UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

CARACTERIZACIÓN FENOTÍPICA Y MOLECULAR DE *Trueperella pyogenes*: PERFIL DE RESISTENCIA ANTIMICROBIANA Y ANÁLISIS PROTEÓMICO

Phenotypical and molecular characterization of Trueperella pyogenes: antimicrobial profile and proteomic analysis

Tesis presentada por la Licenciada en Veterinaria D. Ángela Galán Relaño

para optar al Grado de Doctor en Veterinaria por la Universidad de Córdoba

Departamento de Sanidad Animal

Córdoba, 2019

TITULO: *Caracterización fenotípica y molecular de Trueperella pyogenes: perfil de resistencia antimicrobiana y análisis proteómico*

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FACULTAD ED VETERINARIA
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Ángela Galán Relaño

Tesis Doctoral

Córdoba, 2019

La realización de esta Tesis Doctoral ha sido posible gracias al proyecto “Estrategias de Control frente a la Linfadenitis del Cerdo Ibérico en extensivo”, financiado por el *Ministerio de Agricultura, Alimentación y Medio Ambiente (Rf. 20140020001824)*, y al Proyecto “Aproximaciones multiómicas al estudio de las resistencias a antibióticos en patógenos Gram-positivos”, dentro del *XXII Programa Propio de Fomento de la Investigación, Modalidad 4.2. Ayudas para potenciar el establecimiento de SINERGIAS en el desarrollo de proyectos I+D precompetitivos.*

TÍTULO DE LA TESIS: CARACTERIZACIÓN FENOTÍPICA Y MOLECULAR DE *Trueperella pyogenes*; PERFIL ANTIMICROBIANO Y ANÁLISIS PROTEÓMICO

DOCTORANDO/A: ANGELA GALÁN RELAÑO

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).

La tesis doctoral que presenta la Licenciada en Veterinaria, Dña. Angela Galán Relaño son el resultado de los trabajos desarrollados dentro del Proyecto "Estrategias de Control frente a la Linfadenitis del Cerdo Ibérico en extensivo", financiado por el *Ministerio de Agricultura, Alimentación y Medio Ambiente (Rf. 20140020001824)*, y el Proyecto "Aproximaciones multiómicas al estudio de las resistencias a antibióticos en patógenos Gram-positivos", dentro del *XXII Programa Propio de Fomento de la Investigación, Modalidad 4.2. Ayudas para potenciar el establecimiento de SINERGIAS en el desarrollo de proyectos I+D precompetitivos.*

Fruto del trabajo desarrollado, se ha publicado un trabajo en *Veterinary Microbiology*, revista indexada del *Journal of Citation Report*, que se presenta como indicio de calidad, y otros se encuentran en proceso de revisión. Asimismo, se han publicado diferentes comunicaciones a congresos de ámbito nacional e internacional. Esta tesis se presenta con mención internacional y para ello, la doctoranda ha realizado una estancia durante tres meses en la Università degli studi di Parma, Dipartimento di Scienze Medico Veterinarie, Unità Operativa Clinica Medica, Parma (Italy).

El principal objetivo del trabajo ha sido aportar información relevante para el control de las enfermedades causadas por *Trueperella pyogenes* en ganado porcino y rumiantes, y para ello se han analizado un conjunto de aislamientos procedentes de ambas especies para conocer el perfil de sensibilidad de este microorganismo a diferentes antimicrobianos y analizar las proteínas superficiales, mediante análisis proteómicos basados en la digestión de con tripsina y análisis con técnicas de LC/MS/MS para identificar proteínas superficiales que puedan ser utilizados para la elaboración de nuevas vacunas recombinantes. La doctoranda ha realizado un trabajo de elevada calidad científica, avalado por las publicaciones presentadas en revistas internacionales y nacionales, así como en comunicaciones a congresos, y reúne a nuestro juicio los méritos para optar al Grado de Doctor en Veterinaria. **Por todo ello, se autoriza la presentación de la tesis doctoral.**

Córdoba, 5 de noviembre 2019

Firma de las directoras

Fdo.: Carmen Tarradas Iglesias

Fdo.: Lidia Gómez Gascón

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Introducción / Introduction

España se ha convertido en los últimos 25 años en una de las principales potencias ganaderas de la Unión Europea, tanto por la cantidad, como por la calidad y la diversidad de sus productos. La cabaña anual ronda los 90 millones de cabezas de ganado -porcino, bovino, ovino, caprino y gallinas ponedoras-, a las que hay que sumar 1,4 millones de toneladas de carne avícola. La importancia de la ganadería en España reside en la sostenibilidad socioeconómica de zonas rurales, así como a la preservación de nuestro ecosistema (MAPA, 2019).

Actualmente, el porcino es el primer sector ganadero alcanzando el 36,4 por ciento de la Producción Final Ganadera y durante los últimos años este sector ha crecido notablemente en producción, censo y número de explotaciones. Este aumento de la producción ha superado la ya elevada tasa de autoabastecimiento (170,6 % en 2016, Fuente: SG Estadística, AEAT, INE), siendo España la cuarta potencia mundial productora (después de China, EE. UU. y Alemania) de carne porcina, por tanto el segundo puesto a nivel europeo, con una producción del 17,5 por ciento de las toneladas total producidas (datos 2016, Fuente: SG Estadísticas), otro dato a tener en cuenta es que nuestro país se ha consolidado como el segundo exportador de toda la Unión Europea detrás de Alemania (MAPA, 2019)

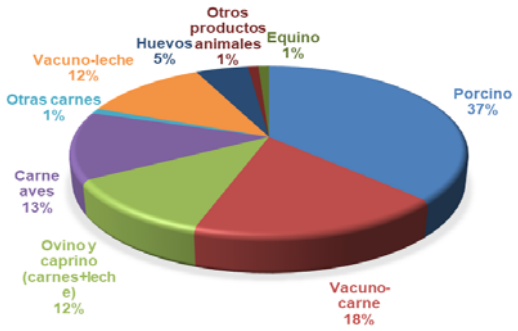

Figura 1.- Valor de la producción final ganadera 2016 (MAPAMA)

Tras el porcino y la avicultura se sitúa el ganado vacuno, que ocupa en nuestro país el 5,9 por ciento de la Producción de la Rama Agraria de España, representando

en 2016 aproximadamente el 16,7% de la Producción Final Ganadera. En cuanto al sector ovino-caprino, representa en España el 12% de la Producción Final Ganadera, si se tiene en cuenta el conjunto del subsector cárnico y lácteo. Nuestro país es el segundo en importancia de la Unión Europea en producción de ganado ovino tras el Reino Unido y el segundo país comunitario también en censo de ganado caprino, con unos 3 millones de animales por detrás de Grecia (Datos 2016 – Estadística) (MAPAMA, 2016).

El sector agroalimentario (incluyendo producción primaria, industria de transformación y comercialización) generó en el año 2018 el 9,2% del PIB (100.742 millones de euros) y el 12,3% (2,5 millones puestos de trabajo) del empleo total, siendo la quinta economía de la UE-28 que más contribuye a la generación de rentas del sector. Otro de los aspectos importantes, como se ha indicado anteriormente es su aportación a las exportaciones, en 2018 se realizaron ventas al exterior por valor de 49.502 millones de euros, aportando el 16,9 por ciento del total de bienes exportados por España, hecho que la sitúa como la cuarta economía exportadora de productos agroalimentarios de la UE-28. El dinamismo exportador se refleja en un superávit comercial del sector de 12.118 millones de euros, que es el segundo más elevado de los países de la UE-28, sólo por detrás de los Países Bajos (https://cincodias.elpais.com/cincodias/2019/07/17/economia/1563373301338688.html).

Estos datos demuestran la importancia económica de los animales de abasto en nuestro país y obligan a los responsables de la producción, sanidad y manejo a promover una gestión rentable, asegurando la calidad y seguridad alimentaria de los productos derivados (FAO, 2019). Entre los factores que disminuyen la rentabilidad, destaca la presencia de enfermedades transmisibles, víricas, bacterianas o parasitarias, algunas de gran impacto económico y sanitario, ya que originan pérdidas directas por disminución de las producciones, alteración de los productos derivados, y presencia de microorganismos o residuos tóxicos que pueden suponer un riesgo para los consumidores (MAPAMA, 2019). Dentro de las enfermedades bacterianas, nuestro trabajo está centrado en *Trueperella pyogenes*, un microorganismo que produce diferentes manifestaciones clínicas en ganado porcino y rumiantes (Ribeiro *et al*., 2015), para aportar información que puede ser de gran valor para el control de las enfermedades causadas por este patógeno.

Desde un punto de vista taxonómico, esta especie ha sido reclasificada en varias ocasiones; hasta el año 2006, estaba incluida en el género *Actinomyces,* cuando fue recalificada dentro del género *Arcanobacterium*, descrito por primera vez por Collins *et al.* en 1982, para agrupar a todas las cepas asignadas a la especie *Corynebacterium haemolyticum* (MacLean *et al.*, 1946) que compartían características morfológicas, bioquímicas y moleculares (Yassin *et al.*, 2011). Este género comprendía inicialmente nueve especies: *A*. *haemolyticum*, *A. hippocoleae*, *A. phocae*, *A. pluranimalium*, *A. abortisuis*, *A. bernardiae*, *A. bialowiezense*, *A. bonasi* y *A. pyogenes*. Posteriormente, en base a estudios quimiotaxonómicos y filogenéticos (fracción 16S del ARNr, composición fosfolipídica y presencia de vitaminas K2 o menaquinonas), se comprobó que existían diferencias entre especies, y se propuso que las especies *A. haemolyticum*, *A. hippocoleae*, *A. phocae* y *A. pluranimalium* permanecieran en el género *Arcanobacterium*, mientras que el resto de las especies debían ser incluidas en un nuevo género, denominado *Trueperella (*derivado de la palabra griega *puon* y latina *pyum*, que significa productora de pus), en honor al microbiólogo alemán Hans Georg Trüper (1936-2016), denominándose de esta forma *T. abortisuis*, *T. bernardiae*, *T. bialowiezense*, *T. bonasi* y *T. pyogenes*, respectivamente, clasificación que se mantiene desde el año 2011 (Yassin *et al*., 2011).

Ambos géneros, *Arcanobacterium* y *Trueperella* se encuentran dentro del orden Actinomycetales, y están relacionados con otros grupos bacterianos importantes tanto para el hombre como para los animales, como *Mycobacterium*, *Corynebacterium*, *Actinomyces*, *Rhodococcus*, *Dermatophilus* o *Nocardia* (Quinn *et al*., 2011). *T. pyogenes* es un bacilo corto (0,5 - 2 µm), Grampositivo, pleomórfico, inmóvil, no formador de esporas y anaerobio facultativo. Es un microorganismo que requiere para su crecimiento medios adicionados de sangre o suero y unas condiciones de microaerofilia (5% de CO_2), y temperaturas de 35 a 37 ºC (Rodríguez *et al.,* 2015).

Trueperella pyogenes se considera un patógeno extracelular que se puede adherir a células epiteliales, aunque también puede invadirlas, a pesar de no ser capaz de replicarse en ellas (Jost y Billington., 2005). Entre los principales factores de virulencia destaca la Piolisina (PLO), que tiene la capacidad de adherirse a las células del hospedador a través de receptores de colesterol, formando oligómeros para crear poros en la membrana citoplasmática y, de esta manera, causar la muerte por lisis de las células (Jost y Billington, 2005; McVey *et al*., 2013; Rodríguez *et al.,* 2015). Esta

proteína es de gran utilidad para fines diagnósticos (Machado y Bicalho, 2014; Ribeiro *et al*., 2015), ya que todos los aislamientos presentan el gen que la codifica (gen *plo*), y se ha demostrado que es muy conservado, por lo que también se ha propuesto como un futuro candidato vacunal (Billington *et al.*, 1997; Ding y Lämmler, 1996; Jost *et al.*, 1999; Risseti *et al.*, 2017; Zhang *et al.*, 2017b). Se considera un factor de virulencia de *T. pyogenes* y, hasta la fecha, el gen que codifica esta proteína (*plo*) se ha detectado en todas las cepas salvajes de la especie (Rzewuska *et al*., 2019). Esta citolisina, que pertenece al grupo de hemolisinas activadas por grupos tiol (https://www.uniprot.org/uniprot/A0A1V0D766), se puede poner de manifiesto utilizando eritrocitos de diferentes especies animales como vacas, ovejas, cerdos, caballos, pollos, conejos y humanos y es la única hemolisina reconocida en la especie (Rodríguez *et al.,* 2015).

Además, esta bacteria presenta proteínas de unión a la matriz extracelular (como la CbpA) y fimbrias (como FimA, FimB, FimC, FimE, FimG), implicadas en la unión de *T. pyogenes* a las células del hospedador y directamente relacionadas con su patogenicidad (Jost & Billington, 2005; Lui *et al.*, 2018). Otro factor de virulencia presente en este microorganismo son las neuroaminidasas (NanH y NanP), que eliminan residuos terminales de ácido siálico de carbohidratos y glicoproteínas, disminuyendo la viscosidad del moco y aumentando la susceptibilidad de los anticuerpos (IgA) a la acción de proteasas bacterianas (Jost & Billington, 2005). También se ha descrito que producen proteínas de unión al fibrinógeno y fibronectina, exoenzimas y proteasas, que permiten a la bacteria invadir células epiteliales, sobrevivir dentro de los macrófagos, proliferar en el tejido conectivo e inducir la formación de abscesos con cápsulas de tejido conectivo denso que dificultan en gran medida el tratamiento terapéutico (Zastempowska *et al.,* 2012).

T. pyogenes es un microorganismo ubicuo que se puede encontrar en la piel, orofaringe y las vías respiratorias superiores, mucosa urogenital y gastrointestinal de los animales sin producir manifestaciones clínicas (Jost y Billington, 2005). Asimismo, este agente puede contaminar los utensilios de la granja o ser vehiculado por moscas domésticas (como *Hydrotoea irritans)*, que se comportarían como vectores mecánicos (Risseti *et al.,* 2017). Se ha demostrado que es capaz de infectar a una gran variedad de animales domésticos, incluyendo perros y gatos, y animales silvestres (Ribeiro *et al.,* 2015; Brinton *et al.,* 1993; Billington *et al.,* 2002; Wareth *et al.,* 2018). En el hombre, se

han descrito casos esporádicos en personas que habitan en áreas rurales y que tienen estrecho contacto con los animales, manifestándose clínicamente con la producción de abscesos subcutáneos que pueden progresar a enfermedad sistémica (Plamondon *et al.,* 2007; Levy *et al.,* 2009; Kavitha *et al.,* 2010). Además, si bien los datos publicados en humanos son limitados, también se incluyen endocarditis, úlceras en miembros inferiores y neumonías. Según platean algunos autores, estos casos deberían ser considerados como zoonosis, aunque la transmisión del animal al hombre aún no se ha demostrado (Rzewuska *et al*., 2019). No obstante, en el año 2007 se publicó el primer caso de infección humana causada por *T. pyogenes* sin contacto identificado con animales, en el que tampoco se pudo determinar el origen de la infección (Plamondon *et al.,* 2007).

Está ampliamente demostrado que *T. pyogenes* puede causar una gran variedad de manifestaciones clínico-lesionales en la mayoría de las especies domésticas como mamitis, metritis, artritis, linfadenitis, otitis, peritonitis, piodermitis, onfaloflebitis, endocarditis, osteomielitis, bronconeumonías e infecciones urinarias y genitales (Ribeiro *et al.*, 2015; Rzewuska *et al.*, 2016). En ocasiones, se pueden producir infecciones sistémicas, en función de la edad del animal, de su estado inmune, que puede verse alterado por la presencia de otras enfermedades inmunosupresoras (Galán-Relaño *et al.*, 2015; Cardoso-Toset *et al.,* 2015), o de factores ambientales, que permiten la supervivencia de la bacteria. Esta enfermedad puede ser diagnosticada en el animal vivo o durante la inspección de canales en el matadero, que pueden ser decomisadas total o parcialmente y dar lugar a importantes pérdidas económicas para las explotaciones tanto de rumiantes como de cerdos (Martínez *et al.*, 2007; Lara *et al.*, 2011; Rzewuska *et al.*, 2012; Cardoso-Toset *et al.*, 2015; Ribeiro *et al.*, 2015).

En el ganado vacuno este patógeno está asociado, en general, a problemas de metritis y endometritis, con tasas de formas clínicas tras el parto que pueden considerarse bastante elevadas (23-52%) (Rzewuska *et al*., 2019). Estas patologías, ya sean clínicas o subclínicas, están asociadas a una disminución en la capacidad reproductiva y de la producción de leche (de Boer *et al.*, 2015; Wagener *et al.*, 2014; Carneiro *et al.*, 2016). Otros autores aseguran que *T. pyogenes* se puede considerar como uno de los agentes primarios de las mamitis, relacionado con lesiones piógenas graves del tejido mamario y la producción de una secreción láctea purulenta y

maloliente, especialmente en caso de co-infección con microorganismos anaerobios (Ishyama *et al.,* 2017).

En pequeños rumiantes*, T. pyogenes* es uno de los principales agentes involucrados en el pedero y la presencia de abscesos purulentos superficiales con múltiples localizaciones. Estas lesiones deben diferenciarse de las producidas por *Corynebacterium pseudotuberculosis*, con el que hay que establecer el diagnóstico diferencial, ya que es una de las principales causas de formación de abscesos en ganado ovino y caprino (Wani *et al.,* 2015). En cerdos, *T. pyogenes* es un agente común en cuadros de neumonía, pleuritis, endocarditis, osteoartritis, poliartritis, mastitis, infecciones del tracto reproductivo y septicemia. También en esta especie es frecuente la formación de abscesos, tanto superficiales como profundos, que dan lugar al decomiso de los animales en el matadero (Jarosz *et al*., 2014; Rzewuska *et al*., 2019).

Para llevar a cabo el diagnóstico, las muestras deben ser tomadas directamente de exudados purulentos, aspirados, tejidos o raspados de la pared de los abscesos o lesiones piogranulomatosas, mediante hisopos o jeringas, acompañadas de tejidos fijados en formalina tamponada al 10 por ciento para su estudio histopatológico (Rodríguez *et al.,* 2015). Para el aislamiento de la bacteria se debe recurrir a la siembra en medios selectivos, enriquecidos con sangre o suero, como agar sangre o agar Columbia, adicionado de un suplemento de antibiótico que inhiba el crecimiento de bacterias Gram negativas, y de sangre ovina desfibrinada estéril al 5 por ciento incubando en condiciones de microaerofilia (5% CO_2) a 37 ºC durante 48 horas, donde se observarán las colonias típicas (Quinn *et al.,* 2011; Rodríguez *et al.,* 2015). *T. pyogenes* crece en medios con sangre donde se observan colonias filamentosas, de tamaño pequeño e irregular, de color blanco y producen beta-hemólisis. Al microscopio óptico se observan bacilos Gram positivos pleomórficos en forma de letras chinas e irregulares (Fig. 2).

Fig. 2.- Placa de Agar Sangre con colonias típicas de T. pyogenes (izqda.) y observación al microscópico óptico (100x) de inmersión con la tinción de Gram (drcha.)

La identificación se basa en la morfología y características culturales, así como en pruebas bioquímicas, para ello se recomienda realizar la tinción de Gram (+), las pruebas de catalasa (-), citocromo-oxidasa (-), producción de ácido a partir de xilosa, hidrólisis de gelatina, la prueba de β-glucuronidasa (+) (Kavitha *et al.,* 2010) y la prueba de Christie Atkins Munch-Peterson (test CAMP, mostrando actividad hemolítica sinérgica con *Staphylococcus aureus*) (Fig. 3). Además, se pueden identificar con técnicas bioquímicas comerciales (API®Coryne, bioMériux, Marcy-l'Etoile, Francia). No obstante, y debido a la limitada capacidad discriminatoria de las pruebas bioquímicas, se requiere para la identificación métodos moleculares, como son la secuenciación de la fracción 16S ARNr o mediante pruebas de PCR (Reacción en Cadena de la Polimerasa), para la detección de la fracción 16S-23S ADNr, concretamente el gen *plo*, que codifica la pyolisina (Rodríguez *et al.,* 2015), o la detección de otros factores de virulencia (Zastempowska y Lassa, 2012).

Fig. 3.- Actividad hemolítica sinérgica con S. aureus (siembra en estría en la zona central de la placa). Detalle de la prueba con cuatro aislamientos de T. pyogenes)

A pesar de su importancia clínica y su frecuencia de presentación, existe un desconocimiento sobre los mecanismos de acción patógena y a su papel como agente etiológico primario en las enfermedades asociadas a este agente (Jost y Billington, 2005; Risseti *et al.*, 2017; Rzewuska *et al.*, 2016). De hecho, esta especie se puede aislar como único agente causal o bien en infecciones mixtas, debido al carácter multifactorial de las enfermedades con las que se relaciona, tanto en rumiantes (Santos *et al.,* 2010; Zastempowska y Lassa, 2012; Ribeiro *et al.*, 2015; Risseti *et al*., 2017; Ashrafi Tamai *et al.,* 2018; Dong *et al.*, 2019; Rzewuska *et al.,* 2019) como en cerdos (Contzen *et al*., 2011; Lara *et al*., 2011; Cardoso-Toset *et al.,* 2015).

El control de los procesos causados por *T. pyogenes* se basa en la aplicación de un tratamiento antimicrobiano terapéutico vía local o sistémica, y la adopción de buenas prácticas de manejo (Ribeiro *et al*., 2015; Abebe *et al.,* 2016). La elección del

antimicrobiano debe basarse en pruebas de susceptibilidad *in vitro*, que proporcionan información para el uso clínico de antimicrobianos (Ribeiro *et al*., 2015; Zhang *et al*., 2017), y evitar así el uso inapropiado o indiscriminado de los mismos, que pueden dar lugar por una parte a fallos terapéuticos, pero también a la selección y diseminación de la resistencia a los antibióticos (RAM). La resistencia antimicrobiana constituye un grave problema mundial ya que afecta a la salud humana y animal, la agricultura, el medioambiente y el comercio (AEMPS, 2015).

Entendemos por resistencia bacteriana a la capacidad de un microorganismo de evitar los efectos de un antimicrobiano (Gimeno y Ortega*,* 2005). Las resistencias pueden ser un hecho natural (el microorganismo es resistente debido a una falta de especificidad por el antibiótico) o adquirido de forma secundaria, tras mutaciones esporádicas o por transmisión de fragmentos de ADN que codifican la resistencia a determinados antibióticos. Se han identificado diferentes mecanismos de resistencia, como son la inactivación enzimática del antibiótico, cambios en la permeabilidad de la membrana interna, bombas de flujo que eliminan el antibiótico desde el interior de la célula bacteriana, y alteraciones de los precursores de la pared celular, de la membrana y de los ribosomas. Es frecuente que una bacteria que tiene altos niveles de resistencias a antibióticos posea simultáneamente varios mecanismos de resistencia (Gimeno y Ortega, 2005).

