo deberían considerarse negativos aquellos intentos de cultivo en los que se hayan empleado cuatro tipos distintos de medio (p. ej. de Juan et al., 2006b). Las técnicas de PCR vendrían a solucionar en parte estos inconvenientes, pero la mera detección de DNA micobacteriano en una muestra no confirma la viabilidad de los microorganismos. Además, algunas de las PCR utilizadas pueden dar lugar a falsos positivos (Möbiusa et al., 2008). Los recientes desarrollos en PCR cuantitativa permiten estudios de intensidad de excreción o de contaminación (Kawaji et al., 2007).

El genotipado de cepas es esencial en epidemiología. En el caso de MAP la técnica más utilizada cuando se parte de DNA extraído de cultivos es la IS900-RFLP. La electroforésis de campo pulsado (PFGE) también da buenos resultados para llevar a cabo un tipado detallado. Gracias a la RFLP han sido diferenciados dos grupos de cepas, las "bovinas" o tipo II y las "ovinas", así denominados por la aparente mayor prevalencia de cada tipo en la especie hospedadora. Las cepas comprendidas en el Tipo II son de crecimiento más rápido, y son comúnemente aisladas de vacas, cabras, ciervos y otras especies, incluyendo la humana, mientras las cepas comprendidas en el Tipo I/III son de crecimiento más lento, con preferencia de hospedador por la especie ovina, y aparentemente más virulentas para este tipo de hospedador (Sevilla, 2007). Posteriormente se han desarrollado procedimientos más sencillos para diferenciar esos dos grandes grupos o incluso para distinguir entre los tipos I y III, como la IS1311 PCR-REA, DMC-PCR y RDA-PCR (de Juan et al., 2006a, Castellanos et al., 2007, Sevilla, 2007). Estos últimos son aplicables a DNA extraído de muestras, lo que supone una ventaja al no depender del cultivo (Nebbia et al., 2000).

Reservorios silvestres de paratuberculosis

La evidencia epidemiológica y de tipado molecular existente sugiere que algunas especies silvestres tengan un papel relevante como reservorios de paratuberculosis (Machackova et al., 2004, Florou et al., 2008). Los ungulados silvestres tales como ciervo, gamo corzo *Capreolus capreolus*, e incluso el jabalí (Machackova et al., 2004, Álvarez et al., 2005, Balseiro et al., 2008, Robino et al., 2008), lagomorfos como el conejo, o las liebres *Lepus* sp. (Daniels et al., 2003, Florou et al., 2008), roedores (Florou et al.,2008), e incluso carnívoros silvestres tales como el zorro (Anderson et al., 2007, Florou et al., 2008), albergan y excretan el agente causal de la paratuberculosis. Las similitudes de tipado entre cepas MAP de silvestres y de rumiantes domésticos apuntan hacia una

probable transmisión interespecífica (Florou et al., 2008 , Robino et al., 2008). Además, las pérdidas causadas por paratuberculosis suponen un problema importante para la producción de ciervo, una industria creciente a nivel mundial (de Lisle et al., 2003) y también en España (Fernández-de-Mera et al., 2007). El posible efecto de la paratuberculosis sobre la propia fauna silvestre es actualmente desconocido. En Europa, una de las situaciones mejor conocidas es la italiana, donde existen elevadas prevalencias (hasta >80%) en cérvidos (Nebbia et al., 2000, Robino et al., 2008). Otra situación interesante se encuentra en Escocia, donde existen prevalencias elevadas de MAP en conejos que comparten pastos con ganado ovino y bovino (Daniels et al., 2003).

Algunos rumiantes silvestres españoles, como el ciervo, son objeto de un manejo cinegético pseudoganadero que, unido a las particularidades de los sistemas mediterráneos (ciclos de sequía), causa sobreabundancia y agregación de animales en torno a puntos de agua y comederos, lo que a su vez se asocia con numerosos problemas sanitarios (Gortázar et al., 2006), y particularmente con micobacteriosis como la tuberculosis bovina (Vicente et al., 2007). Los resultados preliminares conocidos en España indican el interés de estudiar el papel del ciervo y otros rumiantes silvestres en la epidemiología de MAP en nuestro País (Balseiro et al., 2008; Reyes-García et al. 2008). Además, el conejo es localmente abundante en España, y la situación escocesa invita a comprobar si existe un paralelismo en España. El hecho de que muchas enfermedades de la fauna silvestre son compartidas con el hombre y con los animales domésticos, y el hecho de que los pastos y puntos de agua compartidos facilitan el contacto entre fauna silvestre y rumiantes domésticos, convierte el estudio de enfermedades como la paratuberculosis en una prioridad (Gortázar et al., 2007).

Patogenia de MAP

La patogenia de MAP se ha revisado en la tesis doctoral de Iker Sevilla (NEIKER/UPV, 2007). Su entrada en el organismo está mediada principalmente por las células M epiteliales que recubren las cúpulas de las placas de Peyer del íleon y yeyuno (García Marín et al., 1992). Algunos mecanismos conocidos de la interacción MAP–hospedador incluyen: la expresión de la proteína de membrana de 35 kDa de la bacteria (Bannantine et al., 2003); el puente de unión de fibronectina (Secott et al., 2004); la introducción de MAP en fagosomas de macrófagos adyacentes a la placa de Peyer (revisado en Sevilla, 2007); la apoptosis (Bannantine et al., 2002); la respuesta inmune celular mediada por LTh1 (Zhao et al., 1997); la acidificación del fagosoma (Weiss et al., 2004) y la liberación de súperóxido dismutasa (Liu et al., 2001), que a su vez suprime la presentación de antígenos a los LT (Weiss et al., 2001), y altera la expresión de moléculas del complejo mayor de

histocompatibilidad de clase II y de integrinas tipo LFA-1 (Alzuherri et al., 1997). La iniciación de la respuesta inmune adquirida frente a MAP podría verse obstaculizada por una producción defectuosa de citosinas proinflamatorias (como el factor de necrosis tumoral, TNF), y por un incremento en la expresión de interleuquina-10 (IL-10) (Coussens et al., 2004). Las células NK podrían jugar un papel en la transición de inmunidad innata a inmunidad adquirida, por medio de la producción de interferón en respuesta a los antígenos micobacterianos (Storset et al., 2003).

Genómica de la relación patógeno-hospedador en MAP

Existen dos campos de especial interés en la relación patógeno – hospedador, por un lado el conocimiento de los mecanismos que modulan la colonización y la multiplicación del patógeno (factores de virulencia), y por otro el componente genético del hospedador, que influye en la susceptibilidad y/o resistencia al patógeno. En la infección natural por *Mycobacterium bovis* en ungulados silvestres, los estudios realizados en el IREC han identificado, mediante técnicas genómicas, distintos genes que se encuentran inhibidos o sobre-expresados en respuesta a *M. bovis* en tejidos linfoides (Naranjo et al. 2006 y 2007 en jabalí, Fernández-de-Mera et al., 2008 en ciervo). A nivel de la población de hospedadores, se han encontrado efectos tanto genómicos (heterocigosis) como de algunos genes en particular, como MHC-II, sobre la mayor o menor susceptibilidad a la infección (Acevedo-Whitehouse et al., 2005 en jabalí, Fernández-de-Mera, 2007 en ciervo).

Hasta la actualidad no se han realizado suficientes estudios que contemplen un potencial componente genético del hospedador que pueda explicar la resistencia/susceptibilidad al desarrollo de la enfermedad (Sevilla, 2007). Mediante la combinación de herramientas de ecología, patología y genética, esta tesis pretende aportar información básica y aplicada para contribuir al conocimiento de la paratuberculosis en España. Particularmente, se pretende estudiar el papel del conejo como posible reservorio local de MAP en la península ibérica; la utilidad de los cánidos ibéricos (lobo y zorro) como posibles centinelas de la circulación de MAP, el papel del ciervo como reservorio de MAP en la península ibérica y la expresión de genes relacionados con la inmunidad, en respuesta a la infección de rumiantes por patógenos intracelulares de los géneros *Anaplasma* y *Mycobacterium.*

Hipótesis y objetivos

La existencia de al menos dos hospedadores potenciales: gamo (Balseiro et al., 2008) y ciervo (Reyes-García et al., 2008), con elevadas prevalencias de contacto aparente con MAP es indicativa del interés que tiene una prospección más amplia de la situación de la fauna silvestre española en relación con la paratuberculosis. Además, el contacto entre fauna silvestre y rumiantes domésticos es creciente como consecuencia de la expansión demográfica y geográfica de algunas especies y de los cambios en los sistemas de explotación. El hecho de que muchas enfermedades de la fauna silvestre sean compartidas con el hombre y con los animales domésticos, y de que los pastos y puntos de agua compartidos facilitan el contacto entre fauna silvestre y rumiantes domésticos, convierte el estudio de enfermedades como la paratuberculosis en una prioridad (Gortázar et al., 2007).

La paratuberculosis es una enfermedad importante en el ganado ovino, caprino y bovino en España. En los Alpes se han descrito prevalencias de infección de hasta el 33% (Robino et al., 2008), mientras que en otro lugares se señala una prevalencia muy baja (0-0.5%, Álvarez et al., 2005; Kopecna et al., 2008). Debido a la alta densidad poblacional del ciervo en algunas regiones de la península ibérica, que llega a alcanzar los 70 individuos por km^2 (Acevedo et al., 2008), en esta tesis hemos partido de la hipótesis de que la infección por MAP podría ser detectada en un elevado numero de ciervos ibéricos. Además, otras especies, como el jabalí o los carnívoros, también podrían constituir indicadores de paratuberculosis.

En consecuencia, la hipótesis de partida puede formularse de la siguiente forma:

Dado que muchas enfermedades de la fauna silvestre son compartidas con el hombre y con los animales domésticos, y de que los pastos y puntos de agua compartidos facilitan el contacto entre fauna silvestre y rumiantes domésticos, es posible que algunos mamíferos silvestres, particularmente cérvidos y lagomorfos, participen activamente en la epidemiología de MAP, complicando su control en las especies de producción. Esto estaría favorecido por la alta densidad poblacional del ciervo en algunas regiones de la península ibérica y considerando que en algunas regiones, como en los Alpes, se han descrito elevadas prevalencias de infección.

Por ello, es necesario realizar un estudio de la ecología, patología y genética de estos mamíferos silvestres y su relación con la posible diseminación de MAP de acuerdo con los siguientes objetivos.

Objetivos

1. Recopilar la información existente en la literatura científica sobre paratuberculosis en animales domésticos y en fauna silvestre.

Particularmente con objeto de poder abordar las siguientes cuestiones: (1) ¿Cuál es el papel que los hospedadores silvestres juegan como reservorios para el ganado domestico? (2) ¿Es MAP un factor significativo en la dinámica de la población de algunas especies de la fauna silvestre? (3) ¿Cuál es la relevancia de MAP en fauna silvestre, en relación con la especificidad del diagnostico de tuberculosis?

2. Optimizar los métodos de diagnóstico de paratuberculosis para su aplicación a fauna silvestre.

Estas técnicas están desarrolladas en el ciervo (principalmente por investigadores neozelandeses) y en el conejo (por investigadores escoceses), pero existen lagunas importantes en cuanto a la interferencia de otras micobacteriosis en el diagnóstico, así como en el diagnóstico de paratuberculosis en otros taxones. Por ello, también se pretende evaluar la eficacia de los ensayos serológicos en cuanto a la interferencia de otras micobacteriosis en el diagnóstico.

3. Estudiar el papel de la fauna silvestre española como reservorio de paratuberculosis.

Este estudio es de gran importancia, dado que si la paratuberculosis fuese mantenida principalmente por rumiantes domésticos, las prevalencias en fauna silvestre serían mayores en presencia de éstos, a igualdad de otros factores de riesgo. En cambio, si se detectasen prevalencias elevadas de contacto con MAP en fauna silvestre en ausencia de ganado doméstico, la fauna silvestre podría constituir un reservorio.

Situaciones complejas tales como las propias de los países mediterráneos, con distintos rumiantes domésticos afectados y más de una especie como hospedador silvestre potencial, resultan especialmente interesantes. Particularmente, se realizará un análisis de la prevalencia de paratuberculosis en conejos, ciervos y cánidos silvestres.

4. Identificar nuevos mecanismos moleculares de importancia en la interacción MAP – hospedador.

Para ello, se realizará un análisis de la relación patógeno–hospedador utilizando como modelo el ciervo ibérico y empleando las modernas técnicas de la genómica funcional y la transcriptómica.

El análisis genómico propuesto permitiría la eventual identificación de nuevos mecanismos moleculares de importancia en la interacción MAP–hospedador, que puedan contribuir en el futuro al control de esta enfermedad.

CAPITULO 1

Revisión: Paratuberculosis en fauna silvestre

Gamos y vacas en el Parque Nacional Doñana. Los rumiantes domésticos pastan en estrecho contacto con la fauna silvestre, facilitando el intercambio mutuo de patógenos. Foto: IREC.

En esta revisión tratamos de recopilar la información existente sobre paratuberculosis en animales silvestres y su diagnóstico. Además, intentamos responder a tres preguntas fundamentales sobre *Mycobacterium avium paratuberculosis* (MAP) en fauna silvestre: (1) ¿Cuál es el papel que los hospedadores silvestres juegan como reservorios para el ganado domestico? (2) ¿Es MAP un factor significativo en la dinámica de la población de algunas especies de la fauna silvestre? (3) ¿Cuán relevante es MAP en fauna silvestre, en relación con la especificidad del diagnostico de tuberculosis?

Para contestar a estas preguntas hemos analizado un total de 66 artículos, 14 sobre animales en cautividad y 52 de vida libre (uno con ambas situaciones de animales en cautividad y de vida libre, considerado por ese motivo como dos estudios), sobre un total de 178 especies. Para cada especie hemos reunido los datos cuantitativos sobre muestreo, prevalencia y diagnostico.

El cultivo es la prueba diagnóstica mas utilizada (58.5%), seguido por serología, histopatología y PCR. Además, se observa que el tamaño medio de muestra es mayor en el caso del cultivo, aumentando en el tiempo desde una media de 190 muestras por estudio antes del año 2000, hasta una media de 429 después del 2000. La prevalencia media de infección o de contacto con MAP, combinando los cuatro métodos, fue del 2,41%. Sin embargo, la prevalencia media estimada por PCR fue tres veces mayor respecto a cada una de las otras técnicas. Ello sugiere una mayor sensibilidad de la PCR o, alternativamente, la existencia de frecuentes falsos positivos.

Wildlife and paratuberculosis: a review

(Artículo en preparación)

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Abstract

Paratuberculosis, also known as Johne's disease, is an infectious granulomatous enteritis caused by *Mycobacterium avium paratuberculosis* (MAP). Paratuberculosis causes significant economic losses to the livestock industries due to productivity losses, infertility and direct costs of diagnosis and control. However, paratuberculosis in free-living wildlife and captive wild animals has not been as extensively studied as in traditional livestock. We here review the current literature i) to determine the potential impact of MAP infection in wildlife species; ii) to analyze whether wildlife reservoirs are relevant regarding MAP control in domestic ruminants; iii) and to assess the importance of MAP as the cause of potential interferences with tuberculosis diagnosis in wildlife. A total of 66 references on MAP in free-living or captive wildlife were reviewed for information on MAP pathology, epidemiology and distribution in wildlife hosts. The mean MAP prevalence reported in wildlife was 2.41% (1.76-3.06). We conclude that although MAP should be considered an important disease in farmed wild ruminant species (mainly cervids), its impact on free-ranging species could be considered questionable. In wildlife, MAP reservoirs do locally exist. However, their current significance for MAP control in domestic ruminants is quite limited. Interestingly, the most critical aspect derived of MAP infection in wildlife could be the fact that MAP infection interferes with TB diagnosis.

Capítulo 1

Table of contents

1. Introduction

Paratuberculosis, also known as Johne's disease, is an infectious granulomatous enteritis caused by *Mycobacterium avium paratuberculosis* (MAP). MAP infection of domestic ruminants occurs worldwide, although there is a lack of precise data, at a country level, as paratuberculosis is not subjected to compulsory declaration and, currently, available diagnostic tests have limited sensitivity and specificity (see section "diagnosis and cross reactions"). However, current estimates suggest that over 50% of dairy cattle herds in Europe and North America are infected (USDA-APHIS-VS-CEAH, 2008; Nielsen and Toft 2007b). Paratuberculosis causes significant economic losses to the livestock industries due to productivity losses, infertility and direct cost of diagnosis and control (Kennedy et al.,2001; Weber et al., 2006; Juste, in press). In addition, a possible involvement of MAP in the causation of Crohn's disease, a chronic inflammatory disease of the intestine in humans, is still under discussion (Grant, 2005; Uzoigwe et al., 2007).

MAP is a small, gram-positive, acid-fast and facultative anaerobic intracellular bacterium belonging to the *Mycobacterium avium* complex (Thorel et al., 1990). Characteristics distinguishing MAP

from other *Mycobacterium* species include its extremely slow growth, its inability to produce mycobactin and its possession of the insertion element IS*900* that occurs as 14-18 copies within the MAP genome (Collins et al., 1989). The subspecies designation of MAP is based on DNA-DNA hybridization studies and numerical taxonomy analyses (Thorel, et al., 1990). Molecular characterization divided *M. a. paratuberculosis* strains into three main groups: sheep or Type I, cattle or Type II and intermediate or type III. (de Juan et al., 2006a; de Juan et al., 2005; Pavlik et al., 1999), with types I and III being sometimes grouped as type I/III due to their closer genetic homology. Cattle are generally infected with 'bovine' strains of MAP, which is also the type commonly found in wildlife, and to a lesser extent, with intermediate strains; sheep are generally infected with 'ovine' strains and goats can be infected by all three types groups of strains (de Juan et al., 2005).

Moore (1924), predicted that paratuberculosis was destined to become for future generations a bigger problem than tuberculosis is nowadays. Moreover, there are still no ideal, cost-effective methods for the control of paratuberculosis in domestic ruminants. The lack of efficient diagnostic tools and the high costs involved in testing and culling approaches have shifted the focus from eradication to control, including vaccination, given the better benefit/cost ratios of more conservative strategies (Juste 2011). However, induction of cross-reacting responses in diagnostic tests for tuberculosis due to vaccination against paratuberculosis has limited the implementation of this control tool (Mackintosh et al., 2005, Bezos et al., 2012; Chartier et al., 2012; Stringer et al., 2011; Muskens et al., 2002), especially in cattle, usually subjected to tuberculosis eradication campaigns. The existence of wildlife reservoirs of MAP could also contribute to this shift from eradication to control under certain epidemiological settings.

MAP is an example of infectious disease agents where a wildlife reservoir does not consist of a single host species, but rather of a matrix of potential reservoir hosts and the environment itself (Haydon et al., 2002). However, paratuberculosis in free living wildlife and captive wild animals has not been as extensively studied as in traditional livestock (Ayele et al., 2001). Due to the development and intensification of deer farming in Europe (Power et al., 1993; Fawcett et al., 1995; Machackova et al., 2004) and worldwide (de Lisle et al., 1993; Manning et al.; 1998; Mackintosh et al., 2004; O'Brien et al., 2006), the prevalence of paratuberculosis in these ruminants has recently increased (Machackova et al., 2004; Mackintosh et al., 2004). The high MAP prevalence in farmed

deer could potentially contribute to contaminate wildlife populations, if infected deer are released (Fernández-de-Mera et al., 2009).

The aims of this review were i) to determine the potential impact of MAP infection in animal health in wildlife species, ii) to analyze whether wildlife reservoirs are relevant regarding MAP control in domestic ruminants, and iii) to assess the importance of MAP as the cause of interference with tuberculosis diagnosis in wildlife.

Articles included in this review

Literature on MAP in wildlife was searched in PubMed, Google scholar and ISI – Web of Science. We found a total of 66 references on MAP in free-living or captive wildlife. From these publications, we collected data of MAP pathology, epidemiology and distribution in wildlife hosts. The references are listed in the online supplementary file (Table1). Among those articles reporting infection or contact prevalence, we identified 15 articles dealing with captive wildlife and 52 ones dealing with free-ranging wildlife (one with both wild and captive animals), totaling 178 species belonging to 14 orders. Studies on artiodactyls from Europe and North America dominated. For each paper and (if more than one host was studied) for each host, we gathered quantitative data on sampling, diagnosis and prevalence.

2. Impact of MAP infection in wildlife

2.1. Etiology and transmission

MAP has demonstrated its ability to infect a wide range of wild ruminant species, including wild cervids and wild bovids, non-rumiant wild species like the European wild rabbit (*Oryctolagus cuniculus*) and carnivores (Crawford et al., 2006; Kopecna et al., 2008a; Vasnick et al., 2005; Witte et al., 2009; Greig et al., 1999; Maio et al., 2011; Nugent et al., 2011; Anderson et al., 2007; Carta et al., 2011).

Usually MAP strains cultured from wildlife species belong to the cattle (II) type, although occasional isolation of type I/III strains have been reported from deer, house mouse (*Mus*

musculus), mouflon (*Ovis aries*), western grey kangaroos (*Macropus filiginosus fuliginosus*) and tammar wallabies (*Macropus eugenii decres*) (Collins et al., 2002; Florou et al., 2008 2007, Kopecna et al., 2008a, Cleland et al., 2010, Fritsch et at., 2011). These findings may suggest a wider host range of type II strains, although the stricter requirements of type I/III strains for their isolation in-vitro could bias the culture rates towards the former.

As in domestic ruminants, the most common route of infection in wildlife is the faecal–oral one, but other routes are also known including the intrauterine one and pseudo-vertical transmission through colostrum and milk (Lambeth et al., 2004, Mackintosh et al. 2004, Stabel, 2008). Intrauterine transmission is more common in farmed deer than in cattle and sheep (Mackintosh and Griffin 2010). Excretion in urine has been reported in MAP infected rabbits (Daniels et al., 2003a).

In addition to close contact between domestic and wild species, transmission of MAP could happen due to environmental contamination by infected wildlife, as MAP is highly resistant to heat, disinfectants and environmental agents, remaining infective for a long time in the ground (Chiodini and Hermon-Taylor, 1993). In environments with infected ruminants, MAP has been detected on the surface and in the intestinal tracts of invertebrates, including dipterans at different stages of development, cockroaches and earthworms (Fischer et al., 2001, 2003a, 2003b, 2004, 2005, 2006; Machackova et al., 2004).

However, isolation of the bacteria does not necessarily indicate that a certain species should be considered as a competent reservoir capable of maintaining the infection by itself (Corner, 2006). Assessment of the tissue colonization and eventual presentation of clinical disease can help to determine the role of a wildlife species in the epidemiology of paratuberculosis.

2.2. Clinical signs of paratuberculosis in wildlife

Following oral ingestion by competent hosts, MAP localises in the small intestine mucosa, particularly in its terminal part, where it is phagocytised by macrophages but preventing phagosome maturation. There, it can proliferate in large numbers and infiltrate the intestinal submucosa (Hole, 1953; Gilmour et al., 1976). Paratuberculosis can be tuberculoid or multibacillary, depending on the more cellular or more humoral immune response of the host (Balseiro et al., 2008). Unlike cattle,

where the disease occurs in advanced age (2-5 years), deer show clinical signs earlier, at about 3 months of age (Mackintosh et al., 2004, 2007, 2008b).

Paratuberculosis in red deer and elk is characterised by chronic granulomatous enteritis followed by severe clinical signs with loss of condition and weight (Mackintosh et al., 2008b, Jessup et al., 1981; Cook et al., 1997; Manning et al., 2003). Diarrhoea, emaciation and poor condition have also been observed in MAP infected Rocky Mountain bighorn sheep (*Ovis canadensis*; Williams et al., 1979), and Key deer (*Odocoileus virginianus clavium*; Quist et al., 2002). Thus, clinical signs of paratuberculosis in wild ruminants are similar to those described in cattle (Whitlock and Buergelt, 1996). In contrast, non-ruminant species do not usually exhibit the classical clinical signs of paratuberculosis (Daniels et al., 2003a). Subclinically infected animals will probably remain infected for all their life and some may intermittently shed the bacteria in faeces, contributing to spread of the infection (Storset et al., 2001). Young animals are particularly susceptible through contact with infected mothers (Farina and Scatozza, 1998).

In cattle, when the infection becomes disseminated, MAP may be detectable in several extra-intestinal sites, including supra-mammary, pulmonary, hepatic and head lymph nodes (LN) (González et al., 2005). However, in sheep and goats, instead, such lesions are frequently found in the intestinal lymphoid tissue (Perez et al., 1996; Corpa et al., 2000a). Regarding wildlife species, in MAP infected fallow deer, visible lesions were observed in the intestines and intestinal LN of individuals with diffuse lesions. These included intestinal wall thickening and LN enlargement; while lymphangiectasis was not observed (Balseiro et al., 2008). Macroscopical or microscopical lesions compatible with paratuberculosis in culture positive animals from other non-ruminant wild species are uncommon, with the exception of wild rabbits. In this species, visible lesions have been observed in mesenteric lymph nodes, caecal appendix, sacculus rotundus and cecum. These lesions consisted of LN enlargement, thickening of the caecal appendix and the sacculus rotundus wall, and multiple granulomatous to abscess-like tuberculous LN lesions (Greig et al., 1997; Maio et al., 2011). Histologically, paratuberculosis-derived lesions have been classified in fallow deer (Balseiro et al., 2008) and in rabbits (Maio et al., 2011) according to the classification proposed for domestic ruminants (González et al., 2005, Perez et al., 1996; Corpa et al., 2000a).

2.3. Importance of wildlife as a reservoir of MAP

Infected wildlife has been usually detected in areas with significant prevalences of infected domestic ruminants (Greig et al., 1999; Nebbia et al., 2000). MAP has been confirmed in 178 vertebrate species including zoo animals and true wildlife. However, only five of these species are regarded as true MAP reservoirs, and all but one are ruminants, including the white-tailed deer (*Odocoileus virginianus*) in the eastern USA (Chiodini and Van Kruiningen, 1983), red deer and roe deer in the Alps (Nebbia et al., 2000, Robino et al., 2008), and fallow deer in Asturias, Spain (Balseiro et al., 2008). In the latter study, the prevalence observed in fallow deer (29%) was similar to the one observed in local cattle. Among the infected fallow deer, one fifth had multibacillary lesions suggesting significant MAP excretion, indicating a potential epidemiological significance. In few occasions research has been enough to discard a wildlife MAP host as a significant MAP reservoir. This was recently achieved after studying a large sample of wild red deer from the iberian peninsula (Carta et al., 2012). The fact that deer act as reservoirs in some regions but not in others shows how environmental and host population factors drive MAP infection in wildlife. The reservoir status of most free-living wild ruminant species remains unknown.

Another true reservoir situation occurs in wild rabbits in Scotland (Greig et al., 1999; Beard et al., 2001b, Daniels et al., 2003a, b; Judge et al., 2006) and possibly also in other regions including areas of New Zeland (Nugent, personal communication), Spain (Maio et al., 2011) and the Czech Republic (Kopecna et al., 2008a). Instead, in other regions wild rabbits are considered only as potencial mechanical vectors for the MAP (Salgado et al.,2011). Rabbits have a high prevalence of MAP and a relatively high level of infection in their tissues compared to other non-ruminant wildlife hosts, excreting up to 7.6×10^5 CFU/g (Daniels et al., 2003a) which is, however, lower than the 10^8 CFU/g reported in faeces from clinically affected cattle (Cranwell, 1997; Whittington et al., 2000a). Furthermore, livestock pastures often have high levels of rabbit faecal contamination. This combination presents a risk of transmission of MAP from rabbits to livestock via the faecal–oral route. An estimate of the input of MAP onto pasture suggested that sheep and cattle potentially contributed 4 and 125 times more organisms per hectare per day, respectively, than rabbits (Judge et al., 2007). In New Zealand, a reservoir status can be suspected for other wildlife (e.g. the feral ferret, *Mustela putorius furo*), too, but scientific evidence is insufficient (de Lisle et al., 2002).

Other wild mammals might eventually act as MAP indicator species, but not as reservoirs. This could be the case of carnivores in the USA (Anderson et al., 2007), although this indicator role should be evaluated carefully when cross-reactions with other mycobacteria are possible, such as in carnivores investigated in Southwestern Europe (Carta et al., 2011). In a similar way, wild suids, such as the Eurasian wild boar, can get infected (Alvarez et al., 2005), but are not useful as MAP indicators if other mycobacterial infections are prevalent in the study region (Boadella et al., 2011a, 2011b). Literature on MAP in avian species is scarce, and a mean prevalence of 4.5% (range 1-60%) is reported (Corn et al., 2005; Gronesova et al., 2008; Kopecna et al., 2008a; Beard et al., 2001a; Nugent et al., 2011).

2.4. Control of MAP in wildlife

Regarding MAP control, there have been a small number of vaccine efficacy studies in deer of various live attenuated and killed MAP vaccines, which showed that oil-adjuvanted killed vaccines gave some protection against clinical disease but did not prevent infection (Mackintosh et al., 2003, 2005, 2008a). Other means for MAP control in wildlife include culling. For instance, the effectiveness of culling European wild rabbits for MAP control in Scotland has been modelled, resulting that a sustained annual cull of 40% of the rabbits would be required to significantly reduce MAP infection prevalence in this reservoir host (Davidson et al., 2009).

