UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

LINFADENITIS DEL CERDO EN EXTENSIVO: APORTACIONES AL DIAGNÓSTICO E IMPLICACIONES EN LA CADENA ALIMENTARIA

Lymphadenitis in free-range pigs: contributions to diagnosis and implications for the food chain

Tesis presentada por el Licenciado en Veterinaria D. Fernando Cardoso Toset
para optar al Grado de Doctor en Veterinaria por la Universidad de Córdoba

Departamento de Sanidad Animal
Córdoba, 2016

TITULO: *Linfadenitis del cerdo en extensivo: aportaciones al diagnóstico e implicaciones en la cadena alimentaria*

AUTOR: *Fernando Cardoso Toset*

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

www.uco.es/publicaciones
publicaciones@uco.es

TÍTULO DE LA TESIS: Linfadenitis del cerdo en extensivo: aportaciones al diagnóstico e implicaciones en la cadena alimentaria.

DOCTORANDO/A: Fernando Cardoso Toset.

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

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El trabajo presentado por el doctorando D. Fernando Cardoso Toset, Licenciado en Veterinaria, reúne las condiciones y calidad científica necesarias para optar al Grado de Doctor en Veterinaria. Fruto del trabajo desarrollado, se han publicado en revistas indexadas del *Journal of Citation Reports* los tres artículos exigidos por la normativa (pertenecientes al primer cuartil), que constituyen el cuerpo de esta Tesis Doctoral. Asimismo, a partir del trabajo desarrollado por el doctorando se han publicado diferentes comunicaciones a congresos de ámbito nacional e internacional.

Esta Tesis Doctoral está enfocada al estudio de la linfadenitis del cerdo criado en sistemas extensivos, con especial atención a la identificación de los principales agentes causales, la evaluación de técnicas de diagnóstico directas e indirectas y el análisis de la viabilidad de los microorganismos no tuberculosos durante la curación de los productos del cerdo Ibérico. Los trabajos presentados ofrecen herramientas rápidas y eficaces para el diagnóstico de estos procesos; asimismo, se aportan evidencias de que los principales agentes implicados no sobreviven al proceso de curación de los productos del cerdo Ibérico. Consideramos que la aplicación de estas técnicas supone un avance para el sector porcino en extensivo y cumple los objetivos marcados en los proyectos de los que deriva esta tesis.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 10 de octubre de 2016

Firma del/de los director/es

Fdo.: Inmaculada Luque Moreno

Fdo.: Jaime Gómez-Laguna

UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

DEPARTAMENTO DE SANIDAD ANIMAL

LINFADENITIS DEL CERDO EN EXTENSIVO: APORTACIONES AL DIAGNÓSTICO E IMPLICACIONES EN LA CADENA ALIMENTARIA

Lymphadenitis in free-range pigs: contributions to diagnosis and implications for the food chain

Fernando Cardoso Toset

Tesis Doctoral

Córdoba, 2016

La realización de los estudios incluidos en esta Tesis Doctoral ha sido posible gracias al proyecto “BIODHESA: Impacto y medidas de control de la linfadenitis del cerdo Ibérico en la Dehesa” financiado por el Centro para el Desarrollo Tecnológico Industrial (CDTI; referencia IDI-20111633) y al proyecto “Linfadenitis caseosa en ganado porcino en extensivo: desarrollo y evaluación de herramientas diagnósticas rápidas” financiado por la Consejería de Economía, Innovación, Ciencia y Empleo de la Junta de Andalucía (referencia: AGR-2685).

Índice/Contents

Capítulo I/ Chapter I

Introducción general

El papel del sector porcino en la economía española

El sector porcino constituye un importante motor de la economía española, que representa más del 37% de la Producción Final Ganadera y el 14% de la Producción Final Agraria, con un volumen de facturación que roza los 6.000 millones de euros[1]. En el año 2015 España alcanzó un censo de 28,3 millones de animales, superando a Alemania (27,5 millones) y situándose por primera vez como el país con mayor censo porcino de la Unión Europea[1]. Los datos sugieren que la producción europea aumentará un 3,9% durante los primeros tres trimestres de este año respecto al mismo periodo del año 2015. Desde España, se prevé que el crecimiento de sacrificios y el aumento total de la producción aumente este año un 3,5% y un 3,9% respectivamente, pudiendo representar el año 2017 un momento crucial para este sector[2]. De este modo, el porcino supone el sector más importante de nuestra ganadería; dentro de España, Andalucía ocupa el cuarto puesto en producción de carne de cerdo (7,6%)[1].

España es considerada la cuarta potencia productora a nivel mundial (después de China, EEUU, y Alemania), con una producción que supone el 16,9 % de la producida en la UE. Debido a que el sector porcino en Europa presenta un alto nivel de autoabastecimiento (alrededor del 111%), la exportación supone un elemento clave para el equilibrio del mercado, siendo la UE la principal potencia exportadora a nivel internacional. España se sitúa como tercer exportador de porcino de la UE, por detrás de Alemania y Dinamarca[1].

---

1. Ministerio de Agricultura Alimentación y Medio Ambiente. 2015. Caracterización del sector porcino español año 2015 (http://www.magrama.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/sectores-ganaderos/porcino/).
2. Interempresas: http://www.interempresas.net/Industria-Carnica/Articulos/151854-Espana-alcanza-cifras-record-en-produccion-porcina-y-de-vacuno.html.

En este contexto, el sector porcino español se ha establecido como un amplio tejido industrial, formado por miles de pequeñas y medianas empresas repartidas por todo el territorio nacional, que proporcionan empleo a millones de familias (unos 175.000 empleos directos y unos dos millones de puestos de trabajo indirectos), actuando como un claro impulsor de la economía española (Buxadé, 2014). Al igual que las restantes producciones ganaderas, el sector porcino debe hacer frente a las nuevas exigencias del mercado, relacionadas con la seguridad alimentaria, el cuidado del medio ambiente y el bienestar animal (Langreo y González, 2007).

Aproximación a los sistemas de producción porcina extensiva

Dentro del sector porcino existen dos subsectores claramente diferenciados y bien representados en nuestro país, los sistemas de producción *intensiva*, caracterizados por utilizar cerdos de razas mejoradas genéticamente (Landrace, Large White, Duroc, Pietrain, etc), criados en el interior de naves con un manejo nutricional basado en la utilización de alimentos concentrados o piensos compuestos, y los sistemas de producción *extensiva o semiextensiva*, en los que una parte importante del ciclo de cría y engorde de los animales ocurre en el exterior, aprovechando los recursos naturales (Paramio et al., 2012).

Algunos ejemplos de cría porcina extensiva fuera de nuestras fronteras son la cría del cerdo Alentejano en Portugal (Oliveira et al., 2014), el cerdo Negro de Nebrodi en Sicilia (Di Marco et al., 2012) o la cría de razas criollas en Latinoamérica (Velasco, 2006). En España, el sistema de cría extensiva supone alrededor del 10% de la producción nacional y se encuentra estrechamente ligado al cerdo Ibérico y sus cruces, que comprende una amplia gama de variedades, líneas y estirpes genéticas (Paramio et al., 2012).

Una particularidad del sistema extensivo en España es su vinculación al ecosistema de la Dehesa, que permite la cría tradicional del cerdo Ibérico, basada en un largo proceso de crianza y en el aprovechamiento de los recursos naturales durante la montanera, donde los cerdos se alimentan principalmente de bellota, pero también de pasto, raíces, insectos, pequeños vertebrados e incluso algún tipo de carroña, dado el carácter omnívoro de la especie porcina (Astorga et al., 2010).

Figura 1. Cerdos ibéricos criados en el ecosistema extensivo de la Dehesa.

Si bien estos sistemas permiten a los animales gozar de un alto nivel de bienestar animal y la obtención de productos derivados de elevada calidad organoléptica (Ventanas, 2001), también favorecen el contacto entre distintas especies animales, tanto domésticas (ganado vacuno, caprino y ovino) como silvestres (ciervos, jabalíes, tejones etc.) y condicionan que los animales se vean expuestos a múltiples factores ambientales que favorecen la diseminación de patógenos, sobre los cuales resulta difícil actuar (Hermoso de Mendoza et al., 2003; Astorga et al., 2010; Gortázar et al., 2011).

Figura 2. Coexistencia de distintas especies en el ecosistema de la Dehesa.

Es por tanto necesario establecer programas sanitarios y medidas de manejo para el control de las principales dominantes patológicas de este tipo de ganado, ocupando las enfermedades transmisibles, infecciosas y parasitarias, un lugar importante, no sólo por las pérdidas económicas que originan y su capacidad de diseminación dentro y entre granjas, sino también por las repercusiones sobre la Salud Pública y la Seguridad Alimentaria, hechos que obligan a establecer sistemas de vigilancia y control a nivel nacional e internacional (Directiva 2003/99/CE de 17 de noviembre de 2003, sobre la vigilancia de las zoonosis y los agentes zoonósicos y Reglamento (CE) 2160/2003 de 17 de noviembre de 2003, sobre el control de la *Salmonella* y otros agentes zoonósicos específicos transmitidos por alimentos).

La linfadenitis del cerdo en extensivo

Entre las enfermedades que afectan al ganado porcino criado en sistemas extensivos, destacan aquellas enfermedades que cursan de forma subclínica y son detectadas en la inspección *postmortem* que se realiza en el matadero (Reglamento 2004/854/CE, por el que se establecen normas específicas para la organización de controles oficiales de los productos de origen animal destinados al consumo humano). Entre éstas destaca la linfadenitis porcina,

que podemos definir como un proceso que cursa con la inflamación de los nódulos linfáticos superficiales y profundos como respuesta a la infección por diferentes microorganismos, que pueden extenderse a otros órganos por difusión linfohemática dando lugar a la aparición de estas mismas lesiones en otros órganos, principalmente pulmón, hígado y/o bazo (Martin-Hernando et al., 2007; Lara et al., 2011; Johansen et al., 2014). Estas lesiones se caracterizan macroscópicamente por su aspecto nodular, que puede ser de tipo caseoso, purulento o proliferativo, y también son conocidas como lesiones compatibles con tuberculosis en la bibliografía (TBL, del inglés *Tuberculosis-Like Lesions*) (Bollo et al., 2000; Santos et al., 2010).

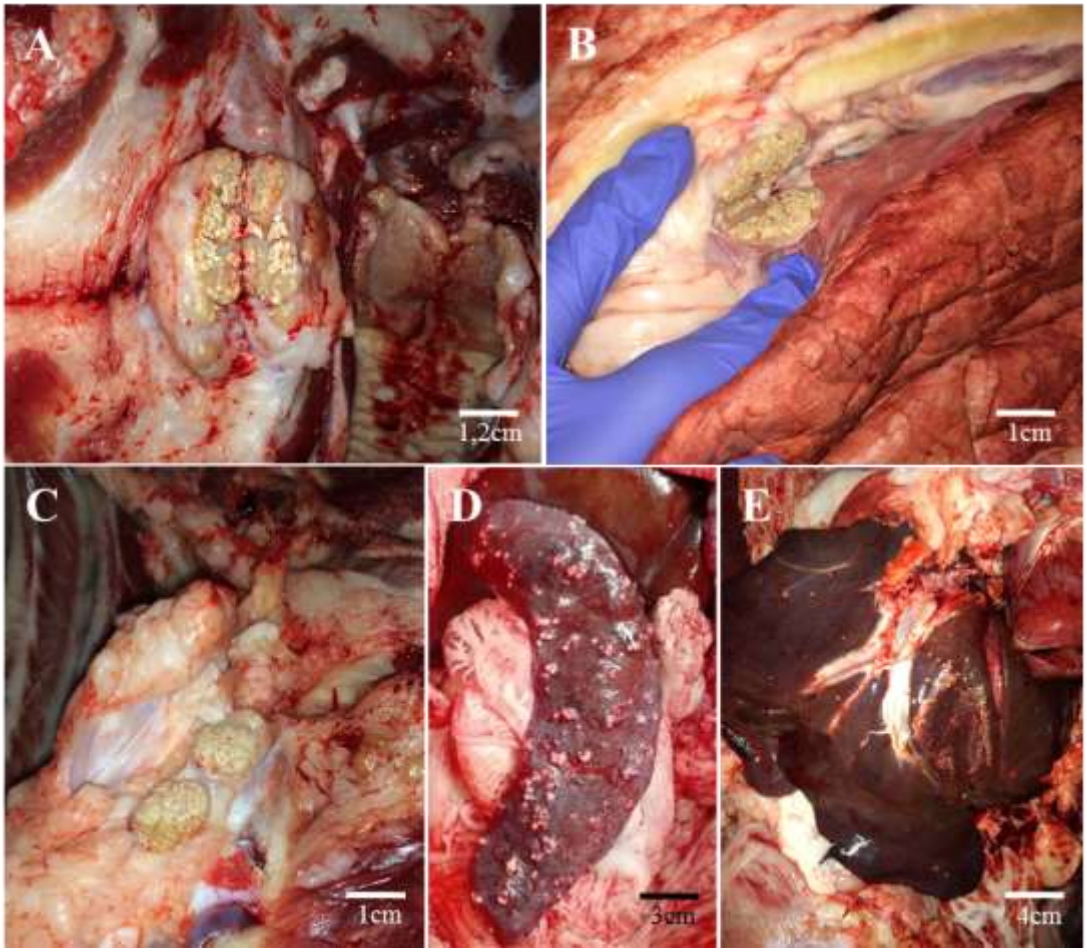


Figura 3. Lesiones de linfadenitis porcina generalizada observadas en el matadero. **A**. Nódulo linfático retrofaríngeo medial. **B**. Nódulo linfático traqueobronquial. **C.** Nódulos linfáticos axilares. **D**. Lesiones multifocales a coalescentes en bazo. **E**. Lesiones miliares en hígado.

Estas lesiones se han asociado históricamente a la infección por *Mycobacterium* spp. (Parra et al., 2003; Ramis y Pallarés, 2009); sin embargo, diferentes estudios destacan la participación de otros agentes en su desarrollo (Komijn et al., 2007; Lara et al., 2011). En países endémicos de tuberculosis bovina *Mycobacterium bovis* se aísla con frecuencia en este tipo de lesiones, mientras que en países o áreas geográficas libres de esta enfermedad, así como en explotaciones porcinas de cría intensiva, se han relacionado principalmente con la infección por *Mycobacterium avium* y *Rhodococcus equi* (Makrai et al., 2008; Pérez de Val et al., 2013; Philip et al., 2013), así como por especies bacterianas de los géneros *Corynebacterium*, *Trueperella* y *Streptococcus*, entre otros (Contzen et al., 2011; Lara et al., 2011).

Sin embargo, el diagnóstico microbiológico de la linfadenitis porcina se ha centrado fundamentalmente en la detección de micobacterias patógenas, ya que tienen un impacto sanitario evidente (Agdestein et al., 2011; Muñoz-Mendoza et al., 2013). Esta situación, podría estar infravalorando la importancia de otros agentes que pueden actuar de forma aislada o concomitante, aumentando la gravedad y complejidad de este proceso (Komijn et al., 2007; Müller et al., 2011).

El carácter inaparente o subclínico que suele caracterizar a esta patología y la ausencia de protocolos de diagnóstico que permitan identificar los agentes causales de forma rápida y eficaz, suponen una importante limitación para implementar medidas de control que reduzcan su incidencia (Bailey et al., 2013; Johansen et al., 2014).

Impacto de la linfadenitis porcina

El impacto generado por la linfadenitis porcina ocurre principalmente a dos niveles: económico y sanitario. El impacto económico directo viene determinado fundamentalmente

por el decomiso de canales y vísceras consideradas no aptas para el consumo humano durante la inspección *postmortem* (Linares et al., 2013; García-Diez y Coelho, 2014). En el ganado porcino criado en sistemas intensivos, se han notificado casos relacionados con cambios concretos en el manejo o alimentación, asociados principalmente a *M. avium* y que suelen ser detectados en el matadero (Pérez de Val et al., 2013; Johansen et al., 2014).

Los datos económicos disponibles derivados del impacto de la linfadenitis porcina son escasos y en su mayor parte se refieren a sistemas de cría intensiva. Así, en un estudio sobre la presencia de lesiones compatibles con tuberculosis llevado a cabo en mataderos de la República Checa durante el periodo 1990-1999, solamente un 0,32% de los cerdos sacrificados mostraron lesiones compatibles con linfadenitis granulomatosa (Pavlik et al., 2003). Este hallazgo es similar al obtenido en España, donde la linfadenitis fue observada de forma esporádica en sistemas intensivos (Martínez et al., 2007). En Finlandia, se estimó que el decomiso de canales porcinas debido a estas mismas lesiones suponía unas pérdidas anuales de aproximadamente 0,5 millones de euros (Tirkkonen et al., 2010). Por otro lado, en un estudio reciente llevado a cabo en Portugal, los decomisos asociados a esta patología llegaron a representar la segunda causa más frecuente de decomiso (22,7%) en cerdos criados en sistemas intensivos (García-Diez y Coelho, 2014).

En sistemas extensivos, esta patología adquiere mayor relevancia debido a las particularidades de la cría extensiva que, como se ha indicado anteriormente, facilitan la entrada y transmisión de los patógenos implicados en el proceso, originando la recurrencia de esta patología en áreas geográficas o explotaciones concretas (Hermoso de Mendoza et al., 2003), siendo especialmente vulnerables aquellas situadas en torno a áreas silvestres o parques naturales (Linares et al., 2013).

Así, un estudio retrospectivo realizado en nuestro país mostró que el 85 % de los decomisos totales acontecidos durante un periodo de cinco años estuvo asociado a casos de linfadenitis porcina (Linares et al., 2013), datos que ponen de manifiesto la importancia del proceso dentro del cómputo total de decomisos en este sector.

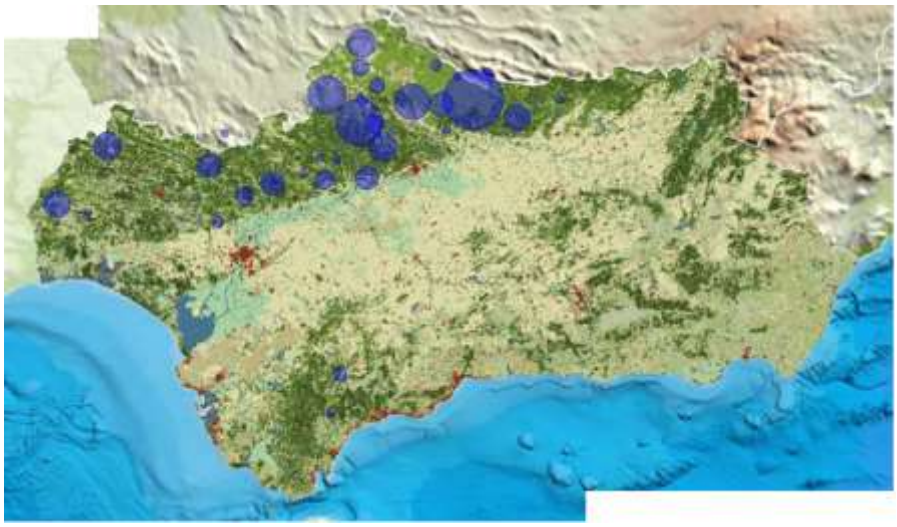


Figura 4. Principales áreas geográficas afectadas por lesiones de linfadenitis porcina en Andalucía (Linares et al., 2013).

A los costes directos derivados del decomiso de canales y vísceras, habría que sumar los producidos por el retraso en el crecimiento de los animales afectados, los relacionados con la aplicación de medidas profilácticas y los derivados de las posibles limitaciones para el comercio de animales vivos (Hermoso de Mendoza et al., 2003). Además, habría que considerar el papel epidemiológico que puede jugar el cerdo en el mantenimiento y transmisión de los microorganismos implicados en la linfadenitis porcina a otras especies con las que convive en sistemas extensivos, hecho que adquiere una mayor importancia en el caso de las micobacterias patógenas (Parra et al., 2003; Di Marco et al., 2012).

En segundo lugar, dentro del impacto generado por la linfadenitis en la cadena de producción del porcino criado en extensivo, cabe destacar la relevancia sanitaria de algunos microorganismos implicados en el proceso, entre los que encontramos patógenos zoonósicos

ampliamente reconocidos, emergentes y oportunistas (Hermoso de Mendoza et al., 2003; Lara et al., 2011), aspecto que comentaremos más adelante.

Etiología de la linfadenitis porcina

Como se ha comentado anteriormente, existe un amplio número de microorganismos que han sido aislados a partir de cuadros de linfadenitis porcina, incluyendo especies del género *Mycobacterium* y otros microorganismos pertenecientes a distintos géneros bacterianos, siendo los principales grupos descritos las micobacterias del complejo *Mycobacterium tuberculosis* (CMT), las micobacterias del complejo *Mycobacterium avium* (CMA) y *R. equi* (Lara et al., 2011).

El **género *Mycobacterium*** engloba una amplia variedad de especies de bacterias saprofitas, patógenos oportunistas y patógenos estrictos del hombre y de los animales. Este género se incluye en el grupo de bacterias Gram positivas con alto contenido en G+C, dentro del orden *Corynebacteriales*, donde se encuentra relacionado taxonómicamente con otros géneros aislados en la linfadenitis porcina, como son *Corynebacterium* o *Rhodococccus* (Goodfellow y Jones, 2015). Son bacterias de forma bacilar, ácido-alcohol resistentes (BAAR), aerobias y no formadoras de esporas (Quinn et al., 2011). Se han utilizado varios criterios para clasificar las especies de este género, siendo uno de los más empleados su velocidad de crecimiento en medios de cultivo sólido, pudiéndose distinguir micobacterias de crecimiento rápido (aquellas que dan lugar a colonias visibles en menos de 7 días) y micobacterias de crecimiento lento (que tardan más de 7 días en producir colonias visibles). Dentro de estas últimas se incluyen las micobacterias de mayor importancia en medicina humana y veterinaria, las llamadas micobacterias tuberculosas que forman parte del CMT y las micobacterias atípicas no tuberculosas, entre las que se encuentran las especies del CMA (Álvarez et al., 2011).

Dentro del **CMT** se encuentran agrupadas las micobacterias patógenas responsables de la tuberculosis humana y animal, que se diferencian en función de su hospedador principal y estudios filogenéticos: *M. tuberculosis, M. bovis, M. bovis* BCG, *M. caprae, M. africanum, M. microti, M. pinnipedii, M. canettii, M. mungi, M. suricattae, M. orygis* (*oryx bacillus*) y el *dassie bacillu* (Pearson et al., 2013; Rodríguez-Campos et al., 2014). De todas ellas, *M. bovis* es la especie que posee un mayor rango de hospedadores, siendo capaz de infectar al hombre y a un gran número de especies animales domésticas, como vacas, cabras, ovejas, caballos y cerdos, entre otras, y silvestres como ciervos, jabalíes, liebres o tejones, entre otras (Broughan et al., 2013). Esta capacidad de adaptación permite a *M. bovis* infectar simultáneamente a diversas especies de un ecosistema creando lo que ha sido denominado como complejo epidemiológico multi-hospedador (Gortázar et al., 2011). Otra especie que ha recibido una especial atención es *M. caprae*, cuyo hospedador principal es el ganado caprino y que puede infectar a distintas especies, incluyendo los suidos (García-Jiménez et al., 2013b). Trabajos recientes han evidenciado la importancia de los reservorios ambientales y la transmisión indirecta en la infección por micobacterias del CMT (Santos et al., 2015b; Barasona et al., 2016; Barbier et al., 2016).

Por otro lado, el **CMA** engloba a *M. avium* y *M. intracellulare*, siendo este último poco relevante en veterinaria. Al igual que ocurre con las especies del CMT, las especies del CMA poseen un amplio rango de hospedadores, que incluye ciervos, ovejas, vacas, roedores, cerdos y humanos, entre otros (Álvarez et al., 2011). *M. avium* agrupa a las subespecies *silvaticum, paratuberculosis* (agente causal de la paratuberculosis en rumiantes), *avium* (agente causal de la tuberculosis aviar) y *hominissuis*, siendo esta última la más frecuente en el cerdo (Pérez de Val., 2013). Estudios recientes muestran prevalencias de este agente en jabalíes de la Península Ibérica que van del 4,55% en la costa Atlántica (Muñoz-Mendoza et al., 2013) al 11% en el sur de España (García-Jiménez et al., 2015). Los miembros de este

complejo poseen una mayor divergencia genética y se caracterizan por su ubicuidad, siendo capaces de multiplicarse y sobrevivir largos periodos en el medio ambiente y distintas fuentes de infección como el agua, pienso, suelo, polvo y turba (Agdestein et al., 2014).

El **género *Rhodococcus*** se incluye dentro de la familia *Nocardiaceae* y engloba varias especies consideradas patógenos oportunistas, siendo *R. equi* la más frecuente y de mayor poder patógeno para el hombre y los animales (Weinstock et al., 2002; Quinn et al., 2011). Este microorganismo es un cocobacilo aerobio y Gram positivo, inmóvil y débilmente AAR, caracterizado por su naturaleza telúrica y asociación clínica a cuadros de bronconeumonía supurativa en potros (Makrai et al., 2008), aunque también se ha demostrado su implicación en la linfadenitis porcina (Pate et al., 2004; Shitaye et al., 2006; Komijn et al., 2007).

Además de estos agentes, en la bibliografía consultada se describe el aislamiento de otros microorganismos a partir de lesiones de linfadenitis porcina, entre los que cabe destacar las corinebacterias (*Corynebacterium* spp., *Trueperella* spp.) y el género *Streptococcus* (Álvarez et al., 2011; Jarosz et al., 2014).

Las **bacterias corineformes o corinebacterias** suponen un amplio grupo de bacterias Gram positivas aeorobias, pleomórficas y no formadoras de esporas, entre las que encontramos los géneros *Corynebacterium* y *Trueperella*, que aparecen en las preparaciones microscópicas como pequeñas bacterias inmóviles, con forma de porra, cocoide o bacilar, agrupadas de forma irregular (morfología corineforme) (Funke y Bernard, 2007). Dentro del **género *Corynebacterium***, las especies *C. pseudotuberculosis* y *C. ulcerans* han sido aisladas a partir de casos de linfadenitis porcina (Contzen et al., 2011; Oliveira et al., 2014). Cabe destacar también la especie ***Trueperella pyogenes*** (anteriormente denominada *Arcanobacterium pyogenes*) que supone un comensal habitual en las superficies epiteliales y mucosas de animales domésticos y silvestres, incluido el cerdo, en el que se aísla

frecuentemente a partir de lesiones supurativas que involucran múltiples localizaciones orgánicas, incluyendo la piel, las articulaciones, las glándulas mamarias o los nódulos linfáticos (Lara et al., 2011; Verjan et al., 2015).

El **género *Streptococcus*** está formado por cocos Gram positivos inmóviles no formadores de esporas, que aparecen organizados en forma de cadenas o pares en las preparaciones microscópicas y que poseen un metabolismo fermentativo (Quinn et al., 2011). La mayoría de estreptococos implicados en la linfadenitis porcina están situados dentro del grupo piogénico o piógeno, que incluye a especies que causan infecciones piógenas en el hombre y los animales, siendo algunas de ellas *S. agalactiae, S. dysgalactiae, S. equi, S. porcinus y S. uberis* (Whiley y Hardey, 2015).

Destacan por su incidencia los estreptococos beta-hemolíticos, descritos como la causa más frecuente de linfadenitis en jabalíes criados en extensivo en Brasil, siendo los estreptococos alfa-hemolíticos menos frecuentes (Lara et al., 2011). Entre estos últimos, cabe destacar la presencia de *S. suis* por ser un patógeno zoonósico capaz de causar infecciones graves en el cerdo y en el ser humano (Goyette-Desjardins et al., 2014).

Por último, también se han aislado de forma esporádica *Staphylococcus* spp., *Actinomyces* spp., y bacterias Gram negativas como *Enterobacter cloacae, Escherichia coli, Pseudomonas* spp., *Proteus mirabilis* o *Klebsiella pneumoniae,* entre otros (Santos et al., 2010; Lara et al., 2011; Mann et al., 2014).

Lesiones asociadas a la linfadenitis porcina

Debido a la ausencia de manifestaciones clínicas evidentes, las lesiones asociadas a la linfadenitis porcina suelen ser detectadas tras el sacrificio de los animales en el matadero (Di Marco et al., 2012).

Aunque los mecanismos de acción patógena de cada microorganismo pueden variar, de forma general se considera que las principales vías de entrada de los patógenos implicados en este proceso son la oronasal y la cutánea (Christensen et al., 2007; Jarosz et al., 2014). En este sentido, cabe señalar que la infección por vía oronasal cobra mayor importancia en los sistemas de cría extensiva, dadas sus particularidades epidemiológicas y la costumbre del cerdo de hozar dentro o muy cerca del barro, alimentos o cadáveres potencialmente contaminados con estos agentes (Hermoso de Mendoza et al., 2003; Barasona et al., 2016). La infección cutánea está ligada a la invasión oportunista de lesiones traumáticas en la piel o mucosas, a menudo debidas a errores de manejo, por ejemplo, el empleo de métodos de inyección inapropiados (Verjan García et al., 2015).

Tras la infección, estos microorganismos se localizan preferentemente en los nódulos linfáticos asociados a la puerta de entrada y/o tonsilas, provocando una reacción inflamatoria primaria de tipo purulento o granulomatoso, que puede dar lugar a tres tipos de lesiones: abscesos, piogranulomas o granulomas. En función de las características del agente causal y el estado inmunológico del animal, la infección puede evolucionar hacia el encapsulamiento y la calcificación de la lesión primaria o hacia la diseminación del agente a uno o varios territorios orgánicos, provocando una generalización del proceso (Bollo et al., 2000).

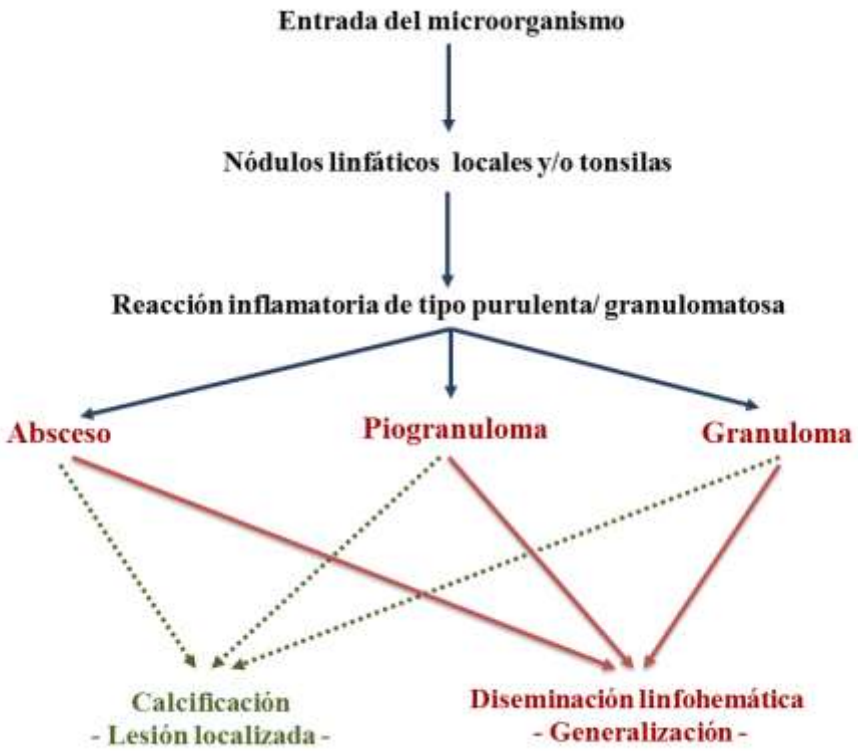


Figura 5. Evolución de las lesiones de linfadenitis porcina.

Macroscópicamente, los **abscesos** son lesiones constituidas por material purulento de aspecto cremoso y color amarillento o verdoso, rodeado de una fuerte cápsula fibrosa. Los **piogranulomas** se caracterizan por la formación de pequeños nódulos, generalmente mal definidos y dotados de un material cremoso de color blanco-amarillento. Por último, los **granulomas** son lesiones bien encapsuladas y fáciles de enuclear, con la presencia de focos blanco-amarillentos distintivos de aspecto caseoso y calcificación relativamente prominente que tienden a unirse por coalescencia.