Tabla 1.- Líneas estratégicas del *Plan estratégico y de acción para reducir el riesgo de selección y diseminación de resistencias a los antibióticos* (AEMPS, 2014)

1. Vigilancia del consumo de antibióticos y las resistencias antimicrobianas	**2. Controlar las resistencias bacterianas**	**3. Identificar e impulsar medidas alternativas y/o complementarias de prevención y tratamiento**
4. Definir las prioridades en materia de investigación.	**5. Formación e información a los profesionales sanitarios.**	**6. Comunicación y sensibilización de la población en su conjunto y subgrupos de población.**

Los Organismos Internacionales que velan por la Salud Pública, advierten de las graves consecuencias de la aparición de resistencias o multirresistencias (resistencia a tres o más clases de antimicrobianos). Según los datos recogidos por la red europea de

vigilancia de resistencia a los antimicrobianos (EARS-Net) de 2015, se estima que aproximadamente 33.000 personas mueren cada año como consecuencia directa de una infección por bacterias resistentes a los antibióticos y que la carga de estas infecciones es comparable a la producida por la influenza, la tuberculosis y el VIH/SIDA combinados (https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(18)30605-4/fulltext). En el año 2011, la Comisión Europea estableció el *Plan de Acción sobre Resistencia a los Antibióticos* (CEC-748, 2011), que se recoge en el *Plan estratégico y de acción para reducir el riesgo y disminución de la resistencia a los antibióticos* (2014), denominado por las siglas PRAN y puesto en marcha por la Agencia Española del medicamento y productos sanitarios (AEMPS, 2014). Este Plan tiene 6 líneas estratégicas y abarca a todas las circunscripciones cuyo principal objetivo es el control de enfermedades (Tabla 1).

De acuerdo con estas líneas estratégicas para el control de resistencias antimicrobianas e impulso de medidas para la prevención o tratamiento de enfermedades, el desarrollo de técnicas de diagnóstico rápidas y eficaces y el estudio de la sensibilidad antimicrobiana a productos de uso común supone una de las funciones más importantes de los laboratorios de microbiología clínica (Taroco *et al*., 2006; Rodloff *et al.,* 2008) y cumple los objetivos establecidos por el PRAN en los siguientes puntos:

- Dirigir la terapéutica una vez identificado el agente etiológico.
- Generar una base de datos para seleccionar los antimicrobianos a utilizar en un tratamiento empírico.
- Desarrollar políticas de uso de antimicrobianos.
- Vigilar la aparición de mecanismos de resistencia bacteriana.
- Detectar precozmente la diseminación epidémica de una cepa, tanto a nivel hospitalario como comunitario.

Existen varias técnicas para evaluar la sensibilidad de un agente patógeno a diferentes antimicrobianos, como son las pruebas de difusión disco-placa, los métodos de dilución en caldo y la detección de genes de resistencia (Taroco *et al.,* 2006; Santos *et al*., 2010; Malinowski *et al.,* 2011; Zastempowska y Lassa 2012; de Boer *et al*., 2015; Ribeiro *et al*., 2015).

La prueba de *difusión disco-placa* se basa en la capacidad de un antimicrobiano a una concentración determinada de inhibir el crecimiento de una bacteria y se realiza en placas de cultivo con el medio agar Mueller-Hinton, adicionado o no de sangre. Este medio de cultivo se recomienda internacionalmente para la realización de las pruebas de sensibilidad *in vitro* en el laboratorio de diagnóstico. Se utilizan discos de papel de unos 6 mm de diámetro impregnados con una concentración determinada del antimicrobiano y se depositan sobre la superficie del agar, previamente inoculado con una concentración definida de la bacteria fresca en cultivo puro. Tras la incubación en estufa a 35 o 37 ºC, durante 18 a 24 horas, se miden las zonas de inhibición alrededor de los discos y se comparan con los valores publicados por organismos internacionales, como el *Clinical and Laboratory Standards Institute* (CLSI, https://clsi.org/), en América o *The European Committee on Antimicrobial Susceptibility Testing* (EUCAST, http://www.eucast.org/), en Europa, que publican guias para la realización de las pruebas e interpretación de los resultados, de manera que sean comparables entre países. El diámetro de la zona de inhibición es proporcional a la susceptibilidad de la cepa bacteriana comprobada. Este método es el más utilizado en los laboratorios de diagnóstico.

Por otro lado, la *prueba de dilución* consiste en enfrentar diferentes concentraciones del antimicrobiano (generalmente, diluciones dobles seriadas en un rango que oscila de 0,06 a 64 μg/mL) a la bacteria, a una concentración definida, medida en unidades formadoras de colonias (10^4 a 10^5 ufc/mL). En este caso, las pruebas se realizan en caldo Mueller-Hinton, adicionado o no de sangre, y puede realizarse en placas ELISA (prueba de microdilución en caldo). También pueden utilizarse placas de medio agar Mueller-Hinton, utilizando una placa por cada concentración de antimicrobiano. La concentración mínima inhibitoria (CMI) se define como la mínima concentración de antibiótico que en un periodo de tiempo predeterminado, es capaz de inhibir el crecimiento *in vitro* de un inóculo bacteriano previamente estandarizado. La prueba de dilución presenta una ventaja sobre la prueba de difusión anteriormente citada, y es que permite cuantificar y calcular la CMI; de forma que podremos conocer las concentraciones de un determinado principio activo necesarias para conseguir alcanzar niveles terapéuticos en sangre o en tejidos (de Boer *et al*., 2015; Galán-Relaño *et al*., 2019).

Para interpretar los resultados debemos conocer los puntos de corte aceptados, que suelen estar publicados y se revisan periódicamente por la *CLSI* o el *EUCAST.* La distribución CMI, junto con los estudios farmacocinéticos, son pasos críticos para establecer puntos de corte clínicos (Dalhoff *et al*., 2009). Además, el EUCAST también ha establecido los denominados puntos de corte epidemiológicos (por sus siglas en inglés, *epidemiological cut-offs* ECOFF), que separan las poblaciones que carecen o no expresan mecanismos de resistencia de aquellas que los presentan y expresan. Los puntos de corte nos permiten categorizar a los aislamientos como sensibles, intermedios o resistentes en función de la probabilidad del éxito o del fracaso terapéutico (Canton, 2010):

- Sensible: cuando un aislado bacteriano es inhibido *in vitro* por una concentración de un antimicrobiano que se asocia a una alta probabilidad con el éxito terapéutico.
- Intermedio: cuando un aislado bacteriano es inhibido *in vitro* por una concentración de un antimicrobiano que se asocia a un efecto terapéutico incierto.
- Resistente: cuando un aislado bacteriano es inhibido *in vitro* por una concentración de un antimicrobiano que se asocia a una alta probabilidad con el fracaso terapéutico.

No obstante, la información que se dispone actualmente sobre el comportamiento de *T. pyogenes* frente a los antimicrobianos utilizados en ganadería es limitada (Rogovsky *et al.,* 2018), y hasta hace tres años, no existían normas publicadas para la realización de las pruebas de sensibilidad *in vitro* para esta especie, que permitieran comparar los resultados entre estudios. En el año 2016, la CLSI publicó un nuevo documento para estandarizar los métodos para la realización de las pruebas e interpretación de resultados con bacterias poco frecuentes y de difícil crecimiento, aisladas de animales, el VET06 (*Methods for Antimicrobial Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria Isolated From Animals, 1st Edition*), que junto con el documento CLSI M45 (*Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria*, 3rd edition), se aplican para el estudio de bacterias corineformes, incluida *T. pyogenes*. El documento VET06 establece puntos de corte para la categoría susceptible para cuatro antimicrobiano, penicilina, ampicilina, eritromicina y trimethoprim/sulfametoxazol, pero no para otros antimicrobianos de uso frecuente en ganadería (CLSI, 2017).

Atendiendo a estas premisas, las pruebas para valorar la sensibilidad o resistencia de *T. pyogenes* aislada de animales se basan en los métodos e interpretación de resultados de otras bacterias Gram positivas que afectan a los animales o al hombre, como *Streptococcus o Staphylococcus*, o bien en función de la especie animal de aplicación o por la eficacia clínica demostrada en el control de los brotes (Zastempowska y Lassa, 2012; de Boer *et al.,* 2015; Ribeiro *et al.,* 2015; Rhodes *et al.,* 2015; Moreno *et al.,* 2017). Estos datos dificultan la comparación de los resultados entre los estudios (Feßler y Schwarz, 2017).

Los resultados publicados muestran una gran variabilidad, y en algunos casos valores elevados, de CMI de diferentes clases de antimicrobianos (β-lactámicos, macrólidos, lincosamidas, tetraciclinas, aminoglucósidos, fenicoles, sulfonamidas, diaminopirimidinas y fluoroquinolonas) frente a *T. pyogenes* (Feßler y Schwarz, 2017). Entre todos los antimicrobianos, resulta de interés el grupo de las tetraciclinas, los estudios realizados muestran valores de resistencia de *T. pyogenes* que oscilan entre el 50 y el 85 por ciento, con valores más altos para cepas aisladas de ganado porcino que en bovino (Guérin-Faublée *et al.*, 1993; Yoshimura *et al.*, 2000; Trinh *et al.*, 2002), que podría explicarse por el mayor consumo de antimicrobianos en ganado porcino intensivo frente a los rumiantes (Trinh *et al.*, 2002).

Las tetraciclinas han sido el tratamiento de elección para el control de enfermedades en animales y en el hombre, y según diferentes investigadores, es una la de las causas de que la mayoría de las bacterias de importancia en medicina veterinaria presenten mecanismos de resistencia frente a estos antimicrobianos. De hecho, según los datos publicados por la EFSA y ECDC (2019) donde se aportan datos de resistencia a los antimicrobianos en bacterias zoonóticas e indicadoras en Europa, 2017, incluyendo *Salmonella, Campylobacter, y Escherichia coli* en alimentos, animales y humanos, nos permiten comprobar que los porcentajes de cepas resistentes de *E. coli* obtenido de cerdos frente a Tetraciclina supera el 88 por ciento y en terneros el 52 por ciento (https://www.efsa.europa.eu/en/interactive-pages/AMR-Report-2017). Si revisamos los datos de consumo en animales de producción (Fig. 5), comprobamos que las tetraciclinas son uno de los grupos de antimicrobianos más consumidos en España, aunque según los últimos datos, este consumo ha descendido en el año 2016 (http://www.resistenciaantibioticos.es/es/system/files/field/files/informe_jiacra-espana.pdf?file=1&type=node&id=410&force=0, pp. 36)

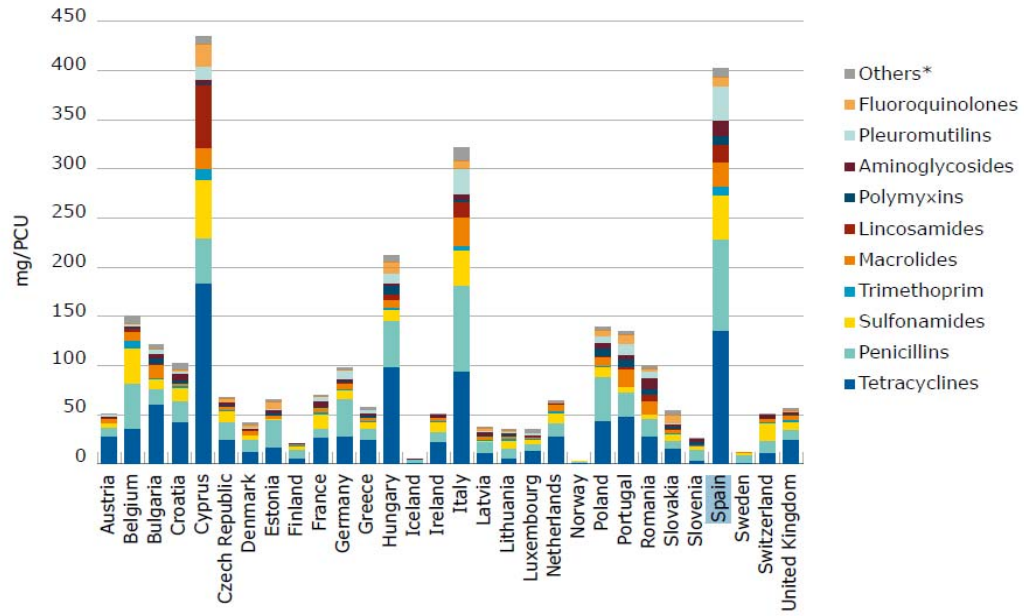

Figura 4.- Venta de antibióticos en la UE en el año 2015 por unidad ganadera (EMA, 2017).

Los mecanismos de resistencia pueden ser naturales o adquiridos, siendo estos últimos de gran importancia, ya que son producidos por una modificación genética de la bacteria (Daza, 1998). Estos genes, si se expresan, confieren protección frente al antimicrobiano mediante diferentes mecanismos, como la destrucción del antimicrobiano, la impermeabilización de la membrana bacteriana, o las mutaciones en la diana o la bomba de eflujo (McCarter, 2005). En *Trueperella pyogenes*, se han detectado genes de resistencia a los grupos macrólido-lincosamida-estreptogramina (*gen ermB* y *gen ermX*), a tetraciclina (tetW, tetZ) y a aminoglucósidos (aadA1, aadA5, aadA24 y aadB en casetes cromosómicos) (Billington *et al.,* 2002; Trinh *et al.,* 2002; Liu *et al.,* 2009; Zastempowska y Lassa, 2012;). Además, se ha comprobado que presentan genes de resistencia a los fenicoles (*cmlA6*), trimetoprim (*dfrB2a*) o a β-lactamicos (*blaP1*) (Liu *et al.,* 2009; Zhao *et al.,* 2011). Además de la importancia que puede tener para el control de los procesos causados por esta bacteria, existe el riesgo de que pueda convertirse en reservorio de genes de resistencia a antibióticos (Baele *et al.*, 2001) para otros patógenos. Por ejemplo, la proteína codificada por el gen *tet*(W) en *T. pyogenes* tiene una similitud del 80% con el *tet*(W) de *B. fibrisolvens*, una bacteria anaerobia del rumen bovino (Billington *et al.*, 2002).

El conocimiento del perfil de resistencia antimicrobiana de las bacterias patógenas para animales es un paso decisivo para conseguir unos resultados eficaces. Además, debemos optar por reducir su uso mejorando el estado sanitario de los animales, y reforzando la bioseguridad, de forma que se reduzcan las oportunidades para el mantenimiento y contagio en la explotación, y evitar la transmisión. Para ello, se debe realizar un adecuado diagnóstico, identificar el agente causal y determinar el perfil de resistencia a diferentes antimicrobianos, antes de seleccionar el más apropiado, considerando la vía, dosis y frecuencia de administración. También debemos mantener un programa de vigilancia, evaluar nuevas opciones de tratamientos y asegurar de forma estricta los tiempos de supresión (Gimeno y Ortega, 2005).

La AMPS (2015) dentro de las lineas estratégicas de actuación para reducir el uso de antimicrobianos (Tabla 1) proponía *Identificar e impulsar medidas alternativas y/o complementarias de prevención y tratamiento* (línea prioritaria 3). Entre ellas, la vacunacion como medida preventiva es una de las más recomendables. No obstante, hasta la fecha no existen vacunas comerciales para el control de los procesos producidos por *T. pyogenes,* utilizándose para el control de brotes vacunas inactivadas experimentales, seleccionando los aislamientos obtenidos a partir de animales enfermos o decomisados, y que hayan sido correctamente identificados (Cardoso-Toset et al., 2014), y realizando el seguimiento de los animales vacunados, con estudios microbiológicos de animales que presenten signos clínicos compatibles con un proceso crónico, así como la recopilación de los datos de decomisos obtenidos en el matadero y la posible intervención de otras enfermedades que puedan tener efectos negativos sobre la respuesta inmune, con el objetivo de detectar posibles fallos vacunales y poder renovar las cepas en caso necesario (Cardoso-Toset et al., 2019). No obstante, estas vacunas presentan algunas desventajas relacionadas con la estabilidad, el coste y el manejo, ya que son necesarias varias dosis y revacunaciones anuales.

La aplicación de vacunas eficaces constituye sin duda uno de los grandes retos de la investigación mundial, y una de las lineas más interesantes de trabajo es el descubrimiento y posterior utilización de antígenos comunes, muy conservados, y que tengan gran capacidad inmunógena, que serán utilizados en vacunas de nueva generación (Gómez-Gascón *et al.,* 2013). Para ello, es importante la formulación de la vacuna, siendo necesario evaluar las propiedades intrínsecas del antígeno y seleccionar el adyuvante que se incorporará para obtener una respuesta inmune adecuada (Gomez-

Gascón *et al.,* 2018). Hasta la fecha, existen limitados estudios en *Trueperella pyogenes*, aunque recientemente se ha probado una vacuna de ADN utilizando el gen de la PLO, cuyos resultados preliminares en ratones han dado buenos resultados (Huang *et al.,* 2016).

Para la búsqueda de candidatos vacunales, el análisis proteómico constituye una técnica de elección para la detección de antígenos superficiales en bacterias de importancia en medicina humana y veterinaria (Rodríguez-Ortega *et al., 2008*, Baums *et al., 2009*; Gómez-Gascón *et al.,* 2012). La proteómica es una rama de la genómica que estudia los *proteomas*, conjunto de proteínas que se expresan a partir del genoma de un organismo. La principal herramienta de la investigación proteómica es la espectrometría de masas (EM), una tecnología que incluye la instrumentación (espectrómetros de masas), métodos de adquisición y software de análisis de datos. En estudios previos, hemos aplicado técnicas proteómicas, basadas en el "afeitado" con tripsina de células vivas seguido de la cromatografía líquida/espectrometría de masas (LC/MS), acoplada a espectrómetros de masa en tándem (LC/MS/MS), que han permitido obtener nuevos candidatos vacunales para el desarrollo de vacunas recombinantes frente a *Streptococcus pneumoniae* y *Streptococcus suis* (Olaya-Abril *et al.,* 2012; Gómez-Gascón *et al.,* 2013).

En relación a los antígenos que pueden utilizarse, las proteínas de superficie bacteriana, que desempeñan un papel fundamental en la interacción célula-hospedador y están expuestas al sistema inmune, pueden ser buenos candidatos para la fabricación de vacunas (Rodríguez-Ortega *et al.,* 2006). Como en otras bacterias, estas juegan un papel clave en la interacción con sus hospedadores, ya que participan en procesos importantes para el desarrollo de las infecciones, como son la adhesión e invasión de células hospedadoras, toxicidad y evasión del sistema inmune. Pero también, al estar más expuestas, tienen una alta posibilidad de entrar en contacto con los elementos del sistema inmune del individuo, y por lo tanto ser reconocidas como antígenos (Navarre *et al.,* 1999). Entre las proteínas de las bacterias que cumplen esta función se encuentran las proteínas secretadas (ya sean solubles, liberadas libremente, o encapsulado dentro de una estructura vesicular) y las proteínas ancladas a la superficie, entre las que se encuentran las de la membrana lipídica externa y las de pared celular (motivo LPXTG) (Gómez-Gascón et al., 2018).

Otro de los puntos críticos para el desarrollo de nuevas vacunas es el adyuvante a utlizar, ya que pueden influir en la respuesta inmune inducida por la vacuna. En medicina veterinaria, existen varios adyuvantes para su uso en vacunas, a base de sal mineral, emulsiones de aceite en agua ó agua en aceite y saponinas. En la mayoría de las ocasiones, se utilizan los adyuvantes en base a la bibliografía consultada, buscando un tipo de respuesta de tipo 1 o 2, sin tener en cuenta las características del antígeno con el que se va a combinar, y esto puede tener consecuencias negativas en el producto final (Li *et al.,* 2016; 2007). Por ello, una vez seleccionado el antígeno, se deben valorar en estudios *in vitro* e *in vivo* la eficacia de los productos obtenidos (Gómez-Gascón *et al.,* 2014, 2018).

Estos antecedentes y los resultados obtenidos hasta la fecha para el control de las infecciones producidas por *T. pyogenes* en nuestras explotaciones, han servido de base para plantearnos la hipótesis de partida de nuestro trabajo, con la finalidad de conseguir un antimicrobiano adecuado e identificar una proteina que pueda ser considerada como candidato en la fabricación de futuras vacunas frente a este patógeno

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Capítulo II / Chapter II:

Objetivos / Objetives

El **principal objetivo** de esta tesis doctoral es aportar información relevante para el control de las enfermedades causadas por *Trueperella pyogenes* en ganado porcino y rumiantes, especies donde esta bacteria tiene mayor importancia sanitaria y económica. Para desarrollar este objetivo, se han llevado a cabo tres estudios con los siguientes objetivos específicos:

Objetivo 1: Estudio de la sensibilidad antimicrobiana y diversidad genética de cepas de *Trueperella pyogenes* aisladas de ganado porcino.

Estudio 1: "Antimicrobial susceptibility and genetic characterization of *Trueperella pyogenes* isolates from pigs reared under intensive and extensive farming practices" Este trabajo ha sido publicado en la revista Veterinary Microbiology, vol. 232, pp: 89-95, https://doi.org/10.1016/j.vetmic.2019.04.011 y se presenta como indicio de calidad para la lectura y defensa de la tesis doctoral (Con un factor de impacto según el Journal Citation Report (2017), de 2,524, situado en el primer decil (8/140) dentro de la categoría de Ciencias Veterinarias.

Objetivo 2: Estudiar la posible relación epidemiológica entre aislamientos de *Trueperella pyogenes* obtenidos de porcino de rumiantes

Estudio 2: "Antimicrobial susceptibility of *Trueperella pyogenes* isolated from food-producing ruminants" Este trabajo ha sido enviado a Veterinary Microbiology en agosto de 2019 y se encuentra actualmente en revisión.

Estudio 3: "Perfil de sensibilidad antimicrobiana de *Trueperella pyogenes*: aportaciones para el control en animales de abasto". Este trabajo ha sido presentado en el XXIV Simposio de AVEDILA (Asociación de Especialistas en Diagnóstico Laboratorial Veterinario) en forma de comunicación oral. Noviembre 2019, Pamplona, España.

Objetivo 3: Identificar y seleccionar las proteínas de superficie comunes de *Trueperella pyogenes* con el fin de desarrollar una vacuna eficaz frente a la infección producida por este microorganismo.

Estudio 4: "Study of *Trueperella pyogenes* pan-surfome as source of putative vaccine candidates" Este trabajo está enviado a PLOS One y se encuentra actualmente en revisión.

The **main objective** of this work is to provide relevant information for the control of diseases caused by *Trueperella pyogenes* in pigs and ruminants, species where this bacterium has greater sanitary and economic importance. To develop this objective, three studies have been carried out with the following specific objectives:

Objective 1: Study of antimicrobial susceptibility and genetic diversity of strains of *Trueperella pyogenes* isolated from pigs.

Study 1: "Antimicrobial susceptibility and genetic characterization of *Trueperella pyogenes* isolates from pigs reared under intensive and extensive farming practices".

