universidad de león

Salmonelosis porcina en España:

factores de riesgo en reproductores, estrategias de control en cerdos de cebo y la importancia del sacrificio

Salmonella infection in pig production in Spain:

risk factors in breeding pigs, control strategies in finishing pigs and the role of the slaughtering in the pork contamination

Héctor Argüello Rodríguez, 2013

Memoria presentada por Héctor Argüello Rodríguez y dirigida por los doctores Ana Carvajal Urueña y Pedro Rubio Nistal para optar al grado de Doctor por la Universidad de León, dentro del programa de doctorado “Sanidad Animal y Reproducción".

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Héctor Argüello Rodríguez disfrutó de una beca de la Consejería de Educación de la Junta de Castilla y León y del Fondo Social Europeo.

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A mi familia,

A mis tíos Chano y Lardo. A mi madre.

RESUMEN

Salmonella es uno de los agentes causales de toxi-infecciones alimentarias más relevantes en la Unión Europea (UE). El éxito de los programas de control de este microorganismo en avicultura ha hecho disminuir la incidencia de las infecciones en el hombre y, al mismo tiempo, ha convertido a la carne de cerdo en una importante fuente de infección, segunda en importancia, solo superada por los huevos.

De acuerdo con las directrices de la UE, en un futuro próximo se establecerán objetivos de reducción de prevalencia de *Salmonella* en el ganado porcino y programas nacionales de vigilancia y control de *Salmonella*. Sin embargo, dichos programas no son preceptivos por el momento aunque algunos países ya los han implantado desde hace años. España no dispone de un programa nacional de control de *Salmonella* si bien en la última década se han realizado numerosos estudios que han arrojado información relevante sobre la situación de la salmonelosis porcina en España. El objetivo general de la presente tesis se ha dirigido hacia el incremento del conocimiento relativo a la epidemiología y el control de la infección por *Salmonella* en el ganado porcino. Los estudios realizados se desarrollan en tres capítulos que abarcan las explotaciones de reproductores, el control de la infección en las granjas de cebo y las etapas posteriores a la granja: transporte, espera y sacrificio. En total, la tesis incluye seis publicaciones científicas originales y dos revisiones, una publicada como artículo de revisión en una revista científica y la otra incluida como capítulo de un libro.

Como parte de los estudios basales de prevalencia de *Salmonella* en cerdos en la UE, se llevó a cabo un estudio en granjas de reproductores en España en el que además de las muestras de heces y el cuestionario incluidos en las directrices europeas, se recogieron muestras de sangre. Los resultados de bacteriología y serología mostraron la elevada prevalencia de la infección en las granjas de reproductores. En cuanto a los serotipos detectados, si bien los dos más prevalentes coincidieron con los previamente identificados en las granjas de cebo, existieron variaciones que podrían ser indicativas de una relevancia moderada de los reproductores en la infección de los cerdos de cebo. El análisis de factores de riesgo a partir de los resultados de bacteriología, mediante una regresión logística binomial, reveló que el empleo de pienso granulado y la reposición externa de los verracos estaban asociados a la presencia de *Salmonella* en la explotación. Las granjas fueron clasificadas en tres niveles en función de la proporción de sueros positivos a *Salmonella* y los resultados fueron incluidos en un modelo de regresión logística ordinal. La seroprevalencia varío entre

trimestres, siendo más elevada en los meses fríos (de octubre a marzo). El alojamiento individual se relacionó con granjas de elevada seroprevalencia mientras que el suelo total o parcialmente enrejillado se asoció a granjas de baja seroprevalencia.

Debido a la elevada prevalencia de *Salmonella* en granjas de engorde en España, en la presente tesis se han valorado dos estrategias de control en cebo. El primero de los estudios consistió en la evaluación del efecto de acidificantes, incorporados en el pienso o agua de bebida durante el último tercio del engorde. En las tres pruebas de campo realizadas la seroprevalencia del grupo experimental fue significativamente inferior a la del grupo control al final del tratamiento. Así mismo, en dos de las granjas se consiguió una reducción en el porcentaje de cerdos eliminadores al final del tratamiento. El segundo estudio determinó la eficacia de una vacuna inactivada de *S.* Typhimurium aplicada al comienzo del periodo de engorde. La vacuna redujo la eliminación fecal de *Salmonella* en las granjas infectadas por *S.* Typhimurium pero no tuvo efecto protector en una granja con infección heteróloga.

En el tercer capítulo de la tesis se evaluaron las etapas relacionadas con el sacrificio de los cerdos en el matadero mediante un estudio de monitorización, de la granja al matadero, y un estudio de evaluación de la contaminación de las diferentes instalaciones, equipos y procedimientos de los mataderos. En dichos estudios, el origen de la contaminación observada en las canales porcinas se evaluó empleando métodos de tipificación molecular. Los resultados mostraron un aumento de la prevalencia de la infección durante las fases de transporte y, sobre todo, de espera en el matadero así como una elevada prevalencia de la contaminación en canales. Esta contaminación se asoció tanto a la propia infección de los cerdos en granja como a las nuevas infecciones ocurridas durante el transporte y la espera en corrales y a la contaminación durante las diferentes actividades, particularmente actividades manuales, en la línea de matanza. Los serotipos y los genotipos encontrados variaron entre visitas al mismo matadero e incluso dentro de un mismo día, demostrando un flujo continuo de *Salmonella* en estas instalaciones. En su conjunto, estos resultados demuestran la gran importancia que las fases posteriores a la granja tienen en el control de *Salmonella*. Finalmente, se evaluó el efecto de una estrategia de sacrificio logístico, organizado en función de la seroprevalencia, en la contaminación por *Salmonella* de las canales porcinas sin que pudieran observarse diferencias significativas. Los resultados obtenidos sugieren que para que el sacrificio logístico sea una estrategia de control eficaz deben mejorarse los protocolos de limpieza de las instalaciones y las actividades del matadero.

SUMMARY

Salmonella is one of the major food-borne pathogens in the European Union (EU). The success of the control programmes in poultry farming has allowed a decrease in the incidence of human cases and pork and pork products have increased their relative relevance. Nowadays, the pig reservoir is reported as the second most common source of human salmonellosis after laying hens.

Only few European countries have already implemented control programmes of *Salmonella* in swine production. Nevertheless in the next future, the EU will address the prevalence reduction objectives in swine and each member state will implement a compulsory national surveillance and control programme. Although there is not a national control programme established in Spain yet, a number of studies have been performed through the last decade. The general objective of this thesis has been the improvement of the knowledge of the epidemiology and control of *Salmonella* in Spain. The surveys performed are organized in three chapters related to breeding pigs, control measures at finishing farms and the post-harvest stages: transport, lairage and slaughtering. In total, the research includes six original articles and two reviews, one published in a peer reviewed journal and the other as a book chapter.

A study in breeding pigs in Spain was performed as part of the *Salmonella* prevalence baseline study in breeding pigs in the EU. Together with the compulsory faeces and questionnaires, the sampling also included the collection of sera samples within each farm. The bacteriological and serological analysis of these samples revealed a high prevalence of *Salmonella* infection in Spanish breeding pigs. Although the two main serotypes were shared with those previously reported in finishing pigs, the differences observed between both populations could be an insight of the moderate influence of the breeding pigs in the infections occurring at the end of the finishing. The use of pelleted feed as well as purchasing the boar replacement were associated to *Salmonella* shedding in the multivariate analysis using binomial logistic regression. Moreover, herds were classified in three levels according to the number of positive sera. The results were used in an ordinal logistic regression model which revealed that *Salmonella* seroprevalence increased during the cold months (October to March). Individual housing was associated with high seroprevalence herds while fully or partially slatted floors were associated with low seroprevalence herds.

Due to the high *Salmonella* prevalence reported in finishing herds, two control measures were evaluated. In the first study, the usefulness of organic acids added in feed or water throughout the last third of the finishing period was assessed in three different finishing farms. A lower seroprevalence in the treated group was reported regardless the acid used in the three clinical trials. Furthermore, a decreased prevalence of *Salmonella* shedders at the end of the treatment was also found in two of the herds. The second study evaluated the efficacy of an inactivated *S.* Typhimurium vaccine administered at the beginning of the finishing period. Vaccinated pigs showed a reduced shedding in those farms infected by *S.* Typhimurium while no protection was achieved in those suffering from heterologous infections.

The third chapter of the thesis is focused on post-harvest stages of the production by a monitoring study from the farm to the slaughterhouse and a descriptive study which analyses the contamination of the different slaughterhouse facilities, equipment and procedures. The isolates recovered in both studies were further typed by molecuar methods to determine the source of carcass contamination. The results revealed an increase in *Salmonella* contamination during the transport and, above all, at the lairage as well as a relevant prevalence in the pre-chilled carcasses. Carcass contamination was associated to *Salmonella* carriers as well as to the new infections occurred at transport and lairage or cross- contamination from slaughter line activities. The serotypes and genotypes varied among visits and even among different samplings in the same visit to the slaughterhouse, fact which implies a continuous flow of *Salmonella* in the slaughtering facilities. The results gathered here highlight the importance of the post-harvest stages in the control of *Salmonella* in swine. Finally, the effect of a logistic slaughter, in which slaughter was arranged according to farm *Salmonella* seroprevalence, was evaluated. Our results suggest that in order to achieve a reduction in *Salmonella* contamination in carcasses through this approach, cleaning and disinfection of facilities, particularly focused on the lairage, and hygiene at the slaughter line activities must be improved.

REVISIÓN BIBLIOGRÁFICA

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Mes de enero. Representa el fin de un año y el comienzo del año nuevo.

1. Importancia de *Salmonella* en las toxi-infecciones alimentarias

1.1 Impacto en la salud pública

Salmonella enterica subespecie *enterica* (de aquí en adelante *Salmonella*) es un microorganismo zoonótico de gran importancia en sanidad humana y animal. En las últimas dos décadas las mejoras en los sistemas de monitorización de las toxi-infecciones alimentarias han favorecido la identificación del agente o agentes etiológicos implicados en las mismas así como la elaboración de informes y datos estadísticos fiables. Se estima que cada año ocurren un total de 93,8 millones de casos de salmonelosis en el mundo mientras que el número de muertes estimadas por *Salmonella* es de 155.000 (Majowicz et al., 2010). En la Unión Europea (UE), *Salmonella* fue el segundo patógeno más frecuentemente involucrado en toxi-infecciones alimentarias, después de *Campilobacter spp*., y el primero en número de brotes, de acuerdo a los datos publicados por la Agencia Europea de Seguridad Alimentaria (EFSA) en los dos últimos informes correspondientes a los años 2010 y 2011 (EFSA, 2012; EFSA, 2013). Junto con la mejora de los sistemas de monitorización, se ha creado una conciencia social sobre la seguridad alimentaria que ha conllevado a la exigencia, por parte del consumidor, de productos más seguros desde un punto de vista sanitario. Este hecho unido a los datos anteriormente expuestos ha provocado que en los países desarrollados se hayan tomado iniciativas para el control de agentes patógenos alimentarios entre los cuales se encuentra *Salmonella.*

1.2 Salmonella en la Unión Europea

En el año 2003 la UE definió las estrategias para el control de *Salmonella* y otros microorganismos zoonóticos mediante el reglamento 2160/2003. Dicho reglamento estableció las bases para la reducción de *Salmonella* en las etapas de producción previas al sacrificio con el fin de contribuir a la mejora de la protección de la salud pública; los países miembros de la UE deben diseñar y poner en marcha programas de vigilancia y control para la reducción de la prevalencia de *Salmonella* en animales de producción (aves y cerdos). Dichos programas, ya están en marcha en avicultura. En la producción porcina, únicamente algunos países han instaurado programas de control, tanto obligatorios como voluntarios, cuyas características se describirán en detalle al final de esta introducción.

En base al reglamento 2160/2003, los datos de vigilancia de zoonosis y agentes zoonóticos y de brotes de enfermedades transmitidas por los alimentos son recogidos por cada uno de los estados miembros y transferidos a la Comisión Europea, que a su vez los traslada a la EFSA que se encarga de publicar, anualmente, los informes sobre las tendencias y

fuentes de las zoonosis, los agentes zoonóticos y la resistencia a los antimicrobianos en la Comunidad. Según los datos disponibles, desde el año 2004 y hasta el año 2011, el número de casos se ha reducido desde los 195.947 casos descritos en 2004 (tasa de incidencia anual 42,5 casos por 100.000 habitantes) hasta los 95.548 (tasa de incidencia anual 20,7 casos por 100.000 habitantes) del informe correspondiente al año 2011 (EFSA, 2013). Esta tendencia a nivel europeo también ha sido observada en los datos referentes a España, si bien la evolución no siempre se mantiene y el número de casos aumenta en años puntuales (Figura 1).

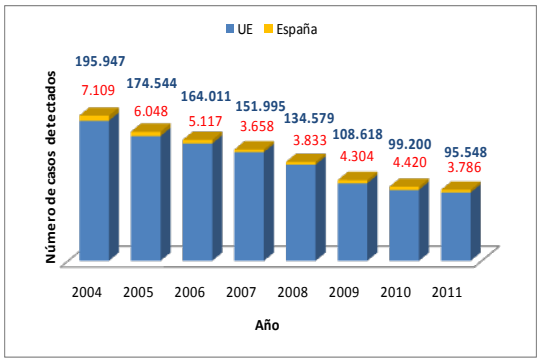

Figura 1a. Nº de casos reportados de salmonelosis humana en la Unión Europea (en azul) y España (en rojo) reportados por la EFSA entre 2004 y 2011

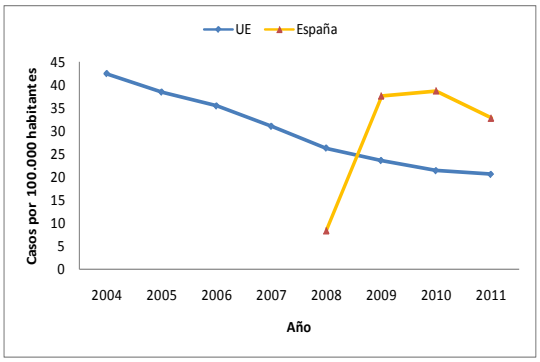

Figura 1b. Tendencia de Nº de casos por 100.000 habitantes reportados por la EFSA en la Unión Europea entre 2004 y 2011. Datos de España desde 2008

Figura 1. Número de casos declarados de salmonelosis entre los años 2004 y 2011 en la UE y España (fuente EFSA).

La disminución en la incidencia de salmonelosis se ha relacionado con la instauración de programas obligatorios de control de *Salmonella* en animales de producción, concretamente en avicultura (EFSA, 2012; EFSA, 2013). Los animales de producción constituyen el principal reservorio de *Salmonella* y son la primera fuente de contaminación tanto de los productos alimenticios de origen animal, principalmente carne y huevos, como de otros alimentos, por ejemplo vegetales, por contaminaciones cruzadas en su cultivo o manipulación. Según el último informe publicado por el Comité de Riesgos Biológicos (BIOHAZ) de la EFSA (EFSA, 2011a), del total de casos de salmonelosis, aproximadamente un 65% fueron debidos al consumo de huevos mientras que un 28%, 4,5% y 2,4% se relacionaron con el consumo de carne de cerdo, pavo y pollo, respectivamente. Del mismo modo, otro estudio externo auspiciado por la EFSA (Pires et al., 2011), estimó, mediante el análisis de serotipos, que las gallinas ponedoras son en la actualidad la principal fuente de infección de *Salmonella,* estando un 43,8 % de los casos de salmonelosis humana relacionados con el consumo de huevos, seguido por la carne de cerdo a la que este estudio atribuye un 26,9 % de los casos. Hay que puntualizar que estos datos son generales para la UE y que existen notables diferencias entre países.

1.3 El control de Salmonella en producción porcina en la UE

El éxito de los programas de control de *Salmonella* en avicultura ha tenido como consecuencia que la proporción relativa de casos debidos al consumo de carne de cerdo se haya visto incrementada y sea, en el momento actual, la segunda fuente más frecuente de salmonelosis humana tras el consumo de huevos como se ha indicado anteriormente. Como paso previo a la instauración de los programas de control, entre los años 2006 y 2008 se llevaron a cabo dos estudios en la UE con el objetivo de establecer los valores basales de prevalencia de esta infección en cerdos de cebo y cerdos reproductores. A partir de estos datos iniciales se podrían establecer los objetivos de reducción de la prevalencia (EFSA, 2008a; EFSA, 2009a). El estudio de prevalencia basal en cerdos de cebo se llevó a cabo sobre muestras de ganglios linfáticos mesentéricos recogidos en matadero, inmediatamente después del sacrificio, mientras que el estudio basal en reproductoras se llevó a cabo sobre muestras de heces recogidas en granja (EC, 2005; EC, 2006). La prevalencia media de cerdos de cebo positivos a *Salmonella* fue del 10,8% (EFSA, 2008a), siendo la prevalencia determinada en España la más alta encontrada dentro de la UE (29% de cerdos de cebo positivos en las muestras de ganglios linfáticos mesentéricos). En el estudio basal en cerdas reproductoras las prevalencias encontradas fueron superiores, siendo la prevalencia media del 28,7%. Nuevamente, la prevalencia en España fue la más elevada de las reportadas con un 64% de las explotaciones positivas a *Salmonella* (EFSA, 2009a).

1.4 Impacto económico

El impacto económico de la salmonellosis porcina es doble. Por una parte hay que tener en cuenta las pérdidas directas que la enfermedad causa a los productores del sector mientras que, por otro lado, se encuentran los costes asociados al problema de salud pública como causa de toxi-infecciones alimentarias.

Aunque la gran mayoría de las infecciones por *Salmonella* en el ganado porcino son asintomáticas y no requieren de tratamiento específico ni provocan muertes, se asocian con disminución de la productividad. Un estudio económico realizado en 48 granjas de engorde de Dinamarca estimó pérdidas de 3 kg por cerdo cebado (Anon., 2000), mientras que otro estudio americano mostró que las granjas con bajas seroprevalencias (menos del 10% de cerdos seropositivos) producían 2,3 Kg más de cerdo al año por metro cuadrado de superficie (Gorton et al., 2000). Finalmente, *Salmonella* está asociada con otros patógenos digestivos como *Brachyspira hyodysenteriae* o *Lawsonia intracellularis* en el denominado complejo entérico porcino (Carvajal et al., 2012). Aparte de las pérdidas directas en la producción, la industria

alimentaria asociada también sufre pérdidas a consecuencia de la detección de *Salmonella* en la carne de cerdo que limita su comercialización, sobre todo hacia mercados del exterior.

De acuerdo a la Organización Mundial de la Salud (OMS), la salmonelosis humana causa importantes pérdidas económicas, ligadas básicamente al estudio del origen de casos y brotes así como a su tratamiento. Los costes incluyen los tratamientos recibidos, las pérdidas laborales y la repercusión que puede tener sobre la industria alimentaria (Socket, 1991). Se calcula que los costes anuales asociados a la enfermedad en personas ascienden a 600 millones de euros en la UE de los cuales 90 millones de euros son costes indirectos atribuibles al consumo de carne de cerdo contaminada (FCC Consortium, 2010).

2. El género *Salmonella*

2.1 Características generales

Salmonella es un género de bacterias perteneciente a la Familia *Enterobacteriaceae*, Orden *Enterobacteriales* y Clase γ Protobacteria (Garrity et al., 2004). Los miembros del género *Salmonella* son bacilos gram-negativos, con un contenido guanina-citosina (G-C) de 50-53%, no productores de endosporas ni cápsula y móviles por la presencia de flagelos perítricos (a excepción del serotipo Gallinarum y de las variantes inmóviles de otros serotipos).

Las bacterias del género *Salmonella* pueden multiplicarse en un amplio rango de temperaturas, desde 5 a 45°C, si bien mutaciones independientes les pueden permitir crecer a temperaturas superiores 48°C y 54°C (Droffner & Yamamoto, 1992). Su temperatura óptima de crecimiento está entre 35 y 37°C y su tiempo de generación a esta temperatura se encuentra en torno a los 22 minutos (D'Aoust, 2000). Son capaces de sobrevivir en un amplio rango de pH, entre 3,8 y 9,5, creciendo mejor en valores de pH próximos a la neutralidad (6,5-7,5). El valor óptimo de actividad de agua (aw) para su multiplicación es de 0,995 aunque crecen en medios con valores de aw de entre 0,945 y 0,999 y son capaces de multiplicarse en alimentos con valores de aw inferiores a 0,93 (Cox, 1999).

La mayoría de las cepas son anaerobias facultativas, utilizan citrato como única fuente de carbono y descarboxilan la lisina, la arginina y la ornitina. Producen sulfuro de hidrógeno, la enzima catalasa, reducen los nitratos a nitritos y su reacción es negativa en la prueba de la citocromo-oxidasa. La reacción de rojo de metilo es positiva y la prueba de indol es negativa. No fermentan la lactosa ni hidrolizan la urea. Estas y otras reacciones bioquímicas características de las bacterias del género *Salmonella* aparecen indicadas en la Tabla 1.

Prueba bioquímica	Reacción	Prueba bioquímica	Reacción
Reducción de nitrato	+	Fermentación	
Oxidasa	-	Glucosa	+
O/F	F	Manitol	+
Hidrólisis de urea	-	Maltosa	+
Indol	-	Lactosa	-
Producción de H_2S	+	Adonitol	-
Uso de citrato	+	Dulcitol	+
Malonato Sódico	-	Sacarosa	-
Crecimiento en KCN	-	Lisina descarboxilasa	+
Rojo de Metilo	+	Ornitina descarboxilasa	+
ONPG	-	Arginina dihidrolasa	+
		Voges Proskauer	-

Tabla 1. Pruebas bioquímicas para *Salmonella enterica* subsp. *enterica* (I) (fuente "*Salmonella* in domestic animals", Wray & Wray, 2000).

Así mismo *Salmonella* es capaz de crecer en medios con altas concentraciones de sales biliares y tolera colorantes como el cristal violeta, la eosina, la fucsina ácida, el azul de metileno o el verde brillante. Todas estas características son tenidas en cuenta a la hora de elaborar protocolos para su aislamiento e identificación (Figura 2).

Figura 2. Medios de aislamiento selectivo de *Salmonella.* A la izquierda medio modificado Rappaport-Valissiliadis (MSRV) que contiene verde malaquita. A la derecha agar XLD, medio selectivo y diferencial donde *Salmonella* produce ácido sulfhídrico (colonias de color negro).

Una de las claves del éxito de *Salmonella* es su ubicuidad y capacidad de adaptación, siendo capaz de sobrevivir en ambientes muy diversos, persistiendo en el medio ambiente durante meses o incluso años en sustratos orgánicos (Schwartz et al., 1999). Su hábitat natural es el tracto gastrointestinal de mamíferos, reptiles, aves e insectos. También se encuentra en el agua, los alimentos o el ambiente como consecuencia de la contaminación por heces (Grimont et al., 2000).

2.2 Taxonomía y Nomenclatura

En la actualidad y pese a los avances en técnicas filogenéticas basadas en la secuenciación, la nomenclatura y clasificación de las bacterias englobadas en el género *Salmonella* continúa siendo muy controvertida. Aunque no está oficialmente reconocido por el Comité Internacional de Taxonomía Bacteriana (ICBT), dentro del género se distinguen dos especies *S. enterica* y *S. bongori.* Estas dos especies están divididas a su vez en 7 subespecies (Popoff & Le Minor, 1987) que se diferencian entre sí mediante técnicas de hibridación ADN/ADN o por sus propiedades bioquímicas (Farmer III, 2003). Dentro de la especie *S. enterica* se agrupan las subespecies *enterica* (subsp. I) *salamae* (subsp. II), *arizonae* (subsp. IIIa), *diarizonae* (subsp. IIIIb), *houtenae* (subsp. IV) e *indica* (subsp. VI). La última clasificación de *Salmonella* mantiene la subsp. "V" para aquellos serovares incluidos en la especie *S. bongori* (Popoff & Le Minor, 1987; Brenner et al., 2000; Grimont & Weill, 2007). Aunque recientemente se ha propuesto una nueva especie en el género, *Salmonella subterránea* (Shelobolina et al., 2004), el análisis de secuencia de su ADN ribosómico 16-S mostró una gran similitud con el de *Salmonella bongori* y *Enterobacter cloacae* y dicha especie no fue tenida en cuenta en la última actualización de la taxonomía de *Salmonella* publicada por Grimont & Weill (2007).

Las subespecies se dividen a su vez en serogrupos y serotipos en función de su fórmula antigénica. La fórmula se elabora mediante la tipificación de los antígenos superficiales somáticos o antígenos O (los cuales dan lugar a los serogrupos en los que se agrupan los serotipos), los antígenos flagelares o antígenos H y, de forma eventual, de los antígenos capsulares (para los serotipos Typhi, Paratyphi y Dublin) (Popoff & Le Minor, 1987). Hay que puntualizar que existen dos tipos de antígenos somáticos, los denominados antígenos somáticos mayores (como el O:4), que definen al serogrupo, y los antígenos somáticos menores, que pueden ser compartidos por varios serogrupos, como por ejemplo el O:12 compartido por los grupos A, B y D. La primera clasificación basada en estos antígenos fue propuesta por White en 1926. Su esquema fue modificado por Kauffmann en 1941 y, actualmente, tanto la Organización Mundial de la Salud (OMS) como los laboratorios de referencia se basan en el esquema denominado Kauffmann-White para la clasificación de las bacterias del género *Salmonella.* Recientemente, Grimont & Weill (2007) han propuesto denominar a este sistema de clasificación esquema de fórmulas antigénicas de Le Minor-Kauffmann-White ya que gran parte de los serotipos descritos han sido identificados por Le Minor. La mayoría de los serotipos de *Salmonella* de interés por su potencial carácter patógeno están englobados en la subespecie *enterica* (I) (Tabla 2). Dada la importancia de los

serotipos de esta subespecie, está permitida la denominación clásica de los mismos, que hace referencia al hospedador principal o al lugar donde se realizó el aislamiento por primera vez para su identificación. Además, para los miembros de esta subespecie se admite acortar la nomenclatura utilizando el nombre del género seguido del nombre del serotipo, sin cursiva y con la primera letra mayúscula (para indicar que no se trata de una especie). Así, *Salmonella enterica* subsp. *enterica* serotipo Typhimurium puede identificarse directamente como *Salmonella* Typhimurium o *S.* Typhimurium. En cualquier caso, se recomienda que si se emplea esta nomenclatura abreviada, la primera vez que se cite en un texto, el nombre del serotipo debe ir precedido por la palabra "serotipo" o por su abreviatura "ser". Para el resto de los miembros del género *Salmonella* se debe emplear la nomenclatura completa: género, especie, subespecie y serotipo designado mediante la fórmula antigénica.

	Nº. de serotipos incluidos	**Principales hábitats**
S. enterica subsp. *enterica* (I)	1.531	Animales de sangre caliente
S. enterica subsp. *salamae* (II)	505	Animales de sangre fría/caliente y medio ambiente
S. enterica subsp. *arizonae* (IIIa)	99	Animales de sangre fría y medio ambiente
S. enterica subsp. *diarizonae* (IIIb)	336	Animales de sangre fría y medio ambiente
S. enterica subsp. *houtenae*	73	Animales de sangre fría y medio ambiente
S. enterica subsp. *indica* (IV)	13	Animales de sangre fría y medio ambiente
S. bongori	22	Animales de sangre fría y medio ambiente
Total	2.579	

Tabla 2. Número de serotipos descritos y principales hábitats para las diferentes especies y subespecies de *Salmonella spp.* (Brenner et al., 2000; Grimont & Weill, 2007).

El rango de especies a las que pueden infectar las bacterias del género *Salmonella* varía en función del serotipo (Kingsley & Bäumer, 2000), lo que permite el empleo del serotipado como herramienta epidemiológica. Algunos serotipos son patógenos estrictos de un único hospedador como por ejemplo *Salmonella* ser. Typhi que es un serotipo específico del hombre o *Salmonella* ser. Abortusequi del caballo. Otros serotipos poseen un hospedador específico pero pueden también ser encontrados en otros hospedadores. Así ocurre con *Salmonella* ser. Cholerasuis que provoca una salmonelosis sistémica en cerdos pero ha sido también descrito en infecciones en el hombre. Finalmente, la mayoría de serotipos son inespecíficos de especie pudiéndose aislar en gran variedad de animales y de ambientes.

3. Diagnóstico e identificación de *Salmonella*

3.1 Diagnóstico indirecto

El diagnóstico indirecto de la infección por *Salmonella* se realiza mediante técnicas serológicas cuyo objetivo es la detección de anticuerpos desarrollados por el sistema inmunitario del hospedador al ser infectado por *Salmonella*. Las técnicas indirectas de detección de *Salmonella* surgieron como alternativa a los métodos bacteriológicos, para tratar de solventar los problemas que planteaba el empleo de éstos últimos en programas de vigilancia y control del patógeno en animales de producción (Mousing et al., 1997): elevado coste y tiempo de aislamiento, baja sensibilidad o la excreción intermitente de *Salmonella* en las heces. El diagnóstico serológico se basa en la detección de las inmunoglobulinas de la clase IgG producidas por los animales infectados que se mantienen en niveles detectables durante largos periodos de tiempo.

La principal técnica indirecta empleada en el diagnóstico de *Salmonella* es el ELISA (Nielsen et al., 1995), aunque también se han descrito otras más simples como la aglutinación rápida en placa o sistemas más novedosos como los sistemas de análisis mediante esferas (Bokken et al., 2003).

3.1.1 Técnica ELISA (Enzime linked Immunosorbent Assay)

La técnica ELISA es una técnica de diagnóstico basada en la detección de antígenos o anticuerpos mediante un ensayo inmunoenzimático realizado sobre un soporte sólido, una microplaca de plástico tratado en cuyos pocillos se producen las diferentes reacciones del ensayo. Existen varios tipos de ELISA, en función de su diseño, pero los empleados en el diagnóstico de *Salmonella* son ELISA indirectos basados en el empleo para el tapizado de las placas de antígenos somáticos (cadena O del lipopolisacarido o LPS), antígenos flagelares o lisados completos de la bacteria.

El análisis serológico mediante ELISA ha tenido una gran aceptación en el diagnóstico de *Salmonella*, enfocado principalmente a programas de control (Nielsen et al., 1995; Christensen et al., 1999; Stege et al., 2000; van der Wolf et al., 2001b), aunque también en estudios de campo sobre prevalencia (Rajic et al., 2007), factores de riesgo (Lo fo Wong et al., 2004), monitorización de la infección (Kranker et al., 2003; Scherrer et al., 2008) o estrategias de control (Creus et al., 2007), ya que su empleo permite determinar el estatus de un rebaño en un momento determinado (Lo Fo Wong & Hald, 2000) y seguir el proceso de infección en estudios longitudinales (Merialdi et al., 2008).