Si bien un absceso resulta relativamente fácil de diferenciar durante un inspección visual, la diferenciación entre un piogranuloma y un granuloma está condicionada al grado de evolución de la lesión, resultando en muchos casos inviable en la práctica rutinaria (Peinado et al., 2006; Gómez-Laguna et al., 2010).

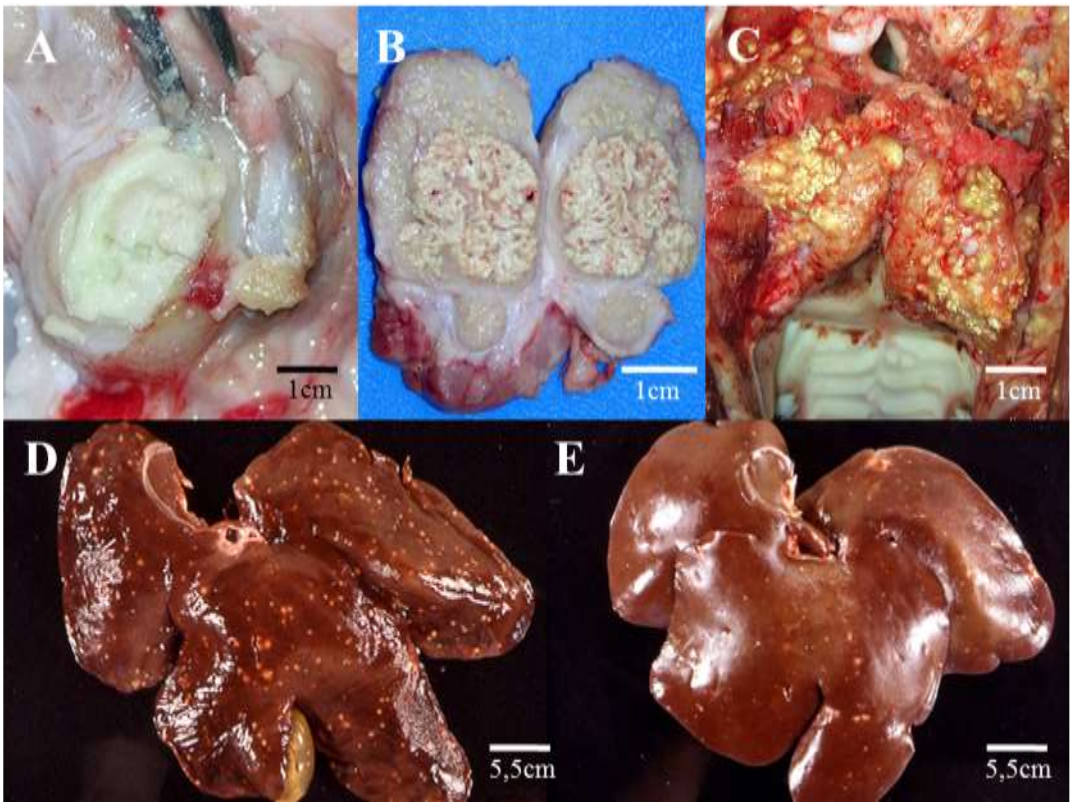


Figura 6. Linfadenitis porcina. **A.** Absceso (nódulo linfático mandibular). **B**. Piogranuloma (nódulo linfático submandibular). **C**. Granuloma (tonsila). **D**. Piogranulomas (hígado). **E**. Granulomas (hígado).

En cualquier caso, las lesiones asociadas a la linfadenitis suelen tener aspecto nodular, presentando un tamaño variable que generalmente oscila entre 1 mm y 4 cm de diámetro (llegando a los 15 cm de diámetro) y pueden aparecer localizadas o diseminadas con una distribución miliar (Martin-Hernando et al., 2007; Di Marco et al., 2013). En algunos trabajos, se han señalado características concretas asociadas a los microorganismos implicados en su desarrollo. Algunos autores han indicado un aspecto proliferativo en las lesiones causadas por micobacterias, mientras que otros han indicado la presencia de capas concéntricas (similares a las producidas por *C. pseudutuberculosis* en rumiantes) en lesiones asociadas al aislamiento de *Corynebacterium* spp. (Contzen et al., 2011; Domingo et al., 2014).

Atendiendo a su distribución orgánica, las lesiones de la linfadenitis porcina suelen localizarse en los nódulos linfáticos de la cabeza (Bollo et al., 2000; Zanella et al., 2008; García-Jiménez et al., 2013a), especialmente en los nódulos linfáticos submandibulares, hecho que determina que hayan sido descritos como el órgano de elección para la realización de pruebas de diagnóstico (Martin-Hernando et al., 2007; Di Marco et al., 2012). No obstante, es posible encontrar este tipo de lesiones en otras localizaciones orgánicas, principalmente pulmón, hígado y bazo (Di marco et al., 2012; Johansen et al., 2014) y de forma esporádica en riñones, válvula ileocecal, glándulas mamarias o en el tracto reproductivo (Martín-Hernando et a., 2007; Agdestein et al., 2011).

Análisis histopatológico de la linfadenitis porcina

Para describir las características histopatológicas de las lesiones de linfadenitis porcina, es importante tener en cuenta el microorganismo involucrado en las mismas. Las lesiones asociadas al CMT han sido descritas en numerosos trabajos de investigación como granulomas con un centro necrótico rodeado de células epitelioides, macrófagos, linfocitos, células plasmáticas y escasas células gigantes multinucleadas de tipo Langhans, con un grado variable de mineralización distrófica y un reducido número de BAAR (lesiones paucibacilares) (de Lisle et al., 2002; Martín-Hernando et al., 2007; Santos et al., 2010; García-Jiménez et al., 2013a). Estos granulomas suelen estar rodeados de una cápsula fibrosa de tejido conjuntivo (Bollo et al., 2000; Gortázar et al., 2011), aunque también se han detectado lesiones no encapsuladas (Di Marco et al., 2012). En base a estas características se establece una clasificación de los granulomas tuberculosos en cuatro estadios diferentes, donde se observa una evolución de los componentes celulares de los mismos desde su formación (estadio I) a los más antiguos (estadio IV) (Wangoo et al., 2005; García-Jiménez et al., 2013a):

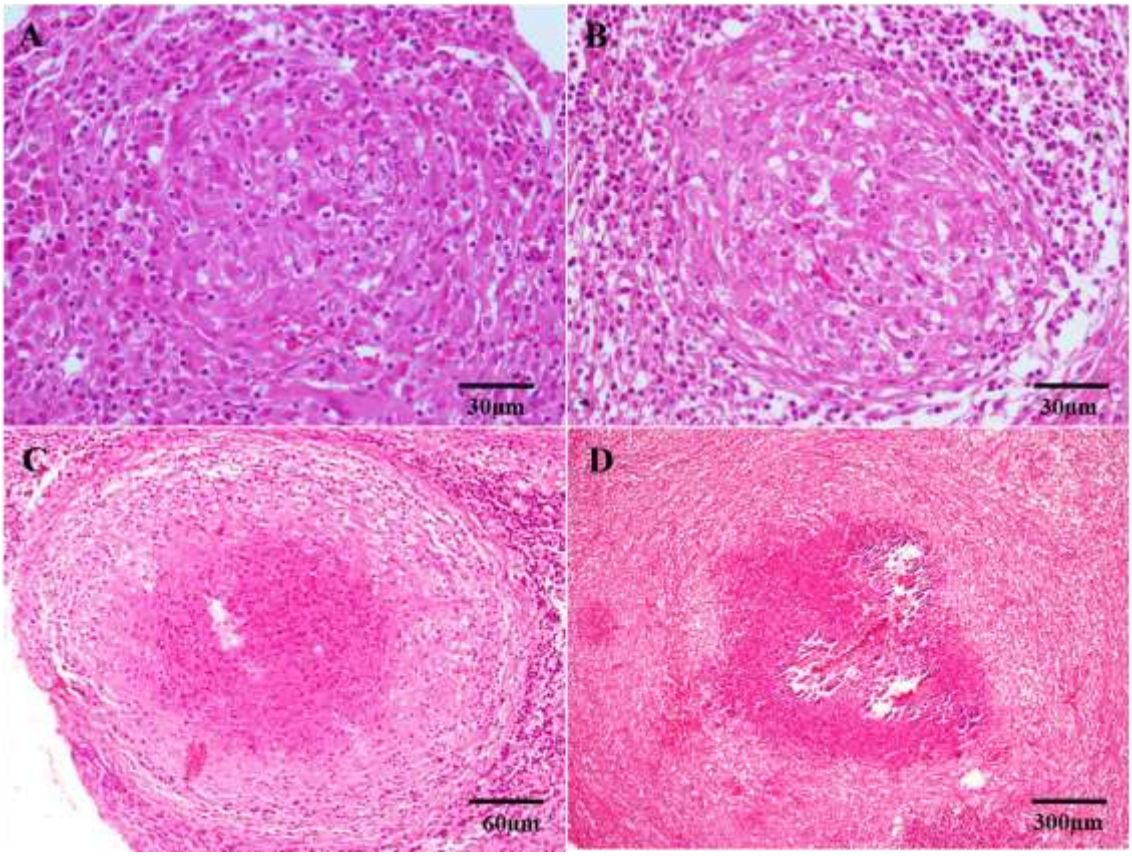


Figura 7. Estadios evolutivos del granuloma. **A**. Estadio I (inicial). **B**. Estadio II (sólido). **C**. Estadio III (necrosis mínima). **D**. Estadio IV (necrosis y mineralización).

- Estadio I (inicial): granulomas caracterizados por el acúmulo de células epitelioides no encapsuladas, con un infiltrado de linfocitos y escasos neutrófilos; ocasionalmente se observan células gigantes multinucleadas y no hay necrosis.

- Estadio II (sólido): granulomas compuestos principalmente por macrófagos epitelioides con un infiltrado de linfocitos, neutrófilos y a menudo células gigantes multinucleadas, rodeados parcial o totalmente por una delgada cápsula de tejido conectivo. Se pueden observar pequeñas áreas de necrosis.

- Estadio III (necrosis mínima): granulomas completamente encapsulados, con áreas centrales de necrosis caseosa y mineralización, rodeados por células epitelioides y

células gigantes multinucleadas. En la zona más periférica se observa infiltrado de linfocitos y neutrófilos dispersos que se extiende hacia la cápsula fibrosa.

- Estadio IV (necrosis y mineralización): granulomas de gran tamaño, irregulares, multicéntricos y densamente encapsuladas, con necrosis caseosa prominente y extensas áreas de mineralización. Rodeando a las áreas de necrosis, se observan macrófagos epitelioides y células gigantes multinucleadas, con abundante infiltrado de linfocitos en la cápsula periférica.

Las lesiones causadas por *M. avium* también han sido descritas como granulomas compuestos por macrófagos, células epitelioides, linfocitos y células gigantes multinucleadas, sin embargo, se ha indicado la presencia ocasional de eosinófilos, miofibroblastos o neutrófilos (Miranda et al., 2012), así como la presencia de abundantes células gigantes tipo Langhans y BAAR (lesiones pluribacilares) (Agdestein et al., 2011; Pérez de Val et al., 2013).

Por último, las lesiones causadas por agentes piógenos (*Trueperella* spp., *Streptococcus* spp., *Corynebacterium* spp., etc.) se caracterizan por la presencia de extensas áreas de necrosis con un marcado infiltrado de neutrófilos, restos celulares y fibrina, que pueden aparecer rodeadas por un infiltrado de macrófagos, linfocitos y células plasmáticas y por una cápsula de tejido conectivo en lesiones crónicas (Martínez et al., 2007; Contzen et al., 2011; Jarosz et al., 2014).

Diagnóstico de la linfadenitis porcina

Debido a la participación de distintos microorganismos en la linfadenitis del cerdo, es necesario llevar a cabo un diagnóstico etiológico con la finalidad de determinar el agente o agentes causales involucrados en el proceso. Para ello disponemos de dos clases de herramientas diagnósticas; las técnicas de **diagnóstico indirecto**, basadas en la detección de la respuesta inmune específica a la que dan lugar las bacterias en el organismo afectado, y las técnicas de **diagnóstico directo**, destinadas a la detección e identificación del microorganismo causal a partir de muestras patológicas (Álvarez et al., 2011). Las técnicas indirectas nos permiten llevar a cabo un diagnóstico *antermortem* de los animales, mientras que las técnicas directas generalmente requieren o bien la toma de muestras de biopsias a partir de las lesiones que presenten los animales o del sacrifico de los mismos para la obtención de muestras clínicas, ofreciendo en este caso un diagnóstico *postmortem*.

Diagnóstico antemortem de la linfadenitis porcina.

Las principales técnicas disponibles para el diagnóstico *antemortem* de la infección por micobacterias patógenas están basadas en la detección de la respuesta inmune celular (asociada a fases más tempranas de la infección), siendo la intradermotuberculinización (IDT) simple o comparada y la detección del gamma interferón (IFN-γ) las pruebas oficiales para la detección de la tuberculosis bovina (OIE, 2009; Bezos et al., 2014).

La **IDT** consiste en la inoculación intradérmica de un extracto de proteínas de micobacterias (PPD bovina, hecha a partir de una cepa de *M. bovis* y PPD aviar, de una cepa de *M. avium*) para después medir el incremento del pliegue al que da lugar, que en animales positivos debe ser fácilmente detectable y puede ir acompañado de dolor en el punto de inoculación (Álvarez et al., 2011). Esta técnica se ha empleado para la detección de animales

infectados por el CMT o el CMA en el ganado porcino, aplicando la prueba sobre la cara posterior de la oreja y llevando a cabo la lectura a las 48-72 horas (Álvarez et al., 2011). Sin embargo, los datos disponibles sobre su utilización son a menudo contradictorios (Nugent et al., 2002; Cvetnic et al., 2007; Aurtenetxe et al., 2008; Jaroso et al., 2010; Magnano et al., 2010; Agdestein et al., 2011; Faldyna et al., 2012; Broughan et al., 2013). A esta variabilidad se unen la dificultad en el manejo, ya que su empleo supone dos actuaciones sobre los animales (administración y lectura), y la dificultad de interpretación de la técnica en razas rústicas de capa oscura y/o pelaje hirsuto (Jaroso et al., 2010; Pesciaroli et al., 2012).

Otras de las técnicas disponibles para el diagnóstico *antemortem* de la tuberculosis, ampliamente utilizada como método complementario de diagnóstico en el ganado bovino, es el ensayo *in vitro* para la **detección de IFN-γ**, en el cual la sangre previamente extraída del animal es estimulada en el laboratorio con los mismos extractos empleados en la IDT (Bezos et al., 2014). En caso de estar infectado, los linfocitos del animal analizado reaccionarán al entrar en contacto con la PPD, aumentando la secreción de IFN-γ, que será detectada posteriormente mediante inmunoensayo (Álvarez et al., 2011). Los ensayos con anticuerpos monoclonales específicos han permitido adaptar este método al porcino (Garrido et al., 2011) mostrando una especificidad del 100% y una sensibilidad del 78,9% respecto al cultivo bacteriológico (Pesciaroli et al., 2012).

Las ventajas de esta técnica son el requerimiento de una única intervención en el animal, la posibilidad de repetir el ensayo varias veces y la detección precoz de la infección (Pesciaroli et al., 2012). Como desventajas al uso del IFN-γ se encuentran la necesidad de emplear las muestras de sangre en un intervalo corto de tiempo desde su recogida, la necesidad de un laboratorio y personal especializado y su coste elevado (Álvarez et al., 2011; Boadella et al., 2011).

Más recientemente, se han incorporado las técnicas basadas en la **detección de la respuesta inmune humoral** (asociada con etapas más avanzadas de infección) para el diagnóstico en especies animales domésticas y silvestres (OIE, 2009; Bezos et al., 2014). Los estudios publicados tras emplear estas técnicas en el jabalí, han demostrado que los anticuerpos generados frente a la infección por *M. bovis* pueden ser detectados con una aceptable sensibilidad y elevada especificidad mediante el uso de test serológicos (Boadella et al., 2011; Richomme et al., 2013; Che'Amat et al., 2015).

Por ello, en los últimos años se han desarrollado varias pruebas serológicas para el diagnóstico en muestras de suero y plasma de suidos, basadas en el empleo de distintos antígenos obtenidos a partir de *M. bovis* (PPDb, MPB83, MPB70, ESAT-6, CFP10) o combinaciones de ellos (Aurtenetxe et al., 2008; García-Bocanegra et al., 2012; Muñoz-Mendoza et al., 2013). No obstante, aún resulta necesario evaluar su uso en ganado porcino doméstico en condiciones de campo (Álvarez et al., 2011).

Entre sus muchas ventajas, las técnicas serológicas suponen una herramienta de diagnóstico económica y fácil de aplicar, pueden ser automatizadas para procesar un gran número de muestras en poco tiempo en el caso de las pruebas ELISA (Boadella et al., 2011) y no requieren de un laboratorio especializado en el caso de las pruebas inmunocromatográficas de flujo lateral (Lyashchenko et al., 2008; Che'Amat et al., 2015). No obstante, los análisis serológicos positivos deben ser considerados como una evidencia de exposición de los animales al agente estudiado y no como la detección de una infección activa (Lyashchenko et al., 2011), están sujetos a posibles reacciones cruzadas con otras bacterias (al igual que las pruebas de tipo celular) y su sensibilidad puede ser reducida durante etapas tempranas de infección o periodos de anergia (Aurtenetxe et al., 2008).

En relación a los microorganismos no pertenecientes al género *Mycobacterium* no existen pruebas inmunológicas fiables para su diagnóstico en el ganado porcino, debido entre otras razones, a que estos animales suelen ser portadores asintomáticos de los mismos (Lowe et al., 2011; Jarosz et al., 2014), hecho que dificulta el diagnóstico *antemortem* de las lesiones internas asociadas a estos microorganismos.

Cabe señalar que algunas técnicas directas pueden ser empleadas para el diagnóstico *antemortem* de la linfadenitis porcina; son ejemplo de ello el aislamiento a partir de biopsias de lesiones superficiales o supurativas en animales vivos (Oliveira et al., 2014) o la detección de los microorganismos excretados a través de distintas vías en los animales infectados (Barasona et al, 2015; Santos et al., 2015a).

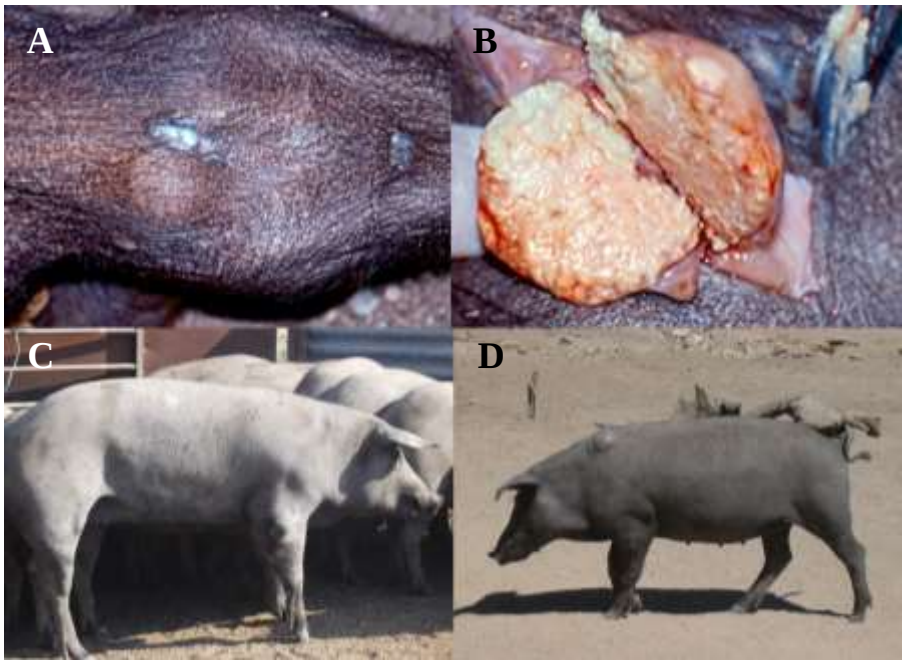


Figura 8. Lesiones superficiales o accesibles asociadas a cuadros de linfadenitis porcina. **A-B**: granuloma palpable a nivel mandibular. **C**. Artritis purulenta ulcerada en miembro anterior. **D**. Lesión cutánea superficial.

Diagnóstico postmortem de la linfadenitis porcina.

Atendiendo a las técnicas de diagnóstico directo, entre las pruebas disponibles para el diagnóstico *postmortem* de los agentes implicados en la linfadenitis porcina, encontramos la tinción selectiva de BAAR (tinción de Ziehl-Neelsen), el estudio histopatológico de las lesiones, el aislamiento selectivo e identificación del agente mediante cultivo bacteriológico y las técnicas de diagnóstico molecular aplicadas a muestras patológicas, principalmente la PCR.

La tinción de **Ziehl-Neelsen** permite realizar un diagnóstico presuntivo de la infección por *Mycobacterium* spp., gracias a la visualización de BAAR en frotis realizados a partir de muestras clínicas (Álvarez et al., 2011). Debido a la presencia de artefactos y a la subjetividad intrínseca de la bacterioscopia, las muestras positivas a esta técnica deben ser confirmadas mediante otras técnicas directas que evidencien la presencia del agente causal con una mayor especificidad (Gómez-Laguna et al., 2010). Además, otras bacterias relacionadas taxonómicamente pueden presentar resultados parcialmente positivos a esta técnica (Quinn et al., 2011). En cualquier caso, la ausencia de BAAR en muestras patológicas no excluye la implicación de *Mycobacterium* spp en muestras compatibles (OIE, 2009).

El aislamiento selectivo del agente mediante **cultivo bacteriológico** (generalmente a partir de animales con lesiones compatibles) y su identificación mediante pruebas bioquímicas o moleculares sigue siendo la prueba de referencia para el diagnóstico de los microorganismos involucrados en la linfadenitis porcina (OIE, 2009; Álvarez et al., 2011, Contzen et al., 2011). No obstante, el cultivo de las micobacterias patógenas está condicionado por varios factores, entre los que se encuentran su lento crecimiento en medios de cultivo específicos (hasta tres meses en el primoaislamiento), la necesidad de emplear

laboratorios especializados con un alto nivel de seguridad biológica o la reducción de su sensibilidad debido a particularidades en la conservación o tratamiento de la muestra (descontaminación previa al cultivo y pérdida de viabilidad) (Álvarez et al., 2011; Corner et al., 2011).

Para el resto de microorganismos involucrados en la linfadenitis porcina se emplean métodos de aislamiento convencional, basados en medios de cultivo generales enriquecidos con sangre y periodos de incubación comprendidos entre las 24-48 horas (Komijn et al., 2007; Martínez et al., 2007; Lara et al., 2011; Oliveira et al., 2014).

El aislamiento del agente permite la obtención de abundante material genético bacteriano que puede servir para realizar posteriores estudios moleculares destinados a identificar la especie implicada o el origen del brote (Álvarez et al., 2011). Entre dichas técnicas, cabe señalar el valor de la secuenciación de los genes ARNr 16S y rpoB para la identificación de corinebacterias patógenas (Contzen et al., 2011; Oliveira et al., 2014) y los estudios de epidemiología molecular basados en espoligotipado y VNTR en las infecciones causadas por *M. bovis* y otras especies del CMT (Bailey et al., 2013).

Las características del **análisis histopatológico** de las lesiones de linfadenitis porcina ya han sido comentadas en profundidad en el apartado anterior. La combinación de esta técnica con el cultivo bacteriológico puede ser empleada para mejorar la sensibilidad del diagnóstico de la tuberculosis y micobacteriosis porcinas (Miranda et al., 2012; Muñoz-Mendoza et al., 2013; Risco et al., 2014). Además, el diagnóstico histopatológico puede ser combinado con otras técnicas directas que permitan evidenciar la presencia del agente en las lesiones, como son la inmunohistoquímica o la tinción de Ziehl-Neelsen (Gómez-Laguna et al., 2010). No obstante, la evaluación de las lesiones es laboriosa y la sensibilidad y especificidad de la técnica puede resultar limitada (Ramis y Pallarés., 2009).

Dado que la vigilancia epidemiológica en el matadero sigue siendo una pieza elemental en los programas de control y erradicación de la tuberculosis animal, es necesario desarrollar nuevos métodos de confirmación que permitan superar las limitaciones del cultivo bacteriológico (Grant y Stewart, 2015). Una alternativa es el desarrollo de técnicas moleculares que permitan la confirmación rápida del agente a partir de las muestras biológicas, siendo la PCR la técnica más ampliamente evaluada (Parra et al., 2008).

La **PCR** permite detectar cantidades muy pequeñas de ADN del agente causal en las lesiones compatibles y obtener un diagnóstico rápido en apenas 24-48 horas (Miranda y et al., 2012; Gómez-Laguna et al., 2010), favoreciendo la toma rápida de decisiones (Miller et al., 2002). A la rápida detección del agente causal, se suma la posibilidad de llevar a cabo una rápida identificación del mismo mediante el desarrollo de técnicas basadas en múltiples dianas de detección (PCR dúplex o PCR múltiple) que permiten distinguir grupos o especies microbianas diferentes en una sola reacción de PCR (Coetsier et al., 2000).

La principal limitación para la implementación de esta técnica ha sido su falta de sensibilidad debido a factores concretos, como son la inhibición de la reacción en muestras complejas, las características de la muestra en las distintas especies animales (fenómenos intensos de fibrosis y calcificación dificultan la extracción de ADN) o la distribución del agente causal en los tejidos, como ocurre en las lesiones tuberculosas del porcino, caracterizadas por un limitado número de bacilos, que dificulta la obtención de cantidades suficientes de ADN a partir de las lesiones patológicas (Bollo et al., 2000; Coetsier et al., 2000; Parra et al., 2008; Santos et al., 2010).

Para mejorar estos valores, se ha evaluado el empleo de métodos de captura selectiva del ADN basados en la incorporación de partículas magnéticas (separación inmunomagnética), obteniendo resultados variables (Parra et al., 2008; Grant y Stewart,

2015), entre los que podemos destacar un aumento considerable en la detección de animales infectados sin lesiones visibles (Stewart et al., 2013). Otra de las principales limitaciones de la PCR es su incapacidad para detectar viabilidad respecto al aislamiento microbiológico, por lo cual se ha recomendado el uso de ambas técnicas en paralelo (OIE, 2009).

En cualquier caso, los valores de sensibilidad de la PCR para el diagnóstico de la tuberculosis a partir de tejidos de distintas especies respecto al cultivo bacteriológico oscilaron entre el 50% y el 80,6% (Bollo et al., 2000; Parra et al., 2008; Santos et al., 2010; Stewart et a., 2013). No obstante, estudios más recientes basados en otros métodos de referencia han obtenido valores de sensibilidad que llegan al 93,6% (Courcoul et al., 2014) así como una alta incidencia de muestras positivas a la PCR que no fueron detectadas mediante las técnicas rutinarias de cultivo (Carvalho et al., 2015).

La PCR también ha sido empleada para la detección e identificación de otros microorganismos involucrados en la linfadenitis porcina a partir de muestras clínicas de otras patologías del cerdo y otras especies animales. Ejemplo de ello es el uso de una PCR múltiple para el diagnóstico de *T. pyogenes* en la endometritis *postpartum* del ganado bovino (Aghamiri et al., 2013) o la detección e identificación de este microorganismo, junto con especies de los géneros *Corynebacterium* y *Streptococcus*, para el diagnóstico de la mastitis bovina (Spittel y Hoedemaker, 2012; Bi et al., 2016). Otros ejemplos son también las técnicas de PCR desarrolladas para el chequeo de cerdos portadores de *S. suis* mediante el análisis de torundas nasales o tonsilares (Marois et al., 2004; Dekker et al., 2016) o las técnicas de PCR desarrolladas para el diagnóstico de *R. equi* a partir de muestras clínicas de potros con bronconeumonía purulenta (Stefánska et al., 2016).

No obstante, aún no se han llevado a cabo ensayos que evalúen el uso de esta metodología para el diagnóstico de los microorganismos distintos a las micobacterias a partir

de lesiones de linfadenitis porcina, siendo estos últimos detectados generalmente mediante técnicas de aislamiento microbiológico convencional (Contzen et al., 2011; Lara et al., 2011).

Implicaciones de la linfadenitis porcina en la cadena alimentaria

Debido al carácter subclínico de la linfadenitis en el cerdo, la detección de los animales afectados se produce generalmente durante la inspección *postmortem* en el matadero, originando el decomiso total o parcial de canales y órganos con lesiones macroscópicas, no aptos para el consumo humano (Blagojevic et al., 2014). No obstante, dado que en algunas infecciones recientes no se detectan lesiones macroscópicas (Lara et al., 2011; Stewart et al., 2013), y que existe la posibilidad de que ocurra contaminación cruzada debido a la entrada de animales infectados o portadores en la cadena de sacrificio (Arai et al., 2015), de forma teórica, estos microorganismos podrían llegar a la cadena alimentaria durante el sacrificio y faenado de canales en el matadero (Hermoso de Mendoza et al., 2003; Lara et al., 2011).

Atendiendo al potencial zoonósico de los microorganismos implicados en la linfadenitis porcina, cabe destacar la posible participación de micobacterias del CMT y del CMA, que pueden originar enfermedad en el hombre con gravedad variable (Biet et al, 2005; Thoen et al., 2006; Johansen et al., 2014). No obstante, se considera que la posibilidad de transmisión de micobacterias por consumo de alimentos en países donde se aplican medidas generales de control y de inspección sanitarias es muy reducida o nula, siendo más probable su valor como zoonosis profesional para el personal expuesto (Hermoso de Mendoza et al., 2003; Pérez de Val et al., 2013; Blagojevic et al., 2014).

En el último informe sobre fuentes y tendencias de zoonosis, agentes zoonósicos y brotes de transmisión alimentaria publicado por la Autoridad Europea de Seguridad Alimentaria (EFSA), *M. bovis* aparece como una infección muy poco frecuente en el hombre

con sólo 134 casos declarados en 2013, número que se ha mantenido estable durante los dos últimos años y que debido al carácter crónico de la enfermedad, es reflejo de procesos adquiridos hace años, no asociados al consumo de alimentos; el resto de agentes tratados aquí, no son considerados en dicho informe (EFSA, 2015).

Aunque algunos autores han sugerido que el consumo de carne de cerdo podría ser una fuente de infección de *M. avium* y *R. equi* para el hombre, especialmente en el caso de personas inmunodeprimidas (Ocampo-Sosa et al., 2007; Tirkkonen et al., 2010; Lara et al., 2015), la teoría más aceptada para un posible contagio con estos agentes sigue siendo la exposición de ambos hospedadores a una fuente ambiental común (Álvarez et al., 2011).

Existen evidencias de que algunos estreptococos y corinebacterias implicados en cuadros de linfadenitis porcina pueden afectar al hombre (Hermida et al., 2004; Kavitha et al., 2010; Pachirat et al., 2012; Goyette-Desjardins et al., 2014; Silva et al., 2015). Entre ellos, existen evidencias de que *S. suis* puede suponer un riesgo para el hombre por el consumo de carne y órganos de cerdo poco cocinados (Huong et al., 2014). De hecho, se ha demostrado la presencia de este microorganismo en la carne de cerdo en países como China y Japón (Cheung et al., 2008; Arai et al 2015). En España, sin embargo, sólo se han descrito algunos casos esporádicos, relacionados con el contacto directo a partir de animales portadores y la infección a través de la vía oronasal o pequeñas heridas en la piel (Hidalgo et al., 2009; Gottschalk et al., 2012; Gómez-Zorrilla et al., 2014).