This work has been published in the journal Veterinary Microbiology, vol 232, pp: 89-95, https://doi.org/10.1016/j.vetmic.2019.04.011 and is presented as a quality parameter for the defence of the doctoral thesis (with an impact factor according to the Journal Citation Report (2017), of 2,524, located in the first decile (8/140) within the category of Veterinary Sciences).

Objective 2: study the possible epidemiological relationship between *Trueperella pyogenes* isolates obtained from pigs and ruminants

Study 2: "Antimicrobial susceptibility of *Trueperella pyogenes* isolated from food-producing ruminants". This manuscript was sent to Veterinary Microbiology in August, 2019 and it is under review.

Study 3: "Antimicrobial susceptibility profile of *Trueperella pyogenes*: contributions to its control in livestock animals". This work has been presented as an oral communication in XXIV Symposium of AVEDILA (Asociación de Especialistas en Diagnóstico Laboratorial Veterinario). November 2019, Pamplona, Spain.

Objective 3: To identify and select common surface *Trueperella pyogenes* proteins to develop an effective vaccine against the infection produced by this microorganism.

Study 4: "Study of *Trueperella pyogenes* "pan-surfome" as source of putative vaccine candidates". This manuscript was sent to PLOS One and it is under review.

Capítulo III / Chapter III:

Estudios / Studies

Objetivo 1/ Objective 1

Objetivo 1: Estudio de la sensibilidad antimicrobiana y diversidad genética de cepas de *Trueperella pyogenes* aisladas de ganado porcino.

Objective 1: Study of antimicrobial susceptibility and genetic diversity of strains of *Trueperella pyogenes* isolated from pigs.

Study 1: Antimicrobial susceptibility and genetic characterization of Trueperella pyogenes isolates from pigs reared under intensive and extensive farming practices

Galán-Relaño *et al.*, 2019, *Veterinary Microbiology*, 232, 89-95

Antimicrobial susceptibility and genetic characterization of Trueperella pyogenes isolates from pigs reared under intensive and extensive farming practices

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Short title: *Trueperella pyogenes* in pigs

Abstract

Trueperella pyogenes is an opportunistic pathogen associated with a variety of diseases and responsible for important economic losses for pig production. Minimal Inhibitory Concentration (MIC) and Pulsed Field Gel Electrophoresis (PFGE) typing analysis were used to determine the MIC distribution and to genetically characterize a total of 180 *T. pyogenes* isolates obtained from slaughtered pigs reared under intensive (TpIN, n=89) and extensive (TpEX, n=91) farming practices. Low MIC_{90} values for penicillin and amoxicillin (0.008 and 0.06 µg/ml, respectively), ceftiofur, gentamicin and enrofloxacin (1 µg/ml, respectively) were obtained, so they could be of choice for the empiric treatment of *T. pyogenes* infections. Except for the penicillin, amoxicillin and ceftiofur, a statistically significant difference was observed in the MIC distribution of all antimicrobials analysed between TpIN and TpEX isolates. Also, MIC_{90} values were higher in TpIN than in TpEX isolates for neomycin and streptomycin (32 µg/ml *vs* 8 µg/ml), sulfamethoxazole/trimethoprim (30.4/1.6 µg/ml *vs* 1.90/0.10 µg/ml) and tylosin (≥1024 µg/ml *vs* 1 µg/ml). A relatively lower genetic diversity was detected in TpIN in comparison with TpEX isolates (GD 0.42 and GD 0.47, respectively). All isolates were distributed in three clusters (A, B, C). TpIN isolates were statistically associated with cluster A (*P = 0.0002*; OR 3.21; CI_{95} 1.74-5.93), whereas the TpEX were distributed throughout the dendrogram, showing more genetic diversity. These data suggest that the antimicrobial susceptibility and genetic variability of the *T. pyogenes* isolates could be influenced by the management systems.

Keywords: Pigs, management sytems, *Trueperella pyogenes*, MIC, PFGE.

1. Introduction

Trueperella pyogenes (formerly *Arcanobacterium pyogenes*) is considered an opportunistic pathogen commonly found on the skin, oropharynx, and upper respiratory, urogenital and gastrointestinal tracts of livestock, that causes different clinical manifestations, in domestic and wildlife animals (Billington et al., 2002; Ribeiro et al., 2015; Rzewuska et al., 2016). Diseases caused by this pathogen are especially important in cattle and swine and are related to a variety of suppurative infections such as metritis, udder lesions, abscesses, pneumonia, arthritis, endocarditis or osteomyelitis (Santos et al., 2010; Zastempowska and Lassa, 2012; Jarosz et al., 2014). The participation of this agent in pyogranulomatous lesions in condemned pigs at slaughterhouses has also been evidenced (Martinez et al., 2007; Cardoso-Toset et al., 2015).

The control of this disease is mainly based on antimicrobial therapy (Ribeiro et al., 2015). Therefore, antimicrobial susceptibility tests provide an empiric basement for clinical use of antimicrobial agents, avoiding therapy failure and the possible development of antimicrobial resistances (Ribeiro et al., 2015; Zhang et al., 2017). The Broth Microdilution Method to determine the Minimum Inhibitory Concentration (MIC) of any antimicrobial agent is worldwide recommended and specific methods to test infrequently isolated or fastidious bacteria have been recently published (CLSI, 2016, 2017). Currently, the monitoring of antimicrobial resistance of pathogens affecting livestock is strongly advisable, with special attention to those antimicrobials of use in human medicine. Nevertheless, only limited information on the antimicrobial susceptibility and valid breakpoints for *T. pyogenes* isolates from animals exist (Feßler and Schwarz, 2017).

Pig production is focused on a limited number of countries, representing Spain the third largest producer in the European Union, behind Denmark and Germany (Eurostat, 2017). Two pig production systems exist in Spain; intensive and free-range systems, being this last one also practised in other countries (Di Marco et al., 2012; Oliveira et al., 2014; Konsgted and Sørensen, 2017). Outdoor production systems with pastures are becoming attractive for consumers, mainly due to environmental sustainability and social benefits of the sector as well as the high quality of the pork products, extremely appreciated by consumers (Estevez et al., 2004). Management systems can influence the health status and prevalence of different diseases (Cardoso-

Toset et al., 2015; Galán-Relaño et al., 2015; Konsgted and Sørensen, 2017), although the behaviour of *T. pyogenes* in both populations is not known.

The molecular characterization of isolates responsible for the emergence of clinical outbreaks is an important step for the implementation of an adequate control program. The Pulsed-Field Gel Electrophoresis (PFGE), has been revealed as one of the most powerful molecular typing methods and has been successfully applied to different bacterial pathogens (Vela et al., 2003). This makes PFGE a suitable option to determine the genetic diversity of *T. pyogenes*.

Therefore, the main objectives of this study were to determine the MIC distribution of 11 antimicrobials against *T. pyogenes* and to explore the genetic diversity of isolates belonging to pigs reared under intensive and extensive production systems. Our results will contribute to the selection of adequate control measures against this pathogen.

2. Material and methods

2.1. Bacteria isolates and identification

A total of 180 *T. pyogenes* isolates were included in this study. They were obtained at different slaughterhouses located in Spain from partial or totally condemned pigs after veterinary inspection (Regulation 2004/854/EC). Pigs were reared under intensive (TpIN; n=89) or extensive (TpEX; n=91) farming conditions and belonged to 18 and 20 different herds, respectively. Samples were obtained from lungs (n=67), lymph nodes (n=56), joints (n=16), liver (n=13), heart (n=9), spleen (n=7), abscesses (n=6), brain (n=3), kidney (n=2) and tonsil (n=1) with macroscopic lesions of pneumonia, endocarditis, arthritis, lymphadenitis, abscess or pyogranuloma-like lesions (Table 1).

Table 1. *Trueperella pyogenes* isolates from pigs reared under intensive and extensive production systems.

Source	Rearing system		Total
	Intensive (TpIN)	Extensive (TpEX)	
Lung	50	17	67
Lymph nodes	5	51	56
Joints	16	0	16
Liver	0	13	13
Heart	9	0	9
Spleen	0	7	7
Abscesses	4	2	6
Brain	3	0	3
Kidney	2	0	2
Tonsil	0	1	1
Total	**89**	**91**	**180**

Samples were plated on Columbia CNA agar (Oxoid ltd., Hampshire, UK) supplemented with 5% sterile defibrinated sheep blood. Plates were incubated under microaerophilic conditions (5% CO_2) at 37 ºC for 24 to 48 h (Feβler and Schwarz, 2017). Coryneform isolates (Gram-positive, catalase variable and oxidase negative irregularly shaped rods) were identified by a species-specific Real Time-PCR assay based on the detection of the pyolysin gene (*plo*), as previously described (Ülbegi-Mohyla et al., 2010). After identification, bacteria were frozen and stored at –80ºC until their study.

2.2. Antimicrobial susceptibility test

The antimicrobial susceptibility of the isolates was determined by broth microdilution method performed as outlined by the Clinical and Laboratory Standards Institute for fastidious organisms (CLSI, 2016, 2017). The following antimicrobial agents (Sigma Aldrich Co., USA) were used: penicillin G, amoxicillin, ceftiofur, apramycin, gentamicin, neomycin, streptomycin, enrofloxacin, oxytetracycline, tylosin and sulfamethoxazole/trimethoprim (19:1). One hundred microliters of serial twofold dilutions of each antimicrobial agent in cation-adjusted Mueller-Hinton broth (MHB) with 5% (v/v) of lysed horse blood were dispensed in U-bottom 96-well microtiter plates (Lab-Center, Spain). An equal volume of adjusted inoculum ($5x10^5$ CFU/ml) was added to each well of the microplate up to a final volume of 200 microliters. Microdilution

plates were read after 24 h incubation at 37 °C with 5% CO_2. *Streptococcus pneumoniae* ATCC 49619 was included as quality control.

For every antimicrobial agent, the Minimum Inhibitory Concentration (MIC) values were obtained. MIC_{50} and MIC_{90} values for the respective antimicrobial agents were also determined (Schwarz et al., 2010). Breakpoints applicable to *Trueperella pyogenes* for penicillin and sulfamethoxazole/trimethoprim were used according to CLSI (2017).

2.3. Pulsed Field Gel Electrophoresis (PFGE) typing

T. pyogenes isolates were grown on blood agar plates with 5% defibrinated sheep blood (Oxoid ltd) and incubated under microaerophilic conditions (5% CO_2) at 37 ºC for 24 to 48 h. Then, isolates were harvested for preparing agarose plugs as previously described by Vela et al., (2003). DNA plugs were equilibrated in restriction buffer for 30 min at 37ºC followed by digestion for 4 h at 37ºC in 150 µl of reaction mixture containing 10 U *Bcu*I enzyme (Thermo Fischer Scientific Inc, USA). Macrorestriction fragments were separated on a 1% agarose gel at 14 °C with 0.5X TBE (Tris–Borate– EDTA) buffer. Electrophoresis was done using a constant voltage of 6 V/cm for 24 h on a CHEF DR-III electrophoresis system (Bio-Rad Laboratories; Hercules, CA, USA). The pulse time was ramped from 0.1 to 10 s and *Salmonella* serotype Branderup strain H9812 was digested with *Xba*I and included for DNA fragment size determination (Hunter et al., 2005).

The PFGE patterns were visually examined and analysed by BioNumerics software (Applied Maths, Belgium). Similarities between restriction endonuclease digestion profiles of the different isolates were expressed using the Dice similarity index, with the numerical taxonomy program BioNumerics (AppliedMaths BVBA, Sint-Martens-Latem, Belgium). A similarity matrix was computed and transformed into an agglomerative cluster using the unweighted pair group method with arithmetic averages (UPGMA). Genetic diversity (GD) was calculated as the ratio between total PFGE patterns and total isolates (Martínez et al., 2002).

2.4. Statistical analysis

The statistical analysis was carried out using SPSS (IBM Company, version 25.0, SPSS Inc., New York, USA), Microsoft Excel 2010 (Microsoft Corporation, USA) and

GraphPad Prism 8 (scientific 2D graphing and statistics software, California corporation). The dependent variable MIC was considered an ordinal numerical variable with different categories depending on the number of double serial dilutions assayed for each antimicrobial.

According to the results obtained from the *in vitro* susceptibility test, the MIC distribution was determined for each antimicrobial against TpIN and TpEX isolates (Table 2) and was graphically represented (Fig. 1) by means of GraphPad Prism. The normality of these distributions was rule out applying the Kolmogorov-Smirnov test ($P < 0.05$) and, subsequently, they were compared using the non-parametric test of Friedman and Wilcoxon, with the pertinent corrections for multiple comparisons ($P < 0.05$).

To determine the possible relationship of the farming system and clusters distribution of the isolates, the Chi-square test was calculated, considering the differences statistically significant when $P < 0.05$. Later, to measure the relation between variables, the Odds Ratio (OR) was estimated with a confidence interval of 95% (CI_{95}).

3. Results

T. pyogenes isolates were obtained from different organic locations from slaughtered animals (Table 1). TpIN isolates were most frequently isolated from lungs with pneumonia ($P < 0.0001$; OR 5.58; CI_{95} 2.91-10.68), whereas TpEX isolates were recovered from lymph nodes with pyogranulomatous lesions or abscess ($P < 0.0001$; OR 21.42; CI_{95} 9.39-48.84).

Results of MIC distribution, MIC_{50} and MIC_{90} values for each antimicrobial against all the *T. pyogenes* isolates are shown in Table 2. Overall, low MIC_{90} values of penicillin (MIC_{90}=0.008 µg/ml), amoxicillin (MIC_{90} = 0.06 µg/ml), ceftiofur, gentamicin and enrofloxacin (MIC_{90}=1µg/ml) were obtained. MIC_{90} for apramycin and streptomycin reached values of 4 µg/ml and 8 µg/ml, respectively. Nevertheless, neomycin, oxytetracycline (MIC_{90} = 16 µg/ml) and sulfamethoxazole/trimethoprim (MIC_{90}=15.2/0.8 µg/ml) needed higher concentrations to inhibit the growth of 90 per cent of *T. pyogenes* isolates. The highest MIC_{90} values were obtained for tylosin (MIC_{9}=512 µg/ml). According to the specific breakpoints for penicillin and sulfamethoxazole/trimethoprim

against *T. pyogenes* proposed by CLSI (2017), the percentage of susceptible and nonsusceptible isolates for these antimicrobials was estimated (Table 2). It stands out the high value of susceptibility to penicillin (97.8%), whereas in case of sulfamethoxazole/trimethoprim, most of the isolates (98.3%) should be considered "nonsusceptible" (CLSI, 2017).

Differences in the MIC distribution of all the analysed antimicrobials against TpIN and TpEX isolates were detected (Table 3, Fig. 1). These differences were statistically significant (*P <0.05*) for all the analysed antimicrobials, except for penicillin, amoxicillin and ceftiofur (Fig. 1). Also, MIC_{90} values were higher in TpIN than in TpEX isolates (Table 3) for neomycin and streptomycin (32 µg/ml *vs* 8 µg/ml), sulfamethoxazole/trimethoprim (30.4/1.6 µg/ml *vs* 1.90/0.10 µg/ml) and tylosin (≥ 1024 µg/ml *vs* 1 µg/ml).

Table 3. Minimum inhibitory concentrations (MICs) ranges, MIC_{50} and MIC_{90} of 11 antimicrobial agents against *Trueperella pyogenes* isolates from pigs reared under intensive (TpIN) (n=89) and extensive (TpEX) (n=91) systems.

Antimicrobial	TpIN isolates (n=89)				TpEX isolates (n=91)			
	MIC (µg/ml) ranges		MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC (µg/ml) ranges		MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
P	0.001	0.12	0.004	0.008	0.001	0.03	0.004	0.008
AML	0.015	0.12	0.03	0.06	0.008	0.12	0.03	0.03
CEF	0.06	64	0.5	1	0.06	64	0,5	1
APR	1	32	2	4	1	8	2	4
CN	0.12	4	1	2	0.06	8	0.25	0.5
N	0.25	32	8	32	0.25	8	4	8
S	0.25	64	8	32	0.06	32	8	8
ENR	0.25	2	1	2	0.06	2	0.5	1
OT	0.12	32	8	16	0.06	32	4	16
TYL	0.06	≥1024	32	≥1024	0.03	128	0.125	1
SXT (1/19)	0.003/0.057	0.8/15.2	0.1/1.9	1.6/30.4	0.003/0.057	3.2/60.8	0.05/0.95	0.1/0.9

P: Penicillin; AML: Amoxicillin; CEF: Ceftiofur; APR: Apramycin; CN: Gentamicin; N: Neomycin; S: Streptomycin; ENR: Enrofloxacin; OT: Oxytetracycline; TYL: Tylosin; SXT: Trimethoprim-Sulfamethoxazole (1/19).

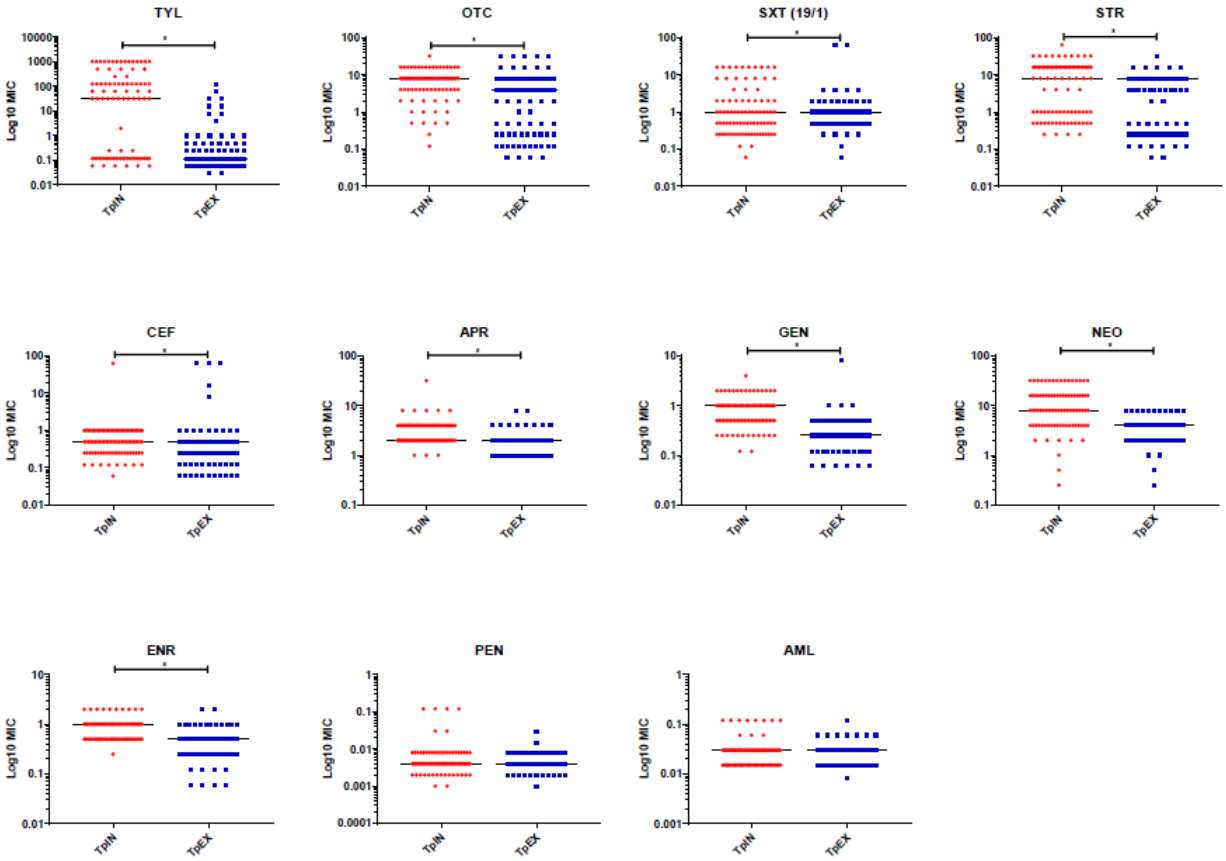

Figure 1. Graphical representation of MIC distribution (Log_{10}) against *Trueperella pyogenes* isolates from pigs reared under intensive (TpIN) (red) and extensive (TpEX) (blue) systems. Every spot represents one single isolate, the median is showed with a horizontal black line. *Statistically significant difference among the MIC distribution of both *T. pyogenes* populations ($P \leq 0,05$). Abbreviations PEN: Penicillin; AML: Amoxicillin; CEF: Ceftiofur; APR: Apramycin; GEN: Gentamicin; NEO: Neomycin; STR: Streptomycin; ENR: Enrofloxacin; OTC: Oxytetracycline; TYL: Tylosin; SXT: Sulfamethoxazole/Trimethoprim (19/1).

By PFGE typing, 70 pulsotypes (37 pulsotypes and 43 pulsotypes in TpIN and TpEX isolates, respectively) were identified (Fig. 2). *T. pyogenes* isolates obtained from

pigs reared under intensive and extensive farming conditions shared only nine pulsotypes, with 60 isolates (33.3%). All the isolates (n=180) could be grouped within three main PFGE clusters at an 85% of genetic similarity (A-C; Fig. 2). Isolates belonging to both pig populations were distributed through the dendrogram, but TpIN isolates were statistically associated with cluster A (*P = 0.0002*; OR 3.21; CI_{95} 1.74-5.93).

PFGE

Table 2. Minimum inhibitory concentrations (MICs), MIC_{50} and MIC_{90} of 11 antimicrobial agents against 180 *Trueperella pyogenes* isolates from pigs (n=180).

Antimicrobial	Nº of isolates with MIC (µg/ml)																								MIC_{50}	MIC_{90}	SU	NS
	≤0.0001	0.0002	0.0005	0.001	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	≥1024	(µg/ml)	(µg/ml)	(%)	(%)
PEN		0	0	3	26	90	53	1	3	0	4	0	0	0	0	0	0	0	0	0					0.004	0.008	176 (97.8)	4 (2.2)
AML							1	53	107	10	9	0	0	0	0	0	0	0	0	0					0.03	0.06	-	-
CEF										12	19	44	60	39	0	0	1	1	0	4					0.5	1	-	-
APR										0	0	0	0	22	103	47	7	0	1	0					2	4	-	-
GEN										6	14	47	60	36	15	1	1	0	0	0					0.5	1	-	-
NEO										0	0	2	2	3	23	78	34	22	16	0					4	16	-	-
STR										2	6	20	23	16	3	15	49	34	11	1					8	8	-	-
ENR										4	4	25	83	52	12	0	0	0	0	0					0.5	1	-	-
OTC										4	12	11	10	6	12	35	62	22	6	0					4	16	-	-
TYL							0	0	2	21	66	13	8	6	1	1	2	2	14	7	15	2	5	15	0.25	512	-	-
SXT (19/1)										1	2	6	51	57	27	14	3	7	10	2					0.05/0.95	0.8/15.2	3 (1.7)	177 (98.3)

PEN: Penicillin; AML: Amoxicillin; CEF: Ceftiofur; APR: Apramycin; GEN: Gentamicin; NEO: Neomycin; STR: Streptomycin; ENR: Enrofloxacin; OTC: Oxytetracycline; TYL: Tylosin; SXT: Sulfamethoxazole/Trimethoprim (19/1); SU: susceptible; NS: nonsusceptible. Breakpoints (indicated with vertical lines) for Penicillin and Sulfamethoxazole/Trimethoprim (19/1) recommended by CLSI (2017) for *Trueperella pyogenes*. No breakpoints for the rest of antimicrobial agents are available for this microorganism.