Nielsen y colaboradores (Nielsen et al., 1995) desarrollaron un ELISA indirecto para el diagnóstico de *Salmonella* que fue incorporado al programa de control de *Salmonella* en ganado porcino de Dinamarca con el fin de monitorizar las granjas de cerdos de engorde (Mousing et al., 1997). Dicho ELISA, denominado Mix-LPS-ELISA, se basa en el empleo, para el tapizado de las placas, de una combinación de antígenos de la cadena O del LPS de los serotipos *S.* Typhimurium y *S.* Cholerasuis, antígenos somáticos 1, 4, 5, 6, 7 y 12, y detecta teóricamente los anticuerpos generados tras la infección por *Salmonella* de los serogrupos B, C1 y D1 (este último por reacción cruzada con el antígeno somático 12) (Chart et al., 1990). La inclusión de estos tres serogrupos permitía detectar las infecciones causadas por el 93% de los serotipos presentes en el ganado porcino de Dinamarca en el momento de su instauración (Baggesen et al., 1996). EL LPS fue seleccionado por su capacidad inmunógena e inductora de anticuerpos específicos así como por su capacidad de adhesión a las placas de inmunoensayo gracias a los polímeros hidrófobos que éstas presentan y por la facilidad de la purificación a gran escala (Nielsen et al., 1995).

Posteriormente y basados en el mismo sistema de detección se han ido desarrollando otros Mix-LPS-ELISA en Holanda (Van den Heijden et al., 1998), Francia (Proux et al., 2000) o España (Collazos, 2008), con variaciones en los serotipos seleccionados para la obtención del LPS y de los serogrupos incluidos. En la actualidad existen varios Mix-LPS-ELISA comerciales, que han sido valorados en diferentes estudios para determinar su sensibilidad, especificidad, concordancia etc., aspectos que serán comentados en el punto que trata sobre los factores que modifican los resultados de las técnicas de diagnóstico indirecto.

Además de los LPS-ELISA se han desarrollado otros ELISA para la detección de anticuerpos frente a *Salmonella.* En algún estudio se han utilizado ELISA basados en el empleo de un lisado completo de la bacteria (Szabo et al., 2008; Roesler et al., 2011) o de otras estructuras inmunógenas como los flagelos. La técnica ELISA basada en la utilización de antígenos flagelares se empleó para la diferenciación de las infecciones causadas por los serotipos *S.* Enteritidis y *S.* Gallinarum (Timoney et al., 1990; Barrow, 1992). Su desarrollo estaba dirigido al control de *Salmonella* en avicultura, donde a consecuencia del corto ciclo productivo de las aves de engorde, es necesario detectar los anticuerpos lo antes posible tras la instauración de la infección. Los flagelos, al encontrarse muy expuestos al medio externo, dan lugar a una respuesta inmunitaria humoral temprana (Van Zijderveld et al., 1992). Además los anticuerpos frente a las flagelinas tienen una elevada especificidad, disminuyendo las reacciones cruzadas con otras enterobacterias (de Vries et al., 1998). Van Zijderveld y colaboradores desarrollaron un ELISA de competición para la detección de anticuerpos frente

a flagelinas del serotipo *S.* Enteritidis (Van Zijderveld et al., 1992) que ha sido empleado en el programa de control de *Salmonella* en avicultura holandés (Edel, 1994). Mediante el tapizado con proteínas flagelares recombinantes de S. Enteritidis y S. Typhimurium se desarrolló un ELISA similar (de Vries et al. 1998). Sin embargo, el empleo de este tipo de ELISA no se ha extendido al porcino porque los resultados no han sido satisfactorios.

3.1.2 Pasos de la técnica LPS-ELISA e interpretación de resultados

Los Mix-LPS-ELISA son ELISA indirectos que se basan en la unión de los anticuerpos, habitualmente de la clase IgG, a los antígenos, cadena O del LPS, que tapizan los pocillos de la placa. Aunque las diluciones de los sueros problema, los conjugados, las incubaciones (tiempo y temperatura) y los substratos empleados para el revelado varían, las fases esenciales de un LPS-ELISA aparecen indicadas en la siguiente figura (Figura 3). Cada una de las etapas se separa de la siguiente por lavados con una solución tampón con un detergente como por ejemplo tampón fosfato (PBS 1X) suplementado con Tween20 (0,05%).

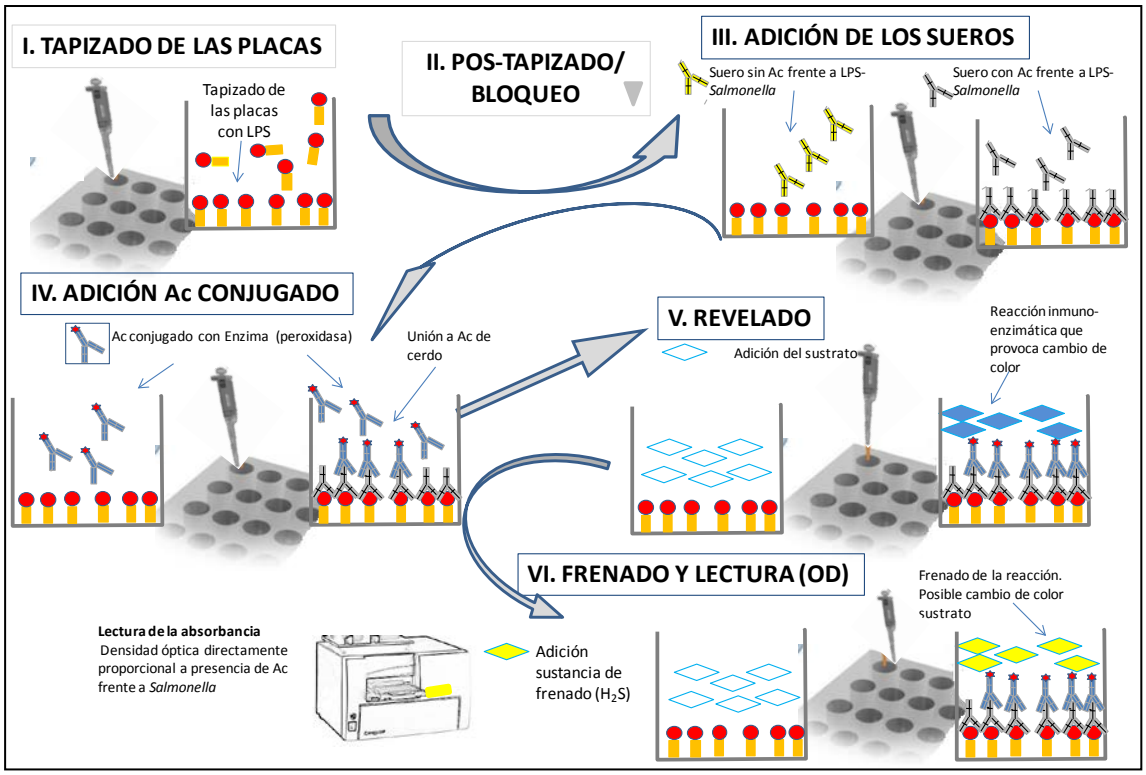

Figura 3. Etapas de un LPS-ELISA indirecto. Las etapas I y II corresponden al tapizado de las placas y no sería necesario llevarlas a cabo con los ELISA comerciales. El resto de etapas es común a todos los ELISAs, variando los anticuerpos empleados en la etapa de conjugado, la enzima con la que van marcados y los sustratos y soluciones de frenado empleados, así como las longitudes de onda a las que se mide la absorbancia. Entre las diferentes etapas hay incubaciones y lavados (excepto entre la etapa V y VI) que también varían en función del ELISA.

I. Tapizado de la placa (sólo para "home-made" ELISAs).

Tapizado de los pocillos de las microplacas con una dilución de los antígenos LPS en una solución tampón (ej. tampón carbonato-bicarbonato pH 9,6).

II. Bloqueo (sólo para "home-made"ELISAs).

Post-tapizado de los pocillos de las microplacas con una solución de albúmina sérica bovina para que aquellas zonas de la superficie del pocillo donde el LPS no ha conseguido adherirse queden cubiertas y en ellas no se puedan fijar los anticuerpos.

III. Adición de muestras.

Adición de muestras de suero o jugo de carne, en la dilución indicada, junto con los controles positivos y negativos correspondientes. Los anticuerpos frente al LPS se fijaran de forma específica en el caso de los sueros positivos.

IV. Adición del conjugado.

Tras la incubación de los sueros, se añade un conjugado anti-inmunoglobulinas de cerdo (normalmente anti-IgG aunque también puede ser específico frente a inmunoglobulinas de la clase IgM o IgA para estudios particulares, en desafíos o estudios de inmunidad). Estos conjugados están marcados con una enzima (peroxidasa generalmente). En aquellos pocillos con presencia de anticuerpos frente al LPS se fijará el conjugado mientras que en el resto será arrastrado durante el lavado posterior.

V. Revelado.

En la etapa de revelado se produce una reacción entre el sustrato adicionado y la enzima que se encuentra unida al conjugado de forma que se produce una reacción colorimétrica.

VI. Frenado y lectura de la densidad óptica.

Después de la incubación de la fase anterior se debe proceder al frenado de la reacción enzimática mediante una solución (por ejemplo H_2S al 5%), que detenga los cambios colorimétricos y mantenga estable, durante la medida, la coloración. En función del color de la reacción, la lectura se realizará a una determinada longitud de onda obteniéndose un valor bruto de absorbancia.

VII. Interpretación de los resultados.

Los valores absolutos de absorbancia son generalmente expresados como porcentaje de densidad óptica (% DO) mediante correcciones establecidas a partir de los valores obtenidos con los controles positivos y negativos. Finalmente, se establece un punto de corte sobre este % DO. Aunque este punto de corte es arbitrario, la mayoría de los estudios serológicos y programas de control emplean valores similares. El punto de corte más bajo empleado es 10% DO. Este fue el punto de corte establecido por Nielsen et al. (1995) en el desarrollo del Mix-LPS-ELISA danés y se emplea en el análisis individual de muestras o en estudios experimentales. Presenta como ventaja su elevada sensibilidad, del 90% según Lo Fo Wong & Hald (2000) pero, por el contrario, es poco específico, 30% según estos mismos investigadores. Por este motivo para los programas de control emplean puntos de corte más altos, que mejoran la especificidad de la técnica, mientras que la sensibilidad disminuye, pudiendo reducirse hasta valores del 50% en función del serotipo (Lo Fo Wong & Hald, 2000). Los puntos de corte más utilizados en el análisis a nivel de rebaño son el 40% DO (Osterkorn et al., 2001; Quirque et al., 2001; Rowe et al., 2003) y el 20% DO (Alban et al., 2002).

3.1.3 Otros métodos de detección indirecta

Sin duda alguna, la técnica ELISA ha sido la más estudiada y empleada en el diagnóstico indirecto de *Salmonella* desde los años noventa. Sin embargo también se han desarrollado otras técnicas que, igualmente, se basan en la unión antígeno-anticuerpo. Destaca la técnica inmunoenzimática de detección de anticuerpos por medio de esferas y citometría de flujo (Bokken et al., 2003). El principio de la técnica se basa en la unión de las cadenas O del LPS a esferas de diferentes tamaños, lo que permite diferenciar el serogrupo al que pertenecen los anticuerpos. La técnica además permite el análisis serológico de varias enfermedades en un mismo procesado. Sin embargo, es una técnica menos desarrollada y contrastada además de más compleja que el ELISA al requerir un citómetro de flujo para el análisis de resultados. Existen otras técnicas inmuno-enzimáticas, como por ejemplo el “surface plasmon resonance" o SPR que detecta anticuerpos frente al LPS de *Salmonella* en chips que son analizados mediante mediciones de intensidad y cambios de ángulo de refracción de luz que están relacionados con la unión de los anticuerpos al chip (Mazumbdar et al., 2008).

Aunque se continúan desarrollando nuevas técnicas que tratan de mejorar las prestaciones del Mix-LPS-ELISA, por el momento ésta sigue siendo la técnica de referencia o “gold standard” en el diagnóstico indirecto de *Salmonella* en el ganado porcino.

3.1.4 Factores que afectan a los resultados de las técnicas de diagnóstico indirecto

Diferentes factores pueden afectar a la sensibilidad y/o especificidad de las técnicas indirectas. Dichos factores deben ser tenidos en cuenta si estas técnicas se emplean en el diagnóstico de *Salmonella*, tanto en planes de control como en estudios científicos.

- *Punto de corte: el* punto de corte seleccionado para realizar el ELISA es el factor que más influye en los parámetros de la técnica y por consiguiente en los resultados obtenidos. El punto de corte determina la sensibilidad y la especificidad de la técnica. Basándonos en esta premisa se pueden establecer variaciones en función de si deseamos tener una mayor sensibilidad (punto de corte establecido a bajos valores de % DO) o una mayor especificidad (punto de corte a elevados valores de % DO).

- *Momento de la infección*: como se ha explicado al inicio del apartado de diagnóstico indirecto, estas técnicas se basan en la detección de los anticuerpos producidos en respuesta a la infección por *Salmonella.* Es por ello que existe una ventana temporal desde que el animal es infectado y hasta que los anticuerpos se producen en niveles suficientes para ser detectados, dando lugar a un resultado falso negativo. Se estima que el tiempo requerido varía entre las dos semanas en infecciones experimentales (van Winsen et al., 2001; Lo Fo Wong et al., 2004) y hasta los dos meses en infecciones naturales (Kranker et al., 2003). Además existen factores inherentes al individuo, a la cepa y al ambiente que pueden provocar variaciones en el desarrollo de la respuesta inmunitaria y por tanto modificar los tiempos de aparición de los anticuerpos.

- *Serotipo:* diversos estudios han demostrado que no todos los serotipos presentan la misma capacidad para infectar cerdos (van Winsen et al., 2001; Collazos, 2008). Como consecuencia, la respuesta inmunitaria también varía y con ella los títulos de anticuerpos producidos. *S.* Typhimurium y S. Cholerasuis inducen una fuerte respuesta humoral en cerdos, fácilmente detectable mediante la técnica LPS-ELISA (van Winsen et al., 2001; Collazos, 2008). Sin embargo, otros serotipos como *S.* Panama, *S.* Goldcoast (van Winsen et al., 2001), *S.* Infantis (Roesler et al., 2011) o *S.* Rissen (Collazos, 2008) inducen respuestas de menor intensidad. Por otra parte, no todos los anticuerpos anti-*Salmonella* son detectables por los antígenos incluidos en las placas de ELISA. Así, si por ejemplo se emplean los antígenos descritos por Nielsen et al. (2001) para el tapizado de las placas, las infecciones por serotipos como *S.* Anatum, perteneciente al serogrupo E1, no pueden ser detectadas.

- *Inmunidad pasiva:* los anticuerpos maternos transferidos durante la lactancia pueden persistir entre 8 y 10 semanas, de modo que pueden ser detectados en animales que no han sido infectados por *Salmonella*.

- *Fallo en la seroconversión:* algunos animales, con independencia del serotipo implicado, no desarrollan respuesta inmunitaria tras la infección (van Winsen et al., 2001). Se estima que este fallo de respuesta ocurre en el 1-2% de los cerdos aunque este valor no es fácil de cuantificar (Nielsen et al., 1995).

- *Tipo de muestra:* para la detección de anticuerpos anti-salmonela se emplean dos tipos de muestras principalmente, jugo de carne o suero. Los valores de absorbancia obtenidos mediante el análisis de jugo de carne son ligeramente inferiores a los obtenidos en suero (Nielsen et al., 1998; Nobman et al., 2011), por lo que para obtener valores comparables hay que realizar diferentes diluciones (Nielsen et al., 1998). Dichos ajustes suelen estar especificados en los test comerciales. Además, respecto a las muestras de jugo de carne, que son las muestras de elección en los programas de control por la facilidad en su recogida, se ha demostrado que existen variaciones en el resultado en función del músculo seleccionado (Nobmann et al., 2011). Así, los títulos de inmunoglobulinas de la clase IgG en el jugo de carne de músculo diafragmático eran mayores que los proporcionados por el jugo de músculo de cuello y de abdomen. Por lo tanto, también la elección del músculo a analizar puede ser relevante.

- *Correlación resultados diferentes ELISA:* finalmente hay que subrayar que diversos trabajos han demostrado la existencia de diferencias entre los resultados ofrecidos por diferentes ELISAs (ya sean comerciales o “home-made”) en el análisis de los mismos sueros (van der Heijden et al., 1998; 2001; Farzan et al., 2007; Collazos, 2008; Szabo et al., 2008). Mediante estos estudios se ha demostrado que la eficiencia de análisis es variable entre distintos test. Por lo tanto y como indicaron Van der Stede et al. (2004), en un estudio realizado en Bélgica con sueros recogidos de 661 cerdos procedentes de 20 granjas, los resultados no solo varían en función del punto de corte empleado sino también en base al test seleccionado. Este hecho debe ser tenido en cuenta a la hora de comparar resultados de muestras procesadas con diferentes ELISAs.

3.2 Diagnóstico directo

3.2.1 Aislamiento bacteriológico

Existe una gran cantidad de información científica acerca del aislamiento de *Salmonella,* con una gran variedad de medios de cultivo, algunos diseñados exclusivamente para este fin. Así mismo, existe un sinfín de procedimientos de aislamiento, con un número variable de pasos, diferentes medios y temperaturas de incubación (Waltman, 2000). El hecho de que exista una gama tan amplia de protocolos es consecuencia de la complejidad de este aislamiento, ya que por lo general, las concentraciones de *Salmonella* tanto en alimentos como en las heces de animales portadores o con infecciones subclínicas suele ser baja. Por ello, el aislamiento en un único paso, sin fase de enriquecimiento, a partir de muestras de heces u otros órganos de animales es en muchas ocasiones inviable, por no ser lo suficientemente sensible.

Con el fin de armonizar y regularizar el aislamiento bacteriológico de *Salmonella* se han diseñado protocolos estandarizados, entre los cuales son de interés en nuestro ámbito los recogidos en la norma ISO 6576:2002 referente al aislamiento de *Salmonella* en muestras de alimentos y piensos y la modificación de esta misma norma introducida en el año 2007 para su adecuación al aislamiento de *Salmonella* en heces de animales (Anexo D de la ISO 6579). También es de interés la norma ISO 6579-2:2012, recientemente publicada y referente al aislamiento de *Salmonella,* identificación y enumeración mediante la técnica del número más probable miniaturizada. Aunque la metodología empleada y sobre todo los medios seleccionados varían de unos estudios a otros, el aislamiento de *Salmonella* por lo general incluye los siguientes pasos: (I) pre-enriquecimiento no selectivo, (II) enriquecimiento selectivo, (III) cultivo en medio selectivo y diferencial y (IV) confirmación mediante pruebas bioquímicas, moleculares y/o serotipificación.

I. Pre-enriquecimiento no selectivo

El objetivo del pre-enriquecimiento de las muestras es permitir la recuperación de la viabilidad de aquellas *Salmonella* que puedan encontrarse dañadas a consecuencia de la aw, el pH, la temperatura, la radiación u otros factores del ambiente. Estas bacterias dañadas, aunque son viables e incluso capaces de provocar enfermedad en condiciones adecuadas, no son capaces de sobrevivir y multiplicarse en medios selectivos, sobre todo cuando son incubadas a temperaturas superiores a 37,5°C (Corry et al., 1969; Aho, 1992). El empleo de un paso previo de pre-enriquecimiento permite la recuperación de las bacterias dañadas antes

iniciar el cultivo en medios selectivos mejorando, por tanto, la sensibilidad de las técnicas de aislamiento.

Para esta fase del aislamiento se han propuesto varios medios de cultivo entre los que se encuentran el agua de peptona tamponada (BPW), el medio M9 o el medio de pre-enriquecimiento universal, siendo el primero el más comúnmente empleado. Los medios de pre-enriquecimiento se caracterizan por no ser ricos en nutrientes, puesto que estos no son necesarios para la recuperación de *Salmonella* (D'Aoust, 1981), por no incluir azucares fermentables y por su gran capacidad tampón, ya que la acidificación excesiva del medio durante la incubación puede disminuir la viabilidad o incluso provocar la muerte de las bacterias presentes (Hilker, 1975), manteniendo el pH al final de la incubación en valores comprendidos entre 5,8-6,4 en el caso del BPW.

El tiempo de incubación se establece entre 18 y 24 horas a 37°C (± 2°C). No es conveniente prolongar las incubaciones por encima de este tiempo ya que se favorece la proliferación de otras bacterias competidoras que pueden disminuir la viabilidad de *Salmonella* aunque tampoco se recomiendan incubaciones inferiores a las 16 horas ya que se reduce la sensibilidad del diagnóstico.

II. Enriquecimiento selectivo

El enriquecimiento selectivo es una etapa crucial en el aislamiento de *Salmonella.* En esta fase, los medios selectivos inhiben el crecimiento de otras posibles bacterias presentes en el medio de pre-enriquecimiento al tiempo que favorecen la multiplicación de *Salmonella* hasta concentraciones que permiten su aislamiento e identificación en la siguiente etapa del protocolo de aislamiento. En la actualidad se emplean tres medios para el enriquecimiento selectivo: caldo tetrationato (MKTT), caldo Rappaport-Valissiliadis (RV) y el medio modificado Rappaport-Valissiliadis (MSRV). En el pasado se incluía también para este fin el caldo selenito pero debido a su menor sensibilidad, su mayor toxicidad y menor estabilidad ha caído en desuso (Waltman, 2000).

El caldo tetrationato es un medio selectivo basado en la combinación de iodina y tiosulfato sódico al que posteriormente se adicionaron sales biliares y verde brillante (MKTT). En la actualidad se suplementa con novobiocina para limitar el crecimiento de flora competidora. La ratio empleada para la inoculación de este medio de cultivo es 1:10, incubándose a 41°C durante 24 y 48 horas. Solo las muestras negativas en la siguiente etapa del protocolo de aislamiento son resembradas, tras la incubación durante 48 horas en MKTT.

El caldo RV es un medio de enriquecimiento que hace referencia a sus creadores. Inicialmente Rappaport y colaboradores (1956) elaboraron un medio de cultivo para *Salmonella* basado en su capacidad para sobrevivir y multiplicarse en medios de alta osmolaridad (conseguida mediante la incorporación de MgCl), en presencia de verde malaquita, a pH relativamente bajo (pH=5,2) y con escaso aporte de nutrientes (5 g de peptona por litro). Posteriormente, Vassiliadis realizó una modificación del medio reduciendo la cantidad de verde malaquita, lo que permitió su incubación a temperaturas de hasta 43°C. La ratio empleada para la inoculación de este medio de cultivo es de 1:100 y al igual que el MKTT se incuba a 41°C durante 24 h, con resiembra tras 48 horas de incubación para las muestras negativas en la siguiente etapa. Diversos estudios han demostrado que el caldo RV es más sensible que el MKTT en el aislamiento de *Salmonella* (Bager & Petersen, 1991). De hecho, el caldo RV fue el medio de cultivo recomendado para el análisis de muestras de heces en la ISO 6579/2002 hasta la inclusión del medio MSRV en la modificación realizada en el año 2007 (Anexo D, ISO 6579/2002).

El medio MSRV es un medio semisólido desarrollado a partir del caldo RV. En comparación con el RV se caracteriza por ser más rico en nutrientes, poseer una mayor capacidad tampón, una menor concentración de MgCl y por incorporar novobiocina. Su presentación en forma de agar semisólido permite el crecimiento y migración de *Salmonella* (ya que la mayoría de serotipos poseen flagelos) y la diferenciación con microorganismos no flagelados que puedan multiplicarse en este medio (Figura 2). La ratio de inoculación, al igual que en el caldo RV, es de 1:100, la temperatura de incubación de 41,5°C y los tiempos de incubación de 24 y 48 horas.

III. Aislamiento en medios selectivos y diferenciales

El paso de enriquecimiento tiene por objetivo la multiplicación de *Salmonella* hasta concentraciones más fácilmente detectables en medios sólidos. Existe una gran variedad de medios de cultivo diseñados para esta etapa del proceso de aislamiento y que se basan en los principios de ser selectivos y diferenciales. Su carácter selectivo, al igual que en la fase anterior, se basa en la incorporación de sustancias inhibidoras del crecimiento de otras bacterias. Por su parte, el carácter diferencial se apoya en la capacidad de *Salmonella* para realizar determinadas reacciones bioquímicas que conllevan la producción de metabolitos que pueden ser identificados mediante cambios de color en el medio de cultivo, como por ejemplo la producción de H_2S (Figura 2). Estas reacciones permiten diferenciar las colonias de *Salmonella* de las de otros microorganismos capaces de crecer en estos mismos medios,

principalmente otras enterobacterias. Entre los medios más empleados en esta etapa se encuentran el agar Rambach, el agar de identificación de *Salmonella* (SM-ID), el agar Hekto-enteric, el agar xilosa-lisina-deoxicolato (XLD), el agar xilosa-lisina-tergitol 4 (XLT4), el agar verde brillante o nuevos medios cromogénicos como el agar "*brilliance*". En este paso del proceso, se recomienda el uso de dos medios diferenciales con propiedades selectivas diferentes y que permitan la identificación de *Salmonella* por mecanismos diferentes. En la Tabla 3 aparecen algunos de los principales medios empleados en esta fase con su mecanismo de diferenciación e identificación de colonias de *Salmonella* y los principales microorganismos capaces de multiplicarse en dichos medios.

Medio de cultivo	***Salmonella spp.***	***Proteus spp.***	**Coliformes**	***Pseudomonas spp.***
Agar Hektoen	Colonias verde azuladas con centro negro (H_2S)	Verde azulado	Rosa	-
BGA	Colonias de color rosa (no fermentan lactosa)	Rojo	Verde	Rojo
Agar Rambach	Rojo carmesí (fermentación de propilenglicol)	Sin color	Violeta	Naranja
SM-ID	Colonia rosa (no fermentan la lactosa ni la sacarosa)	Sin color	Violeta	Naranja
XLD	Colonia negra (H_2S) fondo rojo (lisina)	Amarillo punto negro	Amarillo	-
XLT4	Colonia negra (H_2S) fondo rojo (lisina)	Amarillo punto negro	Amarillo	-
Brilliance agar	Colonias azules (caprilato esterasa y β-glucosidasa)	-	Rosa	-

Tabla 3. Principales medios diferenciales empleados en la identificación de *Salmonella* incluyendo el mecanismo de diferenciación, el color de las colonias de *Salmonella* así como de algunos de los principales microorganismos que pueden aparecer en los medios de cultivo en esta fase del aislamiento.

IV. Identificación

Las colonias sospechosas en los medios selectivos y diferenciales deben ser aisladas, cultivadas en medios no selectivos y sometidas a pruebas bioquímicas y serotipado para su identificación. La identificación también puede realizarse mediante técnicas moleculares (reacción en cadena de la polimerasa o PCR).

Existen multitud de pruebas bioquímicas para la identificación de *Salmonella.* Algunos medios de cultivo complejos permiten conocer características bioquímicas como la fermentación de azúcares (lactosa, glucosa etc.), la utilización de urea, la formación de H_2S, la producción de gas o modificaciones del pH, entre otras. Entre los más utilizados se encuentran el agar hierro triple azúcar (agar TSI), el agar hierro lisina (agar LI) o el agar urea. Otras pruebas

empleadas incluyen la prueba del indol o el test de fluorescencia del metil-lumbiferil-caprilato que permite la detección de *Salmonella* por la emisión de una fuerte fluorescencia azul cuando se observa bajo luz ultravioleta a 366 nm por una reacción enzimática con una esterasa. De forma alternativa se pueden emplear test de baterías bioquímicas como las galerías API (Biomerieux©), el Micro-ID Enterotube II (BBL™) o sistemas automáticos o semiautomáticos como el VITEK (Biomerieux©), AutoMicrobic System (AMS), etc.

El serotipado será abordado con más detenimiento en el punto de tipificación de *Salmonella*. Su empleo permite determinar la fórmula antigénica de los aislados mediante la aglutinación con antisueros específicos frente a los antígenos somáticos y flagelares.

En la pasada década se desarrollaron diferentes técnicas moleculares para la identificación de *Salmonella.* Aunque, como veremos más adelante, estas técnicas están enfocadas a la detección directa de *Salmonella* o a su tipificación, también pueden ser usadas para la confirmación del diagnóstico. Existe gran número de trabajos sobre detección de *Salmonella* mediante PCR y la mayoría se basan en la utilización del gen *invA,* responsable de la codificación de una proteína involucrada en la invasión de células epiteliales (Rahn et al., 1992). Lamentablemente, algunas cepas carecen de este gen y dan lugar a falsos negativos (Galan et al., 1991; Rahn et al., 1992). Otro gen empleado en el diagnóstico mediante PCR es el *ttrRSCA,* un locus involucrado en la codificación de proteínas para la respiración del tetrationato (Malorny et al., 2004), y que ha sido testado en varios ensayos colaborativos con buenos resultados (Malorny et al., 2007).

3.2.2 Factores que afectan a la sensibilidad de las técnicas bacteriológicas

El aislamiento de *Salmonella* tiene una sensibilidad variable en función de diversos factores, habiéndose establecido que esta sensibilidad se encuentra en torno al 80% si se sigue el protocolo estandarizado de la norma ISO (Anexo D, ISO 6579) (EFSA, 2011b). Aparte de los factores inherentes a la técnica, hay que considerar una serie de factores que modifican los valores de sensibilidad y dificultan la comparativa entre resultados de distintos estudios:

- *Protocolo de diagnóstico (etapas y medios empleados):* como se ha indicado en el punto anterior, la sensibilidad de la técnica varía en función de las etapas y los medios empleados en el protocolo seleccionado. Diversos trabajos han tratado de evaluar el efecto provocado por las variaciones en los protocolos de diagnóstico con resultados dispares (Bussee , 1995; Davies et al., 2000a; Mooijman, 2004). Por ello, Davies et al. (2000a) recomiendan que, antes de la realización de un estudio que implique aislamiento bacteriológico, se debe

seleccionar el método de mayor sensibilidad teniendo en cuenta el objetivo del estudio, el tipo de muestras a analizar y las posibilidades de trabajo.

- *Cantidad de muestra:* uno de los factores que más afecta a la sensibilidad de la técnica es la cantidad de muestra procesada. Tanto el peso de la muestra (gramos) como la superficie muestreada (si nos referimos a superficies ambientales o canales) tienen una gran influencia en el resultado obtenido. Funk y colaboradores (Funk et al., 2000) estimaron una sensibilidad para el aislamiento bacteriológico del 9% a partir de muestras de 1 g de heces y del 78% para muestras de 10 g de heces. Igualmente, Lindblad (2007) demostró la influencia de la superficie muestreada en la sensibilidad en la estimación de contaminación en canales.