3. Diagnosis of MAP in wildlife and specificity in diagnostic tests for other mycobacterial diseases.

Due to its chronic nature and long period of incubation, diagnosis of paratuberculosis can be difficult in both domestic and wild species, with no single test achieving a sufficient sensitivity/specificity. Culture followed by molecular identification of isolated strains is considered the gold-standard diagnostic technique in mycobacterial infections. In the case of paratuberculosis diagnosis, usually tissue samples (ileocaecal valve, intestine or mesenteric or ileocaecal LN) or faeces, are used. However, this method has a limited sensitivity, especially for faecal culture and in

subclinically infected animals (Stabel, 1998; Maio et al., 2011). In addition, certain MAP strains may be more difficult to isolate *in-vitro*, decreasing even more the sensitivity. (Table 1)

Histopathology, direct DNA amplification by PCR and serology are also used in the diagnosis of paratuberculosis, in both domestic and wild animals. Histopathology is a particularly valuable additional tool because it allows the classification of the lesions and distinguishes between paubacillary and multibacillary infections (Balseiro et al., 2008). On the other hand, when interpreting serological results, the fact that wildlife hosts are often in contact with more than one species of *Mycobacterium* must be taken into account (Gortazar et al., 2011). Thus, diagnostics based on antibody detection can be affected by a large proportion of cross-reactions and hence, may have a low specificity. As a consequence, the ideal approach for MAP epidemiology studies in wildlife is combining pathology and culture (Carta et al., 2012).

Table 1.

Information collected from the scientific literature on paratuberculosis prevalence in free ranging and captive wildlife. Prevalence data are given for histopathology, PCR, culture and serology.

DIAGNOSTIC TEST									
FREE RANGING	**N**	**Histopathology**		**Direct PCR**		**Culture**		**Serology**	
N OF STUDIES	52	16	30,8	10	19,2	29	55,76	22	42,3
Mean sample size % (range)	52	59,9	(0-584)	36,7	(0-835)	124,6	(0-584)	121,5	(0-1327)
Mean prevalence % (range)	13575	8,6	(0-67)	13,7	(0-100)	9,9	(0-85)	6,5	(0-40)
CAPTIVE									
N OF STUDIES	15	6	40	1	6,66	10	66,66	6	40
Mean sample size % (range)	15	56,9	(0-431)	1	(0-15)	755,6	(0-6495)	48,2	(0-383)
Mean prevalence % (range)	12004	9,3	(0-43)	40	(0-80)	3,3	(0-62,5)	5,6	(0-100)

In line with its consideration of gold-standard, culture was the most often used technique (38 out of 66 studies, 58.5%), followed by serology, histopathology and PCR. Culture is also the test in which the largest mean sample size was analyzed. Sample size has been increasing with time (Pearson correlation coefficient between time (years) and sample size r_s=0.32, n=66, $p<0.05$). In studies carried out before the year 2000 (n=13) sample sizes larger than 1000 (mean 190, range 4-954) were never analyzed, while four of the 53 studies carried out from the year 2000 onwards include over 1000 samples (mean 429, range 1-2296).

With all four diagnostic methods combined, the mean prevalence recorded in wildlife was 2.41% (95%CI 1.76-3.06%; range 0 to 100%). Interestingly, the mean prevalence estimated by PCR was about three times higher than prevalences estimated by any of the other three techniques. This could be due to two not-mutually excluding explanations. First, PCR might be more sensitive than other techniques, and second, false positives due to other mycobacteria might occur, since IS*900*-like sequences have been described in other MAC members too (Naser et al., 1999b; Motiwala et al., 2003).

3.1. Interference of paratuberculosis in the diagnosis of tuberculosis.

In addition to its impact on animal health, the presence of paratuberculosis in wildlife can seriously complicate the *in-vivo* diagnosis of tuberculosis (TB) due to the cross-reactivity between MAP and members of the *Mycobacterium tuberculosis* complex, including *M. bovis*, as it happens in livestock. Most *in-vivo* tuberculosis diagnostic tests are based on the detection of the immune response of the infected animals to certain antigens (mostly bovine purified protein derivative, bPPD) that are partially shared between the *M. avium* and the *M. tuberculosis* complex (Aagaard et al., 2003). Infection of free-ranging wildlife with atypical micobacteria is supposed to be uncommon and, therefore, cross-reactions would be rather rare in these populations. Moreover, the lack of reliable tools to determine the presence of a transient micobacteriosis (animals may respond to mycobacterial antigens after sensitization with these microorganisms even if an infection has not been established) complicates the interpretation of diagnostic results (Chambers et al., 2009). As described before, MAP can infect a wide range of wildlife species and, taking into account the large prevalences described under certain epidemiological settings, it should be considered as a major factor in the development of these false positive responses. The presence of atypical mycobacteria can impair the reliability of studies aiming at determination of TB prevalence in free-ranging wildlife populations (Michel et al., 2007). This fact could be even more important in farmed wild species, (such as deer), in which paratuberculosis-derived false diagnostic responses could impose similar commercial limitations as those existing in cattle herds. Tuberculin test, the most commonly diagnostic technique used in farmed wildlife species (deer) is known to have a limited specificity that can lead to the slaughter of false positive reactors (Buddle et al., 2010). Previous reports have demonstrated that MAP infection can compromise the diagnosis of TB in farmed deer (Fernández-de-Mera et al., 2009) and in free-ranging wildlife (Boadella et al., 2011a, 2011b), as previously

demonstrated in domestic ruminants (Álvarez et al., 2008, 2009; Amadori et al., 2002; Hope et al., 2005). Therefore, this fact could compromise the efficacy of test and slaughter TB eradication campaigns under certain circumstances.

4. Conclusions

Despite the fact that the knowledge on most host species is still incomplete, reviewed data evidenced that MAP circulates almost worldwide among a diversity of wild vertebrates including ruminant and non-ruminant species. Current literature on deer species and on wild rabbits suggests that these species could locally play a significant role in MAP epidemiology. Three questions on wildlife and MAP were proposed in the introduction: (1) Is MAP a significant factor in wildlife population dynamics; (2) What is the role of wildlife hosts in MAP maintenance as reservoirs for domestic ruminants?; and (3) How relevant is MAP infection of wildlife considering TB diagnosis?

Clinical paratuberculosis has occasionally been reported from wildlife, mainly in deer species. However, such cases are rather sporadic and are most unlikely to pose a real threat to free-ranging wildlife populations (Quist et al., 2002, Sleeman et al., 2009). Therefore, in view of the published evidences, MAP should be considered an important disease in farmed wild ruminant species (mainly cervids), but its impact on free-ranging wildlife is questionable.

Regarding wildlife implication in paratuberculosis transmission cycles, MAP sources other than wildlife (including domestic ruminants and the environment), if not properly addressed, could render MAP control in wildlife useless. Clearly, MAP can circulate among wildlife hosts including deer species and rabbits, and available epidemiological data suggest that infection of cattle or sheep through contaminated pastures shared with wildlife is possible. However, MAP excretion by wildlife hosts is lower than excretion by clinically-affected cattle (e.g. Daniels et al., 2003a), and MAP is not currently (or only partially) under control in domestic livestock (Juste, 2011). Therefore, while wildlife MAP reservoirs do locally exist, their current significance for MAP control in domestic ruminants is quite limited. However, infected wild hosts could potentially introduce the pathogen into paratuberculosis-free herds, and therefore, when such a situation is possible; risk posed by wildlife should be considered (Corn et al., 2005).

Hence, the most critical aspect derived of MAP infection in wildlife could be the fact that MAP infection interferes with TB diagnosis, for instance in deer skin tests (Fernández-de-Mera et al.,

2009) or in tests based on serum antibody detection (Boadella et al., 2011, Carta et al., 2012). A main drawback of serological tests is their low specificity, and a possible solution could be the combination of antibody detection assays and the skin test (Jaroso et al., 2010) or pathology tests (Waters et al., 2011). Furthemore, it's also possible to improve the sensitivity of the (bPPD) ELISA test sensitivity using the dual path platform (DPP) VetTB assay and ELISA's test (~97%) (Boadella et al., 2012). Moreover, for eradication of MAP in a farm, to obtain test with high specificity, it is essencial to take into consideration the relationship between stress and no-stress times during each year (Queiros et al., 2012).

This means that, while there would be generally no real need to monitor MAP in wildlife (except for very few local exceptions), the likelihood of MAP interference with TB diagnosis must be considered in attempts to monitor TB (or contact with members of the *M. tuberculosis* complex) in wildlife (Boadella et al., 2011, Boadella et al., 2012).

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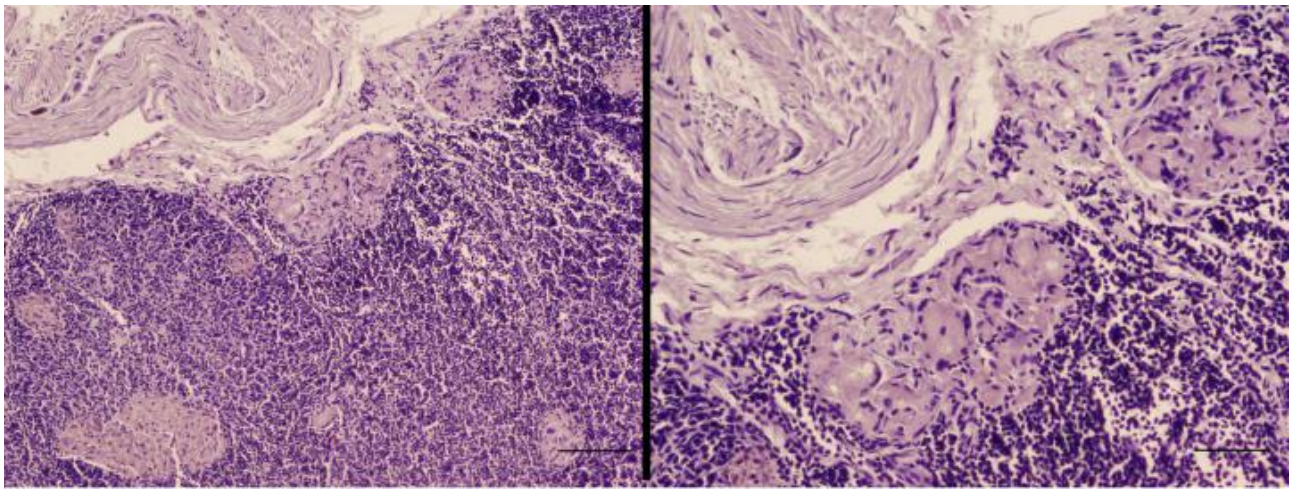
CAPITULO 2

Paratuberculosis del conejo silvestre europeo en la península ibérica



Lesiones de paratuberculosis en la válvula ileocecal de un conejo de Cádiz (Ref. M653/09). Hematoxilina-Eosina; izquierda 100x, 100 micras y derecha 200x, 50 micras. Fotos: Cortesía de Ana Balseiro, SERIDA

Capítulo 2

Entre los animales silvestres no rumiantes, portadores de *Mycobacterium avium paratuberculosis* (MAP), se piensa que los conejos (*Oryctolagus cuniculus*) puedan representar el mayor riesgo de trasmisión al ganado doméstico. En este estudio hemos analizado los cadáveres de 80 conejos cazados en una localidad de la provincia de Cádiz en el Sur de España, y sueros de 157 conejos más, provenientes de otros 7 lugares situados en la península ibérica e Islas Chafarinas (África). En ocho de 10 conejos examinados por histopatología se observó la presencia de lesiones compatibles con MAP. Éstas variaron desde formas focales hasta formas multibacilares. En un conejo con lesiones multibacilares, la presencia de DNA de MAP fue confirmada mediante PCR en tiempo real con los primers para IS-900 e ISMAP02. Sin embargo, no fue posible aislar MAP por cultivo de ninguna de las 47 muestras de tejido (linfonodos mesentéricos y apéndice cecal) inoculadas en medios específicos. En este trabajo se adaptó un ELISA indirecto para la detección de anticuerpos frente a MAP en conejo. Seis de 237 sueros (2.5%) resultaron positivos con el ELISA. Esta prueba detectó anticuerpos en conejos de 3 de los 8 lugares de muestreo. Teniendo en cuenta la creciente importancia de la paratuberculosis para la salud animal, y el hecho de que el conejo es reconocido en Escocia como reservorio de paratuberculosis, estos resultados podrían ser de relevancia para investigaciones futuras.

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Paratuberculosis in European wild rabbits from the Iberian Peninsula

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ABSTRACT

Of the non-ruminant wildlife species known to harbor *Mycobacterium avium paratuberculosis* (MAP), the rabbit (*Oryctolagus cuniculus*) is thought to pose the greatest risk of transmission to cattle. We analyzed 80 hunter-harvested wild rabbits from a core study area in southern Spain, and sera from 157 wild rabbits sampled opportunistically on seven additional sites. Gross lesions compatible with paratuberculosis were observed in two of 80 necropsied rabbits. Histopathology revealed focal to diffuse multibacillary MAP-compatible lesions in 8 of 10 rabbits examined. Presence of MAP was confirmed in one rabbit with gross lesions by positive amplification curves for both IS900 and ISMAP02. However, no isolate was obtained from 47 samples by culture. We adapted an indirect ELISA for the detection of MAP antibodies. At the established cut-off of 0.5, 6 of 237 wild rabbit sera (2.5%) yielded a positive ELISA result. Antibodies were detected in rabbits from 3 of 8 sampling sites. Considering the increasing relevance of MAP infection for animal health, these results open a challenging field for future research.

1. Introduction

Mycobacterium avium paratuberculosis (MAP), an intracellular bacterium, is the causative agent of paratuberculosis or Johne's disease, a chronic granulomatous enteritis of ruminants and other vertebrates. It has been considered as a major disease of ruminants for more than a century and has significant economic and welfare effects on livestock in all continents (Chiodini et al., 1984). Wildlife MAP reservoirs may further limit the success of paratuberculosis control in domestic ruminants. However, the significance of MAP in non-ruminant wildlife is largely unknown, except for the European wild rabbit (*Oryctolagus cuniculus*; Daniels et al., 2003a,b).

In Scotland, extensive studies suggested that rabbits play a role in the epidemiology of the disease, acting as a wildlife reservoir (Daniels et al., 2003a). Natural MAP infection has been reported in wild rabbits, particularly from farms with infected wild and domestic ruminants that shared the same pasture (Angus, 1990; Greig et al., 1997, 1999; Beard et al., 2001b). Experimentally, MAP isolates from naturally infected rabbits are capable of infecting young calves and causing early paratuberculosis histological lesions which suggests that transmission from rabbits to cattle can occur under field conditions (Beard et al., 2001c). Naturally infected rabbits had histopathological changes within the lymph nodes and intestines compatible with ruminant paratuberculosis, demonstrating that MAP not only replicates but can also produce disease in rabbits (Greig et al., 1997). Paratuberculosis lesions in rabbits were categorized as mild or severe (Beard et al., 2001b).

The European wild rabbit is a keystone species in Iberian Mediterranean ecosystems. It is also the most hunted small game species in Spain. Relatively high rabbit densities remain in parts of central and southwestern Spain (Delibes-Mateos et al., 2008). Despite its relevance, current knowledge on MAP epidemiology in rabbits outside Scotland is limited. To the best of our knowledge, the only reference is a clinical case in a wild rabbit from Toledo, central Spain, reported at a conference meeting with histopathological evidence of infection by MAP (Fernández-de-Luco et al., 1995).

Based on the findings in Scotland, on the abundant wild rabbit populations in parts of the Iberian Peninsula, and on the frequent reports of MAP infection or MAP antibodies in ruminants from Portugal (Ferreira et al., 2002; Coelho et al., 2007) and Spain (e.g. Diéguez et al., 2007; Reyes-García et al., 2008), we hypothesized that European wild rabbits may become infected with MAP and can play a role in the epidemiology of paratuberculosis in Mediter-

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ranean habitats. Herein, we provide molecular and histopathology evidence of MAP infection in wild rabbits, propose a pathological classification of rabbit paratuberculosis, and adapt an indirect ELISA, testing sera from several localities to establish the relevance of the European wild rabbit as a potential wildlife reservoir for MAP in Southern Europe.

2. Materials and methods

2.1. Study sites

We analyzed a sample of 80 hunter-harvested wild rabbits from a core study area in Benalup (Cádiz), southern Spain, and sera from 157 wild rabbits sampled opportunistically on seven additional sites throughout the Iberian Peninsula and the Chafarinas Islands, off the North African coast (Fig. 1).

The core study area comprised a 3000 ha hunting estate located in the natural park of 'Los Alcornocales' in Benalup, province of Cádiz (Andalucía, southern Spain). Cork oak (*Quercus suber*), olive tree (*Olea europaea*, var. *sylvestris*) and evergreen oak (*Quercus ilex*) forests mixed with scrubland and pastures compose the main landscape in the hunting estate. Mediterranean climate is dominant in this area, and rains are mainly present during spring and autumn, with annual rainfall ranging 500–1000 mm. Wild ungulates, wild rabbits, wild carnivores and a wide variety of birds are present in the hunting estate.

2.2. Sampling

Eighty European wild rabbits were shot on the core site during the 2008/2009 hunting season. These rabbits were collected from four different situations. Each test group consisted of 20 rabbits. Situation A was a red deer (*Cervus elaphus*) farm with irrigated pastures and high stocking rates where clinical paratuberculosis had been diagnosed in deer and confirmed by culture. Situation B was a second deer farm with no irrigated pastures, lower stocking rates, and no clinical cases of paratuberculosis in deer. Situation C was a deer hunting estate, and situation D was an area with no current presence of ruminants (since 2002). However, ruminants (cattle and roe deer *Capreolus capreolus*) had shared site D with rabbits until 2002.

Rabbit carcasses were divided into three age groups in agreement with their weight: <400 g, young rabbits; ⩾400 g and <1000 g, juveniles; and ⩾1000 g, adults. All carcasses were tagged with an individual identification number, weighed, and sexed. Blood was taken from the thorax and centrifuged to obtain serum. At necropsy, rabbit carcasses were thawed and examined for visible lesions. Separate and clean instruments were used for each animal to reduce the risk of cross-contamination, mesenteric lymph node, caecal appendix, sacculus rotundus, ileum, liver, feces, and spleen were transferred to clean containers and frozen in duplicate at −20 °C until analysis. Small portions of tissue were fixed in 10% formol saline for histopathological analysis.

2.3. Pathology

Gross lesions observed in rabbits were recorded, attention being focused on the gut and associated lymphoid tissues. Samples for histopathological studies were taken from the liver, caecal appendix, ileum, sacculus rotundus and mesenteric lymph nodes of 10 rabbits (two with macroscopic lesions and 8 randomly selected rabbits; Table 1). These samples were dehydrated through graded alcohols and xylol before being embedded in paraffin wax. Several sections (4 μm) were cut from each sample and stained with hae-

Fig. 1. Map of the study area, displaying the sampling localities, sample size, and prevalence.

Table 1
Gross pathology, histopathology (ZN Ziehl-Neelsen staining), ELISA optical density (OD) and microbiology results for a sample of ten hunter-harvested European wild rabbits (*Oryctolagus cuniculus*) from Benalup (Cádiz), southern Spain.

Ref.	Gross pathology	Histopathology	ELISA OD	Microbiology
M653/09	No visible lesion	Multi-focal, ZN negative	0.16	Culture negative
M662/09	Mesenteric lymph nodes with multiple large granulomatous lesions. Thickened wall of the caecal appendix, sacculus rotundus and caecum.	Diffuse multi-bacillary, ZN positive	0.64 (positive)	Culture negative PCR positive
M711/09	No visible lesion	Focal, ZN negative	0.44	Culture negative
M714/09	Mesenteric lymph nodes with small, not encapsulated lesions and thickening of the caecal appendix and the sacculus rotundus.	Multi-focal, ZN positive	2.22 (positive)	Culture negative
M721/09	No visible lesion	Multi-focal, ZN negative	0.09	Culture negative
M726/09	No visible lesion	Multi-focal, ZN negative	0.17	Culture negative
M728/09	No visible lesion	No lesion	0.11	Culture negative
M732/09	No visible lesion	No lesion	0.18	Culture negative
M735/09	No visible lesion	Diffuse intermediate, ZN positive	0.27	Culture negative
M738/09	No visible lesion	Multi-focal, ZN negative	0.12	Culture negative

matoxylin and eosin (HE) and by Ziehl-Neelseńs (ZN) method for acid fast bacteria (AFB).

2.4. Culture

For each culture, two grams of pooled mesenteric lymph node and sacculus rotundus of each animal were processed. Samples were homogenized and decontaminated with 38 ml of a solution (0.75%) of hexadecilpiridinium chloride (HPC; Aduriz et al., 1995). Three drops of the homogenate were inoculated on home-made Herrold's Egg Yolk medium (HEYM) and Löwestein-Jensen medium (LJ) both supplemented with mycobactin J (Allied Monitor), and Middlebrook 7H11 supplemented with 1% Middlebrook OADC Enrichment (Becton, Dickinson and Company, MD, USA) (Sevilla et al., 2007). Tubes were incubated at 37 °C and inspected monthly. They were considered negative if no bacterial growth was observed after 20 weeks.

2.5. PCR

DNA for PCR confirmation of *Mycobacterium avium paratuberculosis* was extracted from formalin-fixed paraffin-embedded (FFPE) intestinal tissue of one rabbit (M662/09). Three FFPE sections (10 μm each) were cut, introduced in a microfuge tube, and submitted to DNA extraction using the WaxfreeTM DNA extraction kit (Trimgen, MD). A negative extraction control was included in all steps to exclude contaminations during the extraction procedure. The final extract (50 μl) was diluted (1:10) in DNase-RNase-free water and used in a triplex real-time PCR targeting the IS900 (Herthnek et al., 2006) and ISMAP02 sequences of MAP, and a synthetic internal amplification control (IAC) to rule out inhibition of the reaction. The 50 μl PCR mixture contained 2.5 μl of the diluted DNA extract, 1× TaqMan Universal MasterMix (Applied Biosystems, CA), 0.4 μM (each) of primers co-amplifying ISMAP02 and the IAC, 0.3 μM (each) of IS900 primers, 0.2 μM (each) of ISMAP02, IAC and IS900 probes and, 2 μl of IAC template DNA. The performance of the PCR was monitored using a negative control (water) and a positive control (DNA from ATCC 19698 strain of MAP). Amplification was carried out in an Applied Biosystems 7500 Real-Time PCR System under the following standard conditions: one cycle at 95 °C for 10 min and 45 cycles with two steps of 95 °C for 15 s and 60 °C for 1 min.

2.6. Production of positive control rabbit sera

In order to obtain serum controls to be used in the ELISA, four adult domestic rabbits living in a MAP free environment were used. These rabbits were females and were 75 days of age when they were inoculated for the first time. Their ears were tattooed for identification, and they were moved to individual cages. On day 0, rabbit 1 and 2 were immunized by the subcutaneous route with 1 ml of an emulsion containing 500 μg paratuberculosis protoplasmatic antigen 3 (PPA-3, an innocuous MAP specific antigen) (Allied Monitor, Fayette, MO, USA) in Freund's Incomplete Adjuvant (Sigma–Aldrich, Madrid, Spain). Rabbits 3 and 4 were immunized by the same route with 50 μg PPA-3. All rabbits were immunized again at the same dose 21 days later at day 21 post immunization (p.i.). At day 43 p.i., rabbit 1 was immunized by the intramuscular route with 500 μg PPA-3, rabbit 2 with 50 μg PPA-3 i.m., rabbit 3 with 50 μg PPA-3 i.m., and rabbit 4 with 500 μg PPA-3 i.m. Serum samples were taken from each animal at 43 d.p.i. and 62 d.p.i. All sera were stored frozen at −20 °C until used for testing. A serum sample obtained from each rabbit in the day of the third inoculation (43 d.p.i) was tested by ELISA at different concentrations. Serum samples from 4 non-immunized domestic rabbits were used as negative controls.

Handling procedures and sampling frequency were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish laws (RD 223/1988; RD 1021/2005), and current guidelines for ethical use of animals in research (ASAB, 2006).

2.7. ELISA test

The ELISA test to detect antibodies against MAP in the rabbit sera was performed adapting protocols reported previously for ruminants (Garrido, 2001; Sevilla, 2007; Reyes-García et al., 2008). Briefly, high absorption capacity Costar polystyrene microtiter plates (Cultek, Madrid, Spain) were coated with 50 μl/well of 0.02 mg/ml PPA-3 diluted in carbonate/bicarbonate buffer (Sigma, Madrid, Spain). The serum samples were adsorbed (1:1, v/v) with a saline suspension of *Mycobacterium phlei* (5 g/l) (Allied Monitor) and left at 4 °C overnight to remove nonspecific anti-*Mycobacterium* spp. antibodies (Milner et al., 1987). After overnight incubation at 4 °C, the plates were washed once with a washing solution (PBS containing 0.05% Tween 20) and blocked with 200 μl/well of blocking solution (5% nonfat dried milk in PBS containing 0.05% Tween 20). After a 1 h incubation period at room temperature, the plates were blocked with 80 μl/well of blocking solution and thereafter 20 μl of each serum, diluted 1:5 (v/v) in PBS solution, and were added into wells of the antigen-coated plate. The plates were incubated at 37 °C for 90 min. Then, the plates were washed four times with the washing solution. Protein G horseradish peroxidase conjugate or anti-rabbit IgG peroxidase conjugate (Sigma) was added (0.002 mg/ml in blocking solution)

and incubated at 37 °C for 1 h. After four washes with washing solution, 200 µl/well of substrate solution (Fast OPD, Sigma) were added. Approximately 20 minutes later, the reaction was stopped with 50 µl/well of H2SO4 3 N and optical density (OD) was measured in a spectrophotometer at 450 nm. Rabbit positive and negative control sera were included in every plate in quadruplicate.

In order to characterize the positive and negative control sera, a titration curve was made with 8 serum samples (rabbits 1–4 at 43 d.p.i; and 4 negative controls) to assess if the four inoculated rabbits developed PPA3 specific antibodies. The sera were serially diluted until 1:1024 in a PBS solution.

3. Results

3.1. Pathology

Macroscopic lesions compatible with paratuberculosis were observed in two of 80 necropsied rabbits: M662/09 and M714/09 (Table 1). These lesions were found in mesenteric lymph nodes (Fig. 2), caecal appendix, sacculus rotundus and cecum. Rabbit M662/09 showed at necropsy swollen mesenteric lymph nodes with multiple granulomatous to abscess-like tuberculous lesions (up to 19 mm diameter). The core of these lesions was composed by a yellowish material varying from solid to purulent/caseous. The intestinal wall of the caecal appendix, sacculus rotundus and caecum was thickened. Rabbit M714/09 presented enlarged mesenteric lymph nodes with multifocal, small (up to 2.5 mm diameter), not encapsulated, pale-yellowish lesions and thickening of the caecal appendix and the sacculus rotundus wall.

Stained sections were examined for typical paratuberculous lesions and AFB. The lesions were classified on the basis of (1) their location, intensity and inflammatory cell type, and (2) their numerical content of mycobacteria. An animal was considered lesion-positive if any sample showed one of the three categories of lesions listed below. The sacculus rotundus was always the most affected tissue.

3.2. Focal lesions

Lesions of this type were present in one animal, and they were formed by small, well-demarcated granulomas located exclusively in the intestinal lymphoid tissue and in the interfollicular areas of the mesenteric lymph nodes. These granulomas consisted of macrophages with abundant, slightly foamy, pale cytoplasm and large nuclei with sparse chromatin. Frequently, lymphocytes and multi-

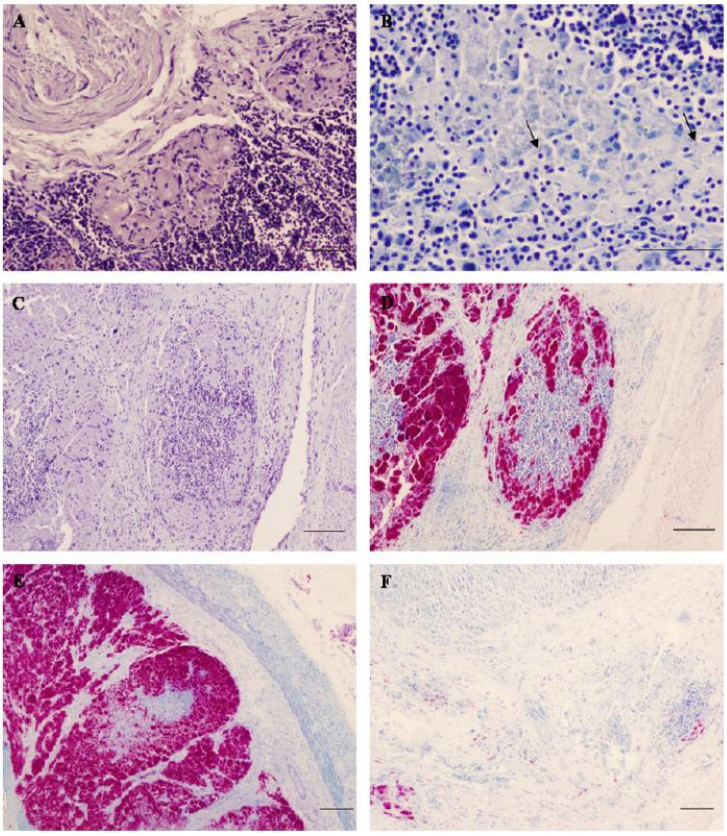

Fig. 2. Photomicrographs of rabbits showing multifocal and diffuse multibacillary lesions of paratuberculosis in intestine. Multifocal lesions: (A) Well-demarcated granulomas, located in the sacculus rotundus lymphoid tissue, consisted mainly of Langhans giant cells and macrophages. HE technique. Bar = 50 µm. (B) Scarce mycobacteria (arrows) are observed within the granuloma. ZN stain. Bar = 50 µm. Diffuse multibacillary lesions: (C) Sacculus rotundus wall and Peyeŕs patch showing a diffuse inflammatory infiltrate consisted of macrophages, lymphocytes and numerous multinucleated Langhans giant cells. HE technique. Bar = 100 µm. (D) Mycobacteria in large number in the mucosa and submucosa. ZN stain. Bar = 100 µm. (E) Ileum showing large amount of Langhans giant cells filled with numerous AFB. ZN stain. Bar = 200 µm. (F) Multifocal granulomatous infiltrates, showing AFB, located in the serosa. ZN stain. Bar = 200 µm.

nucleated Langhans giant cells were found in the granuloma. No entire mycobacteria were observed in ZN stains.