4. Discussion

Infections caused by *T. pyogenes* represent a significant health and economic problem for the pig farming industry (Martinez et al., 2007; Ribeiro et al., 2015; Cardoso-Toset et al., 2015; Moreno et al., 2017). In the present study, the MIC distribution and genetic characterization of a total of 180 *T. pyogenes* isolates from condemned pigs belonging to intensive and free-range systems were analysed. Previous studies have observed differences in lesions at slaughter according to the animal's production system. In our study, TpIN isolates were most frequently isolated from lungs with pneumonia (*P* < *0.0001*; OR 5.58; CI_{95} 2.91-10.68), whereas TpEX isolates were recovered from lymph nodes with pyogranulomatous lesions or abscess (*P* < *0.0001*; OR 21.42; CI_{95} 9.39-48.84), result that could be related to the transmission mechanisms and pathogenesis of disease (Konsgted and Sørensen, 2017).

Since commercial vaccines are not available, the antimicrobial treatment is the main measure of control of diseases caused by this pathogen, although the information about the resistance pattern of this bacterium is limited. There are few studies and most of them have analysed the antimicrobial susceptibility of *T. pyogenes* only from bovine origin, while the analysis of isolates from pigs are more limited (Santos et al., 2010; Zastempowska and Lassa, 2012; Boer et al., 2015; Moreno et al., 2017). A new document published in 2017 proposed *T. pyogenes* specific antimicrobial breakpoints, for penicillin and sulfamethoxazole/trimethoprim (CLSI, 2017). For other antimicrobials analysed in this study no breakpoints are available for this pathogen.

Selection of appropriate antimicrobial treatment for bacterial infections depends on the microorganism involved, the location of infection, and the pharmacokinetics and pharmacodynamics of antimicrobials. According to this, the MIC_{50} and MIC_{90} data can be used as a preliminary step to guide treatment of the diseases caused for this pathogen in pigs (Rhodes et al., 2015; Zhang et al., 2017). Overall, low MIC_{50} and MIC_{90} values of penicillin, amoxicillin, ceftiofur, gentamicin and enrofloxacin were obtained for all the *T. pyogenes* isolates (Table 1), which agree with previous reports for pig isolates (Moreno et al., 2017; Werckenthin et al., 2017). These data indicate that these antimicrobials would be effective at low doses and therefore recommended for their application for the treatment of *T. pyogenes* infections in pigs.

On the other hand, higher concentrations of neomycin, oxytetracycline (MIC_{90} ≥ 16 µg/ml) and sulfamethoxazole/trimethoprim (MIC_{90} ≥ 15.2/0.8 µg/ml) were required to inhibit the growth of all the Tp isolates analysed, highlighting the tylosin, with MIC_{90} values of 512 µg/ml. The neomycin and oxytetracycline have showed similar MIC values in other studies with isolates obtained from ruminants and pigs (Santos et al., 2010; Zastempowska and Lassa, 2012; Tell et al., 2016; Moreno et al., 2017; Pohl et al., 2017). However, lower values of MIC for sulfamethoxazole/trimethoprim and tylosin have been previously detected (Tell et al., 2016; Pohl et al., 2017).

The amount of antimicrobials used represents one of the most relevant differences between extensive an intensive farming practices (Economou and Gousia, 2015), being intensive pig farming one of the livestock activities with the highest antimicrobial use (Moreno, 2014). On the other hand, extensive free-range production systems seem to use relatively small amounts of antimicrobials, mainly for therapeutic use rather than for disease prevention or growth promotion as in intensive systems. In this sense, our findings show significant differences in the MIC distribution for almost all the antimicrobials (except for penicillin and amoxicillin), among of both *T. pyogenes* populations (Fig. 1 and Table 3). Also, differences between the MIC_{90} values were observed for neomycin and streptomycin (32 µg/ml *vs* 8 µg/ml), sulfamethoxazole/trimethoprim (30.4/1.6 µg/ml *vs* 1.90/0.10 µg/ml) and tylosin (≥ 1024 µg/ml *vs* 1 µg/ml) for TpIN in comparison with TpEX isolates. These results could be attributed to the differences in the use of antimicrobials between both pig management practices (Konsgted and Sørensen, 2017).

Consistent with the hypothesis that the wide use of antimicrobials contributes to the emergence and spread of resistance in bacteria (Van Boeckel et al., 2015; Scoppetta et al., 2017), the highest $MIC_{50,90}$ values among TpIN isolates were detected for antimicrobials commonly used in growing and finishing stages in intensive pig farms (Moreno, 2014).

Different farming practices could be risk factors that may alter the epidemiology of microorganisms (Mennerat et al., 2010). Pigs are farmed worldwide under industrial-intensive production systems, being pigs managed under extensive practices in Spain as well. However, no data is available on the comparison of *T. pyogenes* isolates reared in intensive and extensive farming systems. Genetic characterization of *T. pyogenes* by

PFGE typing revealed a comparable relatively high genetic diversity in TpIN and TpEX isolates (GD 0.42 and GD 0.47, respectively). TpIN isolates were statistically associated with cluster A (*P = 0.002*; OR 3.21; CI_{95} 1.74-5.93), although one third (32.6%) of the TpIN were also identified in clusters B and C (Fig. 1). On the other hand, TpEX isolates were distributed through the dendrogram (Fig. 1), showing more genetic diversity. These data suggest a wide genetic variability the of *T. pyogenes* population, higher in TpEX than TpIN isolates and MIC distribution against several antimicrobials among pigs reared in intensive and extensive system, which could be related with the different management practices.

Acknowledgement

This work was supported by the Ministry of Agriculture, Food and Environment [Control strategies against lymphadenitis of the Iberian pig in free-range system, reference number 20140020001824].

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Objetivo 2/Objective 2

Objetivo 2: Estudiar la posible relación epidemiológica entre aislamientos de *Trueperella pyogenes* obtenidos de porcino y rumiantes

Objective 2: Study the possible epidemiological relationship between *Trueperella pyogenes* isolates obtained from pigs and ruminants

Study 2: Antimicrobial susceptibility of Trueperella pyogenes isolated from food-producing ruminants

Galán-Relaño *et al.*, 2019, Under review in *Veterinary Microbiology*

Antimicrobial susceptibility of *Trueperella pyogenes* isolated from food-producing ruminants

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Short title: MIC distribution of *T. pyogenes* in ruminants.

Abstract

A total of 96 *Trueperella pyogenes* isolates, an opportunistic pathogen of food-producing ruminants, obtained from cattle (n=34), sheep (n=35) and goats (n=27), and identified by Real Time PCR (qPCR), were analysed to determine the susceptibility to 12 antimicrobials commonly used in livestock, by the broth microdilution assay. Minimal Inhibitory Concentration (MIC) distributions of apramycin, gentamicin, streptomycin, oxytetracycline, tylosin and erythromycin showed a tendency to bimodality, being unimodal for the rest of antimicrobials. Low MIC_{90} values for penicillin, amoxicillin, ceftiofur, enrofloxacin, and gentamicin (<1 μg/ml) were obtained, so they could be of choice for the first line of empiric treatment of *T. pyogenes* infections. According to the specific *T. pyogenes* breakpoints for penicillin, sulfamethoxazole/trimethoprim and erythromycin, 93.7% of isolates were susceptible to penicillin and 77.2% to erythromycin, whereas the 92.7% were nonsusceptible to sulfamethoxazole/trimethoprim. Significant differences were observed in the MIC distribution of almost all antimicrobials, except enrofloxacin, tylosin and erythromycin against cattle, sheep or goat isolates, although all antimicrobials showed similar MIC_{90} values, except apramycin and oxytetracycline that showed higher values against cattle isolates. These data provide interesting information on the antimicrobials of choice for the treatment of infections caused by *T. pyogenes* in ruminants.

Keywords: *Trueperella pyogenes*; cattle; goat; sheep; MIC distribution.

1. Introduction

Trueperella pyogenes (formerly *Arcanobacterium pyogenes*) is a commensal and opportunistic pathogen of the skin and mucous membranes of the upper respiratory, urogenital and gastrointestinal tracts of a wide range of domestic and wildlife species (Wareth et al., 2018). Sporadic cases of human infections have also been reported in people living in rural areas in contact with animals (Levy et al., 2009; Kavitha et al., 2010).

The clinical manifestations of diseases caused by this pathogen vary from udder lesions and metritis to abscesses, pneumonia, arthritis, endocarditis or osteomyelitis, highlighting its involvement in the production of mastitis in ruminants (Santos et al., 2010; Zastempowska and Lassa, 2012; Ribeiro et al., 2015; Risseti et al., 2017; Ashrafi Tamai et al., 2018). These diseases cause a reduction of meat and milk production and decreasing reproductive and efficiency that can lead to carcase condemnations at slaughterhouse (Dong et al., 2019; Rzewuska et al., 2019).

Nowadays, antimicrobial therapy is the main tool to control this disease (Ribeiro et al., 2015) and must be based on antimicrobial susceptibility tests, which provide a basement for clinical use of antimicrobial (Ribeiro et al., 2015; Zhang et al., 2017). The Broth Microdilution Method is widely used to determine the Minimum Inhibitory Concentration (MIC) of any antimicrobial agent against important pathogens affecting both human and animal species (Clinical and Laboratory Standards Institute, CLSI, 2016, 2017). Moreover, the MIC distribution, together with the pharmacokinetic studies of antimicrobial agents against these pathogens, is are critical steps to establish clinical breakpoints (Dalhoff et al., 2009). Despite the importance of *Trueperella pyogenes* in small and large ruminants, the information on its behaviour against antimicrobials commonly used in livestock is scarce (Rogovskyy et al., 2018).

Therefore, the main objectives of this study are the determination of the MIC distribution of antimicrobials commonly used in livestock farming against *T. pyogenes*, and to study possible differences between small and large food-producing ruminant

species. Our results will contribute to the selection of the adequate antimicrobial agent against this pathogen.

2. Material and Methods

2.1. Bacteria isolates

A total of 96 *Trueperella pyogenes* isolates were analysed from cattle (34), sheep (35) and goats (27) from 22 farms located in different geographic areas of Spain (Figure 1). Samples were obtained from udder lesions (n=51), respiratory disorders (n=22), lymph nodes (13), reproductive disorders (n=5), arthritis (n=2), septicaemia-omphalophlebitis (n=1), ocular lesions (n=1) and encephalitis (n=1) (Table 1).

Table 1. *Trueperella pyogenes* isolates from food-producing ruminants (cattle, sheep and goat) analysed.

Source	Ruminant specie			Total
	Cattle	Sheep	Goat	
Udder lesions	32	6	13	51
Respiratory disorders	1	15	6	22
Lymph nodes	1	9	3	13
Reproductive disorders	0	3	2	5
Arthritis	0	1	1	2
Septicaemia-Omphalophlebitis	0	1	0	1
Ocular lesions	0	0	1	1
Encephalitis	0	0	1	1
Total	34	35	27	96

Samples were plated on Columbia Blood Agar Base with nalidixic acid and colistin sulfate (Oxoid ltd., Hampshire, UK), supplemented with 5% sterile defibrinated sheep blood and incubated under microaerophilic conditions (5% CO_2) at 37 ºC for 48 hours. Coryneform bacteria isolates (Gram-positive, catalase variable and oxidase negative irregularly shaped rods) were further identified by qRT-PCR.

2.2. Real Time Polymerase Chain Reaction qPCR

A species-specific Real Time Polymerase Chain Reaction (qPCR) assay was used. Basing on the detection of the pyolisin gene (*plo*) as previously described (Zastempowska and Lassa, 2012; Risseti et al., 2017) the conventional PCR was

adapted to qPCR. Briefly, primers (forward: 5'-TGTCCGAACGCAAATTGTTA-3'; reverse: 5'-AAAATGTGCCGGTTACCAAG-3') were designed basing on sheep gene sequences deposited in the GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/genbank/) and using the programme Primer3Plus (Primer3Plus Bioinformatics, http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Transcript quantification was carried out using 50 ng of DNA per reaction and the iTaq™ Universal SYBR® Green Supermix kit (BioRad) following the manufacturer instructions.

2.3. Antimicrobials and Minimum Inhibitory Concentration (MIC) determination

A total of 12 antimicrobials agents (Sigma Aldrich Co., USA) were used in this study, belonging to 7 different classes: β-lactams (penicillin-G, PEN, and amoxicillin, AML), cephalosporins (ceftiofur, CEF), aminoglycosides (apramycin, APR; gentamicin, GEN; neomycin, NEO, and streptomycin, STR), fluoroquinolone (enrofloxacin, ENR), tetracycline (oxytetracycline, OTC), sulphonamide (sulfamethoxazole/trimethoprim, SXT) and macrolides (tylosin, TYL, and erythromycin, E).

Double serial dilutions (ranging from 0.0001 to 1024 µg/ml) of each antimicrobial were prepared in cation-adjusted Mueller-Hinton broth (MHB) with 5% (v/v) of lysed horse blood, following the broth microdilution susceptibility test for fastidious organisms (CLSI 2016, 2017). An equal volume of bacterial inoculum was mixed with each antimicrobial dilution up to a final concentration of $5x10^5$ CFU/ml. Every test was carried out in a 96 wells plate and conducted in duplicate. Positive and negative growth controls and quality control (*Streptococcus pneumoniae* ATCC 49619 strain) were included.

The 96 wells plates were incubated at 37°C for 20-24 hours under aerobic conditions and the MIC was determined as the lowest concentration of antimicrobial that inhibited the visible growth of the inoculum. The distribution of MIC for every antimicrobial was obtained (Table 2) and was graphically represented (Figure 1). MIC_{50} and MIC_{90} were also determined for every antimicrobial (Schwarz et al., 2010). For

penicillin, sulfamethoxazole/trimethoprim and erythromycin the breakpoints available for *Trueperella pyogenes* were used, according to CLSI (2017).

2.4. Statistical analysis

The statistical analysis was carried out using SPSS (IBM Company, version 25.0, SPSS Inc., New York, USA), Microsoft Excel 2010 (Microsoft Corporation, USA) and GraphPad Prism 8 (scientific 2D graphing and statistics software, California Corporation). The normality of the MIC distributions was ruled out applying the Kolmogorov-Smirnov test ($P < 0.05$) and were, subsequently, compared using the non-parametric test of Dunn's, with the pertinent corrections for multiple comparisons ($P < 0.05$). To determine the possible relationship of the animal species and the MIC distribution, the Chi-square test was calculated, considering the differences statistically significant when $P < 0.05$. The Odds Ratio (OR) was estimated (confidence interval of 95%, CI95) to measure these associations.

3. Results

The 96 *Trueperella pyogenes* isolates were confirmed with the qPCR designed for this study. Udder lesions were the most frequent pathological disorder in cattle and goats, whereas respiratory disorders were most frequently observed in sheep (Table 1).

MIC distribution (0.0001 to 1024 μg/ml) and MIC_{50} and MIC_{90} values for every studied antimicrobial against all the *T. pyogenes* isolates are shown in table 2. Penicillin, amoxicillin, ceftiofur, gentamycin, neomycin, enrofloxacin and sulfamethoxazole/trimethoprim showed the narrowest ranges of dilutions. MIC values for β-lactams were the lowest ones. MIC distributions of penicillin, amoxicillin, ceftiofur, neomycin, enrofloxacin and sulfamethoxazole/trimethoprim showed a unimodal distribution, whereas MIC distributions of apramycin, gentamicin, streptomycin, oxytetracycline, tylosin and erythromycin showed a tendency to bimodality. (Table 2 and Figure 1).

Table 2. Minimum Inhibitory Concentration (MIC) distributions, MIC_{50} and MIC_{90} of 12 antimicrobials agents against 96 *Trueperella pyogenes* isolates from food-producing ruminants.

Antimicrobial	Nº of isolates with MIC (µg/ml)																							MIC_{50}	MIC_{90}	SU	NS
	≤0.0001	0.0002	0.0005	0.001	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	≥1024	(µg/ml)	(µg/ml)	(%)	(%)
PEN		0	0	5	0	69	14	2	0	6	0	0	0	0	0	0	0	0	0	0					0.004	0.008	90 (93.7)	6 (6.3)
AML		0	0	0	0	0	1	29	57	9	0	0	0	0	0	0	0	0	0	0					0.03	0.06	-	-
CEF		0	0	0	0	0	0	1	0	25	27	26	14	3	0	0	0	0	0	0					0.12	0.5	-	-
APR										0	0	0	4	24	51	11	2	0	0	0	0	0	0	4*	2	4	-	-
GEN		0	0	0	0	0	0	0	0	0	21	25	31	14	3	0	0	0	2	0					0.5	1	-	-
NEO		0	0	0	0	0	0	0	0	0	2	2	1	4	14	45	20	5	3	0					4	8	-	-
STR		0	0	0	0	0	0	0	0	0	21	34	12	5	2	3	8	7	1	0	0	0	0	3*	0.25	16	-	-
ENR		0	0	0	0	0	0	0	0	0	18	58	14	4	2	0	0	0	0	0					0.25	0.5	-	-
OTC		0	0	0	0	0	0	0	0	1	18	12	6	3	9	6	25	6	5	5	0	0	0		2	32	-	-
SXT (19/1)		0	0	0	0	0	0	0	0	0	7	17	42	18	5	2	2	0	3	0	0	0	0		0.475/0.025	1.9/0.1	7 (7.3)	89 (92.7)
TYL		0	0	0	0	0	0	0	0	66	6	2	3	0	0	1	1	2	5	10	0	0	0		0.06	64	-	-
E		0	1	3	11	26	28	5	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	19*	0.008	≥1024	74 (77.2)	22 (22.8)

PEN: Penicillin; AML: Amoxicillin; CEF: Ceftiofur; APR: Apramycin; GEN: Gentamicin; NEO: Neomycin; STR: Streptomycin; ENR: Enrofloxacin; OTC: Oxytetracycline; SXT: Sulfamethoxazole/Trimethoprim (19/1); TYL: Tylosin; E: Erythromycin; SU: susceptible; NS: nonsusceptible. Breakpoints (indicated with vertical lines) for Penicillin, Sulfamethoxazole/Trimethoprim (19/1) and Erythromycin recommended by CLSI (2017) for *Trueperella pyogenes*. No breakpoints for the rest of antimicrobial agents are available for this microorganism. * Number of isolates that grew at the highest concentrations of the tested antimicrobial. In grey, not tested antimicrobials dilutions.

In general, low MIC_{90} values of penicillin (MIC_{90}=0.008 µg/ml), amoxicillin (MIC_{90}=0.06 µg/ml), ceftiofur and enrofloxacin (MIC_{90}=0.5 µg/ml), and gentamicin (MIC_{90}=1 µg/ml) were obtained (Table 2). MIC_{90} for sulfamethoxazole/trimethoprim, apramycin and neomycin reached values of 1.9/0.1 µg/ml, 4 µg/ml and 8 µg/ml, respectively. Nevertheless, streptomycin (MIC_{90}=16 µg/ml), oxytetracycline (MIC_{90}=32 µg/ml) and tylosin (MIC_{90}=64 µg/ml) needed higher concentrations to inhibit the growth of 90 per cent of *T. pyogenes* isolates. The highest MIC_{90} value was obtained for erythromycin, which reached 1024 µg/ml (Table 2).

According to the specific *T. pyogenes* breakpoints for penicillin, sulfamethoxazole/trimethoprim and erythromycin proposed by CLSI (2017), the 93.7% of isolates were susceptible to penicillin and 77.2% to erythromycin. Most of the isolates (92.7%) were considered nonsusceptible to sulfamethoxazole/trimethoprim (Table 2).

Statistically significant differences ($P < 0.05$) in MIC distribution of ceftiofur, apramycin, gentamicin, neomycin, streptomycin and oxytetracycline between cattle and sheep isolates were observed (Figure 1). On the other hand, differences ($P < 0.05$) were also detected in MIC distribution of penicillin and sulfamethoxazole/trimethoprim between cattle and goat. Finally, only differences ($P < 0.05$) were observed in MIC distribution of ceftiofur between sheep and goat isolates. No statistically significant difference was found in case of enrofloxacin, tylosin and erythromycin (Figure 1).

All antimicrobial agents showed similar MIC_{90} values against large and small ruminants, except apramycin and oxytetracycline that showed higher values against cattle isolates (≥ 1024 µg/ml and 64 µg/ml, respectively) in comparison with sheep (2 µg/ml and 8 µg/ml, respectively) and goat isolates (4 µg/ml and 16 µg/ml, respectively) (Table 3).

According to the specific breakpoints (CLSI 2017), *T. pyogenes* showed a homogeneous behaviour independently of the origin of isolates, highlighting the high percentage of nonsusceptible goat isolates to sulfamethoxazole/trimethoprim (100%) (Table 3).

Table 3. Minimum Inhibitory Concentration (MIC) ranges, MIC_{50} and MIC_{90} (µg/ml) of 12 antimicrobials agents against 96 *Trueperella pyogenes* isolates from cattle (n = 34), sheep (n = 35) and goat (n = 27) species.

ntimicrobial	Bovine isolates (n = 34)						Sheep isolates (n = 35)						Goat isolates (n = 27)					
	MIC ranges		MIC_{50}	MIC_{90}	SU	NS	MIC ranges		MIC_{50}	MIC_{90}	SU	NS	MIC ranges		MIC_{50}	MIC_{90}	SU	NS
PEN	0.002	0.06	0.004	0.008	93.7	6.3	0.002	0.06	0.004	0.008	91.4	8.6	0.002	0.06	0.004	0.015	96.3	3.7
AML	0.008	0.06	0.03	0.03	-	-	0.015	0.06	0.03	0.06	-	-	0.015	0.06	0.03	0.03	-	-
CEF	0.06	1	0.12	0.5	-	-	0.015	0.5	0.12	0.25	-	-	0.06	1	0.25	0.5	-	-
APR	0.5	≥1024	2	≥1024	-	-	0.5	4	2	2	-	-	0.5	4	2	4	-	-
GEN	0.12	32	0.5	2	-	-	0.12	1	0.25	0.5	-	-	0.12	1	0.5	0.5	-	-
NEO	0.12	32	8	16	-	-	0.25	8	4	4	-	-	0.25	8	4	8	-	-
STR	0.12	≥1024	0.5	16	-	-	0.12	≥1024	0.25	8	-	-	0.12	16	0.25	8	-	-
ENR	0.06	2	0.25	1	-	-	0.12	2	0.25	0.5	-	-	0.12	0.5	0.25	0.5	-	-
OTC	0.12	64	4	64	-	-	0.06	16	0.5	8	-	-	0.12	64	8	16	-	-
SXT (19/1)	0.12	32	0.95/0.05	7.6/0.4	11.8	88.2	0.12	2	0.475/0.025	0.95/0.05	8.6	91.4	0.25	1	0.475/0.025	0.475/0.025	0	100
TYL	0.06	64	0.06	64	-	-	0.06	64	0.06	32	-	-	0.06	32	0.06	32	-	-
E	0.0005	≥1024	0.008	≥1024	76.4	23.6	0.001	≥1024	0.008	≥1024	77.1	22.9	0.002	≥1024	0.008	≥1024	77.8	22.2

PEN: Penicillin; AML: Amoxicillin; CEF: Ceftiofur; APR: Apramycin; GEN: Gentamicin; NEO: Neomycin; STR: Streptomycin; ENR: Enrofloxacin; OTC: Oxytetracycline; SXT: Sulfamethoxazole/Trimethoprim (19/1); TYL: Tylosin; E: Erythromycin. SU: susceptible; NS: nonsusceptible. Breakpoints for Penicillin, Sulfamethoxazole/Trimethoprim (19/1) and Erythromycin recommended by CLSI (2017) for *Trueperella pyogenes*. No breakpoints for the rest of antimicrobial agents are available for this microorganism.