Por otro lado, la carne de cerdo se emplea habitualmente para la elaboración de productos curados no cocinados listos para el consumo (como son la paleta, el jamón o la caña de lomo), estando este tipo de productos estrechamente ligados a la producción del cerdo Ibérico en extensivo en nuestro país (Ventanas, 2001). Los productos curados son considerados estables y seguros debido a una serie de parámetros físico-químicos intrínsecos

al proceso de curación (Stollewerk et al., 2012). Estudios previos han demostrado la capacidad de este proceso tecnológico para reducir la carga de microorganismos clásicos de transmisión alimentaria (*Salmonella* spp., *Listeria* spp.) (Reynolds., 2001; García-Díez et al., 2016) y algunos virus del cerdo (Mebus et al., 1997). Como parte de la apuesta constante por la calidad y la seguridad alimentaria de este sector, resulta necesario abordar nuevos estudios que arrojen información sobre la capacidad del proceso de curación para eliminar los microorganismos asociados a la linfadenitis del cerdo en extensivo en estos productos.

Referencias

Agdestein A, Johansen TB, Polaček V, Lium B, Holstad G, Vidanović D, Aleksić-Kovačević S, Jørgensen A, Žultauskas J, Nilsen SF, Djønne B. 2011. Investigation of an outbreak of mycobacteriosis in pigs. BMC Vet. Res. 7:63. doi: 10.1186/1746-6148-7-63.

Agdestein, A., Olsen, I., Jorgensen, A., Djonne, B., Johansen, T.B. 2014. Novel insights into transmission routes of *Mycobacterium avium* in pigs and possible implications for human health. Vet. Res. 45:46. doi: 10.1186/1297-9716-45-46.

Aghamiri, S.M., Hagnkhkan, M., Ahmadi, M.R., Gheisari,H.R. 2013. Development of a multiplex PCR for the identification of major pathogenic bacteria of post-partum endometritis in dairy cows. Reprod. Domest. Anim. 49: 233-238.

Álvarez, J., Bezos, J., Casal, C., Boadella, M., de Juán, L. 2011. Micobacteriosis en el porcino: Importancia y pautas para su diagnóstico, identificación y control. Cría y Salud. 35:40-45.

Arai, S., Tohya, M., Yamada, R., Osawa, Ro., Nomoto, R., 2015. Development of loop-mediated isothermal amplification to detect *Streptococcus suis* and its application to retail pork meat in Japan. Int. J. Food. Microbiol. 208, 35-42.

Astorga, R.J., Gómez-Laguna, J., Moreno, P., Hernández, M., Carrasco, L., 2010. 'Sanidad animal en el cerdo ibérico'. En: Especial ''Solo cerdo ibérico 25 Aniversario' Tomo II, Sanidad. Edita AECERIBER. ISBN 84-930710-0-5. Pág. 128-135

Aurtenetxe, O., Barral, M., Vicente, J., De la Fuente, J., Gortázar, C., Juste, R.A. 2008. Development and validation of an enzyme-linked immunosorbent assay for antibodies against *Mycobacterium* bovis in European wild boar. BMC Vet. Res. 43: 1-9.

Bailey, S.S., Crawshaw, T.R., Smith, N.H., Palgrave, C.J. 2013. *Mycobacterium bovis* infection in domestic pigs in Great Britain. Vet. J. 198: 391-397.

Barasona, J.A., Torres, M.J., Aznar, J., Gortázar, C., Vicente, J. 2015. DNA Detection Reveals Mycobacterium tuberculosis Complex shedding routes in its wildlife reservoir the Eurasian wild boar. Transbound. Emerg. Dis. doi: 10.1111/tbed.12458.

Barasona, J.A., Vicente, J., Díez-Delgado, I., Aznar, J., Gortázar, C., Torres, M.J. 2016. Environmental presence of *Mycobacterium tuberculosis* complex in aggregation points at the wildlife/livestock interface. Transbound. Emerg. Dis. doi: 10.1111/tbed.12480.

Barbier, E., Chantemesse, B., Rochelet. M., Fayolle, L., Bochalle, L., Boschiroli, M.L., Hartmann, A. 2016. Rapid dissemination of *Mycobacterium bovis* from cattle during to soil by the earthworm Lumbricus terrestris.

Bezos, J., Casal, C., Romero, B., Schroeder, B., Hardeqqer, R., Raeber, A.J., López, L., Rueda, P., Domínguez, L., 2014. Current ante-mortem techniques for diagnosis of bovine tuberculosis. Res. Vet. Sci. 97: S44-S52.

Bi, Y., Wang, Y.J., Qin, Y., Guix Vallverdú, R., Maldonado García, J., Sun, W., Li, S., Cao, Z. 2016. PLoS One. 11(5): e0155621.

Biet, F., Boschiroli, M.L., Thorel, M.F., Guilloteau, L.A. 2005. Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). Vet. Res. 36: 411-436.

Boadella, M., Lyashchenko, K., Greenwald, R., Esfandiari, J., Jaroso, R., Carta, T., Garrido, J.M., Vicente, J., De la Fuente, J., Gortázar, C. 2011. Serologic tests for detecting antibodies against *Mycobacterium bovis* and *Mycobacterium avium subspecies paratuberculosis* in Eurasian wild boar (*Sus scrofa scrofa*). Journal of Veterinary Diagnostic Investigation. 23: 77-83.

Blagojevic, B., Antic, D., 2014. Assessment of potential contribution of official meat inspection and abattoir process hygiene to biological safety assurance of final beef and pork carcasses. Food. Control. 36: 174-182.

Bollo, E., Ferroglio, E., Dini, V., Mignone, W., Biolatti, B., Rossi, L. 2000. Detection of Mycobacterium tuberculosis complex in lymph nodes of wild boar (Sus scrofa) by a target-amplified test system. J. Vet. Med. B. Infect. Dis. Vet. Public. Health. 47: 337-342.

Broughan, J.M., Downs, S.H., Crawshaw, T.R., Upton, P.A., Brewer, J., Clifton-Hadley, R.S. 2013. *Mycobacterium bovis* infections in domesticated non-bovine mammalian species. Part 1: Review of epidemiology and laboratory submissions in Great Britain 2004-2010. Vet. J. 198: 339-345.

Buxadé, C. El sector porcino: realidades y perspectivas. 2014. Mundo Ganadero. Julio/Agosto: 28-30.

Carvalho, R.C., Furlanetto, L.V., Maruyama, F.H., Araújo, C.P., Barros, S.L., Ramos, CA., Dutra, V., Araújo, F.R., Paschoalin, V.M., Nakazato, L., Figueiredo, E.E. 2015. Evaluation of the efficiency of nested q-PCR in the detection of *Mycobacterium tuberculosis* complex directly from tuberculosis-suspected lesions in post-mortem macroscopic inspections of bovine carcasses slaughtered in the state of Mato Grosso, Brazil**.** Meat. Sci.106: 11-15.

Che' Amat, A., González-Barrio, D., Ortiz, J.A., Díez-Delgado, I., Boadella, M., Barasona, J.A., Bezos, J., Romero, B., Armenteros, J.A., Lyashchenko, K.P., Venteo, A., Rueda, P., Gortázar, C. 2015. Testing Eurasian wild boar piglets for serum antibodies against *Mycobacterium bovis*. Pre. Vet. Med. 121: 93-98.

Cheung, P.Y., Lo, K.L., Cheung, T.T., Yeung, W.H., Leung, P.H., Kam K.M., 2008. *Streptococcus suis* in retail markets: How prevalent is it in raw pork? Int. J. Food. Microbiol. 127: 316-320.

Christensen, B.V., Aalbaek, B., Jensen, H.E. 2007. Pathology of udder lesions in sows. J. Vet. Med. 54: 491-493.

Coetsier, C., Vannuffel, P., Blondeel, N., Denef, J.F., Cocito, C., Gala, J.L. 2000. Duplex PCR for differential identification of *Mycobacterium bovis*, *M. avium*, and *M. avium* subsp. *paratuberculosis* in formalin- fixed paraffin-embedded tissues from cattle. J. Clin. Microbiol. 38: 3048-3054.

Courcoul, A., Moyen, J.L., Brugère, L., Faye, S., Hénault, S., Gares, H., Boschiroli, M.L. 2014. Estimation of sensitivity and specificity of bacteriology, histopathology and PCR for the confirmatory diagnosis of bovine tuberculosis using latent class analysis. Plos. One. 9: e90334.

Contzen, M., Sting, R., Blazey, B., Rau, J. 2011. *Corynebacterium ulcerans* from diseased wild boars. Zoonoses. Public. Health. 58: 479-488.

Corner, L.A.L., Gormley, E., Pfeiffer, D.U. 2012. Primary isolation of *Mycobacterium bovis* from bovine tissues: Conditions for maximizing the number of positive cultures. Vet. Microbiol. 156: 162-171.

Cvetnic, Z., Spicic, S., Benic, M., Katalinic-Jankovic, V., Pate, M., Krt, B., Ocepekm, M. 2007. Mycobacterial infection of pigs in Croatia. Acta. Vet. Hung. 55: 1-9.

Di Marco, V., Mazzone, P., Capucchio, M.T., Boniotti, M.B., Aronica, V., Russo, M., Fiasconaro, M., Cifani, N., Corneli, S., Biasibetti, E., Biagetti M, Pacciarini ML, Cagiola M, Pasquali P, Marianelli C. 2012. Epidemiological significance of the domestic black pig (*Sus scrofa*) in maintenance of bovine tuberculosis in Sicily. J. Clin. Microbiol. 50: 1209-1218.

Directiva 2003/99/CE del Parlamento europeo y del Consejo, de 17 de noviembre de 2003, sobre la vigilancia de las zoonosis y los agentes zoonóticos y por la que se modifica la Decisión 90/424/CEE del consejo y se deroga la Directiva 92/117/CEE del Consejo.

De Lisle, G.W., Bengis, R.G., Schmitt, S.M., O' Brien, D.J. 2002. Tuberculosis in free-ranging wildlife: detection, diagnosis and management. OIE Revue Scientifique et Technique. 21: 317-334.

Dekker, C.N., Bouma, A., Daemen, A.J., van Leengoed, L.A., Jonker, F.H., Wagenaar, J.A., Stegeman, J.A. 2012. Homologous whole bacterin vaccination is not able to reduce *Streptococcus suis* serotype 9 strain 7997 transmission among pigs or colonization. Vaccine. 30: 1379–1387.

Domingo, M., Vidal, E., Marco, A. 2014. Pathology of bovine tuberculosis. Res. Vet. Sci. 97: 520-529.

European Food Safety Authority (EFSA). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA Journal. 13:3991

Faldyna, M., Göpfert, E., Kudlackova, H., Stepanova, H., Kaevska, M., Slana, I., Pavlik, I. 2012. Usability of a gamma interferon release assay in the diagnosis of naturally

infected pigs with *Mycobacterium avium* subspecies *hominissuis*. J. Vet. Diagn. Invest. 3 76-8. doi: 10.1177/1040638711435141.

Funke, G., Bernard, K.A. 2007. Coryneform gram-positive rods. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA (Eds) Manual of clinical microbiology, 9th Ed. American Society for Microbiology, Washington: 485-514.

García-Bocanegra, I., Pérez de Val, B., Arenas-Montes, A., Paniagua, J., Boadella, M., Gortázar, C., Arenas, A. 2012. Seroprevalence and risk factors associated to *Mycobacterium bovis* in wild artiodactyl species from Southern Spain, 2006-2010. Plos. One. 7 (4): e34908.

García-Diez, J., Coelho, A.C. 2014. Causes and factors related to pig carcass condemnation. Vet. Med.-Czech. 59: 194-201.

García-Díez, J., Alheiro, J., Pinto, A.L., Soares, L., Falco, V., Fraqueza, M.J., Patarata, L., 2016. Behaviour of food-borne pathogens on dry cured sausage manufactured with herbs and spices essential oils and their sensorial acceptability. Food. Control. 59, 262-270.

García-Jiménez, W.L, Salgero, F.J, Fernández-Llario, P., Martínez, R., Risco, D., Gough, J., Ortiz-Peláez, A., Hermoso-de-Mendoza, J., Gómez, L. 2013a. Immunopathology of granulomas produced by *Mycobacterium bovis* in naturally infected wild boar. Vet. Immunol. Immunop. 156: 54-63.

García-Jiménez, W.L., Benítez-Medina, J.M., Fernández-Llario, P., Abecia, J.A., García-Sánchez, A., Martínez, R., Risco, D., Ortiz-Peláez, A., Salguero, F.J., Smith, N.H., Gómez, L., Hermoso de Mendoza J. 2013b. Comparative pathology of the natural infections by *Mycobacterium bovis* and by *Mycobacterium caprae* in wild boar (*Sus scrofa*). Transbound. Emerg. Dis. 60: 102-109.

García-Jiménez, W.L., Benítez-Medina, J.M., Martínez, R., Carranza, J., Cerrato, R., García-Sánchez, A., Risco, D., Moreno, J.C., Sequeda, M., Gómez, L., Fernández-Llario, P., reHermoso-de-Mendoza, J. 2015. Non-tuberculous mycobacteria in wild boar (*Sus scrofa*) from Southern Spain: epidemiological, clinical and diagnostic concerns. Transbound. Emerg. Dis. 62:78-80.

Garrido, J.M., Sevilla, I.A., Beltrán-Beck, B., Minguijón, E., Ballesteros, C., Galindo, R.C., Boadella, M., Lyashchenko, K.P., Romero, B., Geijo, M.V., Ruiz-Fons, F., Aranaz, A., Juste, R.A., Vicente, J., de la Fuente, J., Gortázar, C. 2011. Protection against tuberculosis in Eurasian wild boar vaccinated with heat-inactivated Mycobacterium bovis. PLoS One. 6(9): e24905.

Gómez-Laguna, J., Carrasco, L., Ramis, G., Quereda, J.J., Gómez, S., Pallarés, F.J. 2010. Use of real-time and classic polymerase chain reaction assays for the diagnosis of porcine tuberculosis in formalin-fixed, paraffin-embedded tissues. J. Vet. Diagn. Invest. 22: 123-127.

Gómez-Zorrilla, S., Ardanuy, C., Lora-Tamayo, J., Cámara, J., García-Somoza, D., Peña, C., Ariza, J. 2014. *Streptococcus suis* infection and malignancy in man, Spain. Emerg. Infect. Dis. 20: 1067-1068.

Goodfellow, M., Jones, A.L. 2015. Corynebacteriales ord. nov. Bergey's Manual of Systematics of Archaea and Bacteria. 1-14.

Gortázar, C., Torres, M.J., Acevedo, P., Aznar, J., Negro, J.J., de la Fuente, J., Vicente, J. 2011. Fine-tuning the space, time, and host distribution of mycobacteria in wildlife. BMC Microbiol. 11: 27. doi: 10.1186/1471-2180-11-27.

Gottschalk, M. 2012. Streptococcosis. In: Diseases of Swine, 10th Edition. Karriker L.A., Ramirez A., Schwartz K.J., Stevenson G., Zimmerman J.L (Eds). Wiley-Blackwell, NJ, USA.

Goyette-Desjardins, G., Auger, J.P., Xu, J., Segura, M., Gottschalk, M. 2014. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg. Microbes. Infect. doi:10.1038/emi.2014.45.

Grant, I.R., Stewart, L.D. 2015. Methods. Improved detection of *Mycobacterium bovis* in bovine tissues using immunomagnetic separation approaches. Mol. Biol. 1247: 153-61.

Hidalgo, A., Palacios, R., Santos, J. 2009. *Streptococcus suis* meningitis in Spain. Enferm. Infecc. Microbiol. Clin. 27: 195.

Hermida Ameijerias, A., Romero Jung, P., Cabarcos Ortiz de Barrón, A., Treviño castaño M., 2004. A propósito de un caso de neumonía por *Arcanobacterium pyogenes*. An. Med. Interna. 21, 28-30.

Hermoso de Mendoza Salcedo, J., Cerrato, R., Rey Pérez, J., Tato, A., Fernández Llario, P., Alonso Segura, J.M., Casas, E., Hermoso de Mendoza Salcedo, M., Parra, A. 2003. La tuberculosis y el porcino intensivo. El problema y sus alternativas de control. Ganadería. 23: 36-43.

Huong, V.T., Ha, N., Huy, N.T., Horby, P., Nghia, H.D., Thiem, V.D., Zhu, X., Hoa, N.T., Hien, T.T., Zamora, J., Schultsz, C., Wertheim, H.F., Hirayama, K. 2014. Epidemiology, clinical manifestations, and outcomes of *Streptococcus suis* infection in humans. Emerg. Infect. Dis. 20: 1105-1114.

Jaroso, R., Vicente, J., Fernandez-de-Mera, I.G, Aranaz, A., Gortázar, C. 2010. Eurasian wild boar response to skin-testing with mycobacterial and non-mycobacterial antigens. Prev. Vet. Med. 96: 211-217.

Jarosz, L.S., Gradzki, Z., Kalinowski, M. 2014. *Trueperella pyogenes* infections in swine: clinical course and pathology. Pol. J. Vet. Sci. 17: 395-404.

Johansen, T.B, Agdestein, A., Lium, B., Jørgensen, A., Djønne, B. 2014. *Mycobacterium avium* sbsp. *hominissuis* infection in swine associated with peat used for bedding. BMC Infect. Dis. 14: 655. doi: 10.1186/s12879-014-0655-4.

Kavitha, K., Latha, R., Udayashankar, C., Jayanthi, K., Oudeacoumar, P. 2010. Three cases of *Arcanobacterium pyogenes* associated soft tissues infection. J. Med. Microbiol. 59: 736-739.

Komijn, R.E., Wisselink, H.J., Rijsman, V.M.C., Stockhofe-Zurwieden, N., Bakker, D., van Zijderveld, F.G., Eger, T., Wagenaar, J.A., Putirulan, F.F., Urlings, B.A. 2007. Granulomatous lesions in lymph nodes of slaughter pigs bacteriologically negative for *Mycobacterium avium subsp. avium* and positive for *Rhodococcus equi*. Vet. Microbiol. 120: 352-357.

Langreo Navarro, A., Gonzáles del Barrio, A. 2007. El sector porcino en España. Agricultura familiar en España. Fundación de Estudios Rurales (228-232).

Lara, G.H., Ribeiro, M.G., Leite, C.Q., Paes, A.C., Guazzelli, A., da Silva, A.V., Santos, A.C., Listoni, F.J. 2011. Occurrence of *Mycobacterium* spp. and other pathogens in lymph nodes of slaughtered swine and wild boars (*Sus scrofa*). Res. Vet. Sci. 90: 185-188.

Lara, G.H., Takai, S., Sasaki, Y., Kakuda, T., Listonim, F.J., Risseti, R.M, de Morais, A.B, Ribeiro, M.G. 2015. VapB type 8 plasmids in *Rhodococcus equi* isolated from the small intestine of pigs and comparison of selective culture media. Lett. Appl. Microbiol. 61: 306-310.

Linares, R., Cardoso-Toset, F., Gómez-Laguna, J., Carrasco, L., Gómez-Gascón, L., Amarilla, S.P., Luque I. 2013. Retrospective study of condemnation associated with lymphadenitis in pigs reared in free-range systems. Proceedings of the 5th European Symposium of Porcine Health Management. Pag. 136.

Lowe, B.A., Marsh, T.L., Isaacs-Cosgrove, N., Kirkwood, R.N., Kiupel, M., Mulks, M.H. 2011. Microbial communities in the tonsils of healthy pigs. Vet. Microbiol. 147: 346-357.

Lyashchenko, KP., Greenwald, R., Esfandiari, J., Chambers, MA., Vicente, J., Gortázar, C., Santos N., Correia-Neves, M., Buddle, BM., Jackson, R., O'Brien, DJ., Schmitt, S., Palmer MV., Delahay, RJ., Waters WR. 2008. Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. Vet. Microbiol. 132: 283-292.

Lyashchenko, KP., Greenwald, R., Esfandiari, J., Rhodes, S., Dean, G., de la Rua-Domenech, R., Meylan, M., Vordermeier, HM., Zanolari, P., 2011. Diagnostic Value of Animal-Side Antibody Assays for Rapid Detection of *Mycobacterium bovis* or *Mycobacterium microti* Infection in South American Camelids. Clin. Vaccine. Immunol. 18: 2143-2147.

Magnano, G.G., Schneider, M.O., Urbani, C.E., Ambrogi, A., Zapata, L., Jorge, M.C. 2010. Comparación de técnicas diagnósticas de tuberculosis porcina en dos establecimientos de cría confinada en Argentina. In. Vet. 12: 25-31.

Makrai, L., Kobayashi, A., Matsuoka, M., Sasaki, Y., Kakuda, T., Dénes, B., Hajtós, I., Révész, I., Jánosi, K., Fodor, L., Varga, J., Takai, S. 2008. Isolation and characterisation of *Rhodococcus equi* from submaxillary lymph nodes of wild boars (*Sus scrofa*). Vet. Microbiol. 15: 318-323.

Mann, E., Dzieciol, M., Metzler-Zebeli, B.U., Wagner, M., Schmitz-Esser, S. 2014. Appl Environ Microbiol. Microbiomes of unreactive and pathologically altered ileocecal lymph nodes of slaughter pigs. Appl. Environ. Microbiol. 80: 193-203.

Martínez, J., Jaro, P.J., Aduriz, G., Gómez, E.A., Peris, B., Corpa, J.M. 2007. Carcass condemnation causes of growth retarded pigs at slaughter. Vet. J. 174: 160-164.

Marois, C., Bougeard, S., Gottschalk, M., Kobisch, M. 2004. Multiplex PCR assay for detection of *Streptococcus suis* species and serotypes 2 and 1/2 in tonsils of live and dead pigs. J. Clin. Microbiol. 42: 3169–3175.

Martín-Hernando, M.P., Höfle, U., Vicente, J., Ruiz-Fons, F., Vidal, D., Barral M., Garrido, J.M., de la Fuente, J., Gortazar, C. 2007. Lesions associated with *Mycobacterium tuberculosis* complex infection in the European wild boar. Tuberculosis. 87: 360-367.

Mebus, C., Arias, M., Pineda, J.M., Tapiador, J., House, C., Sánchez-Viscaíno, M. 1997. Survival of several porcine viruses in different Spanish dry-cured meat products. Food. Chem. 59: 555-559.

Miller, J.M., Jenny, A.L., Payeur, J.B. 2002. Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* organisms in formalin-fixed tissues from culture-negative ruminants. Vet. Microbiol. 87: 15-23.

Miranda, C., Matos, M., Pires, I., Correia-Neves, M., Ribeiro, P., Alvares, S., Vieira-Pinto M, Coelho, A.C. 2012. Diagnosis of *Mycobacterium avium* complex in granulomatous lymphadenitis in slaughtered domestic pigs. J. Comp. Pathol. 147: 401-405.

Müller, B., de Klerk-Lorist, L.M., Henton, M.M., Lane, E., Parsons, S., Gey van Pittius, N.C., Kotze, A., van Helden, P.D., Tanner, M. 2011. Vet. Microbiol. 147: 340-345.

Muñoz-Mendoza, M., Marreros, N., Boadella, M., Gortázar, C., Menéndez, S., de Juan, L., Bezos, J., Romero, B., Copano, M.F., Amado, J., Sáez, J.L, Mourelo, J., Balseiro, A. 2013. Wild boar tuberculosis in Iberian Atlantic Spain: a different picture from Mediterranean habitats. BMC Vet. Res. 176. doi: 10.1186/1746-6148-9-176.

Nugent, G., Whitford, J., Yockney, I.J., Cross, M.L. 2002. Reduced spillover transmission of Mycobacterium bovis to feral pigs (*Sus scofa*) following population control of brushtail possums (*Trichosurus vulpecula*). Epidemiol. Infect. 140: 1036-1047.

Ocampo-Sosa, A.A., Lewis, D.A, Navas, J., Quigley, F., Callejo, R., Scortti, M., Leadon, D.P., Fogarty, U., Vazquez-Boland, J.A. 2007. Molecular epidemiology of *Rhodococcus equi* based on traA, vapA, and vapB virulence plasmid markers. 196: 763-769.

OIE. 2009. Terrestrial manual. Chapter 2.4.7. Bovine tuberculosis. World Organisation for Animal Health: 1-8.

Oliveira, M., Barroco, C., Mottola, C., Santos, R., Lemsaddek, A., Tavares, L., Semedo-Lemsaddek T. 2014. First report of *Corynebacterium pseudotuberculosis* from caseous lymphadenitis lesions in Black Alentejano pig (*Sus scrofa domesticus*). BMC. Vet. Res. 218: 1-5.

Pachirat, O., Taksinachanekit, S., Mootsikapun, P., Kerdsin, A. 2012. Human *Streptococcus suis* Endocarditis: Echocardiographic Features and Clinical Outcome. Clin. Med. Insights. Cardiol. 6: 119-123.

Paramio, M.T., Manteca, X., Milán, M.J., Piedrafita, J., Izquierod M.D., Gasa, J., Mateu, E., Pares R. 2012. Breve manual de aproximación a la empresa porcina para estudiantes de veterinaria. *Manejo y Producción de Porcino*. Barcelona, Barcelona, España: UAB

Parra, A., Fernández-Llario, P., Tato, A., Larrasa, J., García, A., Alonso, J.M., Hermoso de Mendoza, M., Hermoso de Mendoza, J. 2003. Epidemiology of *Mycobacterium bovis* infection of pigs and wild boars using a molecular approach. Vet. Microbiol. 97: 122-133.

Parra, A., García, N., García, A., Lacombe, A., Moreno, F., Freire, F., Moran, J., Hermoso de Mendoza, J. 2008. Development of a molecular diagnostic test applied to experimental abattoir surveillance on bovine tuberculosis. Vet. Microbiol. 127: 315-324.

Pate, M., Zdovc, I., Pirs, T., Krt, B., Ocepek, M. 2004. Isolation and characterization of *Mycobacterium avium* and *Rhodococcus equi* from granulomatous lesions of swine lymph nodes in Slovenia. Acta. Vet. Hung. 52: 143-150.

Pavlik, I., Matlova, L., Dvorska, L., Bart, J., Oktabcova, L., Docekal, J., Parmova, I. 2003. Tuberculous lesions in pigs in the Czech Republic during 1990-1999: occurrence, causal factors and economic losses. Vet. Med.-Czech. 48: 113-125.

Pearson, S.D., Drewe, J.A., Gey van Pittius, N.C., Warren, R.M., van Helden, P.D. 2013. Novel cause of tuberculosis in meerkats, South Africa. Emerg. Infect. Dis.19: 2004-2007.

Peinado, J., Castillejo, J., Antón Muñoz, F., Redondo, J.L., Toledano, P., Alcalde, J.L., Sánchez, M., Prieto, J.L., Carrasco, L., Méndez, A., Gómez, R., Amaro, M., Ángel Sierra, M., Perea, A. 2006. Criterios para el dictamen en la inspección post mortem ante la presentación de linfadenitis crónicas (purulenta/caseosa) en cerdo. Junta de Andalucía. Consejería de Salud. Pag. 1-10.

Pérez de Val, B., Grau-Roma, L., Segalés, J., Domingo, M., Vidal, E. 2013. Mycobacteriosis outbreak caused by *Mycobacterium avium* subsp. *Avium* detected through meat inspection in five porcine fattening farms. Vet. Rec. 174: 96. doi: 10.1136/vr.101886

Pesciaroli, M., Russo, M., Mazzone, P., Aronica, V., Fiasconaro, M., Boniotti, M.B., Corneli, S., Cagiola, M., Pacciarini, M., Di Marco, V., Pasquali, P. 2012. Evaluation of the interferon-gamma (IFN-γ) assay to diagnose Mycobacterium bovis infection in pigs. Vet. Immunol. Immunopathol. 148:369-372.

Philip, V.Y.T., Denoel, J., Cassart, D., Govaerts, M., Czaplicki, G., Saegerman, C., Laitat, M. 2013. Prevalence of submandibular granulomatous lymphadenitis in pigs slaughtered in Belgium. Journées Recherche Porcine. 45: 277-278.

Quinn, P.J., Markey, B.K., Leonard, F.C., Fitzpatrick, E.S., Fanning, S., Hartigan, P.J. 2011. Veterinary microbiology and microbial disease, 2nd Edition. UK: Wiley-Blackwell: 245-257.

Ramis Vidal, G., Pallarés Martínez, F.J. 2009. Caso Clínico: Diferenciación de lesiones tuberculosas y piogranulomatosas en cerdo ibérico. Anaporc. 57: 40-41.

Reynolds, A.E., Harrison, M.A., Rose-Morrow, R., Lyon, C.E., 2001. Validation of Hams Cured Dry Process for Control of Pathogens. J. Food. Sci. 66, 1373-1379.

Richomme, C., Boadella, M., Courcoul, A., Durand, B., Drapeau, A., Corde, Y., Hars, J., Payne, A., Fediaevsky, A., Boschirol, M.L. 2013. Exposure of wild boar to *Mycobacterium tuberculosis* complex in France since 2000 is consistent with the distribution of bovine tuberculosis outbreaks in cattle. PloS One 8, e77842.

Risco, D., Serrano, E., Fernández-Llario, P., Cuesta, J.M., Gonçalves, P., GarcíaJiménez, W.L., Martínez, R., Cerrato, R., Velarde, R., Gómez, L., Segalés, J., Hermoso de Mendoza, J. 2014. Severity of bovine tuberculosis is associated with co-infection with common pathogens in wild boar. PloS One. 9 (10): e110123.

Rodríguez-Campos, S., Smith, N.H., Boniotti, M.B., Aranaz, A. 2014. Overview and phylogeny of Mycobacterium tuberculosis complex organisms: Implications for diagnostics and legislation of bovine tuberculosis. Res. Vet. Sci. 97: S5-S19.

Santos, N., Geraldes, M., Afonso, A., Almeida, V., Correia-Neves, M., 2010. Diagnosis of tuberculosis in the wild boar (*Sus scrofa*): A comparison of methods applicable to hunter-harvested animals. PLoS One. 5: e12663. doi: 10.1186/s13567-015-0270-4.

Santos, N., Almeida, V., Gortázar, C., Correia-Neves, M. 2015a. Patterns of Mycobacterium tuberculosis-complex excretion and characterization of super shedders in naturally-infected wild boar and red deer. Vet. Res. 46: 129.

Santos, N., Santos, C., Valente, T., Gortázar, C., Almeida, V., Correia-Neves, M. 2015b. Widespread environmental contamination with *Mycobacterium tuberculosis* complex revealed by a molecular detection protocol. PLoS One 10: e0142079.

Shitaye, J.E., Parmova, I., Matlova1, L., Dvorska1, L., Horvathova1, A., Vrbas1, V., Pavlik, I. 2006. Mycobacterial and *Rhodococcus equi* infections in pigs in the Czech Republic between the years 1996 and 2004: the causal factors and distribution of infections in the tissues. Vet. Med.-Czech. 51: 497-511.

Silva, L.G., Genteluci, G.L., Correa de Mattos, M., Glatthardt, T., Sa Figueiredo, A.M., Ferreira-Carvalho, B.T., 2015. Group C *Streptococcus dysgalactiae subsp. equisimilis* in south-east Brazil: genetic diversity, resistance profile and the first report of human and equine isolates belonging to the same multilocus sequence typing lineage. J. Med. Microbiol. 64, 551-558.

Spittel, S., Hoedemaker, M. 2012. Mastitis diagnosis in dairy cows using PathoProof real-time polymerase chain reaction assay in comparison with conventional bacterial culture in a Northern German field study. Berl. Munch. Tierarztl. Wochenschr. 125 (11-12): 494-502.

Stefánska, I., Witkowski, L., Rzewuska, M., Dzieciatkowski, T. 2016. Development and evaluation of the internal-controlled real-time PCR assay for *Rhodococcus equi* detection in various clinical specimens. J. Vet. Med. Sci. 78: 543-549.

Stewart, L.D., McNair, J., McCallan, L., Gordon, A., Grant, I.R. 2013. Improved detection of *Mycobacterium bovis* infection in bovine lymph node tissue using immunomagnetic separation (IMS)-based methods. PLoS. One. 8(3): e58374.

Stollewerk, K., Jofré, A., Comaposada, J., Arnau, J., Garriga, M., 2012. The effect of NaCl-free processing and high pressures on the fate of *Listeria monocytogenes* and *Salmonella* on sliced smoked dry-cured ham. Meat. Sci. 90, 472-477.