3.3. Multifocal lesions

Lesions of this type, which were present in 5 animals (including rabbit M714/09), consisted of well-demarcated granulomas in the intestinal lymphoid tissue (Fig. 2A) and also in the intestinal lamina propria. However, they were not sufficiently numerous to modify significantly the normal architecture of the intestine. Small numbers of granulomas were located in the interfollicular areas of the mesenteric lymph nodes. Entire mycobacteria were demonstrated by ZN stain only in one animal (Fig. 2B).

3.4. Diffuse lesions

Lesions of this type occurred in two animals with severe granulomatous enteritis and lymphadenitis and were divided in two different subtypes, according to the nature of the cells present in the infiltrate and the amount of AFB.

3.5. Diffuse multibacillary lesions

In the animal showing this type of lesion (rabbit M662/09), the intestinal wall was thickened and showed an infiltrate consisting of epithelioid cells, macrophages, lymphocytes and numerous multinucleated Langhans giant cells. The villi were commonly fused due to this infiltrate. The submucosa was affected and the Peyer's patches showed an infiltrate consisting mainly of macrophages and Langhans giant cells that invaded the lymphoid follicles (Fig. 2C). The serosa was less affected but multifocal granulomatous infiltrates were observed. Mesenteric lymph nodes showed a severe and diffuse granulomatous lymphadenitis with macrophages and numerous Langhans giant cells, which caused a significant alteration of the normal lymph node architecture. Mycobacteria in large numbers were demonstrated by ZN stain in the mucosa and submucosa in the intestine (Fig. 2D and E) and in the lymph nodes. Few mycobacteria were observed in the serosa (Fig. 2F). This animal also showed hepatic lesions, consisting of granulomas formed mainly by lymphocytes and macrophages which contained AFB.

3.6. Diffuse intermediate lesions

In the animal showing this type of lesion, the intestinal wall was thickened and the infiltrate contained epithelioid cells, macrophages, lymphocytes and Langhans giant cells. Giant cells were present but always fewer than in the diffuse multibacillary lesions. The submucosa showed an inflammatory infiltrate consisting of plasma cells, macrophages and lymphocytes. The serosa was not affected. In the lymph nodes, a granulomatous lymphadenitis was observed. AFB were demonstrated by ZN staining but in smaller numbers than in lesions of the diffuse multibacillary type.

3.7. PCR and culture

Presence of MAP was confirmed by positive amplification curves for both IS900 (Ct = 37.3236) and ISMAP02 (Ct = 36.1794). As expected, both negative extraction and PCR controls yielded negative results for IS900 and ISMAP02 and a positive result for the IAC (Fig. 3). None of the 47 cultured samples was positive.

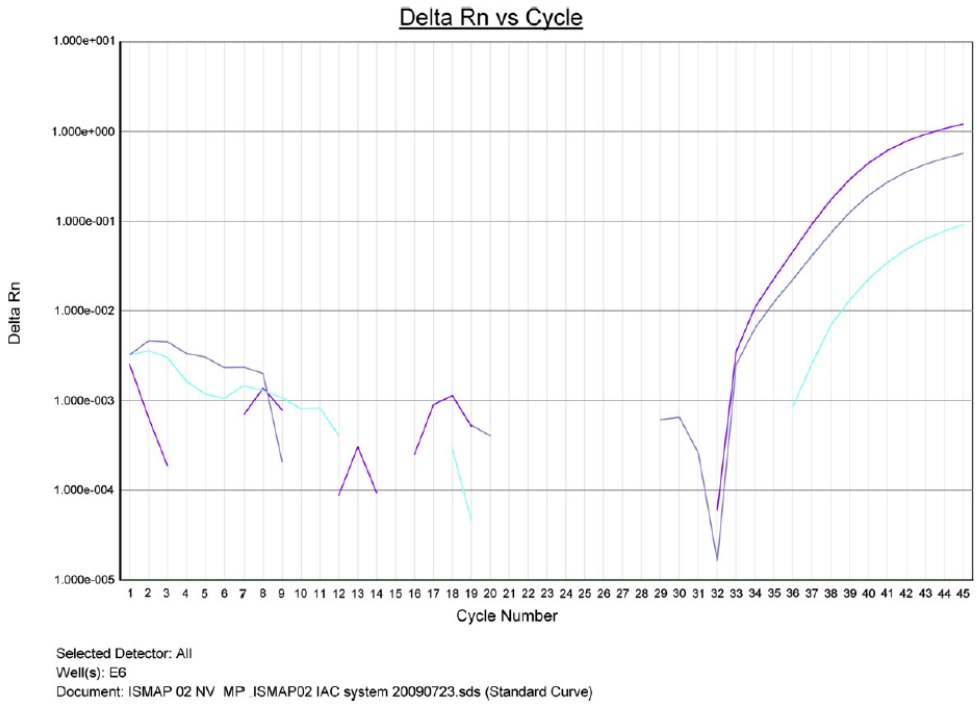

Fig. 3. DeltaRn view of the amplification plot showing positive curves corresponding to IS900 (blue–grey), ISMAP02 (purple) and IAC (blue). (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this paper.)

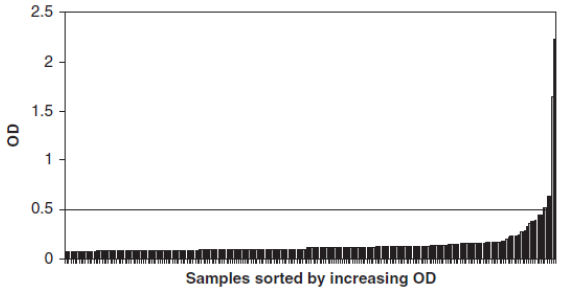

Fig. 4. Distribution of optical density (OD) values for wild rabbits (n = 237) The cut-off value is represented with a horizontal line.

3.8. ELISA

All 4 PPA-3 immunized domestic rabbits developed antibodies at 43 d.p.i. after repeated immunization. Antibody levels after the third immunization (62 d.p.i.) were not higher than those detected after the second immunization (43 d.p.i.), and ranged between 1.04 and 1.89 OD (450 nm) at 1:5 serum dilution. Control sera reacted with both conjugates, Protein G and anti-rabbit IgG. When anti-rabbit IgG was used the antibody response was slightly higher. However, OD values were more homogeneous and discriminated better between positive and negative control sera when using Protein G. Thus, Protein G and not anti-rabbit IgG was used to test the sera from wild rabbits.

In order to assess the magnitude of the antibody response to MAP and to validate the performance of our ELISA test, a titration curve was used. Differences between the mean responses of negative and positive controls were evident up to the 1:64 dilution. However, a neat separation between control positive and control negative rabbit sera was found between dilutions 1:4 and 1:8. Thus, we selected a serum dilution of 1:5 and set the cut-off as OD 0.50, three times the mean OD value of the 4 negative controls.

As reported by others, the distribution of the OD values in the ELISA was not discrete and was continuously distributed (Fig. 4). The two ELISA positive wild rabbits from the core sampling site were confirmed by histopathology. One was also tested and confirmed by PCR. However, of the other 8 rabbits tested by pathology, 6 had MAP-compatible lesions and were ELISA negative (Table 1).

At the (arbitrarily) established cut-off of 0.5, 6 of 237 wild rabbit sera (2.5%; 95% confidence limits: 1.1–5.4%) yielded a positive ELISA result. Positive samples were detected in 3 of the 8 sampling sites. Two of the ELISA positive samples belonged to rabbits from the core sample site, with confirmed clinical paratuberculosis. The highest local prevalence (7.9%) was observed in a site from northern Portugal (Fig. 1).

4. Discussion

Our results provide the first molecular and histopathology evidence of MAP infection in wild rabbits from Mediterranean Europe. We propose a pathological classification of rabbit paratuberculosis and adapt an indirect ELISA showing that a low percentage of sera from Iberian wild rabbits have antibodies reacting against the PPA3 antigen.

The absence of culture positive samples in this study was not surprising. For instance, a low culture success was also observed among wild ungulates cultured for MAP in a known-infected area of southern Spain (Álvarez et al., 2005). Possible explanations for this negative result include sample management with repeated freeze–thawing and the fact that we only cultured mesenteric lymph nodes and the sacculus rotundus and no cecum or the intestinal wall. However, the detection of MAP DNA in FFPE intestinal tissue of one rabbit, along with the description of paratuberculosis-compatible lesions in the same animal (Table 1, Fig. 2), constitutes the first confirmation of paratuberculosis in a European wild rabbit outside Scotland. More studies will be needed in order to establish the actual prevalence of MAP infection in Mediterranean Europe.

The histopathological results confirm the existence of variation in the pathological response in rabbit paratuberculosis, most of the infections being subclinical, as previously outlined by Beard et al. (2001b). The present study confirmed that the histological lesions in this species resemble those observed in small ruminants, cattle and fallow deer (Pérez et al., 1996; Corpa et al., 2000; González et al., 2005; Balseiro et al., 2008). Thus, the classification parameters of paratuberculosis lesions proposed for ruminants appeared to be valid for rabbits. Diffuse lymphocytic lesions, previously described in cattle, goats and sheep were not observed in the present study. Focal lesions have been reported as an initial form of the disease or as latent or persistent lesions that developed when the animal was young and were limited by the immune response (Payne and Rankin, 1961; Larsen et al., 1975; Juste et al., 1994; Clarke, 1997; Kurade et al., 2004). Multifocal lesions, which occur mainly in subclinically infected animals, may represent progression of the infection after failure of the immune response. Multinucleated Langhans giant cells in large numbers, as described by Beard et al. (2001b), were present in all types of lesions, making this a characteristic feature of rabbit paratuberculosis. Langhans giant cell formation may be influenced by host factors and by the infecting strain of MAP (González et al., 2005). The sacculus rotundus and mesenteric lymph nodes would seem to be the site of choice for seeking evidence of paratuberculosis in rabbits, although sections from jejunum were not examined, so a complete comparison cannot be made. Possibly, part of the infected rabbits would have progressed towards a more severe form of paratuberculosis if they lived longer (Mokresh et al., 1989).

The extent of MAP infection in wild animal populations and the role of these animals in maintenance and spread of the disease must be understood to facilitate appropriate and effective disease management programs (Anderson et al., 2007; Judge et al., 2007). The core site in Cádiz is an important livestock breeding area, with high stocking rates of both domestic and wild ruminants. Attempts to control MAP infection prevalence in livestock are ongoing, and the success of such efforts may be limited if other reservoirs complicate the epidemiological scene (Beard et al., 2001a). Moreover, we identified paratuberculous rabbits in one site within the core area, where domestic and wild ruminants had been absent for seven years. This suggests that either rabbits moved more than 5000 m distance from neighboring areas with ruminants (cattle), or they were able to maintain MAP infection for this seven year period, i.e. several wild rabbit generations.

Antibody tests for mycobacteria are likely to produce cross-reactions. For example, sera from known *M. bovis* infected Eurasian wild boar (*Sus scrofa*) have been shown to cross react with the PPA3 antigen (Boadella et al., 2011). However, the 2.5% prevalence estimated by ELISA at our cut off represents probably a conservative estimation. Considering that several rabbits from the core sampling site showed MAP-compatible lesions at histopathology, the true prevalence is probably higher. For comparison, MAP infection prevalence of wild rabbits tested on paratuberculosis infected farms in Scotland ranged from 8% to 100% (Greig et al., 1997, 1999).

Considering the increasing relevance of MAP infection for animal health, the results reported herein open a challenging field for future research.

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CAPITULO 3

Paratuberculosis en canidos silvestres en el suroeste de Europa

Zorro buscando alimento. Los cánidos son predadores y carroñeros, por lo que potencialmente podrían servir de indicadores de la circulación de micobacterias. Foto: IREC.

Capítulo 3

Los carnívoros silvestres están en la cúspide de la pirámide trófica. Son predadores y pueden ser carroñeros. Por lo tanto, tienen más posibilidades de entrar en contacto con agentes patógenos contaminantes del medio ambiente o presentes en animales. Los carnívoros infectados con MAP generalmente no muestran lesiones a nivel anatomopatológico. En Norteamérica se han encontrado niveles relativamente altos de contacto con MAP en mapaches (*Procyon lotor*), zorros (*Vulpes vulpes*), coyotes (*Canis latrans*), gatos (*Felis catus*) y mofetas (*Mephitis mephitis*). Además de esto, los zorros, por ejemplo, pueden realizar grandes desplazamientos, suponiendo potencialmente una capacidad para difusión a larga distancia de la infección. Hemos partido de la hipótesis de que los cánidos silvestres pueden ser usados como centinelas en áreas con alta prevalencia de *Mycobacterium avium paratuberculosis* en fauna silvestres y domestica. Para comprobar esta hipótesis, hemos adaptado un ELISA indirecto para la detención de anticuerpos frente a MAP en sueros de 23 lobos (*Canis lupus*) y 239 zorros, procesando también un pool de muestras para cultivo (n=61) y analizando 15 muestras mediante PCR especifica para MAP y 14 para histopatología. En los lobos, los valores de la densidad óptica (OD) en ELISA estaban distribuidos de forma continua. Diez zorros (4%) tenían una densidad óptica superior al doble de la media, lo que sugiere el contacto con micobacterias. Sin embargo, todas las muestras testadas mediante PCR resultaron negativas para PCR en tiempo real utilizando los cebadores IS900 y ISMAP02. Igualmente, ninguno de los cultivos presentó crecimiento alguno. Tampoco se revelaron lesiones compatibles con paratuberculosis, ni se observaron cambios reseñables en los estudios histopatológicos. En conjunto, estos resultados sugieren que los cánidos silvestres muestran poca o ninguna evidencia de paratuberculosis, y que, en consecuencia, no resultan de utilidad como centinelas para la vigilancia sanitaria de MAP en el suroeste de Europa.

Eur J Wildl Res
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ERRATUM

Erratum to: Lack of evidence of paratuberculosis in wild canids from Southwestern Europe

Tania Carta · O. Aurtenetxe · Raquel Sobrino · L. Mamian · X. Gerrikagoitia · A. Balseiro · A. Oleaga · I. A. Sevilla · M. Barral · J. M. Garrido · Christian Gortazar

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Author order was accidentally changed during copy editing. The first author of this paper is Tania Carta, while co-author Raquel Sobrino is the third author in the manuscript.

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SHORT COMMUNICATION

Lack of evidence of paratuberculosis in wild canids from Southwestern Europe

Raquel Sobrino · O. Aurtenetxe · Tania Carta · L. Mamian · X. Gerrikagoitia · A. Balseiro · A. Oleaga · I. A. Sevilla · M. Barral · J. M. Garrido · Christian Gortazar

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Abstract Wild carnivores are at the top of the trophic chain. They are predators and carrion consumers, and thus, prone to come in contact with disease agents contaminating the environment or infecting live or dead animals. We hypothesized that wild canids could be used as sentinels for the detection of regions with higher *Mycobacterium avium paratuberculosis* (MAP) prevalence in wild and domestic animals. To test this hypothesis, we set up an ELISA to test 262 wolf (*Canis lupus*) and fox (*Vulpes vulpes*) sera for MAP-specific antibodies and processed a subset of samples for culture (n= 61), MAP-specific PCR (15) and histopathology (14). In wolves, the optical density (OD) values in the ELISA were continuously distributed. Ten fox sera (4%) had OD readings of over twice the mean, suggesting contact with mycobacteria. However, all samples tested by PCR were negative for both IS900 and ISMAP02 sequences, and samples cultured for MAP yielded no growth. No visible paratuberculosis or tuberculosis-compatible lesions were recorded. On histopathological examination, no lesions compatible with mycobacterial diseases were observed. These results suggest that wild canids show little or no evidence of paratuberculosis and are unlikely to be useful sentinels for the detection of MAP in Southwestern Europe.

Keywords Carnivore · Johne's disease · *Mycobacterium avium paratuberculosis* · *Mycobacterium bovis* · Wildlife sentinel

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Introduction

Animals may serve as indicators of human health threats in the environment. Examples include the emergence of zoonotic diseases in wildlife populations, concurrent with a novel outbreak of disease in humans, such as West Nile virus, SARS, and avian influenza (Scotch et al. 2009). Wild animals can also act as indicators of diseases circulating among domestic animals or other wildlife. For example, white-tailed deer (*Odocoileus virginianus*) have been used to detect *Anaplasma phagocytophilum* (Rainwater et al. 2006) and *Ehrlichia chaffeensis* (Yabsley et al. 2003), and feral pigs (*Sus scrofa*) to detect bovine tuberculosis (Nugent et al. 2002). These indicator species are known as "sentinels".

Wild carnivores are at the top of the trophic chain. They are predators and carrion consumers, and thus, prone to come in contact with disease agents contaminating the environment or infecting live or dead animals (Anderson et al. 2007; Sobrino et al. 2007); therefore, they could act as sentinels. For example, sea otters (*Enhydra lutris nereis*) can act as sentinels to detect *Toxoplasma gondii* contamination in

coastal environments (Conrad et al. 2005) and coyotes (*Canis latrans*) have been used to detect *Mycobacterium bovis* circulation in wildlife and domestic animals. In Michigan, focusing surveillance on coyotes, rather than on white-tailed deer increased the detection of *M. bovis* by 40% (VerCauteren et al. 2008).

The Iberian Peninsula is one of the last strongholds of the wolf (*Canis lupus*) in Europe, with an estimated population of 2,500 individuals, mainly in the north-west of the peninsula (Blanco 1998). Wolves in Spain depend largely on domestic and wild ungulates as a food source (Cuesta et al. 1991; Barja 2009). Red foxes (*Vulpes vulpes*) are ubiquitous, anthropophilic generalists, with a species abundance ranging from 0.5 to 10 foxes per square kilometer (Gortázar 1997). They are also a game species, which makes sample collection relatively easy. In Spain, the fox behaves as a facultative predator, feeding on rabbits (*Oryctolagus cuniculus*) when they are abundant and shifting to other prey (including carrion of wild and domestic ungulates) when rabbits are scarce (Delibes-Mateos et al. 2008).

Paratuberculosis is chronic enteritis that mainly occurs in wild and domestic ruminants, caused by *Mycobacterium avium paratuberculosis* (MAP), a member of the *Mycobacterium avium* complex (Thorel et al. 1990). MAP also occurs in many non-ruminant mammals and in several bird species. However, the significance of MAP in non-ruminant wildlife is largely unknown (Daniels et al. 2003). Among carnivores, sporadic isolation of MAP has been reported in foxes, Eurasian badgers (*Meles meles*), stoats (*Mustela erminea*), and weasels (*Mustela nivalis*) in Scotland (Beard et al. 2001), and from a red fox in Greece (Florou et al. 2008). In Spain, MAP is widespread among both domestic and wild ruminants (Garrido 2001; Falconi et al. 2010) and has also been recorded in wild rabbits (the authors, submitted). Among red deer (*Cervus elaphus*), for instance, 30% antibody prevalence was reported, and contact with cattle was identified as a risk factor (Reyes-García et al. 2008).

Hence, the available information suggests that wild carnivores could be useful in surveillance schemes for mycobacteria, including MAP. We hypothesized that foxes (and wolves to a lesser extent due to their limited availability) could be used as sentinels to identify regions with higher MAP prevalence among domestic and wild ruminants. To test this hypothesis, we sampled wild canids from Spain, set up an ELISA to test for MAP-specific antibodies and processed a subset of samples for culture, MAP-specific PCR, and histopathology.

Material and methods

In the period of 2004–2009, samples were collected from 24 wolf and 285 fox carcasses from different Spanish regions (Fig. 1). All animals had been legally obtained as road kills (wolves and foxes) or from hunters (foxes only), and were frozen at −20°C until necropsy. Age class, which was assigned as yearling (<1 year) or adult (>1 year) was determined by tooth eruption and the degree of tooth wear (Sáenz de Buruaga et al. 2001). Age or sex was not known for 36 foxes and 18 wolves. In the laboratory, carcasses were thawed and examined at necropsy for visible lesions. Separate, clean instruments were used for each animal to reduce the risk of cross-contamination. Serum samples were obtained by centrifugation of thoracic blood and stored at −20°C until their analysis. Samples of ileocecal valve (ICV) and mesenteric lymph nodes (mLN) were transferred to clean containers and frozen in duplicate at −20°C until analysis. Table 1 presents the number of samples processed for histopathology, ELISA, PCR and culture, respectively.

The ELISA test to detect antibodies against MAP was performed adapting protocols reported previously for ruminants (Garrido 2001; Sevilla et al. 2007; Reyes-García et al. 2008). Briefly, high adsorption capacity Costar polystyrene microtiter plates (Cultek, Madrid, Spain) were coated with 50 µl/well of 0.02 mg/ml paratuberculosis protoplasmatic antigen 3 (PPA-3) diluted in carbonate/bicarbonate buffer (Sigma, Madrid, Spain). The serum samples were adsorbed (1:1, *v/v*) with a saline suspension of *Mycobacterium phlei* (5 g/l) (Allied Monitor, Inc., Fayette, MO, USA) and left at 4°C overnight to remove nonspecific anti-*Mycobacterium* spp. antibodies (Milner et al. 1987). Thereafter, the plates were washed once with a washing solution (PBS containing 0.05% Tween 20) and blocked with 200 µl/well of blocking solution (5% nonfat dried milk in PBS containing 0.05% Tween 20). After a 1-h incubation period at room temperature, sera diluted 1:20 (*v/v*) in PBS solution were added into wells of the antigen-coated plate. The plates were incubated at 37°C for 1 h, before being washed four times with the washing solution. Anti-dog IgG peroxidase antibody produced in rabbits was used as conjugate (Sigma, 0.002 mg/ml in blocking solution) and incubated at 37°C for 1 h. After four washes with washing solution, 200 µl/well of substrate solution (Fast OPD, Sigma) were added. Approximately 20 min later, the reaction was stopped with 50 µl/well of H_2SO_4 3N and optical density (OD) was measured in a spectrophotometer at 450 nm. Since no positive controls were available, we used 2×the mean OD as a conservative arbitrary cutoff (see Fig. 2).

A modified version of the Adiapure ® kit (Adiagene, Saint Brieuc, France) was used for DNA extraction from tissue samples (ICV and mLN). A sample of 2.5 g was weighed in a Stomacher blending bag with filter and 10 ml of sterile water was added. After homogenization in a Stomacher lab blender, 300 µl of filtered liquid was transferred into 2-ml microcentrifuge tubes containing

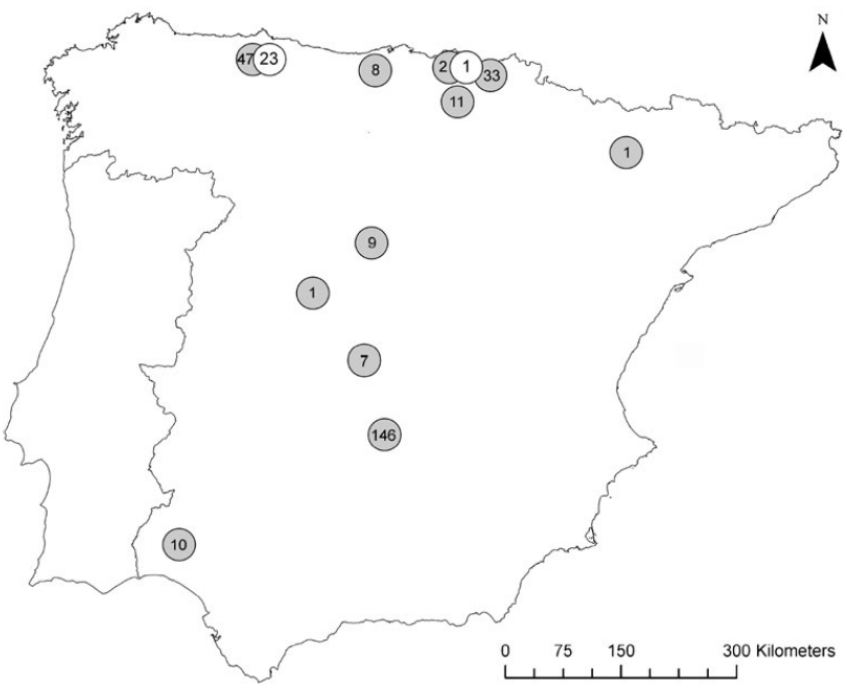

Fig. 1 Map of continental Spain showing the sample size by site and species (foxes, *Vulpes vulpes*, in *gray*; and wolves, *Canis lupus*, in *white*)

300 mg of glass beads. Then 300 µl of L1 buffer (Adiapure) was added, and the tubes were shaken three times at 4,000 rpm for 45 s in a Precess 48 homogenizer (Biorad, Hemel Hempstead, Hertfordshire, UK). After mechanical disruption, samples were centrifuged at 7,500 g for 5 min. We transferred 300 µl of supernatant into a 1.5-ml microcentrifuge tube containing 20 µl of L2 reagent (Adiapure) and the mixture was incubated at 70°C for 10 min. An additional incubation at 95°C for 15 min was carried out. Samples were shortly centrifuged at full speed to collect all the content at the bottom of the tube, and 300 µl of this mixture was transferred into an F1 plate (Adiapure) well. Subsequent steps were performed as indicated by the manufacturer of the kit.

DNA extracts were used in a triplex real-time PCR targeting IS900 (Herthnek et al. 2006) and ISMAP02

Table 1 Number of red fox (*Vulpes vulpes*) and wolf (*Canis lupus*) samples analyzed by each technique for the detection of MAP antibodies by ELISA, MAP DNA by PCR, MAP growth in culture, and paratuberculosis-compatible lesions by histopathology

	Samples tested			
Host species	ELISA	PCR	Culture	Histopathology
Red fox	239	14	56	13
Wolf	23	1	5	1
Total	262	15	61	14

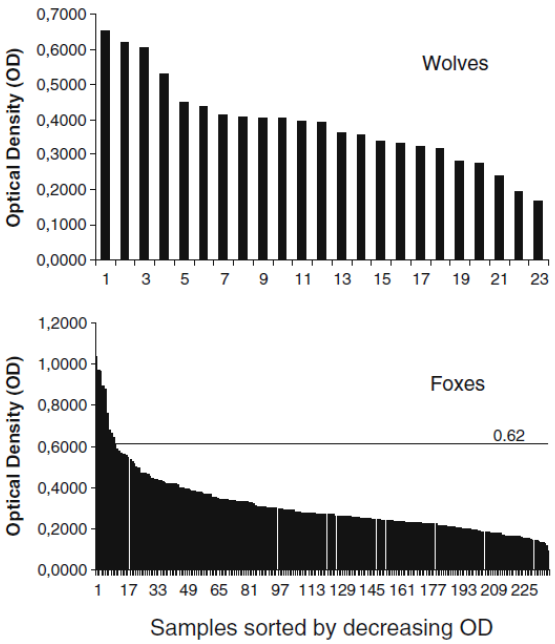

Fig. 2 Distribution of OD values for red fox sera (n=239) and wolf sera (n=23) in the antigen-adsorbed ELISA. Plates were coated with PPA3. The *solid line* in the fox graph represents twice the mean OD, showing that ten samples had ODs above this arbitrary cutoff

sequences of MAP and an internal amplification control (IAC) to rule out inhibition of the reaction (Sevilla et al., submitted). The 50-µl PCR mixture contained 5 µl of template DNA, 1× TaqMan Universal MasterMix (Applied Biosystems, CA), 0.4 µM (each) of primers co-amplifying ISMAP02 and the IAC, 0.3 µM (each) of IS900 primers, 0.2 µM (each) of ISMAP02, IAC and IS900 probes and 2 µl of IAC template DNA (Sevilla et al., submitted). Amplification was carried out in an Applied Biosystems 7500 Real-Time PCR System under the following standard conditions: 1 cycle at 95°C for 10 min and 45 cycles with two steps of 95°C for 15 s and 60°C for 1 min. The performance of the PCR was monitored using a negative and a positive DNA control (ATCC 19698 reference strain).

For each culture, 2 g of a pool of similar volumes of ileocecal valve and mesenteric lymph node of each animal were processed. Samples were homogenized and decontaminated with 38 ml of a solution (0.75%) of hexadecilpiridinium chloride (HPC; Aduriz et al. 1995). Three drops of the homogenate were inoculated on homemade Herrold's Egg Yolk medium (HEYM) and Löwestein–Jensen medium (LJ), both supplemented with mycobactin J (Allied Monitor) and Middlebrook 7H11 supplemented with 1% Middlebrook OADC Enrichment (Becton, Dickinson and Company, MD, USA) (Sevilla et al. 2007). Tubes were incubated at 37°C and inspected monthly. They were considered negative if no bacterial growth was observed after 20 weeks.