4. Discussion

Livestock products consumption is constantly rising, being expected to double their demand by 2030 according to the Food and Agriculture Organization of the United Nations (FAO). The European Union has a substantial population of food-producing ruminants, risen to 88 million cattle and 100 million sheep and goats in 2017 (EUROSTAT, Statistics explained), being the biomass of livestock animals in Spain among the largest of Europe (8th report of European Surveillance of Veterinary Antimicrobial Consumption, ESVAC).

The importance of *Trueperella pyogenes* infections in ruminants has been known for a long time (Rzewuska et al., 2019). The fact that commercial vaccines are not available makes the antimicrobial treatment the main measure of control of diseases caused by this pathogen (Galán-Relaño et al., 2019). Despite this situation, limited information about the *in vitro* susceptibility of this pathogen against antimicrobials traditionally used in veterinary medicine exists, especially in small ruminant (Rogovskyy et al., 2018). Thus, in this study the MIC distribution of 12 antimicrobials commonly used in ruminants farming industry against 96 *T. pyogenes* isolates from cattle, sheep and goats was determined.

Penicillin, sulfamethoxazole/trimethoprim and erythromycin are recommended for primary testing for *Trueperella pyogenes* isolates, and the breakpoints are proposed from 2017 (CLSI, 2017). However, no breakpoints for other antimicrobials exist. MIC distribution, together with pharmacokinetics of the antimicrobial agent, are necessary to establish clinical breakpoints (Dalhoff et al., 2009; Hassan et al., 2018).

A MIC distribution is considered bimodal when it shows two population peaks or groups located in the two values that appear most often throughout the complete distribution.

Bimodal MIC distributions have been previously described with neomycin, streptomycin and oxytetracycline against different microorganisms (Dung et al., 2008; de Boer et al., 2015; Prüller et al., 2015). These results suggest that the isolates that need higher concentrations of antimicrobial are a subpopulation of bacteria that

acquired resistance mechanisms to these antimicrobials (Turnidge and Paterson, 2007; Dung et al., 2008; Prüller et al., 2015). In our study, a bimodal MIC distribution was detected for apramycin, gentamicin, streptomycin, oxytetracycline, tylosin and erythromycin (Table 2 and Figure 1). In addition, tetracyclines have been the most sold antimicrobials in Spain from 2010 to 2016 and macrolides are being used as their substitutes, circumstances that could explain these results (8th report of European Surveillance of Veterinary Antimicrobial Consumption, ESVAC). In contrast, a unimodal distribution of MIC was noted for neomycin, penicillin, amoxicillin, ceftiofur, enrofloxacin and sulfamethoxazole/trimethoprim.

In general, low MIC_{90} values of penicillin (MIC_{90}=0.008 µg/ml), amoxicillin (MIC_{90}=0.06 µg/ml), ceftiofur and enrofloxacin (MIC_{90}=0.5 µg/ml), and gentamicin (MIC_{90}=1 µg/ml) were obtained (Table 2), which agree with previous studies on bovine strains (Zastempowska and Lassa, 2012; de Boer et al., 2015; Pohl et al., 2018) and on pig isolates (Galán-Relaño et al., 2019). Therefore, these antimicrobials agents would be effective at low doses to treat *T. pyogenes* infections in ruminants, according to other studies (Evira, 2018), that recommends, among others, penicillin and enrofloxacin to treat infectious diseases in ruminants.

On the contrary, MIC_{90} for sulfamethoxazole/trimethoprim, apramycin, neomycin and streptomycin showed higher values (1.9/0.1 µg/ml, 4 µg/ml and 8 µg/ml and 16 µg/ml, respectively), highlighting the values for oxytetracycline (MIC_{90} = 32 µg/ml), tylosin (MIC_{90} = 64 µg/ml) and erythromycin (1024 µg/ml) (Table 2). Similar MIC_{90} values for sulfamethoxazole/trimethoprim, streptomycin and oxytetracycline against isolates obtained from cattle and white-tailed deer were obtained (Santos et al., 2010; Tell et al., 2011). However, other studies on cattle isolates showed lower values of MIC_{90} for tylosin and erythromycin, ranging from 0.025 µg/ml to 2 µg/ml (Yoshimura et al., 2000; Liu et al., 2009; Pohl et al., 2018).

According to the specific *T. pyogenes* breakpoints (CLSI, 2017), high values of susceptibility were obtained against penicillin (93.7%) and erythromycin (77.2%), similar to those previously obtained against cattle and pig isolates (Yoshimura et al.,

2000; Dong et al., 2019), although other authors support higher values of resistance in bovine strains (Liu et al., 2009).

Interestingly, a high percentage of nonsusceptible isolates against sulfamethoxazole/trimethoprim (92.7%) was detected, coinciding with previous studies with swine isolates of *T. pyogenes* in Spain (Galán-Relaño et al., 2019), higher than those described previously, that ranged between 0% and 49.3% (Tell et al., 2011; Ribeiro et al., 2015).

Statistically significant differences ($P < 0.05$) in MIC distribution of ceftiofur, apramycin, gentamicin, neomycin, streptomycin and oxytetracycline between cattle and sheep isolates were observed (Figure 1). On the other hand, differences ($P < 0.05$) were also detected in MIC distribution of penicillin and sulfamethoxazole/trimethoprim between cattle and goat. Finally, only differences ($P < 0.05$) were observed in MIC distribution of ceftiofur between sheep and goat isolates. No statistically significant difference was found in case of enrofloxacin, tylosin and erythromycin (Figure 1).

All antimicrobial agents showed similar MIC_{90} values against large and small ruminants, except apramycin and oxytetracycline that showed higher values against cattle isolates (≥ 1024 µg/ml and 64 µg/ml, respectively) in comparison with sheep (2 µg/ml and 8 µg/ml, respectively) and goat isolates (4 µg/ml and 16 µg/ml, respectively) (Table 3). Oxytetracycline has been traditionally used as growth promoter in some countries without control (Ashrafi Tamai et al., 2018), and the amount of antimicrobial used with the intensification of the dairy bovine industry in comparison with sheep and goat farming could influence the appearance of this differences.

According to the specific breakpoints (CLSI 2017), *T. pyogenes* showed a homogeneous behaviour independently of the origin of isolates, highlighting the high percentage of nonsusceptible goat isolates to sulfamethoxazole/trimethoprim (100%) (Table 3). Sulphonamides are the third class of antimicrobials most sent in Spain (8th report of European Surveillance of Veterinary Antimicrobial Consumption, ESVAC) which could explain this result.

These findings indicate that penicillin and amoxicillin are highly active against *Trueperella pyogenes* isolated from ruminants, consistent with the results of the pig study recently published (Galán-Relaño et al., 2019). Thus, these antimicrobials can be used as the first-line of empirical treatment of infections caused by *Trueperella pyogenes* in food-producing animals. The antimicrobial therapy must be based on the knowledge of the susceptibility of the microorganism to the antimicrobial and the existence of specific breakpoints, for which the MIC distribution yields really useful information.

Acknowledgements

This work was supported by the University of Cordoba, XXII Programa Propio de Fomento de la Investigación (2017) “Aproximaciones multiómicas al estudio de las resistencias a antibióticos en patógenos Gram-positivos” (MOD.4.2 P.P.2017 AGR164 AGR256, ref. 500065). Ángela Galán Relaño has a fellowship by the investigation project “Nuevas medidas y técnicas de control de la tuberculosis bovina en Andalucía” (Ayudas al Funcionamiento de los grupos Operativos de la Asociación Europea de Innovación (AEI), convocatoria 2016, referencia: GOP2I-CO-16-0010). The authors thank Jose María Sánchez Carvajal for the technical support for the development of the qPCR used in this work.

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Objetivo 2: Estudiar la posible relación epidemiológica entre aislamientos de *Trueperella pyogenes* obtenidos de porcino y rumiantes

Objective 2: Study the possible epidemiological relationship between *Trueperella pyogenes* isolates obtained from pigs and ruminants

Estudio 3: "Perfil de sensibilidad antimicrobiana de Trueperella pyogenes: aportaciones para el control en animales de abasto".

Galan-Relaño et al., 2019. Este trabajo ha sido presentado en el XXIV Simposio de AVEDILA (Asociación de Especialistas en Diagnóstico Laboratorial Veterinario) en forma de comunicación oral. Noviembre 2019, Pamplona, España

Perfil de sensibilidad antimicrobiana de *Trueperella pyogenes*: aportaciones para el control en animales de abasto

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La producción animal se enfrenta cada vez a mayores exigencias en aspectos relacionados con el bienestar animal, la sostenibilidad y la adecuada gestión de los residuos desechados de la industria agroalimentaria. España es un país eminentemente ganadero y, para mantener un nivel competitivo, debe ir afrontando una serie de retos que le permitan obtener productos rentables reduciendo los riesgos sanitarios y medioambientales (MAPA, 2019). Entre ellos, la reducción del uso de antimicrobianos en animales de abasto se ha convertido en una exigencia, desde la aparición de bacterias que han adquirido resistencias a diferentes antimicrobianos. Estas resistencias provocan, por una parte, fallos en el tratamiento de enfermedades en los animales y, por otra, pueden transmitirse a otras bacterias que afectan a los animales y al hombre. Ejemplos muy llamativos son la *Salmonella* Typhymurium monofásica o los *Staphylococcus aureus* meticilina resistentes, cuya resistencia está asociada a genes, casetes cromosómicos o integrones (Xu *et al.*, 2008).

Desde el año 2014 existe un *Plan estratégico y de acción para reducir el riesgo y disminución de la resistencia a los antibióticos*, denominado por las siglas PRAN y puesto en marcha por la Agencia Española del Medicamento y Productos Sanitarios (AEMPS). Entre las líneas de acción de este plan, se señala la necesidad de conocer los perfiles de sensibilidad de las bacterias antes de aplicar un tratamiento con un antimicrobiano concreto, y basar dicho tratamiento en un buen diagnóstico previo.

Trueperella pyogenes es una bacteria que afecta fundamentalmente al ganado porcino y rumiantes, y está implicada en diferentes cuadros clínicos que originan importantes pérdidas económicas, como son a linfadenitis en porcino y las metritis y las mamitis en rumiantes (Jarosz *et al*., 2014; de Boer *et al.*, 2015; Rzewuska *et al*., 2019). La herramienta fundamental de control de estos procesos es la terapia antimicrobiana, que debe estar basada en estudios se susceptibilidad antimicrobiana para poder conocer la Concentración Mínima Inhibitoria (CMI) (Ribeiro *et al.*, 2015; Zhang *et al.*, 2017). Sin embargo, no fue hasta el año 2016 que se publicaron normas para la realización de estas pruebas, que permiten comparar los resultados de los estudios entre laboratorios. La estandarización de estos estudios es importante, dado que la distribución de CMI, junto con pruebas farmacocinéticas de los agentes antimicrobianos, son pasos cruciales para el establecimiento de puntos de corte específicos para definir un aislamiento como sensible o resistente (Dalhoff *et al.*, 2009).

En los estudios que hemos realizado, se ha analizado un conjunto de aislamientos de *T. pyogenes* (n=276) obtenidos de cerdos (n=180) y de rumiantes (bovino, ovino y caprino, n=96) para conocer los antimicrobianos que pueden ser utilizados de forma empírica para el tratamiento de las infecciones causadas por este patógeno en cada especie animal. En este trabajo realizamos un análisis de todos los aislamientos, comparando los resultados obtenidos entre cerdos y rumiantes y cumpliendo, así, uno de los objetivos marcados en esta tesis doctoral.

En este trabajo, se muestran los resultados de distribución de CMI, CMI_{50}, CMI_{90} y porcentaje de cepas no sensibles a los antimicrobianos en estudio frente al total de aislamientos (n=276), y se comparan en función de la especie de origen de las cepas; ganado porcino (n=180) y rumiantes (n=96).

Los antimicrobianos más eficaces frente al total de cepas (n=276) han sido penicilina y amoxicilina (CMI_{90} de 0.008 µg/ml). Hemos obtenido buenos resultados con ceftiofur, gentamicina y enrofloxacina (1 µg/ml). Por otra parte, detectamos valores de CMI_{90} elevados de tilosina (128 µg/ml), seguidos de los valores de neomicina, estreptomicina, oxitetraciclina y eritromicina, todos de 16 µg/ml (Tabla 1).

En la Tabla 1 se puede observar la distribución de la CMI de los 276 aislamientos, así como los valores de CMI_{50} y CMI_{90} y los porcentajes de aislamientos sensibles y no sensibles a los antimicrobianos para los que hay publicados puntos de corte específicos para *T. pyogenes*.

Aplicando los puntos de corte publicados (penicilina, sulfametoxazol-trimetoprim y eritromicina), hemos detectado cepas no sensibles frente a los tres AMB, siendo más altos estos porcentajes al sulfametoxazol-trimetoprim y eritromicina en porcino, y a la penicilina en rumiantes (Tabla 2). Se puede comprobar que los datos de CMI_{90} y porcentaje de cepas no sensibles, en general, se mantienen cuando se toma como población el total de aislamientos de este microorganismo sin tener en cuenta el animal de origen (n=276) (Tablas 1 y 2).

De acuerdo con nuestros resultados, los antimicrobianos penicilina y amoxicilina pueden ser utilizados como primera línea de tratamiento para el control de las infecciones provocadas por *T. pyogenes* en animales de abasto. Este estudio resalta la importancia de realizar pruebas de sensibilidad *in vitro* para confirmar resultados y asegurar el éxito del tratamiento, además de para monitorizar los microorganismos, ya que se han detectado cepas no sensibles a la penicilina (3.6%) según los puntos de corte actualmente disponibles.

Cuando se comparan los resultados de ambas poblaciones (aislamientos de porcino y aislamientos de rumiantes), se observan diferencias estadísticamente significativas ($P<0.05$) en la distribución de CMI de todos los antimicrobianos, excepto de penicilina y amoxicilina (Figura 1), y se puede apreciar que las cepas aisladas de ganado porcino requieren, en general, concentraciones de antimicrobiano más altas para inhibir su crecimiento que las cepas de rumiantes.

Nuestro trabajo aporta información interesante para la determinación de puntos de corte específicos para *T. pyogenes*, inexistentes hasta la fecha para la mayoría de antimicrobianos e imprescindibles a la hora de un tratamiento eficaz y sostenible frente a este patógeno.

Tabla 1. Concentración Mínima Inhibitoria (CMI), CMI_{50} y CMI_{90} (µg/ml) de los 12 antimicrobianos estudiados frente a 276 cepas de *Trueperella pyogenes* aisladas de animales de abasto.

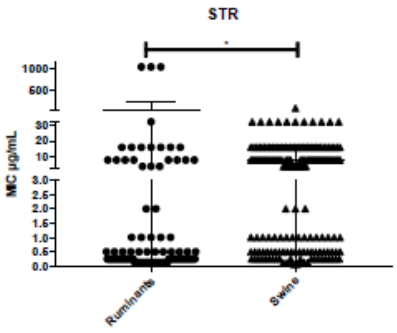
Antimicrobianos	Número de aislamientos con CMI (µg/ml)																								CMI_{50}	CMI_{90}	SU	NS
	≤0.0001	0.0002	0.0005	0.001	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	≥1024	(µg/ml)	(µg/ml)	(%)	(%)
PEN		0	0	8	26	159	67	3	3	6	4	0	0	0	0	0	0	0	0	0					0.004	0.008	266 (96.4)	10 (3.6)
AML		0	0	0	0	0	2	82	164	19	9	0	0	0	0	0	0	0	0	0					0.03	0.06	-	-
CEF		0	0	0	0	0	0	1	0	37	46	70	74	42	0	0	1	1	0	4					0.25	1	-	-
APR										0	0	0	4	46	154	58	9	0	1	0	0	0	0	4*	2	4	-	-
GEN		0	0	0	0	0	0	0	0	6	35	72	91	50	18	1	1	0	2	0					0.5	1	-	-
NEO		0	0	0	0	0	0	0	0	0	2	4	3	7	37	123	54	27	19	0					4	16	-	-
STR		0	0	0	0	0	0	0	0	2	27	54	35	21	5	18	57	41	12	1	0	0	0	3*	1	16	-	-
ENR		0	0	0	0	0	0	0	0	4	22	83	97	56	14	0	0	0	0	0					0.5	1	-	-
OTC		0	0	0	0	0	0	0	0	5	30	23	16	9	21	41	87	28	11	5	0	0	0		4	16	-	-
SXT (19/1)		0	0	0	0	0	0	0	0	1	9	23	93	75	32	16	5	7	13	2	0	0	0		0.95/0.05	3.8/0.2	10 (3.6)	266 (96.4)
TYL		0	0	0	0	0	0	0	2	87	72	15	11	6	1	2	3	4	19	17	15	2	5	15*	0.12	128	-	-
E		0	1	3	11	26	59	23	34	23	3	0	1	6	2	5	1	78*							0.03	16	157 (56.9)	119 (43.1)

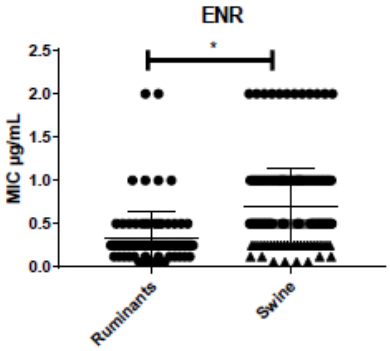
PEN: penicilina; AML: amoxicilina; CEF: ceftiofur; APR: apramicina; GEN: gentamicina; NEO: neomicina; STR: estreptomicina; ENR: enrofloxacina; OTC: oxitetraciclina; TYL: tilosina; SXT: sulfametoxazol/trimetoprim (19/1); SU: susceptible; NS: no susceptible. Puntos de corte para penicilina, sulfametoxazol/trimetoprim (19/1) y eritromicina (indicados con líneas verticales) recomendados por el CLSI (2017) para *Trueperella pyogenes*. No existen puntos de corte disponibles para el resto de antimicrobianos específicos para este microorganismo. * Número de aislamientos que crecieron en las concentraciones testadas más altas de los antimicrobianos. En gris, diluciones no testadas de los antimicrobianos.

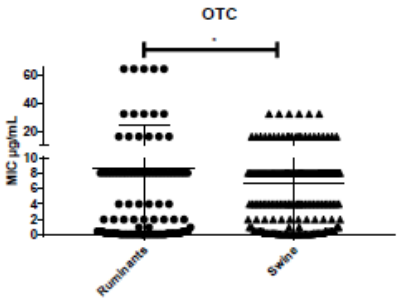
Tabla 2. Concentración Mínima Inhibitoria (CMI) CMI_{50}, CMI_{90} (µg/ml) y porcentaje de aislamientos sensibles y no sensibles (%) a los 12 antimicrobianos estudiados frente a 180 y 96 cepas de *Trueperella pyogenes* aisladas de porcino y rumiantes, respectivamente.

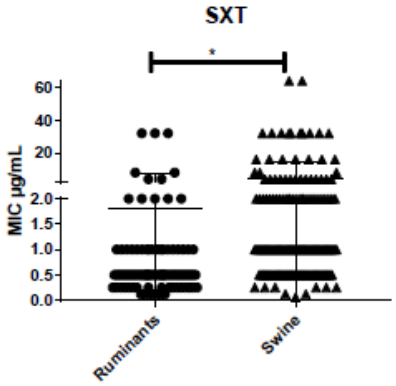
	Aislamientos de porcino (n=180)				Aislamientos de rumiantes (n=96)			
Antimicrobiano	CMI_{50}	CMI_{90}	S	NS	CMI_{50}	CMI_{90}	S	NS
PEN	0,004	0,008	97,8	2,2	0,004	0,008	93,7	6,3
AML	0,03	0,06	-	-	0,03	0,06	-	-
CEF	0,5	1	-	-	0,12	0,5	-	-
APR	2	4	-	-	2	4	-	-
GEN	0,5	1	-	-	0,5	1	-	-
NEO	4	16	-	-	4	8	-	-
STR	8	8	-	-	0,25	16	-	-
ENR	0,5	1	-	-	0,25	0,5	-	-
OTC	4	16	-	-	2	32	-	-
SXT	1	16	1,7	98,3	0,5	2	7,3	92,7
TYL	0,25	512	-	-	0,06	64	-	-
E	0,06	≥16	46,1	54	0,008	≥1024	77,2	22,8

PEN: penicilina; AML: amoxicilina; CEF: ceftiofur; APR: apramicina; GEN: gentamicina; NEO: neomicina; STR: estreptomicina; ENR: enrofloxacina; OTC: oxitetraciclina; SXT: sulfametoxazol/trimetoprim (19/1); TYL: tilosina; E: eritromicina; SU: susceptible; NS: no susceptible.