Thoen, C., LoBue, P., de Kantor, I. 2006. The importance of *Mycobacterium bovis* as a zoonosis. Vet. Microbiol. 112: 339-345.

Tirkkonen, T., Pakarinen, J., Rintala, E., Ali-Vehmas, T., Marttila, H., Peltoniemi, O.A., Mäkinen, J. 2010. Comparison of variable-number tandem-repeat markers typing and IS1245 restriction fragment length polymorphism fingerprinting of *Mycobacterium avium* subsp. *hominissuis* from human and porcine origins. Acta. Vet. Scand. 52:21.

Velasco, C.E. 2006. El cerdo criollo colombiano. Presente y Futuro. Mundo Ganadero. 186: 60-64.

Ventanas, J., 2001. Tecnología del jamón Ibérico: de los sistemas tradicionales a la explotación racional del sabor y el aroma. Ediciones Mundi-Prensa, Madrid, Spain, 423–437.

Verjan García, N., Almario Leiva, G.A., Romero Jola, N.J., Fandiño, L.C. 2015. Múltiples abscesos en un cerdo causados por *Trueperella pyogenes* (*Arcanobacterium pyogenes*): reporte de un caso. Rev. Mex. Cienc. Pecu. 6 (1): 91-98.

Whiley, R. A., Hardie, J. M. 2015. *Streptococcus*. Bergey's Manual of Systematics of Archaea and Bacteria. 1–86; doi: 10.1002/9781118960608.gbm00612.

Wangoo, A., Johnson, L., Gough, J., Ackbar, R., Inglut, S., Hicks, D., Spencer, Y., Hewinson, G., Vordermeier, M. 2005. Advanced granulomatous lesions in *Mycobacterium bovis*-infected cattle are associated with increased expression of type I procollagen, gammadelta (WC1+) T cells and CD 68+ cells. J. Comp. Pathol. 133: 223-234.

Weinstock, D.M., Brown, A.E. 2002. *Rhodococcus equi*: an emerging pathogen. Clin. Infec. Dis. 34: 1379-1385.

Zanella, G., Duvaduchelle, A., Hars, J., Mutou, F., Boschiroli, M.L., Durand, B. 2008. Patterns of lesions of bovine tuberculosis in wild red deer and wild boar. Vet. Rec. 163: 43-47.

Capítulo II/ Chapter II

Objetivos/Objectives

Objetivo 1: Evaluar técnicas de diagnóstico rápido para la detección de la tuberculosis en el cerdo en extensivo.

Objetivo 1a. Evaluar el uso de una PCR dúplex en tiempo real y un ELISA indirecto para el diagnóstico rápido de la tuberculosis en cerdos criados en extensivo.

Estudio 1: "*Evaluation of rapid methods for diagnosis of tuberculosis in slaughtered free-range pigs*" (Cardoso-Toset et al., 2015, The Veterinary Journal. 204, 232-234).

Objetivo 1b. Evaluar cinco ensayos serológicos basados en distintos antígenos para la detección de anticuerpos específicos frente al complejo *Mycobacterium tuberculosis* en cerdos criados en extensivo.

Estudio 2*: "Evaluation of five serologic assays for bovine tuberculosis surveillance in domestic free-range pigs from southern Spain"* (Cardoso-Toset et al., 2016, Preventive Veterinary Medicine, en revisión).

Objetivo 2: Caracterización histopatológica y microbiológica de las lesiones compatibles con tuberculosis de cerdos criados en extensivo sacrificados en el matadero.

Estudio 3: "*Multi-etiological nature of tuberculosis-like lesions in condemned pigs at the slaughterhouse*" (Cardoso-Toset et al., 2015, PLoS One 10, e0139130).

Objetivo 3: Evaluar la viabilidad de *Streptococcus suis*, *Streptococcus dysgalactiae* y *Trueperella pyogenes* durante el proceso de curación de productos del cerdo Ibérico (paletas y lomos) inoculados de forma experimental.

Estudio 4 *"Survival of Streptococcus suis, Streptococcus dysgalactiae and Trueperella pyogenes in dry-cured Iberian pork shoulders and loins"* (Cardoso-Toset et al., 2017, Food Microbiology, 61, 66-71).

Objective 1: Evaluation of rapid methods for the diagnosis of tuberculosis in free-range pigs.

Objective 1a. To evaluate a duplex real-time PCR and an antibody ELISA as rapid diagnostic tools for tuberculosis in slaughtered free-range pigs.

Study 1: "*Evaluation of rapid methods for diagnosis of tuberculosis in slaughtered free-range pigs*" (Cardoso-Toset et al., 2015, The Veterinary Journal. 204, 232-234)

Objective 1b. To evaluate the diagnostic performance of five commercial and in-house serological assays based on different *M. bovis* antigens for detecting antibodies against *Mycobacterium tuberculosis* complex in free-range pigs.

Study 2: *"Evaluation of five serologic assays for bovine tuberculosis surveillance in domestic free-range pigs from southern Spain"* (Cardoso-Toset et al., 2016, Preventive Veterinary Medicine, under review).

Objective 2: Histopathological and microbiological characterization of tuberculosis-like lesions in free-range pigs at slaughterhouse.

Study 3: "*Multi-etiological nature of tuberculosis-like lesions in condemned pigs at the slaughterhouse*" (Cardoso-Toset et al., 2015, PLoS One 10, e0139130).

Objective 3: To evaluate the viability of *Streptococcus suis, Streptococcus dysgalactiae* and *Trueperella pyogenes* along the dry curing process of experimentally inoculated Iberian pork shoulders and loins.

Study 4: *"Survival of Streptococcus suis, Streptococcus dysgalactiae and Trueperella pyogenes in dry-cured Iberian pork shoulders and loins"* (Cardoso-Toset et al., 2017, Food Microbiology, 61, 66-71).

Capítulo III/ Chapter III

Estudios/Studies

Estudio 1/Study 1

Objetivo 1a/Objective 1a

Evaluation of rapid methods for diagnosis of tuberculosis in slaughtered free-range pigs.

F. Cardoso-Toset., I. Luque., S.P. Amarilla., L. Gómez-Gascón., L. Fernández., B. Huerta., L. Carrasco., P. Ruiz., J. Gómez-Laguna.

The Veterinary Journal (2015) 204, 232-234.

Short Communication

Evaluation of rapid methods for diagnosis of tuberculosis in slaughtered free-range pigs.

Fernando Cardoso-Toset [a,b], Inmaculada Luque [a], Shyrley Paola Amarilla [c], Lidia Gómez-Gascón [a], Layla Fernández [b], Belén Huerta [a], Librado Carrasco [c], Pilar Ruiz [d], Jaime Gómez-Laguna [b]

[a] *Department of Animal Health, University of Córdoba, International Excellence Agrifood Campus 'CeiA3', University Campus of Rabanales, 14071 Córdoba, Spain*

[b] *CICAP - Food Research Centre, 14400 Pozoblanco, Córdoba, Spain*

[c] *Department of Anatomy and Comparative Pathology, University of Córdoba, International Excellence Agrifood Campus 'CeiA3', University Campus of Rabanales, 14071 Córdoba, Spain*

[d] *Microbiology service, Reina Sofia University Hospital, Mycobacteria Reference Center, Avd. Méndez Pidal s/n, 14004 Córdoba, Spain*

Abstract

Free-range pigs can be infected by *Mycobacterium tuberculosis* complex (MTC) and may contribute to the spread of bovine tuberculosis (bTB). In the present study, the diagnostic values of bacteriological culture, a duplex real-time quantitative PCR and an antibody ELISA were evaluated in an abattoir study of submandibular lymph nodes and serum samples from 73 pigs with and without lesions consistent with bTB. The duplex qPCR was an accurate method for diagnosis of TB in pigs (specificity 100%; sensitivity 80%). Combining qPCR with histopathology improved sensitivity and had very good concordance ($\kappa = 0.94$) with the reference method. Serological results suggest that the antibody ELISA can be used for monitoring herds but not individuals.

Keywords: Tuberculosis; Pigs; Diagnosis; Real-time quantitative PCR; ELISA

In the Iberian Peninsula, wild boar (*Sus scrofa*) are a reservoir for *Mycobacterium bovis*, the cause of bovine tuberculosis (bTB), along with other members of the *Mycobacterium tuberculosis* complex (MTC) (Parra et al., 2003; Santos et al., 2010). Post-mortem diagnostic tests for TB in livestock include gross pathology, histopathology for detection of tuberculosis-like lesions (TBL) or acid-fast bacilli (AFB) by Ziehl-Neelsen (ZN) staining, bacteriology and PCR (Santos et al., 2010).

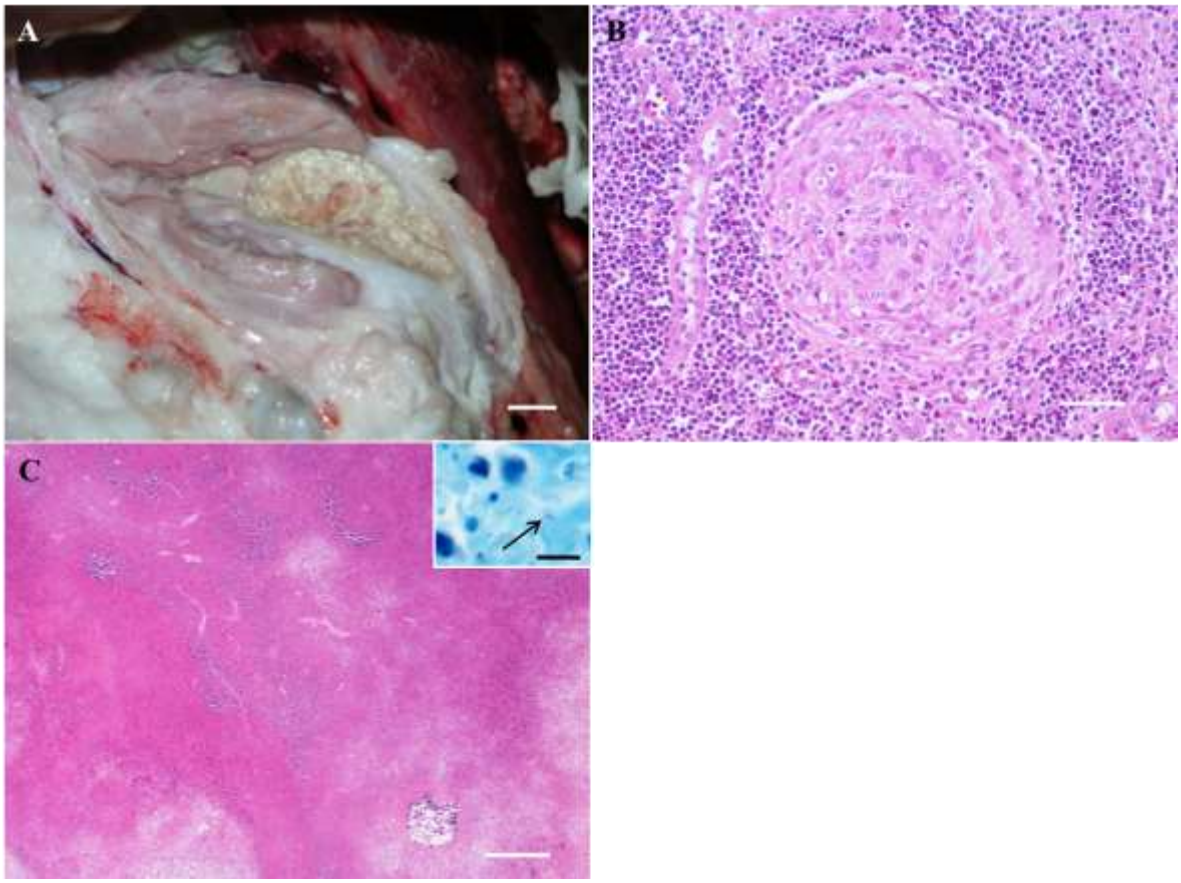
Although bacteriology is considered to be the gold standard for TB confirmation, this technique is time consuming and may produce false negative results (Boadella et al., 2011; Corner et al., 2012). Antibody ELISAs have been used to complement the diagnosis of TB in wild boar (Aurtenetxe et al., 2008; Richomme et al., 2013). The aim of the present study was to evaluate quantitative real-time PCR (qPCR) and an antibody ELISA as diagnostic tools for TB in slaughtered free-range pigs in relation to histopathology and culture.

Submandibular lymph nodes, the most frequently affected site in cases of *M. bovis* infection in pigs (Di Marco et al., 2012), were collected at an abattoir from 100 free-range pigs > 14 months of age without clinical signs raised on Southern Spanish farms with a history of condemnation due to TBL. Pigs were divided into animals with TBL and animals with no visible lesions on gross examination (Di Marco et al., 2012).

Blood samples were collected into plain tubes, allowed to clot and the serum was harvested and stored at -70 °C until testing. Serum samples were tested by means of an indirect ELISA to detect specific antibodies against bovine tuberculin purified protein derivative (bPPD) of *M. bovis* (TB ELISA-VK; Vacunek S.L.)

The presence of epithelioid cells and multinucleated giant (MNG) cells, in the absence of foreign bodies or fungal structures, was considered to be indicative of TB in

routine histological sections stained with haematoxylin and eosin (histopathology I) and the presence of AFB was recorded (histopathology II) (see Appendix: Supplementary Fig. 1).



Supplementary Fig. 1 (A). Granulomatous inflammation of the submandibular lymph node of an affected pig. Bar = 1.3 cm.**Fig. 1 (B)** Submandibular lymph node. Typical tuberculous granuloma made up of numerous epithelioid cells, multinucleated giant cells (*), interspersed lymphocytes and a thin, poorly defined, connective tissue capsule. Haematoxylin and eosin staining. Bar = 30 µm. **(C)** Submandibular lymph node. Extensive area of necrosis with multifocal areas of mineralisation. Haematoxylin and eosin staining. Bar = 300 µm. Inset: Acid-fast bacilli (arrow) identified within a focus of necrosis. Ziehl-Neelsen staining. Bar = 10 µm.

Samples were decontaminated with 0.75% hexa-decyl-pyridinium chloride (Sigma Aldrich) and inoculated in Lowënstein-Jensen medium with pyruvate (Oxoid) (Corner et al., 2012). Colonies consistent with MTC were identified by a multiplex PCR assay based on a MTC-specific 23S ribosomal DNA fragment, gyrB DNA sequence polymorphisms and the RD1 deletion of *M. bovis* BCG (GenoType MTBC, Hain Lifescience) (Richter et al., 2004).

DNA was extracted from 25 mg homogenised tissue from each sample (NucleoSpin Tissue, Macherey-Nagel). A duplex qPCR for MTC and *Mycobacterium avium* complex (MAC) was performed as described by Gómez-Laguna et al. (2010), except that the DNA template was diluted 1:10 in nuclease free-water. All reactions were run in duplicate.

TB positive cases (PC) were defined as animals with TBL and positive MTC isolation, while TB negative cases (NC) were defined as animals with no visible lesions and negative MTC isolation (Aurtenetxe et al., 2008). Sensitivity (Se), specificity (Sp) and 95% confidence intervals (CI_{95}) were assessed using the software WinEpi 2.0[1]. Inter-rate agreement between the different diagnostic methods was calculated by means of Cohen's κ coefficient (GraphPad Software). A combination of tests was also evaluated.

Seventy-three animals matched one of two case definitions and were included in the study; 46/73 were classified as PC and 27/73 were classified as NC. Only *M. bovis* was detected. MTC was detected by duplex qPCR in 40/73 cases; 10/46 samples from PC animals were negative in the duplex qPCR. MTC DNA was amplified from 4/27 NC pigs. AFB or consistent TBL were detected histologically in these samples (see Appendix: Supplementary Table 1).

Supplementary Table 1. Diagnostic tests profiles for animals with uncertain results in some of the diagnostic tests.

Sample ID	Gross lesions + Bacteriology	Histopathology I[a]	Histopathology II[b]	qPCR[c]	ELISA
ID-87	-	+	+	+	+
ID-89	-	-	+	+	-
ID-94	-	+	+	+	+
ID-103	-	+	+	+	-

[a] Histopathology I, animals with granulomas, with presence of epithelioid cells or multinucleated giant (MNG) cells, in the absence of foreign bodies or fungal structures.

[b] Histopathology II, animals with acid-fast bacilli (AFB) detected by Ziehl-Neelsen (ZN) staining.

[c] qPCR, quantitative (real-time) PCR.

[1] See: http://www.winepi.net/

These cases were considered false negative bacteriology results but true positive TB cases by means of qPCR and histopathology. All samples were negative for MAC by both culture and qPCR. In the ELISA, 34/46 (74%) PC and 7/27 (26%) NC had *M. bovis*-specific antibodies. Se and Sp, along with 95% confidence intervals (95% CI) and concordance values, are summarised in Table 1.

Table 1. Estimates of sensitivity and specificity with 95% confidence intervals (95% CI) and concordance values for each diagnostic test with criteria for positive cases based on gross lesions, bacterial culture and PCR.

		Sensitivity		Specificity		Concordance	
Diagnostic tests	Positive/tested samples [a]	%	95% CI	%	95% CI	κ	Agreement
ELISA	41/73	73.9	61.2-86.6	74.1	57.5-90.6	0.46	Moderate
Histopathology I [b]	38/73	73.9	61.2-86.6	85.2	71.8-98.6	0.56	Moderate
Histopathology II [c]	43/73	80.4	69-91.9	77.8	62.1-93.5	0.57	Moderate
qPCR [d]	40/73	77.3	66.3-90.2	85.2	71.8-98.6	0.67	Good
qPCR + Histopathology I	49/73	97.8	93.6-100	85.2	71.8-98.6	0.87	Very good
qPCR + Histopathology II	51/73	97.9	93.7-100	77.8	62.1-93.5	0.78	Good

[a] Tuberculosis positive cases, pigs with compatible gross lesions at post-mortem inspection and *Mycobacterium tuberculosis* complex (MTC) identification by bacterial culture and PCR confirmation.

[b] Histopathology I, animals with granulomas, with presence of epithelioid cells or multinucleated giant (MNG) cells, in the absence of foreign bodies or fungal structures.

[c] Histopathology II, animals with acid-fast bacilli (AFB) detected by Ziehl-Neelsen (ZN) staining.

[d] qPCR, quantitative (real-time) PCR.

In the light of previous studies (Gómez-Laguna et al., 2010; Santos et al., 2010; Corner et al., 2012) and because qPCR showed four false negative bacteriology results, a new criterion was established to describe TB positive cases (Table 2). Use of the duplex qPCR had 100% Sp, 80% Se (95% CI 69-91%) and a good concordance with the case definitions ($\kappa = 0.72$). In comparison, PCR on tissue homogenates from wild boar had a lower Se (67%, 95% CI 41-86%) and a similar Sp (100%, 95% CI 95-100%) to the present study (Santos et al., 2010).

Table 2. Estimates of sensitivity and specificity with 95% confidence intervals (95% CI) and concordance values for each diagnostic test with criteria for positive cases based on gross lesions, bacterial culture and PCR or qPCR.

		Sensitivity		Specificity		Concordance	
Diagnostic tests	Positive/tested samples [a]	%	95% CI	%	95% CI	κ	Agreement
ELISA	41/73	72	59.6-84.4	78.3	61.4-95.1	0.46	Moderate
Histopathology I [b]	38/73	74	61.8-86.2	95.7	87.3-100	0.61	Good
Histopathology II [c]	43/73	82	71.4-92.6	91.3	79.8-100	0.68	Good
qPCR [d]	40/73	80	68.9-91.1	100	-	0.72	Good
qPCR + Histopathology I	50/73	98	94.1-100	95.7	87.3-100	0.94	Very good
qPCR + Histopathology II	52/73	100	-	91.3	79.8-100	0.93	Very good

[a] Tuberculosis positive cases, pigs with compatible gross lesions at post-mortem inspection and *Mycobacterium tuberculosis* complex (MTC) identification by bacterial culture and PCR confirmation or qPCR genome amplification from tissue.

[b] Histopathology I, animals with granulomas, with presence of epithelioid cells or multinucleated giant (MNG) cells, in the absence of foreign bodies or fungal structures.

[c] Histopathology II, animals with acid-fast bacilli (AFB) detected by Ziehl-Neelsen (ZN) staining.

[d] qPCR, quantitative (real-time) PCR.

Porcine tuberculosis is characterised by paucibacillary lesions, which result in low levels of extraction of mycobacterial DNA (Santos et al., 2010). This could explain the lack of successful mycobacterial DNA amplification from 10/46 PC samples in the present study. Combining qPCR and histopathology improved Se (98-100%), while maintaining good Sp (96-91%) and concordance (κ = 0.93-0.94) with respect to the second criterion established for a TB positive case (Table 2).

Serodiagnosis has been proposed for large scale and individual TB testing of wild boar (Boadella et al., 2011; Richomme et al., 2013). In the present study, Se was similar, but Sp was lower than reported by Aurtenetxe et al. (2008) (Table 2). Although our results do not support the use of the antibody ELISA for diagnosis of TB in individual pigs, it could be a valuable tool for the monitoring the TB status of domestic pigs at the herd level.

The results of this study suggest that the duplex qPCR is an accurate method for diagnosis of TB in slaughtered free-range pigs when compared with bacteriology as the reference method. Future efforts should focus on improving Se, while maintaining high Sp. Combining qPCR with histopathology resulted in high diagnostic accuracy.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This study was financially supported by the Council of Economy, Science, Innovation and Employment of the Andalusian Government (AGR-2685-2012) and by the Centre for the Development of Industrial Technology (CDTI) of Spain (project reference IDI-20111633). Cardoso-Toset F was funded by a grant of the Agrifood Campus of International Excellence Programme (ceiA3) from the Ministry of Education, Culture and Sport and by the Santander Universities Global Division.

References

Aurtenetxe, O., Barral, M., Vicente, J., De la Fuente, J., Gortázar, C., Juste, R.A., 2008. Development and validation of an enzyme-linked immunosorbent assay for antibodies against *Mycobacterium bovis* in European wild boar. BMC Veterinary Research 43, 1-9.

Boadella, M., Lyashchenko, K., Greenwald, R., Esfandiari, J., Jaroso, R., Carta, T., Garrido, J.M., Vicente, J., De la Fuente, J., Gortázar, C., 2011. Serologic tests for detecting antibodies against *Mycobacterium bovis* and *Mycobacterium avium subspecies paratuberculosis* in Eurasian wild boar (*Sus scrofa scrofa*). Journal of Veterinary Diagnostic Investigation 23, 77-83.

Corner, L.A.L., Gormley, E., Pfeiffer, D.U., 2012. Primary isolation of *Mycobacterium bovis* from bovine tissues: Conditions for maximizing the number of positive cultures. Veterinary Microbiology 156, 162-171.

Di Marco, V., Mazzone, P., Capucchio, M.T., Boniotti, M.B., Aronica, V., Russo, M., Fiasconaro, M., Cifani, N., Corneli, S., Biasibetti, E., et al., 2012. Epidemiological significance of the

domestic black pig (*Sus scrofa*) in maintenance of bovine tuberculosis in Sicily. Journal of Clinical Microbiology 50, 1209-1218.

Gómez-Laguna, J., Carrasco, L., Ramis, G., Quereda, J.J., Gómez, S., Pallarés, F.J., 2010. Use of real-time and classic polymerase chain reaction assays for the diagnosis of porcine tuberculosis in formalin-fixed, paraffin-embedded tissues. Journal of Veterinary Diagnostic Investigation 22, 123-127.

Parra, A., Fernández-Llario, P., Tato, A., Larrasa, J., García, A., Alonso, J.M., Hermoso de Mendoza, M., Hermoso de Mendoza, J., 2003. Epidemiology of *Mycobacterium bovis* infection of pigs and wild boars using a molecular approach. Veterinary Microbiology 97, 122-133.

Richomme, C., Boadella, M., Courcoul, A., Durand, B., Drapeau, A., Corde, Y., Hars, J., Payne, A., Fediaevsky, A., Boschirol, M.L., 2013. Exposure of wild boar to *Mycobacterium tuberculosis* complex in France since 2000 is consistent with the distribution of bovine tuberculosis outbreaks in cattle. PloS One 8, e77842.

Richter, E., Weizenegger M., Fahr, A.M., Rüsch-Gerdes, S., 2004. Usefulness of the Geno Type MTBC assay for differentiating species of the *Mycobacterium tuberculosis* complex in cultures obtained from clinical specimens. Journal of Clinical Microbiology 42, 4303-4306.

Santos, N., Geraldes, M., Afonso, A., Almeida, V., Correia-Neves, M., 2010. Diagnosis of tuberculosis in the wild boar (*Sus scrofa*): A comparison of methods applicable to hunter-harvested animals. PLoS One 5, e12663.

Estudio 2/Study 2

Objetivo 1b/Objective 1b

Evaluation of five serologic assays for bovine tuberculosis surveillance in domestic free-range pigs from southern Spain

F. Cardoso-Toset., I. Luque., L. Carrasco., F. Jurado-Martos., M.A. Risalde., A. Venteo., J.A. Infantes Lorenzo., J. Bezos., P. Rueda., I. Tapia., C. Gortázar., L. Domínguez., M. Domínguez., J. Gómez-Laguna.

Preventive Veterinary Medicine (2016), under review.

Evaluation of five serologic assays for bovine tuberculosis surveillance in domestic free-range pigs from southern Spain.

Fernando Cardoso-Toset[a,b], Inmaculada Luque[b], Librado Carrasco[c], Francisco Jurado-Martos[a,b], María Ángeles Risalde[d], Ángel Venteo[e], José A Infantes-Lorenzo[f,g], Javier Bezos[f], Paloma Rueda[e], Istar Tapia[e], Christian Gortázar[d], Lucas Domínguez[f], Mercedes Domínguez[g], Jaime Gomez-Laguna[a,c]

[a] *CICAP – Agrifood Research Centre, 14400, Pozoblanco, Córdoba, Spain.*

[b] *Animal Health Department, University of Córdoba, International Excellence Agrifood Campus 'CeiA3', 14071, Córdoba, Spain.*

[c] *Department of Anatomy and Comparative Pathology, University of Córdoba, International Excellence Agrifood Campus 'CeiA3', 14071 Córdoba, Spain.*

[d] *Instituto de Investigación en Recursos Cinegéticos, IREC, (CSIC-UCLM-JCCM) Ronda de Toledo s/n, 13071 Ciudad Real, Spain.*

[e] *Inmunología y Genética Aplicada, S.A. (INGENASA), Calle Hermanos García Noblejas 39, 28037 Madrid, Spain.*

[f] *VISAVET Health Surveillance Centre, Faculty of Veterinary Medicine, Complutense University, 28040 Madrid, Spain.*

[g] *Servicio de Inmunología Microbiana, Centro Nacional de Microbiología, Instituto de Investigación Carlos III, Madrid 28220, Spain*

Abstract

In countries where bovine tuberculosis (bTB) is still prevalent the contact among different animal species in extensive systems contributes to the circulation of *Mycobacterium bovis* (*M. bovis*) and other members of the *Mycobacterium tuberculosis* complex (MTC). Thus, free-range pigs can develop subclinical infections and may contribute to disease spread to bovine and wildlife. Serodiagnosis has been proposed as a screening tool for detecting infected pig herds; however, the value of this method to obtain an accurate diagnosis in this species is still not clear. In this study, sensitivity (Se) and specificity (Sp) estimates of four ELISAs and a lateral flow immunochromatographic antibody assay based on different *M. bovis* antigens, including MPB70 and MPB83 proteins were evaluated in naturally infected

domestic free-range pigs. For this purpose, submandibular lymph nodes and blood samples from 217 pigs from both TB-infected and historically negative farms were sampled at slaughterhouse and analysed by gross examination, histopathology, bacteriological culture and qPCR. Se and Sp estimates of the 5 evaluated assays ranged from 66.1% to 78% (CI_{95} from 54% to 88.5%) and from 98.9% to 100% (CI_{95} from 96.7 to 100%), respectively. Results of our study suggest that all the evaluated assays could be used as a first screening tool to conduct bTB surveillance in domestic pigs at population level. However, animals from seropositive herds should later be surveyed by other methods to reduce false negative results.

Keywords: serologic assays, MTC, tuberculosis-like lesions, pigs, bPPD, MPB83

Introduction

Mycobacterial infections including those caused by *Mycobacterium bovis* (*M. bovis*) and closely related members of the *Mycobacterium tuberculosis* complex (MTC) cause bovine tuberculosis (bTB) with important implications for veterinary and public health (García-Bocanegra et al., 2012). In countries where bTB is still prevalent or is re-emerging the contact among different animal species in extensive systems contributes to the spread of this disease. Recent studies have shown that domestic free-range pigs raised in territories where *M. bovis* is endemic in free-roaming cattle or wildlife can develop tuberculosis-like lesions (TBL) and may act as a reservoir of MTC (Cardoso-Toset et al., 2015b). In fact, the role of free-range pigs as a useful sentinel of bTB has been suggested (Bailey et al., 2013).

Faster and simple detection methods would be critical for monitoring the effects of bTB control measures in domestic pigs. Serodiagnosis based on enzyme-linked immunosorbent assays (ELISAs) and lateral-flow (LF) tests has been proposed as a useful

screening tool for detecting infected swine herds (Lyashchenko et al., 2008; Che'Amat et al., 2015). Due to a limited Se, the value of these assays to obtain an accurate diagnosis in naturally infected pigs is still not clear (Cardoso-Toset et al., 2015a). *M. bovis* purified protein derivative (bPPD) is the antigen most widely used for serodiagnosis of bTB (Bezos et al., 2014). However, assays based on different native or recombinant proteins, such as MPB70 or MPB83 have been developed to improve Se values (García-Bocanegra et al., 2012). Thus, the diagnostic performance of two commercial indirect ELISAs, two in-house indirect ELISAs and a prototype of LF device (LFD) based on different *M. bovis* antigens to detect antibodies against MTC in pigs were evaluated in this study.

Material and Methods

2.1. Animals, study design and sampling

Pigs sampled in this study were domestic free-range Iberian pigs (*Sus scrofa domesticus*) reared outdoors in sparse oak forests (*dehesa*) where they feed on acorns and grass and share natural resources with other wild and domestic animals, including cattle and wildlife. Submandibular lymph nodes (SLN) and blood samples of 217 apparently healthy animals over 14 month-old raised in extensive systems from 12 farms located in Southern Iberian Peninsula (Andalusia region in Spain) were obtained in a convenience sampling after routine inspection at slaughterhouse: to obtain sera from TB positive and negative animals, 129 out of these 217 animals were sampled from 8 farms with a history of condemnation due to TBL and 88 (88/217) animals were sampled from 4 historically TB-free farms. SLNs of each animal were analysed by gross examination, histopathology, bacteriological culture and qPCR to obtain known TB status sera (Cardoso-Toset et al., 2015a). Blood samples were collected into plain tubes, allowed to clot and the serum was harvested and stored at -70 ºC

until testing. Samples from 50 out of the 129 animals from TB positive farms used in this study were previously evaluated in Cardoso-Toset et al. (2015a).

2.2. Antibody detection tests

Serum samples were tested by means of two commercial ELISAs that use bPPD (TB ELISA-VK; Vacunek S.L, Derio, Spain) (bPPD1 ELISA) and recombinant MPB70 and MPB83 proteins (INgezim TB Porcine; Ingenasa, Madrid, Spain) as coating antigens (used in accordance with the manufacturer's instructions), and by means of two in-house indirect ELISA tests based on bPPD (bPPD2 ELISA) and treated bPPD (t-bPPD) (Che' Amat et al., 2015). In addition, a recently developed prototype of coloured latex-based immunochromatographic LFD (INgezim TB-CROM; Ingenasa, Madrid, Spain), coated with MPB83 protein, was also evaluated. All tests were carried out blindly.