For histopathological studies, samples (retropharyngeal and submandibular LN, lungs, heart, spleen, kidney, ICV, and mLN) from 13 animals were fixed in 10% neutral buffered formalin, dehydrated in graded ethanol solutions, embedded in paraffin wax, sectioned at 4-µm thickness and stained with H&E and Ziehl–Neelsen (ZN) for acid-fast bacteria (AFB).

Results

Figure 2 presents the OD of all wild canid sera tested for antibodies against PPA3. In wolves, the OD values in the ELISA were not discrete and were continuously distributed. The mean OD for wolf sera was 0.39, and no OD was higher than twice this value. In fox sera, the mean OD was 0.31 and 10 samples (4.18%) had OD readings of over twice this mean. These included 9 of 156 sera from Southern Spain and 1 of 119 from Northern Spain (Fisher's test, p=0.032). The red fox sample with the highest response had an OD of 1.03.

All samples tested by PCR were negative for both IS900 and ISMAP02 sequences, and all controls yielded the expected result. Inhibition of the reaction was ruled out in all assays by the positive signal observed for the IAC probe. Tissue samples cultured for MAP yielded no isolation, and no bacterial growth was observed.

No visible paratuberculosis or bTB-compatible lesions were recorded during the necropsies. On histopathological examination, no lesions compatible with mycobacterial diseases were observed in the tissues studied. AFB was not demonstrated by ZN stain.

Discussion

Results reported herein led us to reject the initial hypothesis that wild canids could be used as paratuberculosis sentinels in a zone where the prevalence of paratuberculosis in wild ruminants is high (Reyes-García et al. 2008). This contrasts with data from Wisconsin (USA), where MAP-specific DNA was detected in a high proportion of scavenging mammals, including coyotes and red foxes (Anderson et al. 2007).

In the absence of PCR confirmation or MAP isolation by culture, the interpretation of ELISA results is difficult. Wolves were sampled in regions of Northern Spain with very low bTB prevalence in cattle and almost no wildlife TB (Gortázar et al. in press). No wolf serum yielded high ODs, suggesting no contact with MAP or cross-reacting mycobacteria. In the foxes, a few sera (4%) had relatively high ODs, suggesting some contact with mycobacteria. These occurred mainly in two bTB endemic areas of Southern Spain, suggesting that cross-reactions after contact with *M. bovis* could have influenced the ELISA results. Serological cross-reactions of *M. bovis*, and MAP have often been reported (e.g., Buddle et al. 2010). Alternatively, rabbits are more abundant in Southern Spain and consumption of MAP-infected rabbits could also explain the few antibody-positive fox sera.

The absence of mycobacterial isolations was no surprise, considering the low sensitivity of this technique (Anderson et al. 2007). In contrast, the lack of PCR detection of MAP-specific DNA is interesting. The methods used for the carnivores in this survey have a high sensitivity (Herthnek et al. 2006). As was discussed earlier, both wolves and foxes include significant portions of wild ruminants and rabbits in their diet. Thus, exposure to MAP was expected but not confirmed. The contrast with the high-PCR positivity recorded among carnivores in Wisconsin (Anderson et al. 2007) may be explained by the small number of PCR-tested samples in the present study. Alternatively, it might be due to differences in specificity or sensitivity between the PCR protocols used.

Since canids only rarely develop lesions when they have a generalized *M. bovis* infection (Millan et al. 2008), the lack of paratuberculosis and bTB-compatible lesions was not a surprising finding in this study. However, this lack of

visible lesions in a large sample of wild canids, along with the absence of microscopic lesions in the studied subsample, adds to the view that wild canids play no relevant role in the epidemiology of mycobacterial diseases in Southwestern Europe. This finding, in addition to the absence of MAP detection by culture and PCR, suggests that wild canids show little or no evidence of paratuberculosis and are unlikely to be useful sentinels for the detection of MAP in Southwestern Europe. However, studies on other situations or even experimental studies that would be needed before definitive conclusions on the role of canids in MAP epidemiology can be drawn.

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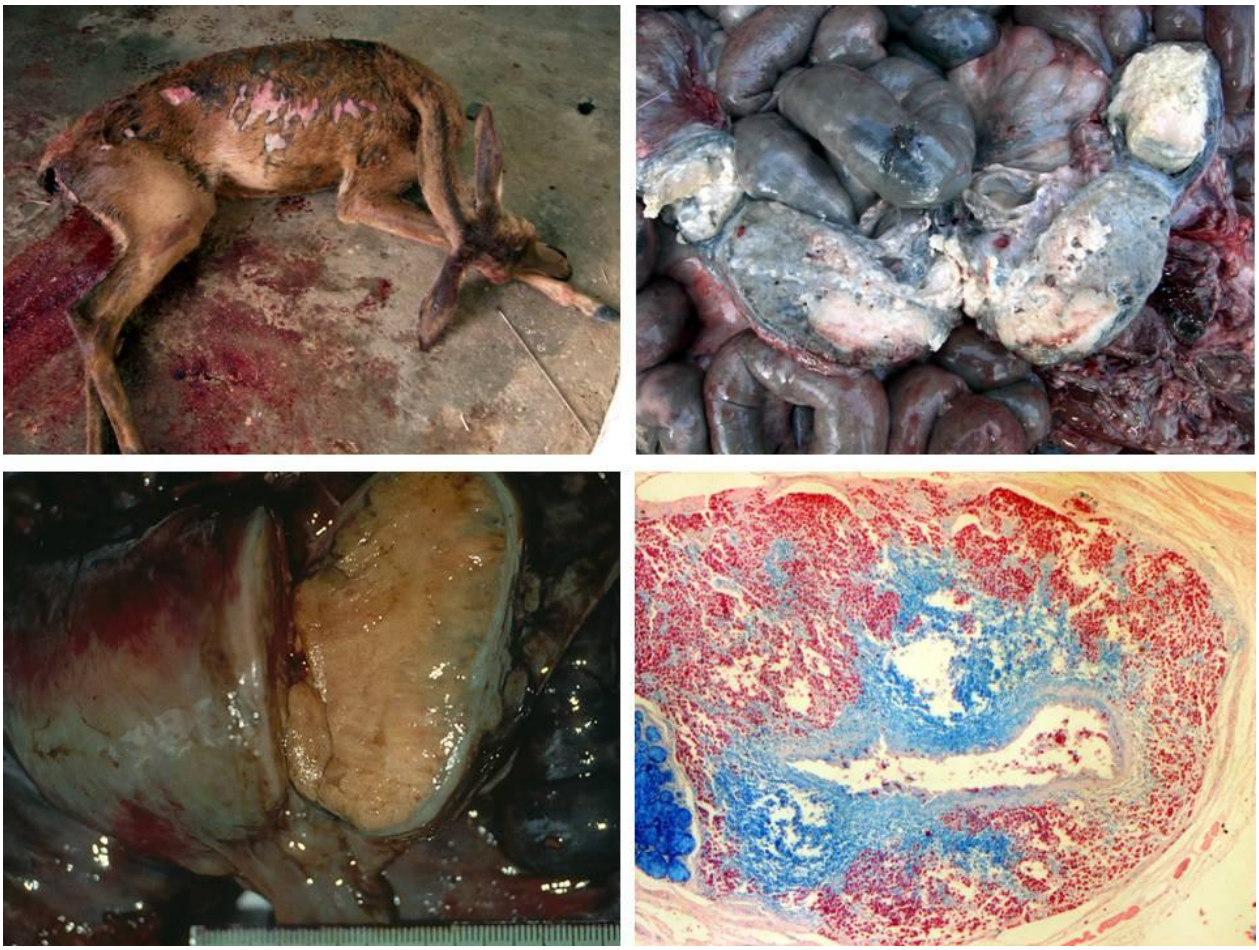
CAPITULO 4

Importancia del ciervo (*Cervus elaphus*) como posible reservorio de MAP en la península ibérica



Fotocomposición sobre paratuberculosis en ciervo. Arriba a la izquierda, primala de granja sacrificada por emaciación y diarreas. Arriba a la derecha, lesiones necróticas difusas en los linfonodos mesentéricos de la misma primala (Fotografías de M. Reglero). Abajo a la izquierda, linfadenitis granulomatosa en un ciervo de los Alpes italianos (Foto: P. G. Meneguz). Abajo a la derecha, corte histológico de la tonsila de una cierva de granja con paratuberculosis, teñida con la técnica de Ziehl - Neelsen (Foto: IREC).

Capítulo 4

El papel que pueda jugar el ciervo (*Cervus elaphus*) como reservorio de *Mycobacterium avium paratuberculosis* (MAP) es todavía desconocido. Algunas referencias encuentran prevalencias de infección de hasta el 33% en los Alpes, mientras que en otro lugares se señala una prevalencia muy baja (0-0.5%). Considerando la alta densidad poblacional del ciervo en algunas regiones de la península ibérica, que llega a alcanzar los 70 individuos por km^2, en esta tesis hemos partido de la hipótesis de que la paratuberculosis podría ser detectada en un elevado numero de ciervos ibéricos. En este trabajo hemos analizado, mediante patología, cultivo y serología, muestras provenientes de 332 ciervos cazados entre 2005 y 2009. La histopatología reveló solamente tres animales (1.12%) que mostraban lesiones focales. No se observó crecimiento en cultivo. En ELISA, las densidades ópticas en placas antigenadas con Paratuberculosis Protoplasmatic Antigen 3 (PPA3) y bovine Purified Protein Derivative (bPPD), respectivamente, estaban significativamente correlacionadas. Ello sugiere una reacción cruzada y, por lo tanto, una falta de especificad del test. Los resultados obtenidos contrastan con la situación descrita en otras regiones, y permiten descartar al ciervo como reservorio de paratuberculosis en el área de estudio.

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Short Communication

No evidence that wild red deer (*Cervus elaphus*) on the Iberian Peninsula are a reservoir of *Mycobacterium avium* subspecies *paratuberculosis* infection

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ABSTRACT

The potential role of red deer (*Cervus elaphus*) as a reservoir of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection is largely unknown. A total of 332 wild red deer were investigated using post-mortem examination, bacteriology and serology. Only three animals (1.12%) were found to have lesions on histopathological examination and no MAP bacteria were recovered on culture. The results suggest it is unlikely that wild red deer make a significant contribution to the maintenance of MAP infection in the region. The cross-reactivity of the ELISAs used indicates this diagnostic modality is ineffective in the detection of MAP infection in this species. The implications of these results for the control of this important pathogen in both livestock and wildlife are discussed.

Paratuberculosis (Johne's disease) is a chronic enteritis of ruminants with a worldwide distribution. It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Wildlife can contribute to the maintenance of MAP infection in livestock (Kopecna et al., 2008). While a high prevalence of MAP infection (33%) has been reported in wild red deer (*Cervus elaphus*) in the Italian Alps (Robino et al., 2008), other studies have found much lower prevalence of infection (0–0.5%) (Álvarez et al., 2005; Kopecna et al., 2008).

Antibodies to paratuberculosis protoplasmatic antigen (PPA)-3 were found in only 4% of Norwegian red deer (Tryland et al., 2004), in contrast to the serological evidence of widespread exposure to MAP or cross-reacting mycobacteria in Spain (30%) (Reyes-Garcia et al., 2008). In southern Spain, MAP was detected in red deer by PCR (Reyes-Garcia et al., 2008), although infection was not confirmed by culture (Álvarez et al., 2005). Throughout the Iberian Peninsula the red deer population is expanding, reaching densities of up to 70 per km^2. We hypothesised that MAP infection could be present in a significant proportion of these animals.

Samples from 332 hunter-killed wild red deer were obtained between 2005 and 2009 (Fig. 1 and Table 1 and Supplementary material). The presence of macroscopically visible lesions suspicious of tuberculosis (Martín-Hernando et al., 2010) was recorded, and samples of the jejunal and ileal mesenteric lymph nodes, and of the ileocaecal valve (ICV) were fixed in formalin and processed for histopathological examination (Balseiro et al., 2008). Samples of pooled mesenteric lymphoid tissue were inoculated onto mycobactin-supplemented Herrold's egg yolk, Löwestein-Jensen media and Middlebrook 7H11 media (Sevilla et al., 2007). An adsorbed ELISA was performed to detect serum antibodies to MAP and *Mycobacterium bovis* (Reyes-Garcia et al., 2008) (see Supplementary material).

No macroscopically visible lesions suspicious of paratuberculosis were observed and there was no evidence of diarrhoea in any of the sampled animals. On histopathological examination, three animals (1.12%; 95%, CI 0.3–3.3%) had small granulomas in the inter-follicular areas of their intestinal lymphoid tissue consistent with paratuberculosis. No acid-fast bacilli were present on Ziehl–Neelsen staining of the lesions, and MAP was not cultured (95%, CI 0–1.2%). Two of these deer were from population 'A' (2/83, 2.4%) and one was from population 'I' (1/20, 5%) (Fig. 1), neither of which population had any contact with deer farms. Lesions consistent with tuberculosis were found in 25 animals (8.4%). Serology detected antibodies to PPA-3 and bovine purified protein derivative (bPPD), and these results were correlated ($r_s = 0.37$; $P < 0.05$) (see Supplementary material).

The results of this survey suggest it is unlikely that wild red deer make a significant contribution to the maintenance of MAP infection in the region. The cross-reactivity of the ELISAs we used indicates this diagnostic modality is ineffective in the detection of MAP infection in this species. The 'negative' results do however

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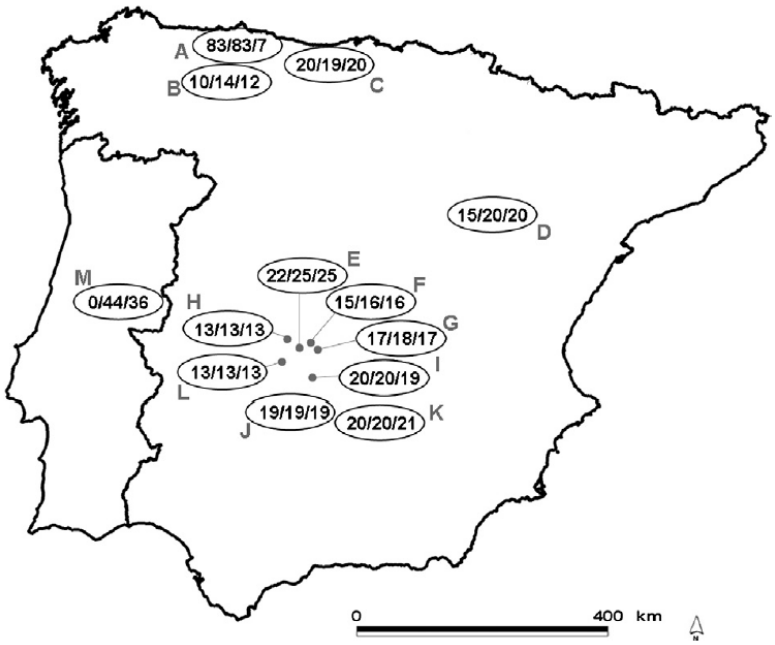

Fig. 1. Map of Iberian Peninsula illustrating the 13 sampling sites (indicated by letters A–M). The numbers within the circles indicate the number of red deer examined using histopathological examination, bacteriological culture and ELISA, respectively. Populations sampled in south-central Spain included sites with high to very high animal densities (mean 20; maximum 69 per km^2). Sampling sites in northern Spain included areas of high animal density where there was close contact with domestic livestock (mainly cattle). No farmed deer were sampled.

Table 1
Details of numbers of samples processed for histopathological examination, bacteriological examination and for ELISA.

Method	Number of samples	Number of sites	Comments
Recording of grossly visible lesions suspicious of paratuberculosis (abdomen)	332	13	See Balseiro et al. (2008)
Recording of grossly visible lesions suspicious of tuberculosis (head, thorax, abdomen)	296	13	See Martín-Hernando et al. (2010)
Histopathology	267	12	Both haematoxylin and Eosin and Ziehl–Neelsen staining carried out
Bacteriological culture	324	13	Mesenteric lymph node or ileo-caecal valve
ELISA	238	13	Using paratuberculosis protoplasmatic antigen-3 (PPA-3) and bovine purified protein derivative (bPPD)

have interesting implications for wildlife disease surveillance. Firstly, given that clinical paratuberculosis occurs in farmed deer, care should be taken to avoid introducing the infection by releasing farmed deer into the wild. Secondly, since paratuberculosis is uncommon in wild deer, it should not interfere with the diagnosis of tuberculosis in this species (Martín-Hernando et al., 2010).

Why MAP infection is much more commonly found in farmed than in wild red deer remains unclear (Fernandez-De-Mera et al., 2009). One possible explanation is related to the fact that calves in the wild are born away from the main herd, only joining the herd some weeks later (Putman, 1988), a behaviour that could decrease the likelihood of neonates becoming infected (Mackintosh et al., 2010). However, the findings of the current survey are difficult to reconcile with the high prevalence of infection reported in red deer in the Alps (Robino et al., 2008).

A further point of interest is the high MAP infection prevalence observed in fallow deer populations on the Iberian Peninsula vs. sympatric red deer (Population A). Balseiro et al. (2008) detected a high MAP infection prevalence among fallow deer, including cases of multibacillary paratuberculosis suggesting significant MAP excretion. However, none of the samples from animals from this location analysed in the context of the present study were found to be infected. The fact that fallow deer are more gregarious and graze, as opposed to browse, more than red deer (Putman, 1988), might account for their greater and or earlier exposure risk. Our results in relation to red deer are similar to those obtained for other wild ruminants such as roe deer (*Capreolus capreolus*), mouflon (*Ovis aries*), and chamois (*Rupicapra pyrenaica*), suggesting that wildlife plays a minor role in the maintenance of MAP infection on the Iberian Peninsula.

Conflict of interest statement

None of the authors of this paper 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tvjl.2011.08.010.

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CAPITULO 5

Análisis de la relación hospedador-patógenos intracelulares (*Anaplasma* y *Mycobacterium)* en el ciervo ibérico

Ciervo común. Los rumiantes silvestres infectados de forma natural pueden aportar información interesante para el conocimiento de las interacciones entre patógenos intracelulares y hospedadores. Foto: Tania Carta (IREC).

Capítulo 5

Los patógenos de los géneros *Anaplasma* y *Mycobacterium* figuran entre los patógenos bacterianos más prevalentes en el ciervo (*Cervus elaphus*) del sur-oeste de España. Se sabe que estos patógenos modifican la expresión génica en los rumiantes. En este trabajo hemos utilizado la hibridación con microarray y la RT-PCR en tiempo real (qRT-PCR) para (1) caracterizar el perfil global de la expresión génica en el ciervo en respuesta a las infecciones por *Anaplasma ovis* y *Anaplasma ovis/Mycobacterium bovis*/ *Mycobacterium avium* subs. *paratuberculosis* (MAP); (2) comparar la expresión génica de la respuesta inmune en ciervos infectados con *A. ovis*, *M. bovis* y *A. ovis/M. bovis/*MAP, así como para (3) caracterizar la diferencia de expresión de genes relacionados con la inmunidad en ciervos y bóvidos infectados con *M. bovis* y *Anaplasma marginale*. Globalmente, el análisis conjunto de la expresión diferencial de genes en ciervos infectados con *A. ovis* y *A.ovis/M. bovis/*MAP reveló la modificación de ciertos procesos biológicos celulares comunes y específicos para estos patógenos. La expresión diferencial de genes relacionados con la inmunidad del hospedador mostró patrones característicos de la respuesta del hospedador al patógeno, así como el efecto de la infección con múltiples patógenos en la respuesta inmune del ciervo. Estas diferencias específicas de la respuesta al patógeno y del hospedador podrían contribuir a mejorar el diagnóstico de las enfermedades causadas por *Anaplasma* y *Mycobacterium*, así como su control en rumiantes.

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Characterization of pathogen-specific expression of host immune response genes in *Anaplasma* and *Mycobacterium* species infected ruminants

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ABSTRACT

Anaplasma and *Mycobacterium* species are among the most prevalent bacterial pathogens in European red deer (*Cervus elaphus*) in south-central Spain and are known to modify gene expression in ruminants. In this study, we used microarray hybridization and real-time RT-PCR analyses to characterize global gene expression profiles in red deer in response to *Anaplasma ovis* and *A. ovis*/*Mycobacterium bovis*/*Mycobacterium avium* sub. *paratuberculosis* (MAP) infections, compare the expression of immune response genes between red deer infected with *A. ovis*, *M. bovis* and *A. ovis*/*M. bovis*/MAP, and characterize the differential expression of immune response genes identified in red deer in cattle infected with *M. bovis* and *Anaplasma marginale*. Global gene differential expression in *A. ovis*- and *A. ovis*/*M. bovis*/MAP-infected deer resulted in the modification of common and pathogen-specific cellular biological processes. The differential expression of host immune response genes showed pathogen and host-specific signatures and the effect of infection with multiple pathogens on deer immune response. These results suggested that intracellular bacteria from *Anaplasma* and *Mycobacterium* genera produce similar genes expression patterns in infected ruminants. However, pathogen and host-specific differences could contribute to disease diagnosis and treatment in ruminants.

1. Introduction

The most prevalent bacterial pathogens in European red deer (*Cervus elaphus*) in south-central Spain are *Mycobacterium* (*Mycobacterium bovis* and *Mycobacterium avium* sub. *paratuberculosis*) and *Anaplasma* (*Anaplasma ovis*, *Anaplasma marginale* and *Anaplasma phagocytophilum*) species [1–7].

The genus *Anaplasma* includes intraerythrocytic rickettsial pathogens of ruminants, *A. marginale*, *A. centrale*, *A. bovis*, and *A. ovis* [8]. Also included in this genus are *A. phagocytophilum*, which infects a wide range of hosts including humans, wild and domesticated animals, and *A. platys* that infects dogs [8]. *A. ovis* is a pathogen of sheep, goats and wild ruminants [6,9–22]. Although ovine anaplasmosis is more frequently associated with hemolytic anemia in goats, *A. ovis* can also cause disease in sheep and wild ruminants, particularly in animals exposed to stress or other predisposing factors [14,17,20,23]. Bovine anaplasmosis is caused by *A. marginale* in tropical and subtropical regions of the world [15,24]. *A. phagocytophilum* is the causative agent of tick-borne fever (TBF) in ruminants and human, equine and canine granulocytic anaplasmo-

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sis [8,25]. Ticks are biological vectors of *Anaplasma* spp. [14,15,22,26]. Mammalian or tick hosts with persistent infection serve as reservoirs of these pathogens in nature [15].

M. bovis, *M. avium* sub. *paratuberculosis* (MAP) and closely related species of the *Mycobacterium tuberculosis* complex are primarily intramacrophagic mycobacteria that constitute an important health problem affecting domestic animals, wild animals and humans worldwide [27–33]. The role of wild animals in the maintenance and spread of *M. bovis* and MAP infections represents important economic, environmental and health risks and complicate control efforts in some regions [2,3,27,29–31,34–37].

Anaplasma and *Mycobacterium* spp. are known to modify gene expression profiles in infected ruminants [38–43]. However, few studies have addressed pathogen-specific differential gene expression associated with the systemic response of ruminants to *Mycobacterium* and *Anaplasma* spp. infections [38].

The objectives of this study were (a) to characterize gene expression profiles in red deer in response to *A. ovis* and *A. ovis*/*M. bovis*/MAP infections, (b) to compare the expression of immune response genes between red deer infected with *A. ovis*, *M. bovis* and *A. ovis*/*M. bovis*/MAP, and (c) to characterize the differential expression of immune response genes identified in red deer in cattle infected with *M. bovis* and *A. marginale* to compare the results between wild and domestic ruminants in response to *Mycobacterium* and *Anaplasma* infections. The study of pathogen-specific gene expression profiles in wild and domestic ruminants infected with *Mycobacterium* and/or *Anaplasma* spp. will expand our current information on the mammalian host responses to pathogen infection and may contribute to the over all understanding of the molecular mechanisms involved in tuberculosis, paratuberculosis and anaplasmosis.

2. Materials and methods

2.1. Animals and sample preparation

Samples were collected in the Doñana Biological Station in southern Spain from hunter-harvested adult (≥5 yr old) male European red deer uninfected ($N = 8$) and naturally infected with *A. ovis* ($N = 7$), *M. bovis* ($N = 3$) or *A. ovis*/*M. bovis*/MAP ($N = 4$). To analyze gene expression profiles in response to pathogen infection in peripheral blood mononuclear cells (PBMC), spleen fragments of approximately 2 cm^3 were dissected and rapidly stored at −80 °C for RNA and DNA extraction. The mesenteric lymph nodes were dissected and used for culture and spoligotyping of mycobacteria.

For cattle, blood samples were obtained from calves uninfected ($N = 7$) and naturally infected ($N = 17$) with *M. bovis* in southern Spain and from cows uninfected ($N = 8$) and experimentally infected ($N = 8$) with *A. marginale* Oklahoma isolate [44] at Oklahoma State University, USA.

Animal experimentation was conducted with the approval and supervision of the Institutional Animal Care and Use Committees.

Total RNA and DNA were extracted from spleen and blood samples using TriReagent (Sigma–Aldrich, St. Louis, MO, USA) according to manufacturer's instructions.

2.2. Diagnosis of pathogen infection

For diagnosis of *Anaplasma* spp. infection, DNA extracted from red deer spleen and cattle blood samples was subjected to major surface protein 4 (*msp4*) PCR and sequence analysis [18,44,45]. One microliter (1–10 ng) DNA was used with 10 pmol of each primer (*A. marginale*/*A. ovis*: MSP45: 5′-GGGAGCTCCTATGAATTACAGAGAATTGT TTAC-3′ and MSP43: 5′-CCGGATCCTTAGCTGAACAGGAATC TTGC-3′; *A. phagocytophilum*: MAP4AP5: 5′-ATGAATTACA GAGAATTGCTTGTAGG-3′ and MSP4AP3: 5′-TTAATTGAAA GCAAATCTTGCTCCTATG-3′) in a 50-μl volume PCR (1.5 mM $MgSO_4$, 0.2 mM dNTP, 1× AMV/*Tfl* reaction buffer, 5u *Tfl* DNA polymerase) employing the Access RT-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler for 35 cycles. After an initial denaturation step of 30 s at 94 °C, each cycle consisted of a denaturing step of 30 s at 94 °C, an annealing for 30 s at 60 °C and an extension step of 1 min at 68 °C for *A. marginale*/*A. ovis* and an annealing-extension step of 1 min at 68 °C for *A. phagocytophilum*. Negative control reactions were performed with the same procedures, but adding water instead of DNA to monitor contamination of the PCR. The program ended by storing the reactions at 10 °C. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 kb DNA Ladder, Promega). Amplified fragments were resin purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both strands by double-stranded dye-termination cycle sequencing (Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University or Secugen SL, Madrid, Spain). At least two independent clones were sequenced for each PCR. The *msp4* coding region was used for sequence alignment. Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) with an engine based on the Clustal W algorithm [46].

For diagnosis of *Mycobacterium* spp. infection in red deer, mesenteric lymph node samples were used for culture and spoligotyping of mycobacteria as reported previously [1]. *Mycobacterium* spp. infection in deer was determined based on the presence of tuberculosis-characteristic lesions, positive mycobacterial cultures and *M. bovis*/MAP spoligotypes [1,7]. In cattle, *M. bovis* infection was diagnosed based on positive reaction to tuberculin skin-test and the presence of tuberculosis-characteristic lesions [47].

2.3. Microarray hybridization and analysis

Total RNA from red deer spleen samples was resin purified (Qiagen Inc., Valencia, CA, USA) and checked using the Experion™ Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA) to evaluate the quality and integrity of RNA preparations. Three samples from *A. ovis*-infected deer and two samples from *A. ovis*/*M. bovis*/MAP-infected

Table 1
Primer sets and real-time RT-PCR conditions used for analysis of differentially expressed immune response genes.