STR
MIC µg/mL
Ruminants
Swine


ENR
MIC µg/mL
Ruminants
Swine


OTC
MIC µg/mL
Ruminants
Swine


SXT
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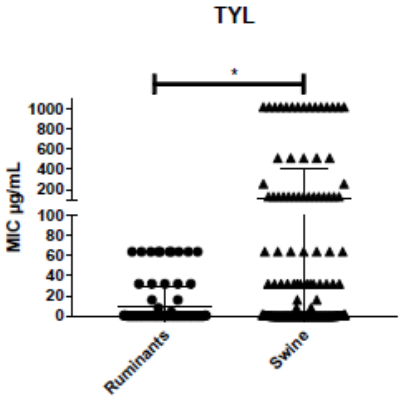
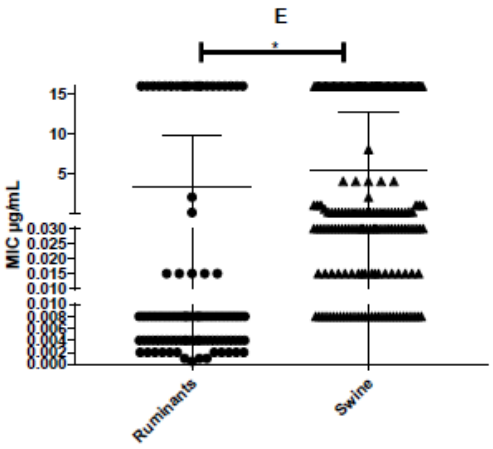
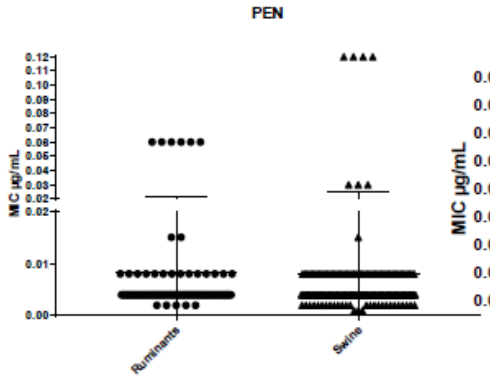
Ruminants
Swine


TYL
MIC µg/mL
Ruminants
Swine


E
MIC µg/mL
Ruminants
Swine


PEN
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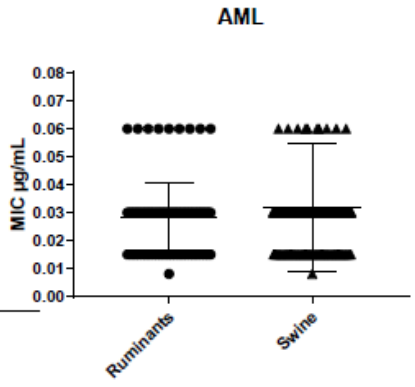
Ruminants
Swine


AML
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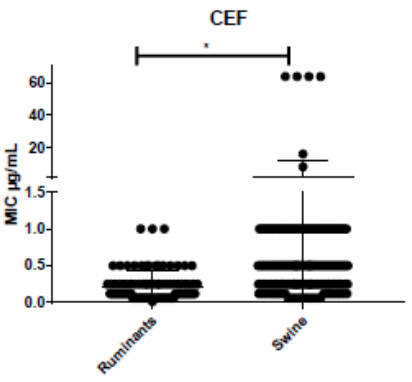
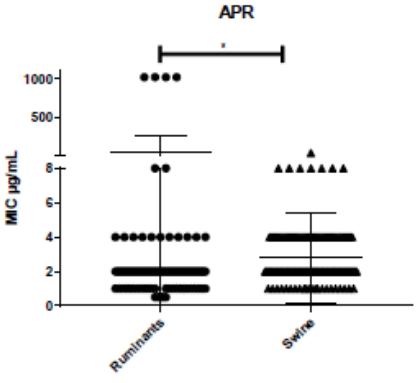
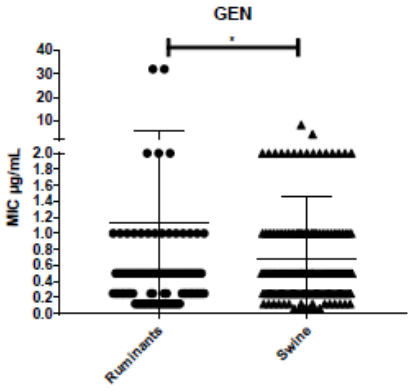
Ruminants
Swine


CEF
MIC µg/mL
Ruminants
Swine


APR
MIC µg/mL
Ruminants
Swine


GEN
MIC µg/mL
Ruminants
Swine

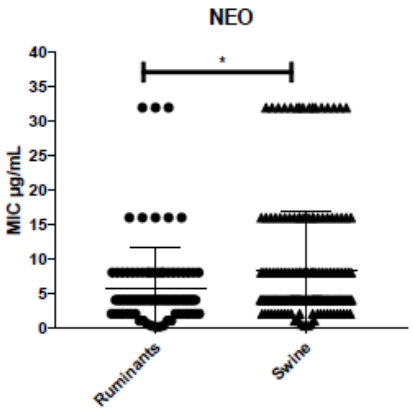

NEO
MIC µg/mL
Ruminants
Swine

Figura 1. Representación gráfica de la distribución de CMI (µg/ml) de los 12 antimicrobianos estudiados frente a los aislamientos de porcino (n=180) (triángulo) y de rumiantes (n=96) (punto). Cada triángulo o punto representa un único aislamiento. La mediana está representada con una línea horizontal. *Diferencia estadísticamente significativa entre la distribución de CMI de ambas poblaciones de *T. pyogenes* (porcino y rumiantes) ($P \leq 0.05$). abreviaturas: PEN: penicilina; AML: amoxicilina; CEF: ceftiofur; APR: apramicina; GEN: gentamicina; NEO: neomicina; STR: estreptomicina; ENR: enrofloxacina; OTC: oxitetraciclina; SXT: sulfametoxazol/trimetoprim (19/1); TYL: tilosina; E: eritromicina; SU: susceptible; NS: no susceptible.

Objetivo 3/Objective 3

Objetivo 3: Identificar y seleccionar las proteínas de superficie comunes de *Trueperella pyogenes* con el fin de desarrollar una vacuna eficaz frente a la infección producida por este microorganismo.

Objective 3: To identify and select common surface *Trueperella pyogenes* proteins to develop an effective vaccine against the infection produced by this microorganism.

Study 4: Study of Trueperella pyogenes pan-surfome as source of putative vaccine candidates

Study of *Trueperella pyogenes* "pan-surfome" as source of putative vaccine candidates

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Short title: Pan-surfome of *T. pyogenes*.

Abstract

Trueperella pyogenes is an opportunistic pathogen responsible for different clinical manifestations in livestock animals, being especially important in swine, in which causes suppurative infections (abscesses, pneumonia, arthritis, endocarditis or lymphadenitis) rising number of condemnations at slaughterhouse. The vaccination is one of the most effective strategies to control these diseases in livestock. The surface proteins, a priori more exposed to antibodies, could be good vaccine candidates. The objective of this work was to study the “pan-surfome” of *T. pyogenes* to identify some new antigen(s) to be used in further studies as vaccine candidates.

Sixteen *T. pyogenes* isolates obtained from slaughtered pigs were studied by proteomics. They were “shaved” (alive cells digestion using trypsin) and analysed by LC/MS/MS to identify the “pan-surfome”. A total of 1144 proteins were detected including 260 surface proteins. Among this group, 47 (18.1%) lipoproteins, 51 (19.6%) cell wall-anchored proteins, 149 (57.31%) membrane proteins and 13 (5%) secreted proteins were found. We classified the surface proteins into three categories. Group A gathered all the proteins identified in more than 70% of isolates, group B in 50-70%, and group C in 30-50%. Apart from being surface expressed and widely distributed, those proteins must fulfil the following criteria to be considered as putative vaccine candidates: tightly expressed and highly conserved.

In this study, the utility of proteomic digestion of living cells (shaving) to detect common surface proteins and to describe the “pan-surfome” of *T. pyogenes* have been showed for the first time. Moreover, 5 cell wall proteins (A0A0M4K9J9, X4QWN2, A0A2G9KBZ5, A0A0M3SNH9, X4R8M3), 3 lipoproteins (A0A0M3SNR1, A0A0M4K9G4, A0A0M4JY33), 3 secreted proteins (X4R0V4, A0A2G9KEL5, Q9S0W7) and 2 membrane proteins (A0A2G9KDB2, A0A0M3SNZ9) were identified in more than 70% of the studied isolates, were tightly expressed and were highly conserved. These proteins could be putative candidates, alone or in combination, to obtain effective vaccines against *T. pyogenes* infections although further studies are necessary.

Keywords: *T. pyogenes*, pan-surfome, surface-anchored proteins, vaccine candidates

1. Introduction

Trueperella pyogenes (formerly *Arcanobacterium pyogenes*) is a common inhabitant of skin and mucous membranes of upper respiratory, gastrointestinal, reproductive and urinary tracts of pigs and ruminants [1]. However, it can be an opportunistic pathogen responsible for purulent infections, such as metritis, mastitis, pneumonia and abscesses, of special importance in livestock [2]. The antimicrobial treatment is the main tool to control the infections caused by this microorganism nowadays [3]. Nevertheless, the increasing bacterial resistance to antibiotics and the difficulty of the antimicrobial to reach the focus of infection (most times abscesses) are causing the search of safe and effective alternatives to control this microorganism infections. Hence, vaccination is one of the most recommended alternatives and should be considered a primary method of *T. pyogenes* infections prevention [2]. Various approaches to stimulate a protective immunity against *T. pyogenes* infection in animals have been tried. Whole-cell vaccines based on killed or attenuated strains or culture supernatant have given inconsistent results [4–6]. Currently, the focus has shifted towards proteins as vaccine candidates, mainly to surface proteins, more exposed and accessible to antibodies. Therefore, they have the best chance to raise a high and effective immune response [7–9]. The studies with *T. pyogenes* are very limited, only the pyolisin (PLO), a recognized virulence factor for this species, has been tested as a possible vaccine, showing promising results (Rzewuska *et al.*, 2019).

Proteomics tools have been applied for the discovery of new antigens as putative vaccines [10,11]. In the last years, the “surfome” analysis through the “shaving” method, consisting in treating live cells with proteases and analysing the resulting peptides by LC/MS/MS, has showed its power to identify, in a fast and reliable way, surface proteins in a sample [12]. This approach has allowed to discover new immunoprotective proteins against different pathogens [13–16]. In this study, the proteomic analysis of different clinical isolates of *T. pyogenes* is developed for the first time, by applying the “shaving” approach, with the objective of describing the “pan-surfome” and identifying common surface proteins, with broad coverage, as vaccine candidates.

2. Material and Methods

2.1. Bacterial strains and culture conditions

Sixteen *Trueperella pyogenes* strains isolated from slaughtered pigs belonging to intensive and extensive production systems were used (Table 1). All strains, maintained at -80 ºC, were plated on Columbia CNA agar (Oxoid ltd.) supplemented with 5% (v/v) sterile defibrinated sheep blood. Plates were incubated under microaerophilic conditions (5% CO_2) at 37 °C for 24-48 h (Feβler and Schwarz, 2017). Then, the whole bacterial growth was inoculated in 45 ml of brain heart infusion (BHI, Oxoid ltd.K) and incubated at 37 ºC for 48 h y aerophilic conditions.

Table 1. *Trueperella pyogenes* isolates obtained from slaughtered pigs analysed

Internal reference	**Proteomics reference**	**Origin***
TAC-20.5-H	A	Liver
M-22	B	Lung
M-29	C	Lymph nodes
M-43	D	Brain
M-66	E	Lung
M-76	F	Articular fluid
428-NLSUB	G	Submandibular lymph node
SRC-47-NL	H	Lymph nodes
424-H	I	Liver
CO-314-B	L	Spleen
TAC-8-NLIS	M	Inguinal superficial lymph node
M-87	N	Heart
M-9	Ñ	Articular fluid
M-85	O	Heart
777-ABS	P	Abscess
M-27	Q	Lung

* All the isolates were recovered from pigs totally or partially condemned at the slaughterhouse.

2.2. Surface digestion of living cells

Forty-five ml of bacteria from exponential growth phase (corresponding approximately to 10^7 cells at OD_{595} = 0.4) were harvested by centrifugation at 3.500 × *g* for 10 min at 4 °C and washed three times with 20 ml of PBS. Cells were resuspended in 0.4 ml of PBS/30% sucrose in a 1.5 ml tube. Proteolytic reactions were carried out with trypsin (Promega) at 5 µg/ml, for 30 min at 37 °C with top-down agitation. The digestion mixtures were centrifuged at 3.500 × *g* for 10 min at 4 °C, and the supernatants (the "surfomes" containing the peptides) were filtered using 0.22-µm pore-size filters (Millipore). "Surfomes" were re-digested with 2 µg trypsin during 2 h at 37 ºC with top-down agitation. Protease reactions were stopped by adding 0.1% formic acid (Promega). Salts were removed using Oasis HLB extraction cartridges (Waters). Peptides were eluted with increasing concentrations of acetonitrile/0.1% formic acid, according to manufacturer's instructions. Peptide fractions were concentrated with a vacuum concentrator (Eppendorf), and kept at -20 ºC until further analysis.

2.3. Liquid Chromatography -Mass Spectrometry analysis (LC/MS/MS)

Peptide separation was performed by nano-LC using a Dionex Ultimate 3000 nano UPLC (Thermo Scientific, San Jose, CA), equipped with a reverse phase C18 75 µm × 50 Acclaim Pepmap column (Thermo Scientific) at 300 nl/min and 40 ºC for a total run time of 85 min. The mix of peptides was previously concentrated and cleaned up on a 300 µm × 5 mm Acclaim Pepmap cartridge (Thermo Scientific) in 2% acetonitrile/0.05% formic acid for 5 min, with a flow of 5 µl/min. Buffer A (0.1% formic acid) and Buffer B (80% acetonitrile, 0.1% formic acid) were used as mobile phase for the chromatographic separation according to the following elution conditions: 4-35% buffer B for 60 min; 35-55% buffer B for 3 min; 55-90% buffer B for 3 min followed by 8 min washing with 90% buffer B, and re-equilibration during 12 min with 4% buffer B.

Peptide positive ions eluted from the column were ionized by a nano-electrospray ionization source and analyzed in positive mode on a trihybrid Thermo Orbitrap Fusion (Thermo Scientific) mass spectrometer operating in Top30 Data Dependent Acquisition mode with maximum cycle time of 3 s. MS1 scans of peptide precursors were acquired

in a 400-1500 m/z range at 120,000 resolution (at 200 m/z) with a 4×10^5 ion count target threshold. For MS/MS, precursor ions were previously isolated in the quadrupole at 1.2 Da, and then CID-fragmented in the ion trap with 35% normalized collision energy. Monoisotopic precursor selection was turned on. Ion trap parameters were: i) the automatic gain control was 2×10^3; ii) the maximum injection time was 300 ms; and iii) only those precursors with charge state 2–5 were sampled for MS/MS. In order to avoid redundant fragmentations a dynamic exclusion time was set to 15 s with a 10-ppm tolerance around the selected precursor and its isotopes.

2.4. Database searching and protein identification

The mass spectrometry raw data were processed using Proteome Discoverer (version 2.1.0.81, Thermo Scientific). Charge state deconvolution and deisotoping were not performed. MS/MS spectra were searched with SEQUEST engine against a database of Uniprot_*Trueperella pyogenes*_Jun2018 (www.uniprot.org), applying the following search parameters: Trypsin digestion with 4 missed cleavages. Methionine oxidation was set as variable modification. A value of 10 ppm was set for mass tolerance of precursor ions, and 0.1 Da tolerance for product ions. Peptide identifications were accepted if they exceeded the filter parameter Xcorr score *versus* charge state with SequestNode Probability Score (+1 = 1.5, +2 = 2.0, +3 = 2.25, +4 = 2.5).

2.5. Bioinformatic analysis of protein sequences

Computational predictions of subcellular localization were carried out by using different algorithms. Briefly, we used the web-based algorithm LocateP [17]. Feature-based algorithms were also used to contrast LocateP predictions: TMHMM 2.0 [18] for searching transmembrane helices; SignalP 3.0 [19] for type-I signal peptides: those proteins containing only a cleavable type-I signal peptide as featured sequence were classed as secreted; LipoP [20] for identifying type-II signal peptides, which are characteristic of lipoproteins. Topological representations of membrane proteins (Figure 1) were performed with the web-based TOPO2 software (http://www.sacs.ucsf.edu/TOPO2/). Moreover, the algorithm VaxiJen (http://www.ddg-

pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), based on proteins physicochemical properties, was used to predict the antigenic properties of the proteins. A Vaxijen model used was “bacterial”, with the threshold fixed on 0,5 [21,22]. Finally, the amino acid composition of the identified secreted proteins was calculated by ProtParam tool (https://web.expasy.org/protparam/) to determine the proportion of proline and alanine, previously related to the protein immunogenicity [21,23]. The graphical representation was carried out using excel (Microsoft® Office Excel).

3. Results

3.1. Describing the “pan-surfome” of *T. pyogenes*

The surface proteome of 16 clinical isolates (Table 1) was obtained by surface tryptic digestion (“shaving approach”). We defined the “surfome” of each isolate as its individual analysis, and the global “pan-surfome” as the complex of all the proteins found in the whole strains collection. The MS analysis resulted in the identification of 1144 proteins of *T. pyogenes* among all the studied strains (Table 2). Related to subcellular location, 260 proteins (22,72%) were annotated as “surface proteins”, 64 proteins (5.59%) were included in the unknown group (no subcellular location prediction) and 820 proteins (71,68%) were classified as cytoplasmic proteins. The list of all the identified proteins, the frequency of identification for each protein among replicates per isolate, and among all the strains analysed are shown in Table 3.

Regarding surface proteins, 4 categories were differentiated; cell wall proteins, those with a LPXTG motif, membrane proteins, those having transmembrane domains, secreted proteins, those possessing a SP1-type signal peptide, and lipoproteins, those predicted as lipid-anchored proteins. Among these categories, 51 (19.6%) proteins were identified as cell wall anchored proteins, 149 (57.31%) as membrane proteins, 13 (5%) as secreted proteins and 47 (18.1%) as lipoproteins. The range with the minimum and maximum numbers of proteins detected per isolate is also shown (Table 2).

Table 2. Summary of identified surface proteins in *Trueperella pyogenes* isolates obtained from pigs by “shaving” cells and LC/MS/MS analysis.

Protein category [a]	Identified proteins among all the isolates	Range of identified proteins per isolate
Surface proteins	**260 (22.72%)**	**87-159**
Lipoprotein	47 (18.1%)	13-29
Cell Wall	51 (19.6%)	13-28
Secretory	13 (5%)	5-10
Membrane	149 (57.31%)	56-92
Unknown	**64 (5.59%)**	**20-48**
Cytoplasmic	**820 (71.68%)**	**289-550**
Total	1144	

[a]Protein categories were established according to web UNIPROT (https://www.uniprot.org/) subcellular localization. Other algorithms were also used to contrast those predictions: TMHMM 2.0 for searching transmembrane helices; SignalP 3.0 for type-I signal peptides which are presented in secreted proteins; LipoP for identifying type-II signal peptides, which are characteristic of lipoproteins. Cell wall proteins were those which had a LPXTG motif. “Pan-surfome” include the sum of those previous categories described (surfaced proteins). The proteins that presented doubtful location were manually inspected. Unknown proteins are those without a subcellular location prediction and cytoplasmic proteins are those without any exporting or sorting signal and predicted as intracellular proteins.

The membrane proteins were those exhibiting the highest expression frequencies: 32 proteins were found in more than 50% of the analysed isolates. In a second place, 8 cell wall anchored proteins were identified in more than 50% of the strains. About lipoproteins and secreted proteins categories, 6 and 5 proteins were found in more than a half of the strains, respectively. However, if we compare the identification frequencies of the different categories in relative terms, the secretory proteins and the cell wall category were the most prevalent, as it showed the highest

number of proteins identified in a high proportion of isolates; 5 out of 13 proteins (38,46%) and 8 out of 51 proteins (15,7%) in ≥50% of the isolates, respectively.

3.2. Classification of proteins *Trueperella pyogenes* proteins from the “pan-surfome” based on their potential as putative vaccine candidates

We selected and classified the identified proteins in three different groups (A, B, C; from best to worst) of a priori potentiality for further immunization and/or vaccination studies based on previous works. Briefly, this classification is based on that a good protein vaccine candidate, in order to increase the chances to raise an effective immune response, must be surface-exposed, tightly expressed, highly conserved and widely distributed among different isolates [13,22–24]. According to the two first parameters, only surface proteins and those expressed in at least 2 out of 3 replicates carried out per isolate, were considered. A special mention is needed for membrane proteins, which are the most embedded in the membrane because they have transmembrane domains (TMD). There are membrane proteins with one TMD, which probably have domains in the extracellular side with hundreds of amino-acids residuals, and membrane proteins with more than one TMD, with less probability of having loops large enough to reach the surface and be accessible to antibodies. For this reason, membrane proteins were divided into two sub-groups; with one TMD (n=8) and with more than one TMD (n=6) (Table 2). Their topology was studied by means of TOPO2 Transmembrane Protein Display to perform their representation (Figure 1) and the TMHMM algorithm to predict TMD presence. The membrane proteins that were included in the ranking were those with the majority of peptides oriented to the external side of the membrane (Figure 1), either with one or more than one TDM.

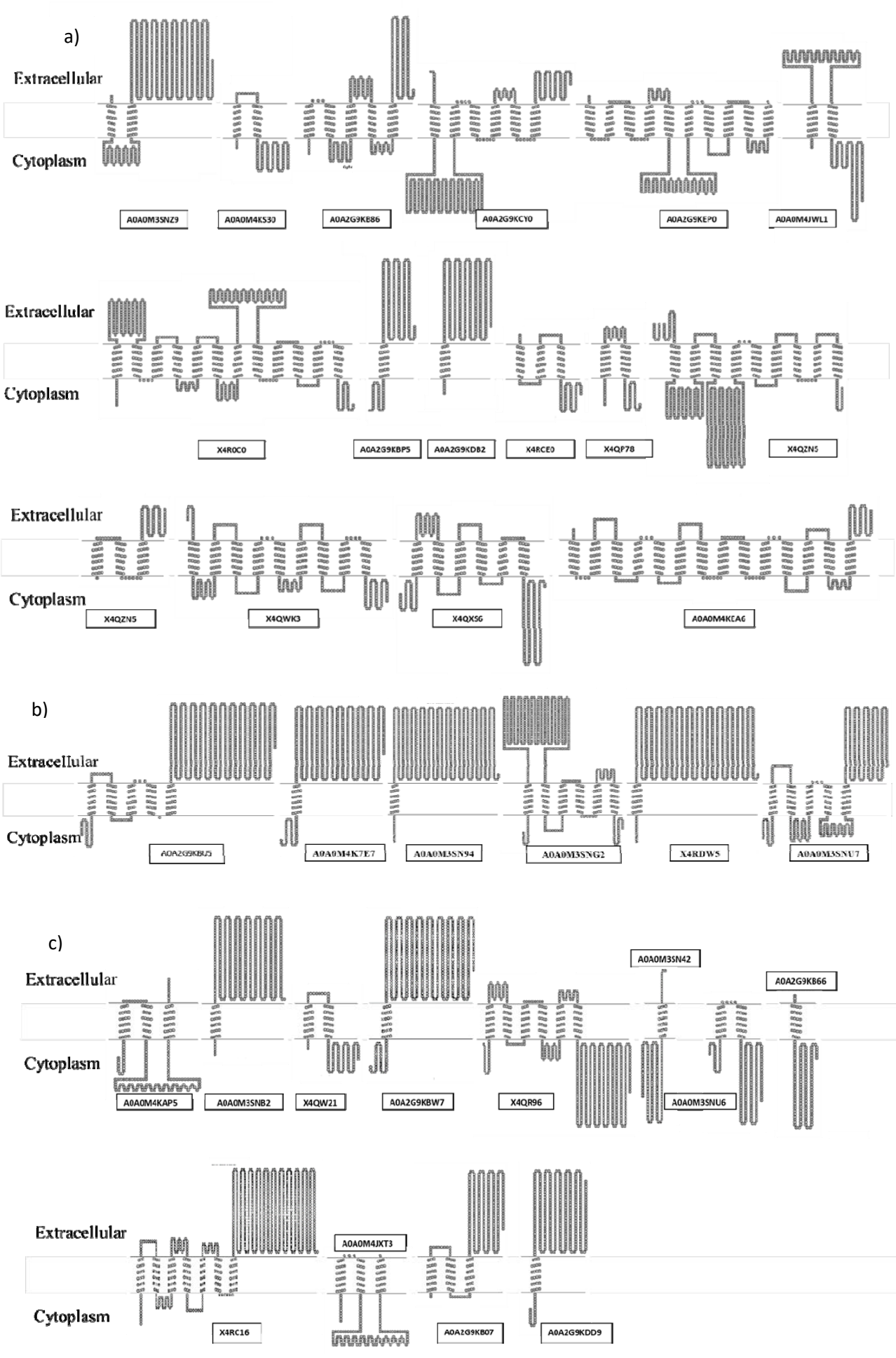

a)
Extracellular
Cytoplasm
A0A0M3SNZ9
A0A0M4KS30
A0A2G9KB86
A0A2G9KCY0
A0A2G9KEP0
A0A0M4JWL1
Extracellular
Cytoplasm
X4R0C0
A0A2G9KBP5
A0A2G9KDB2
X4RCE0
X4QP78
X4QZN5
Extracellular
Cytoplasm
X4QZN5
X4QWK3
X4QXS6
A0A0M4KEA6
b)
Extracellular
Cytoplasm
A0A2G9KBU5
A0A0M4K7E7
A0A0M3SN94
A0A0M3SNG2
X4RDW5
A0A0M3SNU7
c)
Extracellular
Cytoplasm
A0A0M4KAP5
A0A0M3SNB2
X4QW21
A0A2G9KBW7
X4QR96
A0A0M3SN42
A0A0M3SNU6
A0A2G9KB66
Extracellular
Cytoplasm
X4RC16
A0A0M4JXT3
A0A2G9KB07
A0A2G9KDD9

Figure 1. Topological representation of the identified membrane proteins in the “pan-surfome” analysis of the 16 *T. pyogenes* isolates. The TOPO2 Transmembrane Protein Display was used to performance the representation and the TMHMM algorithm to predict transmembrane domains (TMD). a) Proteins included in the group A (70% analyzed isolates). b) Proteins which were included in the group B (50-70% analyzed isolates). c) Proteins included in the group C (30-50% analyzed isolates).