- *Tipo de muestra:* el tipo de muestra seleccionado para el análisis de *Salmonella* condiciona el resultado. Es muy importante conocer y considerar que factores afectan a la presencia o ausencia de *Salmonella* en la muestra en cuestión. Así, por ejemplo, en una muestra de heces recogida en granja, la presencia de *Salmonella* esta únicamente supeditada a la infección de los cerdos en la misma; por el contrario, si la muestra de heces es recogida en el matadero, la presencia de *Salmonella* se puede deber a la existencia de infección en la granja o a nuevas infecciones ocurridas durante el transporte o en el propio matadero (Hurd et al., 2002). Por lo tanto no se puede estimar la prevalencia de *Salmonella* en granja a partir de esa muestra. Lo mismo ocurre con el análisis del contenido de ciego o de los ganglios linfáticos mesentéricos. En este último caso así como en otros órganos como tonsilas, hígado o bazo, entre otros, el resultado se verá influido por la capacidad invasiva del serotipo o de la cepa en cuestión (Collazos, 2008). También se han observado diferencias entre el uso de gasas o de mezclas de heces a partir de muestras individuales, siendo las primeras más sensibles en la detección de *Salmonella* en corrales de cerdas reproductoras (EFSA, 2011b). En el análisis de canales, el tipo de muestra (superficial recogida con gasas, muestra obtenida con trocar, etc.) y la técnica de muestreo (arrastre superficial, abrasión, etc.) va a tener influencia en la sensibilidad de la técnica. En la introducción específica del capítulo correspondiente a los estudios en matadero se amplía la información relativa al efecto asociado al tipo de muestra y sistema de recogida en canales.

- *Muestras individuales o mezclas:* diversos estudios han demostrado que la mezcla de heces individuales incrementa la sensibilidad en la detección de *Salmonella* (Arnold et. al., 2005; 2009; EFSA, 2011b). Por ejemplo, en el estudio basal de cerdos reproductores en la UE se observó que la prevalencia estimada en mezclas de muestras fue superior (10,7%) a la determinada con el análisis de muestras individuales (9,7%). En el análisis de muestras de

canales se ha observado un efecto similar. Sorensen et al. (2007), en un estudio sobre canales en Dinamarca, comprobaron que empleando muestras que contenían gasas recogidas en cuatro canales se obtenía una prevalencia 5 veces superior a la estimada mediante muestras individuales.

3.2.2 Diagnóstico molecular de *Salmonella*

Debido a que los métodos tradicionales de aislamiento bacteriológico de *Salmonella* son largos, tediosos e incluso costosos, se han buscado alternativas que permitan acortar los tiempos de diagnóstico, simplifiquen el análisis, sean económicas y, al mismo tiempo, sensibles y específicas (Löfström et al., 2010). Entre estos nuevos métodos se encuentran los métodos basados en la detección de proteínas y de ácidos nucleicos.

Entre los métodos moleculares de detección directa, el más empleado es sin duda la reacción en cadena de la polimerasa (PCR), tanto la PCR convencional como, en la última década, la PCR en tiempo real (real-time PCR), que disminuye el riesgo de contaminación cruzada y el tiempo de análisis además de simplificar los protocolos, al unir las etapas de amplificación y detección en una única. Existen dos sistemas de detección en real-time PCR (Bustin, 2002): (I) mediante marcadores fluorescentes que se unen específicamente a la doble cadena de ADN y (II) mediante sondas específicas marcadas con un fluoróforo. La principal ventaja de los marcadores fluorescentes es que son sencillos de diseñar y más económicos. Sin embargo, son menos específicos que las sondas específicas de secuencia, por lo que es más complicado demostrar que el producto de PCR deseado es el que realmente se detecta. Por ello, su uso para el diagnóstico es limitado. Por el contrario, el empleo de sondas específicas marcadas elimina la necesidad de confirmación del producto obtenido en la PCR e incrementa la especificidad (Löfström et al., 2010). Independientemente del sistema empleado, diversos estudios han demostrado la utilidad de la real-time PCR para la detección y la cuantificación de *Salmonella* a partir de la fase de pre-enriquecimiento en agua de peptona en muestras de heces y canales de cerdo (Wolffs et al., 2006; Malorny et al., 2008; Kramer et al., 2011).

Otro método de detección molecular es la técnica de hibridación directa. Se basa en la detección de una secuencia concreta mediante un oligonucleótido marcado en 3’ con ácido polideoxiadenílico y otro oligonucleótido específico de una secuencia de ARN ribosómico de *Salmonella* marcado en 5’ con la enzima peroxidasa. Ambas sondas hibridarán con la secuencia complementaria en la molécula diana y podrán ser detectadas mediante un revelado.

A pesar de la gran evolución de las técnicas moleculares en los últimos años, su mayor desventaja frente a los métodos bacteriológicos es que no permiten el aislamiento de la cepa.

Actualmente sigue siendo indispensable el trabajo con los aislados para poder llevar a cabo estudios de tipificación o determinación de resistencias.

4. Tipificación de *Salmonella*

El tipado de los aislados obtenidos en el diagnóstico de *Salmonella* permite resolver múltiples cuestiones relevantes tanto desde un punto de vista microbiológico como epidemiológico. Actualmente la clasificación de *Salmonella* por debajo del nivel de subespecie se realiza mediante el serotipado, como se ha indicado anteriormente. Aparte de este serotipado, existen muchas técnicas fenotípicas o moleculares que permiten clasificar y estudiar la relación filogenética entre las bacterias incluidas en el género *Salmonella*. Estas técnicas varían en su poder discriminativo, desde muy bajo a muy alto, y su empleo está condicionado por el objetivo del estudio (Malorny et al., 2011).

4.1 Métodos fenotípicos

Los métodos fenotípicos de tipificación suelen ser empleados en una primera etapa de caracterización de las cepas de *Salmonella*. Son técnicas que tienen una capacidad de discriminación inferior a la de las técnicas moleculares, pero que, en algunos casos, han sido empleadas desde hace décadas y ofrecen una información muy valiosa desde un punto de vista epidemiológico. Permiten agrupar las cepas en tipos epidemiológicos que pueden estar asociados a determinados patrones de resistencia o de virulencia.

4.1.1 Serotipado

El serotipado es la técnica de caracterización más empleada. Este sistema de tipificación fenotípica, como se ha indicado en el punto de taxonomía, ha permitido organizar la clasificación de *Salmonella* por debajo del nivel de subespecie (Grimont & Weil, 2007), mediante la caracterización del dominio polisacárido del LPS (antígeno O) y de las flagelinas que forman los flagelos de fase 1 y fase 2 (antígenos H1 y H2). Actualmente se han descrito 67 antígenos-O que conforman 46 serogrupos con los que se combinan 144 antígenos H1 y H2 para constituir los más de 2.500 serotipos de *Salmonella* descritos hasta el momento.

Los antígenos somáticos están codificados por el cluster cromosómico *rfb* aunque algunos se pueden formar por conversiones lisogénicas mediadas por fagos (Brussow et al., 2004). De acuerdo con el esquema Kauffmann-White, los 46 serogrupos del género *Salmonella* son designados inicialmente con letras (de la A a la Z) y posteriormente con números (por

ejemplo O:2; O:4; O:67). En la actualidad se considera más correcto designar cada serogrupo en función de su antígeno somático mayor, manteniéndose las letras de forma ocasional y entre paréntesis, por ejemplo O:4 (B) (Grimont & Weill, 2007). Algunas cepas de *Salmonella* carecen del polisacárido O por un defecto en su síntesis. A estas cepas se las conoce como "rugosas" y no son serotipables.

En cuanto a los antígenos flagelares o antígenos H, la mayor parte de las cepas de *Salmonella* son capaces de expresar, alternativamente, dos flagelinas antigénicamente diferentes codificadas por los genes *fliC* y *fljB*. La flagelina de fase 1 es característica del serotipo y se denomina "específica" mientras que la flagelina de fase 2 o "inespecífica" puede ser común a otros serotipos. La flagelina H1 se nombra con letras minúsculas que van de la *a* a la *z*, mientras que las flagelinas H2 se pueden indicar con números, letras minúsculas o con la letra *z* seguida de un subíndice numérico. Algunos serotipos son aflagelados (*S.* Typhi o *S.* Gallinarum) mientras que otros han perdido la capacidad para expresar alguna de las flagelinas y son denominados monofásicos, como por ejemplo la variante monofásica del serotipo *S.* Typhimurium (*S.* 4,5,12:i:-) que ha perdido la capacidad para expresar el gen *fljB* (Hauser et al., 2010).

El serotipado se realiza tradicionalmente por el método clásico de aglutinación con anticuerpos mono o policlonales, mediante la observación directa de las reacciones de aglutinación que se producen al enfrentar la bacteria al antisuero correcto.

Además, desde los años noventa se han desarrollado técnicas de PCR para la realización de un serotipado molecular que se basan en la detección de polimorfismos en los genes responsables de la expresión de los antígenos. Así, mantienen los fundamentos de las técnicas serológicas y algunas de sus características (repetitividad, reproducibilidad y concordancia epidemiológica) presentando algunas ventajas adicionales como son la rapidez, la automatización o la reducción de costes (Malorny et al., 2011). Se evita el cultivo de *Salmonella* en medios de cultivos especiales, necesarios la expresión de los flagelos así como para la reversión de fase, y permite la caracterización de cepas "rugosas", que no son capaces de expresar las fases flagelares y que por consiguiente no se pueden serotipar por métodos clásicos (Hoorfar et al., 1999). Entre los genes seleccionados para la realización del tipado molecular empleando técnicas de PCR se encuentran los genes implicados en la expresión de los diferentes antígenos incluidos en el serotipado, genes *rfb* (Herrera-Leon et al., 2007), *fliC* (Herrera-Leon et al., 2004; McQuinston et al., 2004) y *fljB* (Echeita et al., 2002; McQuinston et al., 2004). Además del serotipado basado en PCR existen nuevos métodos como la hibridación

genómica comparativa, basados en técnicas de real-time PCR (Arrach et al., 2008). Por el momento, es una técnica en desarrollo y no todos los genes empleados han sido validados como específicos de serotipo, siendo necesario profundizar al respecto. También hay avances en nuevas plataformas, por ejemplo Yoshida y colaboradores han desarrollado un chip de ADN o microarray para el serotipado de aislados de *Salmonella* (Yoshida et al., 2007).

4.1.2 Fagotipado

Los fagos son virus que infectan a bacterias, provocando, en la mayor parte de los casos, su destrucción y, consecuentemente, zonas de lisis visibles a simple vista en placas de agar previamente inoculadas con la bacteria.

Debido a su alta especificidad, cada fago solo es capaz de infectar a una determinada cepa o a un conjunto de cepas muy similares, que se incluyen en un mismo grupo llamado fagotipo. Esta especificidad de fagos se ha aprovechado en *Salmonella* para tipificar cepas dentro de un mismo serotipo, ya que se ha comprobado que dentro de un serotipo las cepas se agrupan en fagotipos en función de sus reacciones frente a una batería de fagos. El fagotipado ha sido una herramienta de tipificación muy empleada en estudios epidemiológicos de *Salmonella* (Baggesen & Wegener, 1994; Rabsch et al., 2002). Se han desarrollado esquemas para los serotipos *S.* Typhi, *S.* Paratyphi A y B, *S.* Enteritidis o *S.* Typhimurium (Callow et al., 1959; Anderson et al., 1977). Además se han desarrollado esquemas de fagotipos para otros serotipos de importancia clínica y epidemiológica como *S.* Newport (Petrow et al., 1974), *S.* Hadar o *S.* Virchow (Chambers et al., 1987). El fagotipado se utiliza en laboratorios de referencia, como complemento al serotipado ya que aporta información sobre determinadas características de la cepa como son el perfil de resistencia o la virulencia. Un claro ejemplo lo constituye el fagotipado de aislados de *S.* Typhimurium, con fagotipos como el DT193 o el DT104 de gran relevancia desde el punto de vista epidemiológico y clínico (Skov et al., 2008; Hopkins et al., 2012).

Sin embargo, es importante conocer que el fagotipado es una técnica compleja, que requiere de personal experto y muy entrenado, lo que puede provocar discrepancias entre laboratorios en la determinación del fagotipo de una misma cepa (Baggesen et al., 2010). Además la cesión de los fagos se hace a través del laboratorio de referencia encargado de su producción (Collindale, UK) y por ello es una técnica que está cayendo en desuso y que está siendo progresivamente reemplazada por técnicas moleculares (Baggesen et al., 2010),

4.1.3 Otros métodos fenotípicos

Recientemente se han desarrollado nuevas técnicas de tipificación basadas en el análisis de la bacteria completa, con las que se busca establecer métodos que permitan la identificación rápida de patógenos (Malorny et al., 2011). Estas técnicas se basan en la medición de un amplio abanico de caracteres espectrales que, en conjunto, reflejan la composición química de la bacteria. Entre estas técnicas fenotípicas se incluyen técnicas de espectrometría de masas como el MALDI-TOF (Diekmann & Malorny, 2011) que se basan en el análisis de proteínas de bajo peso molecular o la espectrometría de masas por pirolisis (PyrMS) basada en la degradación térmica de la bacteria. También se han desarrollado técnicas espectroscópicas de libre vibración como la espectroscopía infrarroja transformada de Fourier (FT-IR) que analiza las bacterias mediante la absorción de distintos espectros infrarrojos en función de sus constituyentes celulares, polisacáridos, lípidos y proteínas (Álvarez-Ordoñez et al., 2011a). Todas estas técnicas se caracterizan por la fácil y rápida preparación de las muestras, siempre a partir de un cultivo puro, sin necesidad de tinciones, marcajes o amplificaciones. Aunque relativamente sencillas, su capacidad para tipificar *Salmonella* y sobre todo para sustituir a las técnicas fenotípicas clásicas no está todavía determinada.

4.2 Métodos genotípicos

Los métodos fenotípicos poseen un poder de discriminación reducido que limita su utilidad en estudios de filogenia o de epidemiología para el seguimiento de brotes o de la propagación de clones. Por ello, se han desarrollado técnicas moleculares cuyo poder discriminativo es mayor y que permiten el estudio evolutivo de *Salmonella* y el establecimiento de relaciones entre cepas con un origen común.

4.2.1 Análisis de secuencias multilocus (MLST)

La caracterización mediante el análisis de secuencias multilocus (MLST) surgió a partir de los fundamentos del análisis mediante electroforesis enzimática de secuencias multilocus (MLEE), desarrollado hace más de veinte años. El MLST se basa en el análisis comparativo de secuencias de fragmentos de genes altamente conservados (genes *housekeeping*). Las mutaciones en la secuencia de estos genes son infrecuentes y dan lugar a diferencias con respecto a la cepa original. Desde comienzos de la pasada década, varios trabajos se han centrado en el desarrollo de esta técnica para su aplicación en la tipificación de *Salmonella* (Kotetiskvili et al., 2002; Fahkr et al., 2005; Sukhnanand et al., 2005). Actualmente existe un protocolo estandarizado para el análisis por MLST de *Salmonella* mediante la secuenciación de fragmentos de los genes *thrA, purE, sucA, hisD, aroC, hemD* y *dnaN* (University College, Cork, Irlanda). Dicho protocolo está apoyado por una base de datos

(http://mlst.ucc.ie/mlst/dbs/Senterica) donde se pueden cotejar las secuencias y obtener el tipo epidemiológico o ST correspondiente a los resultados obtenidos. En el año 2011 ya se disponía de información sobre más de 4.000 aislados procedentes de 554 serotipos con 1.092 STs y es probable que en un periodo breve de tiempo ese número aumente progresivamente (Achman et al., 2012). Se han publicado varios estudios siguiendo el esquema de genes anteriormente propuesto (Torpdahl et al., 2005; Cooke et al., 2008; Wiesner et al., 2009). Además, este no es el único esquema de MLST; existe otro más reciente (Tankouo-sandonj et al., 2007) que emplea cuatro genes *atpD, gyrB, fliC, fljB* y cuyo objetivo ha sido desarrollar un sistema más equiparable al serotipado. La técnica de MLST permite observar cambios genéticos a largo plazo por lo que su uso está dirigido hacia estudios de evolución y filogenia. En *Salmonella* también ha adquirido importancia su empleo taxonómico, habiendo sido propuesto como alternativa al serotipado (Achman et al., 2012), ya que se ha comprobado que existen serotipos en los que se engloban cepas no emparentadas filogenéticamente. Así, por ejemplo, se ha comprobado que las cepas del serotipo *S.* Newport, pese a expresar los mismos antígenos somáticos y flagelares, no muestran relación alguna, presentando diferencias notables en sus propiedades y virulencia (Achman et al., 2012). Con la reducción de los costes de secuenciación en los últimos años, el MLST ha pasado a ser una técnica más asequible, lo que ha permitido que un mayor número de investigadores puedan emplearla en sus estudios. Sin embargo y contrariamente a lo que Achman et al. (2012) indican, sigue siendo una técnica más cara y compleja que el serotipado, por lo que su instauración como alternativa al mismo, para la caracterización y clasificación de *Salmonella* a nivel global, no es por el momento una opción viable.

4.2.2 Electroforesis en Gel de Campo Pulsado (PFGE)

La electroforesis en campo pulsado (PFGE) fue descrita en 1983 y su fundamento reside en el estudio de la variación de la secuencia de nucleótidos del ADN revelada por el polimorfismo en los fragmentos de restricción generados por tratamientos con endonucleasas específicas de baja frecuencia de corte (Goering, 2004). Estas enzimas realizan restricciones en puntos muy específicos del genoma de modo que en lugar de obtenerse varios cientos de fragmentos de ADN, como sucedería empleando una endonucleasa convencional, se produce un número limitado de fragmentos que pueden ser resueltos en un gel de agarosa. La corriente electroforética mediante pulsos, en la que la orientación del campo eléctrico se alterna periódicamente, permite la separación de los fragmentos de gran tamaño que se forman tras la acción de estas endonucleasas. Así, mientras que la electroforesis convencional no permite separar moléculas mayores de 40-50 Kb, la electroforesis de campo pulsado

permite resolver patrones desde tamaños de 20-30 Kb hasta 1 Mb, aproximadamente. De esta forma, se pueden obtener patrones de restricción claros y fácilmente distinguibles. El análisis de estos patrones mediante un software apropiado permite construir árboles filogenéticos en los que se ve claramente la mayor o menor proximidad genética entre los aislados analizados (Figura 4).

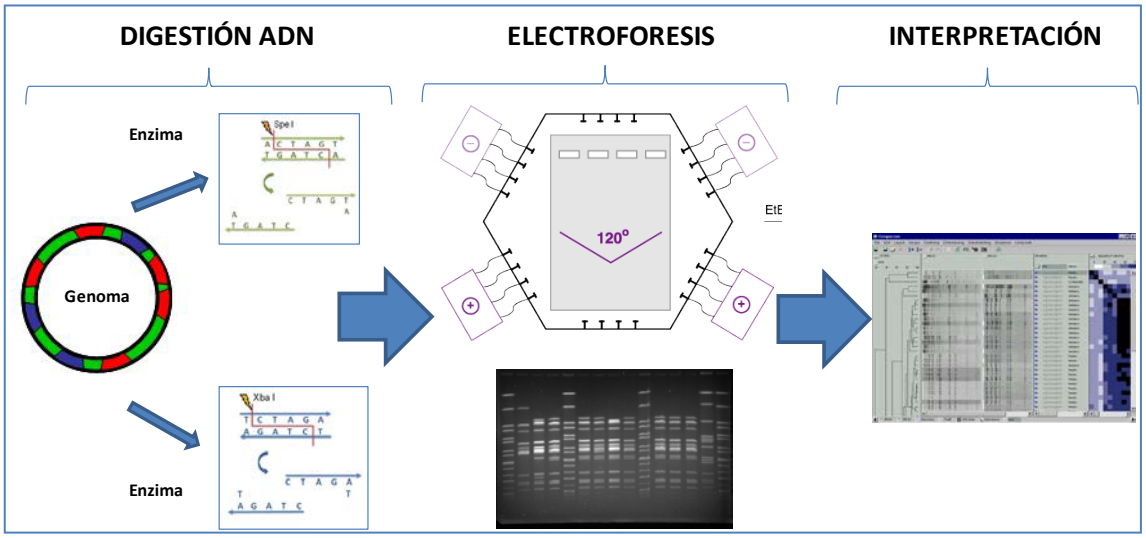

Figura 4. Etapas de la electroforesis en campo pulsado (PFGE). Digestión enzimática con una enzima de restricción, electroforesis de campo pulsado y análisis de los perfiles de bandas obtenidos.

El PFGE ha sido la técnica de tipificación genotípica de elección para *Salmonella* (Olsen et al., 1992; Ridley et al., 1998; Baggesen et al., 2000; Guerra et al., 2000; Lawson et al., 2004; Eriksson et al., 2005; Valdezate et al., 2005; Aarestrup et al., 2007; Kérouanton et al., 2007). Un estudio llevado a cabo por la EFSA con el fin de determinar los principales métodos de caracterización empleados en Europa destacó que esta técnica es utilizada por 19 de los 20 laboratorios nacionales de referencia (EFSA, 2009b). El CDC (Centers for Disease Control and Prevention, Atlanta, EEUU) estandarizó la técnica para varios géneros bacterianos, entre ellos *Salmonella*. Dicho protocolo se emplea dentro del programa de vigilancia conocido como PulseNet iniciado en el año 1996, destacando por su elevada especificidad y reproducibilidad y permitiendo caracterizar la práctica totalidad de los aislados con gran poder de discriminación (Swaminathan et al., 2001; Ribot et al., 2006). Pese a ser una técnica laboriosa y costosa en tiempo, que requiere de una correcta puesta a punto, y que solo puede ser llevada a cabo en laboratorios que dispongan del equipamiento necesario, el PFGE sigue siendo la técnica de referencia o "*gold standard*" en la tipificación genotípica de *Salmonella*.

4.2.3 Protocolo e interpretación de la técnica PFGE

A continuación, se describen resumidamente los pasos de la técnica de PFGE, de acuerdo al protocolo descrito por el CDC (Ribot et al., 2006). La técnica se inicia con **(I)** un cultivo puro de la cepa a analizar, **(II)** cuya concentración celular se ajusta para disponer de la adecuada cantidad de ADN en la restricción y resolución de bandas, siendo comparable esta concentración entre aislados. **(III)** La suspensión bacteriana se embebe en bloques de agarosa, con la finalidad de aislar el ADN en un ambiente protegido de la degradación enzimática y mecánica, mientras el resto de componentes celulares va siendo degradado y eliminado. **(IV)** Seguidamente, dichos bloques se someten a un lisado de las células mediante un tampón con detergentes y proteinasa K, tampón de lisis, que lisa la pared celular y provoca la proteólisis. Este paso se realiza a temperatura elevada con el fin de facilitar el proceso de lisis, acortando el tiempo necesario. **(V)** Posteriormente se realizan lavados para eliminar el exceso de enzimas y sustancias químicas que podrían interferir con la enzima de restricción; se realizan varios lavados con agua bidestilada desionizada y con tampón Tris-EDTA. Entre estos lavados, los bloques se mantienen en baño de agua con agitación continua a 56°C. **(VI)** La digestión del ADN es el siguiente paso y se realiza con la enzima que deseemos utilizar. Las más empleadas en la restricción de *Salmonella* son *Xba*I y *Bln*I y se emplean a concentraciones elevadas para que puedan actuar sobre el ADN embebido en agarosa. Estas enzimas realizan la restricción completa en menos de 4 horas, por lo que este tiempo de incubación es suficiente para finalizar el proceso. **(VII)** Después de la restricción, el patrón de corte obtenido se resuelve por medio de la electroforesis que se lleva a cabo en una cubeta específica, normalmente mediante un sistema CHEF, que permite el cambio del campo migratorio a través de pulsos de 120° y cambios de voltaje, durante el tiempo que dura la migración (aproximadamente 22 horas). **(VIII)** Para el procesado de los resultados se suelen emplear programas bio-informáticos como el Bionumerics (Applied Maths, Belgium), que permiten el procesamiento de las imágenes, la normalización de los resultados (lo que permite extrapolarlos y comparar resultados obtenidos en diferentes geles) y el análisis final en el que se establece la relación entre cepas ya sea por medio de la intensidad y grosor de las bandas (coeficiente de Pearson) o por el tamaño de las mismas (coeficiente de Dice). La representación gráfica se realiza mediante árboles de similitud, en los que los patrones de bandas están agrupados por algoritmos tales como el método de agrupación de pares no ponderado.

Factores que afectan al PFGE

El campo pulsado es una técnica molecular compleja que requiere de una gran precisión en su puesta a punto y estandarización para lograr resultados repetibles. Existen dos grupos de factores que pueden afectar a la repetitividad del PFGE: factores relacionados con la preparación del ADN y factores inherentes al procesado, concretamente la restricción y las condiciones de la electroforesis. La calidad del ADN empleado, su preparación para facilitar la restricción por la enzima de corte y el uso de una concentración adecuada que permita que el bandeo obtenido tras la restricción sea discernible mediante PFGE, son factores que se deben tener en cuenta durante el procesado de la muestra. En cuanto a factores propios de la técnica, hay que saber que la selección de la enzima de restricción es el más determinante. La enzima seleccionada debe producir un número de bandas superior a 10 e inferior a 25-30 y con una distribución por tamaños apropiada (Goering, 2004). El patrón de bandas está relacionado con la frecuencia en la que la enzima reconoce sitios de restricción. Para la mayoría de las enzimas, el número de puntos de reconocimiento viene determinado por el contenido en G+C del cromosoma, de modo que enzimas como *Xba*I rara vez cortan en bacterias Gram negativas, ricas en G-C. En el caso particular de *Salmonella,* hay que tener en cuenta, además, que no todos los serotipos se comportan de la misma forma frente a la enzima de restricción. Así, *Xba*I es una enzima adecuada para algunos serotipos como *S.* Typhimurium pero, sin embargo, no es útil para otros por existir gran homología en el patrón de restricción obtenido (Barret et al., 2004). Finalmente existen variaciones intrínsecas al equipo de campo pulsado y a la electroforesis del gel. Es por ello que en cada gel se incluyen al menos dos controles de tamaño, ya sea un control de peso molecular o la cepa *S.* Branderup H9812 cuyo perfil de bandas está determinado y se emplea como referencia. Otros factores dependientes de la técnica vienen fijados por el protocolo de Ribot y colaboradores (2006) e incluyen tiempos y temperaturas de trabajo así como las condiciones de la electroforesis.

Interpretación de los resultados

Tenover y colaboradores elaboraron unos criterios para la interpretación de los resultados de PFGE y de las relaciones existentes entre aislados o cepas (Tenover et al., 1995). De acuerdo a estos criterios, los cambios genéticos que pueden modificar un perfil de PFGE son inserciones, delecciones, inversiones o sustituciones de bases (transiciones y transversiones). Si estos cambios genéticos ocurren fuera del lugar de restricción provocan modificaciones en el tamaño de los fragmentos y, por lo tanto, en su migración en el gel. Si por el contrario ocurren en el lugar de restricción, darán lugar a modificaciones en el tamaño y

número de bandas observados. Resumidamente, se considera que dos cepas son de un mismo tipo si presentan el mismo patrón de bandas, mientras que diferencias en una banda indican que se trata de subtipos epidemiológicamente relacionados. Siguiendo este mismo criterio, aquellas cepas con dos eventos genéticos aun tendrían algo de relación mientras que cepas con tres o más variaciones en el patrón no presentarían relación alguna.

Sin embargo, estos criterios no son apropiados para el estudio de bacterias patógenas altamente clonales como *E. coli* o *Salmonella.* En contraposición a lo asumido por los criterios de Tenover y colaboradores, no son las mutaciones puntuales sino las inserciones y las delecciones los sucesos genéticos más comunes en enterobacterias. Además, este tipo de bacterias suele presentar plásmidos extracromosómicos en los que también son frecuentes los cambios y que también dan lugar a cambios en el patrón de restricción. Finalmente indicar que estos criterios pueden ser válidos para el análisis de aislados procedentes de eventos epidemiológicamente relacionados, en un periodo de tiempo corto. Sin embargo, el que dos aislados presenten un mismo patrón de bandas no implica que posean un mismo origen en estudios con un contexto más amplio, por ejemplo en estudios epidemiológicos de brotes en los que la pieza clave es la información epidemiológica mientras que el PFGE es una herramienta accesoria que permite clarificar los resultados.

Por lo tanto, la interpretación de los resultados se debe hacer siguiendo las guías establecidas por PulseNet para *Salmonella*. Se considera que aquellos patrones que presentan cambios discernibles deben ser considerados patrones distintos. De este modo, en estudios epidemiológicos, aquellos perfiles que posean un mismo patrón, se denominan indistinguibles, siendo este término más preciso que el de idénticos puesto que solo implica que no se observan variaciones bajo las circunstancias de estudio. Ligeras variaciones en el perfil darán lugar a sub-tipos, que pueden aparecer con frecuencia en estudios prolongados en el tiempo, mientras que cuando las diferencias sean claras los perfiles serán considerados diferentes.

4.2.4 Análisis de variabilidad de secuencias multi-locus (MLVA)

La técnica de análisis de variabilidad de secuencias multi-locus (MLVA) es una de las técnicas de tipificación más recientemente aplicadas para la caracterización de *Salmonella* (Lindstedt et al. 2003). El análisis del genoma de *Salmonella* reveló la existencia de regiones variables de repeticiones en tándem (TRs) o VNTR en múltiples loci. Cada una de estas regiones posee un cierto número de unidades de repetición limitado por las regiones flanqueantes (Figura 5). Variaciones o mutaciones en dichas regiones darán lugar a cambios en el número de nucleótidos de la secuencia del VNTR en cuestión. La técnica de MLVA amplifica,

mediante PCR, fragmentos de ADN de regiones que contienen VNTR y el producto obtenido se analiza empleando geles de agarosa o electroforesis capilar, siendo este segundo sistema mucho más preciso, para determinar el tamaño del fragmento amplificado (Lindstedt et al. 2004).

La primera técnica de MLVA en *Salmonella* se desarrolló con el objetivo de tipificar *Salmonella* Typhimurium DT104 ya que es un fagotipo muy homogéneo en el perfil de PFGE (Lindstedt et al., 2003). Los resultados fueron prometedores y dieron lugar a nuevos estudios con variaciones y mejoras en la técnica (Lindstedt et al. 2004; 2007; Torpdahl et al., 2007), así como a la descripción de nuevos loci VNTR tanto para la caracterización de *S.* Typhimurium (Witonksi et al., 2006) como para otros serotipos (Liu et al., 2003; Ramisse et al., 2004; Malorny et al., 2008). El MLVA se ha revelado como una técnica de tipificación muy potente, incluso más sensible que el PFGE (Achman et al., 2012). Pese a que el PFGE todavía se considera la técnica de referencia en la tipificación genotípica de *Salmonella*, la mayor rapidez y sencillez unido al menor coste del MLVA, hacen que diversos estudios, sobre todo aquellos relacionados con el serotipo *S.* Typhimurium, se hayan decantado por el uso del MLVA, junto con o en substitución del PFGE. El problema que encierra el uso de MLVA es que gran parte de los loci VNTR diana son específicos de serotipo (Lindstedt, 2005) y muchos serotipos no pueden ser analizados por esta técnica al no estar su genoma secuenciado y, por lo tanto, no ser posible identificar estas regiones VNTR.