Genbank accession number	Gene description	Upstream/downstream primer sequences (5′–3′)	PCR conditions[a]
XM_614306	Metallopeptidase domain 9 (meltrin gamma) (ADAM9)	Bov-ADAM9F: TGTCATGGACATGGGGTATG Bov-ADAM9R: CAGCTTTTCCGTAGCTGGTC	55 °C, 30 s/ 72 °C, 30 s
GQ497281	Complement component receptor 2 (CR2) (CD21)	Bov-CR2F: AAGGCTTTCTTTGGCTGTCA Bov-CR2R: TGTGTGCAAAGAAGGAGTGC	55 °C, 30 s/ 72 °C, 30 s
NM_001083689	Forkhead box P1 (FOXP1)	Bov-FOXP1F: TACAGCAGCAACACCTCCTG Bov-FOXP1R: CTTGGAAGGTGCAGAGGAAG	53 °C, 30 s/ 72 °C, 30 s
U20500	Interleukin 1, beta (IL1B)	Ci-IL1F: CAGTGCTACGCACATGTCTC Ci-IL1R: GAAGCTCATGCAGAACACCA	53 °C, 30 s/ 72 °C, 30 s
NM_001076534	Adaptor-related protein complex 3, beta 1 subunit (AP3B1)	Bov-AP3B1F: AGCCCACGCAATACAAAAAC Bov-AP3B1R: TGGCCCCACTCTTCTACATC	55 °C, 30 s/ 72 °C, 30 s
NM_175796	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B)	Bov-ATPSF: CCTCTAAGGTAGCGCTGGTG Bov-ATPSR: GGATTCTGCCCAATAAAGCA	55 °C, 30 s/ 72 °C, 30 s
DQ520243	CD5 molecule-like (CD5L)	Ci-CD5F: CATTATATTATATGCCCCATGCTT Ci-CD5R: GCATGGTGATCAAGCTCGT	55 °C, 30 s/ 72 °C, 30 s
XM_617543	CD80 molecule (CD80)	Bov-CD80F: CAGAGCTGACTTCCCTGTCC Bov-CD80Rv: TGGGACAGTGTTGTGTTGGT	55 °C, 30 s/ 72 °C, 30 s
NM_001034334	Lymphocyte-specific protein tyrosine kinase (LCK)	Bov-LCKF: CTGTAGTTGGGTGGGCTCAT Bov-LCKR: TCTTGATGACCAGGGGAAAG	55 °C, 30 s/ 72 °C, 30 s
BC151458	Presenilin 1 (PSEN1)	Bov-PRESENF: CCATGCCTGGCTTATTGTTT Bov-PRESENR: ATGCCTGCTGGAGTCTCAGT	55 °C, 30 s/ 72 °C, 30 s
NM_001099701	Phosphatase and tensin homolog (PTEN)	Bov-PTENF: GCAGTTACCAGGAGGCTCAG Bov-PTENR: TCACCCCAGAGGCTTAAATG	55 °C, 30 s/ 72 °C, 30 s
NM_174201	Tumor protein p53 inducible nuclear protein 1 (TP53)	Bov-TP53F: ATTTACGCGCGGAGTATTTG Bov-TP53R: CCAGTGTGATGATGGTGAGG	55 °C, 30 s/ 72 °C, 30 s
NM_001078074	Vav 1 guanine nucleotide exchange factor (VAV1)	Bov-VAV1F: GTGTGGTCGACATGGACAAG Bov-VAV1R: GTTCGGCTTCAGCCTTAGTG	55 °C, 30 s/ 72 °C, 30 s
CEU62112	*Cervus elaphus* beta-actin	Ci-ActF: CCCAGATCATGTTCGAGACC Ci-ActR: GGCGTACCCCTCGTAGATG	55 °C, 30 s/ 72 °C, 30 s
GH5465371	*Cervus nippon* cyclophilin	Ci-cycF: AGCACTGGGGAGAAAGGATT Ci-cycR: GTCTTGGCAGTGCAGATGAA	55 °C, 30 s/ 72 °C, 30 s
AF191490	*Bos taurus* beta actin	BovActF: CTAGGCACCAGGGCGTAAT BovActR: CACACGGAGCTCGTTGTAGA	55 °C, 30 s/ 72 °C, 30 s
NM_174152	*Bos taurus* cyclophilin	BovCycF: GGTCATCGGTCTCTTTGGAA BovCycR: TCTTACCTCCAGTGCCGTCT	55 °C, 30 s/ 72 °C, 30 s

[a] PCR conditions are shown as annealing/extension for real-time RT-PCR analysis.

deer were selected for microarray hybridization analysis together with two RNA samples from uninfected control animals. To obtain a comprehensive gene expression profile in response to pathogen infection, the GeneChip® Bovine Genome Array which contains 24,072 *Bos taurus* probe sets that interrogate approximately 23,000 transcripts (Affymetrix, Santa Clara, CA, USA; http://www.affymetrix.com/products_services/arrays/specific/bovine.affx) was used. One microgram total RNA was labeled using the GeneChip® HT IVT Labeling Kit (Affymetrix). The images were processed with Microarray Analysis Suite 5.0 (Affymetrix). All samples demonstrated characteristics of high-quality cRNA (3′/5′ ratio of probe sets for glyceraldehyde-3-phosphate dehydrogenase and beta-actin of 1.5) and were subjected to subsequent analysis. Raw expression values obtained directly from .CEL files were preprocessed using the RMA method [48]. Standard quality controls based on Affymetrix original methods including average background, scale factor, number of genes called present, 3′–5′ ratios computed from the MAS

5.0 algorithm and probe-level models (PLM) based on fitting a model for probe values and analyzing its residuals (Relative Log Expression and Normal Unscaled Standard Error) were performed. The mean expression of each probe set in uninfected controls was compared with that of the infected samples. The microarray data was analyzed using the free statistical language R and the libraries included in Bioconductor Project (www.bioconductor.org). The analysis to select differentially expressed genes was based on adjusting a linear model with empirical Bayes moderation of the variance, a technique similar to ANOVA specifically developed for microarray data analysis by Smyth [49]. The gene ontology enrichment analysis was performed with GOstats package [50]. All the microarray data were deposited at the NCBI Gene Expression Omnibus (GEO) under the platform accession number GPL2112 and the series number GSE21967.

2.4. Real-time RT-PCR

Real-time RT-PCR was performed on RNA samples from infected and uninfected red deer and cattle with immune response gene specific primers (Table 1) using the iScript One-Step RT-PCR Kit with SYBR Green and the iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) following manufacturer's recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample [51]. The mRNA levels were normalized against deer or bovine cyclophilin and beta-actin using the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0) [52]. In all cases, the mean of the duplicate values was used and data from infected and uninfected animals were compared using the Student's *t*-test ($P = 0.05$).

3. Results

3.1. Pathogen infection in red deer and cattle

In the red deer analyzed here, a single *A. ovis msp4* genotype (Genbank accession number HQ014384) was found with a sequence 99.9% identical (with only a single C × T nucleotide change at position 820) to sequences reported previously in roe deer in Spain (EF067341), ticks in Serbia (GQ925817) and goats and/or sheep in Italy and Hungary (AY702923, EU436160, EF190511). Cattle were experimentally infected with the *A. marginale* Oklahoma isolate. *M. bovis* spoligotypes in red deer were identical to spoligotypes recorded in cattle [53]. All naturally infected animals were sampled as adults in pathogen-endemic areas, thus probably showing chronic infections.

3.2. Gene expression profiles in A. ovis- and A. ovis/M. bovis/MAP-infected red deer

Of the approximately 23,000 transcripts that were analyzed in the microarray, 231 showed significant ($P \leq 0.05$) ≥2 fold changes in expression in red deer in response to *A. ovis* infection. Of these genes, 220 were over-expressed and 11 were down-expressed in infected animals. In red deer infected with *A. ovis*/*M. bovis*/MAP, 97 genes were significantly differentially expressed. Of these genes, 91 were over-expressed and 6 were down-expressed in infected animals. Significantly differentially expressed genes were clustered to look for common expression patterns (Fig. 1A and B). Hierarchical clustering with Euclidean distance was used to form the groups and heatmaps were used to visualize them. Multidimensional scaling was applied on the distance matrices used to group samples in order to produce graphical representations that helped to discover distinct expression patterns and correlation between functional groups within clusters, i.e., genes that perform similar functions share comparable expression profiles in infected animals (Fig. 1A and B). The results showed distinct gene expression patterns for infected and uninfected deer and these patterns were similar between *A. ovis*- and *A. ovis*/*M. bovis*/MAP-infected deer (Fig. 1A and B). Accordingly, of the differentially expressed genes, 78 (78 over-expressed and none down-expressed) were common to *A. ovis*- and *A. ovis*/*M. bovis*/MAP-infected deer.

The analysis of biological significance was based on a biological process gene ontology (GO) enrichment analysis aiming at establishing if differentially expressed genes appeared to be significantly concentrated in some GO terms that could be related with the biological processes involved in pathogen infection, i.e., genes that perform similar functions share comparable expression and appear associated with particular biological process GO terms at a probability higher than the probability of being represented in the microarray. GO assignments showed that differentially expressed genes affected cellular biological processes such as metabolic processes, translation, cell adhesion, transport, immune system development, cell activation and proliferation, neuropeptide and cytokine signaling, Jak-stat cascade, lactation, response to organic substances, and calcium ion binding (Table 2). When compared between groups, genes in metabolic processes and translation were over-expressed in both *A. ovis*- and *A. ovis*/*M. bovis*/MAP-infected deer. However, over-expression of genes in other cellular biological processes occurred only in *A. ovis*-infected (cell adhesion, transport, immune system development, cell activation) or *A. ovis*/*M. bovis*/MAP-infected (response to organic substances) deer. Genes in cellular biological processes such as cell proliferation, Jak-stat cascade, and lactation were only down-expressed in *A. ovis*-infected deer while calcium ion binding was down-expressed in *A. ovis*/*M. bovis*/MAP-infected animals only.

3.3. Comparison of immune response genes expression between A. ovis- and A. ovis/M. bovis/MAP-infected deer

The results of the global gene expression analysis suggested common and pathogen-specific mechanisms in red deer in response to *A. ovis* and *A. ovis*/*M. bovis*/MAP infection. Immune response genes were selected to further characterize pathogen-specific gene expression profiles in infected red deer. Unique over-represented immune response genes after conditional test for biological process GO were always over-expressed in infected deer (Fig. 2A–C). The 4 immune response genes over-expressed

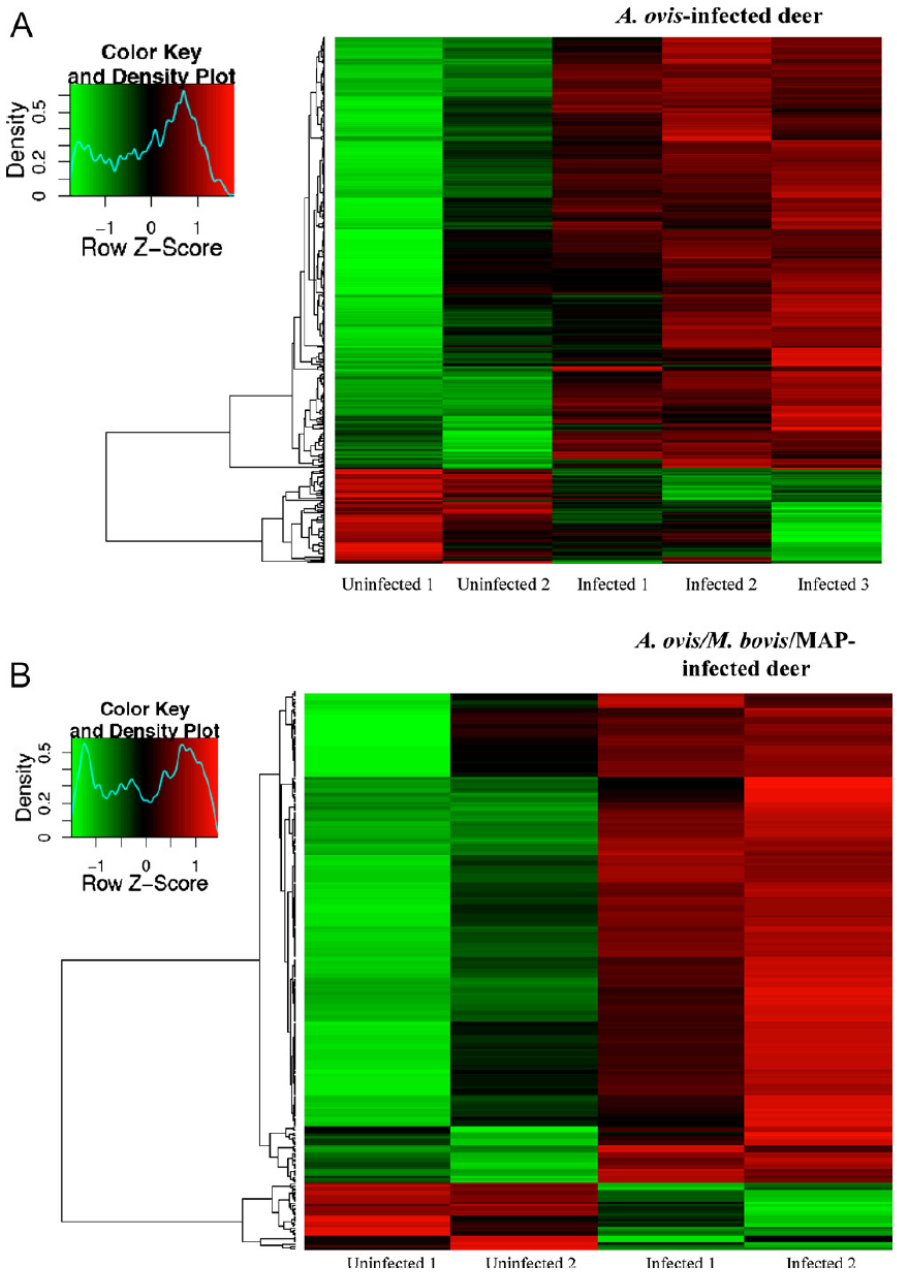


Fig. 1. Red deer transcriptional responses induced by pathogen infection. Clustering and heat map analysis of (A) *A. ovis* and (b) *A. ovis*/*M. bovis*/MAP regulated genes. The expression pattern of genes significantly differentially expressed in response to pathogen infection is represented as a hierarchical clustering, using UPGMA (Unweighted Pair-Group Method with Arithmetic mean) with Euclidean distance measure. The heat map is an intensity plot that represents the clusters within the dataset. The row dendrogram represents the genes clusters with similar patterns within and across different samples. Color corresponds to the expression level of the transcript with low, intermediate and high expression represented by green, black, and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in *A. ovis*/*M. bovis*/MAP-infected deer were part of the 13 genes over-expressed in *A. ovis*-infected deer (Fig. 2A). These genes included those involved in the control of innate immunity (CD21, IL1B, ATP5B, CD80, PSEN1, PTEN), adaptive immunity (ADAM9, FOXP1, AP3B1, CD5L, LCK) or both (TP53, VAV1) (Fig. 2B and C).

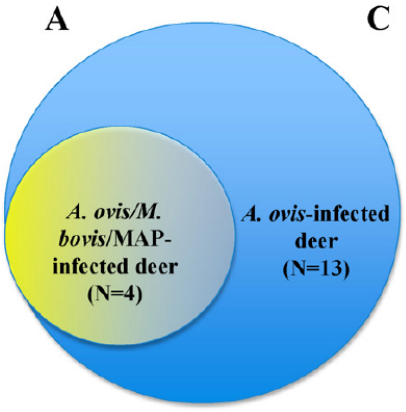
To corroborate and expand the results obtained in the microarray analysis, the mRNA levels of differentially

Table 2
Over represented terms after conditional test for biological process gene ontology.

GOBPID[a]	*P*-value[b]	Odds ratio[c]	ExpCount[d]	Count[e]	Size[f]	Term[g]
Genes over-expressed in A. ovis-infected red deer						
GO:0044237	9.3E–06	2.3	85.2	109	290	Cellular metabolic process
GO:0044238	3.8E–05	2.1	89.1	111	303	Primary metabolic process
GO:0044267	6.2E–05	2.3	39.4	58	134	Cellular protein metabolic process
GO:0006412	2.2E–04	4.4	7.94	17	27	Translation
GO:0044260	3.2E–04	1.9	69.1	88	235	Cellular macromolecule metabolic process
GO:0034960	4.1E–04	1.9	68.5	87	233	Cellular biopolymer metabolic process
GO:0006519	1.2E–03	6.3	4.1	10	14	Cellular amino acid and derivative metabolic process
GO:0019538	2.3E–03	1.8	46.1	61	157	Protein metabolic process
GO:0044248	1.9E–03	3.5	7.6	15	26	Cellular catabolic process
GO:0044249	2.3E–03	1.8	44.7	59	152	Cellular biosynthetic process
GO:0009058	2.8E–03	1.8	45	59	153	Biosynthetic process
GO:0008152	2.9E–03	1.7	101	116	343	Metabolic process
GO:0005975	3.0E–03	3.7	6.5	13	22	Carbohydrate metabolic process
GO:0006520	3. 5E–03	8.7	2.7	7	9	Cellular amino acid metabolic process
GO:0044106	3.5E–03	8.7	2.7	7	9	Cellular amine metabolic process
GO:0043170	6.0E–03	1.6	75.8	90	258	Macromolecule metabolic process
GO:0006650	7.3E–03	Inf	1.2	4	4	Glycerophospholipid metabolic process
GO:0007162	7.3E–03	Inf	1.2	4	4	Negative regulation of cell adhesion
GO:0008643	7.3E–03	Inf	1.2	4	4	Carbohydrate transport
GO:0030258	7.3E–03	Inf	1.2	4	4	Lipid modification
GO:0030384	7.3E–03	Inf	1.2	4	4	Phosphoinositide metabolic process
GO:0042113	7.3E–03	Inf	1.2	4	4	B cell activation
GO:0043283	7.4E–03	1.6	75.2	89	256	Biopolymer metabolic process
GO:0002520	7.7E–03	5	3.5	8	12	Immune system development
GO:0030097	7.7E–03	5	3.5	8	12	Hemopoiesis
GO:0044262	7.7E–03	5	3.5	8	12	Cellular carbohydrate metabolic process
GO:0048534	7.7E–03	5	3.5	8	12	Hemopoietic or lymphoid organ development
GO:0045321	9.9E–03	3.6	5	10	17	Leukocyte activation
GO:0046649	9.9E–03	3.6	5	10	17	Lymphocyte activation
Genes down-expressed in A. ovis-infected red deer						
GO:0001936	0.003	Inf	0.1	2	2	Regulation of endothelial cell proliferation
GO:0007259	0.003	Inf	0.1	2	2	Jak-stat cascade
GO:0046425	0.003	Inf	0.1	2	2	Regulation of jak-stat cascade
GO:0008284	0.004	8.2	0.7	4	13	Positive regulation of cell proliferation
GO:0048518	0.01	3.2	3.8	9	66	Positive regulation of biological process
GO:0042127	0.01	4.9	1.4	5	24	Regulation of cell proliferation
GO:0001935	0.01	34.9	0.2	2	3	Endothelial cell proliferation
GO:0007218	0.01	34.9	0.2	2	3	Neuropeptide signaling pathway
GO:0007595	0.01	34.9	0.2	2	3	Lactation
GO:0018108	0.01	34.9	0.2	2	3	Peptidyl-tyrosine phosphorylation
GO:0018212	0.01	34.9	0.2	2	3	Peptidyl-tyrosine modification
GO:0019221	0.01	34.9	0.2	2	3	Cytokine-mediated signaling pathway
Genes over-expressed in A. ovis/M. bovis/MAP-infected red deer						
GO:0006412	0.0001	5.22	3.9	12	27	Translation
GO:0044267	0.003	2	19.6	30	134	Cellular protein metabolic process
GO:0019538	0.01	1.9	22.9	33	157	Protein metabolic process
GO:0044237	0.01	1.8	42.4	53	290	Cellular metabolic process
GO:0010033	0.01	4.6	2	6	14	Response to organic substance
Genes down-expressed in A. ovis/M. bovis/MAP-infected red deer						
GO:0005509	0.03	13.5	0.7	5	43	Calcium ion binding

[a] Biological process GO term identification.
[b] *P*-value associated to the GO term.
[c] Strength of the association between the probability that the GO term appears within differentially expressed genes with respect to the probability of being represented in the microarray.
[d] Number of differentially expressed genes that are predicted within the GO term tested.
[e] Number of differentially expressed genes that were found within the GO term tested.
[f] Number of genes in the microarray that are listed within the GO term tested.
[g] Biological process GO term tested.

expressed immune response genes were characterized in red deer uninfected ($N=8$) and naturally infected with *A. ovis* ($N=7$), *M. bovis* ($N=3$) or *A. ovis*/*M. bovis*/MAP ($N=4$). Despite differences in the infected/uninfected ratio obtained by both methods that may be due to differences in the sensitivity of the methods and/or the fact that bovine sequences were used in the microarray used to analyze deer RNA, results were similar between microarray and real-time RT-PCR analyses for most genes analyzed in red deer (Table 3). The results also showed that *M. bovis* infection in red deer significantly over-expressed the expression of only two (CD5L and TP53) of the analyzed genes (Table 3).



B

Genes over-expressed in *A. ovis*- and *A. ovis*/*M. bovis*/MAP-infected deer
Metallopeptidase domain 9 (meltrin gamma) (ADAM9)
Complement component receptor 2 (CR2) (CD21)
Forkhead box P1 (FOXP1)
Interleukin 1, beta (IL1B)

C

Genes over-expressed in *A. ovis*-infected deer only
Adaptor-related protein complex 3, beta 1 subunit (AP3B1)
ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B)
CD5 molecule-like (CD5L)
CD80 molecule (CD80)
Lymphocyte-specific protein tyrosine kinase (LCK)
Presenilin 1 (PSEN1)
Phosphatase and tensin homolog (PTEN)
Tumor protein p53 inducible nuclear protein 1(TP53)
Vav 1 guanine nucleotide exchange factor (VAV1)

Fig. 2. Over represented immune response genes over-expressed in infected red deer after conditional test for biological process gene ontology. (A) Venn diagram detailing shared and distinct gene expression in response to *A. ovis* and *A. ovis*/*M. bovis*/MAP infection. (B and C) List of immune response genes over-expressed in (B) *A. ovis*- and *A. ovis*/*M. bovis*/MAP-infected deer and in (C) *A. ovis*-infected deer only. Gene symbols are shown in parenthesis.

3.4. Characterization of the differential expression of immune response genes in cattle infected with M. bovis and A. marginale

To compare the results between wild and domestic ruminants in response to *Mycobacterium* and *Anaplasma* spp. infection, the mRNA levels of differentially expressed immune response genes identified in red deer were characterized in cattle uninfected ($N = 7$) and naturally infected ($N = 17$) with *M. bovis* and uninfected ($N = 8$) and experimentally infected ($N = 8$) with *A. marginale* (Table 3). The results showed that infection with *M. bovis* and *A. marginale* significantly upregulated in cattle the expression of most of the immune response genes identified in red deer (Table 3).

4. Discussion

In this study, microarray hybridization was used first with a small number of samples to characterize gene expression profiles in *A. ovis* and *A. ovis*/*M. bovis*/MAP naturally infected red deer and provided the basis for in-depth studies with immune response genes. Immune response genes were selected after conditional test for biological process GO to further characterize pathogen-specific and host-specific gene expression profiles in a larger number of red deer uninfected and naturally infected with *A. ovis*, *M. bovis* or *A. ovis*/*M. bovis*/MAP and in cattle uninfected and naturally or experimentally infected with *M. bovis* or *A. marginale*. This experimental approach allowed the identification of the biological processes affected by pathogen infection in a small number of red deer and then use this information to further characterize the expression of immune response genes in a larger number of uninfected and infected red deer and cattle to compare results between different ruminant species in response to *Mycobacterium* and *Anaplasma* spp. infection.

Mycobacteria and *A. ovis*/*A. marginale* infect different cell types, primarily macrophages and erythrocytes, respectively [15,24,29]. However, most of the genes that were identified as differentially expressed in *A. ovis*-infected deer were also differentially expressed in *A. ovis*/*M. bovis*/MAP-infected animals. This result suggested that *A. ovis* and *M. bovis*/MAP infection produces similar differential gene expression patterns in red deer or, as with other pathogens [54], gene expression in *A. ovis*/*M. bovis*/MAP co-infected red deer is dominated by *A. ovis*. The expression analysis of immune response genes in red deer infected with *A. ovis*, *M. bovis* and *A. ovis*/*M. bovis*/MAP provided additional support for the last possibility by showing that while most genes were over-expressed in response to *A. ovis* and *A. ovis*/*M. bovis*/MAP infection, only two genes were significantly over-expressed in *M. bovis*-infected animals. These results were similar to those obtained previously in lymph nodes of *M. bovis*-infected red deer [40], and suggested a limited effect of *M. bovis* infection on immune response gene expression and tissue-specific differences between lymph nodes and PBMC in red deer. Nevertheless, some biological processes were affected by differential gene expression in *A. ovis*- or *A. ovis*/*M. bovis*/MAP-infected deer only, thus suggesting pathogen-specific effects on gene expression and/or the effect of multiple infections in red deer. The co-infection with multiple pathogens as in the

Table 3
Differential expression of immune response genes in red deer and cattle infected with *Anaplasma* and/or *Mycobacterium* spp.

Gene	Infected/uninfected mRNA ratio						
	Red deer infected with					Cattle infected with	
	AO (microarray)	AO/MB/MAP (microarray)	AO (RT-PCR)	MB (RT-PCR)	AO/MB/MAP (RT-PCR)	AM (RT-PCR)	MB (RT-PCR)
Metallopeptidase domain 9 (meltrin gamma) (ADAM9)	3.7	2.6	3.7 ± 1.9	2.0 ± 1.6	10.2 ± 0.8*	$2.2 \pm 0.0 \times 10^{4}$*	34.6 ± 11.1*
Complement component receptor 2 (CR2) (CD21)	4.0	3.0	1.2 ± 1.1	1.6 ± 1.8	5.2 ± 1.1*	$3.9 \pm 0.0 \times 10^{4}$*	26.6 ± 21.2*
Forkhead box P1 (FOXP1)	2.2	2.7	0.7 ± 1.1	1.2 ± 0.9	1.6 ± 0.4*	17.5 ± 12.0*	19.5 ± 9.1*
Interleukin 1, beta (IL1B)	3.0	3.0	1.6 ± 1.1*	1.2 ± 0.8	1.2 ± 0.7	$2.9 \pm 0.0 \times 10^{5}$*	ND
Adaptor-related protein complex 3, beta 1 subunit (AP3B1)	2.8	NS	1.1 ± 1.5	1.1 ± 0.9	2.3 ± 1.2	$1.2 \pm 0.0 \times 10^{5}$*	33.9 ± 21.8*
ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B)	2.4	NS	1.4 ± 1.5	2.4 ± 1.6	4.6 ± 1.6*	$2.7 \pm 0.0 \times 10^{6}$*	12.4 ± 9.3*
CD5 molecule-like (CD5L)	2.0	NS	1.2 ± 1.1	1.8 ± 1.4*	2.1 ± 1.2	31.5 ± 22.0*	52.5 ± 36.0*
CD80 molecule (CD80)	2.3	NS	1.3 ± 1.3	1.5 ± 1.1	2.1 ± 0.9*	$2.5 \pm 1.6 \times 10^{3}$	9.1 ± 5.3*
Lymphocyte-specific protein tyrosine kinase (LCK)	3.6	NS	2.1 ± 1.2*	1.2 ± 1.2	0.7 ± 0.8	$7.1 \pm 0.0 \times 10^{2}$*	19.9 ± 11.2*
Presenilin 1 (PSEN1)	2.2	NS	1.0 ± 1.1	1.4 ± 1.2	2.5 ± 1.1	6.4 ± 0.2*	ND
Phosphatase and tensin homolog (PTEN)	2.4	NS	4.6 ± 1.6*	2.1 ± 1.8	2.7 ± 1.8	2.9 ± 0.5*	5.2 ± 4.4
Tumor protein p53 inducible nuclear protein 1 (TP53)	2.5	NS	1.1 ± 0.8	2.7 ± 1.6*	4.3 ± 1.4*	77.6 ± 1.2*	9.5 ± 6.3*
Vav 1 guanine nucleotide exchange factor (VAV1)	2.7	NS	4.9 ± 1.4*	1.3 ± 1.3	1.5 ± 1.5	2.9 ± 0.7*	178.2 ± 93.3*

The mRNA levels were characterized by microarray hybridization and/or real-time RT-PCR in red deer and cattle infected with *Anaplasma* and *Mycobacterium* spp. In the microarray analysis, only significant ($P \leq 0.05$) ≥2 fold changes in expression were considered. In real-time RT-PCR analyses, mRNA levels were characterized in red deer uninfected ($N = 8$) and naturally infected with *A. ovis* ($N = 7$), *M. bovis* ($N = 3$) or *A. ovis*/*M. bovis*/MAP ($N = 4$) and in cattle uninfected ($N = 7$) and naturally infected ($N = 17$) with *M. bovis* and uninfected ($N = 8$) and experimentally infected ($N = 8$) with *A. marginale*. The mean of the duplicate values was used and data from infected and uninfected animals were compared using the Student's *t*-test (*$P < 0.05$). *Abbreviations*: AO, *A. ovis*; MB, *M. bovis*; MAP, *M. avium* sub. *paratuberculosis*; NS, not significant; ND, not determined.

case of *A. ovis*/*M. bovis*/MAP could produces unique gene expression patterns as a result of the interaction between species that are not seen with each individual pathogen infection [55].

When immune response genes differentially expressed in red deer in response to *A. ovis* or *A. ovis*/*M. bovis*/MAP infection were characterized in cattle naturally or experimentally infected with *M. bovis* and *A. marginale*, respectively, the results showed that most genes were significantly over-expressed in infected cattle. This result suggested that different ruminant species respond to *Mycobacterium* and *Anaplasma* spp. with similar expression patterns, at least for the immune response genes analyzed herein. However, when the mRNA levels of immune response genes were compared between *M. bovis*-infected red deer and cattle, the results suggested that cattle might have a stronger immune response to *M. bovis* than red deer, perhaps reflecting the fact that red deer are natural reservoir species for this pathogen [1–3,7].

When compared to previously published results, some of the biological processes affected by differential gene expression in infected red deer were also affected in MAP-infected cattle (i.e., calcium ion binding [43]), *M. bovis*-infected cattle (i.e., transport, metabolic processes, translation, cell proliferation, cell adhesion, calcium ion binding [38,41]) and MAP-infected sheep (i.e., metabolic processes, cell proliferation, cell differentiation, transport [42]). As expected, other biological processes such as immune system development, neuropeptide and cytokine signaling, Jak-stat cascade, and lactation were affected in *A. ovis*-infected deer only. These results corroborated that *A. ovis*, *M. bovis* and MAP infection modify gene expression affecting similar biological processes in ruminants. However, pathogen-specific signatures were also evidenced by differential expression of genes affecting biological processes in *A. ovis*-infected deer only.