To check whether those proteins were highly conserved among the strains, the degree of homology in the amino acid sequence of each proteins was compared with the 10 sequenced strains that are nowadays published in NCBI. All these proteins showed a degree of homology in their amino acid sequence that ranged from 82,5% to 100% among all the completely sequenced isolates of *T. pyogenes* (n=10).

To ensure the widely distribution of these proteins, three groups were established, group A (n=14) included proteins present in more than 70% of isolates, group B (n=14) gathered proteins present in 50-70% of isolates, whereas group C (n=17) included proteins present in 30-50% of isolates (Table 4).

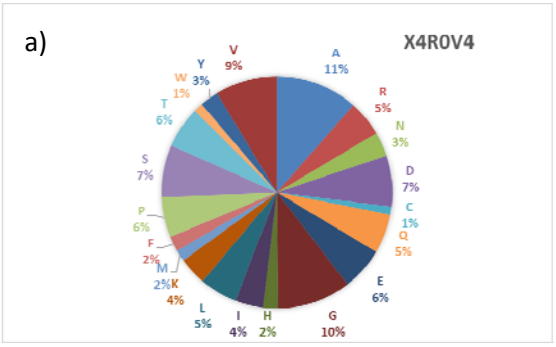
Table 4: Grouping of proteins identified in *T. pyogenes* isolates (n=16) according to their potentiality as putative antigens for further immunization and/or vaccination studies.

Group	Proteins
A (14)[a]	Cell wall: A0A0M4K9J9, X4QWN2, A0A2G9KBZ5, A0A0M3SNH9, X4R8M3 Lipoproteins: A0A0M3SNR1, A0A0M4K9G4, A0A0M4JY33 Secreted proteins: X4R0V4, A0A2G9KEL5, Q9S0W7 Membrane proteins (1 TMD): **A0A2G9KBP5,** A0A2G9KDB2 Membrane proteins (>1 TMD): A0A0M3SNZ9
B (14)	Cell wall: A0A2G9KAP3, A0A2G9KC18, X4RCL8 Lipoproteins: **A0A0M5KPJ2**, A0A0M4KR83, X4QXA0 Secreted proteins: A0A2G9KD79, A0A2G9KA87 Membrane proteins (1 TMD): A0A0M4K7E7**, A0A0M3SN94, X4RDW5** Membrane proteins (>1 TMD): A0A2G9KBU5, A0A0M3SNG2, A0A0M3SNU7
C (17)	Cell wall: A0A0M3SNB7, A0A0M5KIG4, **X4QMI5,** A0A2G9K9K3, A0A2G9KAV2 Lipoproteins: A0A0M4K9R6, A0A0M5KH79, **A0A2G9KEH2**, A0A0M4K5B8, A0A0M5KKG8 Secreted proteins: A0A0M4KB23, **A0A0M4JYB5** Membrane proteins (1 TMD): A0A0M3SNB2, A0A2G9KBW7, **A0A2G9KDD9** Membrane proteins (>1 TMD): X4RC16, A0A2G9KB07

[a] A: >70% analyzed isolates; B: 50-70% analyzed isolates; C: 30-50% analyzed isolates. The listed proteins were identified in at least 2 replicates of the proteomic analysis. We classified the membrane proteins in two different groups, membrane proteins with one transmembrane domain (TMD) and membrane proteins with more than one transmembrane domain. In bold are highlight the proteins which were excluded because did not reach the score 0.5 by using VaxiJen tool.

Furthermore, the amino acid composition of the selected secreted proteins was calculated by using ProtParam tool (https://web.expasy.org/protparam/). The analysis revealed that all (100%) of the most predominant secreted proteins were rich in alanine (9.2%-26.40%), being this amino acid its principal compound. The secreted proteins were also rich in proline, ranging from 2.4 % to 6.9%, although this amino acid was not one of the major compounds (Figure 2).

a)


X4R0V4
A 11%
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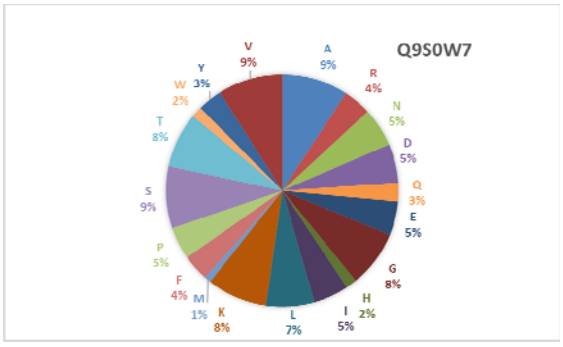
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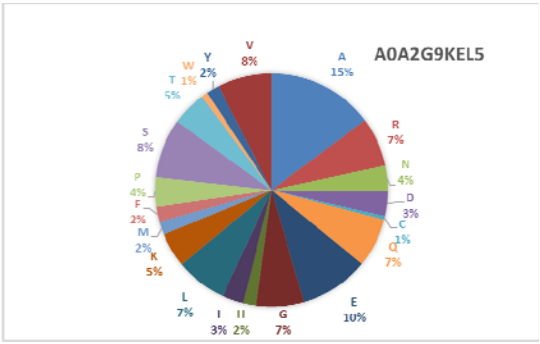
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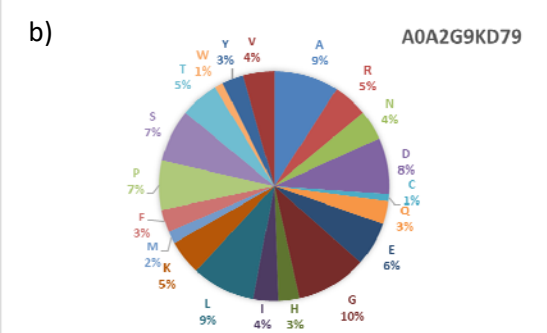
b)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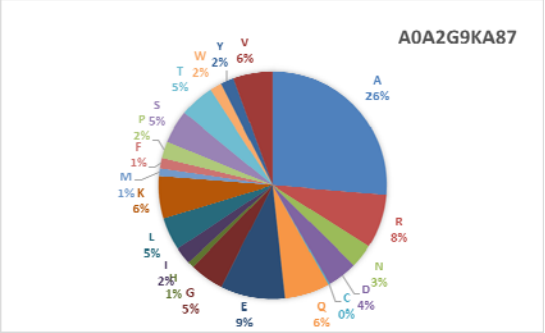
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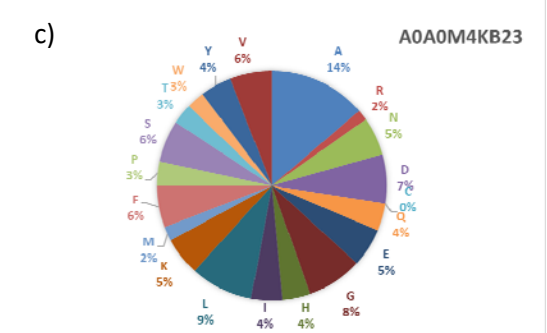
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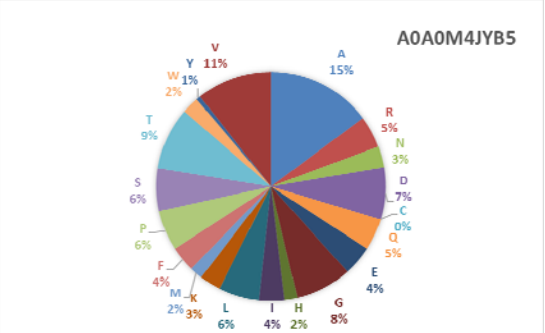
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Figure 2. Amino acid composition of the identified secreted proteins in the "pan-surfome" analysis of the 16 *T. pyogenes* isolates. The amino acid composition was evaluated by using ProtParam tool (https://web.expasy.org/protparam/). Amino acids are indicated by the standard nomenclature one-letter code. a) Proteins included in the group A (70% analyzed isolates). b) Proteins included in group B (50-70% analyzed isolates). c) Proteins included in the group C (30-50% analysed isolates).

Finally, the algorithm VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was used to predict the antigenic properties of the selected proteins. Most of the proteins which were previously selected reached a score of 0.5 (ranged from 0.50 to 0.8727). Some proteins like 1 membrane protein from the group A, 1 lipoprotein, 2 membrane proteins with 1 TMD and 1 membrane protein with >1TMD ranking in the group B and 1 cell wall anchored protein, 1 secreted protein, 1 lipoprotein and 1 membrane protein with 1 TMD from group C, were removed from the rating due to reach a VaxiJen score lower than 0.5 and be classified as a non-antigen (in bold in the Table 4).

Considering the average score per protein category (independently of the group A, B or C), an average of 0.61 was obtained for cell wall proteins, secreted proteins and lipoproteins. A lower score was obtained for the membrane proteins, either with one TMD (0.48) or more than one TMD (0.55) (Table 2).

4. Discussion

The development of recombinant vaccines for the control of animal diseases is a global challenge and the identification of common antigens with high immunogenic capacity is a preliminary step for the design of these new vaccines. Previous studies have demonstrated the utility of the "shaving" approach of proteomics for the identification of bacterial antigens as vaccine candidates [13–16]. We applied this technique for the first time to describe the "pan-surfome" of *T. pyogenes* species, analyzing different clinical isolates obtained from pigs. A total of 260 out of 1144 proteins detected were surface proteins, including cell wall anchored proteins (19.6%), secreted proteins (5%), membrane proteins (57.31%) and lipoproteins (18.1%). Similar results have also been obtained by proteomic analysis in other Gram-positive species,

such as *Streptococcus pneumoniae*, *Streptococcus suis*, *Enterococcus faecalis* and group A *Streptococcus* [10,13,25,26].

Considering that intra-species genetic variability can take place and vary the protein expression pattern among isolates, finding a lot of proteins common to the majority of the analysed isolates would not be expected [10,13,27]. In fact, in *Streptococcus pneumoniae* it has been reported that only the 10.5% of identified surface proteins were common to the 5 the analysed strains [10] and in *Streptococcus suis*, Gómez-Gascón *et al*., did not identify any protein in the 100% of the tested strains (n=35). In our study some surface proteins have been identified in more than 50% of the *T. pyogenes* isolates (Table 3) with differences between categories, being the secreted proteins (38.46% ,5/13) and the cell wall proteins (15.7%, 8/51) the most prevalent ones. These fact suggests that these proteins are most exposed on the surface of Gram-positive bacteria, as already reported [7]. Moreover, this indicates a variable protein expression pattern among all the isolates, which coincides with the findings previously showed by our group, that show a relatively high genetic diversity in this bacteria species [28].

After describing the pan-surfome, we selected and classified the identified proteins in three different groups (A, B, C; from best to worst) for further immunization and/or vaccination studies. This classification is based on that a good protein vaccine candidate, in order to increase the chances to raise an effective immune response, must be surface-exposed, tightly expressed, highly conserved and widely distributed among different isolates. Following the criteria described above, 14 proteins were included in the groups A and B, and 17 proteins gathered in the group C, belonging to cell-wall-anchored protein (n=13), secreted protein (n=7) and lipoprotein (n=9) categories. Although several membrane proteins were presented in more than 30% of isolates the proportion of those proteins included in the ranking (n=14) is relative much lower than for the rest of surface protein categories, since these proteins are only accessible if they have large enough domains to reach the surface through the peptidoglycan layer.

Finally, we used the algorithm VaxiJen, which is based on the physicochemical properties of the proteins to predict the immunogenic capacity of those identified proteins. Proteins that reached a score ≥ 0.5 were included in the classification [21,22]. Most of the selected proteins reached the above described score (ranged from 0,50 to 0,8727), although some proteins were removed because they did not reach the cited VaxiJen score (< 0.5), being classified as “non-antigen” by the software. According to the average score obtained in VaxiJen for cell wall proteins, secreted proteins and lipoproteins (0.,61, respectively), those categories would be considered the best options to get a high and effective immune response. Those finding agree with the statement published by other authors supporting that the surface proteins, the anchored to the cell wall and the secreted ones are the best options to this purpose [7–9].

Furthermore, the amino acid composition of the identified secreted proteins was studied to detect the proportion of proline and alanine, which have been related to higher immunogenicity of the proteins [21,23]. Our results showed that all (100%) of the most predominant secreted proteins detected, were rich in alanine (9.2%-26.40%), being this amino acid their main component. The secreted proteins were also rich in proline, (2.4%-6.9%), although it was not the main component.

From all the proteins detected, 5 cell wall proteins, 3 lipoproteins, 3 secreted proteins, and 2 membrane proteins (Table 4), fulfilled requirements to be appropriate candidates for further vaccination studies. They all were surface proteins, were tightly expressed (at least, in 2 out of 3 replicates carried out per isolate), were widely distributed (present in ≥70% of isolates) and were highly conserved (high homology with NCBI sequenced *T. pyogenes* strains). It should be noted that pyolysin (Q9S0W7) resulted a putative vaccine candidate in this work, which is not surprising since is considered the major virulence factor of *T. pyogenes* and the *plo* gen has been detected in all will-type strains until now [2]. Other studies have showed promising results with a vaccine based on PLO in murine models[29–31]. We consider that our work provides interesting information to obtain an effective vaccine against *Trueperella pyogenes* infections in the livestock.

5. Conclusions

The utility of proteomic digestion of living cells (shaving) to detect common surface proteins and to describe the “pan-surfome” of *Trueperella pyogenes* have been showed. Moreover, 5 cell wall proteins (A0A0M4K9J9, X4QWN2, A0A2G9KBZ5, A0A0M3SNH9, X4R8M3), 3 lipoproteins (A0A0M3SNR1, A0A0M4K9G4, A0A0M4JY33), 3 secreted proteins (X4R0V4, A0A2G9KEL5, Q9S0W7) and 2 membrane proteins (A0A2G9KDB2, A0A0M3SNZ9) were identified in more than 70% of the studied strains, tightly expressed and highly conserved. These proteins could be candidates, alone or in combination, to obtain effective vaccines against *T. pyogenes* infections, although further studies are needed.

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Capítulo IV / Chapter IV:

Conclusiones / Conclusions

1. Según nuestros resultados, los antimicrobianos penicilina (CMI_{90} = 0,008 µg/ml), amoxicilina (CMI_{90} = 0,06 µg/ml) y ceftiofur (CMI_{90} = 1 µg/ml) pueden ser utilizados como primera línea de tratamiento para el control de las infecciones producidas por *Trueperella pyogenes* en ganado porcino criado en sistemas intensivos y extensivos.

2. Se han detectado diferencias estadísticamente significativas en los perfiles de sensibilidad antimicrobiana y diversidad genética (PFGE) entre los aislamientos de *T. pyogenes* obtenidos de cerdos criados en sistemas extensivos e intensivos. Estos datos sugieren que las prácticas de manejo influyen en las características fenotípicas y moleculares de los aislamientos.

3. Analizando los resultados obtenidos con aislamientos obtenidos de rumiantes, se observaron algunas diferencias entre la distribución de CMI de los antimicrobianos entre bovino, ovino y caprino, aunque los valores de CMI_{90} de penicilina, amoxicilina y ceftiofur (≤1 µg/ml) permiten aconsejar su uso para el control de las infecciones por este patógenos en bovino, ovino y caprino. Por otra parte, no se aconseja la utilización de otros antimicrobianos, como la oxitetraciclina (32 µg/ml, tilosina (512 µg/ml) y eritromicina (≥1024 µg/ml).

4. De acuerdo con nuestros resultados, los antimicrobianos penicilina y amoxicilina pueden ser utilizados como primera línea de tratamiento para el control de las infecciones provocadas por *T. pyogenes* en animales de abasto, aunque debe confirmarse en el laboratorio su eficacia, ya que aplicando los puntos de corte publicados (penicilina, sulfametoxazol-trimetoprim, eritromicina), hemos detectado cepas no sensibles frente a los tres antimicrobianos.

5. Nuestro trabajo aporta información interesante para la determinación de puntos de corte específicos para *T. pyogenes*, inexistentes hasta la fecha para la mayoría de antimicrobianos e imprescindibles a la hora de un tratamiento eficaz y sostenible frente a este patógeno.

6. Se demuestra la utilidad de la técnica proteómica de digestión de células vivas (*pelado*) y posterior análisis mediante espectrometría de masas para la descripción del

“pan-surfoma” de *T. pyogenes*, obteniendo un total de 260 proteínas superficiales, que pueden ser utilizadas con fines diagnósticos y de control de la enfermedad.

7. Del conjunto de proteínas superficiales identificadas, 13 (5 proteínas de pared celular, 3 lipoproteínas, 3 proteínas secretadas y 2 proteínas de membrana) están presentes en más del 70 por ciento de las cepas analizados, y se proponen como posibles candidatos para la elaboración de vacunas de subunidades para el control de las enfermedades producidas por *T. pyogenes*.

CONCLUSIONS

1. According to our results, the antimicrobials penicillin (MIC_{90}=0.008 µg/ml), amoxicillin (MIC_{90}=0.06 µg/ml) and ceftiofur (MIC_{90}=1 µg/ml) can be used as the first line of treatment for control of infections caused by *Trueperella pyogenes* in pigs reared in intensive and extensive systems.

2. Statistically significant differences in the profiles of antimicrobial susceptibility and genetic diversity (PFGE) of *Trueperella pyogenes* have been detected between isolates obtained from pigs reared in extensive and intensive systems. These data suggest that the management practices could influence the phenotypic and molecular characteristics of the isolates.

3. Comparing MIC distribution of antimicrobials studied against isolates obtained from cattle, sheep or goats some differences were observed. No significant differences were found between ruminant and swine isolates regarding MIC_{90} values of penicillin, amoxicillin and ceftiofur (≤1µg/ml). Thus, these antimicrobials agents could be of choice for diseases caused by *T. pyogenes* in livestock animals.

4. When the cut-off points published by CLSI (2016) are applied, a variable percentage of isolates not susceptible to penicillin and sulfamethoxazole/trimethoprim was obtained, which demonstrates the importance of the *in vitro* tests to know the efficacy of antimicrobials before applying the treatments. On the other hand, we consider that our results represent an advance to define the cut-off points of a broad group of antimicrobials against this pathogen.

5. The usefulness of the proteomic technique of living cell digestion (shaving) and subsequent analysis by mass spectrometry for the description of “pan-surfome” of *T. pyogenes* is demonstrated, obtaining a total of 260 surface proteins, which can be used with *T. pyogenes* diagnostic and diseases control purposes.

6. Of the set of surface proteins identified, a set of 13 proteins included in Group A (4 cell wall proteins, 2 membrane proteins, 3 secreted proteins and 3 lipoprotein) are present in more than 70 percent of the isolates tested, and are proposed as possible candidates for the development of subunit vaccines for the control of diseases caused by *T. pyogenes*.

Capítulo V / Chapter V:

Resumen / Summary

Esta tesis doctoral incluye parte de los resultados obtenidos de los proyectos “Estrategias de Control frente a la Linfadenitis del Cerdo Ibérico en extensivo”, financiado por el Ministerio de Agricultura, Alimentación y Medio Ambiente (Rf. 20140020001824), y el Proyecto “Aproximaciones multiómicas al estudio de las resistencias a antibióticos en patógenos Gram-positivos”, dentro del XXII Programa Propio de Fomento de la Investigación, Modalidad 4.2. Ayudas para potenciar el establecimiento de SINERGIAS en el desarrollo de proyectos I+D precompetitivos.

T. pyogenes es un microorganismo ubicuo, que se puede encontrar en la piel, orofaringe y las vías respiratorias superiores, mucosa urogenital y gastrointestinal de los animales sin producir manifestaciones clínicas. Se ha demostrado que es capaz de infectar a una gran variedad de animales domésticos, incluyendo perros y gatos, animales silvestres y se han descrito cuadros clínicos en personas en contacto con animales. Este patógeno oportunista causa una gran variedad de manifestaciones clínico-lesionales, fundamentalmente en rumiantes y cerdos, siendo raro en caballos o aves, como mamitis, metritis, artritis, linfadenitis, otitis, peritonitis, piodermitis, onfaloflebitis, endocarditis, osteomielitis, bronconeumonías e infecciones urinarias y genitales, aunque también producir infecciones sistémicas, en función de la edad, el estado inmune del animal, que puede verse alterado por la presencia de otras enfermedades inmunosupresoras y factores ambientales, que permiten la supervivencia de la bacteria. Esta enfermedad puede ser diagnosticada en el animal vivo o bien durante la inspección de las canales en el matadero, y originan el decomiso parcial o total, dando lugar a importantes pérdidas económicas para las explotaciones afectadas de rumiantes o cerdos.