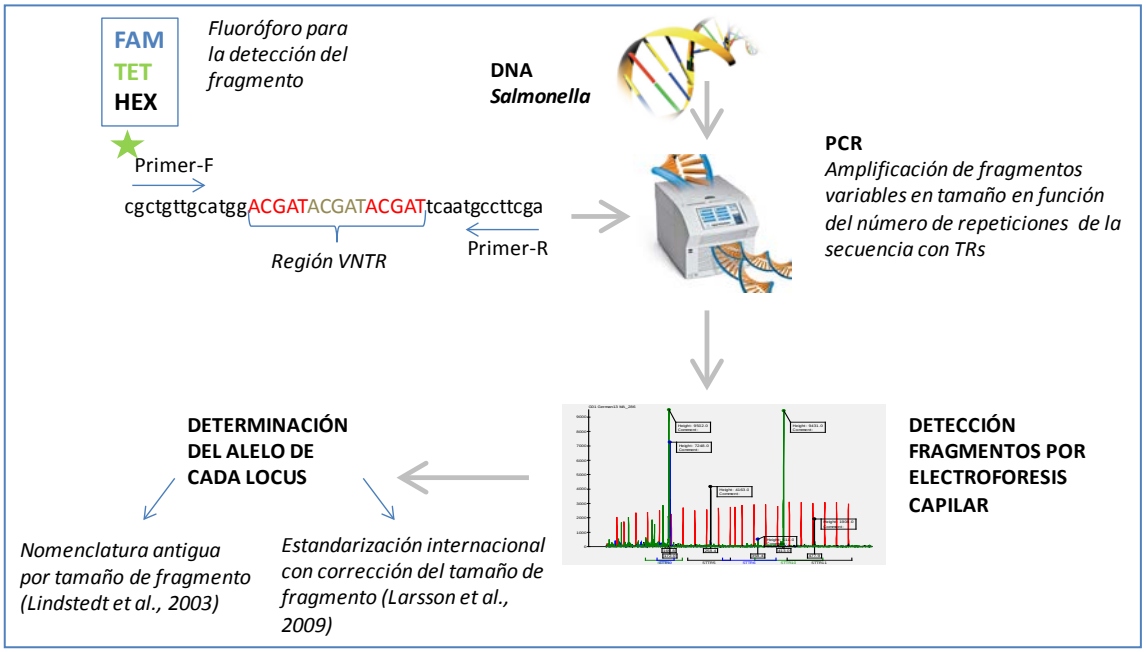

Figura 5. Bases de la técnica de análisis de variabilidad de secuencias multi-locus (MLVA). Amplificación de regiones con repeticiones en tándem que varían de unas cepas a otras en el número de repeticiones y por lo tanto en el tamaño de los fragmentos detectados mediante electroforesis capilar e

identificados por el rango de tamaño de fragmento y el color de marcaje del fluoróforo. Los tamaños de fragmento amplificados se transforman en alelos para la caracterización de los aislados.

Para *S.* Typhimurium existe un protocolo que podría ser definido como estándar, ya que es empleado por la mayoría de los laboratorios de referencia, en el que se evalúa las variaciones de tamaño de 5 loci (Lindstedt et al., 2004). Torpdahl y colaboradores evaluaron la utilidad de esta técnica en el sistema de vigilancia y detección de brotes danés y concluyeron que el MLVA es la técnica más eficaz para la tipificación de *Salmonella* Typhimurium, siendo su poder de discriminación superior al del PFGE (Torpdahl et al., 2007). En el año 2009 se fijó la nomenclatura del MLVA con un nuevo sistema que normalizaba los resultados entre laboratorios para evitar las pequeñas diferencias que se producen por secuenciadores, los polímeros empleados o los fluoróforos y que pueden dar lugar a errores en la designación del número de alelo (Larsson et al., 2009). Para ello, se seleccionaron 31 cepas que han sido empleadas, posteriormente, para estandarizar la técnica entre los diferentes laboratorios.

4.2.5. Otras técnicas genotípicas

Aparte de las técnicas anteriormente descritas, existen otras entre las que destacan el RAPD (análisis de polimorfismos aleatorios de ADN) y nuevas técnicas como el CRISPR (clustered regularly interspaced short palindromic repeats) o el WGS (Whole-Genome Sequencing). El CRISPR es una técnica recientemente descrita en *Salmonella* (Fabre et r écnicaal., 2012), basada en variaciones de tamaño en palíndromos intergénicos. Esta técnica ha demostrado una elevada concordancia con serotipado y MLST permitiendo la tipicficacion y subtipificación de cepas a un mismo tiempo. La tipificación por genomas completos (WGS) es en la actualidad la técnica más novedosa y sin duda con más potencial (Zankari et al., 2013). Se basa en la obtención de la secuencia del genoma completo con la que por ejemplo se pueden caracaterizar cepas con la identificación de SNPs (Single-Nucleotid Polimorfisms), detección de genes de virulencia o de resistencia (Zankari et al., 2013)

Como resumen podemos destacar que existe una amplia diversidad de técnicas de tipificación de *Salmonella*. El rápido desarrollo y abaratamiento de técnicas moleculares nos hace pensar que en un futuro próximo sustituirán a las técnicas clásicas. Actualmente la elección de las técnicas a emplear dependerá de las posibilidades pero sobre todo de los objetivos del estudio. En la Figura 6 se resumen las técnicas comentadas en este apartado.

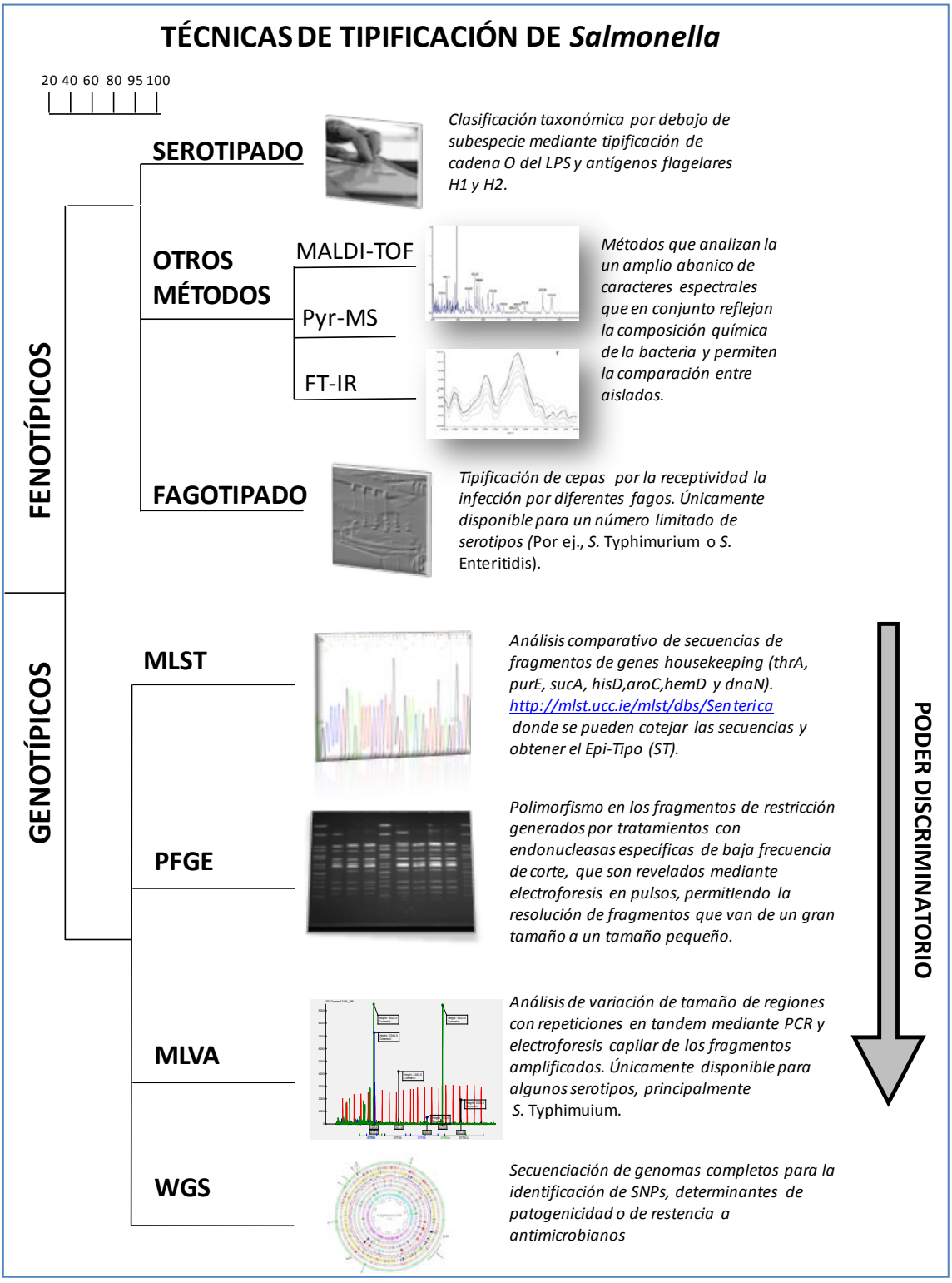

Figura 6. Esquema de las principales técnicas de tipificación de *Salmonella*. Las técnicas genotípicas aparecen ordenadas según su poder discriminatorio.

5. Infecciones por *Salmonella* en el cerdo

La infección por *Salmonella* en el ganado porcino puede ser abordada desde dos perspectivas; por una parte como infección propia del cerdo y por otra como problema de salud pública. Aunque como entidad clínica *Salmonella* puede causar pérdidas económicas significativas, en la actualidad, la infección por *Salmonella* en cerdos tiene una mayor relevancia como problema de salud pública asociado al consumo de carne de cerdo contaminada.

5.1 Presentación clínica

La presentación septicémica de la salmonelosis porcina es provocada principalmente por *S.* Cholerasuis, que es el serotipo adaptado al cerdo. La infección puede aparecer a cualquier edad, si bien en cerdos jóvenes tiene una tasa de mortalidad superior. Esta forma clínica se caracteriza por signos respiratorios: respiración superficial o diafragmática, disnea y tos húmeda, así como cianosis en la parte distal de las extremidades, inapetencia, letargia o fiebre. Estos signos aparecen entre las 24 y las 36 horas tras la instauración de la infección y se suelen resolver en un periodo de 14 días (Reed et al., 1986; Gray et al., 1996). Además, a partir de los 4-5 días post-infección puede presentarse diarrea. La mortalidad y morbilidad de esta presentación septicémica son variables (Reed et al., 1986; Wilcock & Schwartz, 1992). Aparte de *S.* Cholerausis, los cuadros clínicos septicémicos también pueden estar provocados por otros serotipos como *S.* Typhimurium, que en infecciones experimentales induce un cuadro clínico similar, llegando incluso a provocar la muerte de los cerdos (Fedroka-Cray et al., 1994; Collazos, 2008). En cualquier caso, esta forma septicémica de la enfermedad es rara en la UE, donde el serotipo *S.* Cholerasuis es poco prevalente. Por el contrario, en países de América, entre ellos EE.UU., no es infrecuente la aparición de esta forma clínica en las granjas porcinas.

Exceptuando las infecciones provocadas por el serotipo *S.* Cholerasuis, la presentación más común de la infección por *Salmonella* en el ganado porcino es la entérica. Dicha forma clínica se atribuye principalmente al serotipo *S.* Typhimurium (EFSA, 2006; Boyen et al., 2008a), aunque no se puede descartar la participación de otros serotipos potencialmente patógenos. Esta forma entérica suele afectar a cerdos de entre 6 y 12 semanas de edad, aunque se describen casos durante todo el periodo de transición y cebo, siendo su principal signo clínico la enterocolitis que cursa con diarrea acuosa verde-amarillenta. Otros signos clínicos que suelen acompañar a la diarrea son letargia, inapetencia y fiebre. Estos signos aparecen a las 48 horas post-infección, aproximadamente, y suelen resolverse en un periodo de entre 7 y 10 días (Fedorka-Cray et al., 1994). La mortalidad suele ser nula o muy baja pero la

morbilidad de la infección puede ser alta (Wilcock & Schwartz, 1992). La prevalencia de enterocolitis asociada a *Salmonella* es complicada de estimar puesto que la infección suele estar asociada a otros agentes etiológicos englobados en el síndrome entérico porcino y además *Salmonella* puede ser aislada en cerdos asintomáticos.

5.2 Mecanismos de transmisión y eliminación

La principal ruta de transmisión de *Salmonella* es la ruta fecal-oral. Tras su entrada por vía oral, *Salmonella* es capaz de colonizar el tracto gastrointestinal y aparecer en heces en un periodo de tiempo muy corto, que puede ser inferior a los 60 minutos (Hurd et al., 2001). En la fase aguda de la infección, *Salmonella* es excretada en concentraciones muy elevadas, que pueden alcanzar las 10^7 UFC/g de heces (Gutzmann et al., 1976), lo que facilita la contaminación del ambiente y la transmisión entre animales alojados en el mismo lugar. Teniendo en cuenta que la dosis infectiva para las infecciones naturales está en torno a 10^3 UFC/g (Loynachan & Harris, 2005), podemos deducir que la infección por *Salmonella* se transmite con gran facilidad en condiciones de campo a través de esta ruta fecal-oral. Según diversos autores, la eliminación fecal disminuye con el tiempo en intensidad, hasta convertirse en una eliminación intermitente (Wood et al., 1989; Nielsen et al., 1995; Beloeil et al., 2003). Una elevada proporción de los cerdos, tras la infección por *Salmonella* desarrollan un estado de portador, manteniéndose viable la bacteria en determinados órganos o tejidos, particularmente las tonsilas y los ganglios linfáticos mesentéricos. La eliminación fecal en estos animales portadores puede ser nula o muy baja, con valores del orden de 50 UFC/g (Wood & Rose, 1992; Sibley et al., 2003), lo que hace muy complejo el diagnóstico de la infección empleando técnicas directas. Estos cerdos portadores de *Salmonella* tienen una gran importancia epidemiológica puesto que son animales aparentemente sanos pero potenciales eliminadores de la bacteria, bien a dosis bajas o de forma intermitente, o incluso eliminadores de dosis elevadas tras la reactivación de la infección en situaciones de estrés o inmunosupresión.

Por otra parte se ha demostrado que también es posible la transmisión por vía respiratoria, mediante la inhalación de aerosoles o de partículas de polvo contaminadas con *Salmonella* (Fedroka-Cray et al., 1995; Proux et al., 2001; Oliveira et al., 2006). Estos trabajos indican que, a través de la vía respiratoria, *Salmonella* es capaz de invadir las tonsilas y los pulmones, habiendo sido aislada de macrófagos pulmonares. A partir de estos órganos puede diseminarse a otras partes del organismo, incluido el tracto digestivo. Fedorka-Cray et al. (1995) aislaron *Salmonella* del tracto digestivo y de los ganglios linfáticos mesentéricos pocas horas después de la infección por vía aerógena en cerdos esofagoectomizados. Se ha sugerido

que el peso de esta vía aerógena en la transmisión puede ser importante, teniendo en cuenta la gran eficiencia de esta vía de entrada y la capacidad de *Salmonella* para sobrevivir largos periodos de tiempo en aerosoles (McDermid & Lever, 1996).

5.3 Patogenia e inmunidad

El curso de la infección por *Salmonella* está determinado por una serie de factores entre los que se encuentran la vía de entrada, la dosis infectante, el serotipo implicado, la presencia y grado de inmunidad y el grado de resistencia del hospedador.

Cronológicamente, la patogenia de la salmonelosis puede dividirse en dos fases: **(I)** una primera fase localizada en el intestino y en el tejido linfoide asociado al mismo y **(II)** una segunda fase sistémica, que no siempre tiene lugar, en la que *Salmonella* disemina, por el torrente circulatorio, desde los ganglios linfáticos hacia otros órganos (Figura 7)**.**

Si la entrada se ha producido por vía oral, *Salmonella* debe enfrentarse a una serie de condiciones adversas. Para poder establecerse en la porción distal del intestino delgado y comienzo del intestino grueso, el patógeno debe hacer frente a factores como el pH ácido del estómago, las sales biliares secretadas en las porciones anteriores del intestino delgado, la elevada osmolaridad, el peristaltismo o el ambiente anaerobio. *Salmonella* posee diversos mecanismos que le permiten superar estas agresiones tales como la respuesta de tolerancia a ácidos (Álvarez-Ordoñez et al., 2011a), la adaptación a ambientes anaerobios o la batería de componentes genéticos que le permiten sobrevivir en ambientes con elevadas concentraciones de sales biliares y osmolaridad (Álvarez-Ordoñez et al., 2011b). Además la competición por los nutrientes con la microbiota comensal y la producción de bacteriocinas y péptidos antimicrobianos por parte de esta, constituye igualmente un desafío para la supervivencia e instauración de *Salmonella*.

Una vez que alcanza la porción distal del intestino delgado, *Salmonella* es capaz de adherirse a las células epiteliales de la mucosa a través de sus fimbrias (Dibb-Fuller et al., 1999; Vimal et al., 2000). *Salmonella* presenta especial tropismo por las células M de las placas de Peyer presentes en el íleon, aunque también puede invadir enterocitos (Ginocchio et al., 1994) o penetrar entre las células intestinales, alcanzando la lámina propia mediante tránsito para-celular (Boile et al., 2006). La invasión de las células de la pared intestinal está regulada por los genes de virulencia de la isla de patogenicidad de *Salmonella* 1 (SPI-I). Los genes presentes en esta isla codifican un sistema de secreción tipo III mediante el cual el patógeno induce en la célula un proceso conocido como macropicnocitosis, una endocitosis no selectiva de partículas de gran tamaño como pueden ser bacterias. Esta macropicnocitosis está mediada por la

inoculación en la célula hospedadora de una serie de proteínas que inducen la reorganización del citoesqueleto y la endocitosis del microbio (Zhou & Galán, 2001). A consecuencia de la interacción patógeno-células intestinales se concentra en la zona un elevado número de polimorfonucleares y se induce la producción de citoquinas proinflamatorias que van a provocar cambios que pueden afectar a la modulación de la secreción del cloro, contribuyendo directamente a la aparición de la diarrea (EcKmann et al., 1997). La reacción inflamatoria trae como consecuencia un aumento de la permeabilidad vascular que provoca un edema de la mucosa así como una transmigración de células inflamatorias a la luz intestinal. Además, la respuesta inflamatoria, junto con la producción de toxinas, provoca daños en la superficie del epitelio intestinal, que pueden ir desde ulceración hasta destrucción de la mucosa (Ekperigin & Nagaraja, 1998), facilitando la salida de fluidos extravasculares y, consecuentemente el cuadro clínico de diarrea (Zhang et al., 2003).

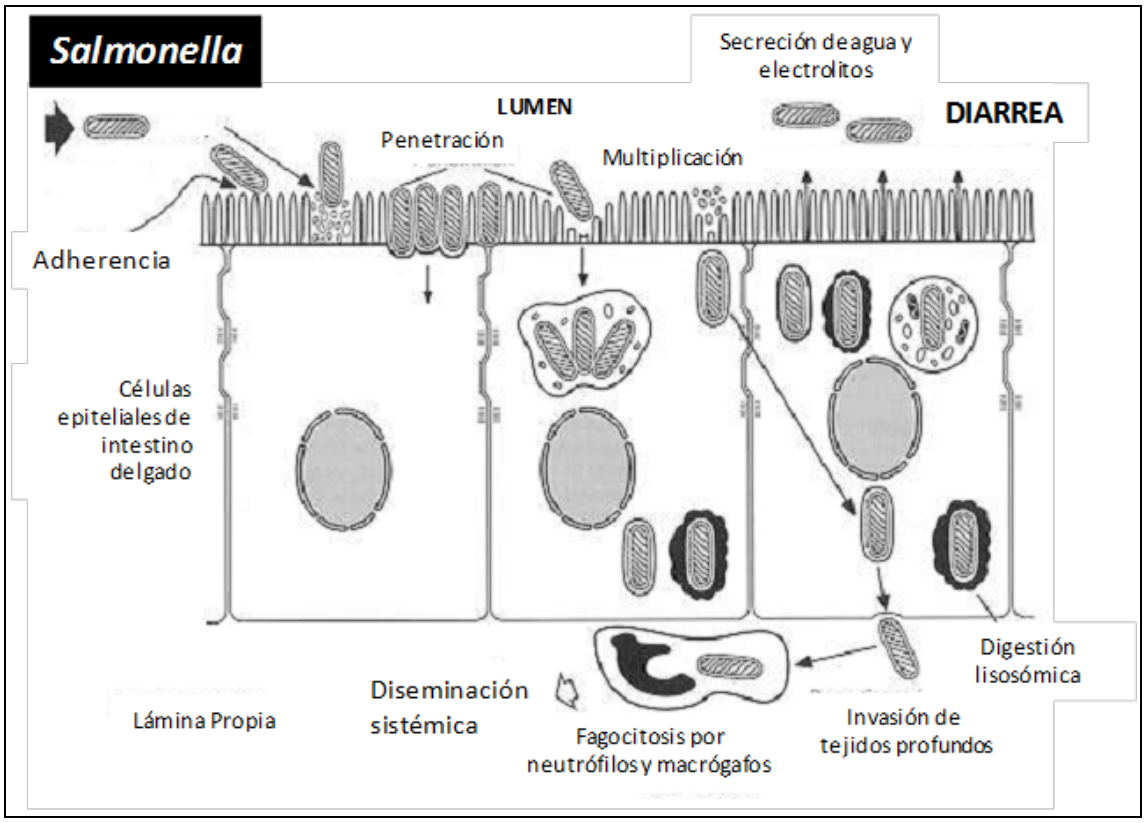

Figura 7. Esquema de la invasión de la mucosa intestinal por *Salmonella*. Adaptación de figura de Ralph A. Gianella, 1996.

La fase sistémica se inicia cuando *Salmonella* alcanza la lámina propia y es fagocitada por los macrófagos, siendo esta etapa clave puesto que el mantenimiento de la infección se basa en la capacidad de *Salmonella* para sobrevivir y multiplicarse en el interior de estos macrófagos. Este mecanismo está regulado por la isla de patogenicidad de *Salmonella* 2 (SPI-II), que permite la traslocación de proteínas bacterianas a través de la membrana vacuolar y

hacia el citoplasma de los macrófagos (Hensel, 2000; Gal-Mor & Finlay, 2006), permitiendo a la bacteria alcanzar el sistema retículo-endotelial como etapa previa a una infección sistémica. La gran mayoría de las infecciones causadas por serotipos diferentes a *S.* Cholerasuis se extienden hasta el tejido linfoide asociado al intestino y los ganglios linfáticos mesentéricos. La diseminación del patógeno suele detenerse aquí gracias a la respuesta inmunitaria inespecífica y adaptativa que trabajan conjuntamente en los ganglios linfáticos mesentéricos (Martins et al., 2012). Esta respuesta inmunitaria es capaz de reducir, aunque no eliminar, la carga de *Salmonella* presente en estos ganglios linfáticos (Martins et al., 2013). En algunas ocasiones, cuando la infección la producen serotipos muy invasivos o cuando afecta a animales debilitados, *Salmonella* puede alcanzar la circulación sanguínea y, así, diferentes órganos internos. Los primeros órganos que coloniza son el hígado y el bazo, donde la bacteria puede ser detectada en concentraciones relevantes en las primeras fases de la infección (Barrow, 1999). Estos órganos pueden constituir una barrera o, por el contrario, un punto donde el microorganismo se multiplica, principalmente en macrófagos. Posteriormente, a través del torrente sanguíneo, puede colonizar otros órganos. En concordancia con estos mecanismos, numerosos estudios basados en infecciones experimentales, principalmente con los serotipos *S.* Cholerasuis y *S.* Typhimurium, han descrito la presencia de *Salmonella*, además de en el tracto gastrointestinal (intestino y ganglios linfáticos mesentéricos), en diversos órganos como el bazo, el riñón, el pulmón, el corazón, el hígado, las tonsilas y diferentes ganglios linfáticos (pulmonares, inguinales, cervicales, etc.) (Wood et al., 1989; Wood & Rose, 1992; Fedorka-Cray et al., 1994; Nielsen et al., 1995; Cote et al., 2004; Loynachan et al., 2004; Boyen et al., 2006; Collazos, 2008). En algunos de estos órganos *Salmonella* se acumula en concentraciones relevantes, siendo los órganos diana para su detección las tonsilas, el ciego, la porción caudal del íleon, el colon y los ganglios linfáticos mesentéricos, retrofaríngeos y mandibulares (Wood et al., 1989; Wood & Rose, 1992; Boyen et al., 2006; Collazos, 2008).

La patogenia de *Salmonella* que acabamos de ver resumidamente va a condicionar la respuesta inmunitaria del hospedador. En el momento en que *Salmonella* alcanza el tracto gastrointestinal, se produce una respuesta del sistema inmunitario compleja y coordinada que varía entre las diferente -.s localizaciones intestinales (Collado-Romero et al., 2010) e incluye tanto respuesta inmunitaria innata como adaptativa. Como se comentó en el párrafo anterior, las células M de las placas de Peyer son la principal diana para la entrada de *Salmonella.* Se ha comprobado que cuando el microorganismo penetra a través de estas células, estimula la producción de inmunoglobulinas del tipo IgA. Una vez que *Salmonella* atraviesa la barrera epitelial del intestino estimula la llegada de macrófagos, neutrófilos y monocitos al tejido

linfoide que se diferenciarán en células dendríticas o macrófagos. Estas células monocíticas estimulan la producción de linfocitos T y proporcionan la primera barrera celular frente a la invasión por *Salmonella.* Además, en esta fase también actúan interleuquinas como la IL-12, IL-1 o las recientemente involucradas IL-17, IL-22 e IL-23 (Raffatelu et al., 2008 Schulz et al., 2008; Godinez et al., 2009), así como el TNFα y el IFNγ.

En etapas posteriores de la infección, la respuesta inmunitaria específica se hace más relevante. La respuesta de base humoral puede ser detectada en la primera semana de infección (Gray et al., 1996a; 1996b), primero por la producción de inmunoglobulinas de la clase IgM y posteriormente por la producción de inmunoglobulinas de las clases IgA e IgG. Mientras que los niveles de IgM e IgA decaen rápidamente, los valores de IgG son más estables y pueden ser detectados durante largos periodos de tiempo. La respuesta celular específica está mediada principalmente por linfocitos T $CD4^+$ y CD8+ (Hess et al., 1996.) El mecanismo por el cual estos linfocitos $CD4^+$ controlan al patógeno se desconoce, pero se sabe que no está relacionado con el TNFα ni el IFNγ.

En resumen, a consecuencia del carácter intracelular del patógeno y de su capacidad para sobrevivir y multiplicarse en células del sistema retículo-endotelial, la respuesta inmunitaria frente a S*almonella* está mediada principalmente por linfocitos $CD4^+$ y $CD8^+$. Las células de respuesta innata, neutrófilos y macrófagos, constituyen una primera barrera y participan en la presentación de este patógeno, aunque no son capaces de controlar la infección. Por su parte, la respuesta humoral es evitada, al menos parcialmente, al encontrarse *Salmonella* "protegida" en el interior de las células que infecta. Únicamente en fases extracelulares o en la luz intestinal, las inmunoglobulinas de la clase IgA son eficaces.

5.4. Principales serotipos de *Salmonella* que infectan al cedo

El cerdo es receptivo a la infección por un gran número de serotipos de *Salmonella*. En el estudio basal llevado a cabo en los países miembros de la UE con el fin de conocer la prevalencia de esta infección en cerdos de engorde se detectaron más de 80 serotipos diferentes en los ganglios linfáticos mesentéricos recogidos en los cerdos inmediatamente tras el sacrificio, siendo los más frecuentes *S.* Typhimurium (40 %), *S.* Derby (14,62 %) y *S.* Rissen (5,8 %) (EFSA, 2008a). Los dos primeros son los principales serotipos aislados en diversos estudios llevados a cabo en el ganado porcino de diferentes países europeos (Baggesen et al., 1996; Van der Wolf et al., 1999; Rowe et al., 2003; Nollet et al., 2005; Arguello et al., 2013a). Los datos más recientes señalan que *S.* Typhimurium continúa siendo el serotipo predominante en las explotaciones porcinas europeas con un 28,6% de los aislados, seguido de

S. 4,5,12:i:- (9,3%) y *S.* Derby (5,7%) (EFSA, 2012). Sin embargo, estos últimos datos se basan en resultados proporcionados por solo diez de los estados miembros de la UE, proviniendo un 68,9 % de los aislados de Alemania, por lo que son menos precisos que los del estudio basal de 2007.

Los datos referentes a España señalan que *S.* Typhimurium y *S.* Derby (25,6% y 11,5% de los aislados españoles respectivamente) son igualmente muy frecuentes en el ganado porcino de nuestro país (EFSA, 2008a; EFSA, 2009a). Sin embargo, cabe destacar que *S.* Rissen es un serotipo muy frecuente en el ganado porcino de España (14,1% de los aislados españoles) y de forma general en toda la península ibérica (Gomes-Neves et al., 2012).

En cuanto a los serotipos aislados en carne de cerdo, el último informe de la EFSA sobre datos del año 2011, refleja que los serotipos identificados en carne de cerdo son similares a los aislados de animales vivos, siendo Typhimurium el más frecuente, seguido de Derby y la variante monofásica *S.* 4,5,12:i:-. En el informe del año anterior (EFSA, 2012) en los productos de carne de cerdo se detectó el serotipo Rissen, asociándose a los países del sur de Europa y siendo el serotipo más frecuente en carne de cerdo en Grecia e Italia en el año 2010 . En este informe no se reportaron datos de carne de cerdo en España.

6. *Salmonella* en explotaciones porcinas

La epidemiología de la salmonelosis porcina es muy compleja por los numerosos factores que están involucrados en la entrada y mantenimiento de la infección en las explotaciones. Entre estos factores cabe destacar las diversas fuentes de infección y el amplio rango de vectores y hospedadores a los que se suma la capacidad de *Salmonella* para mantenerse viable durante largos periodos de tiempo en el ambiente.

6.1 Fase de la producción

La producción porcina se puede dividir en tres fases bien diferenciadas: reproducción, transición y cebo. Cada una de ellas presenta unas características particulares que van a influir en la frecuencia de las infecciones por *Salmonella*.