2.3. Data analysis

TB positive animals were defined as individuals with both TBL and MTC detection by means of culture or qPCR analysis and TB negative animals were defined as pigs without compatible lesions and negative MTC detection from farms without a previous history of TB. Since infection among exposed animals from TB-infected herds could not be completely ruled out, pigs negative to MTC from farms where *M. bovis* was isolated from other pigs were considered as uncertain status animals (Lyashchenko et al., 2011) and discarded. Se and Sp estimates with 95% confidence intervals (CI_{95}) (WinEpi v.2.0, Zaragoza, Spain) and area under curve (AUC; based on ROC curve analysis) (MedCalc statistical, v.16.8, Ostend, Belgium) of the 5 serologic assays were assessed using sera from TB positive and negative animals and MTC detection by bacteriological culture or qPCR as reference standard (García-Bocanegra et al., 2012; Che'Amat et al., 2015). Inter-rater agreement between the

evaluated assays and the reference standards was calculated by means of Cohen's κ coefficient (Epidat v.3.0, Galicia, Spain).

Results

A total of 147 animals matched one out of two case definitions and were included in the analysis as follows: 59 out of 129 (45.7%) animals from TB positive farms were classified as TB positive animals and all the pigs sampled from farms with no TB history were confirmed as TB negative animals (88 animals). Sixty two out of 129 (48.1%) pigs from TB-positive farms were negative to MTC detection and were classified as uncertain status animals. Finally, MTC was detected in 8 animals without compatible lesions from TB-positive farms (8/129; 6.20%).

Regarding the serum antibody tests, Se and Sp, along with CI_{95} of the 5 evaluated assays are summarised in Table 1. Seropositivity in relation to TBL at slaughterhouse is showed in Table 2. Kappa values between the evaluated assays with CI_{95} are showed in Table3.

Table 1. Estimates of sensitivity and specificity with 95% confidence intervals (CI_{95}) of the five tests for the detection of serum antibodies against *Mycobacterium tuberculosis* complex (MTC), using MTC culture and qPCR as reference. A total of 147 animals were included within the description of positive (59) and negative (88) animals.

	Sensitivity		Specificity		AUC
Serologic assays	%	CI_{95}	%	CI_{95}	
MPB70+MPB83 ELISA (INgezim TB Porcine, INGENASA)	78	67.4-88.5	100	100-100	0,902
MPB83 LFD (INgezim TB-CROM, INGENASA)	74.6	63.5-85.7	98.9	96.7-100	-
t-bPPD in-house ELISA	71.2	59.6-82.7	100	100-100	0,785
bPPD1 ELISA (TB ELISA-VK; Vacunek S.L)	71.2	59.6-82.7	100	100-100	0,979
bPPD2 in-house ELISA	66.1	54-78.2	100	100-100	0,706

TB positive animals: pigs with compatible gross lesions at post-mortem inspection and MTC identification by means of bacteriological culture or qPCR genome amplification from tissue. TB negative animals: pigs from TB-free areas without compatible gross lesions at post-mortem inspection and absence of MTC by qPCR genome amplification from tissue.

Discussion

Tuberculosis due to *M. bovis* is one of the most important and oldest known diseases whose eradication is a problem because of the wide host range (Aurtenetxe et al., 2008). Rapid and sensitive techniques to diagnose infected animals are a priority to apply appropriate control measures in countries where bTB is still present in livestock (Che´Amat et al., 2015).

Post-mortem examination with bacteriological confirmation has been widely considered the gold standard for bTB diagnosis (García-Bocanegra et al., 2012; Che'Amat et al., 2015). In addition, qPCR has been proved as an accurate alternative for bTB confirmation when compared with bacteriology (Cardoso-Toset et al., 2015a). Nevertheless, sub-optimal Se values have been reported for both tests and must be taken into account when results of this study are evaluated (Boadella et al., 2011; Cardoso-Toset et al., 2015a). Latent class analysis has been applied as an alternative to determinate Se and Sp of diagnostic methods in the absence of a true standard (Courcoul et al., 2014). However, as recommended by consulted bibliography, culture and qPCR were used as reference standards in the present study.

All the evaluated assays showed a good agreement among them and with bacteriology or qPCR. In this sense, it should be noted that the stablished criteria to consider an animal as infected by MTC in this study (compatible lesions and MTC detection), could be associated with the selection of animals with and advanced stage of disease and could be responsible of an optimistic estimation of Se for all the evaluated assays. In contrast, TB infected pigs are often apparently healthy pigs (Cardoso-Toset., 2015b). For that reason, although pigs sampled in this study were slaughtered animals instead of pigs from field conditions, Se and

Sp estimates should not be influenced by the sampling procedure. Although serology has been suggested as a rapid and cost-effective screening tool to conduct TB surveillance in pigs at population level (Boadella et al., 2011), we consider that seropositive herds should later be surveyed combining other methods as pathology and bacteriology or qPCR (Richomme et al., 2013; Cardoso-Toset el a., 2015a) to reduce false negative results at individual level.

Variability of Se values has been also associated with the antigen used (Bezos et al., 2014). In this study, the use of the MPB70 and MPB83 based ELISA and the MPB83 based LFD provided the highest Se estimates. These results are similar to those obtained in a wide range of species (García-Bocanegra et al., 2012). This fact has been associated with a highest and early detection of antibodies by using the MPB83 protein (Bezos et al., 2014). Since the MPB83 based LFD is easy to perform, stable at room temperature and do not require high logistical demands, results of this study highly recommend its use when a moderate number of pigs is evaluated.

Although a slightly better Se was previously obtained using a bPPD based ELISA in naturally infected wild boar (Boadella et al., 2011), Se and Sp values obtained in our study using both commercial (bPPD1) and in-house (bPPD2) bPPD based tests are in agreement with values obtained by Aurtenetxe et al. (2008). The lower Sp obtained in our previous report (Cardoso-Toset et al., 2015a) was probably related with differences in the established criteria to classify pigs as TB negative animals. The results of both in-house indirect ELISAs (bPPD2 and t-bPPD) showed good agreement with MTC detection by bacteriological culture or qPCR with an increased Se with the use of t-bPPD in comparison to bPPD2.

Previous reports have hypothesized that production of antibodies against MTC in pigs

is closely related to lesion severity (Aurtenetexte et al., 2008; Lyashchenko et al., 2008). In this sense, antibodies against MTC were detected in the 67.53% to 76.62% of pigs showing TBL during meat inspection at slaughterhouse in this study. However, a reduced number of animals without gross compatible lesions showed antibodies against MTC. This result could be associated with infected animals with no developed lesions (early stage of disease), pigs with small or atypically located lesions missed during inspection (Bailey et al., 2013) or non-infected pigs exposed to MTC (Lyashchenko et al., 2011). Future studies focusing on the relationship between MTC infection and production of specific antibodies against this microorganism in pigs are required to better understand this finding.

Table 2. Seropositivity in relation to TB compatible lesions at slaughterhouse in domestic free-range pigs. A total of 129 animals from TB positive farms were included in the analysis and described as pigs with tuberculosis-like lesions at slaughterhouse (TBL; n=77) and pigs with no visible lesions (NVL; n=52).

	TBL [a]		NVL [b]	
Serologic assays	Nº	%	Nº	%
MPB70+MPB83 based ELISA (INgezim TB Porcine, INGENASA)	59/77	76.62	6/52	11.54
MPB83 based LFD (INgezim TB-CROM, INGENASA)	57/77	74.02	8/52	15.38
t-bPPD in-house ELISA	55/77	71.42	5/52	9.61
bPPD1 ELISA (TB ELISA-VK; Vacunek S.L)	55/77	71.42	15/52	28.85
bPPD2 in-house ELISA	52/77	67.53	5/52	9.61

[a]TBL: pigs with TBL detected during meat inspection at slaughterhouse. [b]NVL: pigs without visible compatible lesions during meat inspection at slaughterhouse.

In conclusion, our results support the use of antibody based diagnostic tests as a first screening tool to conduct bTB surveillance in domestic pigs at population level, showing the MPB83 based assays the highest Se estimates. Seropositive populations should be later surveyed by combining other methods to improve the detection of positive animals at individual level.

Table 3. Kappa (κ) values with 95% confidence intervals (CI_{95}) between the evaluated assays.

Test	MPB70+ MPB83-ELISA	MPB83-LFD	t-bPPD-ELISA	bPPD1-ELISA	bPPD2-ELISA	Bacteriology + qPCR
MPB70+MPB83-ELISA	-	0.97 (0.92-1)	0.87 (0.78-0.96)	0.84 (0.74-0.93)	0.85 (0.76-0.94)	0.81 (0.71-0.91)
MPB83-LFD	0.97 (0.92-1)	-	0.92 (0.85-0.99)	0.85 (0.76-0.94)	0.87 (0.78-0.96)	0.76 (0.66-0.87)
t-bPPD-ELISA	0.87 (0.78-0.96)	0.92 (0.85-0.99)	-	0.87 (0.78-0.96)	0.95 (0.89-1)	0.75 (0.64-0.86)
bPPD1-ELISA	0.84 (0.74-0.93)	0.85 (0.76-0.94)	0.87 (0.78-0.96)	-	0.85 (0.75-0.94)	0.75 (0.64-0.85)
bPPD2-ELISA	0.85 (0.76-0.94)	0.87 (0.78-0.96)	0.95 (0.89-1)	0.85 (0.75-0.94)	-	0.70 (0.58-081)
Bacteriology + qPCR	0.81 (0.71-0.91)	0.76 (0.66-0.87)	0.75 (0.64-0.86)	0.75 (0.64-0.85)	0.70 (0.58-0.81)	-

Poor agreement: κ < 0.20; fair agreement: κ = 0.21-0.40; moderate agreement: κ =0.41-0.60; good agreement: κ = 0.61-0.80; very good agreement: κ = 0.81-1.00. *P* value for all the κ estimates was <0.001.

Acknowledgements

This study was financially supported by the Andalusian Government (AGR-2685-2012) and by the European Project WILDTBVAC (FP7-KBBE-613799). Cardoso-Toset F is funded by a grant of the ceiA3 Campus from the Ministry of Education, Culture and Sport and by the Santander Universities Global Division. Gómez-Laguna J is supported by a "Ramón y Cajal" contract of the Spanish Ministry of Economy and Competitiveness. Authors would like to acknowledge the contribution of Sofia Costa and Alba Fresco to the development of the LFD prototype.

References

Aurtenetxe, O., Barral, M., Vicente, J., de la Fuente, J., Gortázar, C., A Juste, R., 2008. Development and validation of an enzyme-linked immunosorbent assay for antibodies against *Mycobacterium bovis* in european wild boar. BMC Vet. Res. 43, 1-

Bailey, S.S., Crawshaw, T.R., Smith, N.H., Palgrave, C.J., 2013.*Mycobacterium bovis* infection in domestic pigs in Great Britain. Vet. J. 198, 391-397.

Bezos, J., Casal, C., Romero, B., Schroeder, B., Hardeqqer, R., Raeber, A.J., López, L., Rueda, P., Domínguez, L., 2014. Current ante-mortem techniques for diagnosis of bovine tuberculosis. Res. Vet. Sci. 97, S44-S52.

Boadella, M., Lyashchenko, K., Greenwald, R., Esfandiari, J., Jaroso, R., Carta, T., Garrido, J.M., Vicente, J., de la Fuente, J., Gortázar, C., 2011. Serologic test for Detecting Antibodies against *Mycobacterium bovis* and *Mycobacterium avium* subspecies paratuberculosis in Eurasian wild boar (*Sus scrofa scrofa*). J. Vet. Diagn. Invest, 23, 77-83.

Cardoso-Toset, F., Luque, I., Amarilla, S.P., Gómez-Gascón, L., Fernández, L., Huerta, B., Carrasco, L., Ruiz, P., Gómez-Laguna, J., 2015a. Evaluation of rapid methods for diagnosis of tuberculosis in slaughtered free-range pigs. Vet. J. 204, 232-234.

Cardoso-Toset, F., Gómez-Laguna J., Amarilla, S.P., Vela, A.I., Carrasco, L., Fernández-Garayzábal, J., Astorga, R.J., Luque, I., 2015b. Multi-Etiological Nature of Tuberculosis-Like Lesions in Condemned Pigs at the Slaughterhouse. PLoS One. 10 (9), e0139130.

Courcoul, A., Moyen, J.L., Brugère L., Faye, S., Hénault, S., Gares, H., Boschiroli, M.L., 2014. Estimation of Sensitivity and Specificity of Bacteriology, Histopathology and PCR for the Confirmatory Diagnosis of Bovine Tuberculosis Using Latent Class Analysis. PLoS One. 9 (3), e90334.

Che' Amat, A., González-Barrio, D., Ortiz, J.A., Díez-Delgado, I., Boadella, M., Barasona, J,A., Bezos, J., Romero, B., Armenteros, J.A., Lyashchenko, K.P., Venteo, A., Rueda, P., Gortázar, C., 2015. Testing Eurasian Wild boar piglets for serum antibodies against *Mycobacterium bovis*. Prev. Vet. Med. 121, 93-98.

García-Bocanegra, I., Pérez de Val, B., Arena-Montes, A., Paniagua, J., Boadella, M., Gortázar, C., Arenas, A., 2012. Seroprevalence and risk factors associated to *Mycobacterium bovis* in wild artiodactyl species from southern Spain, 2006-2010. PloS One, 7 (4), e34908.

Lyashchenko, KP., Greenwald, R., Esfandiari, J., Chambers, MA., Vicente, J., Gortázar, C., Santos N., Correia-Neves, M., Buddle, BM., Jackson, R., O'Brien, DJ., Schmitt, S., Palmer MV., Delahay, RJ., Waters WR., 2008. Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free-ranging wildlife. Vet. Microbiol. 132, 283-292.

Lyashchenko, KP., Greenwald, R., Esfandiari, J., Rhodes, S., Dean, G., de la Rua-Domenech, R., Meylan, M., Vordermeier, HM., Zanolari, P., 2011. Diagnostic Value of Animal-Side Antibody Assays for Rapid Detection of *Mycobacterium bovis* or *Mycobacterium microti* Infection in South American Camelids. Clin. Vaccine. Immunol. 18, 2143-2147.

Richomme, C., Boadella, M., Courcoul, A., Durand, B., Drapeau, A., Corde, Y., Hars, J., Payne, A., Fediaevsky, A., Boschiroli, M.L., 2013. Exposure of Wild Boar to *Mycobacterium tuberculosis* Complex in France since 2000 Is Consistent with the Distribution of Bovine Tuberculosis Outbreaks in Cattle. PLoS One 8(10), e77842.

Estudio 3/Study 3

Objetivo 2/Objective 2

Multi-etiological nature of tuberculosis-like lesions in condemned pigs at the slaughterhouse

F. Cardoso-Toset., J. Gómez-Laguna, S.P. Amarilla, A.I. Vela, L. Carrasco, J.F. Fernández-Garayzábal, R.J. Astorga., I. Luque.

PLoS One (2015) 10 (9): e0139130: 1-12.

Multi-etiological nature of tuberculosis-like lesions in condemned pigs at the slaughterhouse

Fernando Cardoso-Toset[a,b], Jaime Gómez-Laguna[b], Shyrley P. Amarilla[c], Ana I. Vela[d,e], Librado Carrasco[c], Jose F. Fernández-Garayzábal[d,e], Rafael J. Astorga[a], Inmaculada Luque[a]

[a]*Department of Animal Health, Faculty of Veterinary Medicine, 'International Excellence Agrifood Campus, CeiA3', Córdoba, Spain*

[b]*Department of R&D, CICAP - Food Research Centre, Pozoblanco, Córdoba, Spain*

[c]*Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, 'International Excellence Agrifood Campus, CeiA3', Córdoba, Spain*

[d]*Department of Animal Health, Faculty of Veterinary Medicine, Complutense University, Madrid, Spain.*

[e]*VISAVET Health Surveillance Centre, Faculty of Veterinary Medicine, Complutense University, Madrid, Spain*

Abstract

Tuberculosis-like lesions (TBL) in pigs have been associated with microorganisms other than mycobacteria. In this work a histopathological and microbiological evaluation of TBL in pigs is shown. A total of 352 samples belonging to 171 pigs totally condemned at slaughterhouse due to generalized TBL were sampled and selected for analysis. Pyogranulomatous (56.2%) and granulomatous lesions (20.2%) were observed in all analysed organs. Most of the granulomas observed in both lymph nodes and lungs belonged to more advanced stages of evolution (stages III and IV) whereas in the liver and the spleen most of lesions belonged to intermediate stages (stages II and III). Different microorganisms were simultaneously detected from TBL in the 42.7% of the animals. *Mycobacterium tuberculosis* complex (MTC) (38%), coryneform bacteria (40.3%) and streptococci (28.1%) were the main groups of microorganisms detected after bacteriological analysis, with *Trueperella pyogenes* and *Streptococcus suis* as the most frequently isolated species. Mycobacteria belonging to MTC were the most frequently detected pathogens in granulomatous and pyogranulomatous lesions in submandibular lymph nodes (32.7%) and coryneform bacteria were the

microorganisms more frequently isolated from lungs (25.9%) and spleen samples (37.2%). These results may provide new insights into the pathogenesis and diagnosis of this pathology. The importance of coryneform bacteria and streptococci in such processes must be evaluated in future studies.

Introduction

Tuberculosis-like lesions (TBL) can be an important cause of condemnation in swine abattoirs representing significant important economic losses (Lara et al., 2011). In pigs these lesions are described as necrotic-calcified, proliferative or purulent gross lesions compatible with tuberculosis (TB) (Santos et al., 2010; Di Marco et al., 2012). Although TBL in pigs are frequently limited to head lymph nodes, different body locations such as other lymph nodes and thoracic or abdominal organs can be also affected (Martín-Hernando et a., 2007; Di Marco et al., 2012).

Granulomatous and pyogranulomatous lesions can be identified in TBL according to the cellular components (Gómez-Laguna et al., 2010). Granulomas, as the main lesions associated with TB, have been widely classified within different stages of evolution that may help in the interpretation of disease progression (Co et al., 2004; Wangoo et al., 2005; Martín-Hernando et al., 2007; García-Jiménez et al., 2013). More advanced stages of granulomas have been associated with primary sites of infection (Martín-Hernando et al., 2007; Di Marco et al., 2012), but also with a lower bacterial load (Co et al., 2004; Di Marco et al., 2012).

Mycobacterium avium complex (MAC), *Mycobacterium tuberculosis* complex (MTC) and *Rhodococcus equi* have been reported as the species most frequently associated with

TBL, originating indistinguishable gross lesions in pigs worldwide (Parra et al., 2003; Komijn et al., 2007; Makrai et al., 2008; Gómez-Laguna et al., 2010; Miranda et al., 2012; Bailey et al., 2013). Other genera such as *Corynebacterium* spp., *Streptococcus* spp., or *Staphylococcus* spp., have also been isolated in caseous lymphadenitis in pigs, highlighting the potential diversity of pathogens that might be associated with TBL in this species (Lara et al., 2011; Contzen et al., 2011; Oliveiraet al., 2014). This diversity of microorganisms together with the zoonotic nature of several of them, are factors that should be considered by public health authorities (Lara et al., 2011).

Detailed studies evaluating the relative importance of microorganisms other than *Mycobacterium* spp. identified from TBL in pigs are scarce (Komijn et al., 2007; Lara et al., 2011). In this work a histopathological and microbiological evaluation of TBL in pigs is shown. Results of this study can help to better understand the interaction among microorganisms in pigs affected by TBL to improve the knowledge on the pathogenesis and diagnosis of this pathology.

Material and Methods

Ethics statement

This study did not involve purposeful killing of animals. Samples were collected from pigs after routine slaughter and meat inspection procedures. No ethical approval was deemed necessary.

Study design and sampling

A total of 171 pigs totally condemned due to the identification of generalized disease according to the European regulation for meat inspection (Regulation 2004/854/EC) were

sampled at two different slaughterhouses between January 2011 and June 2014. All animals were apparently healthy free-range pigs over 14 month-old raised in extensive systems from 56 farms located in South West Iberian Peninsula (Andalusia and Extremadura regions in Spain). After meat inspection procedures selected organs affected by TBL were sampled according to previous reports (Santos et al., 2010; Di Marco et al., 2012), including submandibular lymph nodes, lungs, liver and spleen to evaluate disseminated lesions (Parra et al., 2003; Martín-Hernando et al., 2007; Di Marco et al., 2012) (Fig 1.A-B). From these animals, a total of 352 samples were removed at the slaughterhouse and transported to the laboratory for analysis. To avoid cross contamination, different sets of sterile instruments and vials were used to collect and transport samples from each animal. Whenever possible, one well-defined lesion was selected in each organ to be divided into two portions: one portion was subjected to histopathological analysis and the other was immediately submitted to bacterial culture and frozen at -20 ºC to perform qPCR assays (Miranda et al., 2012). However, when small-sized disseminated lesions were observed, lesions that were similar in appearance and concentrated in one locality were selected and submitted to each analysis.

Histopathological analysis

Submandibular lymph nodes, lungs, liver and spleen tissue samples were fixed in 10% neutral buffered formalin and 4 µm sections were stained with haematoxylin and eosin for histopathological examination and by the Ziehl-Neelsen (ZN) method to detect acid-fast bacilli (AFB) by light microscopy (Santos et al., 2010; García-Jiménez et al., 2013). Each sample was classified according to the identification of specific structures, namely, epithelioid cells, multinucleated giant (MNG) cells, lymphocytes and/or neutrophils infiltration, connective tissue capsule formation, antigen-antibody deposits, necrosis and mineralization. The presence of granulomas with epithelioid cells and MNG cells in the

absence of foreign bodies or fungal structures was considered compatible with TB. Granulomas were classified into four stages (I–IV) based on the pathological characterization of TB granulomas previously described (Wangoo et al., 2005; García-Jiménez et al., 2013) (Fig 1.D-G). Lesions characterized by a necrotic core with an abundant neutrophil infiltration surrounded by epithelioid cells and a rim of connective tissue with infiltrate of mononuclear cells were described as pyogranulomas (Fig 1.C).

TB diagnosis

The presence of MTC and MAC in the lesions was tested by a duplex real time PCR (qPCR) previously validated by our research group (Cardoso-Toset et al., 2015). Fat and connective tissue were removed from affected organs and lesions were subsequently minced into small pieces with sterile scissors. For every sample, up to 2 g of tissue were homogenized in a stomacher with 10 mL of sterile distilled water for a duration of 2 min. The obtained solution was centrifuged for 10 min at 1,400 *g* resulting in a pellet for each sample. Genomic DNA was extracted from 25 mg of tissue homogenate using NucleoSpin® Tissue DNA isolation kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions. DNA yields and quality were determined using a NanoDrop 3300 spectrophotometer (Thermo scientific).

All reactions were run in duplicate in a Agilent Technologies Mx3000P thermocycler under the following conditions: initial denaturation at 95 ºC for 10 min, 40 cycles of amplification consisting of denaturation at 95 ºC for 30 sec, primer annealing at 65 ºC for 30 sec, and extension at 72 ºC for 30 sec. To check the specificity of the amplified products, DNA from *M. bovis* and *M. avium* isolates and non template controls were included in each assay and used as positive and negative controls, respectively.

ZN staining was performed in tissue samples negative to qPCR and examined for AFB presence as described by Santos et al. (2010).

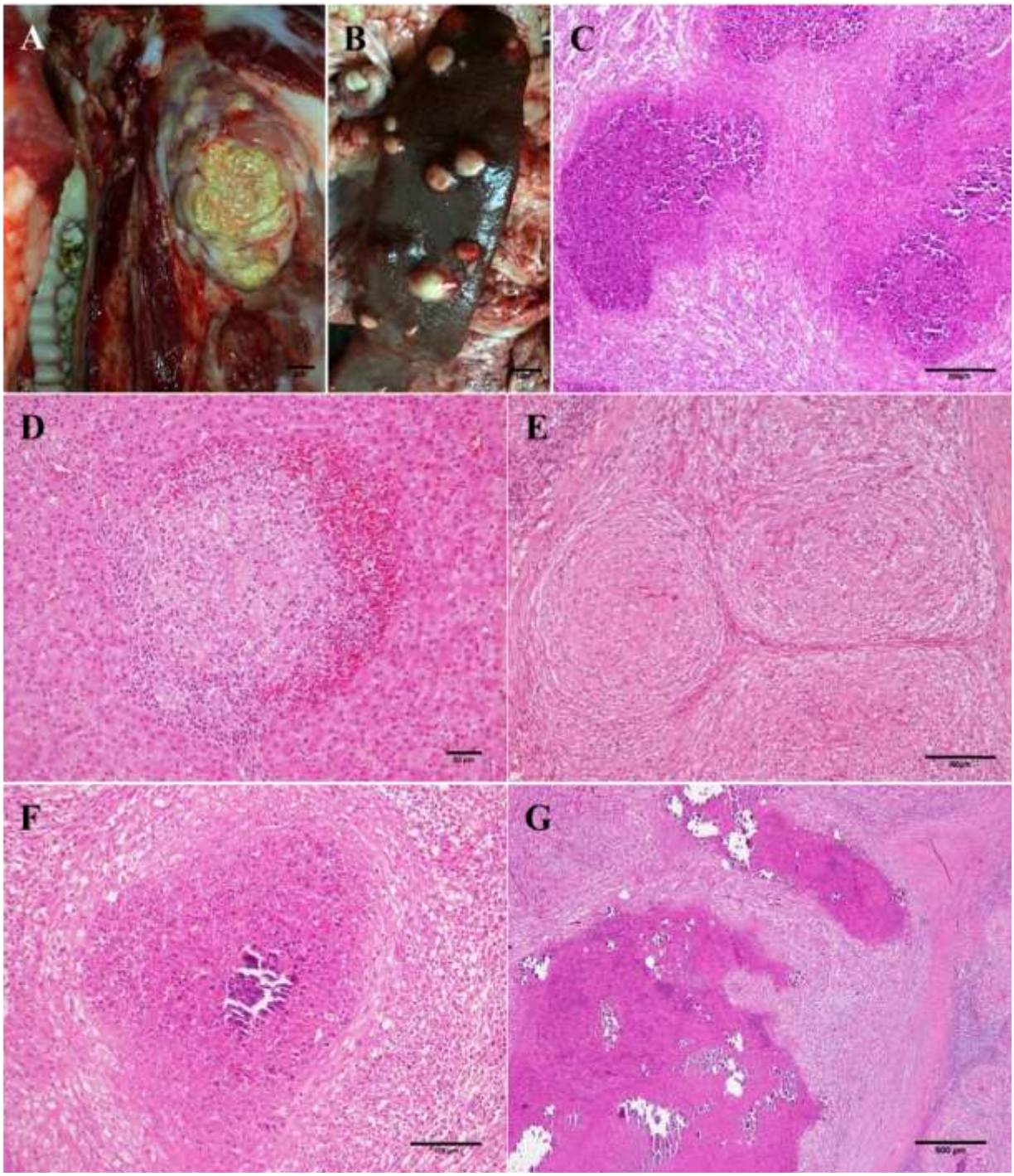


Fig. 1 A-G. A) TBL in the submandibular lymph node of an affected pig. Bar, 1cm. B) TBL in the spleen of an affected pig. Bar, 1cm. C) Microscopic image of a TBL lesions in the lymph node of an affected animal showing a profuse neutrophils infiltration. HE. Bar, 200µm. D) Clustered epithelioid macrophages surrounded by lymphocytes and erythrocytes in a stage I granuloma in the liver. HE. Bar, 50µm. E) Coalescent stage II granulomas in the lymph node of a pig showing epithelioid macrophages completely enclosed by a thin capsule, with peripheral infiltration of scattered lymphocytes. HE. Bar, 100µm. F) Stage III granuloma with a central necrotic core, partially mineralized, surrounded by a dense connective tissue capsule infiltrated by lymphocytes and scattered neutrophils. HE. Bar, 100µm. G) Thickly encapsulated, large, irregular, multicentric granulomas with prominent caseous necrosis and multifoci islands of mineralization (stage IV granulomas). HE. Bar, 500µm.

Bacterial isolation

A swab from each sample submitted to bacterial culture was plated on Blood Agar Base and Columbia Blood Agar Base with nalidixic acid and colistin sulfate (Oxoid ltd., Hampshire, UK), supplemented with 5% sterile defibrinated sheep blood and incubated both in aerobic and microaerophilic (5% CO_2) conditions at 37 ºC for 48 h. One representative colony of the most abundant morphologically distinct colonies were selected, subcultured and grown in the same conditions for further biochemical identification. Gram staining, bacterial morphology and production of catalase and cytochrome oxidase were performed as preliminary identification tests according to standard procedures (Smibert et al., 1994).

Further biochemical identification was performed using commercial identification galleries (API®Coryne, API®20Strep, API®20E and API®20NE, bioMérieux, Marcy-l'Etoile, France) according to manufacturer's instructions. Isolates were identified as a particular species only if identification scores in the multi-substrate identification systems were excellent, very good or good (99.9-99.0% ID); otherwise, identification was made only at the genus level (spp.). Latex agglutination test for the identification of streptococcal groups (Streptococcal grouping kit, Oxoid ltd, Hampshire, UK), and Christie Atkins Munch-Petersen test (CAMP test) were used for identification if necessary according to previous reports (Funke et al., 1997; Facklam et al., 2002; Ülbegi-Mohyla et al., 2009). Pure cultures of each isolate were stored at -70 ºC.

16S rRNA gene sequencing.

Coryneform bacteria isolates (Gram-positive, catalase variable and oxidase negative irregularly shaped rods) were identified applying 16S rRNA gene sequencing due to the limited capacity of biochemical methods to discriminate between species (Adderson et al.,

2008). The 16S rRNA gene of each isolate was amplified by PCR and further sequenced to determine genotypic identity (Vela et al., 2003). The determined sequences consisted of about 1,400 nucleotides and were compared with the sequences of other Gram-positive species available in the GenBank database, by using the FASTA program (http://www.ebi.ac.uk/fasta33).

Results

Histopathological analysis.

A total of 352 samples belonging to 171 slaughtered pigs with TBL were evaluated. Pyogranuloma was the lesion most frequently detected in all the examined organs (198/352; 56.2%) (Table 1) and around 60% of the animals (104/171; 60.8%) (Table 5).

Table1. Type of lesions identified from samples.

	Total	**SLN[a]**	**Lungs**	**Liver**	**Spleen**
	Nº (%)	**Nº (%)**	**Nº (%)**	**Nº (%)**	**Nº (%)**
Pyogranuloma	198 (56.2)	94 (60.3)	42 (49.4)	33 (48.5)	29 (67.4)
Granuloma	71 (20.2)	38 (24.4)	14 (16.5)	14 (20.6)	5 (11.6)
Necrosis or calcification	12 (3.4)	8 (5.1)	0 (0)	4 (5.9)	0 (0)
Other lesions	33 (9.4)	2 (1.3)	23 (27.1)	7 (10.3)	1 (2.3)
No lesions	38 (10.8)	14 (8.9)	6 (7.1)	10 (14.7)	8 (18.6)
Total	352 (100)	156 (100)	85 (100)	68 (100)	43 (100)

[a]SLN: submandibular lymph nodes.

Granulomatous lesions were observed in 71/352 (20.2%) of samples, being described in 38/171 of animals (22.2%). The presence of concomitant pyogranulomatous and granulomatous lesions in different organs was observed in 14 out of 171 animals (8.2%). Necrotic foci or lesions showing an intense mineralization and fibrosis, with absence of epitheiloid cells or MNGCs (13/352; 3.7%) were separately considered due to the impossibility of identifying these lesions as pyogranuloma or granuloma. Other type of

lesions such as interstitial pneumonia, catarrhal-purulent bronchitis, periportal fibrosis, periesplenitis and interstitial and multifocal hepatitis were also detected in the absence of granulomatous or pyogranulomatous lesions (71/352, 20.2% samples; and 1/171, 0.6% animals). Finally, in a reduced number of cases, lesions could not be detected (38/352, 10.8% samples; and 9/171, 5.3% animals).

Granulomas were mostly evidenced in samples from submandibular lymph nodes and to a lesser extent in liver, lungs and spleen (Table 1). Attending to the stages of the evolution of granulomas, 31% of granulomas belonged to the initial stages (I and II), whereas 69% of the granulomas were included within the stages III and IV. This pattern was confirmed for lymph node and lung samples, whereas in the spleen and the liver most of the granulomas belonged to the stages II and III (Table 2).

Table 2. Distribution of granulomas per examined organ and evolutionary stage.