A pesar de su importancia clínica y su frecuencia de presentación, existe un desconocimiento sobre los mecanismos de acción patógena y a su papel como agente etiológico primario en las enfermedades asociadas a este agente. Por otra parte, la información que se dispone actualmente sobre el comportamiento de *T. pyogenes* frente a los antimicrobianos utilizados en ganadería es limitada, y hasta hace tres años, no existían normas publicadas para la realización de las pruebas de sensibilidad in vitro para esta especie, que permitieran comparar los resultados entre estudios. En el año 2017, la CLSI publicó un nuevo documento para estandarizar los métodos para

la realización de las pruebas e interpretación de resultados con bacterias poco frecuentes y de difícil crecimiento, aisladas de animales (VET06, CLSI M45). El documento VET06 establece puntos de corte para la categoría susceptible para la penicilina, ampicilina, eritromicina y trimethoprim/sulfametoxazol, pero no para otros antimicrobianos de uso frecuente en ganadería. Por último, existen importantes lagunas en el control basado en la vacunación, ya que las vacunas que se han utilizado tradicionalmente han sido vacunas inactivadas, que se aplican para el control de los brotes causados en una explotación, pero no son eficaces para otros brotes.

Por ello, el principal objetivo de este trabajo es aportar información relevante para el control de las enfermedades causadas por *Trueperella pyogenes* en ganado porcino y rumiantes, especies donde esta bacteria tiene mayor importancia sanitaria y económica. Se han llevado a cabo diferentes estudios para cumplir los siguientes objetivos específicos

Objetivo 1: Estudio de la sensibilidad antimicrobiana y diversidad genética de cepas de *Trueperella pyogenes* aisladas de ganado porcino.

Estudio 1: “Antimicrobial susceptibility and genetic characterization of Trueperella pyogenes isolates from pigs reared under intensive and extensive farming practices” Este trabajo ha sido publicado en la revista Veterinary Microbiology, vol 232, pp: 89-95, https://doi.org/10.1016/j.vetmic.2019.04.011 y se presenta como indicio de calidad para la lectura y defensa de la tesis doctoral (Con un factor de impacto según el Journal Citation Report (2017), de 2,524, situado en el primer decil (8/140) dentro de la categoría de Ciencias Veterinarias.

En este trabajo se analizaron 180 aislamientos de *T. pyogenes*, obtenidos de cerdos sacrificados en matadero y criados bajo sistemas intensivos (TpIN, n = 89) y extensivos (TpEX, n = 91). Se utilizaron análisis de tipificación mediante electroforesis en gel de campo pulsado (PFGE) para caracterizar genéticamente las cepas y análisis de concentración inhibitoria mínima (MIC) para determinar la distribución de MIC. Se obtuvieron valores bajos de MIC90 para penicilina y amoxicilina (0,008 y 0,06 µg / ml, respectivamente), ceftiofur, gentamicina y enrofloxacina (1 µg / ml, respectivamente),

por lo que podrían aconsejarse para el tratamiento empírico de las infecciones por *T. pyogenes*. A excepción de la penicilina, la amoxicilina y el ceftiofur, se observó una diferencia estadísticamente significativa en la distribución MIC de todos los antimicrobianos analizados entre los aislados TpIN y TpEX. Además, los valores de MIC90 fueron más altos en TpIN que en los aislados de TpEX para neomicina y estreptomicina (32 µg / ml frente a 8 µg / ml), sulfametoxazol / trimetoprima (30,4 / 1,6 µg / ml frente a 1,90 / 0,10 µg / ml) y tilosina (≥ 1024 µg / ml frente a 1 µg / ml). En base a los resutlados de PFGE, se detectó una diversidad genética relativamente menor en TpIN en comparación con los aislados de TpEX (GD 0,42 y GD 0,47, respectivamente). Todos los aislamientos se distribuyeron en tres grupos genéticos (A, B, C). Los aislados de TpIN se asociaron estadísticamente con el grupo A (P = 0,0002; OR 3,21; CI95 1,74-5,93), mientras que los TpEX se distribuyeron en todo el dendrograma, mostrando una mayor diversidad genética. Estos datos sugieren que la susceptibilidad a los antimicrobianos y la variabilidad genética de los aislados de T. pyogenes podrían estar influidos por los sistemas de manejo.

Objetivo 2: Estudiar la posible relación epidemiológica entre aislamientos de *Trueperella pyogenes* obtenidos de porcino y rumiantes. Se han llevado a cabo dos estudios

Estudio 2: “Antimicrobial susceptibility of Trueperella pyogenes isolated from food-producing ruminants” Este trabajo ha sido enviado a Veterinary Microbiology en agosto de 2019, y está en revisión cuando se presenta la tesis.

Se han analizado un total de 96 aislados de *Trueperella pyogenes*, obtenidos de bovinos (n = 34), ovinos (n = 35) y caprinos (n = 27), e identificados por PCR en tiempo real (qPCR), para determinar la susceptibilidad a 12 antimicrobianos de uso frecuente en ganadería, mediante el ensayo de microdilución en caldo. Nuestros resultados muestran que las distribuciones de concentración inhibitoria mínima (MIC) de apramicina, gentamicina, estreptomicina, oxitetraciclina, tilosina y eritromicina mostraron una tendencia a la bimodalidad, siendo unimodal para el resto de los antimicrobianos. Se obtuvieron valores bajos de MIC90 para penicilina, amoxicilina, ceftiofur, enrofloxacina y gentamicina (<1 µg / ml), por lo que podrían ser la primera

línea de tratamiento empírico para el control de las enfermedades producidas por T. pyogenes en rumiantes. De acuerdo con los puntos de corte específicos de T. pyogenes para penicilina, sulfametoxazol/ trimetoprim y eritromicina, el 93,7% de los aislamientos fueron susceptibles a la penicilina y el 77,2% a eritromicina, mientras que el 92,7% no fueron susceptibles al sulfametoxazol/trimetoprim. Se observaron diferencias significativas en la distribución MIC de casi todos los antimicrobianos, excepto la enrofloxacina, la tilosina y la eritromicina frente a cepas aisladas de bovinos, ovinos o caprinos, aunque todos los antimicrobianos mostraron valores de MIC90 similares, excepto la apramicina y la oxitetraciclina que mostraron valores más altos para la inhibición de cepas de origen bovino. Estos datos muestran información interesante sobre los antimicrobianos de elección para el tratamiento de infecciones causadas por *T. pyogenes* en rumiantes.

Estudio 3: "Perfil de sensibilidad antimicrobiana de *Trueperella pyogenes*: aportaciones para el control en animales de abasto". Este trabajo ha sido presentado en el XXIV Simposio de AVEDILA (Asociación de Especialistas en Diagnóstico Laboratorial Veterinario) en forma de comunicación oral. Noviembre 2019, Pamplona, España.

En este trabajo realizamos un análisis de todos los aislamientos, comparando los resultados obtenidos entre cerdos y rumiantes y cumpliendo, así, uno de los objetivos marcados en esta tesis doctoral. Se muestran los resultados de distribución de CMI, CMI50, CMI90 y porcentaje de cepas no sensibles a los antimicrobianos en estudio frente al total de aislamientos (n=276), y se comparan en función de la especie de origen de las cepas; ganado porcino (n=180) y rumiantes (n=96).

Los antimicrobianos más eficaces frente al total de cepas (n=276) han sido penicilina y amoxicilina (CMI90 de 0.008 µg/ml). Hemos obtenido buenos resultados con ceftiofur, gentamicina y enrofloxacina (1 µg/ml). Por otra parte, detectamos valores de CMI90 elevados de tilosina (128 µg/ml), seguidos de los valores de neomicina, estreptomicina, oxitetraciclina y eritromicina, todos de 16 µg/ml De acuerdo con nuestros resultados, los antimicrobianos penicilina y amoxicilina pueden ser utilizados como primera línea de tratamiento para el control de las infecciones provocadas por *T.*

pyogenes en animales de abasto. Este estudio resalta la importancia de realizar pruebas de sensibilidad in vitro para confirmar resultados y asegurar el éxito del tratamiento, además de para monitorizar los microorganismos, ya que se han detectado cepas no sensibles a la penicilina (3.6%) según los puntos de corte actualmente disponibles.

Cuando se comparan los resultados de ambas poblaciones (aislamientos de porcino y aislamientos de rumiantes), se observan diferencias estadísticamente significativas ($P<0.05$) en la distribución de CMI de todos los antimicrobianos, excepto de penicilina y amoxicilina, y se puede apreciar que las cepas aisladas de ganado porcino requieren, en general, concentraciones de antimicrobiano más altas para inhibir su crecimiento que las cepas de rumiantes.

Nuestro trabajo aporta información interesante para la determinación de puntos de corte específicos para *T. pyogenes*, inexistentes hasta la fecha para la mayoría de antimicrobianos e imprescindibles a la hora de un tratamiento eficaz y sostenible frente a este patógeno.

Objetivo 3: Identificar y seleccionar las proteínas de superficie comunes de *Trueperella pyogenes* con el fin de desarrollar una vacuna eficaz frente a la infección producida por este microorganismo.

Estudio 4: “Study of *Trueperella pyogenes* pan-surfome as source of putative vaccine candidates” Este trabajo está enviado a PLOS One, y está en revisión cuando se presenta la tesis.

En este estudio se obtuvo el “pan-surfoma” de 16 aislamientos clínicos de *T. pyogenes* mediante digestión de superficie con tripsina con el objetivo de identificar algún antígeno nuevo para futuros estudios vacunales. Los aislamientos clínicos fueron “pelados” (digestión con tripsina de células vivas) y analizados mediante LC/MS/MS para identificar el “pan-surfoma”. Se identificaron un total de 1144 proteínas, 260 de las cuales resultaron ser de superficie (22,72%), 820 se incluyeron en la categoría de proteínas citoplasmáticas (71,68%) y, finalmente, no fue posible identificar la categoría subcelular (desconocida) de 64 proteínas (5.59%).

Todas las proteínas de superficie se clasificaron en los tres grupos A, B y C (de mejor a peor) en función de su potencial para ser incluidas en futuros estudios de inmunización y vacunación. Únicamente se tuvieron en cuenta las proteínas de superficie, dado que son las más expuestas al sistema inmune. De las 260 proteínas de superficie, 47 fueron lipoproteínas (18.1%), 51 resultaron proteínas de anclaje a la pared (19,6%) y 149, proteínas de membrana (57,31%), aunque también se identificaron 13 proteínas secretadas (5%). Una vez debidamente identificadas, se clasificaron según los siguientes parámetros: ser expresadas de forma constante en la población (al menos en 2 de las 3 réplicas realizadas para cada aislamiento), ser altamente conservadas (gran homología con las cepas de T. pyogenes secuenciadas del NCBI) y estar ampliamente distribuidas en entre los aislamientos (% de los aislamientos en el que las proteínas están presentes). De este modo, las proteínas se clasificaron en tres grupos; el grupo A (n=14) incluye todas las proteínas presentes en más del 70% de los aislamientos, el grupo B (n=14) entre 50 y 70%, y el grupo C (n=17) entre 30% y 50%. Todas las proteínas del ranking mostraron una homología en su secuencia de aminoácidos de entre el 82.5% y el 100% con los aislamientos de T. pyogenes que han sido secuenciados por completo (n=10).

Finalmente, todas estas proteínas se analizaron usando el algoritmo VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), que se basa en las propiedades fisicoquímicas de las proteínas para predecir la propiedad antigénica de las del ranking. Aquellas proteínas con una puntuación inferior a 0.5, se descartaron de la selección. En total, el grupo A quedó con 13 proteínas, el grupo B, con 10, y el grupo C, con 13.

En conclusión, queda demostrada la utilidad de la técnica proteómica de digestión de células vivas (pelado), y del subsecuente análisis mediante espectrometría de masas, para la descripción del “pan-surfoma” de *T. pyogenes*. A pesar de la contaminación con proteínas citosólicas, debida a la lisis, se ha identificado un gran número de proteínas de superficie. Las 13 incluidas en el grupo A (4 proteínas de pared, 2 proteínas de membrana, 3 proteínas secretadas y 3 lipoproteínas) son atractivas para el desarrollo de vacunas recombinantes o de subunidades, aunque son recomendables más estudios en profundidad.

This doctoral thesis includes part of the results obtained from the projects “Control Strategies against Lymphadenitis of Iberian Pig in extensive”, financed by the Ministry of Agriculture, Food and Environment (Rf. 20140020001824), and the Project “Multiomic approaches to the study of antibiotic resistance in Gram-positive pathogens ”, within the XXII Own Program for the of Research Promotion, Modality 4.2. Aid to enhance the establishment of SINERGIAS in the development of pre-competitive R&D projects.

T. pyogenes is a ubiquitous microorganism, which can be found in the skin, oropharynx and upper respiratory tract, urogenital and gastrointestinal mucosa of animals without producing clinical manifestations. It has been shown to be able to infect a wide variety of domestic animals, including dogs and cats, wild animals and clinical profiles have been described in people in contact with animals. This opportunistic pathogen causes a wide variety of clinical-lesion manifestations, mainly in ruminants and pigs, being rare in horses or birds, such as mastitis, metritis, arthritis, lymphadenitis, otitis, peritonitis, pyodermitis, omphalophlebitis, endocarditis, osteomyelitis, bronchopneumonia and infections urinary and genital. This microorganism also produces systemic infections, depending on animal`s age or immune status, which can be altered by the presence of other immunosuppressive diseases and influenced by environmental factors. These diseases can be diagnosed in farms or, more frequently, in the slaughterhouse, which leads to the partial or total carcasses condemnation and, thus, to significant economic losses.

Despite its clinical importance and frequency of presentation, there is a lack of knowledge about the mechanisms of pathogenic action and the role of *Trueperella pyogenes* as a primary etiological agent in the diseases associated with this agent. On the other hand, the information currently available on the behaviour of *T. pyogenes* against antimicrobials used in livestock is limited, and until three years ago, there were no published standards for conducting the *in vitro* susceptibility tests for this species, which would allow to compare the results between studies. In 2017, the CLSI published a new document to standardize the methods for testing and interpreting results with rare and hard-growing bacteria, isolated from animals (VET06, CLSI M45). Document VET06 establishes cut-off points for the susceptible category for penicillin, ampicillin,

erythromycin and sulfamethoxazole/trimethoprim, but not for other antimicrobials often used in livestock. Finally, there are important gaps in vaccination-based control, only inactivated vaccines have been applied for outbreaks control in farms and are not effective under other conditions.

Therefore, the **main objective** of this work is to provide relevant information for the control of diseases caused by *Trueperella pyogenes* in pigs and ruminants, species where this bacterium has greater sanitary and economic importance. To develop this objective, three studies have been carried out with the following specific objectives:

Objective 1: Study of antimicrobial susceptibility and genetic diversity of strains of *Trueperella pyogenes* isolated from pigs.

Study 1: "Antimicrobial susceptibility and genetic characterization of *Trueperella pyogenes* isolates from pigs reared under intensive and extensive farming practices".

This work has been published in the journal Veterinary Microbiology, vol 232, pp: 89-95, https://doi.org/10.1016/j.vetmic.2019.04.011 and is presented as a quality parameter for the defense of the doctoral thesis (with an impact factor according to the Journal Citation Report (2017), of 2,524, located in the first decile (8/140) within the category of Veterinary Sciences).

In this work, the Minimal Inhibitory Concentration (MIC) and Pulsed Field Gel Electrophoresis (PFGE) typing analysis were used to determine the MIC distribution and to genetically characterize a total of 180 *T. pyogenes* isolates obtained from slaughtered pigs reared under intensive (TpIN, n=89) and extensive (TpEX, n=91) farming practices. Low MIC_{90} values for penicillin and amoxicillin (0.008 and 0.06 µg/ml, respectively), ceftiofur, gentamicin and enrofloxacin (1 µg/ml, respectively) were obtained, so they could be of choice for the empiric treatment of *T. pyogenes* infections. Except for the penicillin, amoxicillin and ceftiofur, a statistically significant difference was observed in the MIC distribution of all antimicrobials analysed between TpIN and TpEX isolates. Also, MIC_{90} values were higher in TpIN than in TpEX isolates for neomycin and streptomycin (32 µg/ml vs 8 µg/ml), sulfamethoxazole/trimethoprim (30.4/1.6 µg/ml vs 1.90/0.10 µg/ml) and tylosin (≥1024 µg/ml vs 1 µg/ml). A relatively

lower genetic diversity was detected in TpIN in comparison with TpEX isolates (GD 0.42 and GD 0.47, respectively). All isolates were distributed in three clusters (A, B, C). TpIN isolates were statistically associated with cluster A (P = 0.0002; OR 3.21; CI95 1.74-5.93), whereas the TpEX were distributed throughout the dendrogram, showing more genetic diversity. These data suggest that the antimicrobial susceptibility and genetic variability of the *T. pyogenes* isolates could be influenced by the management systems.

Objective 2: To study the possible epidemiological relationship between isolates of Trueperella pyogenes obtained from pigs and ruminants. Two studies have been carried out

Study 2: "Antimicrobial susceptibility of *Trueperella pyogenes* isolated from food-producing ruminants". This manuscript was sent to Veterinary Microbiology in August, 2019 and is under review.

In this work, a total of 96 *Trueperella pyogenes* isolates, an opportunistic pathogen of food-producing ruminants, obtained from cattle (n=34), sheep (n=35) and goats (n=27), and identified by Real Time PCR (qPCR), were analysed to determine the susceptibility to 12 antimicrobials commonly used in livestock, by the broth microdilution assay. Minimal Inhibitory Concentration (MIC) distributions of apramycin, gentamicin, streptomycin, oxytetracycline, tylosin and erythromycin showed a tendency to bimodality, being unimodal for the rest of antimicrobials. Low MIC_{90} values for penicillin, amoxicillin, ceftiofur, enrofloxacin, and gentamicin (<1 µg/ml) were obtained, so they could be of choice for the first line of empiric treatment of *T. pyogenes* infections. According to the specific *T. pyogenes* breakpoints for penicillin, sulfamethoxazole/trimethoprim and erythromycin, 93.7% of isolates were susceptible to penicillin and 77.2% to erythromycin, whereas the 92.7% were nonsusceptible to sulfamethoxazole/trimethoprim. Significant differences were observed in the MIC distribution of almost all antimicrobials, except enrofloxacin, tylosin and erythromycin against cattle, sheep or goat isolates, although all antimicrobials showed similar MIC_{90} values, except apramycin and oxytetracycline that showed higher values against cattle

isolates. These data provide interesting information on the antimicrobials of choice for the treatment of infections caused by *T. pyogenes* in ruminants.

Study 3: “Antimicrobial sensitivity profile of *Trueperella pyogenes*: contributions for control in food-producing animals”. This work has been presented at the XXIV Symposium of AVEDILA (Association of Veterinary Laboratory Diagnostic Specialists) in the form of oral communication. November 2019, Pamplona, Spain.

In this work an analysis of all isolates was carried out, comparing the results obtained between pigs and ruminants and thus fulfilling one of the objectives set out in this doctoral thesis. The results of the distribution of MIC, MIC50, MIC90 of every antimicrobial against total isolates (n = 276) are shown, and they are compared according to the species of origin of the strains; pigs (n = 180) and ruminants (n = 96).

The most effective antimicrobials against the total of isolates (n = 276) have been penicillin and amoxicillin (MIC90 of 0.008 µg / ml). We have obtained good results with ceftiofur, gentamicin and enrofloxacin (1 µg / ml). On the other hand, high MIC90 values of tylosin (128 µg / ml), neomycin, streptomycin, oxytetracycline and erythromycin (16 µg / ml, respectively) were detected. According to our results, penicillin and amoxicillin can be used as the first line of treatment for the control of infections caused by *T. pyogenes* in food-producing animals. This study highlights the importance of performing *in vitro* sensitivity tests to confirm results and ensure the success of the treatment, in addition to monitoring microorganisms, since non-penicillin-sensitive strains (3.6%) have been detected according to the cut-off points currently available.

When the results of both populations are compared (pig isolates and ruminant isolates), statistically significant differences ($P < 0.05$) are observed in the MIC distribution of all antimicrobials, except penicillin and amoxicillin. Pigs isolates needed, in general, higher concentrations of antimicrobial to inhibit their growth than ruminant strains.

Our work provides interesting information for the determination of specific cut-off points for *T. pyogenes*, nonexistent to date for most antimicrobials and essential when it comes to an effective and sustainable treatment against this pathogen.

Objective 3: To identify and select common surface *Trueperella pyogenes* proteins to develop an effective vaccine against the infection produced by this microorganism.

Study 3: "Study of *Trueperella pyogenes* "pan-surfome" as source of putative vaccine candidates".

For this study, the "pan-surfome" of 16 clinical isolates was obtained by surface tryptic digestion with the aim of identifying some new antigen(s) to be used in further studies as vaccine candidates. The clinical isolates were "shaved" (alive cells digestion using trypsin) and analysed by LC/MS/MS to identify the "pan-surfome". A total of 1144 proteins were identified, 260 were annotated as surface proteins (22,72%), 820 were included in cytoplasmatic category (71,68%) and finally, 64 proteins (5,59%) resulted in no subcellular location prediction (unknown).

The total of surface proteins was classified in three groups A, B, C (from best to worst) of a priori potentiality for further immunization and/or vaccination studies on the basis of previous works. Only surface proteins were taken into account due to be the most exposed ones to the immune system. From the 260 surface ones, 47 were lipoproteins (18,1%), 51 (19,6%) cell wall-anchored proteins and 149 (57,31%) membrane proteins, also 13 (5%) proteins secreted were identified. Then, they were ranked according to the following parameters: being tightly expressed (at least, in 2 out of 3 replicates carried out per isolate), highly conserved (high homology with ncbi sequenced *T. pyogenes* strains) and widely distributed among isolates (% of isolates in which the proteins are present). This way, the proteins were classified into three groups; group A (n=14) gathered all the proteins identified in more than 70% of isolates, group B (n=14), in 50-70%, and group C (n=17) in 30-50%. All the proteins listed in the ranking in all the groups shown a degree of homology in their amino acid sequence which range from 82,5% to 100% among all the completely sequenced isolates of *T. pyogenes* (n=10).

Finally, all those proteins were analyzed by using the algorithm VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) which is based on the physicochemical properties of the proteins to predict the protective antigenicity of those proteins included on the ranking. Proteins with a score under 0,5 were discarded from the ranking. In total, group A included 13 proteins, group B, 10 and group C, 13.

In conclusion, the usefulness of the proteomic technique of living cell digestion (shaving) and subsequent analysis by mass spectrometry for the description of “pan-surfome” of *T. pyogenes* is demonstrated. Despite the contamination with cytosolic proteins, due to lysis, a high number of surface proteins were identified, being the set of 13 proteins included in Group A (4 cell wall proteins, 2 membrane proteins, 3 secreted proteins and 3 lipoprotein) attractive to develop recombinant vaccines or subunit ones, although further studies are necessary.