As shown herein and in previous studies, the expression of immune response genes was affected by *Anaplasma* and *Mycobacterium* spp. infection in red deer and other ruminants [38–43]. However, the set of differentially expressed genes varied between pathogens and hosts. Herein, 9 of the 13 immune response genes characterized were differentially expressed in *A. ovis*-infected deer only, again highlighting pathogen-specific signatures and the effect of co-infection with multiple pathogens on red deer host immune response. Interestingly, the ADAM9 gene that was differentially expressed in *A. ovis*/*M. bovis*/MAP-infected deer and in *A. marginale*- and *M. bovis*-infected cattle has

been shown to be involved in the formation of multicellular giant cells found during active infection with *M. tuberculosis* [56]. Other genes also upregulated in *Mycobacterium* and *Anaplasma* infected deer and cattle such as IL1B and CD80 have been suggested to play a role in the pathogenesis of mycobacterial infection [57] and in antigen-specific CD4(+) T-cell responses to *A. marginale* [58], respectively.

5. Conclusions

In summary, the results of this study showed that global gene differential expression in *A. ovis*- and *A. ovis*/*M. bovis*/MAP-infected red deer resulted in the modification of common and pathogen-specific cellular biological processes. In particular, the differential expression of host immune response genes showed host and pathogen-specific signatures and the effect of infection with multiple pathogens on red deer host immune response. Some of the differentially expressed immune response genes constitute new findings that expanded our knowledge on the effect of *Anaplasma* and *Mycobacterium* spp. infection on host innate and adaptive immunity. These results suggested that intracellular bacteria from *Anaplasma* and *Mycobacterium* genera produce similar genes expression patterns in infected ruminants while pathogen and host-specific mechanisms underline differences that could contribute to the diagnosis and treatment of diseases such as tuberculosis, paratuberculosis and anaplasmosis in ruminants.

Conflict of interest statement

None declared.

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DISCUSIÓN GENERAL

La revisión bibliográfica ha sido esencial para tener una visión más clara y actual sobre la paratuberculosis en fauna silvestre. Se trata de una infección común, de amplia distribución geográfica, presente en un gran número de hospedadores silvestres. Sin embargo, sólo unas pocas especies son consideradas verdaderos reservorios, y nunca (salvo en granjas de ciervo) se considera que la paratuberculosis constituya un factor importante de mortalidad en fauna silvestre. En consecuencia, el aspecto más importante de MAP en la fauna silvestre española es su posible interferencia con el diagnóstico y el seguimiento de la tuberculosis (Boadella et al. 2011). El problema fundamental en el estudio de esta enfermedad es la ausencia de un único test que combine una alta sensibilidad junto a una alta especificidad. Por el contrario, es necesario combinar varias pruebas en paralelo, que en su conjunto darán fiabilidad a los resultados. Esta aproximación es la aplicada en esta tesis, donde se han combinado los estudios anatomopatológicos con el cultivo, la serología y, en determinadas ocasiones, la PCR y otras técnicas de genómica funcional. Esta combinación, sumada a algunos trabajos previos del IREC y de otros grupos de investigación españoles, permite tener una visión muy completa sobre la paratuberculosis en la fauna ibérica.

Los trabajos sobre MAP en fauna ibérica pueden, a su vez, dividirse en tres grupos, tal y como se ha hecho en esta tesis doctoral. En primer lugar estarían los estudios epidemiológicos descriptivos sobre aquellas especies que podrían jugar un papel como reservorios silvestres de paratuberculosis. En segundo lugar estarían aquellas otras especies que, sin tener potencial como reservorio, podrían constituir buenos indicadores de la presencia de paratuberculosis en su entorno. Finalmente, encontraríamos los trabajos relacionados con la interacción entre MAP u otros patógenos intracelulares y sus hospedadores naturales, que incluyen especies silvestres.

Reservorios silvestres de MAP en España

En el primer grupo, el de los potenciales reservorios, situaríamos, de acuerdo con la literatura internacional, algunos rumiantes silvestres, principalmente cérvidos (Chiodini and Van Kruiningen, 1983; Nebbia et al., 2000) y al conejo (Daniels et al., 2003). En España existen dos situaciones, muy localizadas geográficamente, en las que puede hablarse de la posible existencia de un reservorio silvestre de MAP: la reserva de caza del Sueve en Asturias y una parte del parque natural de Los Alcornocales, en Cádiz. Ambas son zonas de abundante pluviometría y próximas al mar, caracterizadas por un intenso aprovechamiento de los pastos por rumiantes domésticos (ovejas y vacas en el Sueve y ciervos en Los Alcornocales). Esos pastos son compartidos con rumiantes silvestres (ciervo y gamo) en Asturias, y por conejos en Cádiz. En ambos casos, la paratuberculosis afecta tanto a domésticos como a silvestres, dando lugar a situaciones multi-hospedador (Raizman et al., 2005).

El caso asturiano había sido estudiado sobre todo en relación con el gamo, encontrándose una elevada prevalencia de gamos con lesiones y algunos individuos con lesiones difusas multibacilares, compatibles con una fuerte excreción de MAP (Balseiro et al., 2008). Particularmente, los ciervos estudiados en la presente tesis, que incluían 83 animales muestreados precisamente en el entorno del Sueve, no presentaron tales lesiones, indicando un nulo papel de los mismos en la epidemiología de MAP en el Sueve (Carta et al., 2012).

En Cádiz se han diagnosticado repetidamente casos clínicos de paratuberculosis en una granja de ciervos. Estos casos dieron lugar a la puesta en marcha de un estricto programa de testado y eliminación, que ha dado lugar al cese de los citados casos clínicos (Fernández-de-Mera et al., 2009; Queirós et al., 2012). La investigación sobre los conejos capturados en los pastizales de esta granja ha permitido confirmar la existencia de infección por MAP y, al igual que en el caso del gamo, la presencia de conejos con lesiones difusas multibacilares (Maio et al., 2011). Teniendo en cuenta la creciente importancia de paratuberculosis para la salud animal, y el hecho de que el conejo sea reconocido en Escocia como reservorio de MAP, estos resultados podrían ser de utilidad en investigaciones futuras.

En consecuencia, el conejo en Cádiz y el gamo en Asturias podrían dificultar el control de la paratuberculosis en los rumiantes domésticos. Sin embargo, éstas son situaciones puntuales, muy

locales, donde un hospedador susceptible coincide con la presencia de altas densidades de hospedadores domésticos y en un ambiente seguramente adecuado para la supervivencia de MAP. Además, el control de paratuberculosis se basa en actuaciones sobre el ganado infectado, y sólo en segundo lugar tiene sentido ampliar el control a eventuales reservorios silvestres.

La situación más habitual es por el contrario bien distinta. El rebeco cantábrico y el corzo, por ejemplo, tienen contacto con MAP en proporciones comparables a las encontradas en especies ganaderas en régimen extensivo. Sin embargo, estas especies no constituyen un reservorio significativo de MAP para el ganado doméstico (Boadella et al., 2010, Falconi et al., 2010). En el ciervo, la paratuberculosis constituye un problema relevante en las granjas pero no tanto en las poblaciones naturales (Fernández de Mera et al., 2009). En efecto, el análisis histopatológico y microbiológico de una amplia muestra de ciervos ibéricos no permitió ningún aislamiento, ni la observación de casos de infección multibacilar (Carta et al., 2012).

Los resultados negativos en el ciervo tienen importancia epidemiológica. En primer lugar, dada la baja prevalencia de MAP en esta especie, MAP no interfiere en el uso de lesiones como indicador en la vigilancia de tuberculosis (Martin-Hernando et al., 2010; Boadella et al., 2011). En segundo lugar, sabiendo que la enfermedad se manifiesta casi exclusivamente en ciervos de granja, conviene tomar precauciones en relación con las sueltas de estos animales al medio natural.

La duda que surge es ¿Por qué hay prevalencia de MAP en ciervos de granja, pero no en ciervos de vida libre, considerando la abundancia de estos últimos? Las explicaciones pueden ser debidas al hecho que las ciervas silvestres, para parir, se alejan del grupo, disminuyendo la posibilidad de infecciones neonatales (Mackintosh et al., 2010). Sin embargo, ello no explica la elevada prevalencia que se encuentra en los Alpes (Robino et al., 2008). Otro punto interesante es que Balseiro y colaboradores (2008) encontraron MAP en gamos, incluyendo lesiones multibacilares, que suponen una fuerte excreción de MAP al medio. Sin embargo, nuestro estudio incluyó ciervos de la misma localidad que los gamos y no encontró nada similar. Cabe entonces la posibilidad de que los gamos, por motivos fisiológicos o de comportamiento, resulten más susceptibles a MAP que los ciervos. Por ejemplo, los gamos son mucho más gregarios que los ciervos (Putman, 1988). Por otra parte, es posible que MAP requiera condiciones ambientales determinadas, y que los ecosistemas xerófilos mediterráneos no resulten muy adecuados para su difusión.

Fauna silvestre como centinelas de MAP

El segundo aspecto abordado en la tesis es el estudio de la potencialidad de algunas especies de fauna silvestre como indicadoras de paratuberculosis. En el jabalí, la detección de anticuerpos frente al (supuestamente específico) antígeno PPA3 se produce principalmente en ejemplares infectados por *Mycobacterium bovis*. No obstante, la seropositividad a PPA3 es muy baja en áreas no endémicas de tuberculosis. Esto sugiere una nula relevancia del jabalí como reservorio de MAP y una escasa utilidad como especie centinela o indicadora (Boadella et al. 2011).

En cuanto a los carnívoros, los estudios sobre zorro y lobo que forman parte de esta tesis permiten concluir que, al igual que el jabalí, no sirven de indicadores de paratuberculosis (Carta et al. 2011). Los análisis en tejones tampoco encontraron MAP por histopatología y cultivo (Balseiro et al. 2011a, 2011b).

Los resultados negativos de prevalencia obtenidos en carnívoros silvestres ibéricos contrastan con los niveles relativamente altos encontrados en mapaches, zorros, coyotes, gatos silvestres y mofetas en Wisconsin, EEUU (Anderson et al., 2007). Las diferencias de prevalencia entre nuestros resultados y el estudio de Wisconsin podrían explicarse por las diferencias en el alcance del muestreo o por una distinta sensibilidad de los análisis de PCR, según los protocolos utilizados en los dos estudios.

Interacción entre MAP y sus hospedadores silvestres

Las especies de los géneros *Anaplasma* y *Mycobacterium* se encuentran entre los patógenos bacterianos más prevalentes en el ciervo en España. Ambos géneros tienen la capacidad de modificar la expresión génica en los rumiantes infectados. En esta tesis utilizamos la hibridación en microarrays y la RT-PCR en tiempo real para caracterizar la expresión génica global del ciervo en respuesta a infecciones naturales con *Anaplasma ovis* y co-infecciones *A. ovis*/*Mycobacterium bovis*/*Mycobacterium avium paratuberculosis* (MAP).

Las respuestas específicas de cada patógeno y cada especie hospedadora pueden eventualmente aportar conocimientos básicos para el futuro desarrollo de mejoras en el diagnóstico y tratamiento de estas infecciones (Galindo et al., 2010). El análisis realizado permitió valorar el efecto de la co-infección con múltiples patógenos intracelulares sobre la respuesta inmune del ciervo. Sin embargo una de las limitaciones de este estudio fue que no pudimos contar con ciervos infectados sólo con MAP, y, por lo tanto, no se descartan posibles coinfecciones con otros patógenos intracelulares, limitando consiguientemente las oportunidades para encontrar respuestas específicas a la infección por MAP.

SÍNTESIS (SPANISH)

Esta tesis aborda el papel de los animales silvestres en relación con la paratuberculosis, en la península ibérica bajo la hipótesis de que debido a su alta densidad poblacional de ciervos, la paratuberculosis podría ser detectada en un elevado numero de ciervos ibéricos. Además, otras especies, como el jabalí, los conejos o los carnívoros, también podrían constituir indicadores de paratuberculosis. En concreto, se plantean los siguientes objetivos:

1. Actualizar todas las informaciones vigentes sobre paratuberculosis en fauna silvestre

2. Analizar especificidad y sensibilidad de los métodos de diagnóstico en la detención de MAP;

3. Ver si MAP sea un factor significativo en la dinámica de la población de algunas especies de la fauna silvestre española

4. Caracterizar la diferencia de expresión de genes relacionados con la inmunidad en ciervos y bóvidos infectados con *M. bovis* y *Anaplasma marginale*.

La tesis se estructura en cinco capítulos. El primero de ellos es una revisión bibliográfica sobre la paratuberculosis en la fauna silvestre, que permite conocer el estado actual de conocimientos sobre su posible implicación en el mantenimiento de MAP, su efecto sobre sus poblaciones, y las consecuencias de paratuberculosis sobre la vigilancia sanitaria de otras micobacteriosis, particularmente la tuberculosis. Los siguientes tres capítulos describen el papel del conejo como posible reservorio local de paratuberculosis en la península ibérica; la ausencia de utilidad de los cánidos ibéricos (lobo y zorro) como posibles centinelas de la circulación de MAP; y el hecho de que, contrariamente a lo esperado, el ciervo no constituye un reservorio de paratuberculosis en el área de estudio. El quinto y último capítulo analiza la expresión de genes relacionados con la inmunidad en respuesta a la infección de rumiantes por patógenos de los géneros *Anaplasma* y *Mycobacterium*.

Capítulo 1

En la revisión bibliográfica que constituye el capítulo 1 de esta tesis se recopila la información existente sobre paratuberculosis en animales silvestres y su diagnóstico.

Esta revisión demuestra que MAP circula en casi todo el mundo, entre diferentes vertebrados silvestres. De todas las especies estudiadas, solamente cinco son considerados como reservorios, incluyendo varios rumiantes como el ciervo de cola blanca en Norteamérica, ciervo y corzo en los Alpes, y gamo en Asturias, y el conejo en Escocia y, probablemente, en Nueva Zelanda, España y República Checa. La literatura sobre cérvidos y conejos sugiere que puedan participar en la epidemiología de MAP, pero siempre dependiendo de la región geográfica. La excreción de MAP por parte de los animales silvestres es menor que la excreción por bovinos clínicamente afectados (Daniels et al., 2003a). No obstante, el principal reservorio mundial de MAP lo constituyen los propios rumiantes domésticos, por lo que el papel de los rumiantes silvestres como reservorio tendría una relevancia muy local, en lugares donde los rumiantes domésticos sean objeto de control y coexistan con un reservorio silvestre (Raizman et al., 2005). En ningún caso los investigadores han llegado a concluir que paratuberculosis constituya un factor limitante para la dinámica de una población silvestre. Los principales problemas de la presencia de paratuberculosis se relacionan con animales en cautividad o con animales expuestos a una elevada contaminación ambiental por MAP con origen en los rumiantes domésticos.

En ningún caso los investigadores han llegado a concluir que la paratuberculosis constituya un factor limitante para la dinámica de una población silvestre. Los principales problemas se relacionan con animales en cautividad (por ejemplo en granjas de ciervo, Kopecna et al., 2008b; Mackintosh et al., 2004) o con animales expuestos a una elevada contaminación ambiental por MAP con origen en los rumiantes domésticos (Maio et al., 2011).

En cambio, sí es importante la reacción cruzada entre antígenos del complejo *Mycobacterium avium* y antígenos del complejo *M. tuberculosis* (en pruebas serológicas) y la falta de especificidad en PCR y en patología. Estos problemas son debidos a las similitudes entre MAP y otras micobacterias, incluyendo a miembros del complejo *Mycobacterium tuberculosis* como *M. bovis*,

agente causal de la tuberculosis bovina. En consecuencia, MAP puede comprometer el diagnostico y control de la tuberculosis, por ejemplo en ciervos de granja o en traslados de fauna silvestre (Fernandez-de-Mera et al., 2009).

Igualmente, MAP limita la utilidad del diagnóstico de las micobacteriosis en la fauna silvestre mediante pruebas de detención de anticuerpos (Boadella et al., 2011, Carta et al., 2012). Esto coincide con cuanto ocurre en el ámbito de los rumiantes domésticos, donde es sabido que la infección por MAP puede limitar la especificad y la sensibilidad de las pruebas de tuberculosis y por lo tanto afectar a las campañas de erradicación de tuberculosis (Alvarez et al., 2008, 2011).

Capítulo 2

La paratuberculosis, o enfermedad de Johne, es una enteritis crónica compartida entre el ganado doméstico y fauna silvestre. Además de numerosos rumiantes silvestres, también el conejo puede actuar como reservorio de *Mycobacterium avium paratuberculosis* (MAP). No obstante, apenas se conoce el papel epidemiológico del conejo en relación con MAP fuera de Escocia.

A través de la biología molecular y la histopatología, se ha evidenciado la presencia de MAP en conejos del Sur Europa y se ha desarrollado una prueba ELISA indirecta para la detención de anticuerpos específicos frente a MAP. Este trabajo también describe las diferentes formas patológicas presentes en conejos con paratuberculosis. Se han muestreado 80 conejos del sur de España, y sueros de 157 conejos más, provenientes de otros 7 lugares. Se han inmunizado 4 conejos domésticos mediante inoculación de un antígeno específico (Paratuberculosis Protoplasmátic Antigen 3, PPA3), al fin de disponer de controles positivos en la prueba ELISA. Se utilizaron sueros de conejos de granja sin contacto con MAP como controles negativos, al fin de establecer un punto de corte. Eligiendo el punto de corte de 0,5 de densidad óptica (DO), se identificaron como positivos con la prueba ELISA 6 de los 237 sueros de conejos silvestres testados (2,5%). Se detectaron anticuerpos específicos anti-MAP en conejos provenientes de 3 de las 8 áreas de estudio.

En la necroscopia, 2 de 80 conejos presentaron lesiones macroscópicas compatibles con paratuberculosis. La histopatología reveló presencia de paratuberculosis con lesiones que iban de focales hasta multibacilares en 8 de los 10 conejos examinados. La presencia de MAP fue

confirmada mediante RT-PCR en un conejo con lesiones macroscópicas, evaluando la curva de amplificación de los fragmentos IS-900 e ISMAP02.

Por lo tanto, en este estudio, se ha confirmado por primera vez la infección y la clínica en conejos silvestres del sur de Europa, y se ha comprobado que el contacto del conejo silvestre con MAP está muy extendido. Considerando la creciente importancia de MAP en la salud animal, estos resultados abren nuevas ventanas para futuras investigaciones.

Capítulo 3

Los carnívoros silvestres siendo predadores y tal vez carroñeros tienen más posibilidades de entrar en contacto con agentes patógenos contaminantes del medio ambiente o presentes en animales.

Por ello, en el capítulo 3 de esta tesis hemos partido de la hipótesis de que los cánidos silvestres pueden ser usados como centinelas en áreas con alta prevalencia de MAP en fauna silvestre y domestica. Para comprobar esta hipótesis se muestrearon, entre 2004 y 2009, 24 lobos y 285 zorros procedentes de distintas regiones peninsulares. En el laboratorio los cadáveres fueron necropsiados, y las muestras conservadas en congelación y en formol para histopatología. En la necropsia no se detectaron lesiones compatibles con MAP. Se adaptó un ELISA indirecto para la detención de anticuerpos frente a MAP en suero, procesando también un pool de muestras para cultivo (n=61) y analizando 15 muestras mediante PCR especifica para MAP y 14 para histopatología.

Los resultados obtenidos muestran como los cánidos silvestres no sean útil como centinelas de paratuberculosis para la vigilancia sanitaria en el suroeste de Europa.

Capítulo 4

En este trabajo, que forma el capítulo 4 de esta tesis, hemos analizado mediante patología, cultivo y serología, muestras provenientes de 332 ciervos cazados en distintas regiones de la península ibérica. Entre 2005 y 2009 hemos podido obtener muestras de 332 ciervos. Éstos animales se inspeccionaron en busca de lesiones macroscópicas compatibles con tuberculosis (Martín-Hernando

et al., 2010). Se tomaron muestras de los linfonodos de la cadena mesentérica, incluyendo el linfonodo ileocecal, así como la válvula ileocecal para histopatología (Balseiro et al., 2008). Un pool de linfonodos mesentéricos se utilizó para cultivo (Sevilla et al., 2007).

La histopatología reveló solamente tres animales (1.12%) que mostraban lesiones focales compatibles con paratuberculosis en el área interfolicular del tejido linfoide intestinal. En estas lesiones no se encontraron organismos ácido-alcohol resistentes en las tinciones de Ziehl Neelsen. Se encontraron lesiones compatibles con tuberculosis en 25 animales (8.4%). No se observó crecimiento de MAP en cultivo. En ELISA, las densidades ópticas en placas antigenadas con Paratuberculosis Protoplasmatic Antigen 3 (PPA3) y bovine Purified Protein Derivative (bPPD), respectivamente, estaban significativamente correlacionadas. Ello sugiere una reacción cruzada y, por lo tanto, una falta de especificad del test.

Los resultados obtenidos contrastan con la situación descrita en otras regiones, y permiten descartar al ciervo como reservorio de paratuberculosis en el área de estudio. Los únicos ciervos significativamente afectados por la paratuberculosis serían por tanto los ciervos de granja (Fernández-de-Mera et al., 2009).

Capítulo 5

Los patógenos de los géneros *Anaplasma* y *Mycobacterium* figuran entre los patógenos bacterianos más prevalentes en el ciervo del sur-oeste de España. Se sabe que estos patógenos modifican la expresión génica en los rumiantes.

En este trabajo, que conforma el capítulo 5 de esta tesis, hemos utilizado la hibridación con microarray y la RT-PCR en tiempo real para caracterizar el perfil global de la expresión génica en el ciervo en respuesta a las infecciones por *Anaplasma ovis* y *Anaplasma ovis*/*Mycobacterium bovis*/ *Mycobacterium avium paratuberculosis* (MAP); comparar la expresión génica de la respuesta inmune en ciervos infectados con *A. ovis*, *M. bovis* y *A. ovis*/*M. bovis*/MAP, así como para caracterizar la diferencia de expresión de genes relacionados con la inmunidad en ciervos y bóvidos infectados con *M. bovis* y *Anaplasma marginale*.

Síntesis

Globalmente, la expresión diferencial de genes en ciervos infectados con *A. ovis* y *A.ovis/M. bovis/*MAP resultó en la modificación de procesos biológicos celulares comunes y específicos para estos patógenos.

La expresión diferencial de genes relacionados con la inmunidad del hospedador mostró patrones característicos de la respuesta del hospedador al patógeno, así como el efecto de la infección con múltiples patógenos en la respuesta inmune del ciervo.

Estas diferencias específicas del patógeno y del hospedador podrían contribuir a mejorar el diagnóstico de las enfermedades causadas por *Anaplasma* y *Mycobacterium*, así como su control en rumiantes.

SYNTHESIS (ENGLISH)

This thesis attempts to evaluate the role of wildlife in relation to pararatuberculosis in the Iberian Peninsula under the assumption that the contact between wildlife and domestic ruminants is increasing due to the demographic and geographic expansion of some species and to changes in management systems.

In particular, we established the following objetives:

1. To collect and review the information of the scientific literature about paratuberculosis in domestic animals and wildlife.

2. To optimize some paratuberculosis diagnostic methods for application in wildlife.

3. To study the putative role of wildlife as a reservoir for paratuberculosis.

4. To identify new molecular mechanisms important in the *Mycobacterium avium paratuberculosis* (MAP) - host interaction.

The thesis is divided into five chapters. The first one is a review on paratuberculosis in wildlife, which identifies the current state of knowledge about their possible involvement in the maintenance of MAP, its effect on populations, and the impact of paratuberculosis on health surveillance for other mycobacterial infection, particularly tuberculosis. The next three chapters describe the role of rabbits as a possible local paratuberculosis reservoir in the iberian peninsula, the putative role of the iberian canids (wolf and fox) as sentinels for MAP circulation, and the fact that, contrary to our expectations, deer are not a reservoir of paratuberculosis in the study area. The fifth and final chapter analyzes the expression of genes related to immunity in response to infection of ruminants with pathogens of the genera *Anaplasma* and *Mycobacterium*.

Chapter 1 reviews the information about paratuberculosis in wild animals and the diagnostic methods. This review indicates that MAP circulates in most of the world, among different wild vertebrates. Among all species studied, only five are considered as reservoirs, including several ruminants, such as white-tailed deer in North America, red deer and roe deer in the Alps, and fallow deer in Asturias, Spain, and the rabbit in Scotland and probably in New Zealand, Spain and Czech Republic. The literature suggests that deer and rabbits can participate in the epidemiology of MAP, but always depending on the geographic region. The excretion intensity of MAP by wild animals is lower than the excretion by clinically affected cattle. Indeed, the main reservoir of MAP is domestic ruminants, so the role of wildlife as a reservoir would have a very local relevance, in places where domestic ruminants are controlled for MAP infection and coexist with a wild reservoir. No study concluded that paratuberculosis constitutes a limiting factor for the dynamics of a wild population. The main problems with the presence of paratuberculosis are related to captive animals or animals exposed to high environmental contamination by MAP originating from domestic ruminants. The cross-reaction between antigens of *Mycobacterium avium* complex and *M. tuberculosis* complex antigens (serology) and the lack of specificity in PCR and pathology is important. Consequently, MAP can compromise the diagnosis and control of tuberculosis, for example in farmed deer or in wildlife disease surveillance.

Paratuberculosis is a chronic enteritis shared between domestic livestock and wildlife. In addition to numerous wild ruminants, rabbits could also act as a reservoir of *Mycobacterium avium paratuberculosis* (MAP). Using molecular biology and histopathology techniques, **chapter 2** describes the presence of MAP in rabbits in southern Europe and the development of an indirect ELISA for the detention of specific antibodies against MAP. This chapter also describes the different pathological forms present in rabbits with paratuberculosis. 6 of the 237 tested sera from wild rabbits (2.5%) were identified as positive with the ELISA. Specific antibodies anti-MAP were detected in rabbit from 3 of the 8 areas studied. Necropsy revealed that 2 of 80 rabbits had gross lesions compatible with paratuberculosis. Histopathology revealed the presence of paratuberculosis lesions ranging from focal to multibacillary in 8 of the 10 rabbits examined. The presence of MAP was confirmed by RT-PCR. Therefore, in this study we confirmed, for the first time, the clinics and infection of MAP in wild rabbits from southern Europe. We have shown that the contact of wild

rabbit with MAP is widespread. Considering the growing importance of MAP in animal health, these findings open new windows for future research.

In **chapter 3** of this thesis we tested the hypothesis that wild canids, given their trophic ecology, could be used as sentinels in areas with high prevalence of MAP in wildlife and domestic animals. To test this hypothesis, we sampled 24 wolves and 285 foxes from different regions of the Peninsula, between 2004 and 2009. Our results rejected the hypothesis that wild canids may be useful as sentinels of paratuberculosis in southwestern Europe.

In **chapter 4** we analyzed samples from 332 red deer sampled in different regions of the iberian peninsula. Histopathology revealed only three animals (1.12%) showing focal lesions compatible with paratuberculosis. No MAP growth was observed in culture. In ELISA, the optical densities of plates coated with Protoplasmatic Paratuberculosis Antigen 3 (PPA3) Purified Protein Derivative and bovine (BOPD), respectively, were significantly correlated. This suggests a cross-reaction and, therefore, a lack of specificity of the test. The results contrast with the situation described in other regions, and led us to reject the hypothesis that the deer could act as a reservoir for paratuberculosis in the study area.

Pathogens of the genera *Anaplasma* and *Mycobacterium* are among the most prevalent bacterial pathogens of deer from the south-west of Spain. These pathogens are known to alter gene expression in ruminants. In **chapter 5** of this thesis, we used microarray hybridization and real time qRT-PCR to characterize the global profile of gene expression in deer in response to infections *Anaplasma ovis* and Anaplasma ovis / Mycobacterium bovis / *Mycobacterium avium paratuberculosis* (MAP), to compare gene expression of immune response in deer infected with *A. ovis*, *M. bovis* and *A. ovis* / *M. bovis* / MAP, and to characterize the differential expression expression of genes related to immunity in deer and cattle infected with *M. bovis* and *Anaplasma marginale*. Overall, the differential gene expression in deer infected with *A. ovis* / *M. bovis* / MAP resulted in the modification of common cellular and biological processes specific to these

pathogens. These specific differences of the pathogen and host may contribute to improve the diagnosis of diseases caused by *Anaplasma* and *Mycobacterium* and its control in ruminants.

CONCLUSIONES

1.- El diagnóstico de la paratuberculosis o de la infección por *Mycobacterium avium paratuberculosis* (MAP) en animales silvestres requiere combinar varias técnicas, incluyendo fundamentalmente la histopatología y el cultivo. El trabajo con fauna silvestre genera muestras más heterogéneas y, a menudo, de peor calidad que el trabajo con especies ganaderas. En esta tesis se han combinado los estudios anatomopatológicos con el cultivo, la serología y, en determinadas ocasiones, la PCR. Esta combinación permite tener una visión muy completa sobre la paratuberculosis en la fauna Ibérica.

2.- El conejo (*Oryctolagus cuniculus*) es localmente un potencial reservorio silvestre de MAP en ambientes termomediterráneos de la península ibérica. En este trabajo se ha observado que el conejo puede desarrollar lesiones difusas multibacilares de paratuberculosis, y puede presentar prevalencias de lesión elevadas, al menos en ambientes intensamente utilizados por rumiantes domésticos. En consecuencia, confirmamos el potencial del conejo como reservorio silvestre de paratuberculosis.