De forma resumida, los trabajos que han estudiado la distribución de *Salmonella* en las diferentes fases de la producción porcina indican que en las explotaciones dedicadas a la reproducción o durante la fase de reproducción en el caso de granjas de ciclo completo, la prevalencia de anticuerpos anti-*Salmonella* es por lo general muy elevada, a consecuencia del

contacto del animal con el patógeno a lo largo de su vida productiva (Nollet et al., 2005). Los datos de los informes de la EFSA sobre explotaciones de reproducción en Europa revelaron una elevada prevalencia tanto en muestras procedentes de mezclas (EFSA, 2009a) como en muestras individuales (EFSA, 2011b), pese a que se ha mantenido que la prevalencia en muestras de heces debía ser baja ya que la mayoría de individuos se encuentran en estado portador.

Existen pocos estudios acerca del impacto de *Salmonella* durante la lactación. Los resultados indican que la infección en lactantes no es muy común si bien se acepta que podría estar subestimada por la pequeña cantidad de heces disponible para realizar el análisis (Funk et al., 2000). Roesler et al. (2005) estimaron que en una granja con infección endémica entre el 2% y el 9% de los lechones en lactación eliminaban *Salmonella*. En otra granja francesa donde un tercio de las cerdas en lactación eliminaban *Salmonella,* tan solo el 1,2% de las muestras recogidas en lechones fueron positivas (Beloeil et al., 2003). Otro estudio desarrollado en cinco granjas en EE.UU. proporcionó prevalencias de lechones eliminadores de entre el 0,5% y el 7% (Funk et al., 2001a).

De forma similar, la prevalencia en cerdos destetados es baja. Estudios de monitorización de la infección en esta etapa, muestran que los patrones de excreción varían notablemente a consecuencia del amplio número de factores que pueden modificar el curso de la misma: factores de estrés asociados al destete, serotipo, cepa, genética de los animales y, muy particularmente, la duración de la inmunidad transferida por la madre, particularmente la duración e intensidad del aporte de IgA. Una vez que esta inmunidad pasiva se reduce, los cerdos incrementan su receptividad a la infección, facilitándose la transmisión horizontal y pudiendo alcanzarse prevalencias de eliminadores próximas al 50% (Kranker et al., 2003).

Sin duda alguna, el engorde o cebo es la fase de producción en la que se detectan más infecciones y seguramente en la que se infectan la mayoría de los animales (Berends et al., 1996; Kranker et al., 2003; Beloeil et al., 2004; Funk et al., 2005). No existe un único patrón de infección durante esta etapa que se extiende habitualmente entre 3 y 4 meses, pudiéndose infectar los cerdos en las primeras fases (Berends et al., 1996) o durante el resto del tiempo que permanecen en las instalaciones (Kranker et al., 2003; Beloeil et al., 2004; Funk et al., 2005). Los cerdos infectados en etapas previas de la producción pueden introducir el patógeno en la nave de engorde y actuar como fuente de infección para otros cerdos (Wood et al., 1989; Fedroka-Cray et al., 1994; Bager et al., 1994; Letellier et al, 1999). Así mismo, también es muy

importante la presencia de *Salmonella* en el ambiente de las granjas de engorde como fuente de infección en esta etapa (Davies et al., 2000b; Lo Fo Wong et al., 2004).

6.2 Factores asociados al tipo de explotación, instalaciones y manejo

Existen diversos factores relacionados con la producción porcina tales como el tipo de producción, el manejo de los animales o las instalaciones, entre otros, que pueden tener influencia en la infección por *Salmonella*. Muchos de ellos han sido identificados como factores de riesgo o factores protectores en diversos estudios epidemiológicos analíticos. Dichos factores serán revisados con mayor detenimiento en la introducción del capítulo relativo a la infección por *Salmonella* en explotaciones de cerdos reproductores.

Nollet y colaboradores realizaron un estudio de distribución de *Salmonella* en explotaciones de ciclo cerrado o completo. Aunque no pudieron demostrar la transmisión directa de la madre a los lechones, si que pudieron comprobar la similitud entre las cepas identificadas en las diferentes fases, por lo que concluyeron que la transmisión entre las mismas era una opción probable (Nollet et al., 2005). Teniendo en cuenta que las diferentes fases de producción se encontraban físicamente muy cerca unas de otras, es factible que la infección de las cerdas reproductoras pudiera repercutir en las etapas posteriores. Por el contrario, controlar la entrada de *Salmonella* en las explotaciones con este tipo de producción es más sencillo ya que el movimiento de animales es más limitado. En contraposición, en los sistemas de producción en puntos múltiples, la importancia de la transmisión entre fases parece ser mucho menor. La separación entre las diferentes etapas puede ser muy grande, de hasta cientos de kilómetros, de modo que las infecciones que acontecen en la fase de reproducción pueden tener poco o nada que ver con las que ocurren en la fase de engorde. Por otra parte, la mezcla de cerdos de diferentes orígenes y de diferentes estatus sanitarios facilita la difusión de *Salmonella* y de otros microorganismos patógenos en los cebaderos multiorigen (Davies et al., 1997a).

Otros factores relativos al tipo de instalaciones, medidas de bioseguridad, número de proveedores, tamaño o alimentación también pueden influir, facilitando o dificultando la presencia de la infección en la granja. En lo que respecta al estatus sanitario, diversos autores han descrito que el riesgo de encontrar cerdos infectados por *Salmonella* es inferior en explotaciones porcinas con un elevado estatus sanitario (van der Wolf et al., 2001a; Oliveira et al., 2005; Mejia et al., 2006). Otros estudios han ligado la presencia de *Salmonella* a la presencia de patógenos como *Lawsonia intracellularis* o al virus del síndrome respiratorio y reproductivo porcino (Beloeil et al., 2004).

6.3 Supervivencia de *Salmonella* en el medio

Siendo una bacteria carente de cápsula externa y no productora de esporas, podría pensarse que *Salmonella* es un microorganismo poco resistente en el medio. Sin embargo, diversos estudios han demostrado que es capaz de sobrevivir en el ambiente durante periodos más o menos prolongados en función del sustrato donde se encuentra y la disponibilidad de nutrientes, la humedad relativa, la temperatura y la exposición a la radiación. Esta capacidad de supervivencia en el medio es una pieza clave en la epidemiología de la infección.

Se ha comprobado que *Salmonella* puede sobrevivir durante largos periodos de tiempo en heces (Fedroka-Cray et al., 1995), efluentes (Wray, 1999), agua (Davies and Evison, 1991) o incluso en suelo (Davies and Wray, 1996). Por ello no es de extrañar que pueda permanecer viable en la materia orgánica y polvo de las explotaciones (Berends et al., 1996; Rajik et al., 2005). En un estudio donde se contaminaron muestras de forraje, polvo y pienso con 10^6-10^8 UFC/g de *S.* Typhimurium se comprobó que el tiempo de supervivencia de *Salmonella* puede llegar incluso a los 4 años (Mitscherlin & Marth, 1984). También se ha comprobado que es capaz de permanecer viable hasta 4 horas en acero inoxidable (Scott & Bloomfield, 1990), incrementándose ese tiempo con la presencia de materia orgánica, lo que refleja la importancia de una correcta limpieza de los utensilios y superficies en las instalaciones de sacrificio. Su supervivencia se ve reducida en ambientes con baja actividad de agua, carentes de materia orgánica y también cuanto mayor es la temperatura y exposición a radiación ultravioleta. Finalmente hay que indicar *Salmonella* es capaz de formar bio-películas o biofilms (Marin et al., 2009), otra estrategia para su supervivencia en el medio ambiente. La formación de estos biofilms permite a la bacteria adaptarse a condiciones muy diversas, desde las que pueden acontecer en las células epiteliales del intestino a las del acero inoxidable en camiones de transporte o en el equipamiento del matadero. La formación de biofilms permite al patógeno resistir tanto a agentes ambientales adversos como a detergentes y desinfectantes así como a la desecación (Vestby et al., 2009).

6.4 El pienso como fuente de infección

La alimentación ha sido descrita como fuente de infección de *Salmonella*, siendo variable su impacto en diferentes países o regiones (Osterberg et al., 2006). En un informe realizado por la EFSA, se estimó que en el año 2005, en la UE, la proporción de materias primas de origen vegetal y destinadas a la producción animal contaminadas con *Salmonella* oscilaba entre el 0% y 6,7% (EFSA, 2006). La proporción de piensos de cebo contaminados en este mismo estudio varió entre el 0% y el 1,7%, si bien estos resultados deben interpretarse con cautela debido a la limitada representatividad de los muestreos. En un estudio realizado en

España, el 3,5% de las muestras de las materias primas resultaron positivas aunque solo una pequeña proporción, 0,3%, estaban contaminadas por serotipos de *Salmonella* con importancia en salud pública (Torres et al., 2011).

6.5 El papel del hombre y de otros hospedadores

El hombre también ha sido señalado como un vector que puede participar en la transmisión indirecta y la diseminación de *Salmonella* en las explotaciones porcinas (Berends et al., 1996). Se ha comprobado que en las explotaciones donde hay un mayor flujo de personal, existe también un mayor riesgo de presencia de *Salmonella* (Funk et al., 2001b). Igualmente, la implantación de medidas higiénicas relativas al correcto lavado de manos y normas de bioseguridad para el personal de la granja y los visitantes reducen el riesgo de presencia de *Salmonella* (Funk et al., 2001b; Lo Fo Wong et al., 2004).

Otras especies diferentes de los cerdos, tales como roedores, aves, insectos y animales domésticos, pueden participar, igualmente, en la introducción, diseminación y perpetuación de la infección en granja (Davies et al., 1997a; Murray et al., 2000). Hay que tener en cuenta que *Salmonella* es un microorganismo capaz de sobrevivir en un amplio rango de hospedadores, tanto de sangre caliente como de sangre fría (Grimont & Weill, 2007), que en muchos casos actúan como reservorio del patógeno (Wells et al., 2004; Foti et al., 2009) y que lo pueden transmitir a los cerdos bien directamente o indirectamente, a través de la contaminación del pienso o del agua de bebida (Harris et al., 1997). Los estudios en ratas y ratones han demostrado que estas especies pueden eliminar *Salmonella* en elevadas concentraciones, hasta 10^5 UFC/pellet de heces (Henzler & Opitz, 1992), habiendo estado ligados los serotipos encontrados en roedores con los de los animales domésticos (Barber et al., 2002). Del mismo modo, las aves pueden estar infectadas o ser portadoras de una gran diversidad de serotipos de *Salmonella* con prevalencias en las aves silvestres presentes en las explotaciones que pueden llegar al 50% (Craven et al., 2000; Barber et al., 2002). Los insectos, como las moscas, pueden actuar como vectores mecánicos de *Salmonella*, habiéndose aislado la bacteria de moscas en granjas con elevada prevalencia de infección (Letellier et al., 1999). Finalmente algunos animales domésticos como perros y gatos también pueden ser portadores dentro de las explotaciones (Evans & Davies, 1996; Funk et al., 2001a; Barber et al., 2002); especial mención merecen los segundos por alimentarse de roedores. Sin embargo, los gatos también pueden jugar un papel positivo al controlar los roedores y aves presentes en las granjas (Nollet et al., 2004).

7. Programas de Vigilancia y Control

7.1 Conceptos

La Organización Mundial de Sanidad Animal (OIE) define la vigilancia como "la investigación continua de una población dada y de su medio ambiente, para detectar cambios en la prevalencia de una enfermedad o en las características de un patógeno". La vigilancia implica la recogida y análisis sistemático de datos con la publicación regular de la información obtenida, de forma que permita la toma de decisiones en base a dichos resultados (Anon., 2010). Según Zepeda & Salmanl (2003) un programa de vigilancia y control debe estar amparado por un marco legal e incluir los siguientes elementos::

Definición del objetivo.

I. Selección del peligro.
II. Definición del caso.
III. Definición de la población en estudio.
IV. Definición de la muestra (tamaño muestral y método de muestreo).
V. Recogida y análisis de datos.
VI. Comunicación de los resultados.
VII. Definición del umbral al que se toman medidas de control.
VIII. Definición de las medidas de control.
IX. Definición de las estrategias de valoración y seguimiento.

Conjuntamente a los elementos citados, todo programa de control debe desarrollarse bajo las premisas de exactitud, precisión, rapidez y eficiencia (Thurmond, 2003). La exactitud viene determinada por el método de muestreo y el sistema elegido para realizar el diagnóstico (sensibilidad y especificidad). La precisión se refiere a la repetitividad y reproducibilidad de la técnica de diagnóstico, así como del esquema de muestreo. La rapidez hace referencia a la capacidad del sistema de vigilancia para detectar, comunicar e implementar medidas de control. Finalmente, la eficiencia incluye aspectos relacionados con el presupuesto y la relación coste-eficacia del sistema de vigilancia.

7.2 Programas de control y vigilancia

Como se indicó en el punto 1.2 de esta revisión, la UE sentó las bases para el control de *Salmonella* y otros microorganismos zoonóticos en el reglamento 2160/2003. Numerosos países han establecido programas de vigilancia de *Salmonella* en el ganado porcino basados en la recogida de información en tanto en granja como en matadero. Algunos de estos programas de vigilancia, además, incluyen estrategias de control para mitigar el riesgo de salud pública. A

continuación se recogen las bases de los programas de control de *Salmonella* en ganado porcino vigentes en la actualidad en la Unión Europea (Tabla 4).

7.2.1 Programas de erradicación

En Suecia, Finlandia y Noruega, la prevalencia de *Salmonella* en cerdos es muy baja. Según los resultados del estudio basal en cerdos de cebo llevado a cabo en la UE, la prevalencia estimada en ganglios linfáticos mesentéricos en cerdos de cebo en Suecia fue del 1% mientras que *Salmonella* no fue detectada en las muestra recogidas en Noruega o Finlandia (EFSA, 2008a). Estos países tienen en marcha programas erradicación de *Salmonella.* Dichos programas incluyen el muestreo en mataderos, recogiendo muestras de ganglios linfáticos mesentéricos y de canales. Adicionalmente se recogen muestras de heces a nivel de granja, en todas las fases de la producción (Bengtsson et al., 2009; Hofshagen et al., 2007; Huttunen et al., 2006) y cuando se detecta la presencia de la bacteria, independientemente del serotipo aislado, se ponen en marcha una serie de medidas. Las granjas afectadas son sometidas a restricciones que incluyen la prohibición del movimiento de animales, con excepción del transporte para sacrificio o sanitario. El ambiente y los animales son muestreados para la detección bacteriológica de *Salmonella*. Los cerdos infectados por *Salmonella* son sacrificados y destruidos y se aplican cuidadosos protocolos de limpieza y desinfección. Las restricciones son levantadas tras dos muestreos consecutivos de todos los animales de la granja en los que no se detecte *Salmonella*. A su vez, se realiza una investigación epidemiológica para determinar el posible origen de la infección.

7.2.2 Programas de control

La prevalencia de la infección por *Salmonella* en el ganado porcino impide la instauración de estrategias o programas de erradicación en la mayoría de los países industrializados, siendo substituidos por programas de vigilancia y control que reduzcan al máximo el riego para la salud pública. Existen dos estrategias bien diferenciadas en la vigilancia y control de *Salmonella.* Por una parte, en los EE.UU. esta vigilancia y el control de la salmonelosis porcina se realiza en el matadero mientras que la estrategia europea focalizó inicialmente sus esfuerzos en la producción primaria, en las granjas (Stark et al., 2002), habiendo evolucionado hacia una estrategia mixta que también incluye al matadero (Alban et al., 2012).

Programa de control en Dinamarca

El programa danés de control de *Salmonella* en ganado porcino fue puesto en marcha en el año 1995, a consecuencia del incremento de casos de salmonelosis humana relacionados con el consumo de cerdo en el año 1993. La base del programa fue descrita por Baggesen et al. (1996) y desde su instauración hasta el momento actual ha sufrido diversas modificaciones y actualizaciones (Baggesen et al., 1996; Alban et al., 2012). El programa danés incluye la realización de análisis en granja y matadero. Existe un programa de vigilancia serológica y bacteriológica de reproductoras y serológica de cerdos de cebo que se suma a la evaluación de los mataderos mediante el control bacteriológico de las canales.

Los análisis serológicos permiten clasificar las granjas con cerdas reproductoras en base a un índice que se calcula a partir del número de cerdos seropositivos en muestras de jugo de carne. Este índice se calcula mediante el número de muestras seropositivas obtenidas en los tres meses previos, empleando medias móviles (6:3:1). En aquellas granjas con un índice de 5 o superior, se realizan análisis bacteriológicos y, adicionalmente, se limita el movimiento de animales. Las granjas de engorde a las que se destina la progenie de esas explotaciones deben ser granjas con moderada o elevada contaminación por *Salmonella* (Anon, 2009). Por otra parte, todas aquellas granjas que producen más de 200 cerdos de cebo al año están incluidas en el programa de control. El número de sueros que debe ser recogido en estas explotaciones anualmente para el cálculo del índice serológico se establece en función del tamaño de granja (Tabla 4). Empleando los resultados obtenidos en los tres últimos meses (3:1:1), las explotaciones son clasificadas en tres niveles en función del porcentaje de cerdos seropositivos: baja prevalencia (< 40% de sueros positivos), prevalencia moderada, aunque aceptable (≥40% y <65% de sueros positivos) y prevalencia elevada (≥65%) (Alban et al., 2012). En las granjas de moderada y elevada prevalencia se recomienda la instauración de medidas de control y se impone una penalización económica del 2% del valor de la canal para las granjas de nivel 2 y de entre un 4% y 8% para las granjas de elevada prevalencia. Además en las granjas de nivel 3 es obligatorio llevar a cabo un sacrificio logístico, con transporte y alojamiento separado físicamente. Los cerdos son sacrificados al final de la jornada de matanza y es obligatoria la descontaminación con agua caliente de las canales (Alban & Sorensen, 2010). Los análisis bacteriológicos en cerdos de cebo fueron suprimidos del programa de control en 2011. Desde entonces, aquellas granjas altamente contaminadas tienen que demostrar la ausencia de *Salmonella* durante un periodo de 5 años (Alban et al., 2012).

Además del programa de vigilancia en granja, el programa de control danés analiza canales cada día de matanza en aquellos mataderos que sacrifican más de 200 cerdos al día. El número de canales analizadas varía en función del número de cerdos sacrificados. Las muestras no son individuales, sino que se componen de cinco canales cada una y son recogidas después de 12 horas de oreo. En los mataderos más pequeños se emplea una modificación de dicho sistema y se analiza una canal cada tres meses. Este sistema de vigilancia permite analizar las dinámicas de cada matadero mensualmente. En aquellos mataderos en los que el promedio de canales positivas supere el 2,2% en cuatro de los últimos seis meses analizados es obligatorio implantar un programa de control de *Salmonella* basado en la mejora de la higiene del proceso de sacrificio (Sorensen & Mogelmose, 2005).

Programa Irlandés

En 1997, se implantó en Irlanda un programa voluntario de control de *Salmonella* en cerdos (Quirke et al., 2001). El programa irlandés está basado en el análisis serológico de los animales empleando muestras recogidas en el matadero y, al contrario que en el programa danés, las granjas no son categorizadas en niveles de contaminación. Todas las granjas incluidas deben disponer de un programa de control de *Salmonella* y una prevalencia establecida. Se recogen muestras de suero de 6 cerdos cada mes y el estatus se determina por medio de la ponderación de los resultados de los últimos tres meses. Si la prevalencia se encuentra por encima del 50% en los tres últimos resultados, la granja pierde su situación de garantía de calidad lo que implica penalizaciones económicas. Estas granjas contaminadas pasan a ser monitorizadas por bacteriología y sus los cerdos se sacrifican al final del día y se mantienen en corrales de espera separados del resto de animales. Igualmente, el programa incluye muestreos en el matadero. Si la prevalencia en canales supera el 10%, se deben establecer las siguientes medidas: **I)** revisión profunda de su HACCP; **II)** aumento del muestreo en la línea de matanza; **III)** mejora de las medidas generales de higiene; **IV)** implementar medidas específicas para granjas con prevalencia superior al 50%; v) si las medidas en la línea no son eficaces, modificarlas o disminuir su velocidad; **VI)** tratamiento con calor de las partes más peligrosas de la canal.

Programa de control en Alemania

En el año 2002, se puso en marcha en Alemania un programa voluntario de control de *Salmonella* en producción porcina (Osterkorn *et al.,* 2001; Blaha, 2004), impulsado por la industria alimentaria alemana y denominado "QS *Salmonella* Monitoring Programme". Al igual que los anteriores, este programa de control está basado en la monitorización serológica de

los animales en el matadero, en este caso a través de la recogida de 60 muestras de jugo de carne de cada explotación. El punto de corte utilizado en el Mix-LPS-ELISA es de un 40% de DO y la clasificación de las explotaciones en función de su categoría de riesgo es la siguiente: categoría I o de bajo riesgo con menos del 20% de animales positivos, categoría II o de riesgo medio, entre el 20% y el 40% de animales positivos y categoría III o de alto riesgo con más del 40% de muestras positivas. Entre las medidas asociados al programa de control está el sacrificio separado de los animales procedentes de explotaciones clasificadas en la categoría III, así como facilitar a los productores herramientas para la reducción de la contaminación por *Salmonella* en las explotaciones de las categorías II y III. Respecto a esta última medida, es importante señalar que cada vez son más los ganaderos que solicitan la ayuda de los servicios veterinarios para que les asesoren sobre cómo mejorar la higiene y la bioseguridad de la explotación con el objeto de volver a englobarse en la categoría I o, al menos, de evitar la categoría III.

Programa de control británico

En el año 2002, en Gran Bretaña por iniciativa de la industria se implantó el programa denominado "Zoonosis Action Plan", conocido por la abreviatura ZAP. Cada granja incluida en el programa realiza tres análisis serológicos de 15 muestras en meses consecutivos. Las explotaciones se clasificaban en función de su seroprevalencia, empleando un punto de corte del 40% DO, en granjas con baja seroprevalencia o ZAP1 (< 50% de seropositivos), en las que no se requiere ningún plan de acción, en granjas de seroprevalencia moderada (50-75%) o ZAP 2, en las que deben tomarse medidas que permitan su paso a ZAP 1 en un periodo de 17 meses y en granjas con elevada seroprevalencia (≥ 75%) o ZAP 3, en las que debe desarrollarse un plan de acción para la reducción de *Salmonella* que permita su vuelta a la categoría ZAP 1 en un periodo inferior al año (11 meses).

En el año 2008 se introdujo un nuevo programa en el Reino Unido, "Zoonoses National Control Programme" o ZNCP, diseñado para reducir la seroprevalencia en granja. Este programa es similar al ZAP pero el punto de corte se ha reducido hasta el 10% de OD. Las granjas no se categorizan por niveles y todas ellas deben disponer de un programa de acción frente a *Salmonella*, estimándose su prevalencia en función de los resultados proporcionados por las muestras recogidas en los 12 meses inmediatamente anteriores (Snary et al., 2010).

Programa holandés

En febrero de 2005 comenzó el programa de control de *Salmonella* holandés que se basa en la categorización de las explotaciones de cerdos de cebo en función de su seroprevalencia. Las granjas se clasifican empleando muestras recogidas en la granja (tres semanas antes del sacrificio) o en el matadero (el día del sacrificio). Se toman un total 36 muestras por año en cada explotación, 12 por trimestre, y se emplea un sistema de clasificación semejante al del programa alemán. Además, el programa holandés incluye muestreos aleatorios de la superficie de las canales, un total de 10 muestras cada dos semanas y en cada matadero.

Programas de Control en otros países europeos

En Austria se han implementado programas regionales de control que proporcionan una buena base para la instauración de un programa nacional de control de *Salmonella*. En otros países europeos como Bélgica o Francia los programas de control se han limitado a estudios piloto.

7.2.3 Avances sobre el control en España

Actualmente, no existe todavía un programa de control de *Salmonella* en el ganado porcino en España. Teniendo en cuenta las directrices marcadas por la UE en el reglamento 2160/2003, dicho programa debería instaurarse en un futuro próximo, al igual que han sido puestos en marcha los programas de gallinas reproductoras y ponedoras, de pollos de engorde y de pavos (Anon., 2012a; Anon., 2012b; Anon., 2012c; Anon., 2012d).

Pese a que no hay un programa nacional de control, se han realizado diversos estudios con el fin de determinar la prevalencia de *Salmonella* en ganado porcino. El primer estudio publicado a este respecto, fue el realizado por Mejía y colaboradores cuyo objetivo fue determinar la prevalencia de *Salmonella* en Cataluña a través de un muestreo que incluyó 113 explotaciones de engorde y 74 de reproductoras (Mejía et al., 2006). *Salmonella spp.* fue aislada en el 20% de las granjas de engorde y el 24% de las de reproducción. Los valores de seroprevalencia corroboraron los resultados bacteriológicos y se detectaron animales seropositivos en el 69% de las explotaciones, con un 26% de granjas con el 50% o más de cerdos seropositivos. Al año siguiente se publicó un estudio de prevalencia, completado con resultados de resistencia antimicrobiana, en Andalucía (Astorga et al., 2007), detectándose un 33% de granjas positivas, y el primer trabajo de prevalencia bacteriológica a nivel nacional en el que un 43,1% de las granjas de cebo analizadas resultaron positivas a *Salmonella* (Garcia-

Feliz et al., 2007). Este último dato quedó refrendado por el estudio de la EFSA desarrollado al año siguiente en todos los países miembros de la UE. En dicho estudio, España ocupó el primer lugar siendo el 29% de las muestras de ganglios linfáticos mesentéricos de cerdos de cebo recogidas en el matadero positivas (EFSA, 2008a). En lo que respecta a las granjas de cerdas reproductoras, al igual que en los cerdos de cebo, España ocupó el primer puesto de prevalencia bacteriológica en el estudio basal de la UE con un 64% de las granjas de multiplicación (150 de las analizadas) y un 53% de las de producción (209) positivas a *Salmonella.* Finalmente, en 2011 se publicó el primer trabajo específico de granjas de cerdo Ibérico en el que se estimó una prevalencia del 33% (Gómez-Laguna et al., 2011), un dato similar a los descritos en cerdo blanco. Respecto a los resultados de análisis serológicos, no existen estudios a nivel nacional. Aparte del trabajo de Mejía y colaboradores ya comentado en Cataluña, existen otros dos trabajos centrados en las regiones de Castilla y León y Andalucía respectivamente (Collazos, 2008; Hernández et al., 2013a). En Castilla y León la proporción de explotaciones de cebo seropositivas, al menos un suero positivo, fue del 85 % cuando se empleó un punto de corte de 40 % DO y del 95 % para un punto de corte del 20 % DO. En cerdos Ibéricos, Hernández et al. (2013a) detectaron anticuerpos específicos frente a *Salmonella* en un 73% de las explotaciones porcinaspositivas a *Salmonella*.

Aparte de estos estudios de prevalencia, se han realizado investigaciones para conocer y controlar la naturaleza de la infección, entre los que se incluye un estudio de factores de riesgo en cerdos de cebo (García-Feliz et al., 2009), un pequeño trabajo sobre la transmisión vertical en granjas de producción en puntos múltiples (Arguello et al., 2010), un estudio sobre el control de la infección mediante acidificantes (Creus et al., 2007) y probióticos (Collazos, 2008), varios estudios sobre resistencias a antimicrobianos en aislados de *Salmonella* de cerdos de cebo (Astorga et al., 2007; García-Feliz et al., 2008), estudios de tipificación de cepas de campo (Vidal et al., 2005), un estudio sobre la eficacia de los sistemas de limpieza y desinfección de granjas de cebo, camiones y corrales de espera (Argüello et al., 2011) y recientemente el primer trabajo centrado en las fases de transporte y matadero de cerdos Ibéricos (Hernández et al., 2013b).

Toda esta información demuestra la existencia de una base sólida de conocimiento del problema asociado a las infecciones por *Salmonella* en el sector porcino en España, con gran cantidad de información disponible para la instauración de un programa de control a nivel nacional. Este programa será obligatorio de acuerdo a los reglamentos europeos pero también muy necesario dada la importancia del sector porcino y de sus exportaciones en nuestro país y los resultados de los estudios de prevalencia comentados.

PLANTEAMIENTO Y OBJETIVOS

Calendario Agrícola Panteón de los Reyes de Léon. Siglo XII (San Isidoro, León, España).

Mes de mayo. Comienza la guerra. Representa al soldado con el caballo y escudo.

El grupo de investigación en infecciones digestivas del ganado porcino de la Universidad de León (DIGESPOR) ha desarrollado diversas investigaciones relativas a las infecciones por *Salmonella* en los cerdos desde el año 2001, participando en proyectos a nivel regional, nacional e internacional que han permitido presentar tres tesis doctorales. La primera de las tesis realizadas abordó aspectos relacionados con la puesta a punto y evaluación de las técnicas de diagnóstico bacteriológico y de tipificación de aislados (Vidal, 2005). En una segunda etapa, se incluyeron estudios relacionados con las técnicas de diagnóstico serológico y el control de la infección a través del empleo de probióticos (Collazos, 2008). Finalmente, la última de las tesis realizadas hasta el momento se centró en el estudio de la prevalencia, serotipos y factores de riesgo en las explotaciones de cerdos de cebo de España así como en la evaluación de la resistencia antimicrobiana de los aislados de origen porcino (García-Feliz, 2011).

Con estos antecedentes, el objetivo general de la presente tesis se ha dirigido hacia el incremento del conocimiento relativo a la epidemiología y el control de la infección por *Salmonella* en el ganado porcino. La tesis se desarrolla en tres capítulos que abarcan las explotaciones de reproductores, el control de la infección en granjas de cebo y las etapas posteriores a la granja: transporte, espera y sacrificio. En cada uno de los capítulos se incluye una introducción específica (en dos de los capítulos esta introducción ha sido publicada, como artículo de revisión y como capítulo de libro respectivamente) así como las publicaciones correspondientes a las investigaciones desarrolladas.

No existía información sobre la situación de las granjas de reproductoras ni sobre los mataderos en España ya que la mayoría de las investigaciones se habían centrado en el engorde donde se había reportado una alta prevalencia (García-Feliz et al., 2007; EFSA, 2008a). Por ello, decidimos investigar la prevalencia y los factores de riesgo asociados a la infección en granjas de reproductoras con el fin de conocer el impacto de la infección en esta etapa e identificar posibles actuaciones que faciliten su instauración o que, por el contrario, pudieran ser de utilidad para su control. Además, conocida la elevada prevalencia de la infección en las explotaciones con cerdos de cebo, decidimos valorar algunas estrategias de control en esta fase. Finalmente, consideramos relevante analizar las etapas posteriores a la granja, con el fin de estimar la frecuencia de la contaminación de las canales porcinas, los serotipos implicados, las fuentes de infección en el matadero y evaluar posibles medidas correctoras para reducir la contaminación de la carne de cerdo por *Salmonella.*

De una forma más específica los objetivos fijados en cada uno de los capítulos fueron los siguientes:

Capítulo I

1. Determinar la prevalencia bacteriológica y serológica de la infección por *Salmonella* en las explotaciones porcinas de reproductores en España así como los serotipos y serogrupos circulantes.
2. Identificar factores de riesgo para la infección por *Salmonella* en explotaciones porcinas de reproductores en España.