	Total	**SLN[a]**	**Lung**	**Liver**	**Spleen**
	Nº (%)	**Nº (%)**	**Nº (%)**	**Nº (%)**	**Nº (%)**
Stage I	2 (2.8)	0 (0)	1 (7.7)	1 (7.1)	0 (0)
Stage II	20 (28.2)	10 (25.6)	3 (23.1)	5 (35.7)	2 (40)
Stage III	27 (38)	16 (41)	3 (23.1)	6 (42.9)	2 (40)
Stage IV	22 (31)	13 (33.3)	6 (46.1)	2 (14.3)	1 (20)
Total	71 (100)	39 (100)	13 (100)	14 (100)	5 (100)

[a]SLN: submandibular lymph nodes.

TB diagnosis

Genome of MTC was amplified on samples from 65 animals (65/171 animals; 38%). In 44 out of these 65 animals generalized TBL affecting submandibular lymph nodes and other organs (lungs, liver or spleen) were detected. In 25 out of these 44 animals (56.8%) MTC was detected only in submandibular lymph nodes; in 6/44 (13.6%) mycobacteria were

detected in submandibular lymph nodes and lungs, liver and/or spleen; whereas in 13/44 animals (29.5%) mycobacteria were detected only in lungs, liver or spleen (data not shown). MAC was detected only in one case associated with pyogranulomatous lesions in a liver.

AFB were recorded in 15/269 (5.6%) of qPCR negative samples and in 9/105 (8.6%) of qPCR negative animals by ZN staining.

Bacterial isolation

A total of 235 isolates were obtained after bacteriological culture (Table 3). Due to the high number of bacterial species detected in low percentages the analysis was focused on coryneform bacteria and streptococci as the main groups of microorganisms detected in this study besides MTC. Coryneform bacteria were identified in 100 out of 235 isolates (42.5%) and were recovered from 69 animals (40.3%). Most coryneform microorganisms were identified as *Trueperella pyogenes* (formerly *Arcanobacterium pyogenes*) (72%), which was isolated from a significant number (69.6%) of examined animals (Table 3). Streptococci were also identified in a notable number of isolates (65/235; 27.7%) and animals (48/171; 28.1%). *Streptococcus suis* was the species most frequently identified within this group (40% of isolates and 47.9% of animals in which streptococci were isolated) followed by *Streptococcus porcinus* and *Streptococcus dysgalactiae* spp. *equisimilis* (Table 3).

Organic distribution of identified microorganisms.

The frequency of detection of microorganisms in TBL from the examined organs is shown in Table 4. MTC, coryneform bacteria and streptococci were detected in all analysed organs. MTC was more frequently detected in submandibular lymph nodes (32.7%), whereas coryneform bacteria were more frequently isolated from lungs (25.9%) and spleen (37.2%). *T. pyogenes* was the species identified in over 60% of cases associated with coryneform

bacteria in all examined organs (data not shown). Streptococci were equally isolated from lesions of all examined organs.

Table 3. Microorganisms isolated from lesions.

	Isolates		**Positive animals**	
	Nº	**%**	**Nº**	**%**
Coryneform bacteria	**100**	**42.5**	**69**	**40.3**
Trueperella pyogenes	72/100	72	48/69	69.6
Corynebacterium suicordis	12/100	12	10/69	14.5
Rhodococcus equi	3/100	3	3/69	4.3
Corynebacterium xerosis	4/100	4	2/69	2.9
Corynebacterium spp.	2/100	2	1/69	1.4
Corynebacterium ulcerans	1/100	1	1/69	1.4
Corynebacterium urealyticum	1/100	1	1/69	1.4
Other coryneform bacteria[a]	5/100	5	5/69	7.2
Streptococci	**65**	**27.7**	**48**	**28.1**
Streptococcus suis	26/65	40	23/48	47.9
Streptococcus porcinus	12/65	18.5	7/48	14.6
Streptococcus dysgalactiae spp. *equisimilis*	6/65	9.2	5/48	10.4
Streptococcus equi spp. *Zooepidemicus*	5/65	7.7	4/48	8.3
Streptococcus agalactiae	4/65	6.1	4/48	8.3
Streptococcus alactolyticus	4/65	6.1	2/48	4.2
Streptococcus uberis	2/65	3.1	2/48	4.2
Other streptococci[b]	6/65	9.2	6/48	12.5
***Enterococcus* spp.[c]**	19	8.1	14	8.2
***Carnobacterium* spp.[d]**	17	7.2	14	8.2
***Aerococcus* spp.[e]**	13	5.5	11	6.4
***Staphylococcus* spp.[f]**	7	3	7	4.1
Pasteurella multocida	4	1.7	4	2.3
Others[g]	10	4.2	7	4.1
Total	235	100	171	100

[a]*Rhodococcus boritolerans, Dietzia timorensis, Pseudoclavibacter* spp, *Brevibacterium* spp and *Actinomyces masicol* (1 isolate/each); [b]*Streptococcus* spp. (3 isolates), *S. mitis, S. rattus* and *S. bovis* (1 isolate/each); [c]*E. faecium* (8 isolates) *E. durans* (3 isolates), *E. faecalis* (6 isolates), *E. gallinarum* and *E. avium* (1 isolate/each). [d]*C. maltaromaticum* (16 isolates) and *C. divergens* (1 isolate). [e]*A. urinae* (7 isolates), *A. viridans* (4 isolates) and *A nurinaequi* (2 isolates); [f]*S. sciuri, S. xylosus* (2 isolates/each), *Staphylococcus* spp., *S. aureus* and *S. haemolyticus* (1 isolate/each); [g]*Leuconostoc* spp. (4 isolates), *Escherichia coli* (2 isolates) *Mezorhizobium* spp., *Halospirulina* spp., *Glanulicatella* spp. and *Lactococcus lactis* (1 isolate/each).

Table 4. Frequency of detection of microorganisms from TBL within the examined organs.

	Total	**SLN[a]**	**Lungs**	**Liver**	**Spleen**
	Nº (%)	**Nº (%)**	**Nº (%)**	**Nº (%)**	**Nº (%)**
MTC	82 (23.3)	51 (32.7)	12 (14.1)	14 (20.6)	5 (11.6)
Coryneform bacteria	98 (27.8)	46 (29.5)	22 (25.9)	14 (20.6)	16 (37.2)
Streptococci	62 (17.6)	30 (19.2)	16 (18.8)	10 (14.7)	6 (13.9)
Others	63 (17.9)	28 (17.9)	15 (17.6)	13 (19.1)	7 (16.3)

[a]SLN: submandibular lymph nodes.

Microorganisms and type of lesions at individuals.

The bacteria identified from different type of lesions are summarized in Table 5. Twenty-three animals (13.4%) yielded negative results both by microbiological and qPCR studies despite having presented microscopic lesions. In 75 (43.9%) of the pigs, a single microorganism was identified, whereas in 73 (42.7%) of the animals two or more microorganisms were detected. The isolation of coryneform bacteria from MTC positive pigs was frequent (10.5%), with *T. pyogenes* being identified in 77.8% (14/18) of these cases. Moreover, different species of coryneform bacteria were isolated from MTC negative pigs (16.9%), with *T. pyogenes* as the main species identified (17/29; 58.6%).

Mycobacteria belonging to MTC were identified in 19/38 (50%) of pigs in which granuloma was the unique detected lesion (Table 5). Bacteria other than mycobacteria were also detected from granulomatous lesions in 13/38 of animals (34.2%), whereas no microorganisms were identified in 6/38 (15.8%). However, when pyogranuloma was considered as a sole lesion, most animals were negative to MTC (70/104; 67.3%) (Table 5). Coryneform bacteria (50/104; 48.1% animals) and less frequently streptococci (26/104; 25% animals) were the main microbial agents isolated from these lesions.

Table 5. Frequency of detected microorganisms and type of lesions identified at individual level.

	Total		**Granuloma**		**Pyogranuloma**		**Concomitant lesions[a]**		**Other lesions[b]**	
	Nº	**%**	**Nº**	**%**	**Nº**	**%**	**Nº**	**%**	**Nº**	**%**
MTC positive animals	**65**	**38**	**19**	**50**	**34**	**32.7**	**7**	**50**	**5**	**33.3**
Coryneforms	18	10.5	2	5.2	16	15.4	0	0	0	0
Streptococci	6	3.5	1	2.6	2	1.9	0	0	3	20
Others	9	5.3	3	7.9	4	3.8	1	7.1	1	6.7
Coryneforms + streptococci	10	5.8	4	10.5	4	3.8	2	14.3	0	0
Coryneforms + others	4	2.3	1	2.6	2	1.9	1	7.1	0	0
Streptococci+ others	3	1.7	2	5.3	1	1	0	0	0	0
No isolation	15	8.8	6	15.8	5	4.8	3	21.4	1	6.7
MTC negative animals	**106**	**62**	**19**	**50**	**70**	**67.3**	**7**	**50**	**10**	**66.7**
Coryneforms	29	16.9	1	2.6	24	23.1	1	7.1	3	20
Streptococci	17	9.9	1	2.6	10	9.6	2	14.3	4	26.7
Others	22	12.9	6	15.8	12	11.5	1	7.1	3	20
Coryneforms + streptococci	4	2.3	1	2.6	3	2.9	0	0	0	0
Coryneforms +streptococci + others	1	0.6	0	0	1	1	0	0	0	0
Coryneforms + others	3	1.7	2	5.3	0	0	1	7.1	0	0
Streptococci + others	7	4.1	2	5.3	5	4.8	0	0	0	0
No isolation	23	13.4	6	15.8	15	14.4	2	14.3	0	0
Total	171	100	38	100	104	100	14	100	15	100

[a]Granulomatous and pyogranulomatous lesions detected in the same animal. [b]Necrotic or calcified foci (5/15) and other lesions or no lesions (10/15).

Discussion

Tuberculosis-like lesions include a wide range of lesions compatible with TB (Santos et al., 2010; Di Marco et al., 2012). However, previous studies have shown that pathogens other than mycobacteria may cause indistinguishable gross TBL in pigs (Komijn et al., 2007). The involvement of different pathogens in the development of these lesions needs to be evaluated to assess an accurate diagnosis of TBL in pigs and to establish effective control measures against this pathology (Lara et al., 2011).

In this study, pyogranuloma was the predominant lesional pattern (104/171 animals; 60.8%) with granuloma being detected only in 38 out of 171 animals (22.2%). The high number of pyogranulomatous lesions detected in the present study (198/352 samples; 56.2%) suggests the importance of pyogenic bacteria in the etiology of TBL in pigs. In fact, a wide spectrum of bacteria belonging to twenty different genera was detected. MTC was detected in 65 (38%) of the animals, together with an important participation of coryneform bacteria and streptococci (40.35% and 28.1% positive animals respectively). These findings reinforce the multi-etiological nature of TBL.

The detection of multiple microbial agents was frequent (42.7% of analysed animals) highlighting the importance of performing a thorough microbiological examination of TBL for disease surveillance (Lara et al., 2011). MTC, coryneform bacteria (including *T. pyogenes*, *Corynebacterium* spp., and related genera) and streptococci were the pathogens more frequently detected from TBL. Other microorganisms also identified but with a lower frequency (*Staphylococcus* spp., *Pasteurella multocida*, *Enterococcus* spp., *Carnobacterium* spp., *Aerococcus* spp.) can be isolated from the environment, faeces, skin and mucous membranes of pigs (Radostitis et al, 2007), but their importance in this process is unknown.

Therefore, our analysis was focused on the most representative groups of pathogens identified in the study.

MTC and MAC may play different roles in TBL according to the prevalence of bovine TB. In this sense, *M. avium* is the main mycobacteria recovered from TBL in officially bovine TB free countries (Johansen et al., 2014) as well as in fattening pigs reared in intensive systems (Pérez de Val et al., 2014). However, in countries where TB is still prevalent in cattle and wildlife MTC is frequently detected from TBL in free-range pigs (Parra et al., 2003; Santos et al., 2010; Bailey et al., 2013). Pigs of this study were bred in a free-ranged system sharing natural resources with other wild and domestic animals in a geographical area in which a high prevalence of TB infected wild boars has been described (Vicente et al., 2006). Contact and cross infection between these populations may occur as has been reported in other areas of Spain (Parra et al., 2003). Accordingly, MTC was predominately detected from TBL in our study.

The frequency of isolation of *Rhodococcus equi* in our study (4/171, 2.3% animals) was much lower than previously reported in pigs, negative to mycobacteria, and reared in intensive farms in the Netherlands (Komijn et al., 2007). Despite further studies being deemed necessary to elucidate these differences, several factors such as breed robustness, herd management and the ecology of the bacteria may play a role. Corynebacteria other than *Rhodococcus equi* have been sporadically related with severe caseous lymphadenitis in pigs, including *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* (Contzen et al., 2011; Oliveira et al., 2014).

In the present study, *Corynebacterium suicordis* was the main species isolated within this genus, but its relative importance was low. *T. pyogenes* was the predominant species within this group. This pathogen is involved in miscellaneous pyogenic infections in pigs and

ruminants, including metritis, udder lesions, abscesses, pneumonia, arthritis, endocarditis, lymphadenitis and osteomyelitis (Martínez et al., 2007; Rzewuska et al., 2012; Ribeiro et al., 2015). Although *T. pyogenes* has previously been associated with caseous lymphadenitis in pigs (Lara et al., 2011), its relative importance was low in comparison with our study.

Similarly to Lara et al. (Lara et al., 2011) streptococci were detected in a high percentage of animals. Interestingly, *S. suis* was the species most frequently identified within this group. This microorganism has been associated with a wide variety of diseases in pigs such as meningitis, arthritis, bronchopneumonia, endocarditis, poliserositis and septicaemia and has been considered as an emerging zoonotic agent in humans secondary to exposition to pigs and pork products (Goyette-Desjardins et al., 2014). The other two streptococcal species more frequently isolated, *Streptococcus porcinus* and *Streptococcus dysgalactiae* spp. *equisimilis*, are also frequently isolated from pigs with suppurative infections (Katsumi et al., 1998; Martínez et al., 2007).

Negative bacteriological results were observed in accordance with previous reports (Santos et al., 2010; Lara et al., 2011; Costa et al., 2013). These results may be attributed to false negative results of bacteriology or animals with advanced lesions in which viable microorganisms could not be obtained. In this sense, the 21.7% (5/23) of animals that showed microscopic lesions but were negative to both bacterial culture and qPCR, showed AFB suggesting a possible mycobacterial involvement in several of them.

Coryneform bacteria isolation from MTC negative TBL was the pattern most frequently observed from animals affected by pyogranulomatous lesions (24/104; 23.1%). These results support the idea that pyogenic bacteria can originate TBL in pigs without the

involvement of mycobacteria. In this sense, although false negative results of the qPCR analysis should be considered, AFB were only recorded in lesions of two of these animals. Alternatively, simultaneous detection of coryneform bacteria and MTC was also frequent, suggesting a possible involvement of this microbial association in pigs affected by TBL.

Granulomas were predominately observed in submandibular lymph nodes and to a lesser extent in other body locations. The submandibular lymph node was the organ in which MTC was more frequently detected, followed by the liver, lungs and spleen. Interestingly, MTC was identified in a similar rate from both liver and lung samples, with most of the granulomatous lesions belonging to stages III and IV. These results support that both respiratory and digestive routes of infection play an important role in pigs, as previously suggested (Parra et al., 2003; Martín-Hernando et al., 2007).

Despite several authors have suggested that generalized TB in swine is frequent (Di Marco et al., 2012; Domingo et al., 2014), others have reported a restriction of TBL to head lymph nodes or less frequently to the respiratory tract (Parra et al., 2003; Zanella et al., 2008; Santos et al., 2010). Our results are in agreement with this latter statement, since MTC was detected only in submandibular lymph nodes in more than half of the animals. Interestingly, pyogenic bacteria, including *T. pyogenes*, *Streptococcus* spp., and *Corynebacterium* spp., were isolated from TBL observed in other organs from these MTC-positive animals (data not shown). These findings should be taken into account to avoid misdiagnosis of generalized TB based on gross inspection and to carry out further studies to determine the true role of these agents, especially *T. pyogenes* in pyogranulomatous lesions in pigs

Conclusions

The results of this study show that a wide spectrum of microorganisms different to mycobacteria can be isolated from TBL in pigs, with coryneform bacteria and streptococci as the microorganisms most frequently detected besides MTC. The high frequency of detection of *T. pyogenes* in pyogranulomatous lesions is also shown. These results should be considered to prevent misdiagnosis of TB based on gross lesions and to establish specific control measures against these pathogens in pigs.

References

Adderson EE, Boudreaux JW, Cummings JR, Pounds S, Wilson DA, Procop GW et al. Identification of clinical coryneform bacterial isolates: comparison of biochemical methods and sequence analysis of 16S rRNA and rpoB genes. J. Clin. Microbiol. 2008; 46: 921-927.

Bailey SS, Crawshaw TR, Smith NH, Palgrave CJ. *Mycobacterium bovis* infection in domestic pigs in Great Britain. Vet J. 2013; 198: 391-397.

Cardoso-Toset F, Luque I, Amarilla SP, Gómez-Gascón L, Fernández L, Huerta B, et al. Evaluation of rapid methods for diagnosis of tuberculosis in slaughtered free-range pigs. Vet. J. 2015; doi:10.1016/j.tvjl.2015.01.022.

Co DO, Hogan LH, Kim SL, Sandor M. Mycobacterial granulomas: keys to a long-lasting host-pathogen relationship. Clin. Immunol. 2004; 113: 130-136.

Contzen M, Sting R, Blazey B, Rau J. *Corynebacterium ulcerans* from diseased wild boars. Zoonoses. Public. Health. 2011; 58: 479-488.

Costa P, Ferreira AS, Amaro A, Albuquerque T, Botelho A, Couto A, et al. Enhanced detection of tuberculous mycobacteria in animal tissues using a semi-nested probe-based real-time PCR. PLoS One. 2013; doi:10.1371/journal.pone.0081337.

Di Marco V, Mazzone P, Capucchio MT, Boniotti MB, Aronica V, Russo M, et al. Epidemiological significance of the domestic black pig (*Sus scrofa*) in maintenance of bovine tuberculosis in Sicily. J. Clin. Microbiol. 2012; 50: 1209-1218.

Domingo M, Vidal E, Marco A. Pathology of bovine tuberculosis. Res. Vet. Sci. 2014; 97: 520-529.

Facklam R. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin. Microbiol. 2002; 15: 613-630.

Funke G, Graevenitz von A, Clarridge JE, Bernard KA. Clinical microbiology of coryneform bacteria. Clin. Microbiol. Rev. 1997; 10: 125-157.

García-Jiménez WL, Salgero FJ, Fernández-Llario P, Martínez R, Risco D, Gough J, et al. Immunopathology of granulomas produced by *Mycobacterium bovis* in naturally infected wild boar. Vet. Immunol. Immunop. 2013; 156: 54-63.

Gómez-Laguna J, Carrasco L, Ramis G, Quereda JJ, Gómez S, Pallares FJ. Use of real-time and classic polymerase chain reaction assays for the diagnosis of porcine tuberculosis in formalin-fixed, paraffin-embedded tissues. J. Vet. Diagn. Invest. 2010; 22: 123-127.

Goyette-Desjardins, G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg. Microbes. Infect. 2014; doi:10.1038/emi.2014.45.

Johansen TB, Agdestein A, Lium B, Jorgensen A, Djonne B. *Mycobacterium avium* subsp. *hominissuis* Infection in Swine Associated with Peat Used for Bedding. Biomed. Rest. 2014; doi: 10.1155/2014/189649.

Katsumi M, Kataoka Y, Takahashi T, Kikuchi N, Hiramune T. Biochemical and Serological examination of β-hemolytic Streptococci isolated from slaughtered pigs. J. Vet. Med. Sci. 1998; 60: 129-131.

Komijn RE, Wisselink HJ, Rijsman VMC, Stockhofe-Zurwieden N, Bakker D, van Zijderveld, FG, et al. Granulomatous lesions in lymph nodes of slaughter pigs bacteriologically negative for *Mycobacterium avium subsp. avium* and positive for *Rhodococcus equi*. Vet. Microbiol. 2007; 120: 352-357.

Makrai L, Kobayashi A, Matsuoka M, Sasaki Y, Kakuda T, Dénes B, et al. Isolation and characterisation of *Rhodococcus equi* from submaxillary lymph nodes of wild boars (*Sus scrofa*). Vet. Microbiol. 2008; 15: 318-323.

Martín-Hernando MP, Höfle U, Vicente J, Ruiz-Fons F, Vidal D, Barral M, et al. Lesions associated with *Mycobacterium tuberculosis* complex infection in the European wild boar. Tuberculosis. 2007; 87: 360-367.

Martínez J, Jaro PJ, Aduriz G, Gómez EA, Peris B, Corpa JM. Carcass condemnation causes of growth retarded pigs at slaughter. Vet. J. 2007; 174: 160-164.

Miranda C, Matos M, Pires I, Correia-Neves M, Ribeiro P, Alvares S, et al. Diagnosis of *Mycobacterium avium* complex in granulomatous lymphadenitis in slaughtered domestic pigs. J. Comp. Pathol. 2012; 147: 401-405.

Oliveira M, Barroco C, Mottola C, Santos R, Lemsaddek A, Tavares L, et al. First report of *Corynebacterium pseudotuberculosis* from caseous lymphadenitis lesions in Black Alentejano pig (*Sus scrofa domesticus*). BMC. Vet. Res. 2014; 218: 1-5.

Parra A, Fernández-Llario P, Tato A, Larrasa J, García A, Alonso JM, et al. Epidemiology of *Mycobacterium bovis* infection of pigs and wild boars using a molecular approach. Vet. Microbiol. 2003; 97: 122-133.

Pérez de Val B, Grau-Roma L, Segalés J, Domingo M, Vidal E. Mycobacteriosis outbreak caused by *Mycobacterium avium* subsp. *avium* detected through meat inspection in five porcine fattening farms. Vet. Rec. 2014; 174: 1-3.

Radostitis OM, Gay ME, Hinchcliff KW, Constable PD. Veterinary medicine: A textbook of the disease of cattle, horses, sheep, pigs, goats and horses. 10 ed. WB Saunders, London; 2007. p. 2156.

Ribeiro MG, Risseti RM, Bolaños CAD, Caffaro KA, de Morais ACB, Lara GHB, et al. *Trueperella pyogenes* multispecies infections in domestic animals: a retrospective study of 144 cases (2002 to 2012). Vet. Quart. 2015; doi: 10.1080/01652176.2015.1022667.

Rzewuska M, Stefanska I, Osinska B, Kizerwetter-Swida M, Chrobak D, Kaba J, et al. Phenotypic characteristics and virulence genotypes of *Trueperella* (*Arcanobacterium*) *pyogenes* strains isolated from European bison (*Bison bonasus*). Vet. Microbiol. 2012; 160: 69-76.

Santos N, Geraldes M, Andreia A, Almeida V, Correia-Neves M, et al. Diagnosis of tuberculosis in the wild boar (*Sus scrofa*): A comparison of methods applicable to hunter-harvested animals. Plos One. 2010; doi: 10.1371/journal.pone.0012663.

Smibert RM, Krieg NR. Methods for general and molecular bacteriology. American Society for Microbiology, Washington DC; 1994.

Ülbegi-Mohyla H, Hassan AA, Kanbar T, Alber J, Lämmler C, Prenger-Berninghoff E, et al. Synergistic and antagonistic hemolytic activities of bacteria of genus *Arcanobacterium* and CAMP-like hemolysis of *Arcanobacterium phocae* and *Arcanobacterium haemolyticum* with *Psychrobacter phenylpyruvicus*. Res. Vet. Sci. 2009; 87: 186-188.

Vela AI, Mateos A, Collins MD, Briones V, Hutson R, Domínguez L, et al. *Corynebacterium suicordis* sp. nov., from pigs. Int. J. Syst. Evol. Microbiol. 2003; 53: 2027–2031.

Vicente J, Hoflë U, Garrido JM, Fernández de Mena IG, Juste R, Barral M, Gortazar C. Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. Vet. Res. 2006; 37: 107-119.

Wangoo A, Johnson L, Gough J, Ackbar R, Inglut S, Hicks D, et al. Advanced granulomatous lesions in *Mycobacterium bovis*-infected cattle are associated with increased expression of type I procollagen, gammadelta (WC1+) T cells and CD 68+ cells. J. Comp. Pathol. 2005; 133: 223-234.

Zanella G, Duvaduchelle A, Hars J, Mutou F, Boschiroli ML, Durand B. Patterns of lesions of bovine tuberculosis in wild red deer and wild boar. Vet. Rec. 2008; 163: 43-4.

Estudio 4/Study 4

Objetivo 3/Objective 3

Survival of *Streptococcus suis, Streptococcus dysgalactiae* and *Trueperella pyogenes* in dry-cured iberian pork shoulders and loins

F. Cardoso-Toset., I. Luque., A. Morales-Partera., A. Galán-Relaño., B. Barrero-Domínguez., M. Hernández., J. Gómez-Laguna.

Food Microbiology (2017) 61: 66-71

Survival of *Streptococcus suis, Streptococcus dysgalactiae* and *Trueperella pyogenes* in dry-cured iberian pork shoulders and loins.

F. Cardoso-Toset[a,b], I. Luque[a], A. Morales-Partera[b], A. Galán-Relaño[a], B. Barrero-Domínguez[a] , M. Hernández[b], J. Gómez-Laguna[b].

[a] *Animal Health Department, University of Córdoba, International Excellence Agrifood Campus 'CeiA3', 14071, Córdoba, Spain.*

[b] *CICAP – Food Research Centre, 14400, Pozoblanco, Córdoba, Spain*

[c] *Anatomy and Comparative Pathology Department, University of Córdoba, International Excellence Agrifood Campus 'CeiA3', 14071, Córdoba, Spain.*

Abstract

Dry-cured hams, shoulders and loins of Iberian pigs are highly appreciated in national and international markets. Salting, additive addition and dehydration are the main strategies to produce these ready-to-eat products. Although the dry curing process is known to reduce the load of well-known food borne pathogens, studies evaluating the viability of other microorganisms in contaminated pork have not been performed. In this work, the efficacy of the dry curing process to eliminate three swine pathogens associated with pork carcass condemnation, *Streptococcus suis, Streptococcus dysgalactiae* and *Trueperella pyogenes*, was evaluated. Results of this study highlight that the dry curing process is a suitable method to obtain safe ready-to-eat products free of these microorganisms. Although salting of dry-cured shoulders had a moderate bactericidal effect, results of this study suggest that drying and ripening were the most important stages to obtain dry-cured products free of these microorganisms.

Keywords: *T. pyogenes, S. suis, S. dysgalactiae,* dry curing process.

Introduction

Iberian dry-cured pork products have an important demand of consumers in national and international markets (Gamero-Negrón et al., 2015). The traditional elaboration of these products is based on salting (dry-cured hams and shoulders), addition of a mixture of ingredients and spices (dry-cured loins) and subsequent dehydration and ripening to obtain shelf stable ready-to-eat (RTE) products (Soto et al., 2008; García-Gil et al., 2014).

Dry-cured products are considered to be safe due to several factors such as their low pH and water activity (a_w), their high salt concentration or the addition of nitrites, spices and other ingredients that contribute to their stability (Stollewerk et al., 2012). Previous reports have evaluated the efficacy of the dry curing process to control food-borne pathogens such as *Listeria monocytogenes, Salmonella* spp. or *Staphylococcus aureus* (Reynolds et al., 2001; García-Díez et al., 2016). However, the survival of other pathogens that can infect pigs and may contaminate pork meat at slaughterhouse has not been evaluated.

Streptococcus suis, Streptococcus dysgalactiae and *Trueperella pyogenes* are swine pathogens that can be isolated from healthy pigs (Lara et al., 2011; Lowe et al., 2011) and that can be also involved in a high rate of suppurative lesions of growing pigs that typically result in partial or total carcass condemnation at slaughterhouse (Martínez et al., 2007; Cardoso-Toset et al., 2015). For that reason, cross contamination of pork along the slaughtering, meat inspection and operational handling can occur (Arai et al., 2015). In fact, previous reports have shown that these microorganisms can be isolated from pork carcasses and raw pork (Martel et al., 2003; Cheung et al., 2008; Blagojevic et al., 2014; Brinch Kruse et al., 2015).

S. suis has been recognised as an emerging human pathogen that causes severe

infections in pigs and humans in close contact with pigs and pork-derived products, including meningitis, arthritis, septicaemia, pneumonia and endocarditis (Goyette-Desjardins et al., 2014). Although 35 serotypes have been described on the basis of their polysaccharide capsular antigens, *S. suis* serotype 2 is the most prevalent and pathogenic in both species (Arai et al., 2015). The natural habitat of this pathogen is the tonsils and nasal cavities of pigs, but also their genital and digestive tracts (Goyette-Desjardins et al., 2014).

S. dysgalactiae is another *Streptococcus* species that can be divided into two subspecies based on whole-cell protein profiles and biochemical properties: *S. dysgalactiae* spp. *dysgalactiae*, which includes strains of animal origin and *S. dysgalactiae* spp. *equisimilis* which includes strains of both animal and human origin (Martínez et al., 2007; Silva et al., 2015). This subspecies has increasingly been recognized as etiological agent of several human invasive infections worldwide, including pharyngitis, septic arthritis, pneumonia, endocarditis, meningitis, streptococcal toxic shock-like syndrome, cellulitis and necrotizing fasciitis (Takahashi et al., 2011; Silva et al., 2015).

Finally, *T. pyogenes* (formerly *Arcanobacterium pyogenes*) is a well-recognized coryneform bacterium related to miscellaneous opportunistic pyogenic infections among cattle, sheep, pigs and goats, species in which this microorganism is usually found on the skin, oropharynx, upper respiratory, urogenital and gastrointestinal tracts (Ribeiro et al., 2015). In contrast, *T. pyogenes* has been sporadically associated with human infections, including endocarditis, pneumonia and sepsis (Hermida et al., 2004; Levy et al., 2009).

In this study the survival of *S. suis*, *S. dysgalactiae* and *T. pyogenes* along the dry curing process of experimentally inoculated pork shoulders and loins is evaluated. Results of

this study can help to better understand the efficacy of this procedure to obtain safe RTE products free of these potential biological hazards.

Material and Methods

Preparation of the experimental dry-cured pork shoulders

Dry-cured shoulders were selected instead of dry-cured hams due to their shorter ripening period (14-18 months vs more than 24 months), which makes it possible to draw conclusions about dry-cured products in less time (Gamero-Negrón et al., 2015). Seventeen homogeneous sized pork forequarters with an average weight of 4 Kg and in a pH range from 5.5 to 6.0 were obtained from freshly slaughtered Iberian free-range pigs at a local slaughterhouse. After collection, shoulders were frozen to -20 ºC until analysis. Before inoculation, pieces were thawed at refrigeration temperature (4 ºC) for 4 days.

After inoculation (described in 2.2.2), pork shoulders were salted with a traditional 100 % NaCl formulation on plastic vats in a cold room held at 3-4 °C and 85 % relative humidity (RH) during 5 days (1.25 day/kg) following routine procedures (García-Gil et al., 2014). A small portion of salt was mixed with nitrificant salts and applied by rubbing and kneading to each shoulder as curing agents (Armenteros et al., 2012). After salting, pieces were pressure washed with warm water to remove the remaining salt from their surfaces and held at 3-4 °C and 85 % RH for 40 days (post-salting phase). Temperature was thereafter increased from 4 to 24 °C at 0.5 °C/day during 40 days and RH was progressively reduced to 65 % (drying stage). The final ripening was carried out during 14 months at room temperature (14-24 ºC) (Table 1).

Preparation of the experimental dry-cured pork loins

Ten pork loins (*Longissimus dorsi*) from freshly slaughtered Iberian free-range pigs (two loins/pig) were obtained after routine slaughter procedures at a local slaughterhouse and stored at -20 ºC until analysis. Before inoculation, loins were thawed at refrigeration temperature (4 ºC) for 2 days and divided into three pieces. A total of 28 pieces with an average weight of 0.53 Kg ± 0.06 Kg and a pH value from 5.70 to 6.15 were obtained.