3.- En la península ibérica los cánidos silvestres zorro (*Vulpes vulpes*) y lobo (*Canis lupus*) no constituyen buenos indicadores de la presencia de MAP. Al contrario de lo descrito para otras regiones geográficas, los cánidos silvestres estudiados en esta tesis no mostraron evidencia específica de paratuberculosis y por lo tanto no resultan de utilidad como centinelas de MAP en el suroeste de Europa.

4.- En la península ibérica la paratuberculosis no interfiere en el uso de las lesiones visibles como herramienta en la vigilancia de tuberculosis en el ciervo (*Cervus elaphus*). Las poblaciones ibéricas de ciervo apenas presentaron lesiones compatibles con paratuberculosis, y tuvieron una nula prevalencia de infección de acuerdo con los resultados de cultivo. Este resultado contrasta

vivamente con las altas prevalencias registradas en granjas de ciervo, en poblaciones naturales de ciervo del arco alpino, y en gamos (*Dama dama*) simpátricos con ciervos de la reserva del Sueve en Asturias.

5.- El estudio de la expresión diferencial de genes en ciervos naturalmente infectados con los patógenos intracelulares *Anaplasma ovis* y *A.ovis/Mycobacterium bovis/*MAP aporta conocimientos básicos para el futuro desarrollo de mejoras en el diagnóstico y tratamiento de estas infecciones. En esta tesis se ha comprobado que la hibridación en micro-arrays y la RT-PCR en tiempo real son útiles para caracterizar la expresión génica global del ciervo en respuesta a infecciones naturales con *Anaplasma ovis* y co-infecciones *A. ovis*/*Mycobacterium bovis*/*Mycobacterium avium paratuberculosis* (MAP). Este análisis permitió valorar el efecto de la co-infección con múltiples patógenos intracelulares que resultó en la modificación de procesos biológicos celulares comunes y específicos para estos patógenos.

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Anexo 1

Shows the number of studies on MAP in free-ranging wild animals that used histopathology, PCR, culture and serology, as well as the sample sizes and estimated prevalences.

Columns 1-2-3-4: Scientific classifications of all species studied and their relation with MAP infection/prevalence; **Column 5**: Location of study and author; **Column 6**: Number of animals analyzed for each publication; **Column 7**: Status (free-living/captive); **Column 8**: Histopathology (yes/no); **Columns 9-10**: Number of animals tested by histopathology and percentage of positivity; **Column 11**: PCR (yes/no); **Columns 12-13**: Number of animals tested by PCR and percentage of positivity; **Column 14**: Culture (yes/no); **Columns 15-16**: Number of animals tested by culture and percentage of positivity; **Column 17**: Serology (yes/no); **Column 18-19**: Number of animals tested by serology and percentage of positivity; **Column 20**: clinical cases for each publication; **Column 21**: Combination of four test (histopathology, PCR, culture and serology).

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Anseriformes	Anatidae	Tadorninae	**Paradise shelducks** (*Tadorna variegata*)	New Zealand (Nugent et al., 2011)	17	wild	0	0		0	0		1	17	17,6	0	0		0	17,6
Artiodactyla	Bovidae	Bovinae	**Common Eland** (*Taurotragus oryx*)	Kenya (Paling et.al., 1988)	14	wild	0	0		0	0		0	0		1	14	0,0	0	0,0
Artiodactyla	Bovidae	Bovinae	**Water buffalo** (*Bubalus bubalis*)	Kenya (Paling et.al., 1988)	4	wild	0	0		0	0		0	0		1	4	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Chamois** (*Rupicapra rupicapra*)	Italy (Fattori et al., 2010)	47	wild	0	0		1	12	0,0	0	0		1	47	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Chamois** (*Rupicapra rupicapra*)	Spain (Falconi et al., 2010)	236	wild	0	0		0	0		0	0		1	236	9,7	0	9,7

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Caprinae	**Mouflon** (*Ovis musimon*)	Spain (Lopez-Olvera et al., 2009)	101	wild	0	0		0	0		0	0		1	101	1,0	0	1,0
Artiodactyla	Bovidae	Hippotraginae	**Scimitar Oryx** (*Oryx dammah*)	Kenya (Paling et.al., 1988)	20	wild	0	0		0	0		0	0		1	20	0,0	0	0,0
Artiodactyla	Bovidae		**Bison** (*Bison bison*)	Canada (Sibley et al., 2007)	835	wild	0	0		1	835	3,1	0	0		0	0		0	3,1
Artiodactyla	Bovidae		**Alpine ibex** (*Capra ibex*)	Switzerland (Giacometti et al., 1995)	89	wild	0	0		0	0		0	0		1	89	0,0	0	0,0
Artiodactyla	Bovidae		**Bison** (*Bison bison*)	USA (Ellingson et al., 2005)	25	wild	0	0		1	25	100,0	0	0		0	0		25	100,0
Artiodactyla	Camelidae		**Guanacos** (*Lama guanicoe*)	Chile (Salgado et al.,2009)	501	wild	0	0		0	0		1	501	4,2	0	0		0	4,2
Artiodactyla	Camelidae		**Bactrian camel** (*Camelus bactrianus*)	Kenya (Paling et.al., 1988)	102	wild	0	0		0	0		0	0		1	102	7,8	0	7,8
Artiodactyla	Cervidae	Capreolinae	**Moose** (*Alces alces*)	Norway (Tryland et al., 2004)	537	wild	0	0		0	0		0	0		1	537	1,9	0	1,9
Artiodactyla	Cervidae	Capreolinae	**Reindeer** (*Rangifer tarandus tarandus*)	Norway (Tryland et al., 2004)	325	wild	0	0		0	0		0	0		1	325	3,4	0	3,4
Artiodactyla	Cervidae	Capreolinae	**Whitetailed deer** (*Odocoileus virginianus*)	USA (Davidson et al., 2004)	313	wild	0	0		0	0		1	313	0,3	1	313	1,9	0	1,1

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Cervidae	Capreolinae	**Florida Key deer** (*Odocoileus virginianus clavium*)	USA (Pedersen et al., 2008)	97	wild	0	0		0	0		1	90	26,6	1	97	2,1	2	14,3
Artiodactyla	Cervidae	Capreolinae	**Florida Key deer** (*Odocoileus virginianus clavium*)	USA (Quist et al, 2002)	170	wild	1	170	1,2	0	0		1	96	1,0	1	142	4,9	2	3,0
Artiodactyla	Cervidae	Capreolinae	**Whitetailed deer** (*Odocoileus virginianus*)	USA (Raizman et al., 2005)	309	wild	0	0		0	0		1	309	0,6	0	0		0	
Artiodactyla	Cervidae	Capreolinae	**Whitetailed deer** (*Odocoileus virginianus*)	USA (Shulaw et al., 1986)	954	wild	0	0		0	0		0	0		1	954	2,5	0	2,5
Artiodactyla	Cervidae	Capreolinae	**Whitetailed deer** (*Odocoileus virginianus*)	USA (Sleeman et al., 2009)	83	wild	1	83	1,2	0	0		1	83	0,0	1	83	0,0	1	0,4
Artiodactyla	Cervidae	Capreolinae	**Whitetailed deer** (*Odocoileus virginianus*)	USA (Wolf et al., 2008)	114	wild	0	0		0	0		0	0		1	114	1,8	0	1,8
Artiodactyla	Cervidae	Cervinae	**Aixs deer** (*Axis axis*)	USA (Riemann et al., 1979)	52	wild	0	0		0	0		1	52	9,6	0	0		0	9,6
Artiodactyla	Cervidae	Cervinae	**Fallow Deer** (*Dama dama*)	USA (Riemann et al., 1979)	37	wild	0	0		0	0		1	37	8,1	0	0		0	8,1
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Italy (Andreoli et al., 2005)	3	wild	0	0		1	3	0,0	0	0		0	0		0	0,0
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Italy (Fattori et al., 2010)	97	wild	0	0		1	26	0,0	0	0		1	97	5,2	0	2,6

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Italy (Nebbia et al, 2000)	19	wild	0	0		1	19	89,5	1	14	21,4	1	10	10,0	6	40,3
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Italy (Robino et al.,2008)	77	wild	1	77	35,1	1	77	39,0	1	77	16,9	0	0		0	30,3
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Norway (Tryland et al., 2004)	416	wild	0	0		0	0		0	0		1	371	1,4	0	
Artiodactyla	Cervidae	Cervinae	**Fallow Deer** (*Dama dama*)	Spain (Álvarez et al., 2005)	94	wild	1	94	0,0	0	0		1	94	1,1	0	0		0	0,5
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Spain (Álvarez et al., 2005)	101	wild	1	101	0,0	0	0		1	101	0,0	0	0		0	0,0
Artiodactyla	Cervidae	Cervinae	**Fallow Deer** (*Dama dama*)	Spain (Balseiro et al., 2008)	95	wild	1	95	29,4	0	0		0	0		0	0		0	29,4
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Spain (Carta et al., 2012)	332	wild	1	267	1,1	0	0		1	324	0,0	1	238	39,1	0	19,6
Artiodactyla	Cervidae	Cervinae	**Fallow Deer** (*Dama dama*)	Spain (Marco et al., 2002)	33	wild	0	0		0	0		0	0		1	33	0,0	5	0,0
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Spain (Reyes-Garcia et al., 2008)	852	wild	0	0		0	0		0	0		1	852	30,2	0	30,2
Artiodactyla	Cervidae	Cervinae	**Tule elk** (*Cervus elaphus nannodes*)	USA (Crawford et al., 2006)	37	wild	0	0		0	0		1	37	2,7	1	0		0	2,7

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Cervidae	Cervinae	**Tule elk** (*Cervus elaphus nannodes*)	USA (Manning et al,2003)	45	wild	0	0		0	0		1	45	13,0	1	45	40,0	0	26,5
Artiodactyla	Cervidae	Cervinae	**Elk** (*Cervus elaphus nelsoni*)	USA (Rhyan et al., 1997)	289	wild	1	289	0,0	0	0		0	0		0	0		0	0,0
Artiodactyla	Cervidae	Odocoileinae	**Pampas deer** (*Ozotoceros bezoarticus celer*)	Argentina (Uhart et al., 2003)	14	wild	0	0		0	0		0	0		1	14	0,0	0	0,0
Artiodactyla	Cervidae	Odocoileinae	**Roe Deer** (*Capreolus capreolus*)	Italy (Fattori et al., 2010)	428	wild	0	0		1	205	1,0	0	0		1	428	0,0	0	0,5
Artiodactyla	Cervidae	Odocoileinae	**Roe Deer** (*Capreolus capreolus*)	Italy (Robino et al., 2008)	69	wild	1	69	37,7	1	69	29,0	1	69	11,6	0	0		0	26,1
Artiodactyla	Cervidae	Odocoileinae	**Roe Deer** (*Capreolus capreolus*)	Norway (Tryland et al., 2004)	49	wild	0	0		0	0		0	0		1	49	12,2	0	12,2
Artiodactyla	Cervidae	Odocoileinae	**Roe Deer** (*Capreolus capreolus*)	Spain (Boadella et al., 2010)	519	wild	0	0		0	0		0	0		1	519	9,2	0	9,2
Artiodactyla	Cervidae		**Grey brocket deer** (*Mazama gouazoubira*)	Bolivia (Deem et al., 2004)	17	wild	0	0		0	0		0	0		1	17	0,0	0	0,0
Artiodactyla	Suidae		**Wild boar** (*Sus scrofa*)	Czech Republic (Trcka et al., 2006)	274	wild	0	0		0	0		1	274	0,0	0	0		0	0,0
Artiodactyla	Suidae		**Wild boar** (*Sus scrofa*)	Italy (Zanetti et al., 2008)	48	wild	0	0		1	48	20,8	1	48	6,3	0	0		0	13,5

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Suidae		**Wild boar** (*Sus scrofa*)	Spain (Álvarez et al., 2005)	65	wild	1	65	0,0	0	0		1	65	1,5	0	0		0	0,8
Artiodactyla	Bovidae	Bovinae	**Antelopes** (*Boselaphus tragocamelus)*	India (Kumar et al., 2010)	42	wild	0	0		0	0		1	42	23.8	0	0		0	
Artiodactyla	Bovidae		**Indian bison** (*Bos gourus*)	India (Singh et al., 2011)	13	wild	1	13	46,1	0	0		1	13	61.8	0	0		0	46,1
Carnivora	Canidae	Caninae	**Red Fox** (*Vulpes vulpes*)	Greece (Florou et al., 2008)	10	wild	0	0		1	10	0,0	1	10	10,0	0	0		0	5,0
Carnivora	Canidae	Caninae	**Red Fox** (*Vulpes vulpes*)	Spain (Carta et al., 2010)	285	wild	0	0		0	0		1	56	0,0	1	239	4,2	0	2,1
Carnivora	Canidae	Caninae	**Wolf** (*Canis lupus*)	Spain (Carta et al., 2010)	24	wild	0	0		0	0		0	0		1	23	0,0	0	0,0
Carnivora	Canidae	Caninae	**Red Fox** (*Vulpes vulpes*)	UK (Beard et al., 2001a)	27	wild	1	26	46,2	0	0	0,0	1	27	85,2	0	0		0	42,6
Carnivora	Canidae	Caninae	**Red Fox** (*Vulpes vulpes*)	USA (Anderson et al., 2007)	7	wild	0	0		1	7	71,0	0	0	0,0	0	0		0	35,5
Carnivora	Canidae		**Coyote** (*Canis latrans*)	USA (Anderson et al., 2007)	59	wild	0	0		1	59	47,0	1	59	1,7	0	0		0	24,3
Carnivora	Felidae		**Feral cat** (*Felis catus*)	USA (Anderson et al., 2007)	5	wild	0	0		1	5	20,0	0	0	0,0	0	0		0	10,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Carnivora	Felidae		**Feral cat** (*Felis catus*)	USA (Corn et al., 2005)	18	wild	0	0		0	0		1	18	11,0	0	0		0	11,0
Carnivora	Felidae		**Feral cat** (*Felis catus*)	USA (Palmer et al., 2005)	25	wild	1	0	0,0	0	0		0	0		1	25	28,0	0	14,0
Carnivora	Felidae		**Feral cat** (*Felis catus*)	USA (Pedersen et al., 2008)	3	wild	0	0		0	0		1	3	0,0	0	0		0	0,0
Carnivora	Mephitidae		**Striped skunk** (*Mephitis mephitis*)	USA (Anderson et al., 2007)	5	wild	0	0		1	5	60,0	0	0	0,0	0	0		0	30,0
Carnivora	Mephitidae		**Striped skunk** (*Mephitis mephitis*)	USA (Corn et al., 2005)	10	wild	0	0		0	0		1	10	10,0	0	0		0	10,0
Carnivora	Mustelidae	Mustelinae	**Stoat** (*Mustela erminea*)	UK (Beard et al., 2001a)	37	wild	1	13	7,7	0	0	0,0	1	37	46.0	0	0		0	0,0
Carnivora	Mustelidae	Mustelinae	**Weasel** (*Mustela nivalis*)	UK (Beard et al., 2001a)	4	wild	1	4	50,0	0	0	0,0	1	4	50,0	0	0		0	33,3
Carnivora	Mustelidae		**Badger** (*Meles meles*)	Greece (Florou et al., 2008)	3	wild	0	0		1	3	0,0	1	3	0,0	0	0		0	0,0
Carnivora	Procyonidae		**Raccoon** (*Procyon lotor*)	USA (Anderson et al., 2007)	73	wild	0	0		1	73	40,0	0	0	0,0	0	0		0	20,0
Carnivora	Procyonidae		**Raccoon** (*Procyon lotor*)	USA (Corn et al., 2005)	42	wild	0	0		0	0		1	42	19,0	0	0		0	19,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Carnivora	Procyonidae		**Raccoon** (*Procyon lotor*)	USA (Palmer et al., 2005)	6	wild	1	0	0,0	0	0		0	0		1	6	0,0	0	0,0
Carnivora	Procyonidae		**Raccoon** (*Procyon lotor*)	USA (Pedersen et al. 2008)	30	wild	0	0		0	0		1	30	3,3	0	0		0	3,3
Carnivora	Ursidae		**Brown bear** (*Ursus arctos*)	Slovakia (Kopecna et al., 2006)	20	wild	1	20	5,0	0	0		1	20	10,0	0	0		0	7,5
Carnivora	Mustelidae		**Ferret** (*Mustela putorius furo*)	New Zealand (Nugent et al., 2011)	44	wild	0	0		0	0		1	43	17,4	0	0		0	17,4
Carnivora	Mustelidae	Mustelinae	**Stoats** (*Mustela erminea*)	New Zealand (Nugent et al., 2011)	5	wild	0	0		0	0		1	5	0,0	0	0		0	0,0
Charadriiformes	Laridae		**Black-headed Gull** (*Larus ridibundus*)	Slovakia (Gronesova et al., 2006)	11	wild	0	0		1	11	18,2	0	0		0	0		0	18,2
Charadriiformes	Scolopacidae		**Ruff** (*Philomachus pugnax*)	Slovakia (Gronesova et al., 2006)	9	wild	0	0		1	9	11,1	0	0		0	0		0	11,1
Charadriiformes	Laridae		**Black-backed gulls** (*Larus dominicanus*)	New Zealand (Nugent et al., 2011)	5	wild	0	0		0	0		1	5	20,0	0	0		0	20,0
Cingulata	Dasypodidae	Dasypodinae	**Armadillo** (*Dasypus novemcinctus*)	USA (Corn et al., 2005)	23	wild	0	0		0	0		1	23	17,4	0	0		0	17,4
Columbiformes	Columbidae		**Rock pigeon** (*Columba livia*)	UK (Beard et al., 2001a)	59	wild	1	59	0,0	0	0	0,0	1	59	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Columbiformes	Columbidae		**Wood pigeon** (*Columba palumbus*)	UK (Beard et al., 2001a)	15	wild	1	15	0,0	0	0	0,0	1	15	0,0	0	0		0	0,0
Didelphimorphia	Didelphidae	Didelphinae	**Opossum** (*Didelphis virginiana*)	USA (Anderson et al., 2007)	63	wild	0	0		1	63	24,0	0	0	0,0	0	0		0	12,0
Didelphimorphia	Didelphidae	Didelphinae	**Opossum** (*Didelphis virginiana*)	USA (Corn et al., 2005)	54	wild	0	0		0	0		1	54	3,7	0	0		0	3,7
Didelphimorphia	Didelphidae	Didelphinae	**Opossum** (*Didelphis virginiana*)	USA (Palmer et al., 2005)	3	wild	1	0	0,0	0	0		0	0		1	3	0,0	0	0,0
Diprotodontia	Macropodiadae		**kangaroo** (*Macropus giganteus*)	Australia (Abbott et al., 2002)	300	wild	1	94	0,0	0	0		1	205	0,48	0	0		0	0,0
Diprotodontia	Phalangeridae		**Common brushtail possum** (*Trichosurus vulpecula*)	New Zealand (Nugent et al., 2011)	73	wild	0	0		0	0		1	73	25,0	0	0		0	25,0
Erinaceomorpha	Erinaceinae		**Hedgehogs** (*Erinaceus europaeus occidentalis*),	New Zealand (Nugent et al., 2011)	42	wild	0	0		0	0		1	42	35,7	0	0		0	35,7
Falconiformes	Accipitridae		**Australasian harriers** (*Circus approximans*)	New Zealand (Nugent et al., 2011)	3	wild	0	0		0	0		1	3	0,0	0	0		0	0,0
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	UK (Greig et al., 1997)	33	wild	1	33	67,0	0	0		0	0		0	0		0	67,0
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	UK (Greig et al., 1999)	210	wild	0	0		0	0		1	130	7,1	0	0		0	7,1

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Lagomorpha	Leporidae		**Brown hare** (*Lepus europaeus*)	Greece (Florou et al., 2008)	44	wild	0	0		1	44	0,0	1	44	2,7	0	0		0	1,4
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	Spain (Maio et al., 2011)	237	wild	1	80	2,5	0	0		0	0		1	237	2,5	0	2,5
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	UK (Angus et al., 1990)	32	wild	1	32	3,1	0	0		0	0		0	0		0	3,1
Lagomorpha	Leporidae		**Brown hare** (*Lepus europaeus*)	UK (Beard et al., 2001a)	6	wild	1	4	0,0	0	0	0,0	1	6	16,6	0	0		0	5,5
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	UK (Beard et al., 2001b)	110	wild	1	98	18,4	0	0		1	110	15,5	0	0		0	
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	UK (Judge et al., 2006)	378	wild	1	378	0,0	1	0		1	378	34,1	1	0		0	17,1
Lagomorpha	Leporidae		**Eastern cottontail** (*Sylvilagus floridanus*)	USA (Corn et al., 2005)	56	wild	0	0		0	0		1	56	1,8	0	0		0	1,8
Lagomorpha	Leporidae		**Eastern cottontail** (*Sylvilagus floridanus*)	USA (Corn J et al., 2005)	56	wild	0	0		0	0		1	56	16,6	0	0		0	16,6
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	USA (Palmer et al., 2005)	8	wild	1	0	0,0	0	0		0	0		1	8	0,0	0	0,0
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	USA (Raizman et al., 2005)	218	wild	0	0		0	0		1	218	0,9	0	0		0	0,9

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	Australia (Abbott et al., 2002)	300	wild	1	47	0,0	0	0		1	252	0,0	0	0		0	0,0
Lagomorpha	Leporidae		**Brown hare** (*Lepus europaeus*)	Chile (Salgado et al., 2011)	385	wild	1	385	0,0	0	0		1	385	16,6	0	0		0	8,3
Lagomorpha	Leporidae		**Hare** (*Lepus europaeus occidentalis*),	New Zealand (Nugent et al., 2011)	76	wild	0	0		0	0		1	76	6,6	0	0		0	6,6
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	New Zealand (Nugent et al., 2011)	113	wild	0	0		0	0		1	113	25,7	0	0		0	25,7
Passeriformes	Corvidae		**Magpies** (*Pica pica*)	Greece (Florou et al., 2008)	30	wild	0	0		1	30	0,0	1	30	0,0	0	0		0	0,0
Passeriformes	Corvidae		**Carrion Crow** (*Corvus corone*)	UK (Beard et al., 2001a)	60	wild	1	60	1,7	0	0	0,0	1	60	60,0	0	0		0	30,0
Passeriformes	Corvidae		**Jackdaw** (*Corvus monedula*)	UK (Beard et al., 2001a)	38	wild	1	38	2,6	0	0	0,0	1	38	0,0	0	0		0	0,0
Passeriformes	Corvidae		**Rook** (*Corvus frugilegus*)	UK (Beard et al., 2001a)	53	wild	1	53	0,0	0	0	0,0	1	53	5,7	0	0		0	0,0
Passeriformes	Emberizidae		**Reed Bunting** (*Emberiza schoeniclus*)	Slovakia (Gronesova et al., 2006)	6	wild	0	0		1	6	0,0	0	0		0	0		0	0,0
Passeriformes	Hirundinidae		**Swallow** (*Hirundo rustica*)	Slovakia (Gronesova et al., 2006)	3	wild	0	0		1	3	0,0	0	0		0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Passeriformes	Panuridae		**Bearded Tit** (*Panurus biarmicus*)	Slovakia (Gronesova et al., 2006)	7	wild	0	0		1	7	0,0	0	0		0	0		0	0,0
Passeriformes	Paridae		**Blue Tit** (*Parus caeruleus*)	Slovakia (Gronesova et al., 2006)	3	wild	0	0		1	3	0,0	0	0		0	0		0	0,0
Passeriformes	Passeridae		**House sparrow** (*Passer domesticus*)	Greece (Florou et al., 2008)	32	wild	0	0		1	32	0,0	1	32	0,0	0	0		0	0,0
Passeriformes	Passeridae		**House sparrow** (*Passer domesticus*)	UK (Beard et al., 2001a)	47	wild	1	47	0,0	0	0	0,0	1	47	0,0	0	0		0	0,0
Passeriformes	Passeridae		**House sparrow** (*Passer domesticus*)	USA (Corn et al., 2005)	60	wild	0	0		0	0		1	60	1,7	0	0		0	1,7
Passeriformes	Sturnidae		**Common Starling** (*Sturnus vulgaris*)	Slovakia (Gronesova et al., 2006)	3	wild	0	0		1	3	33,3	0	0		0	0		0	33,3
Passeriformes	Sturnidae		**European starling** (*Sturnus vulgaris*)	USA (Corn et al., 2005)	40	wild	0	0		0	0		1	40	17,5	0	0		0	17,5
Passeriformes	Sylviidae		**Reed Warbler** (*Acrocephalus scirpaceus*)	Slovakia (Gronesova et al., 2006)	10	wild	0	0		1	10	0,0	0	0		0	0		0	0,0
Passeriformes	Sylviidae		**Savi's Warbler** (*Locustella luscinioides*)	Slovakia (Gronesova et al., 2006)	3	wild	0	0		1	3	33,3	0	0		0	0		0	33,3
Passeriformes	Cracticidae		**Magpies** (*Gymnorhina tibicen*)	New Zealand (Nugent et al., 2011)	4	wild	0	0		0	0		1	4	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Rodentia	Cricetidae	Arvicolinae	**Bank vole** (*Clethrionomys glareolus*)	UK (Beard et al., 2001a)	19	wild	1	19	5,2	0	0	0,0	1	19	0,0	0	0		0	1,7
Rodentia	Cricetidae	Arvicolinae	**Field vole** (*Microtus agrestis*)	UK (Beard et al., 2001a)	7	wild	1	7	0,0	0	0	0,0	1	7	0,0	0	0		0	0,0
Rodentia	Cricetidae		**Hispid cotton rat** (*Sigmodon hispidus*)	USA (Corn et al., 2005)	41	wild	0	0		0	0		1	41	2,4	0	0		0	2,4
Rodentia	Muridae	Murinae	**House mouse** (*Mus musculus*)	Greece (Florou et al., 2008)	149	wild	0	0		1	149	0,0	1	149	1,3	0	0		0	0,7
Rodentia	Muridae	Murinae	**House mouse** (*Mus musculus*)	UK (Beard et al., 2001a)	89	wild	1	89	0,0	0	0	0,0	1	89	0,0	0	0		0	0,0
Rodentia	Muridae	Murinae	**Norway rat** (*Rattus norvegicus*)	UK (Beard et al., 2001a)	35	wild	1	23	0,0	0	0	0,0	1	35	8,6	0	0		0	0,0
Rodentia	Muridae	Murinae	**Wood mouse** (*Apodemus sylvaticus*)	UK (Beard et al., 2001a)	88	wild	1	88	1,1	0	0	0,0	1	88	3,4	0	0		0	0,0
Rodentia	Muridae	Murinae	**Norway rat** (*Rattus norvegicus*)	USA (Corn et al., 2005)	4	wild	0	0		0	0		1	4	25,0	0	0		0	25,0
Rodentia	Muridae	Murinae	**Mouse** (species unknown)	USA (Palmer et al., 2005)	9	wild	1	0	0,0	0	0		0	0		1	9	11,1	0	5,6
Rodentia	Muridae		**Black rat** (*Rattus rattus*)	Greece (Florou et al., 2008)	55	wild	0	0		1	55	1,8	1	55	1,8	0	0		0	1,8

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	N° Animales	Wild/ Capt.	Histop.	n° an.	% histo+	PCR	n° an.	% PCR+	Culture	n° an.	% Cult+	Serology	n° an.	% Serol.+	Clin. case	Mean 4 TEST
Rodentia	Muridae	Murinae	**Norway rat** (*Rattus norvegicus*)	New Zealand (Nugent et al., 2011)	4	wild	0	0		0	0		1	4	0,0	0	0		0	0,0
Soricomorpha	Soricidae		**Southeastern shrew** (*Sorex longirostris*)	USA (Corn et al., 2005)	4	wild	0	0		0	0		1	4	25,0	0	0		0	25,0
TOT				**127**	**13577**	**wild**	**40**	**3035**	**8,6**	**33**	**1909**	**13,7**	**78**	**6230**	**9,9**	**38**	**6401**	**6,5**	**41**	**10,2**

Anexo 2

Data collected from the studies on MAP in captive wild animals (including zoos and game parks) that used histopathology, PCR, culture and serology, as well as the sample sizes and estimated prevalences.

Columns 1-2-3-4: Scientific classifications of all species studied and their relation with MAP infection/prevalence; **Column 5**: Location of study and author; **Column 6**: Number of animals analyzed for each publication; **Column 7**: Status (free-living/captive); **Column 8**: Histopathology (yes/no); **Columns 9-10**: Number of animals tested by histopathology and percentage of positivity; **Column 11**: PCR (yes/no); **Columns 12-13**: Number of animals tested by PCR and percentage of positivity; **Column 14**: Culture (yes/no); **Columns 15-16**: Number of animals tested by culture and percentage of positivity; **Column 17**: Serology (yes/no); **Column 18-19**: Number of animals tested by serology and percentage of positivity; **Column 20**: clinical cases for each publication; **Column 21**: Combination of four test (histopathology, PCR, culture and serology).