Capítulo II

3. Investigar la eficacia de una administración estratégica de ácidos, en agua o en pienso, durante la última parte del periodo de cebo, para minimizar la infección por *Salmonella* en la granja y, como consecuencia, el riesgo de enviar cerdos infectados al matadero.
4. Investigar la eficacia de una vacuna inactivada de *Salmonella* Typhimurium aplicada al inicio del cebo para prevenir o reducir la infección por *Salmonella* en la granja y, como consecuencia, el riesgo de enviar cerdos infectados al matadero.

Capítulo III

5. Determinar la prevalencia de la contaminación por *Salmonella* en canales porcinas así como los serotipos implicados y las principales fuentes de contaminación por *Salmonella* para estas canales.
6. Determinar el efecto que las fases posteriores a la granja: transporte, espera y sacrificio tienen en la infección o en la contaminación de los cerdos y de sus canales.
7. Identificar potenciales puntos críticos de control en los mataderos que pudieran ser de utilidad para el control.
8. Investigar la eficacia de un sistema de sacrificio logístico organizado en función de resultados serológicos para prevenir o reducir la contaminación por *Salmonella* de las canales porcinas.

CAPÍTULO I / CHAPTER I

SALMONELLA EN REPRODUCTORAS

SALMONELLA IN BREEDING PIGS

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Mes de octubre. El porquero está alimentando a dos pequeños lechones.

1. Introduction

1.1. The role of the breeding pigs in swine salmonellosis

Focused in food safety, the control of *Salmonella* must be carried out taking into account the philosophy "*from farm to fork*" which implies the participation of all sectors involved throughout the food chain. Due to the complexity of its epidemiology and ecology, the control of *Salmonella* is a great challenge. As it has been proposed by Davies et al. (2004), the food supply should be seen as a linear series of sectors engaged in production, harvest, distribution and consumption and the goal of control programmes is to define the optimal combination of interventions at each sector that delivers the maximum risk reduction at minimal cost.

There is some public debate regarding the role of the breeding pigs in swine salmonellosis and their impact in the final pork contamination. Vertical transmission is frequent in poultry production where the infected laying hens transfer the *Salmonella* to their progeny (Berchiery et al., 2001). Nevertheless, the vertical transmission neither during the pregnancy nor during the suckling seems to be common in swine. This idea is supported by several studies which did not find any relation among the prevalence and strains detected in breeding and finishing pigs concluding that vertical transmission is not the main source of *Salmonella* in finishers (Berends et al., 1996; Stege et al., 2000; Funk et al., 2001a; Arguello et al., 2010; Wilkins et al., 2010). In contrast, a limited number of studies have reported the transmission of the pathogen from infected sows to their offspring. Letellier et al. (1999) described the relationship between the *Salmonella* strains from the growing and finishing pigs and those from the sows. Similarly, Kranker et al. (2003) found a positive correlation among the seroprevalence in breeding herds and the faecal shedding in weaned pigs. Finally, in a risk factor study, frequent removal of dung during lactation was associated with a lower prevalence of *Salmonella* in finishing pigs (Beloeil et al., 2004).

Regarding the prevalence of *Salmonella* infection in sows, a number of studies have demonstrated that the faecal shedding is higher in breeding pigs than in finishing pigs (Merialdi et al., 2008; EFSA, 2009a; Christensen et al., 2002; Wilkins et al., 2010; Arguello et al., 2013). A few studies have focused on the pattern of *Salmonella* infection in sows. According to Funk et al. (2001a), shedding increases during the last part of the gestation. In contrast, an increase in *Salmonella* shedding after weaning and more frequently in sows with more than five offspring was reported by Nollet et al. (2005). These results suggest that probably there is not a unique pattern of excretion for *Salmonella* infection in breeding farms.

1.2. Risk factors associated with *Salmonella* infection in swine

One of the greatest challenges in the control of *salmonella* is the identification of effective interventions and control measures that can be taken at the herd level. Therefore, management strategies and production processes that can increase the risk of introduction, transmission and maintenance of *Salmonella* need to be investigated and their rectification is of paramount importance (Lo Fo Wong et al., 2004). Most of the risk factors research has been focused on finishing farms (Berends et al., 1996; Van der Wolf et al., 1999; 2001b; Leontides et al., 2003; Lo Fo Wong et al., 2004; Nollet et al. 2004; Rajic et al 2007; Hautekiet et al. 2008; García-Feliz et al. 2009) or farrow-to-finish herds (Beloeil et al., 2004; 2007), while until the baseline study performed in the EU according to the EFSA guidelines (EFSA, 2009a; 2011c), only one study was focused on breeding pigs (Kranker et al., 2001). More recently, an analysis from the data obtained during the EFSA baseline study in breeding pigs at the EU level (EFSA, 2011b) as well as two Portuguese studies have been published regarding risk factors for *Salmonella* in breeding pig farms (Correia-Gomes 2012; 2013).

In the present introduction a brief review of the main risk factors described by the different studies performed in the last two decades will be pointed out.

1.2.1 Feed

The feed plays a double role in the epidemiology of *Salmonella* in swine production. On the one hand it can be the source of infection while on the other it can also be a protective factor. The role of some feed interventions such as the addition of acidic compounds in the water or feed in the control of swine salmonellosis will be address in chapter II, where the control measures at finishing farms will be discussed in depth.

Salmonella can be isolated from both pelleted and non-pelleted feed as well as wet and dry feed. However, it is seldom isolated from feed stuffs when leaving the feed mills being generally contaminated during the transport and storage at the farm level (Fedorka-Cray et al., 1997; Lo Fo Wong et al., 2004).

In a relevant number of risk factor studies, the use of pelleted feed has been associated with the faecal shedding of *Salmonella* while a protective effect was associated to the consumption of non-pelleted or coarsely ground meal (Kranker et al., 2001; Van der Wolf et al., 2001b; Leontides et al., 2003; Beloeil et al., 2004; Lo Fo Wong et al., 2004; Nollet et al., 2004; Rajic et al., 2007;; Hautekiet et al., 2008; García-Feliz et al., 2009). The pelleting and heat-treatment procedures reduce the *Salmonella* contamination in compound feed. However, it has been proved that non-pelleted feed results in a microbiological ecosystem that provides

Salmonella with poor growing conditions compared to pelleted feed. Non-pelleted feed is linked with a slower gastric passage rate together with a higher porridge-like consistency of the stomach content, factors which favour the microbial fermentations in the stomach. Moreover, coarsely-ground meal might not be digested as well as finely ground pelleted feed and at least part of the carbohydrates will be fermented in the large intestine (Mikelsen et al., 2004). As consequence, the growth of the acid-lactic microbiota is promoted and volatile fatty acids concentration is increased creating a hostile environment for *Salmonella* (low pH, organic acids, competitive exclusion etc.).

The use of liquid feed and fermented liquid feed has been analyzed by most of the risk factors studies (Van der Wolf et al., 2001b; Lo Fo Wong et al., 2004) and even there is a specific risk factor study focused on the use of liquid feed (Farzan et al., 2006). The protective effect of liquid-feeding on *Salmonella* can be a result of the reduced pH and the presence of lactic and short-chain volatile fatty acids produced by the fermentations of the large amount of lactic-acid producing bacteria and yeasts present (Prohaszka et al., 1990; van Winsen et al., 2002). Many studies have reported a decreased risk for *Salmonella* infection when wet feed is used (Van der Wolf et al., 1999, 2001b; Beloeil et al., 2004; Lo Fo Wong et al., 2004). However, the only risk factor study at the country level in Spain failed to demonstrate this association because few fattening farms used this type of feed (García-Feliz et al., 2009). It is important to remark that liquid-feeding systems are expensive and only economically profitable at large size farms.

1.2.2. Size

The size is a complex factor in which many other factors can also be involved. Apart from the number of pigs, the size is affected by other variables such as the number of barns, the number of compartments per barn and the number of pens per compartment as well as the density of pigs or the number of caretakers (Funk et al., 2001b). All these factors can be associated with the presence of *Salmonella.* The large number of variables involved could be the reason why the results from risk factor studies are contradictory regarding the analysis of the farm size. In the research performed by Van der Wolf et al. (2001b), low size farms (lower than 800 finishing pigs) reported high *Salmonella* seroprevalence while other studies have reported a higher risk of infection by *Salmonella* in herds with higher sizes (Cartensen & Christensen, 1998; Farzan et al., 2006; Hautekiet et al., 2008; García-Feliz et al., 2009). Other studies have not found any association between the size and the risk of *Salmonella* (Lo Fo Wong et al., 2004; Rajic et al., 2007).

1.2.3 Herd management

All-in/All-out

The introduction of all-in/all-out flow (AIAO) facilitates the disruption of transmission among production stages and consecutive reared batches. The instauration of AIAO implies the cleaning and disinfection of the facilities together with a period of time during which the compartment/barn/farm is kept empty. This flow management cannot prevent the establishment of the infection but can be useful to prevent the contamination between consecutive batches of reared pigs as well as to decrease the proportion of *Salmonella* carriers. Several studies have linked the AIAO flow with a lower *Salmonella* prevalence (Beloeil et al., 2004; Lo Fo Wong et al., 2004; Farzan et al., 2006; Hautekiet et al., 2008) while in others no differences were detected among farms performing AIAO and those which do not use this management (Davies et al., 1997b; Nollet et al., 2004; Rajic et al., 2007; García-Feliz et al., 2009).

Farrow-to-finish/Multi-site-production

The effect of the type of production has been evaluated in several studies. According to Rajic et al. (2005), barns from farrow-to-finish farms had lower odds of *Salmonella*-positivity than those from multisite or individual grow-to-finish farms. Davies et al (1997a) also reported that in North Carolina the proportion of *Salmonella* positive farms was lower among farrow-to-finish farms than among multisite operations with AIAO pig flow of finishing pigs.

Cleaning and disinfection

Theoretically, the combination of cleaning and disinfection of the facility between groups of pigs and the segregation of age groups decreases the potential for *Salmonella* exposure and infection of new arrivals. It has been generally accepted, although rarely demonstrated, that improved hygiene due to the implementation of strict cleaning and disinfection protocols together with AIAO pig flow reduces *Salmonella* contamination within the farm environment and the pig population (Funk et al., 2004). In this study, no significant difference in *Salmonella* shedding was observed among farms that do not clean those that only scrap the pens and those that use pressure washing with or without disinfection. Similarly, Belgian researchers investigating herd-risk factors on 62 farrow-to-finish farms did not find any association between any of the evaluated hygiene measures and the presence of *Salmonella* (Nollet et al., 2004). Thus, there is a need to evaluate the effectiveness of the current cleaning, disinfection and pig flow practices in terms of *Salmonella* control.

Biosecurity

The role of biosecurity in the control of *Salmonella* has been already mentioned. Therefore we will only include several results regarding risk factors analysis.

Vectors are an important source of *Salmonella* contamination in swine herds and have been included in most of the questionnaires performed within risk factors studies. For instance, the presence of cats was associated with the presence of *Salmonella* (Nollet et al., 2004) although it was a protective factor in another study (Funk et al., 2001b). The lack of control measures to prevent the entrance of birds in the facilities has also been associated with *Salmonella* seropositivity (Bahnson et al., 2006). In contrast other studies have not found any association between the control of vectors such as birds or rodents and *Salmonella* (Beloeil et al., 2004).

The role of caretakers and visitors has also been analyzed. Several studies have stressed the role of personnel entering the farms in the spreading of the infection (Berends et al., 1996; Funk et al., 2001b). Furthermore, biosecurity aspects related to visits, such as the presence of toilets to wash hands (Funk et al. 2001b; Lo Fo Wong al., 2004) or the use of footbaths at the entrance of the buildings (Hausekiet et al., 2008) have been associated with a reduction in the *Salmonella* seroprevalence.

The high sanitary status seems to be a protective factor according to a number of risk factor studies (Kranker et al., 2001; Lo Fo Wong et al., 2004). The presence of diarrhoea has been associated with a higher risk of *Salmonella* excretion (Van der Wolf et al., 2001b) while Beloeil et al. (2007) reported an increased *Salmonella* seropositivity in those farms suffering from diseases such as porcine proliferative enteritis or respiratory and reproductive syndrome (PRRS). Finally, in the same study the participation in an integrated Quality Control production system was associated with a decreased risk of *Salmonella* infection.

Facilities

Slatted floor minimizes the contact with faeces reducing the risk of transmission of gastrointestinal diseases and therefore it has been identified as a protective factor by several studies (Davies et al., 1997a; Davies et al., 1997b; Funk & Gebreyes, 1994; Nollet et al., 2004). In contrast, other studies did not found any association between the type of floor and the presence of *Salmonella* (Van der Wolf et al., 2001b; Lo Fo Wong et al., 2004; Rajic et al., 2007; García-Feliz et al., 2009).

The drinkers design can also have some influence. In the study performed by Bahnson et al. (2006) the use of nipple drinkers was associated with a lower risk compared to bowl drinkers which can be easily contaminated by faeces.

Pen separation determines the pen to pen transmission; if pen walls are solid and high enough, the nose to nose contact can be avoided and therefore *Salmonella* transmission can be limited (Dahl et al., 1996). This idea is supported by another risk factor study (Rajic et al., 2007).

The presence of a clothe-change facilities and the supply of farm clothes and boots for caretakers and visitors was associated to a lower *Salmonella* seroprevalence in Denmark (Lo Fo Wong et al., 2004) and Belgium (Hautekiet et al., 2008). However, this association could not be evidenced in a study performed in the Netherlands (Van der Wolf et al., 2001b).

2.4 Season

Salmonella infection seems to be clearly affected by seasonality and theoretically, higher prevalence would be expected in warm months, during the summer. Nevertheless, there is some variability among the studies which have analyzed this factor. Higher prevalence was found in winter and spring months in one study (Funk et al., 2001b), in autumn and winter in another one (Christensen & Rudemo, 1998) and during summer months in a third (Hautekiet et al., 2008). The results of the baseline study in European finishing pigs (EFSA, 2008b) are another example of variability of these results. *Salmonella* prevalence in mesenteric lymph nodes was affected by the season but this relationship was also influenced by the country. The effect of this factor is probably modified by the temperature regulation within the pig facilities. According to this, the regulation of temperature at adequate ranges for pig rearing was a protective factor for *Salmonella* infection in swine farms (Funk et al., 2001b; Hautekiet et al., 2008).

Pigs replacement policy

Lo Fo Wong et al. (2004) reported an increased *Salmonella* seroprevalence in those finishing farms purchasing pigs to three or more suppliers. Similarly, Gilt replacement was a significant factor in the global EU data (EFSA, 2011b). According to Davies et al. (2000b), transport can increase the *Salmonella* shedding in purchased gilts. These results demonstrate that checking relevant infectious diseases, including *Salmonella* status is of paramount importance when new pigs are purchased in herds for breeding purposes.

1.3. Risk factors in breeding herds

Until the baseline cross-sectional study performed in the EU (EFSA 2009a; 2011b), there was only one specific study focused on the evaluation of risk factors in breeding herds. In this study, ready mixed pelleted feed and health status were associated with higher odds of *Salmonella* infection (Kranker et al., 2003). The baseline survey on *Salmonella* in breeding pigs within the EU was performed through 2008 and the analysis of the data collected in breeding farms revealed that the presence of *Salmonella* in faecal pooled samples was associated with the size of the herd, with the density or the number of pigs per pen, with the type of floor and with the pig replacement policy (Table 1).

Finally, two new risk factors studies have been published using the data of the baseline study for *Salmonella* in breeding pigs in Portugal. One of the studies analysed the risk factors using a generalised linear mixed model (Correia-Gomes et al., 2013) and concluded that *Salmonella* shedding was more frequent in mating pens as compared with maternity pens, in farms using feed from external or mixed source as compared with those using home-made feed, in those farms without a rodent control program, with more than 90% of boars from an external source or using semen from insemination centres and in farms with a size of 170 or more sows. The same data was analysed using a Bayesian hierarchical model taking into consideration the serotype involved in the infection (Correia-Gomes et al., 2012). *Salmonella* was linked to the age of the sow, the source of semen, the size of the herd and the number of pigs per pen in *S.* Typhimurium infected herds while control of rodents, region of the country, source of semen, breeding sector room and source of feed were the significant associations found in those farms infected by other *Salmonella* serotypes different from *S.* Typhimurium.

Risk factor[a]	Comparison	OR[b]	95% CI		DF[c]	*P*-value
SAME EFFECT IN BREEDING AND PRODUCTION HOLDINGS						
Holding size	100-399 vs <100	1.84	1.34	2.53	3	<.0001
	400-999 vs <100	3.23	2.26	4.62		
	>999 vs <100	5.25	3.48	7.93		
Number of Pigs in Pen	Per 10 pigs	1.03	1.00	1.06	1	0.0230
Production stage	Pregnant vs Maiden gilts	0.81	0.67	0.96	4	<.0001
	Farrowing and lactating vs Maiden gilts	0.65	0.53	0.80		
	Service area vs Maiden gilts	1.06	0.86	1.30		
	Mixed vs Maiden gilts	0.86	0.67	1.10		
Origin of feed	Other vs Commercial compound	0.51	0.27	0.97	3	0.0001
	Feed with maize vs Commercial compound	0.62	0.36	1.04		
	Home-mill vs Commercial compound	0.58	0.45	0.74		
Type of diet	Others vs Cobbs/rolls/nuts/pellets	0.67	0.43	1.05	3	<.0001
	Meal/mash vs Cobbs/rolls/nuts/pellets	0.52	0.41	0.67		
	Porridge/liquids vs Cobbs/rolls/nuts/pellets	0.46	0.34	0.62		
DIFFERENT EFFECT IN BREEDING AND PRODUCTION HOLDINGS						
BREEDING HOLDINGS						
Floor type	Outdoors in fields or paddocks vs Slatted floor	1.18	0.57	2.45		1)
	Solid floor other bedding vs Slatted floor	1.83	0.43	7.84		
	Solid floor with straw vs Slatted floor	0.63	0.38	1.03		
	Solid floor without bedding vs Slatted floor	1.30	0.77	2.20		
	Partly slatted floor vs Slatted floor	1.01	0.76	1.35		
	Other vs Slatted floor	3.82	1.21	12.10		
Gilt replacement policy	10-90% Gilts homebred vs >90% Gilts homebred	0.32	0.18	0.58		2)
	>90% Gilts purchased vs >90% Gilts homebred	0.76	0.52	1.10		
PRODUCTION HOLDINGS						
Floor type	Outdoors in fields or paddocks vs Slatted floor	3.32	2.04	5.39		1)
	Solid floor other bedding vs Slatted floor	2.37	1.08	5.19		
	Solid floor with straw vs Slatted floor	1.12	0.75	1.67		
	Solid floor without bedding vs Slatted floor	2.07	1.49	2.89		
	Partly slatted floor vs Slatted floor	1.43	1.15	1.77		
	Other vs Slatted floor	1.04	0.40	2.68		
Gilt replacement policy	10-90% Gilts homebred vs >90% Gilts homebred	1.37	0.96	1.95		2)
	>90% Gilts purchased vs >90% Gilts homebred	1.15	0.90	1.46		

[a] Odds ratio estimates and standard errors were assessed using a mixed-effects model with the effect of holdings included as a random intercept and with the factor 'country' included as a fixed effect. The between holding variance (on the log-odds scale) is 4.20 with a 95% confidence interval of [3.79; 4.67]. Both the holding (random intercept) and the country effects were statistically significant (*P*-value<0.0001). Single country effects are not shown.

[b] All odds ratios were adjusted for the factor 'sample type', which was also retained in the final model.

[c] DF=degrees of freedom.

1) Type III *P*-value of the of the main effect: 0.0002 (6 DF); type III *P*-value of the interaction: 0.0001 (6 DF)

2) Type III *P*-value of the of the main effect: 0.078 (2 DF); type III *P*-value of the interaction: 0.0198 (2 DF)

Table 1. Final logistic mixed-effects model (a) for factors associated with *salmonella* positive pens including interaction terms. Extracted from the Salmonella EU baseline survey, 2008.

2-. Publications included in Chapter 1

Publication 1. *Salmonella* in Spanish breeding pigs, prevalence and risk factors associated.

2.1 Publication 1

Status: **Submitted for publication**

Journal: **BMC Veterinary Research**

Salmonella in Spanish breeding pigs, prevalence and risk factors associated

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ABSTRACT

Salmonella is one of the major concern food-borne pathogens worldwide. It has been identified at all the stages of pork production and control programs for reducing the prevalence of *Salmonella* in pig primary production will be soon compulsory at the European Union according to the regulation EC No 2160/2003. The data provided by the cross-sectional study in breeding pigs performed in the EU was an excellent opportunity to analyse the prevalence, serotypes and risk factors for *Salmonella* infection in Spanish breeding pigs.

Through 2008, ten pooled-faecal samples and ten blood samples were collected from 364 pig herds with breeding pigs. The results obtained confirmed the high spread of *Salmonella* in the pig population of the country. Apparent bacteriological herd prevalence was 58.2% (95% CI: 52.9-63.3). Using serological data at 40% OD cut-off at least one positive sera was detected in 76.6% of the farms (95% C.I: 71.8-80.9) while at 20% OD cut-off the proportion of herds with seropositive individuals was 87.3% (95% C.I: 83.9-90.7). *S.* Rissen and *S*. Typhimurium were the two main serotypes detected and six of the ten most prevalent serotypes were shared with those previously reported in Spanish finishing pigs.

Risk factors for *Salmonella* shedding identified by multivariate analysis included the use of pelleted feed (OR=1.7; 95%CI: 0.989-2.783) as well as the purchasing of the boar replacement (OR=1.8; 95%CI: 1.068-2980). According to serological data, herds were classified into three categories by two different schemes and using 40% DO and 20% OD as cut-offs. Multivariate analysis was performed using ordinal logistic regression and the same variables were retained in the model regardless of the classification system used. Seroprevalence varied among trimesters, being higher in autumn and winter. The risk of *Salmonella* contamination was higher in those farms using individual housing of pigs while the presence of fully or partially slatted floors was associated to a lower seroprevalence.

Keywords: *Salmonella, breeding pig, prevalence, risk factors*

1. INTRODUCTION

Food-borne diseases are of major concern in developed countries. Among the diseases embraced, human salmonellosis is in the European Union (EU) the second most prevalent food-borne disease in number of cases and the first in number of outbreaks, according to the last report published by EFSA (EFSA, 2013). Pork and pork products are one of the main sources of human contamination (EFSA, 2013) and a recent survey reflects the increase of their relative importance, consequence of the success in the reduction of *Salmonella* prevalence in avian production (Pires et al., 2011).

At the level of the EU and according to the regulation EC No 2160/2003, the control of *Salmonella* must be performed under the philosophy "from farm to fork" which implies the participation of all the sectors involved throughout the food chain, particularly the primary production, in order to reduce the prevalence and the risk to the public health. This regulation established that community targets have to be set up for the reduction of the prevalence in broiler chickens, layer hens, turkeys and pig primary production. In a preliminary step, baseline studies were conducted across the EU to determine the prevalence of *Salmonella* in each of these populations and national control programs have been approved for breeding flocks of *Gallus gallus*, laying hens, broilers and turkeys.

Although two baseline surveys were performed in pig primary production to determine *Salmonella* prevalence in slaughtered and breeding pig populations (Commission Decisions 2006/668/EC and 2008/55/EC), the reduction targets have not been defined and the control programs are still not compulsory for pig farms. Consistent with Van der Wolf et al. (2001), programs to reduce *Salmonella* in pork and pork products should include monitoring and interventions at the farm. According to this, risk factors for *Salmonella* infection in pigs have to be determined. By the identification of proper farm procedures related to husbandry, farm design characteristics or production management, adequate actions to reduce pig prevalence can be implemented. A number of studies have pointed out risk factors for *Salmonella* infection in finishing farms (Berends et al., 1996; van der Wolf et al., 1999; 2001; Lo Fo Wong et al., 2004; García-Feliz et al., 2009). Other studies have reported risk factors in farrow-to-finish farms (Stege et al., 2001; Beloeil et al., 2004; 2007) but there is few information regarding *Salmonella* risk factors in breeding pigs (Kranker et al., 2001; Correia-Gomes et al., 2012; 2013).

The present study aims to investigate prevalence, serotypes and herd-level risk factors for *Salmonella* infection in breeding pig farms using the data obtained during the baseline

survey performed in Spain, that included the compulsory analysis of faecal samples as well as additional blood samples.

2. MATERIALS AND METHODS

2.1. Study design

The survey took place between January and December 2008 and followed the technical specifications described in the Commission Decision 2008/55/EC. Farms with breeding pigs were the unit of study and were classified either as breeding herds, those selling gilts or boars for breeding purposes, and production herds, those selling pigs for fattening or slaughtering. Two population frames were established by including all the holdings with more than fifty breeding pigs within the country, a total of 415 breeding herds and 12,449 production herds, respectively. These farms embraced more than 80% of the Spanish breeding pig population. The number of farms to be sampled within each category was determined using the correction for a finite population, with 50% as the expected prevalence of *Salmonella* positive farms with a 95% CI and an absolute error of 7.5%. Estimated sample sizes were increased by 10% to account for non-response as indicated in the Commission Decision 2008/55/EC. Farms were selected using a proportional random stratified approach according to the farm size (50-99, 100-399, 400-999 and more than 999 pigs per herd) and administrative regions (17 different regions).

Within each herd, ten faecal samples (a pool of approximately 25 g of fresh faecal material) from ten randomly selected pens and ten blood samples from randomly chosen animals were collected. This sample size allowed the detection of at least one positive sample in those farms with a within herd prevalence ≥25% with 95% confidence.

Data on factors potentially associated to *Salmonella* outcome were gathered during the sampling of the holdings through a mandatory questionnaire according to the Commission Decision 2008/55/EC. The questionnaire included information about farm identification and location, date of sampling, type of production, farm structure, husbandry factors, feeding practices and antimicrobial usage. In total 18 variables were included, all of them closed. More details about the variables analyzed are presented in Table 1.

Table 1. Summary of variables derived from the questionnaire and assessed as potential risk factors for *Salmonella* faecal shedding in Spanish breeding pigs.

A-. Variables related to sampling	
A.1 Date of sampling	
Month	
Trimester	January to March (1) April to June (2) July to September (3) October to December (4)
B-. Variables related to herd	
B.1. Type of breeding farm	Selection (S); Multiplication (M); Both (SM)
B.2. Type of production farm	Farrow to finish (FF); Farrow to wean (PGT); Both (MX)
B.3. Farm size	50-99 (1); 100-399 (2); 400-999 (3); >999 (4)
C-. Variables related to the replacement policy	
C.1. Gilt replacement policy	> 90% purchased (1); > 90% homebred (2); 10-90% homebred (3)
C.2. Boar replacement policy	> 90% purchased (1); > 90% homebred (2); 10-90% homebred (3)
D-. Variables related to farm structure	
D.1. Outdoor access[1]	Yes/No
D.2. Individual housing	Yes/No
D.3. Floor type	Fully slatted floor (1); Partially slatted floor (2); Solid floor without bedding (3); Solid floor with deep straw (4); Solid floor with straw (5); Solid floor with compost (6); Solid floor with savings (7); Solid floor with peat (8); Outdoor in fields or paddocks (9); Others (10)
E-. Variables related to husbandry	
E.1. All-in/all-out management[2]	Yes/No
E.2. Diarrhea symptoms present in the herd during the last three weeks[3]	Yes/No
F-. Feeding practices	
F.1. Pelleted-feed	Yes/No
F.2. Liquid feed	Yes/No
F.3. Feed origin	Commercial Compounds (1); Home meal (2); Cereals together commercial compounds (3); Others (4)
F.4. Organic acids	Yes/No
F.5. Probiotics	Yes/No
F.6. Other additives	Yes/No
G-. Variables related to antimicrobial usage	
G.1. Antimicrobials administered during the last four weeks[4]	Yes/No

[1] Outdoor-access is considered as YES when all the breeding pigs sampled fulfill this requirement.
[2] All All-in/all-out management is considered as YES when all the animals fulfill this requirement.
[3] Diarrhea is considered as YES when at least one of the animals presented it.
[4] Antimicrobial usage is considered when at least one antimicrobial has been used in any treatment performed the last three weeks.

2.2. Sample analysis

Faecal samples were submitted to the National Reference Laboratory (Algete, Madrid). *Salmonella* detection was performed at this laboratory using the method described by Annex D of ISO 6579. A single confirmed *Salmonella* isolate from each positive sample was serotyped by slide agglutination in accordance with the White-Kauffmann-Le Minor scheme (Grimond and Weill, 2007). The reported sensitivity of cultured pooled faecal samples was 80% and the specificity was 100% (Hoorfar and Mortensen 2000; Arnold et al. 2005).

Sera samples were submitted to the Veterinary Faculty of the University of León where they were analyzed for antibodies against *Salmonella* O-antigens by a commercial indirect mix-LPS-ELISA according to the instructions of the manufacturer (Herdcheck Swine *Salmonella* Antibody Test Kit, Idexx laboratories Inc.). The indirect mix-LPS-ELISA test is designed to detect antibodies against *Salmonella* serogroups B, C1, C2 and D (O-antigens 1, 4, 5, 6, 7, and 12). For each sera, the sample to positive (*S/P*) ratio was determined by relating its absorbance value at 650 nm to that of the positive control. Adjusted OD was estimated by dividing the *S/P* value by a correction factor of 2.5. For each sample, ELISA results were interpreted using two cut-offs, 40% and 20% OD and herds were classified in three levels, level 1 or low *Salmonella* risk, level 2 or moderate *Salmonella* risk and level 3 or high *Salmonella* risk, based on the number of positive sera. Using the 40% OD as cut-off, herds were classified as described by Mousing et al. (1997) as level 1 if the number of positive sera was ≤ 20%, level 2 between 21% and 40% of positive sera and level 3 if the number of positive sera was >40%. For the 20% OD cut-off, herds were classified according to Alban et al. (2002) as level 1 (≤ 20% positive sera), level 2 (≥21%-<70%) and level 3 (≥70% positive sera).

2.3. Statistical analysis

Data were introduced into an Excel file (Microsoft® Excel 2007). Their quality was assessed and obvious typing errors were checked against original records and corrected. A descriptive analysis was performed using EpiInfo 7.0 (CDC, USA) for all the variables to identify those that might be of little value for modeling such as variables with large numbers of missing observations, low variability or ambiguous answers (Dohoo et al., 2003). A herd was considered positive when at least one of the samples was positive. All statistical analysis was done at $\alpha = 0.05$. Prevalence of *Salmonella* positive herds was compared between breeding and production herds by using the chi-square test while the relationship between within-herd prevalence and the *Salmonella* serotype involved in the infection was investigated using ANOVA.