After inoculation (described in 2.2.3), pork loins were rubbed with a seasoning mixture of curing agents (salt, nitrates and nitrites), spices and condiments including paprika (*Capsicum annuum*) and powdered garlic (*Allium sativum*) as detailed by Soto et al., (2008) in a relation of 48 g/Kg and macerated at 4 ºC for 5 days to allow the penetration of the seasoning mixture into the meat. Afterwards, every loin was stuffed into a collagen case (90 mm in diameter) and ripened. Products were kept at 6-7 ºC and 85 % RH for a week. Then temperature was increased to 8-10 ºC and RH was reduced to 75 % during 50 days. Finally, loins were maintained to 10-12 ºC and 75 % RH until the end of the ripening process (77 days) (Table 1).

Table 1. Conditions used along the processing of dry-cured shoulders and loins

	Stage	Time (days)	Temperature (ºC)	Relative humidity (%)
Dry-cured shoulders	Refrigeration	1	4	-
	Salting	6	4	85
	Post-salting	40	4	85
	Drying	40	4-24	85-65
	Final ripening	420	14-24	-
Dry-cured loins	Refrigeration	1	4	-
	Post-maceration	5	4	-
	Drying ^{1st} stage	7	6-7	85
	Drying ^{2st} stage	50	8-10	75
	Final Ripening	20	10-12	75

Bacterial strains and inoculum preparation

T. pyogenes (C48ABS), *S. suis* serotype 2 (S17G) and *S. dysgalactiae* spp. *equisimilis* (S32P) strains used in this study were previously isolated from pig carcasses condemned at slaughterhouse and identified by biochemical and molecular techniques (Blume et al., 2009; Cardoso-Toset et al., 2015). Bacteria were stored at -80 ºC in Brain Heart Infusion (BHI) broth (Oxoid, UK) containing 20% glycerol (Sharlau, Spain) until use.

Strains were plated on Columbia blood agar base supplemented with 5% sterile defibrinated sheep blood (Oxoid, UK) and incubated for 24 h at 37 ºC under a 5% CO_2 enrichment atmosphere. Four colonies of *T. pyogenes* were transferred to 30 mL of BHI broth supplemented with 10% bovine serum albumin (Sigma Aldrich, Spain) and incubated overnight to obtain and OD_{600} of 0.6 (5 x 10^8 CFU/ml). When streptococci were evaluated, 4 colonies were transferred to 30 ml of Todd-Hewitt broth medium (THB, Oxoid, UK) and incubated overnight at 37 ºC under aerobic conditions (pre-inoculum). Then, 0.1 ml were transferred to 9 ml of THB and incubated under the same conditions until the OD_{600} was 0.5 for *S. suis* and 0.4 for *S. dysgalactiae* (5 x 10^8 UFC/ml each). Each inoculum was subjected to ten-fold serial dilutions in a 0.9% NaCl and 0.1% sterile peptone water solution to obtain a bacterial suspension of 5-6 log CFU/ml as final inoculum. Bacterial counts were checked by plating on Columbia agar (Oxoid, UK) in each assay.

Inoculation of pork shoulders

Fifteen pork shoulders were inoculated with the evaluated microorganisms (five shoulders/pathogen) and two shoulders were inoculated with sterile PBS and kept as controls. To evaluate the efficacy of the dry-curing process to eliminate the presence of these pathogens in deeper structures of the pork shoulders (e.g. after lympho-hematic spread), the

microorganisms were injected into the meat. Five areas of 5 x 5 cm^2 were systematically marked on the meat of the inner and external side of each piece using a sterile template and sterile pins (Reynolds et al., 2001). Then, for every pathogen 1 ml of a 5 log CFU/ml inoculum was injected in each area using sterile syringes and needles (BD, Spain) and dipping approximately 2 cm into the meat. Later on, pieces were kept at room temperature (24 ºC) for 10 min to allow the attachment and absorption of the inoculum. Pieces were maintained at 4 ºC for 24 h until analysis.

Inoculation of pork loins

For each microorganism, eight pieces were inoculated by immersion for 2 min in a 6 log CFU/ml peptone water solution. Four pieces were immersed in a sterile peptone water solution and used as controls. After immersion, loins were placed on plastic racks at room temperature (24 ºC) for 10 min to allow microbial attachment and stored at 4 °C on cling film. After chilling for 24 h, each loin was irrigated with water for 15 s to select superficially located attached bacteria according to Warriner et al. (2001).

Sampling and microbiological analysis

Sampling and microbiological analysis of shoulders was performed at four stages: 24 h post-refrigeration (T1), end of post-salting stage (T2), final of drying stage (T3) and post-ripening/final product (T4). One out of the five 5 x 5 cm^2 inoculated areas of each shoulder (five shoulders/pathogen and two controls) was randomly selected in each sampling.

Dry-cured loins were also tested at four stages: 24 h post-refrigeration (T1), post-seasoning (T2), after the first week of drying (T3) and post-ripening/final product (T4). In each sampling four pieces per pathogen and two controls were randomly selected and analysed.

Before the microbiological analysis, any fungal growth on the surface of the inoculated product was removed rubbing with sterile gauzes impregnated with sterile saline solution and the external case of the dry-cured loins was discarded. Then, 10 g of each sample were aseptically collected and homogenised with 90 ml of BHI (*T. pyogenes*) or 90 ml of THB (*S. suis* and *S. dysgalactiae*) in a Masticator Classic (IUL S.A, Spain) for 1 minute (dilution 1/10). The homogenate was serially diluted (1/10) in peptone water and appropriate dilutions were plated onto Columbia agar plates supplemented with 5% sterile defibrinated sheep blood and nalidixic acid and colistine sulphate (Oxoid, Uk). Plates were incubated in a 5% CO_2 atmosphere at 37 ºC and evaluated after 24-48 h. Compatible colonies were confirmed as explained in 2.2.1. The homogenates were also incubated and plated at the same conditions to evaluate the presence of viable cells of each pathogen in cases in which the bacterial load was under the plate detection limit (< 2 log CFU/g).

Physicochemical analysis

Water activity, pH and total chlorides (expressed on a dry matter basis) of dry-cured shoulders and a_w, pH and weight loss values (expressed as percentage of the initial weight) of dry-cured loins were measured at the end of the experiments to evaluate the stability and quality of the final product. The evolution of pH and a_w values of dry-cured loins was also evaluated during each processing stage. The pH values were measured using a portable Crison penetration electrode connected to a Crison pH-meter PH25 (Crison Instruments S.A, Spain). Water activity (a_w) was measured by a computer-based dew point method (Aqualab, USA). Total chlorides were determined according to ISO 1841–2 (1996) using a potentiometric titrator 785 DMP Titrino (Metrohm, Herisau, Switzerland).

Statistical analysis

Results of bacterial load are presented as log CFU/g and expressed at mean ± standard deviation (SD). For statistical purposes, absence of each pathogen in 10 g of product was considered to be zero log CFU/g and presence of pathogens when counts were below the plate detection limit (< 2 log CFU/g) was considered to be 1.95 log CFU/g (Stollewerk et al. 2012). The values were evaluated for approximate normality of distribution by the D'Agostino & Pearson omnibus normality test (GraphPad Prims v5.0, USA). When data did not followed a normal distribution, differences among the means of control and treated groups were assessed by Kruskal-Wallis test or by Friedman test, for repeated measures, followed by Dunn's Multiple Comparison test (GraphPad Prims v5.0, USA). Comparison between two groups was performed with Mann Whitney-U non-parametric test or Wilcoxon signed rank test for non-parametric paired values (GraphPad Prims v5.0, USA). Comparison between two groups with a normal distribution of the data was assessed by paired-t test (GraphPad Prism 5, USA). Correlation between bacterial load, a_w and pH values were assessed by Pearson test (GraphPad Prism 5, USA). Differences with a $P < 0.05$ were considered to be statistically significant.

Results

Behaviour of selected pathogens in pork shoulders

The average bacterial load of the three inoculated pathogens along the dry-cured shoulders processing are showed in Table 2 and Figure 1. Bacterial counts of *S. suis* and *T. pyogenes* showed a similar behaviour, being reduced from the initial stage (T1) to the first month of drying (T3). Although *S. dysgalactiae* showed a slight increase after the first month of drying (T3), no bacterial growth was obtained at the final stage in any case. At the end of

the experiment, the final product was free of the three inoculated pathogens, showing a statistically significant reduction in the bacterial counts from T1 to T4 (P <0.05; Table 2).

Table 2. *S. suis*, *S. dysgalactiae* and *T. pyogenes* average bacterial load and logarithmic reduction along dry-cured shoulders processing. Results of bacterial load are expressed as log CFU/g (means± standard deviation).

	S. suis			***S. dysgalactiae***			***T. pyogenes***		
PP	Count	Log reduction		Count	Log reduction		Count	Log reduction	
		Units	%		Units	%		Units	%
T1	5.26 ± 0.41[a]	-	-	5.14 ± 0.37[a]	-	-	5.21 ± 0.54[a]	-	-
T2	3.88 ± 0.31[b]	1.38	26.23	3.93 ± 0.49[a,b]	1.21	23.54	3.37 ± 1.89[a,b]	1.84	35.31
T3	2.99 ± 0.82[a,b]	2.27	43.15	4.45 ± 1.17[a]	0.69	13.42	2.65 ± 2.57[a,b]	1.92	36.85
T4	0.00 ± 0.00[b]	5.26	100	0.00 ± 0.00[b]	5.14	100	0.00 ± 0.00[b]	5.21	100

PP: processing phase. T1: 24-hours post refrigeration. T2: end of the post-salting phase. T3: end of the drying phase. T4: end point (final product).
[a-b] Values within a column with different superscripts differs significantly at P< 0.05.

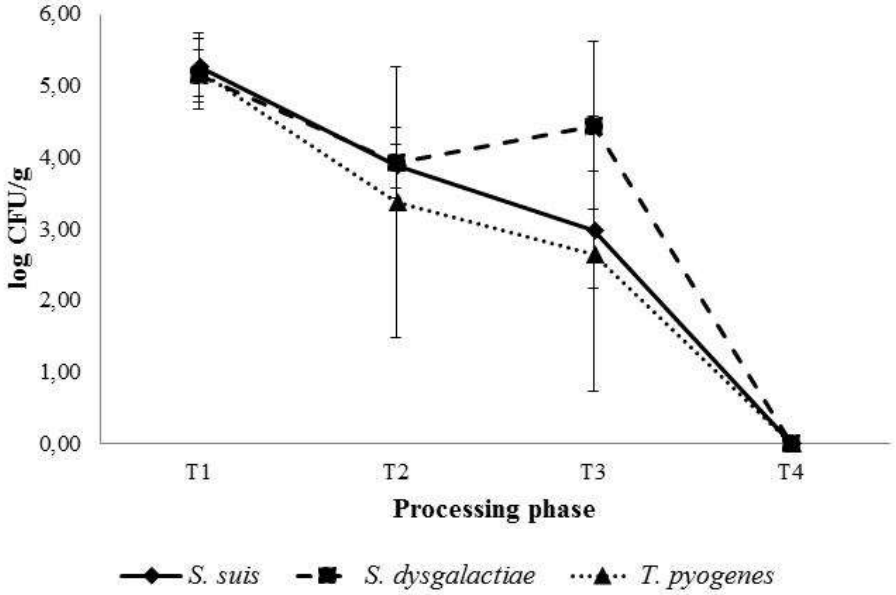


Figure 1. *S. suis*, *S. dysgalactiae* and *T. pyogenes* average bacterial load reduction along dry-cured shoulders processing. T1: 24-hours post refrigeration. T2: end of the post-salting stage. T3: drying stage. T4: final product.

Behaviour of selected pathogens in pork loins

The bacterial load values of the three evaluated pathogens along the dry-cured loins processing are showed in Table 3 and Figure 2. The three examined microorganisms

presented a statistically significant reduction of the bacterial load at the initial stages (from T1 to T3) (P <0.05; Table 3), showing a similar pattern among them. At the end of the experiment, the final product was free of the three inoculated pathogens, showing a drastic reduction in the bacterial counts from T3 to T4 (P <0.05; Table 3).

Table 3. *S. suis*, *S. dysgalactiae* and *T. pyogenes* average bacterial load and logarithmic reductions along dry-cured loins processing. Results of bacterial loads are expressed as log CFU/g (means± standard deviation).

	S. suis			***S. dysgalactiae***			***T. pyogenes***		
PP	Count	Log reduction		Count	Log reduction		Count	Log reduction	
		Units	%		Units	%		Units	%
T1	4.91 ± 0.18^{a}	-	-	4.18 ± 0.08^{a}	-	-	4.33 ± 0.31^{a}	-	-
T2	4.27 ± 0.94^{a,b}	0.64	13	3.68 ± 0.31^{b}	0.49	11.72	4.12 ± 0.37^{a}	0.21	4.85
T3	3.91 ± 0.65^{b}	1	20.37	2.93 ± 0.24^{c}	1.24	29.66	2.96 ± 0.85^{b}	1.37	31.64
T4	0.00 ± 0.00^{c}	4.91	100	0.00 ± 0.00^{d}	4.18	100	0.00 ± 0.00^{c}	4.33	100

PP: processing phase. T1: 24-hours post refrigeration. T2: post-seasoning. T3: end of the first week of drying. T4: end point (final product).
$^{a-d}$ Values within a column with different superscripts differs significantly at P< 0.05.

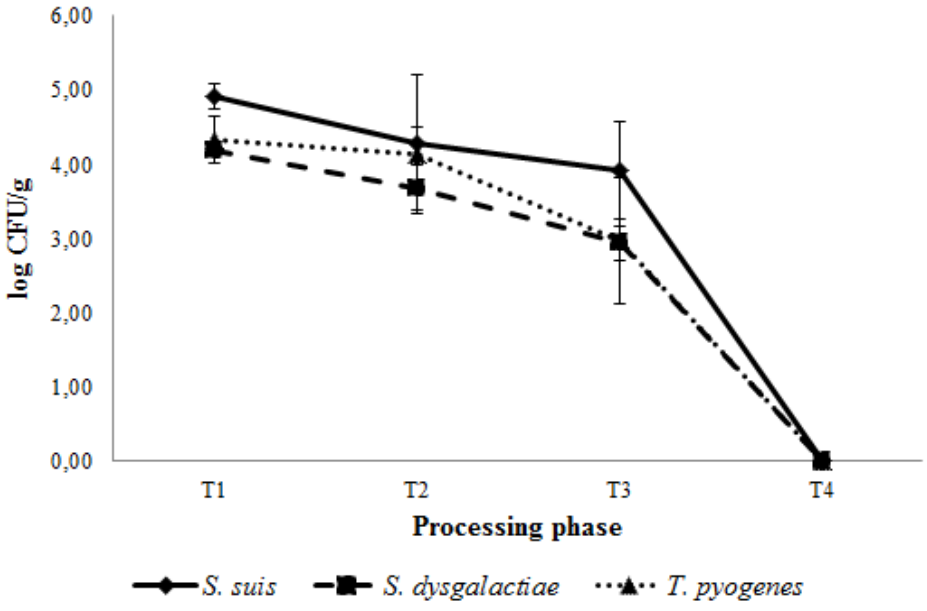


Figure 2. *S. suis*, *S. dysgalactiae* and *T. pyogenes* average bacterial load reduction along dry-cured loins processing. T1: 24-hours post refrigeration. T2: post-seasoning. T3: end of the first week of drying. T4: final product.

Physicochemical parameters of dry-cured shoulders

Results of the physicochemical parameters evaluated in dry-cured shoulders are represented in Table 4. The average end-point pH values ranged from 6.10 to 6.25. In addition, the average end-point a_w values were similar among inoculated and control groups, ranging from 0.830 to 0.851. A higher variability was observed in the salt content within and between groups with average values ranging from 13.68 % to 15.00 % of dry matter.

Table 4. End-point physicochemical parameters of the dry-cured shoulders. Results are expressed as means± standard deviation. DM: dry matter.

Parameter	**Inoculated pathogen**			
	S. suis	***S. dysgalactiae***	***T. pyogenes***	Controls
a_w	0.848±0.02	0.830±0.03	0.851±0.02	0.840±0.01
pH	6.25 ± 0.08	6.23 ± 0.14	6.10±0.24	6.25±0.14
NaCl (%DM)	15.00±1.26	13.68±2.69	14.66±0.60	14.37±0.60

Physicochemical parameters evaluated in dry-cured loins

The evolution of the physicochemical parameters evaluated along the dry-cured loins processing is showed in Table 5. A progressive reduction of a_w and pH was obtained from T1 to T4, with a final average value of a_w ranging from 0.877 to 0.883 and a final average pH value ranging from 5.49 to 5.58 (P<0.05; Table 5).

A significant positive correlation was observed between bacterial loads, a_w and pH values dynamics along the study (Table 6). As usual when dry-cured loins are fully processed, the average weight loss values at the end of the experiment reached around 40 %.

Table 5. Changes of physicochemical parameters along the manufacture of dry-cured loins. Results are expressed as means ± standard deviation.

Parameter	Inoculated pathogen			
PP	***S. suis***	***S. dysgalactiae***	***T. pyogenes***	Controls
a_w[a]				
T1	0.989±0.00[a]	0.986±0.01[a]	0.984±0.01[a]	0.985±0.01
T2	0.979±0.00[a]	0.981±0.01[b]	0.981±0.02[b]	0.980±0.00
T3	0.966±0.01[b]	0.976±0.01[b]	0.980±0.02[a,b]	0.968±0.00
T4	**0.877±0.02[c]**	**0.878±0.01[c]**	**0.883±0.02[c]**	**0.875±0.02**
pH[a]				
T1	5.89±0.14[a]	5.85±0.13[a]	5.89±0.21	5.67±0.03
T2	5.86±0.10[a,b]	5.75±0.13[a]	5.81±0.14	5.55±0.04
T3	5.75±0.01[a,b]	5.72±0.11[a]	5.70±0.09	5.49±0.06
T4	**5.58±0.21[b]**	**5.53±0.06[b]**	**5.57±0.21[*]**	**5.49±0.06**
Weight loss (%)				
T4	**39.88±2.88**	**43.18±4.31**	**43.55±3.33**	**43.90±0.37**

PP: processing stage. T1: 24 h-post refrigeration; T2: post-seasoning; T3: one week of drying; T4: end point (final product).
[a-c] Values within a column with different superscripts differs significantly ($P< 0.05$).
[*]Statistical differences between T1 and T4 $P=0.061$.

Table 6. Correlation between bacterial load, a_w and pH values along dry-cured loins processing.

	a_w	pH		a_w	pH		a_w	pH
S. suis	0.960[**]	0.700[**]	*S. dysgalactiae*	0.959[**]	0.768[**]	*T. pyogenes*	0.931[**]	0.596[*]
a_w	-	0.716[**]	a_w	-	0.702[**]	a_w	-	0.435

[**]$P<0.01$; [*]$P<0.05$

Discussion

Previous studies about the efficacy of the dry curing process against pathogenic microorganisms have been focused on selected food-borne pathogens (Reynolds et al., 2001; García-Díez et al., 2016). However, the viability of other swine pathogens that may contaminate pork at slaughterhouse has not been evaluated. In this work, the efficacy of the dry curing process to control *S. suis*, *S. dysgalactiae* and *T. pyogenes* in experimentally inoculated pork shoulders and loins is evaluated.

Four different processing stages were selected to evaluate the behaviour of the three inoculated pathogens along the dry curing process. T1 was used to assess the initial load of the inoculated bacteria. When dry-cured shoulders were evaluated, T2 was used to determine the effect of salting on the viability of the inoculated microorganisms. When dry-cured loins were evaluated, T2 was used to determine the effect of the seasoning mixture on the viability of the inoculated bacteria. Furthermore, T3 and T4 were used in both products to evaluate the effect of the drying stage and the survival of the inoculated pathogens in the final product respectively.

When dry-cured shoulders were evaluated, the bacterial count reduction of the three inoculated pathogens after salting and post-salting procedures was similar to the values obtained by others authors in dry-cured hams and shoulders inoculated with different food-borne pathogens (Reynolds et al., 2001; Latha et al., 2009). This finding can be explained by the antimicrobial effect of salt against a broad diversity of pathogens, including those evaluated in this work (Wijnker et al., 2006).

Although variations of the inoculation procedures and particularities of the dry curing process should be taken into account, these results suggest that salting has a moderate bactericidal effect and is an adequate strategy to reduce the proliferation of a multiplicity of microorganisms in contaminated meat products, including *S. suis*, *S. dysgalactiae* and *T. pyogenes*. For that reason, the food safety of reformulated salt-reduced dry-cured pork products should be properly evaluated as suggested by Stollewerk et al. (2012). Considering that the dry-cured shoulders of this study were inoculated in depth, this bacterial reduction was probably related to an adequate salt diffusion into the meat (post-salting stage) and it was perhaps reinforced by the addition of nitrificant salts, which has been proved as a

successful strategy to inhibit the growth of Gram positive bacteria such as *Clostridium botulinum* (Armenteros et al., 2012).

The behaviour of the inoculated pathogens during the drying stage of dry-cured shoulders was more heterogeneous. Although a reduction of the *S. suis* counts was evidenced between T2 and T3 phases, no changes or a slight increase were detected in the bacterial counts of *T. pyogenes* and *S. dysgalactiae* respectively. This variability was also observed between the samples obtained within the same group, in which a marked increase of the standard deviation was recorded. These results can be related to the heterogeneous structure of this product (Ventanas et al., 2001). In this sense, it should be noted that the salt concentration in the final product was variable between groups. Although undesirable, salt uptake within a batch of dry-cured salted hams or shoulders can be often highly variable (García-Gil et al., 2012). This variability has been associated with a heterogeneous behaviour along the drying process (García-Gil et al., 2014). In our study, the bacterial load reduction evidenced after drying was more evident in groups with a higher salt content at the end of the experiment.

When dry-cured loins were evaluated, the behaviour of the three inoculated pathogens showed a lower variability than observed in dry-cured shoulders. This is probably influenced by the higher homogeneity of this product. When the addition of the seasoning mixture was evaluated, a weak count reduction was observed. This result suggests a high resistance of the three inoculated pathogens to the mix of curing salts, spices and condiments used under these processing conditions. Differences observed at this stage are probably influenced by the rubbing procedure.

After a week of drying (T3) the average bacterial count of the three evaluated pathogens presented a statistically significant reduction from the initial bacterial load. Different parameters of the dry curing process such as pH and a_w may play a significant role in the viability of food-borne pathogens (Stollewerk et al., 2012). Whereas a high a_w was still observed at this stage, a decrease of pH values was recorded. This drop of pH values has been associated with the proliferation of desirable lactic acid bacteria (LAB), which may produce antimicrobial peptides able to inhibit undesirable pathogenic or spoiling bacteria in meat products (Lorenzo et al., 2010). Futures studies should be conducted to address the role of LAB in the viability of food-borne pathogens in dry-cured products.

None of the three evaluated pathogens could be recovered from dry-cured loins or dry-cured shoulders at the end of the experiment. In this sense, the marked a_w reduction observed at the final stage reinforces the hypothesis that dehydration during final drying and ripening is crucial to eliminate the examined pathogens. Future studies including different strains of each pathogen could be valuable to discard possible intra-species variability in the susceptibility of the three inoculated pathogens to the dry curing process.

Conclusion

Results of this study suggest that the dry curing process is a suitable method to obtain safe ready-to-eat pork products free of *S. suis*, *S. dysgalactiae* and *T. pyogenes*. Although salting and the addition of curing agents were able to reduce the bacterial load of these microorganisms in dry-cured pork shoulders and loins, the results of this study suggest that drying and ripening were the most important stages to eliminate them in the final product.

Acknowledgements

This study was financially supported by the Council of Economy, Science, Innovation and Employment of the Andalusian Government (AGR-2685-2012) and by the Centre for the Development of Industrial Technology (CDTI) of Spain (project reference IDI-20111632/20111633). Cardoso-Toset F was funded by a grant of the Agrifood Campus of International Excellence Programme (ceiA3) from the Ministry of Education, Culture and Sport and by the Santander Universities Global Division.

References

Arai, S., Tohya, M., Yamada, R., Osawa, Ro., Nomoto, R., 2015. Development of loop-mediated isothermal amplification to detect *Streptococcus suis* and its application to retail pork meat in Japan. Int. J. Food. Microbiol. 208, 35-42.

Armenteros, M., Aristoy, M.C., Toldrá, F., 2012. Evolution of nitrate and nitrite during the processing of dry-cured ham with partial replacement of NaCl by other chloride salts. Meat. Sci. 91, 378-381.

Blagojevic, B., Antic, D., 2014. Assessment of potential contribution of official meat inspection and abattoir process hygiene to biological safety assurance of final beef and pork carcasses. Food. Control. 36, 174-182.

Blume, V., Luque, I., Vela, A.I, Borge, C., Maldonado, A., Domínguez, L., Tarradas, C., Fernández-Garayzábal, JF., 2009. Genetic and virulence-phenotype characterization of serotypes 2 and 9 of *Streptococcus suis* swine isolates. Int. Microbiol. 12, 161-166.

Brinch Kruse, A., Halberg Larsen, M., Baek Skou, P., Alban, L., 2015. Assessment of human health risk associated with pyaemia in Danish finisher pigs when conducting visual-only inspection of the lungs. Int. J. Food. Microbiol. 196, 32-39.

Cardoso-Toset, F., Gómez-Laguna, J., Amarilla, S.P., Vela, A.I, Carrasco, L., Fernández-Garayzábal, J.F, Astorga, R.J., Luque, I., 2015. Multi-etiological nature of tuberculosis-like lesions in condemned pigs at the slaughterhouse. PLoS One. 10, e0139130.

Cheung, P.Y., Lo, K.L., Cheung, T.T., Yeung, W.H., Leung, P.H., Kam K.M., 2008. *Streptococcus suis* in retail markets: How prevalent is it in raw pork? Int. J. Food. Microbiol. 127, 316-320.

García-Díez, J., Alheiro, J., Pinto, A.L., Soares, L., Falco, V., Fraqueza, M.J., Patarata, L., 2016. Behaviour of food-borne pathogens on dry cured sausage manufactured with herbs and spices essential oils and their sensorial acceptability. Food. Control. 59, 262-270.

García-Gil, N., Santos-Garcés, E., Muñoz, I., Fulladosa, E., Arnau, J., Gou, P., 2012. Salting, drying and sensory quality of dry-cured hams subjected to different pre-salting treatments: Skin trimming and pressing. Meat. Sci. 90, 386-392.

García-Gil, N., Muñoz, I., Santos-Garcés, E., Arnau, J., Gou, P., 2014. Salt uptake and water loss in hams with different water contents at the lean surface and at different salting temperatures. Meat. Sci. 96, 65-72.

Gamero-Negrón, R., Sánchez del Pulgar, J., García, C., 2015. Immune-spaying an alternative to surgical spaying in Iberian X Duroc females: Effect on quality characteristics and fatty acid profile in dry-cured shoulders and loins. Meat. Sci. 104, 52-57.

Goyette-Desjardins, G., Auger, J.P., Xu, J., Segura, M., Gottschalk, M., 2014. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution

based on serotyping and sequence typing. Emerg. Microbes. Infec. doi: 10.1038/emi.2014.45.

Hermida Ameijerias, A., Romero Jung, P., Cabarcos Ortiz de Barrón, A., Treviño castaño M., 2004. A propósito de un caso de neumonía por *Arcanobacterium pyogenes*. An. Med. Interna. 21, 28-30.

Latha, C., Sherikar, A.T., Waskar, V.S., Dubal, Z.B., Ahmed, S.N., 2009. Sanitizing effect of salts on experimentally inoculated organisms on pork carcasses. Meat. Sci., 83, 796-799.

Lara, G.H., Ribeiro, M.G., Fujimura Leite, C.Q., Paes, A.C., Guazzelli, A., Viera da Silva, A., Barreto Santos, A.C., Paganini Listoni, F.J., 2011. Ocurrence of *Mycobacterium* spp. and other pathogens in lymph nodes of slaughtered swine and wild boars (*Sus scrofa*). Res. Vet. Sci. 90, 185-188.

Levy, C.E., Pedro, R.J., von Nowakonski, A., Holanda, L.M., Brocchi, M., Ramos, M.C., 2009. *Arcanobacterium pyogenes* sepsis in farmer, Brazil. Emerg. Infect. Dis. 15, 1131-1132.

Lorenzo, J.B., García-Fontán, M.C., Cachaldora, A., Franco, I., Carballo, J., 2010. Study of the lactic acid bacteria throughout the manufacture of dry-cured lacón (a Spanish traditional meat product). Effect of some additives. Food. Control. 27, 229-235.

Lowe, B.A., Marsh, T.L., Isaacs-Cosgrove, N., Kirkwood, R.N., Kiupel, M., Mulks, M.H., 2011. Microbial communities in the tonsils of healthy pigs. Vet. Microbiol. 147, 346-357.

Martel, A., Devriese, L.A., Descostere, A., Haesebrouck, F., 2003. Presence of macrolide resistance genes in streptococci and enterococci isolated from pigs and pork carcasses. Int. J. Food. Microbiol. 84, 27-32.

Martínez, J., Jaro, P.J., Aduriz, G., Gómez, E.A., Peris, B., Corpa, J.M., 2007. Carcass condemnation causes of growth retarded pigs at slaughter. Vet. J. 174, 160-164.

Reynolds, A.E., Harrison, M.A., Rose-Morrow, R., Lyon, C.E., 2001. Validation of Hams Cured Dry Process for Control of Pathogens. J. Food. Sci. 66, 1373-1379.

Ribeiro, M.G., Risseti, R.M., Bolaños, C.A.D., Caffaro, K.A., de Morais, A.C.B., Lara, G.H.B., Zamprogna, T.O., Paes, A.C., Listoni, F.J.P., Franco, M.M.J., 2015. *Trueperella pyogenes* multispecies infections in domestic animals: a retrospective study of 144 cases (2002 to 2012). Vet. Quart. 35, 82-87.

Silva, L.G., Genteluci, G.L., Correa de Mattos, M., Glatthardt, T., Sa Figueiredo, A.M., Ferreira-Carvalho, B.T., 2015. Group C *Streptococcus dysgalactiae subsp. equisimilis* in south-east Brazil: genetic diversity, resistance profile and the first report of human and equine isolates belonging to the same multilocus sequence typing lineage. J. Med. Microbiol. 64, 551-558.

Soto, E., Hoz, L., Ordóñez, J.A., Hierro, E., Herranz, B., López-Bote, C., Cambero, M.I., 2008. Impact of feeding and rearing systems of Iberian pigs on volatile profile and sensory characteristics of dry-cured loin. Meat. Sci. 79, 666-676.

Stollewerk, K., Jofré, A., Comaposada, J., Arnau, J., Garriga, M., 2012. The effect of NaCl-free processing and high pressures on the fate of *Listeria monocytogenes* and *Salmonella* on sliced smoked dry-cured ham. Meat. Sci. 90, 472-477.

Takahashi, T., Ubukata, K., Watanabe, H., 2011. Invasive infection caused by *Streptococcus dysgalactiae subsp. equisimilis*: characteristics of strains and clinical features. J. Infect. Chemother. 17, 1-10.

Ventanas, J., 2001. Tecnología del jamón Ibérico: de los sistemas tradicionales a la explotación racional del sabor y el aroma. Ediciones Mundi-Prensa, Madrid, Spain, 423–437.

Warriner, K., Eveleigh, K., Goodman, J., Betts, G., Gonzales, M., Waites, W.M., 2001. Attachment of bacteria to beef from steam-pasteurized carcasses. J. Food. Protect. 64, 934-937.

Wijnker, J.J., Koop, G., Lipman, L.J., 2006. Antimicrobial properties of salt (NaCl) used for the preservation of natural casings. Food. Microbiol. 23, 657-662.

Capítulo IV/ Chapter IV

Conclusiones/Conclusions

1. La qPCR dúplex utilizada en este estudio es un método adecuado para el diagnóstico rápido de la tuberculosis en el ganado porcino. La combinación en paralelo de la qPCR dúplex y el análisis histopatológico mejora la sensibilidad del diagnóstico (*objetivo 1a;* Cardoso-Toset et al., 2015, The Veterinary Journal, 204: 232-234).