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Alcelaphinae	**Blue gnu** (*Connochaetes taurinus*)	Belgium (Vansnick et al., 2005)	8	captive	0	0		0	0		0	0		1	8	0,0	0	0,0
Artiodactyla	Bovidae	Alcelaphinae	**Blesbok** (*Damaliscus pygargus*)	Belgium (Vansnick et al., 2005)	3	captive	0	0		0	0		0	0		1	3	100,0	0	100,0
Artiodactyla	Bovidae	Alcelaphinae	**Whitetailed Gnu** (*Connochaetes gnou*)	Belgium (Vansnick et al., 2005)	3	captive	0	0		0	0		0	0		1	3	0,0	0	0,0
Artiodactyla	Bovidae	Alcelaphinae	**Blue Wildebeest** (*Connochaetes taurinus*)	USA (Witte et al., 2009)	17	captive	0	0		0	0		1	17	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Alcelaphinae	**Blesbok** (*Damaliscus pygargus*)	USA (Witte et al., 2009)	14	captive	0	0		0	0		1	14	14,3	0	0		0	14,3

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Alcelaphinae	**Jimela topi** (*Damaliscus korrigum*)	USA (Witte et al., 2009)	4	captive	0	0		0	0		1	4	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Antilopinae	**Saiga antelope** (*Saiga tatarica*)	Belgium (Vansnick et al., 2005)	3	captive	0	0		0	0		0	0		1	3	0,0	0	0,0
Artiodactyla	Bovidae	Antilopinae	**Slender-horned gazelle** (*Gazella leptoceros*)	Belgium (Vansnick et al., 2005)	6	captive	0	0		0	0		0	0		1	6	0,0	0	0,0
Artiodactyla	Bovidae	Antilopinae	**Blackbuck** (*Antilope cervicapra*)	USA (Witte et al., 2009)	39	captive	0	0		0	0		1	39	2,6	0	0		0	2,6
Artiodactyla	Bovidae	Antilopinae	**Addra gazelle** (*Nanger dama*)	USA (Witte et al., 2009)	62	captive	0	0		0	0		1	62	3,2	0	0		0	3,2
Artiodactyla	Bovidae	Antilopinae	**Angolan springbok** (*Antidorcas marsupialis*)	USA (Witte et al., 2009)	28	captive	0	0		0	0		1	28	32,1	0	0		0	32,1
Artiodactyla	Bovidae	Antilopinae	**Cuvier's gazelle** (*Gazella cuvieri*)	USA (Witte et al., 2009)	58	captive	0	0		0	0		1	58	1,7	0	0		0	1,7
Artiodactyla	Bovidae	Antilopinae	**Grant's gazelle** (*Nanger granti*)	USA (Witte et al., 2009)	47	captive	0	0		0	0		1	47	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Antilopinae	**Nubian Soemmerring's gazelle** (*Nanger soemmerringii*)	USA (Witte et al., 2009)	53	captive	0	0		0	0		1	53	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Antilopinae	**Red-fronted gazelle** (*Eudorcas rufifrons*)	USA (Witte et al., 2009)	18	captive	0	0		0	0		1	18	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Antilopinae	**Russian saiga** (*Saiga tatarica*)	USA (Witte et al., 2009)	8	captive	0	0		0	0		1	8	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Antilopinae	**Saharan Dorcas gazelle** (*Gazella dorcas*)	USA (Witte et al., 2009)	28	captive	0	0		0	0		1	28	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Antilopinae	**Sand gazelle** (*Gazella subgutturosa*)	USA (Witte et al., 2009)	106	captive	0	0		0	0		1	106	0,9	0	0		0	0,9
Artiodactyla	Bovidae	Antilopinae	**Slender-horned gazelle** (*Gazella leptoceros*)	USA (Witte et al., 2009)	55	captive	0	0		0	0		1	55	3,6	0	0		0	3,6
Artiodactyla	Bovidae	Antilopinae	**South African sable antelope** (*Hippotragus niger*)	USA (Witte et al., 2009)	31	captive	0	0		0	0		1	31	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Antilopinae	**Southern gerenuk** (*Litocranius walleri*)	USA (Witte et al., 2009)	10	captive	0	0		0	0		1	10	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Antilopinae	**Thomson's gazelle** (*Eudorcas thomsonii*)	USA (Witte et al., 2009)	69	captive	0	0		0	0		1	69	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**African cape buffalo** (*Syncerus caffer caffer*)	Belgium (Vansnick et al.,2005)	20	captive	0	0		0	0		0	0		1	20	0,0	0	0,0
Artiodactyla	Bovidae	Bovinae	**Anoa** (*Bubalus depressicornis*)	Belgium (Vansnick et al., 2005)	5	captive	0	0		0	0		0	0		1	5	0,0	0	0,0
Artiodactyla	Bovidae	Bovinae	**Banteng** (*Bos javanicus*)	Belgium (Vansnick et al., 2005)	5	captive	0	0		0	0		0	0		1	5	0,0	0	0,0
Artiodactyla	Bovidae	Bovinae	**Common Eland** (*Taurotragus oryx*)	Belgium (Vansnick et al., 2005)	33	captive	0	0		0	0		0	0		1	33	3,0	0	3,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Bovinae	**Forest buffalo** (*Syncerus caffer nanus*)	Belgium (Vansnick et al.,2005)	7	captive	0	0		0	0		0	0		1	7	0,0	0	0,0
Artiodactyla	Bovidae	Bovinae	**Nilgai** (*Boselaphus tragocamelus*)	Belgium (Vansnick et al., 2005)	11	captive	0	0		0	0		0	0		1	11	9,1	0	9,1
Artiodactyla	Bovidae	Bovinae	**Sitatunga** (*Tragelaphus spekii gratus Sclater*)	Belgium (Vansnick et al., 2005)	12	captive	0	0		0	0		0	0		1	12	0,0	0	0,0
Artiodactyla	Bovidae	Bovinae	**Banteng** (*Bos javanicus*)	Germany (Weber et al., 1992a)	15	captive	0	0		0	0		1	15	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Banteng** (*Bos javanicus*)	USA (Witte et al. ,2009)	37	captive	0	0		0	0		1	37	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Bongo** (*Tragelaphus eurycerus*)	USA (Witte et al., 2009)	27	captive	0	0		0	0		1	27	3,7	0	0		0	3,7
Artiodactyla	Bovidae	Bovinae	**Common Eland** (*Taurotragus oryx*)	USA (Witte et al., 2009)	34	captive	0	0		0	0		1	34	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**East African sitatunga** (*Tragelaphus spekii*)	USA (Witte et al., 2009)	33	captive	0	0		0	0		1	33	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Eastern yellow-backed duiker** (*Cephalophus silvicultor*)	USA (Witte et al., 2009)	12	captive	0	0		0	0		1	12	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Forest buffalo** (*Syncerus caffer nanus*)	USA (Witte et al., 2009)	5	captive	0	0		0	0		1	5	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Bovinae	**Giant eland** (*Taurotragus derbianus*)	USA (Witte et al., 2009)	16	captive	0	0		0	0		1	16	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Grey Rhebok** (*Pelea capreolus*)	USA (Witte et al., 2009)	7	captive	0	0		0	0		1	7	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Indian gaur** (*Bos frontalis*)	USA (Witte et al.,2009)	36	captive	0	0		0	0		1	36	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Lowland wisent** (*Bison bonasus*)	USA (Witte et al.,2009)	20	captive	0	0		0	0		1	20	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Nilgai** (*Boselaphus tragocamelus*)	USA (Witte et al., 2009)	12	captive	0	0		0	0		1	12	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**South African greater kudu** (*Tragelaphus strepsiceros*)	USA (Witte et al., 2009)	30	captive	0	0		0	0		1	30	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Bovinae	**Water buffalo** (*Bubalus bubalis*)	USA (Witte et al., 2009)	4	captive	0	0		0	0		1	4	25,0	0	0		0	25,0
Artiodactyla	Bovidae	Caprinae	**Bighorn sheep** (*Ovis canadensis*)	Belgium (Vansnick et al., 2005)	7	captive	0	0		0	0		0	0		1	7	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Capricorn** (*Ibex ibex*)	Belgium (Vansnick et al., 2005)	4	captive	0	0		0	0		0	0		1	4	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Goat** (*Capra hircus*)	Belgium (Vansnick et al., 2005)	6	captive	0	0		0	0		0	0		1	6	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Goat** (*Capra hircus*)	Belgium (Vansnick et al., 2005)	5	captive	0	0		0	0		0	0		1	5	0,0	0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Caprinae	**Himalayan tahr** (*Hemitragus jemlahicus*)	Belgium (Vansnick et al., 2005)	14	captive	0	0		0	0		0	0		1	14	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Mouflon** (*Ovis musimon*)	Belgium (Vansnick et al.,2005)	6	captive	0	0		0	0		0	0		1	6	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Mountain Goat** (Oreamnos americanus)	Belgium (Vansnick et al., 2005)	3	captive	0	0		0	0		0	0		1	3	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Muskox** (*Ovibos moschatus Wardi*)	Belgium (Vansnick et al., 2005)	11	captive	0	0		0	0		0	0		1	11	0,0	0	0,0
Artiodactyla	Bovidae	Caprinae	**Chamois** (*Rupicapra rupicapra*)	Czech Republic (Kopecna et al., 2008a)	162	captive	0	0		0	0		1	162	2,5	0	0		0	2,5
Artiodactyla	Bovidae	Caprinae	**Mouflon** (*Ovis musimon*)	Czech Republic (Kopecna et al., 2008a)	866	captive	0	0		0	0		1	866	3,2	0	0		0	3,2
Artiodactyla	Bovidae	Caprinae	**Bighorn sheep** (*Ovis canadensis*)	Germany (Weber et al., 1992a)	6	captive	0	0		0	0		1	6	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Caprinae	**Mouflon** (*Ovis musimon*)	Germany (Weber et al., 1992a)	14	captive	0	0		0	0		1	14	21,4	0	0		0	21,4
Artiodactyla	Bovidae	Caprinae	**Wild goats** (*Capra aegagrus*)	Germany (Weber et al., 1992a)	9	captive	0	0		0	0		1	9	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Caprinae	**Bharal** (*Pseudois nayaur*)	USA (Witte et al., 2009)	52	captive	0	0		0	0		1	52	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Caprinae	**Caucasian tur** (*Capra caucasica*)	USA (Witte et al., 2009)	61	captive	0	0		0	0		1	61	1,6	0	0		0	1,6

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Caprinae	**Chinese goral** (*Naemorhedus griseus*)	USA (Witte et al., 2009)	33	captive	0	0		0	0		1	33	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Caprinae	**Himalayan tahr** (*Hemitragus jemlahicus*)	USA (Witte et al., 2009)	39	captive	0	0		0	0		1	39	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Caprinae	**Japanese serow** (*Capricornis crispus*)	USA (Witte et al., 2009)	11	captive	0	0		0	0		1	11	9,1	0	0		0	9,1
Artiodactyla	Bovidae	Caprinae	**Markhor** (*Capra falconeri*)	USA (Witte et al., 2009)	62	captive	0	0		0	0		1	62	9,7	0	0		0	9,7
Artiodactyla	Bovidae	Caprinae	**Mouflon** (*Ovis musimon*)	USA (Witte et al.,2009)	214	captive	0	0		0	0		1	214	4,7	0	0		0	4,7
Artiodactyla	Bovidae	Caprinae	**Nilgiri tahr** (*Hemitragus hylocrius*)	USA (Witte et al.,2009)	14	captive	0	0		0	0		1	14	7,1	0	0		0	7,1
Artiodactyla	Bovidae	Caprinae	**Nubian ibex** (*Capra nubiana*)	USA (Witte et al.,2009)	51	captive	0	0		0	0		1	51	5,9	0	0		0	5,9
Artiodactyla	Bovidae	Caprinae	**Rocky Mountain bighorn** (*Ovis canadensis*)	USA (Witte et al., 2009)	3	captive	0	0		0	0		1	3	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Caprinae	**Sichuan takin** (*Budorcas taxicolor*)	USA (Witte et al., 2009)	25	captive	0	0		0	0		1	25	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Caprinae	**Sudan Barbary sheep** (*Ammotragus lervia*)	USA (Witte et al., 2009)	33	captive	0	0		0	0		1	33	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Cephalophinae	**Blue duiker** (*Philantomba monticola*)	Belgium (Vansnick et al., 2005)	3	captive	0	0		0	0		0	0		1	3	0,0	0	0,0
Artiodactyla	Bovidae	Cephalophinae	**Maxwell's duiker** (*Philantomba maxwellii*)	USA (Witte et al., 2009)	7	captive	0	0		0	0		1	7	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Cephalophinae	**Blue duiker** (*Philantomba monticola*)	USA (Witte et al., 2009)	6	captive	0	0		0	0		1	6	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Cephalophinae	**Southeastern crowned duiker** (*Sylvicapra grimmia*)	USA (Witte et al., 2009)	16	captive	0	0		0	0		1	16	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Hippotraginae	**Sable antelope** (*Hippotragus niger*)	Belgium (Vansnick et al., 2005)	4	captive	0	0		0	0		0	0		1	4	0,0	0	0,0
Artiodactyla	Bovidae	Hippotraginae	**Arabian Oryx** (*Oryx leucoryx*)	Belgium (Vansnick et al., 2005)	10	captive	0	0		0	0		0	0		1	10	0,0	0	0,0
Artiodactyla	Bovidae	Hippotraginae	**Addax** (*Addax nasomaculatus*)	USA (Witte et al., 2009)	42	captive	0	0		0	0		1	42	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Hippotraginae	**Angolan roan antelope** (*Hippotragus equinus*)	USA (Witte et al., 2009)	17	captive	0	0		0	0		1	17	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Hippotraginae	**Arabian oryx** (*Oryx leucoryx*)	USA (Witte et al., 2009)	40	captive	0	0		0	0		1	40	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Hippotraginae	**East African oryx** (*Oryx beisa*)	USA (Witte et al., 2009)	80	captive	0	0		0	0		1	80	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	N° Animales	Wild/ Capt.	Histop.	n° an.	% histo+	PCR	n° an.	% PCR+	Culture	n° an.	% Cult+	Serology	n° an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae	Hippotraginae	**Gemsbok** (*Oryx gazella*)	USA (Witte et al., 2009)	40	captive	0	0		0	0		1	40	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Hippotraginae	**Scimitar Oryx** (*Oryx dammah*)	USA (Witte et al., 2009)	59	captive	0	0		0	0		1	59	0,0	0	0		0	0,0
Artiodactyla	Bovidae	Reduncinae	**Ellipsen waterbuck** (*Kobus ellipsiprymnus*)	USA (Witte et al., 2009)	125	captive	0	0		0	0		1	125	6,4	0	0		0	6,4
Artiodactyla	Bovidae		**Bison** (*Bison bison*)	Belgium (Vansnick et al., 2005)	43	captive	0	0		0	0		0	0		1	43	2,3	0	2,3
Artiodactyla	Bovidae		**European bison** (*Bison bonasus*)	Belgium (Vansnick et al., 2005)	19	captive	0	0		0	0		0	0		1	19	15,7	0	15,7
Artiodactyla	Bovidae		**Yak** (*Poephagus mutus grunniens*)	Belgium (Vansnick et al., 2005)	23	captive	0	0		0	0		0	0		1	23	13,0	0	13,0
Artiodactyla	Bovidae		**Alpine ibex** (*Capra ibex*)	Germany (Weber et al., 1992a)	11	captive	0	0		0	0		1	11	9,1	0	0		0	9,1
Artiodactyla	Bovidae		**Bison** (*Bison bison*)	USA (Buergelt et al., 2000)	70	captive	1	70	43,0	0	0		0	0		0	0		17	43,0
Artiodactyla	Bovidae		**Alpine ibex** (*Capra ibex*)	USA (Witte et al., 2009)	61	captive	0	0		0	0		1	61	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Dik-dik** (*Madoqua kirkii*)	USA (Witte et al., 2009)	11	captive	0	0		0	0		1	11	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae		**Kenya impala** (*Aepyceros melampus*)	USA (Witte et al., 2009)	55	captive	0	0		0	0		1	55	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Kenyan Guenther's dik-dik** (*Madoqua guentheri*)	USA (Witte et al., 2009)	7	captive	0	0		0	0		1	7	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Klipspringer** (*Oreotragus oreotragus*)	USA (Witte et al., 2009)	9	captive	0	0		0	0		1	9	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Lechwe** (*Kobus leche*)	USA (Witte et al., 2009)	50	captive	0	0		0	0		1	50	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Nile lechwe** (*Kobus megaceros*)	USA (Witte et al., 2009)	43	captive	0	0		0	0		1	43	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Nyala** (*Tragelaphus angasii*)	USA (Witte et al., 2009)	12	captive	0	0		0	0		1	12	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Pyrenean Ibex** (*Capra pyrenaica*)	USA (Witte et al., 2009)	3	captive	0	0		0	0		1	3	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Red-flanked duiker** (*Cephalophus rufilatus*)	USA (Witte et al.,2009)	4	captive	0	0		0	0		1	4	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Royal antelope** (*Neotragus pygmaeus*)	USA (Witte et al.,2009)	6	captive	0	0		0	0		1	6	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Siberian ibex** (*Capra sibirica*)	USA (Witte et al., 2009)	43	captive	0	0		0	0		1	43	2,3	0	0		0	2,3

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Bovidae		**Steenbok** (*Raphicerus campestris*)	USA (Witte et al., 2009)	13	captive	0	0		0	0		1	13	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Suni** (*Neotragus moschatus*)	USA (Witte et al., 2009)	10	captive	0	0		0	0		1	10	0,0	0	0		0	0,0
Artiodactyla	Bovidae		**Uganda kob** (*Kobus kob*)	USA (Witte et al., 2009)	36	captive	0	0		0	0		1	36	0,0	0	0		0	0,0
Artiodactyla	Cervidae	Capreolinae	**Moose** (*Alces alces*)	Belgium (Vansnick et al., 2005)	7	captive	0	0		0	0		0	0		1	7	0,0	0	0,0
Artiodactyla	Cervidae	Capreolinae	**Pudu** (*Pudu pudu*)	Belgium (Vansnick et al., 2005)	7	captive	0	0		0	0		0	0		1	7	28,5	0	28,5
Artiodactyla	Cervidae	Capreolinae	**Reindeer** (*Rangifer tarandus tarandus*)	Belgium (Vansnick et al., 2005)	22	captive	0	0		0	0		0	0		1	22	0,0	0	0,0
Artiodactyla	Cervidae	Capreolinae	**Whitetailed deer** (*Odocoileus virginianus*)	Canada (Woodbury et al., 2008)	16	captive	1	16	6,3	1	15	80,0	1	16	62,5	1	15	13,3	0	40,5
Artiodactyla	Cervidae	Capreolinae	**Whitetailed deer** (*Odocoileus virginianus*)	USA (Chiodini and Van Kruiningen, 1983)	10	captive	1	10	0,0	0	0		1	10	20,0	0	0		N/K	10,0
Artiodactyla	Cervidae	Capreolinae	**Whitetailed deer** (*Odocoileus virginianus*)	USA (Hattel et al., 2004)	160	captive	1	160	1,3	0	0		0	0		0	0		0	1,3
Artiodactyla	Cervidae	Capreolinae	**Pudu** (*Pudu pudu*)	USA (Witte et al., 2009)	13	captive	0	0		0	0		1	13	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Cervidae	Capreolinae	**Reindeer** (*Rangifer tarandus tarandus*)	USA (Witte et al., 2009)	5	captive	0	0		0	0		1	5	0,0	0	0		0	0,0
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Belgium (Godfroid et al., 2000)	24	captive	0	0		0	0		0	0		1	24	12,5	1	12,5
Artiodactyla	Cervidae	Cervinae	**Père David's deer** (Elaphurus davidianus)	Belgium (Vansnick et al., 2005)	29	captive	0	0		0	0		0	0		1	29	0,0	0	0,0
Artiodactyla	Cervidae	Cervinae	**Sika Deer** (*Cervus nippon*)	Belgium (Vansnick et al., 2005)	4	captive	0	0		0	0		0	0		1	4	0,0	0	0,0
Artiodactyla	Cervidae	Cervinae	**Tule elk** (*Cervus elaphus nannodes*)	Belgium (Vansnick et al., 2005)	15	captive	0	0		0	0		0	0		1	15	0,0	0	0,0
Artiodactyla	Cervidae	Cervinae	**Tule elk** (*Cervus elaphus nannodes*)	Canada (Rohonczy et al., 1996)	431	captive	1	431	0,9	0	0		1	429	2,6	0	0		0	1,7
Artiodactyla	Cervidae	Cervinae	**Fallow Deer** (*Dama dama*)	Czech Republic (Kopecna et al., 2008a)	1381	captive	0	0		0	0		1	1381	5,7	0	0		0	5,7
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Czech Republic (Kopecna et al., 2008a)	2 296	captive	0	0		0	0		1	2296	0,5	0	0		0	0,5
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Czech Republic (Kopecna et al., 2008b)	272	captive	0	0		0	0		1	242	23,1	1	272	3,7	0	13,4
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Czech Republic (Machackova et al., 2005)	272	captive	0	0		1	0	0,0	1	242	23,1	272	1	3,7	0	0,0
Artiodactyla	Cervidae	Cervinae	**Fallow Deer** (*Dama dama*)	Germany (Weber et al., 1992b)	62	captive	0	0		0	0		1	62	16,1	0	0		0	16,1

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	Ireland (Power et al,1993)	70	captive				0	0		1	70	4,0	0	0		3	4,0
Artiodactyla	Cervidae	Cervinae	**Hog deer** (*Axis porcinus*)	USA (Witte et al.,2009)	56	captive	0	0		0	0		1	56	3,6	0	0		0	3,6
Artiodactyla	Cervidae	Cervinae	**Aixs deer** (*Axis axis*)	USA (Witte et al.,2009)	34	captive	0	0		0	0		1	34	8,8	0	0		0	8,8
Artiodactyla	Cervidae	Cervinae	**Calamian deer** (*Axis calamianensis*)	USA (Witte et al.,2009)	60	captive	0	0		0	0		1	60	0,0	0	0		0	0,0
Artiodactyla	Cervidae	Cervinae	**Eld's deer** (*Rucervus eldii*)	USA (Witte et al.,2009)	62	captive	0	0		0	0		1	62	0,0	0	0		0	0,0
Artiodactyla	Cervidae	Cervinae	**Fallow Deer** (*Dama dama*)	USA (Witte et al., 2009)	22	captive	0	0		0	0		1	22	4,5	0	0		0	4,5
Artiodactyla	Cervidae	Cervinae	**Indian sambar** (*Rusa unicolor*)	USA (Witte et al., 2009)	90	captive	0	0		0	0		1	90	2,2	0	0		0	2,2
Artiodactyla	Cervidae	Cervinae	**Javan rusa** (*Rusa timorensis*)	USA (Witte et al., 2009)	42	captive	0	0		0	0		1	42	2,4	0	0		0	2,4
Artiodactyla	Cervidae	Cervinae	**Père David's deer** (Elaphurus davidianus)	USA (Witte et al., 2009)	30	captive	0	0		0	0		1	30	9,1	0	0		0	9,1
Artiodactyla	Cervidae	Cervinae	**Red deer** (*Cervus elaphus*)	USA (Witte et al., 2009)	153	captive	0	0		0	0		1	153	0,7	0	0		0	0,7

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Cervidae	Cervinae	**Sika Deer** (*Cervus nippon*)	USA (Witte et al., 2009)	118	captive	0	0		0	0		1	118	1,7	0	0		0	1,7
Artiodactyla	Cervidae	Cervinae	**Thorold's deer** (*Przewalskium albirostris*)	USA (Witte et al., 2009)	38	captive	0	0		0	0		1	38	0,0	0	0		0	0,0
Artiodactyla	Cervidae	Muntiacinae	**Formosan Reeves' muntjac** (*Muntiacus reevesi*)	USA (Witte et al., 2009)	15	captive	0	0		0	0		1	15	13,3	0	0		0	13,3
Artiodactyla	Cervidae	Muntiacinae	**North Indian muntjac** (*Muntiacus muntjak*)	USA (Witte et al., 2009)	23	captive	0	0		0	0		1	23	0,0	0	0		0	0,0
Artiodactyla	Cervidae	Muntiacinae	**Tufted deer** (*Elaphodus cephalophus*)	USA (Witte et al., 2009)	29	captive	0	0		0	0		1	29	14,3	0	0		0	14,3
Artiodactyla	Cervidae	Odocoileinae	**Roe Deer** (*Capreolus capreolus*)	Czech Republic (Kopecna et al., 2008a)	835	captive	0	0		0	0		1	835	0,2	0	0		0	0,2
Artiodactyla	Cervidae	Odocoileinae	**Chaco pampas deer** (*Ozotoceros bezoarticus*)	USA (Witte et al., 2009)	9	captive	0	0		0	0		1	9	0,0	0	0		0	0,0
Artiodactyla	Cervidae		**Barasingha** (*Rucervus duvaucelii*)	USA (Witte et al., 2009)	30	captive	0	0		0	0		1	30	3,3	0	0		0	3,3
Artiodactyla	Cervidae		**Mexican red brocket** (*Mazama americana*)	USA (Witte et al., 2009)	10	captive	0	0		0	0		1	10	20,0	0	0		0	20,0
Artiodactyla	Giraffidae		**Okapi** (*Okapia johnstoni*)	Belgium (Vansnick et al., 2005)	25	captive	0	0		0	0		0	0		1	25	0,0	0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	Nº Animales	Wild/ Capt.	Histop.	nº an.	% histo+	PCR	nº an.	% PCR+	Culture	nº an.	% Cult+	Serology	nº an.	% Serol.+	Clin. case	Mean 4 TEST
Artiodactyla	Giraffidae		**Masai giraffe** (*Giraffa camelopardalis*)	USA (Witte et al., 2009)	29	captive	0	0		0	0		1	29	0,0	0	0		0	0,0
Artiodactyla	Giraffidae		**Okapi** (*Okapia johnstoni*)	USA (Witte et al., 2009)	15	captive	0	0		0	0		1	15	0,0	0	0		0	0,0
Artiodactyla	Moschidae		**Siberian musk deer** (*Moschus moschiferus*)	USA (Witte et al., 2009)	21	captive	0	0		0	0		1	21	0,0	0	0		0	0,0
Artiodactyla	Suidae		**Wild boar** (*Sus scrofa*)	Czech Republic (Kopecna et al., 2008a)	805	captive	0	0		0	0		1	805	0,1	0	0		0	0,1
Artiodactyla	Suidae		**Wild boar** (*Sus scrofa*)	Czech Republic (Trcka et al., 2006)	568	captive	0	0		0	0		1	568	0,2	0	0		0	0,2
Carnivora	Canidae		**Raccoon dog** (*Nyctereutes procyonoides*)	Czech Republic (Kopecna et al., 2008a)	4	captive	0	0		0	0		1	4	0,0	0	0		0	0,0
Carnivora	Mustelidae	Lutrinae	**Eurasian otter** (*Lutra lutra*)	Czech Republic (Kopecna et al., 2008a)	4	captive	0	0		0	0		1	4	0,0	0	0		0	0,0
Carnivora	Mustelidae		**Badger** (*Meles meles*)	Czech Republic (Kopecna et al., 2008a)	82	captive	0	0		0	0		1	82	0,0	0	0		0	0,0
Carnivora	Mustelidae		**Beech marten** (*Martes foina*)	Czech Republic (Kopecna et al., 2008a)	55	captive	0	0		0	0		1	55	0,0	0	0		0	0,0
Carnivora	Mustelidae		**European polecat** (*Mustela putorius*)	Czech Republic (Kopecna et al., 2008a)	9	captive	0	0		0	0		1	9	0,0	0	0		0	0,0

ORDER	FAMILY	SUBFAMILY	SPECIES	REF	N° Animales	Wild/ Capt.	Histop.	n° an.	% histo+	PCR	n° an.	% PCR+	Culture	n° an.	% Cult+	Serology	n° an.	% Serol.+	Clin. case	Mean 4 TEST
Columbiformes	Columbidae		**Pigeon** (*Columba livia f. domestica*)	Czech Republic (Kopecna et al., 2008a)	2113	captive	0	0		0	0		1	2113	0,0	0	0		0	0,0
Lagomorpha	Leporidae		**Brown hare** (*Lepus europaeus*)	Czech Republic (Kopecna et al., 2008a)	25	captive	0	0		0	0		1	25	0,0	0	0		0	0,0
Lagomorpha	Leporidae		**Wild rabbit** (*Oryctolagus cuniculus*)	Czech Republic (Kopecna et al., 2008a)	5	captive	0	0		0	0		1	5	0,0	0	0		0	0,0
Passeriformes	Corvidae		**Malayan lesser chevrotain** (*Tragulus javanicus*)	USA (Witte et al., 2009)	4	captive	0	0		0	0		1	4	0,0	0	0		0	0,0
Rodentia	Cricetidae	Arvicolinae	**Common voles** (*Microtus arvalis*)	Czech Republic (Kopecna et al., 2008a)	59	captive	0	0		0	0		1	59	1,7	0	0		0	1,7
Rodentia	Muridae	Murinae	**House mouse** (*Mus musculus*)	Czech Republic (Kopecna et al., 2008a)	34	captive	0	0		0	0		1	34	0,0	0	0		0	0,0
Rodentia	Muridae	Murinae	**Norway rat** (*Rattus norvegicus*)	Czech Republic (Kopecna et al., 2008a)	17	captive	0	0		0	0		1	17	5,9	0	0		0	5,9
Soricomorpha	Soricidae		**Lesser white-toothed shrews** (*Crocidura suaveolens*)	Czech Republic (Kopecna et al., 2008a)	39	captive	0	0		0	0		1	39	2,6	0	0		0	2,6
TOT				**157**	**12109**	**captive**	**5**	**757**	**10,3**	**2**	**15**	**40,0**	**121**	**13706**	**3,3**	**308**	**695**	**5,6**	**21**	**14,8**

Figura 1

Números de trabajos revisados sobre MAP en fauna silvestre de vida libre