An univariate analysis using chi-square test was performed to explore the association between each variable and the bacteriological farm status. All the variables with a relaxed significance ($p \leq 0.25$) were selected for further analysis in a multivariate model. Collinearity between selected variables was assessed by a pair-wise calculation of the Spearman rank correlations. When two potential risk factors were highly correlated (correlation coefficient >0.5), only one was used in the multivariable analysis (i.e. the one with the smallest *p*-value in the univariate analysis). A multivariate logistic model was constructed using a stepwise backward elimination procedure in SPSS Statistics v.19 (SPSS Inc.). When only significant variables ($p < 0.05$) were left in the model, a stepwise forward selection process was performed offering previously deleted variables to the final model one at each time. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to measure the strength of the association (Martin et al., 1987). Confounding was assessed every time a non-significant variable was dropped from the model by comparing the changes in the coefficients for the variables retained. Variables that modified the coefficients by 25% or more were classified as confounding and retained in the model (Dohoo et al. 2003). Finally, two-way interactions among independent variables remaining in the model were tested by addition into the model and retained if they were significant ($p < 0.05$).

Taking into account that farms were classified into three categories of *Salmonella* contamination according to serological data, an ordinal logistic regression model using ~~PROC~~ CATMOD procedure of SAS (release 9.1; SAS Institute, 2009) was constructed to analyse potential risk factors related to *Salmonella* seropositivity. All the variables were offered to the full model and retained if $p < 0.05$. A stepwise forward selection process was performed offering previously deleted variables to the final model one at one, to prove if the model was improved according to the probability ratio test. Confounding and two-ways interactions were assessed as described for the multivariate logistic model.

3. RESULTS

3.1. Prevalence results

Apparent prevalence of *Salmonella* infection in Spanish herds with breeding pigs determined by bacteriological and serological analysis is shown in Table 2.

From the 364 herds included in the study, bacteriological analysis could be performed in 359 farms. *Salmonella* was detected in at least one of the ten faecal samples collected in 209 herds and the apparent herd prevalence was 58.2% (95% CI: 52.9-63.3). The mean number of positive samples per positive herd was 2.99 (median 2). Most of the herds presented just one positive sample (31.6 % of the positive herds) and less than the 25% of the farms presented four or more positive samples (Figure 1).

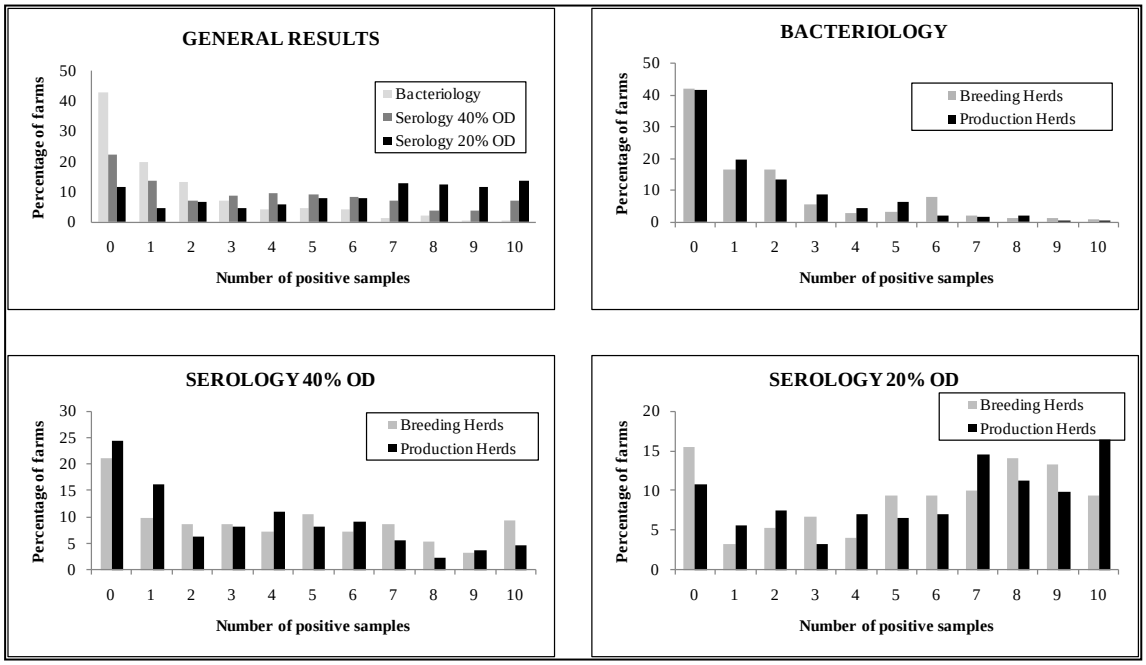
Table 2. Prevalence of *S. enterica* detected by bacteriological or serological examination of Spanish pig herds with breeding pigs. Herds were classified as breeding herds (150) and production herds (213).

	Selection herds	Production herds	Global results
Bacteriological examination			
Prevalence of positive herds[2]	58.7%	58.4%	58.2%
(95% CI)	(50.1-65.9)	(51.7-65.1)	(51.7-65.1)
Serological examination using 40% OD cut-off			
Prevalence of positive herds[3]	78.7%	75.1%	76.6%
(95% CI)	(72.0-85.4)	(69.3-80.9)	(71.8-80.9)
Level 1 herds[4]	40%	45.1%	43%
(95% CI)	(32.2-47.8)	(38.4-51.8)	(37.5-48.5)
Level 2 herds[4]	30.6%	26.8%	25%
(95% CI)	(23.2-38.0)	(20.8-32.8)	(23.6-33.6)
Level 3 herds[4]	29.4%	28.1%	28.6%
(95% CI)	(22.1-36.7)	(22.1-34.1)	(23.6-33.6)
Serological examination using 20% OD cut-off			
Prevalence of positive herds[2]	84.7%	89.2%	87.32%
(95% CI)	(78.9-90.4)	(85.0-93.3)	(83.9-90.7)
Level 1 herds[5]	24%	23.9%	24%
(95% CI)	(17.2-30.8)	(18.3-29.6)	(19.6-28.4)
Level 2 herds[5]	26.2%	29.3%	26.2%
(95% CI)	(19.2-33.2)	(18.3-29.6)	(21.7-30.7)
Level 3 herds[5]	46.7%	51.6%	49.8%
(95% CI)	(38.7-54.7)	(44.8-58.4)	(44.6-54.9)

[1]Breeding herds were those selling gilts or boars for breeding purposes while production herds were those selling pigs for fattening or slaughtering.
[2]A herd was considered as positive by bacteriological examination when at least one of the 10 faecal samples collected was positive;
[3]A herd was considered as positive by serological examination when at least one of the 10 serum samples collected was positive;
[4]Using 40% OD as cut-off herds were classified as level 1 (≤20%), level 2 (>20%-≤40%) or level 3 (>40%).
[5]Using 20% OD as cut-off herds were classified as level 1 (≤20%), level 2 (≥21%-<70%) or level 3 (≥70%).

Sera samples were collected in 363 of the 364 herds included in the study. Using 40% OD as cut-off at least one seropositive pig was detected in 278 of the 363 herds evaluated (76.6%, 95% C.I: 71.8-80.9). Within each positive farm, the number of positive samples varied from 1 (48 farms, 17.3%) to 10 (24 farms, 8.6%). The mean number of positive sera in seropositive herds was 4.75 (median 4) (Figure 1). Using 20% OD as cut-off, one or more seropositive pigs were detected in 317 of the 363 herds (87.3%, 95% C.I: 83.9-90.7). Within each positive farm, the number of positive samples varied from 1 (17 farms, 5.3%) to 10 (49 farms, 15.5%). The mean number of positive sera in seropositive herds was 6.48 (median 7) (Figure 1).

Figure 1. Percentage of *S. enterica* positive samples within each analysed farm detected by bacteriological or serological examination of Spanish pig herds with breeding pigs. Herds were classified as breeding herds (150) and production herds (213).



Breeding herds were those selling gilts or boars for breeding purposes while production herds were those selling pigs for fattening or slaughtering. Serological results were interpreted using two cut-offs, 40% and 20% OD.

No differences in the prevalence of *Salmonella* positive herds between breeding and production herds was observed neither by bacteriology (χ^2= 0.005, p = 0.943) or serology (χ^2= 0.62, p = 0.432 and χ^2=1.63, p = 0.263 for 40% OD and 20% OD cut-offs, respectively).

3.2. Serotype results

Main serotypes detected in this study are indicated in Table 3. Thirty-four different serotypes were identified in the 625 positive samples recovered from the 209 positive herds. Infection by a single serotype was detected in 62.2% of the positive herds (130 of 209), while

two different serotypes were present in 54 herds (25.8%), three different serotypes in 20 herds (9.6%) and four and five serotypes in four (1.9%) and one herd (0.5%) respectively. The most prevalent serotype was *S.* Rissen, detected in 26.8% of positive herds (56 of 209), followed by *S.* Typhimurium (22%) and *S.* Anatum (15.3%). Mean number of positive samples in herds infected by the most frequent serotypes are shown in Table 3. Although mean number of positive faecal samples was lower in *S.* Typhimurium infected farms than in *S.* Rissen, *S.* Anatum and *S.* Derby infected herds, differences did not reach statistical significance (F= 2.19, p = 0.09). Mean number of positive sera was similar in *S.* Rissen, *S.* Typhimuirum, *S.* Anatum and *S.* Derby infected farms.

Table 3. Main *Salmonella* serotypes recovered in Spanish herds with breeding pigs, within-farm prevalence according to bacteriology and serology (40% and 20% OD cut-offs) and farm classification according to seroprevalence in herds infected by these serotypes.

	Bacteriological results			Serology 40%OD				Serology 20%OD			
Serotype1	**No. herds**	**Mean No. of positive samples**	**No. herds in which only this serotype was present**	**Mean No. of positive sera**	**Level 12**	**Level 22**	**Level 32**	**Mean No. of positive sera**	**Level 13**	**Level 23**	**Level 33**
Rissen	56 (26.8%)	3.48	19 (33.9%)	4.55	14 (25%)	14 (25%)	28 (50%)	5.75	13 (23.2%)	19 (33.9%)	24 (42.9%)
Typhimurium	46 (22.0%)	2.93	24 (52.1%)	5.07	9 (19.6%)	11 (23.9%)	26 (56.5%)	5.38	14 (30.4%)	11 (23.9%)	21 (45.6%)
Anatum	32 (15.3%)	4.06	12 (37.5%)	3.66	12 (37.5%)	8 (25%)	12 (37.5%)	5.84	6 (18.8%)	9 (28.1%)	17 (53.1%)
Derby	29 (13.9%)	4.10	12 (41.4%)	4.66	6 (20.7%)	8 (27.6%)	15 (51.7%)	5.55	9 (31.0%)	4 (13.8%)	16 (55.2%)
London	19 (9.1%)	3.32	10 (37.5%)	1.89	11 (57.9%)	5 (26.3%)	2 (10.5%)	4.84	5 (26.3%)	8 (42.1%)	6 (31.6%)
Goldcoast	15 (7.2%)	3.27	5 (33.3%)	2.53	8 (53.4%)	4 (26.6%)	3 (20%)	4.2	5 (33.3%)	5 (33.3%)	5 (33.3%)
Bredeney	14 (6.7%)	3.2	5 (35.7%)	2.5	8 (57.2%)	3 (21.4%)	3 (21.4%)	5.5	3 (21.4%)	4 (28.6%)	7 (50%)
Meleagridis	14 (6.7%)	4.0	5 (35.7%)	5.5	5 (35.6%)	2 (14.3%)	8 (57.1%)	7.4	4 (28.6%)	-	10 (71.4%)
Muenchen	14 (6.7%)	4.14	6 (42.9%)	2.36	8 (57.2%)	3 (21.4%)	3 (21.4%)	5.78	2 (14.3%)	5 (35-7%)	7 (50%)
Wien	10 (4.8%)	3.0	4 (40%)	3.6	3 (30%)	3 (30%)	4 (40%)	4.7	2 (20%)	-	8 (80%)

[1]10 main serotypes are included although another 24 different serotypes were detected during the study;
[2]Using 40% OD as cut-off herds were classified as level 1 (≤20%), level 2 (>20%-≤40%) or level 3 (>40%); [3]Using 20% OD as cut-off herds were classified as level 1 (≤20%), level 2 (≥21%-<70%) or level 3 (≥70%).

3.3. Risk factors analysis

Several variables were discarded due to their low variability. Probiotics were only used in 3 herds (0.83%) and liquid feed was reported in 15 herds (4.1%). In contrast, only 7 herds (1.9%) did not report the use of antimicrobials during the last four weeks. Moreover, recategorisation was performed for variables such as type of floor (fully slatted/partially slatted/others) and feed origin (commercial/home-mill). Those farms located in regions with less than 10 farms (8 regions and 26 farms) as well as those reporting a replacement policy in which the number of homebred boars or gilts was between 10 and 90% (64 farms) were discarded. The variables type of breeding herd and type of production herd were not included in any of the models as they were linked only to breeding or production holdings, respectively. Another three herds did not fulfill the questionnaires and were discarded.

Table 4. Variables identified as being significantly associated (p<0.25) in the univariate analysis and variables retained by the final multivariate model (p<0.5) of herd-level risk factors for *Salmonella* faecal shedding in Spanish farms with breeding pigs.

Variables selected in the univariable analysis				
Variable	**Levels**	**No. of herds**	**No of positive herds**	**p-value**
Use of acids	No	311	186	0.168
	Yes	46	22	
Boar replacement policy	>90% Homebred	125	83	0.037
	>90% Purchased	127	67	
Gilt replacement policy	>90% Homebred	86	56	0.118
	>90% Purchased	206	112	
Use of pelleted feed	No	201	123	0.176
	Yes	148	79	
Trimester	Jan-March	69	43	0.123
	Apr-June	101	58	
	July-Sept	95	62	
	Oct-Dec	94	46	
Farm size	50-99	48	34	0.235
	100-399	118	64	
	400-999	101	59	
	> 999	83	46	
Region	16 different	-	-	0.032

Variables retained in the model					
Variable	**Levels**	**B (SE)**	**OR**	**95% CI**	**p-value**
Boar replacement	**>90% Homebred**	**0.579 (0.262)**	**1.784**	**1.068-2.980**	**0.027**
	>90% Purchased	**0 (0)**	**1**	**0**	
Type of ration	**Pelleted feed**	**0.506 (0.264)**	**1.659**	**0.989-2.783**	**0.055**
	Non-pelleted feed	**0 (0)**	**1**	**0**	

The univariate analysis resulted in eight variables identified as associated (p <0.25) with the bacteriological status of the farm (Table 4). There was no evidence of colinearity amongst any of these variables and, in a second step, a multivariate logistic regression was performed. The model fitted with the data adequately (Hosmer and Lemeshow χ^2= 0.253 with 2 d.f., p= 0.881). Two factors were retained in the final model as associated with higher odds of faecal shedding of *Salmonella*: boar replacement policy (p = 0.027) and the use of pelleted-feed (p = 0.055) (Table 4). No significant interaction or confounding were observed and none of the variables re-introduced improved the final model.

Two separate models were constructed for serological data using 40% and 20% OD as cut-offs and the corresponding classification systems proposed by Mousing et al. (1997) and Alban et al. (2002). The ordinal logistic regression models were statistically significant (p = 0.047 and p = 0.011, respectively) and retained three of the variables, regardless of the cut-off used: trimester, individual housing and type of floor (Table 5). None of the variables improved the final model when they were re-introduced and no significant interaction or confounding was evidenced.

Table 5. Variables retained by the final multivariate ordinal logistic regression model (*p*<0.5) of herd-level risk-factors for Salmonella seroprevalence in Spanish farms with breeding pigs. Farms were classified into three levels of Salmonella contamination according to their seroprevalence.

		Classification by 40% OD[1]					Classification by 20% OD[1]				
Variable	**Levels**	**No. herds Level 1**	**No. herds Level 2**	**No. herds Level 3**	**Chi^2**	***p*-Value**	**No. herds Level 1**	**No. herds Level 2**	**No. herds Level 3**	**Chi^2**	***p*-Value**
Trimester					25.46	<0.001				17.60	0.0073
	Jan-March	17	18	22			9	12	27		
	Apr-June	28	35	22			30	24	28		
	July-Sept	33	29	16			26	29	23		
	Oct-Dec	20	16	39			32	32	20		
										17.52	<0.001
Individual Housing					13.25	<0.001					
	Yes	16	35	23			17	17	32		
	No	83	65	76			82	82	67		
Floor					11.97	0.0018				17.49	0.0016
	Slatted	10	16	13			15	10	13		
	Part-Slatted	59	39	47			62	50	43		
	Rest	30	44	38			21	38	43		

[1] By 40% OD cut-off, herds were classified as level 1 if the number of positive sera was ≤ 20%, level 2 between 21% and 40% of positive sera and level 3 if the number of positive sera was >40%. For the 20% OD cut-off, herds were classified as level 1 (≤ 20% positive sera), level 2 (≥21%-<70%) and level 3 (≥70% positive sera).

4. DISCUSSION

Salmonella has been identified at all the stages of pork production. This means that efforts to decrease the *Salmonella* burden on society should be targeted at various stages of the production chain (Lo Fo Wong et al., 2004). Although their relevance is still under discussion, breeding pigs may be a source of dissemination of *Salmonella* throughout the pig-production chain (EFSA,2009a). The cross-sectional study in breeding pigs performed in the EU (EFSA, 2009) provided an excellent opportunity to analyse the prevalence, serotypes and risk factors for *Salmonella* infection in Spanish breeding pigs which were not previously reported at national level.

Two approaches were used to study *Salmonella* infection in Spanish breeding pig farms: the compulsory bacteriological analysis together with further non-compulsory serological analysis. The serological diagnosis of swine salmonellosis is usually performed by Mix-LPS-ELISA and offers several advantages respect to the bacteriological culture; it is more sensitive, it overcomes the false negative results due to intermittent shedding and thus it is more consistent along the time and finally it is much more cost effective than the bacteriological culture (Van der Wolf et al., 1999). There are also some disadvantages of these methods: a positive result does not demonstrate that the infection is still present at the time of sampling, it does not detect infections occurring shortly, within one or two weeks before sampling, and it only detects antibodies against *Salmonella* serogroups B, C1, C2 and D (Van der Wolf et al., 1999).

The EFSA baseline study of *Salmonella* infection in breeding pigs was the first cross-sectional study performed in this pig population in Spain. The apparent prevalence of the infection was the higher reported in any of the 24 countries participating in the study and two of each three and three of each four herds had at least one positive sample in the bacteriological and serological analysis respectively. This finding confirms that *Salmonella* is spread among the Spanish population of breeding pigs as it has been previously demonstrated in finishing pigs (Mejía et al., 2006; García-Feliz et al., 2007; EFSA, 2008a). Similarly to studies from other countries (Christensen et al., 2002; Arguello et al., 2013), the prevalence detected in breeding pigs was higher than those reported in finishing pigs, independently if breeding pigs were reared for breeding or for production purposes. Undoubtedly, breeding pigs can constitute a reservoir of *Salmonella* which could be transmitted to their progeny although the relevance of this transmission from breeding pigs to latter stages of the production, growing

and fattening, is unclear (Berends et al., 1996; Lettelier et al., 1999; Stege et al., 2000; Funk et al., 2001; Kranker et al., 2003) and probably needs further research.

Salmonella Derby was the most prevalent serotype detected in breeding pigs in the global EU data (EFSA, 2009) while it only ranked fourth in Spanish population. On the other hand, *S.* Rissen, a serotype particularly associated with pig production in the Iberian peninsula (Spain and Portugal) (Vieira-Pinto et al., 2005; García-Feliz et al., 2007; Arguello et al., 2012), was the most prevalent among Spanish farms with breeding pigs. *S.* Typhimurium, the main serotype in finishing pigs in Spain and Europe (García-Feliz et al., 2007; EFSA, 2008), was the second most prevalent serotype in both, the Spanish and the European breeding pigs. The comparison of the ten main serotypes identified in breeding pigs in Spain with those previously described in fattening pigs in the country (García-Feliz et al., 2007) revealed that six of them were shared by both populations and could be a sign of the relationship between breeding and finishing pigs. The most relevant changes were the lower impact of the monophasic variant of *S.* Typhimuirum, *S.* 4,5,12:i:-, in breeding pigs as compared with fattening pigs and, on the other side, the fact that *S.* Anatum seems to be less spread among finishing pigs as compared with breeding pigs. At the herd-level, 92% of the *Salmonella* positive herds were infected by at least one serotype from *Salmonella* serogroups B, C1, C2 or D1, and therefore could be theoretically detected as positive by the commercial Mix-LPS-ELISA used in the present research. This data is slightly lower than the 94.5% described by Baggesen et al. (1996) among finishing farms in Denmark but provide enough sensitivity for the use of these serological methods in the diagnosis of *Salmonella* infection in Spanish pig breeding farms.

The identification of control measures, management procedures or production processes that potentially promote the spread and maintenance of *Salmonella* is the aim of analytic studies (Dohoo et al., 2003). The present cross-sectional study is the first large-scale research conducted in Spain to elucidate risk factors associated with *Salmonella* infection using a sample representative of the entire breeding pig population. Risk factor analysis from bacteriological results was performed by binomial logistic regression similarly to previous studies (van der Wolf et al., 1999; Bahnson et al., 2006; Rajic et al., 2007; García-Feliz et al., 2009) and identified two factors associated with *Salmonella* shedding: type of ration and boar replacement policy. On the other hand, risk factor analysis using serological results as dependent variable was performed by ordinal logistic regression since farms were classified into three different categories of *Salmonella* contamination, low, moderate and high, considering the proportion of seropositive sera. The cut-off used to classify sera samples as positive or negative to *Salmonella* is a relevant point when interpreting serological results.

Most of the *Salmonella* risk factor studies using serology have fixed their cut-off at 10% OD (Stege et al., 2001; Van der Wolf et al., 2001; Lo Fo Wong et al., 2004; Farzan et al., 2005), although two studies have used a 40% OD cut-off (Beloeil et al., 2007; Hotes et al., 2010). In our study, as a consequence of the high seroprevalence observed, we declined to use 10% OD as cut-off. Instead, two more relaxed cut-offs, frequently used in control programmes, 20% OD and 40% OD were selected (Mousing et al., 1997; Quirque et al., 2001; Alban et al., 2002) and the final models provided the same results identifying three variables related to *Salmonella* seropositivity: trimester, type of floor and individual housing.

According to our results, the use of pelleted feed has been associated with an increased risk of *Salmonella* faecal shedding in pig farms by several studies (Beloeil et al., 2004; Lo Fo Wong et al., 2004; Rajic et al., 2007; Hautekiet et al., 2008; Hotes et al., 2010) including two risk factor studies in breeding pigs (Kranker et al., 2001; EFSA, 2011). This finding was also asserted by a risk factor study performed in finishing herds in Spain (García-Feliz et al., 2009). The differences in coarseness of the ground grain seems to be the main reason that explains this effect (Mikkelsen et al., 2004). Non-pelleted and ground meal feed results in a microbiological ecosystem that provides *Salmonella* poor growing conditions compared to pelleted feed (Lo Fo Wong et al., 2004). Therefore, the use of meal instead of pelleted feed could be a reliable recommendation for those breeding herds infected by *Salmonella.* The second factor retained in the model for *Salmonella* shedding was the boar replacement policy. The purchase of more than 90% of the replacement boars was associated to *Salmonella* infection in Spanish breeding pig herds. This is not the first time in which external boar replacement has been highlighted as a risk factor for breeding pig herds (Correia-Gomes et al., 2013). From our point of view, this effect could be related to the introduction of pathogens through purchased pigs. Davies et al. (2000) reported an increase in *Salmonella* shedding in purchased gilts after their transport and similarly a risk factor study in finishing herds described that recruiting pigs from more than three suppliers was associated with *Salmonella* infection at the farm (Lo Fo Wong et al., 2004). This result demonstrates that checking relevant infectious diseases, including *Salmonella* status, is of paramount importance when new pigs are purchased in herds for breeding purposes. However, this finding was not corroborated by a similar effect of gilt replacement policy although this variable was a significant factor associated with *Salmonella* shedding in breeding pig herds in the analysis of the global data at the EU level (EFSA, 2011).

Three variables, trimester, type of floor and individual housing, were associated with *Salmonella* seroprevalence in Spanish farms with breeding pigs. In accordance with Lo Fo

Wong et al. (2004), the same variables were retained in the model regardless of the cut-off and classification scheme used showing a high concordance between both systems used to define the degree of *Salmonella* contamination. In the present research, a higher seroprevalence was detected during the autumn and winter (October to March). Although theoretically, summer conditions and high temperatures would favour the survival of *Salmonella* in the environment (EFSA, 2008b), the delay in seroconversion (Kranker et al., 2003) could explain why the higher seroprevalence was detected in subsequent months. A similar result was reported by Smith et al. (2010) although just the opposite, a higher seroprevalence during summer months, was found by Hautekiet et al. (2008). Contradictory results regarding the seasonality of *Salmonella* infection among different countries of the EU were also described in the EFSA baseline study in fattening pigs (EFSA, 2008b). It has been proposed that the effect of the season is consequence of several factors such as mean temperatures, rainfalls or hours of sunshine (Smith et al., 2010) and it is probably modified by the environmental temperature of the farm (Hald and Andersen, 2001; Hautekiet et al., 2008).

Totally or partially slatted floors allow faecal matter to drain away, thereby reducing the risk for other pigs within the same pen to come into contact with contaminated material. In the present study, fully or partially slatted floors were linked to low or moderately contaminated farms according to serological data. The effect of this factor was also highlighted by the base-line study in breeding pig across the EU but only in the production holdings (EFSA, 2011) as well as by other studies in finishing pigs (Davies et al., 1998; Nollet et al., 2004; Hotes et al., 2010). Finally, individual housing was more frequent in highly seroprevalence or level 3 farms. Although *Salmonella* transmission is mainly associated to the fecal-oral route (Fedorka-Cray et al. 1994) and pig to pig contact favours the spreading of the bacteria (Dahl et al., 1996), individual housing increases the stress of the animals and this fact could be linked to a higher susceptibility to infectious diseases. Anyhow, the individual housing is not allowed in breeding pigs in Europe, with the only exception of the lactation period, since the first of January 2013 (council directive 2001/88/EC) and so the relevance of this result is negligible.

In spite of the relatively high number of farms included in the present study, it failed to identify associations with several risk factors previously reported in the literature such as herd size, all-in/all-out management, gilt replacement policy, outdoor production or feed origin. It is worth mentioning that the lack of association in a particular analytic study does not imply that the factor is not related to *Salmonella* infection.

5. CONCLUSION

The analysis of the faeces and blood samples collected in Spanish farms with breeding pigs demonstrated that *Salmonella* is spread in this population as it has been already demonstrated for fattening pigs. Further research about the role of these breeding pigs in the contamination of pork products by *Salmonella* is needed to elucidate the importance of this finding. The multivariate analysis showed that the use of pelleted feed and the purchasing in replacement policy was associated with higher odds of *Salmonella* faecal shedding. In contrast, fully or partially slatted floors were associated with a lower risk of *Salmonella* contamination according to serological data. Differences in seroprevalence were also detected among different trimesters of the year.

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CAPÍTULO II/CHAPTER II

MEDIDAS DE CONTROL DE SALMONELLA EN CERDOS DE ENGORDE

SALMONELLA CONTROL MEASURES IN FINISHING PIGS

Calendario Agrícola Panteón de los Reyes de León. Siglo XII (San Isidoro, León, España).

Mes de octubre. El porquero está alimentando a dos pequeños lechones.

1-. Introduction

Adapted from:

Hector Arguello, Pedro Rubio and Ana Carvajal (2012). *Salmonella* Control Measures at Farm in Swine Production. In: Salmonella - Distribution, Adaptation, Control Measures and Molecular Technologies, Dr Bassam Annous (Ed.), ISBN: 978-953-51-0661-6, InTech.

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Swine *Salmonella* control programs are not compulsory at the moment but the EU Regulation 2160/2003 has established the need for developing proper and effective measures to detect and control *Salmonella* at all relevant stages of pork production chain and particularly at the primary production level in order to reduce the prevalence and the risk that *Salmonella* poses to public health (EU Regulation 2160/2003). From our point of view, *Salmonella* control should start at the end of the pork production chain (slaughterhouses and finishing farms) and go back to the first steps of the production system (breeding herds and feed suppliers). The compulsory or voluntary *Salmonella* control programmes that have already been established in several European countries base their *Salmonella* evaluation on serological and carcass microbiological contamination results principally (Alban et al., 2002; Nielsen et al., 2001). At the

farm level, these control programmes include the implementation of specific measures to reduce the *Salmonella* prevalence in those herds identified as highly contaminated according to their serological results.

The present revision of literature aims to give a view of the most relevant control measures that can be used to reduce *Salmonella* prevalence in swine farms including a deep review of scientific research as well as our personal experience with control strategies at swine finishing farms. They will be presented into three different categories: (I) measures related to feeding practices, (II) vaccination and (III) generic measures of hygiene and biosecurity.

1.1. *Salmonella* control in the pork production chain

Focused in food safety, the control of *Salmonella* must be carried out taking into account the philosophy "*from farm to fork*" which implies the participation of all sectors involved throughout the food chain. Due to the complexity of its epidemiology and ecology, *Salmonella* control is a great challenge. As it has been proposed by Davies et al. (2004) the food supply should be seen as a linear series of sectors engaged in production, harvest, distribution and consumption and the goal of control programmes is to define the optimal combination of interventions at each sector that delivers the maximum risk reduction at minimal cost. It has been demonstrated that the risk of *Salmonella* contamination increases across the pork production chain and reaches its maximum in the slaughterhouse (Duggan et al., 2010; Argüello et al., 2011; Visscher et al., 2011). Therefore, the slaughter process seems to be the main target to

implement control measures and its importance has been pointed by many studies (Hurd et al., 2002, Argüello et al., 2011). According to this, several countries, led by Denmark with more than twenty years of experience with a national swine *Salmonella* control program are pointing nowadays their reduction strategies towards slaughterhouse interventions (Alban & Stark, 2005; Goldbach et al., 2006). Nevertheless there are some aspects that make us to include pig finishing farms, together with the slaughterhouses, as the primary control points in the pork production chain. On the one hand sometimes it is difficult to implement slaughterhouse strategies due to policy restrictions at this level; and if we consider that has been clearly demonstrated that infected pigs are the main source of *Salmonella* at the slaughterhouse (Visscher et al., 2011), thus in order to reduce the risk of *Salmonella* transmission in the food chain including feasible cost and success, finishing farms should be taken into consideration.

Although sows have been implicated as the primary source of infection to finishers (Lettelier et al., 1999; Kranker et al., 2003), other studies have demonstrated that this transmission can be controlled or interrupted by proper handling practices (Dahl et al., 1997). Several surveys have also supported that vertical transmission is not the main source of *Salmonella* in finishers (Berends et al., 1996; Stege et al., 2000; Funk et al., 2001a; Argüello et al., 2010). According to this, finishing farms should be the main target of *Salmonella* control programmes at the primary level.