2. Las pruebas serológicas pueden ser utilizadas para la vigilancia epidemiológica de la tuberculosis en el cerdo criado en extensivo, obteniendo los ensayos basados en la proteína MPB83 los mejores resultados. No obstante, se aconseja que los rebaños seropositivos sean evaluados utilizando una combinación de técnicas que mejore los valores de sensibilidad (*objetivo 1a;* Cardoso-Toset et al., 2015, The Veterinary Journal. 204, 232-234 y *objetivo 1b*; Cardoso-Toset et al., 2016, Preventive Veterinary Medicine, en revisión).

3. La linfadenitis del cerdo en extensivo presenta un carácter multietiológico destacando la implicación de las micobacterias del complejo *Mycobacterium tuberculosis*, las corinebacterias (especialmente *Trueperella pyogenes*) y distintas especies de estreptococos (*objetivo 2;* Cardoso-Toset et al., 2015, PLoS One 10, e0139130).

4. El proceso de curación de paletas y lomos del cerdo Ibérico permite obtener productos libres de *Streptococcus suis*, *Streptococcus dysgalactiae* y *Trueperella pyogenes*, siendo el factor determinante la disminución progresiva de la actividad agua (*objetivo 3;* Cardoso-Toset et al., 2017, Food Microbiology, 61, 66-71).

1- The duplex qPCR evaluated in this study is an accurate method for the diagnosis of tuberculosis in slaughtered free-range pigs. Parallel combination of duplex qPCR and histopathology improves the sensitivity of the diagnosis (*objective 1a;* Cardoso-Toset et al., 2015, The Veterinary Journal, 204, 232-234).

2- Serological techniques can be employed for the epidemiological surveillance of tuberculosis in free-range pigs, obtaining the best results with the assays based on the MPB83 protein. Nonetheless, it is advisable to check seropositive herds by a combination of diagnostic methods which help to improve sensitivity values (*objective 1a;* Cardoso-Toset et al., 2015, The Veterinary Journal. 204, 232-234 and *objective 1b*; Cardoso-Toset et al., 2016, Preventive Veterinary Medicine, *under review*).

3- Tuberculosis-like lesions in free-range pigs present a multi-etiological nature, highlighting the involvement of mycobacteria from *Mycobacterium tuberculosis* complex, coryneform bacteria (specially, *Trueperella pyogenes*) and different species of *Streptococcus* (*objective 2;* Cardoso-Toset et al., 2015, PLoS One 10, e0139130).

4- The dry curing process of Iberian pork shoulders and loins allow obtaining products free of *Streptococcus suis*, *Streptococcus dysgalactiae* and *Trueperella pyogenes*, with a prominent role for the progressive decrease in the water activity (*objective 3;* Cardoso-Toset et al., 2017, Food Microbiology, 61, 66-71).

Capítulo V/ Chapter V

Resumen/Summary

Esta tesis doctoral se presenta como compendio de publicaciones de los trabajos derivados del proyecto "Biodhesa: Impacto y medidas de control de la linfadenitis del cerdo Ibérico en la Dehesa" (CDTI; Ref. IDI-20111633) y el proyecto "Linfadenitis caseosa en ganado porcino en extensivo: desarrollo y evaluación de herramientas diagnósticas rápidas" (Consejería de Economía, Innovación, Ciencia y Empleo de la Junta de Andalucía, Ref. AGR-2685), dirigidos a reducir el impacto económico que supone la linfadenitis porcina del ganado criado en sistemas extensivos.

La linfadenitis se puede definir como el proceso que cursa con la inflamación de los nódulos linfáticos superficiales o profundos como respuesta a la infección por diferentes microorganismos, y se caracteriza macroscópicamente por la aparición de granulomas, piogranulomas o abscesos, que pueden extenderse desde los nódulos linfáticos a otros órganos por difusión linfohemática, dando lugar a la aparición de lesiones similares en otros órganos. También son conocidos en la bibliografía internacional como lesiones compatibles con tuberculosis (del inglés *tuberculosis-like lesions*, TBL). Debido al carácter subclínico de estas infecciones, en la mayoría de los casos las lesiones pasan desapercibidas y no son detectadas hasta la inspección *postmortem* en el matadero, siendo la generalización de las mismas la responsable del decomiso total de las canales afectadas.

Entre los agentes causales de la linfadenitis porcina destaca la participación de las micobacterias del complejo *Mycobacterium tuberculosis* (CMT), las micobacterias del complejo *Mycobacterium avium* (CMA) y otros géneros bacterianos, como *Rhodococcus*, *Corynebacterium* o *Streptococcus*, que poseen un riesgo zoonósico variable.

El primer objetivo de esta tesis doctoral ha sido la validación de técnicas de diagnóstico rápido de la tuberculosis (TB) en ganado porcino. Para ello se han desarrollado dos trabajos, en el primero (Cardoso-Toset et al., 2015, Vet. J., 204: 232-234) se evaluó la capacidad de una PCR dúplex a tiempo real (qPCR) para la detección de CMT en tejidos y un

ELISA indirecto que valora la presencia de anticuerpos frente a la PPD bovina en suero. Para ello, se obtuvieron muestras de nódulos linfáticos submandibulares y suero de 73 cerdos, clasificados como positivos (n=46) o negativos (n=27) en función de la presencia de lesiones compatibles y la detección del agente mediante análisis bacteriológico. En un segundo trabajo (Cardoso-Toset et al., 2016, Prev.Vet. Med., en revisión), se realizó una comparación de test serológicos basados en diferentes antígenos de *M. bovis* para el diagnóstico de la TB en el ganado porcino. Para ello, se obtuvieron 217 sueros de cerdos criados en extensivo procedentes de rebaños positivos y negativos (zonas libres de enfermedad) que se evaluaron mediante cuatro técnicas de ELISA indirecto (detección de anticuerpos frente a PPDb, MPB70 y MPB83) y un prototipo de ensayo inmunocromatográfico de flujo lateral (MPB83). Los resultados obtenidos evidenciaron que la qPCR dúplex es una herramienta adecuada para el diagnóstico rápido de la TB porcina (sensibilidad 80%, CI_{95} 68,9-99,1%; especificidad 100%, $\kappa = 0,72$ CI_{95} 0,56-0,87). Los resultados del análisis serológico (sensibilidad 66,1-78%, CI_{95} 54,0-88,5%; especificidad 98,9-100%, CI_{95} 96,7-100%; $\kappa = 0,70$-$0,81$, CI_{95} 0,58-0,91) mostraron que los ensayos evaluados son adecuados para la monitorización inicial de rebaños infectados por micobacterias del CMT, siendo los test basados en la proteína MPB83 los que presentaron los mejores resultados. No obstante, se aconseja, en función de los resultados, que los rebaños positivos sean analizados utilizando una combinación de técnicas que mejoren los valores de sensibilidad.

El segundo objetivo ha consistido en identificar los principales microorganismos aislados de cuadros de linfadenitis del cerdo en extensivo, con especial interés en la interacción entre dichos microorganismos y sus patrones de diseminación orgánica. Para ello, se tomaron muestras (n=352) de tejidos con TBL obtenidos de 171 animales decomisados por linfadenitis generalizada en dos mataderos diferentes, que fueron procesadas para su análisis histopatológico, microbiológico y de biología molecular (Cardoso-Toset et al., 2015,

PlosONE, 10, e0139130). Las lesiones piogranulomatosas fueron las más frecuentes (60,8% de los animales, 56,2% de las muestras), seguidas de los granulomas (22,2% de los animales, 20,2% de las muestras), con diferentes estadios de desarrollo. Se identificó la presencia simultánea de dos o más microorganismos diferentes en el 42,7% de los animales, siendo los más frecuentes las micobacterias del CMT (38,0%), corinebacterias (40,3%) y estreptococos (28,1%). *Trueperella pyogenes* y *Streptococcus suis* fueron los principales microorganismos no tuberculosos aislados de las lesiones en estudio. Estos resultados evidencian la existencia de un amplio grupo de microorganismos diferentes a las micobacterias asociados a las lesiones compatibles con tuberculosis en el cerdo en extensivo. Entre las conclusiones, se aconseja profundizar en el estudio del papel de las corinebacterias y los estreptococos en el desarrollo de este tipo de lesiones para prevenir errores en el diagnóstico.

Como tercer y último objetivo de este trabajo se ha evaluado cómo el proceso de curación de lomos y paletas del cerdo Ibérico afecta a la viabilidad de los principales microorganismos no tuberculosos aislados en nuestros estudios, *Streptococcus suis*, *Streptococcus dysgalactiae* y *Trueperella pyogenes*. Para ello, se realizó un ensayo experimental infectando paletas (n=17) y lomos (n=28) con un inóculo bacteriano de 5 log UFC/ml de cada patógeno y posteriormente fueron sometidos al procesado habitual que se lleva a cabo en la industria, realizando controles en diferentes fases del proceso de curación (Cardoso-Toset et al. 2017, Food. Microbiol. 61, 66-71). Al final del proceso de curación, ninguno de los microorganismos inoculados fue aislado en el producto final, poniéndose de manifiesto la capacidad del mismo para obtener productos libres de estos microorganismos. La disminución progresiva de la actividad agua durante la fase final del secado y maduración fue considerada el factor más importante para obtener productos seguros.

This PhD Thesis is presented as a compendium of publications from the research project "BioDhesa: Impact and control measures of lymphadenitis in the Iberian pig in the Dehesa" (CDTI; Ref IDI-20111633) and "Caseous lymphadenitis in free-range pigs: development and evaluation of rapid diagnostic tools" (Regional Ministry of Economy, Innovation, Science and Employment of Junta de Andalucía, Spain, Ref AGR-2685), aimed at reducing the economic impact of porcine lymphadenitis in extensive systems.

Lymphadenitis, also known in the literature as tuberculosis-like lesions (TBL), can be defined as the process that causes inflammation of superficial or deep lymph nodes in response to infection by different microorganisms, and is macroscopically characterized by the development of granulomas, pyogranulomas or abscesses, which may extend from the lymph nodes to other organs by hemolymphatic spread, leading to similar lesions in lungs, liver and spleen, among other organs. Due to the subclinical character of lymphadenitis, these lesions are not detected in most cases until the *postmortem* inspection at the slaughterhouse, with the dissemination of the lesions as the responsible for the total condemnation of the affected carcasses.

Among the causative agents of swine lymphadenitis highlights the participation of mycobacteria from the *Mycobacterium tuberculosis* complex (MTC), mycobacteria from the *Mycobacterium avium* complex (MAC) and other bacterial genera such as *Rhodococcus*, *Corynebacterium* or *Streptococcus*, which have a varying zoonotic risk .

The first aim of this PhD thesis was to evaluate the diagnostic performance of rapid methods for the diagnosis of tuberculosis (TB) in free-range pigs. To achieve this objective, two studies were performed. In the first one (Cardoso-Toset et al., 2015, Vet. J., 204, 232-234) the diagnostic values of a duplex real time PCR (qPCR) for MTC detection from tissues and a commercial indirect ELISA to detect specific antibodies against bPPD in serum samples were evaluated. Serum and submandibular lymph nodes samples from 73 pigs, classified as

positive (n=46) or negative (n=27) depending on the presence of TBL and detection of the agent by bacteriological analysis, were obtained. In a second study (Cardoso-Toset et al., 2016, Prev.Vet. Med., *under review*), a comparison of serological tests based on different antigens against MTC for the diagnosis against TB in pigs was performed. Serum samples from 217 free-range pigs from positive and negative (disease-free areas) herds were evaluated by four indirect ELISAs (with PPDb, MPB70 and MPB83 as coating antigens) and a prototype of immunochromatographic lateral flow assay (MPB83). The results from these studies showed that the duplex qPCR is a suitable tool for the rapid diagnosis of swine TB (sensitivity 80.0 %, CI_{95} 68.9 to 99.1 %; specificity 100 %, CI_{95}; $\kappa = 0.72$, CI_{95} 0.56 to 0.87). The results of the serological analyses (sensitivity 66.1 to 78 %, CI_{95} 54.0 to 88.5 %; specificity 98.9 to 100 %, CI_{95} 96.7 to 100%; $\kappa = 0.70$ to 0.81, CI_{95} 0.58 to 0.91) showed that the evaluated tests are suitable for the initial monitoring of herds infected by mycobacteria from MTC, with the tests based on the MPB83 protein showing the best results. However, according to these results, it is advisable to check seropositive herds with a combination of techniques that help improving the sensitivity values.

The second aim of this PhD thesis was to identify the main microorganisms associated with TBL in free-range pigs, with special interest on the interaction among them and their patterns of organic dissemination. In this study, a total of 352 samples belonging to 171 free-range pigs totally condemned due to generalized lymphadenitis were sampled at two slaughterhouses from southern Spain and selected for histopathological, microbiological and molecular biology analyses (Cardoso-Toset et al., 2015, PlosONE, 10, e0139130). After histological analysis, pyogranulomatous lymphadenitis was the predominant lesional pattern (60.8% of animals and 56.2% of samples), and in a lesser extent granulomas (22.2% of animals and 20.2% of samples), which showed different stages of evolution. Two or more microorganisms were simultaneously detected from these lesions in the 42.7% of the animals,

being MTC (38.0%), coryneform bacteria (40.3%) and streptococci (28.1%) the main groups of microorganisms detected after bacteriological analysis. In this sense, *Trueperella pyogenes* and *Streptococcus suis* were the most frequently non mycobacterial isolated species. Results of this study show that a wide spectrum of microorganisms different to mycobacteria can be isolated from TBL in free-range pigs. The role of corynebacteria and streptococci in such processes must be evaluated in future studies to prevent misdiagnosis based in indistinguishable gross lesions at slaughterhouse.

As a third and final objective of this PhD Thesis the impact of dry-curing process of loins and shoulders from Iberian pig on the viability of the main nontuberculous microorganisms isolated in our studies, *Streptococcus suis*, *Streptococcus dysgalactiae* and *Trueperella pyogenes* was evaluated. Loins (n=28) and shoulders (n=17) were experimentally inoculated with a bacterial inoculum of 5 log CFU/ml of each pathogen and then were subjected to the routine process performed at the industry, analyzing the viability of the selected microorganisms at different stages of the curing process (Cardoso-Toset et al. 2017, Food Microbiol. 61, 66-71). At the end of the curing process of loins and shoulders none of the inoculated microorganisms was recovered from the final product. Our results highlight a prominent role of the gradual decrease in water activity during the drying and ripening stages of the dry-curing process to obtain safe ready-to-eat products.

Capítulo VI/Chapter VI

Otras aportaciones científicas/Other contributions

- Diagnóstico laboratorial de la linfadenitis caseosa en el cerdo ibérico. XVI Simposio Anual de AVEDILA. 24-25 noviembre, Tenerife (España), 2011. Pag. 63.
- Lesional and microbiological analysis of lymphadenitis in free-range pigs. 4th European Symposium of Porcine Health Management. April 25-27, Bruges (Belgium), 2012. Pag. 98.
- Evaluación de las herramientas diagnósticas medidas de control de los principales microorganismos asociados a las linfadenitis caseoas en el cerdo Ibérico. II Congreso Científico de Investigadores en Formación en Agroalimentación de la eidA3. April 9-10, Córdoba, 2013.
- Retrospective study of condemnation associated with lymphadenitis in pigs reared in free-range systems. 5th European Symposium of Porcine Health Management. May 22-24, Edinburgh (UK), 2013. Pag. 136.
- Comparision of methods for tuberculosis diagnosis in pigs at slaughterhouse. 6th European Symposium of Porcine Health Management. May 7-9, Sorrento (Italy), 2014. Pag. 133.
- Protective effect of bacterins against lymphadenitis in free-range pig farms. 6th European Symposium of Porcine Health Management. May 7-9, Sorrento (Italy), 2014. Pag. 246.
- Evaluación de la PCR a tiempo real para el diagnóstico de la tuberculosis porcina. XIX Simposio Anual de AVEDILA. 13-14 noviembre, Zaragoza (España), 2014. Pag. 70.
- Evaluation of four ELISA assays to diagnose *Mycobacterium tuberculosis* complex infection in pigs. 8th European Symposium of Porcine Health Management. June 7-10, Dublin (Irland), 2016. Pag. 262.

- Development of a Pulse-Field Gel Electrophoresis (PFGE) method for molecular typing of *Trueperella pyogenes* isolates. 8th European Symposium of Porcine Health Management. June 7-10, Dublin (Irland), 2016. Pag. 258.

Indicios de calidad

INDICIOS DE CALIDAD DE LA TESIS APORTADOS POR EL DOCTORANDO

APELLIDOS	NOMBRE
CARDOSO TOSET	FERNANDO

TÍTULO DE LA TESIS

Linfadenitis del cerdo en extensivo: aportaciones al diagnóstico e implicaciones en la cadena alimentaria.

X Especificar la publicación que aporta como indicios de calidad de la tesis, establecidos en el artº. 25.a de las actuales normas reguladoras de los estudios de doctorado:

- Título: ***Evaluation of Rapid Methods for Diagnosis of Tuberculosis in Slaughtered free-range pigs***.
- Autores (p.o. de firma): *F. Cardoso-Toset, I. Luque, S.P. Amarilla, L. Gómez-Gascón, L. Fernández, B. Huerta, L. Carrasco, P. Ruiz. J. Gómez-Laguna.*
- Revista (año,vol.,pág.):*The Veterinary Journal* (2015) 204:232-234.
- Base de Datos Internacional o Nacional) en las que está indexada: ISI Web of Citation.
- Área temática en la Base de Datos de referencia: Veterinary Sciencies.
- Índice de impacto de la revista en el año de publicación del Artículo: 1,680.
- Lugar que ocupa/Nº de revistas del Área temática: 26/138.

- Título: ***Multi-Etiological Nature of Tuberculosis-Like Lesions in Condemned Pigs at the Slaughterhouse***
- Autores (p.o. de firma): *F. Cardoso-Toset, J. Gómez-Laguna, S.P. Amarilla, A.I Vela, L. Carrasco, J. F. Fernández-Garayzábal, R.J. Astorga. I. Luque.*
- Revista (año,vol.,pág.): *PloS One* (2015) 10 (9) e0139130, doi: 10.1371/journal.pone.0139130. eCollection 2015.
- Base de Datos Internacional o Nacional en las que está indexada: ISI Web of Citation.
- Área temática en la Base de Datos de referencia: Multidisciplinary Sciences.
- Índice de impacto de la revista en el año de publicación del Artículo: 3,057
- Lugar que ocupa/Nº de revistas del Área temática: 11/63.

Título: ***Survival of Streptococcus suis, Streptococcus dysgalactiae and Trueperella pyogenes in dry-cured Iberian pork shoulders and loins.***
- Autores (p.o. de firma): *F. Cardoso-Toset, I. Luque, A. Morales-Partera, A.Galán-Relaño, B. Barrero-Domínguez, M. Hernández, J. Gómez-Laguna.*
- Revista (año,vol.,pág.): *Food Microbiology* (2017) 61: 66-71.
- Base de Datos Internacional o Nacional) en las que está indexada: ISI Web of Citation.
- Área temática en la Base de Datos de referencia: Food Science & Technology.
- Índice de impacto de la revista en el año de publicación del Artículo: 3,682 (2015; últimos datos disponibles).
- Lugar que ocupa/Nº de revistas del Área temática: 12/125 (2015; últimos datos disponibles).

X Internacionalización tesis : presenta la tesis con mencion internacional y aporta contribuciones cientificas no incluidas como publicaciones

Córdoba, a 10 de Octubre de 2016 Firma del interesado,

Fdo.: Fernando Cardoso Toset

SR/A. COORDINADOR/A DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO

UNIVERSITY OF SURREY

Faculty of Health and Medical Sciences
University of Surrey
Guildford, Surrey GU2 7XH
UK

University of Surrey, 4 December 2015.

This letter is to confirm that Mr Fernando Cardoso Toset (DNI 30998832-F) enrolled on the Biosciences and Agrifood Sciences Doctoral Program (regulated by RD 1393/2007), at University of Cordoba (Spain), has carried out a 3 month research project at the Nutritional Sciences Department, University of Surrey, UK from August 28, 2015 to December 4, 2015.

This project has provided Fernando with the necessary knowledge and practical skills to perform the screening and evaluation of antimicrobial peptides produced by lactic acid bacteria (LAB) against pathogens of interest to Veterinary Medicine, such as *Mycobacterium* spp, *Streptococcus* spp, *Campylobacter* spp and *Staphylococcus aureus*. This study has given Fernando the opportunity to acquire more experience in laboratory methodologies to monitor the antagonistic activity of LAB when co-culturing with these pathogens.

His time spent on the project has been very productive. He has showed great commitment to this study and generated some excellent data that will eventually contribute to a peer reviewed publication. In fact, these results will be used as preliminary data for a grant application that I will submit early next year. I have always found Fernando to be very helpful, hardworking and conscientious. He has interacted very well with the other members of the lab and is an excellent team player. I wholeheartedly recommend him as an excellent researcher and colleague.

Dr Jorge Gutierrez Merino

Department of Nutritional Sciences
School of Biosciences and Medicine
Faculty of Health and Medical Sciences
University of Surrey

THE QUEEN'S ANNIVERSARY PRIZES
FOR HIGHER AND FURTHER EDUCATION
2011

Agradecimientos

Son muchas las personas que me han acompañado, ayudado, motivado o inspirado a lo largo de este proyecto de tesis doctoral. Del mismo modo, han sido muchos los lugares, vivencias y responsabilidades que han marcado este periodo. Si alguna vez pensé que esta diversificación de esfuerzos era un reto demasiado grande, hoy me doy cuenta de que ha sido la mayor de mis fortunas, pues me ha permitido compartir esta experiencia con un gran número de profesionales y personas de gran valor.

En primer lugar quiero agradecer su dedicación infinita a mis directores de tesis doctoral, los doctores Inmaculada Luque Moreno y Jaime Gómez Laguna. Sin duda, he sido muy afortunado al contar con vosotros para el desarrollo de este proyecto. Dicen que bien acaba lo que bien empieza, y no imagino un mejor comienzo que disponer de personas con vuestro grado de compromiso y vuestro saber hacer.

Inma, este folio se me queda pequeño para escribir todo lo que he aprendido contigo estos años. En primer lugar, nada de esto hubiera sido posible si no hubieras apostado por mí desde el primer día. Contigo he aprendido el valor de implicarse y sacrificarse por lo que uno cree, me has inspirado a ver siempre el lado bueno de las cosas, a centrarme en lo que de verdad importa, pero sobre todo, contigo he aprendido el valor de creer en tu equipo. Contigo es fácil sentir aquello que tantas veces te he oído decir, "ante todo, somos personas". **Jaime**, muchas gracias por haber sabido ser director, jefe, compañero y amigo al mismo tiempo, no todos saben y a ti se te da "de muerte". Gracias por haber sabido motivarme a dar un paso adelante cuando hacía falta y a la vez dejar siempre un espacio para desarrollar mi propio punto de vista. Inma, Jaime, estoy seguro de que este es solo uno de los muchos proyectos juntos que tenemos por delante. Como alguien dijo una vez, "nos costó mucho coincidir, como para dejarnos ir tan fácilmente". Gracias mil veces.

Agradecer al **Departamento de Sanidad Animal** de la Universidad de Córdoba el haber puesto a mi disposición todos los medios necesarios para acometer este proyecto. Carmen Tarradas, Alfonso Maldonado, Rafael Astorga, Belén Huerta, Lidia Gómez, Ángela Galán, Belén Barrero, Ana Lucía Solarte y Fabiana de Aguilar, muchas gracias a todos por vuestra ayuda. Ha sido y sigue siendo un placer trabajar con vosotros; es un privilegio cruzar ese pasillo lleno de ilusión y sonrisas.

Lidia, esta aventura no hubiera sido lo mismo sin ti, nunca olvidaré nuestras tardes juntos o nuestro tan comentado viaje a Dinamarca. Te admiro muchísimo, en lo profesional y sobre todo en lo personal; siempre sabes dar lo mejor de ti y por eso conseguirás todo lo que te propongas. Muchas gracias también al resto de "**las niñas**" por vuestra disposición a ayudar y por vuestra ilusión en todo lo que hacéis, no perdáis nunca esas ganas.

Agradecer también su ayuda a los compañeros del **Departamento de Anatomía y Anatomía Patológica Comparada** de la Universidad de Córdoba, los doctores Librado Carrasco y Paola Amarilla. He tenido la suerte de poder plasmar parte de vuestro conocimiento y experiencia en esta tesis doctoral. **Librado**, muchas gracias por facilitarme, entre otras muchas cosas, algunas de las imágenes y figuras que forman parte de ese manuscrito.

Dar las gracias al **Campus de Excelencia Internacional Agroalimentario (ceiA3)** y a su convocatoria "doctores en empresa" cofinanciada por Santander Universidades por haberme otorgado la oportunidad y el soporte económico para desarrollar este proyecto de investigación en el seno del sector agroalimentario.

Agradecer al **Centro de Investigación y Calidad Agroalimentaria del Valle de los Pedroches (CICAP)** el haberme facilitado todos los medios necesarios para llevar a cabo este

proyecto. Agradecer a todo el equipo su ayuda y en especial a mis compañeros de Departamento, Manuela Hernández, Antonio Serrano, Rosa García, Lourdes Sánchez, Blas Plazuelo, Ana Alicia Bolivar, Francisco Jurado; gracias por haber conseguido que todo funcione día tras día y así, haberme permitido llegar hasta aquí. **Manoli**, muchas gracias por facilitarme esos tres meses de estancia y por haber sabido entender lo importante que eran unos días de dedicación exclusiva a este proyecto. **Ana Alicia**, gracias por motivarme cada día y hacerme sentir como "tu científico favorito" cuando me hacía falta.

Muchas gracias al **Centro de Vigilancia Sanitaria Veterinaria de la Universidad Complutense de Madrid (VISAVET)** por su contribución a este trabajo. Agradecer de forma especial su labor a los doctores Lucas Domínguez, Ana Isabel Vela y José Francisco Fernández Garayzábal, así como a los compañeros Almudena Casamayor, Elisa Pulido y Verónica Sánchez por sus enseñanzas y su calurosa acogida durante mi estancia en el centro.

Agradecer también al **Departamento *Food Nutrition* de la Universidad de Surrey** y en especial al doctor Jorge Gutiérrez, el haberme acogido durante tres meses, permitiéndome optar al reconocimiento europeo de esta tesis doctoral y el haber puesto a mi disposición todos los medios y recursos necesarios para mi formación.

Dar las gracias a Pilar Ruiz y al personal del **Servicio de Microbiología del Hospital Reina Sofía** de Córdoba, por su valiosa formación y contribución al primer trabajo presentado en esta tesis doctoral.

Agradecer a **INGENASA S.A** y en especial a Paloma Rueda y Ángel Venteo los medios y el asesoramiento prestado para el desarrollo de estos estudios.

Dar las gracias al **Instituto de Investigación en Recursos Cinegéticos (IREC)** por los análisis y el asesoramiento realizado para el segundo estudio de esta tesis doctoral. Destacar la

colaboración de los doctores Christian Gortázar y María de los Ángeles Risalde en la evaluación de dichos resultados.

Agradecer también al **Centro para el Desarrollo Tecnológico Industrial (CDTI)** y a la **Consejería de Economía, Innovación, Ciencia y Empleo de la Junta de Andalucía** la concesión de los medios económicos que han dado soporte a los proyectos de los cuales derivan los trabajos presentados en esta tesis doctoral.

Dar las gracias también a los **ténicos, ganaderos y operarios** que colaboraron en este trabajo durante la selección de explotaciones y las tareas de recolección de muestras en el matadero.

Marisol Guerrero, Ana Cumplido, Rebeca Caballero, Juan Trevilla, Blanca Pages, Francisco Perán, Carmen Fernández, Maite Urbano, Matilde Blasco, Laura Barbosa y Ángela Morales, muchas gracias a todos por vuestra paciencia para escuchar mis "historias de doctorando" y soportar mis múltiples agobios y fines de semanas de encierro. No fueron pocas las veces que no pude estar con vosotros porque tenía que hacer "cosas de la tesis". Os estaré eternamente agradecido por haber sabido entenderlo, sé que no siempre ha sido fácil.

Marisol, hemos sido muy afortunados de poder compartir este proyecto juntos, como tantos otros. Estoy muy orgulloso de ti por todo lo que has conseguido y por todo lo que representas. Has sido un apoyo fundamental para mí durante este proyecto. Muchísimas gracias.

Cumpli, Fran, Juan, muchas gracias por haber creado para mí esos espacios de risas y desconexión mental que solo nosotros sabemos tener. No fueron pocas las veces que la dedicación a este proyecto me alejó un poco de vosotros, pero fueron las mismas en que os acercasteis más a mí. Gracias de todo corazón por vuestra paciencia y por creer en mí. **Laura**,

muchas gracias por enseñarme el valor de simplificar las cosas, todavía no he conseguido hacerlo tan bien como tú, pero te prometo que lo sigo intentando.

Ángela, gracias por tu ayuda y por haber hecho de mí llegada a CICAP algo tan especial. Rara es la semana que no me acuerdo de alguna de nuestras aventuras, siempre con una sonrisa. **Richard Blakey**, gracias por tu paciencia infinita con las traducciones de última hora.

Por último, agradecer **a mis padres y a mi hermano** su papel dentro de este proyecto. Nada de esto hubiera sido posible sin el apoyo y el ejemplo de **mi familia**. Esta tesis es un fiel reflejo de los valores que me habéis inculcado desde siempre, esfuerzo, trabajo, dedicación, respeto e ilusión. Agradecer a mis padres el haberme facilitado los espacios de desarrollo personal que he necesitado durante estos años y en especial agradecer a mi madre su infinita paciencia ante mis idas y venidas. Cada día de estos años he sentido vuestro apoyo, entrega y dedicación a este proyecto. El resultado de este trabajo es tan vuestro como mío.

A todos, muchísimas gracias.

Listado de abreviaturas/List of abbreviations

ADN	Ácido desoxirribonucleico
AFB	*Acid-fast bacilli*
ARNr 16S	16S ribosomal ribonucleic acid
a_w	*Water activity*
BAAR	Bacilos ácido-alcohol resistentes
BCG	Bacilo de Calmette-Guérin
BHI	*Brain heart infusión*
bTB	*Bovine tuberculosis*
CAMP	Christie Atkins Munch-Petersen test
CFP-10	Culture filtrate protein 10
CFU	*Colony-forming unit*
CI_{95}	*95% confidence intervals*
CMA	Complejo *Mycobacterium avium*
CMT	Complejo *Mycobacterium tuberculosis*
DM	*Dry matter*
DNA	*Deoxyribonucleic acid*
EEUU	Estados Unidos
EFSA	*European Food Safety Authority*
ELISA	*Enzyme linked immunosorbent assay*
ESAT-6	*Secretory antigenic target 6*
G+C	Guanina+citosina
gyrB	*DNA gyrase subunit B*
IC_{95}	Intervalo de confianza 95%
IDT	Intradermotuberculinización
IFN-γ	Interferón gamma
κ	*Kappa value*
LFD	*Lateral-flow device*
MAC	*Mycobacterium avium* complex
MNG	*Multinucleated giant cells*
MTC	*Mycobacterium tuberculosis* complex
NaCl	*Sodium chloride*
NC	*Negative cases*
OD_{600}	*Optical density measured at a wavelength of 600 nm*
OIE	Organización mundial de la Sanidad Animal

PBS	*Phosphate-buffered saline*
PC	*Positive cases*
PCR	*Polymerase chain reaction*
PP	*Processing phase*
PPD	*Purified protein derivative*
PPDa	Derivado proteico aviar purificado
PPDb	Derivado proteico bovino purificado
qPCR	*Real-time polymerase chain reaction*
RD1	*Region of difference 1*
RH	*Relative humidity*
rpoB	*β-subunit of RNA polymerase*
RTE	*Ready-to-eat*
Se	*Sensitivity*
SD	*Standard desviation*
SLN	*Submandibular lymph nodes*
Sp	*Specificity*
TB	Tuberculosis
TBL	*Tuberculosis-like lesions*
t-bPPD	*Treated bovine purified protein derivative*
THB	*Todd-hewitt broth*
VNTR	*Variable number of tandem repeats*
UE	Unión Europea
ZN	Ziehl-Neelsen