1.2. Feeding practices for *Salmonella* control in swine farms

The role of feed in the control of *Salmonella* in swine farms includes two different views. On the one hand, feed can be a source of *Salmonella* contamination while on the other hand there are several feeding practices that are useful tools to be used in the control of *Salmonella.* Several studies have demonstrated the relative low importance of feed as a primary source of infection to pigs (Harris et al., 1997). *Salmonella* is rarely detected after feed processing at the feed mills due to the thermal treatment coupled with good manufacturing practices, and moreover *Salmonella* serotypes sporadically isolated from feed are not related to those usually identified at the farm level (Harris et al., 1997; Davies et al., 2004; Torres et al., 2011). However, most researchers agree that feed can be easily contaminated at the farm level.

Feeding practices include many strategies for the control of *Salmonella*. Most of them are based on the same principle: the modification of the intestinal environment and the promotion of the beneficial microbial flora within the gastrointestinal tract, creating a healthier environment. Even when the feeding practices that are exposed in this chapter are very different, the main mechanisms elicited to reduce or prevent *Salmonella* contamination are shared. Briefly, these feeding control strategies reduce directly or indirectly the pH within the intestinal tract and create an environment which is adverse to *Salmonella* and favours the growth of other bacteria. In a second step, this beneficial gut microflora contributes to maintain a

hostile environment to *Salmonella* by lowering the pH and/or producing several anti-*Salmonella* compounds and metabolites.

1.2.1. Feed composition and feed physical structure

It is well documented that feed presentation, pelletized or not, coupled with the milling type, coarse or fine, has an influence on the gut microflora and therefore determines the success in the establishment and multiplication of *Salmonella* in the intestinal tract of swine. In the revision of risk factors was introduced the idea that although pelleting and thermal-treatment processes can reduce the *Salmonella* contamination in compound feed, it has been demonstrated that non-pelleted feed has a clear protective effect against *Salmonella* compared to the use of pelleted feed (Jørgensen et al., 1999; Kjeldsen & Dahl, 1999; Kranker et al., 2001; Leontides et al., 2003; Lo Fo Wong et al., 2004; Rajic et al., 2007; García-Feliz et al., 2009). In a similar way, coarsely ground meal has been demonstrated to have a protective effect compared to fine grounded meal (Jørgensen et al., 1999; Kjeldsen & Dahl, 1999; Jørgensen et al., 2001; Mikkelsen et al., 2004). It is important to remark that more than defining pelleted or fined ground meal as risk factors that promote the presence of *Salmonella* at farm level, we should define non-pelleted feed or coarsely meal as efficient protective elements against *Salmonella* in swine farms.

As we have already indicated, the anti-*Salmonella* activity seems to be related to the changes in the intestinal microflora that are associated with these types of feed. The effect of feed grinding and feed processing on physicochemical properties and microbial

populations in the gastrointestinal tract of pigs were evaluated by Mikkesen et al. (2004). Those pigs fed a coarse non-pelleted feed showed a significant increase in the number of total anaerobic bacteria within the stomach as well as higher concentrations of various organic acids and lower pH compared to those pigs fed other diets suggesting a higher microbial fermentation in the stomach, fact that was also asserted by a slower gastric passage rate. These environmental conditions in the stomach would reduce the population of *Salmonella* populations by 1000-fold (Mikkesen et al., 2004). Other effects were also observed, to a leser extent, in other parts of the gastrointestinal tract with a lower number of coliform bacteria in the distal small intestine, in the colon and in the caecum and higher concentrations of butyric acid (Mikkesen et al., 2004). Apart from these findings, it is well known that the digestibility of non-pelleted and coarse feed is lower than that of fine pelleted feed. Consequently, higher amounts of carbohydrates reach the last part of the small intestine and the large intestine providing a source of energy for anaerobic bacteria settled there.

1.2.2. Dry or liquid feed

It is well documented that liquid feed has a protective effect against *Salmonella* as compared to dry feed (Van der Wolf et al., 2001b; Højberg et al., 2003). Basically, this feeding strategy can be accomplished by using non-fermented liquid feed or fermented feed. In the first case, water or food industry derivates such as serum from dairy industry are added to the mixed feed immediately before its administration while when using fermentation, the feed and the

water are mixed and stored at a certain temperature for a period of time, prior to its use. Traditionally, liquid feeding systems are much extended in areas where liquid co-products from the human food industry are abundant and cheap. Industries involved in potato, vegetable, milk and fish processing, starch and sugar manufacture, baking, brewing and bio-ethanol production generate co-products that can be valuable and cost-saving inclusions in liquid diets.

The beneficial effects of liquid diets in the gastrointestinal tract are related to the stimulation of epithelial cells growth, the reduction of the intestinal pH and the increase in the lactic acid microbial flora. Its anti-*Salmonella* activity is based on the effect of the fermented feed against *Salmonella* itself since fermented liquid feed contains high concentrations of acids including lactic acid and short chain fatty acids and decreases the pH level in the gastrointestinal tract which in turn influence the ecology of the gastrointestinal microflora. In a study carried out in Canada, Farzan et al. (2006) compared *Salmonella* infection between 20 liquid-feeding farms and 61 dry-feeding farms. The use of liquid feed was associated to a lower number of *Salmonella* positive farms by both serological and bacteriological analysis. Moreover, a reduced usage of antimicrobials and consequently an improved pig health status was reported in those farms using liquid feed. Van Winsen et al. (2002) carried out a clinical trial comparing two groups of pigs, one fed with a dry-diet and the other with a *Lactobacillus plantarum* supplemented fermented liquid diet. A reduction in the total counts of *Enterobacteriaceae* within the gastrointestinal tract was reported

in those animals receiving the supplemented fermented liquid food and was associated with an increase in the concentration of un-dissociated lactic acid and short chain fatty acids in the stomach content. According to these results, many risk factor studies have also described the protective effect of the liquid feed in *Salmonella* infection in swine farms (Beloeil et al., 2004; Lo Fo Wong et al., 2004; Farzan et al., 2010; Hotes et al., 2010).

It is important to remark that in contrast to other feeding practices, the use of liquid feed and particularly of fermented liquid feed has been associated with an improvement in the growth performance. However, this feed system is not feasible economically in all herds due to the investment needed for storage capacity, mixers, pumps, pipelines and computers (Van der Wolf *et al.,* 2001b).

1.2.3. Probiotics

Feeding antibiotics is one of the most effective strategies of prophylactically controlling gastrointestinal infections but this practice is in decline because of the concern with antibiotic resistance in human medicine (Fairbrother et al., 2005). Even more, the European Union banned the use of antibiotics as growth promoters in food animals in 2003 (EC, 1831/2003), on the basis of the "*precautionary principle*". One of the most promising and attractive alternatives to in-feed antibiotics is the use of probiotics and according to this, several researchers have also proposed their utility in the control of *Salmonella* infections in swine farms.

Probiotic treatment is based on the oral administration of viable bacteria, generally non-pathogenic anaerobic bacteria, with the objective to establish the first indigenous flora in newborn piglets or remove the pathogenic flora already established in growers or finishers. The two main actions of probiotics include the nutritional effect and the sanitary effect (Anadón et al., 2006). The nutritional effect is attributed to a reduction of the metabolic reactions that produce toxic substances, a stimulation of the indigenous enzymes and a production of vitamins. The sanitary effect of probiotics is linked to several actions including the creation of a restrictive environment by reducing the pH at the intestinal tract, the competition for gut surface adhesion, the production of anti-bacterial substances such as bacteriocins, the competition for the nutrients, the improvement of the epithelial gut cells health and the stimulation of the immune system acting as bio-regulators of the gut microflora and reinforcing the host natural defences.

At the moment most of the probiotics that are in use consist in a well-defined mix of microorganisms. The main bacterial genera used in these probiotics include *Clostridium, Enterococcus, Bacteroides, Streptococcus, Pediococcus, Bifidobacterium* or *Lactobacillus* as well as yeast such as *Saccharomyces (S. cerevesiae)* or *Kluyveromyces*. According to the guidelines from the EFSA, the identification of all the bacteria included in the mixture and the determination of the absence of antimicrobial resistance genes or plasmids and toxic metabolites are recommended for all probiotic products in the market (Anadón et al., 2006).

Despite the fact that anti-*Salmonella* activity of several lactic acid bacteria has been already demonstrated using *in vitro* procedures (Hume et al., 2001; Harvey et al., 2002; Casey et al., 2004), the literature regarding the efficacy of probiotics in clinical trials is scarce, above all in pig surveys. According to the idea that the efficiency of probiotics is strongly related to the host animal where they have been developed (Ozawa et al., 1983), we will focus the discussion on trials performed in pigs even when there is not too much data available. Genovese et al. (2000 and 2003) evaluated the effect of an undefined mixture of lactic acid bacteria of porcine origin previously developed by Harvey et al. (2002) on caecal colonization and faecal shedding of *S.* Cholerasuis in neonatal and weaned pigs. Their results showed a significant decrease in colonization as well as a reduced shedding after experimental infection with *S.* Cholerasuis in treated animals as compared with the control group. In a similar way, Fedorka-Cray et al. (1999) demonstrated the usefulness of a mixed and undefined culture from caecal mucosa of a 6-week-old healthy pig for the control of *Salmonella* infection. A 2- to 5-log reduction of *Salmonella* in the caecal content or ileocolic junction was observed in the pigs that received this probiotic mixture when compared with the controls. Moreover, 28% of the gut tissues from the treated pigs were positive versus 79% from the control pigs. More recently, the effect of a defined mixture of lactic acid bacteria of porcine origin containing *Lactobacillus murinus*, *L. pentosus, L. salivarus* and *Pediococcus pentosaceus* developed by Casey et al. (2004) was evaluated in weaned pigs (Casey et al., 2007). The study design included three groups of five pigs: two treated groups that

were administered the probiotic directly or fermented prior to its use and a control group in which milk was used as a placebo. All the animals from the treated groups were administered $4x10^9$ colony forming units (CFU) of the probiotic bacteria during 6 days. On day 7, all the pigs were challenged with 10^9 CFUs of *S.* Typhimurium and were monitored for 23 days. Probiotic treated animals showed reduced incidence, severity and duration of diarrhoea as well as a lower concentration of *Salmonella* in faeces. In contrast Zsabó et al. (2009) did not find differences in clinical symptoms after a probiotic treatment based on *Enterococcus faecium* and subsequent challenge with *S.* Typhimurium DT104. Moreover the invasiveness was greater in the treated group than in the control one, showing that not all the potential probiotic bacteria offer protection against *Salmonella.*

Our research group has evaluated hundreds of lactic acid bacteria recovered from faeces, intestinal content or intestinal mucosa of healthy pigs and selected according to their potential probiotic properties including their anti-*Salmonella* effect. Among those with high anti-bacterial activity against *Salmonella* we found isolates from the *Streptococcus* and *Lactobacillus* genera, including *L. reuteri, S. gallolyticus* subsp. *gallolyticus, L. delbrueckii, S. alactolyticus, L. animalis, L. salivarius*, *L ruminis* and *L. murinus* (Collazos *et al.*, 2008a*)*. In general, *L. reuteri* and *L. animalis* isolates are particularly resistant to the gastrointestinal environment of swine. Although *L. delbrueckii* isolates exhibited a strong anti-*Salmonella* activity, they were particularly sensitive to gastric conditions. When a defined probiotic mixture of five lactobacilli

containing *L. reuteri, L. delbrueckii, L. animalis, L. murinus* and *L. ruminis* was administered to 5-weeks old piglets for 7 days before the challenge with *S.* Typhimurium (10^9 CFU) a significant reduction in the pathogen shedding and its dissemination to different organs and tissues as well as an alleviation of the clinical signs of the infection as compared with the pigs from the control group was demonstrated (Collazos et al., 2008b). Similar results were previously reported by Casey et al. (2007).

In spite of all these promising results from experimental trials, there is very little experience regarding the effect of such probiotic treatments in *Salmonella* infected swine farms. Moreover, at least two relevant questions regarding the probiotic use in the real practice still arise: (1) how can or should be administered the probiotic and (2) at which growth stage should it be used in order to reduce *Salmonella* contamination at the time of the slaughtering. Regarding the first question, two main possibilities should be considered. On the one hand, direct administration of the probiotic bacteria should be very effective and consequently high ratios of viable bacteria would reach the gastrointestinal tract. However it is almost impossible to use this administration in field conditions at farm level, particularly if the product is going to be used in growers or finishers. On the other hand, probiotic bacteria could be mixed with feed or drinking water allowing a very easy administration that could be extended for large periods of time. According to this, De Angelis et al. (2006) proposed that one of the main prerequisites for the selection of probiotic bacteria in swine is that these bacteria should

be able to survive and maintain their health-promoting properties during feed manufacturing and storage. Our research group have evaluated the survival of five lactic acid bacteria of porcine origin incorporated into pelleted feed and stored for 24 days at farm conditions. Although one of the evaluated isolates was not included because its performance in the previous steps of fermentation and lyophilisation, was not satisfactory, stable numbers of the other four bacteria were recovered from pelleted feed stored in the farm until the end of the experiment allowing us to conclude that pelleted feed can apparently be used as a vehicle to administer probiotics in swine (Argüello et al., 2013b). Regarding the second question elicited, the moment of administration of the probiotic in a *Salmonella* control strategy in a swine farm, to our knowledge there is no field study in a *Salmonella* infected farm that can be used to give a well-grounded answer. In general, probiotics can be used to establish the flora in a newborn piglet, strengthen colonization resistance to pathogenic bacteria, or to compete with potential pathogenic bacteria already established in the gastrointestinal tract. Hence, the administration of probiotics is recommended during critical periods such as weaning (3 or 4 age-weeks) or at the beginning of the fattening period, when intestinal disorders are common. Focusing on the control of *Salmonella* infection in swine farms, both periods seem to be also suitable to establish a health intestinal status which would increase the resistance to *Salmonella* colonization. However, special attention should be paid in order to avoid infections by *Salmonella* during the fattening period. Other option would be the administration of the probiotic during this fattening period or even at the end of the

fattening period to reduce the risk of *Salmonella* transmission in the food chain.

1.2.4. Acids

A well studied strategy for the control of *Salmonella* infection on swine farms is the addition of acidic compounds to feed or drinking water. The main idea is not new and acids have been evaluated to replace growth promoters and to improve the hygiene and quality of the gut microflora since the 1980's (Giesting & Easter, 1985). It has been demonstrated that the un-dissociated form of various acids can freely cross the bacterial cell membrane and enter the bacterial cell, causing cell death (Van Immersel et al., 2006). Moreover, acids decrease the pH at the gastrointestinal tract and they could serve as carbon source, taking part in several bacterial metabolic routes.

The anti-*Salmonella* effect of many acids have been tested and evaluated in several experimental and field studies. The fact that short chain volatile fatty acids are produced by anaerobic bacteria of the gut microflora has focused many studies on their effectiveness against *Salmonella*. Propionic acid has shown satisfactory results against *Salmonella* in poultry (Hume et al., 1993). To our knowledge, there is no reported clinical trial based on the use of butyric acid, nevertheless its activity against *Salmonella* has been documented *in vitro.* The increase of butyric acid concentration in the gut has been associated with a decrease in *Enterobacteriacea*e and *Salmonella* populations (Van Immerseel et al., 2006) and an inhibition of the pathogenicity island I of *Salmonella*, involved in the gut cells invasion,

after exposure to butyric acid has been reported (Gantois et al., 2005). Acetic acid is probably the most evaluated short chain volatile fatty acid in clinical trials. However, several studies have concluded that this acid does not show a relevant anti-*Salmonella* activity (Dahl et al., 1996; Van Immerseel et al., 2006) and further, it increases the development of resistance against acids by the mechanisms defined as acid tolerance response (Known et al., 1998). The anti-*Salmonella* effect of lactic and propionic acids have also been evaluated in several studies with promising results (Wingstrand et al*.,* 1996; Tsiloyiannis et al., 2001; Creus et al., 2007). Apart from these five acids described here, many other studies have been carried out using other products such as citric acid, fumaric acid, malic acid and many other acid products that can be found in the market. At the same time, some of these acids have been coated in an attempt to avoid an early absorption in the small intestine. The most relevant results of clinical trials evaluating the use of acids in the control of *Salmonella* infection in swine farms are summarized in Table 1 (Dahl et al., 1996; van Wolf et al., 2001c; Tsiloyiannis et al., 2001; Anderson et al., 2004; Creus et al., 2007; Boyen et al., 2008b; De Busser et al., 2008).

Table 5. Summary of experimental and field trials carried out using acid treatments to control *Salmonella.*

Study	Trial Type	Production Stage	Acid selected	Vehicle	Concentration Used	Treatment duration	Results and discussion
Anderson, 2004	Clinical trial	Weaning and fattening	Sodium chlorate	Water	30-80 mg/kg bw	36 h.	24 h. of administration in weaned pigs are enough to reduce the qualitatively recovery of *Salmonella* from gut and rectum. Proportions of *Salmonella* positive pigs were not significant reduced in finishers
Boyen,2008	Clinical trial	6-week old piglets	1-. Coated butyric 2-. Coated caprilyc 3- Uncoated butyric 4-. Uncoated caprilyc	Feed	1-. Butyric 0.02% 2-. Caprylic 0.03% 3-. Butyric 0.01% 4-. Caprylic 0.017%	12 days	Treatment with coated butyric acid decreased the intestinal *Salmonella* load and shedding. (the concentration of butyric acid used in the uncoated treatment was half the coated).
Creus, 2007	Field trial	Finishers	Formic-propionic (50:50)	Feed	a) 1.2 % b) 0.8 %	a) 14 weeks b) 8 weeks	a) Reduction of percentage of *Salmonella* carriage in lymph nodes. b) Clear serological reduction and partial reduction of carriers in lymph nodes or cecal content.
Dahl, 1996	Field trial	Finishers	Formic, propionic, ammoniumformiate ammoniumpropionate	Feed	0.4%	14 days	No differences in shedding or serological prevalence. The treatment was not effective in previously infected pigs.
De Busser, 2008	Field trial	Finishers	-	Water	-	14 days	No beneficial effect in samples collected (carcass, lymph nodes or rectum)
Tsiloyiannis, 2001	Field trial	Weaners	Separately diets of: - Propionic acid (1 %) / Malic acid (1.2 %) / Formic acid (1.2 %) / Lactic acid (1.6 %) / Citric acid (1.5 %) / Fumaric acid (1.5 %)	Feed	Cited in acids colums	14 days	Thse study was carried out in a famr with clinical post-weaning diarrhoea syndrome caused by ECET. All the treatments reduced the numbers of ECET and showed ain improved growing specially the lactic acid group.
Wolf, 2000	Field trial	Finishers	Acid mixture: Lactic (8 %), formic (23 %), ammonium formiat (28 %), acetic (4 %), propionic (3 %) sorbic (1 %).	Water	0.2 %	12 weeks	The overall prevalence in control group was three times the treated groups, but just in a situation with clinical problems would justify the use of acids to the authors.

In summary and taking into account all the information provided by the different studies, it seems that the success in the control of *Salmonella* infection by using acids is related to several factors. The concentration given must be related to the pH value (Boyen et al., 2008) and the duration of the treatment should be higher than a few weeks. No differences have been demonstrated between their administration in the feed or water. While the incorporation of the acids in the drinking water allows an easy regulation of the concentration and duration of the treatment, it has been associated with damages in the supply water circuits (Van der Wolf et al., 2001c). Moreover, it has been proposed that the success of these acid treatments administered at the end of the fattening period is related to the establishment of the *Salmonella* infection before the acid addition (Dahl et al, 1996; Creus et al., 2007).

1.2.5. Other feed strategies

Other products such as prebiotics, mainly fructo-oligosacharides that cannot be digested by the animal but serve as carbon source for intestinal bacteria, or herbal extracts with significant anti-*Salmonella* activities have been proposed as potential options in the control of *Salmonella* infection in swine farms. However, further studies evaluating their usefulness in *Salmonella* infected swine units are required.

1.3. Vaccination

Immune response stimulation by vaccines has been a useful mechanism to battle against pathogens. In this subheading of the chapter, vaccinology to control *Salmonella* in pigs will be reviewed

including the different types of vaccines tested against *Salmonella* in swine, discussing their efficacy, advantages and disadvantages. In order to develop a useful vaccine against *Salmonella,* the mechanisms involved in the defence of the host as well as those by which the bacteria is able to establish the infection in the host have to be taken into consideration. Hence, a brief revision of the *Salmonella* transmission*,* pathogenesis and host immune response will be included to improve the reader comprehension about vaccination theories.

The stimulation of the immune system by vaccines against *Salmonella* in swine aims to prevent gut colonization and faecal shedding as well as the development of a carrier state; in a word, bring to end the infection cycle at the farm level (Haesebrouck et al., 2004). The disappearance of clinical symptoms is not the goal of this vaccination since most of the infections by *Salmonella* are not associated with clinical disease in pigs.

Several vaccines have been tested against *Salmonella* including live vaccines, attenuated or genetically modified, inactivated vaccines and also subunit vaccines. Live vaccines have the ability to arouse the best immune response; they stimulate the production of IgA in the intestinal mucosa since they can be used by oral administration and on the other hand they are theoretically able to produce a strong cell-mediated immune response. Besides, antibody titres seem to be lower than those induced by inactivated vaccines (Springer et al., 2001; Husa et al., 2009) and this fact is relevant if the vaccine is going to be used in the course of control

programmes based on serological detection and quantification of the infection.

1.3.1 Live vaccines

Live vaccines against *Salmonella* included (I) attenuated vaccines obtained by the dwindling of at least one of the virulence mechanisms of the bacteria without localizing or characterizing the molecular basis of attenuation; and (II) genetically modified vaccines which in contrast to attenuated vaccines are those in which identified genes for the bacterial metabolism such as *aroA* (Lumsden et al., 1991), global regulator genes or virulence genes such as *spv* genes located in *Salmonella* virulence plasmid (Kramer et al., 1992) have suffered induced mutations to attenuate the bacteria.

Many studies have tested live vaccines in both challenge and clinical trials. We will focus our attention on those studies that have reported bacteriological results and therefore have measured the impact of vaccination in the *Salmonella* shedding and *Salmonella* infection in the gut or the associated lymphoid tissue. Several live vaccines including those based on modifications of their genome such as *aroA* mutants, *Δcya-Δcrp, gyrA-cpxA-rpoB* or *adenine-histidine* auxotrophy organisms (Lumsden et al., 1991; Lumsden et al., 1992; Springer et al., 2001; Denagamage et al., 2007; Selke et al., 2007; Husa et al., 2009) have demonstrated a reduction in the faecal shedding and isolation of *Salmonella* from the gut and lymphoid tissues. When piglets were vaccinated with these vaccines and challenged with the bacteria, a diminution in the infection pressure based on a reduction of the *Salmonella* faecal shedding and isolation

from the gut and the lymphoid tissue associated was demonstrated. Nevertheless in most of these challenge experiments, the monitoring of the piglets was only carried out during the subsequent days or weeks after the experimental infection and therefore there are doubts regarding the duration of this protection. The experience from field trials has provided scarce but very interesting data; an *adenine-histidine* auxotrophy *S*. Typhimurium vaccine was tested for a period of six months in a farrow-to-finish farm. The prevalence of *Salmonella* infection in the unit decreased from 65% to 23% in 6 weeks. Unfortunately, this study does not include a control group and comparison was made using historical data. More recently, Farzan & Frienship (2010) have evaluated a commercial *S.* Cholerasuis live vaccine in a clinical field trial. The prevalence of *Salmonella* shedding animals decreased as immunized pigs aged but the results were not conclusive since this fact was also reported to a lesser degree in the control pigs. The point that the pigs were probably infected before their vaccination together with the coexistence of three different serotypes of *Salmonella* involved in the infection at the farm could explain at least part of the low efficacy of vaccination against *Salmonella* found in this study. Finally another study using a *S.* Cholerasuis live vaccine (Maes et al., 2001) showed a reduction in the positive ileocaecal lymph nodes (ILN) in the vaccinated group, 0.6%, compared to the control 7.2% while 24% and 9% of the vaccinated and control animals were positive in serology at 24 weeks (cut-off > 10).

1.3.2 Inactivated vaccines

Although theoretically live vaccines offer the best protection, they have also several disadvantages; firstly they are not as secure as inactivated vaccines since reversion to virulence can theoretically occur. Besides, transport and storage conditions are more demanding and finally if they are going to be administered orally several factors such as handling, withdrawal of antimicrobial treatments during administration or negative effects such pyrexia or reduced daily gain have to be taken into consideration (Husa et al., 2008). For these reasons, there is still interest in *Salmonella* inactivated vaccines, which are easier to administer, more secure and also cheaper than attenuated life vaccines. In general, inactivated vaccines are useful against extracellular or toxin producer bacteria because humoral immune response can easily and effectively protects the host. It could be expected that no protective or a very limited effect would be seen with intracellular bacteria since the cell-mediated response is not stimulated directly. However, it is important to take into account that at least part of the infection cycle of *Salmonella* takes place in the extracellular space being vulnerable to the action of specific antibodies.

Inactivated vaccines are easy to produce and there are a number of clinical field and experimental trials to evaluate their effectiveness against *Salmonella* in different stages of the swine production including breeding herds, nursery pigs and finishers. A homologous inactivated *S.* Typhimurium vaccine was applied to sows in a research performed by Roesler et al. (2006). The results of this

vaccination were measured in the offspring and revealed a decreased in the prevalence of *Salmonella* shedders as well as in the prevalence of seropositive piglets. According to these results, vaccination with an inactivated vaccine could be a proper tool to control *Salmonella* transmission from the sows to their progeny, easy to apply and cheap. On the contrary, Farzan & Friendship (2010) failed to demonstrate a clear protection in piglets after vaccination with an autogenous *S.* Typhimurium bacterin probably because the vaccine failed to elicit cross-protection against other serovars and piglets were suffering a multiple-serovar infection.

In spite of their limitations, inactivated vaccines as well as subunit vaccines can increase in usefulness by taking advantage of the improvements in DIVA vaccines (Differenciating Infected from Vaccinated Animals) which have already been tested (Selke et al., 2007; Leyman et al., 2011) and also in adjuvants which should be able to increase the immunostimulation boost of these vaccines. The application of such technology in conjunction with the ongoing developments in identifying new virulence determinants such as purified recombinant proteins, synthetic peptides or plasmid DNA could induce protective immunity by the selective activation of immune effectors mechanisms. The next generation of *Salmonella* vaccines could be based on these premises, to overcome the problems discussed above and improve the protections elicited by vaccines against *Salmonella.*

Despite the fact that the vaccine field has been the target of many surveys since decades, there are still many gaps. Most of the

investigations regarding *Salmonella* immunity have been done in a murine model without taking account that *S.* Typhimurium is the host-specific pathogen for this specie. Moreover, most of the challenge trials carried out in piglets do not perform an extended monitoring of the animals until the market-weigh. Further research should be done to increase the knowledge in the immune response against *Salmonella* in production animal species and to non-host specific serotypes as well as in vaccine field trials in both finishers and sows (transmission of the immunity to the piglet).

1.4. Hygiene, handling practices and biosecurity

At farm level there are many factors that can modify the epidemiology of the infection determining the success of *Salmonella* colonization. Throughout this chapter we have mentioned that *Salmonella* needs to overcome the hostile environment of the gastrointestinal tract of the host as well as the immune response mechanisms elicited in order to establish an infection. In the pig *Salmonella* can survive in the gut associated lymphoid tissue with reactivation of infection and shedding in favourable conditions, a fact which implies that infected animals are always a risk of infecting other animals throughout their lives. Moreover, *Salmonella* is perfectly adapted to the external environment and is able to survive outside the host for extended periods of time. These two premises, the carriage of *Salmonella* by apparently healthy animals and the ability of these bacteria to survive in the environment, determine the importance of hygiene and biosecurity practices in the control of the infection. None of the control measures aforementioned will be

successful if they are not accompanied by adequate hygiene and biosecurity practices on the farm.

Hygiene standards are based on cleaning and disinfection procedures. All-in/all-out flow, where each room or building is completely emptied and sanitized between groups of pigs, are used frequently in finishing units in swine production and it is during the period of time comprised between two consecutive batches when the effort must be paid in order to prevent the infection of the incoming pigs. *Salmonella* can survive in the environment for long periods of time, for instance 14 days on smooth metallic surfaces, one year in wet soil or even up to two and four years in dry excrements and dust respectively (Murray, 2000). Its ability to persist in the environment enhances its transmission capacity. Apart from the direct transmission from pig to pig, the environment is the most important source of *Salmonella* infection in finishing units, being more relevant than contaminated sows at breeding herds (Berends et al., 1996). As was demonstrated by Dahl et al. (1997) pigs coming from infected breeding herds, allocated in an environment perfectly cleaned and free of *Salmonella,* can arrive at the slaughterhouse without any positivity in bacteriological or serological samples. Hence, special attention should be paid to avoid the presence of *Salmonella* in the environment.

An effective cleaning protocol should cover the following premises: (I) clean the facilities with pressured water to remove the organic matter with special attention to holes and corners where it can be accumulated, (II) apply detergents together with the

pressured water to enhance the organic matter removal and finally (III) apply a disinfectant after the proper cleaning protocol. Regarding useful disinfectants, it can be said that *Salmonella* is susceptible to most of the disinfectants used, such chlorine, iodine derivates, phenols, peroxides or quaternary ammonium compounds. However it is surprising that being susceptible to most disinfectants, *Salmonella* can be found after cleaning and disinfection protocols routinely applied at farm level (Argüello et al., 2011).

We have evaluated the effectiveness of routinely cleaning and disinfection procedures against *Salmonella* in swine farms (Argüello et al., 2011). A total number of thirty-six pig finishing farms performing a strict all-in/all-out management (AI/AO) were studied by collecting twelve samples within each farm including samples from pen floors (5 samples), pen walls (5 samples), corridors (1 sample) and dust (1 sample). All the farms were studied after cleaning and disinfection procedures, just before the entrance of a new batch of animals. Despite the fact that cleaning procedures were classified as satisfactory by clinicians and a phenol derivate disinfectant was used, *Salmonella* was still detected in one of each five investigated farms (22.2%). *Salmonella* was recovered mainly from floor samples (6 out of 8 positive farms were positive in floor samples) followed by pen walls (three farms). It is remarkable that in two of the positive farms the contamination was only detected in corridors. In contrast, *Salmonella* was not isolated from dust samples in any of the farms. In a similar farm environmental study performed in Germany, Gotter et al. (2011) reported *Salmonella* positive results in 22% of the pens

floors, 28% of the pen walls and 32% of the central hallway. Gebrelles et al. (1999) also found that 80% of the pens were contaminated after cleaning and disinfection procedures in swine farms. Moreover, *Salmonella* serotypes isolated were related to new infections in the incoming pigs. Regarding these results, it is important to note that it has been described that holes in floors and walls make difficult the penetration of disinfectant solutions along with the biofilm formation by *Salmonella* can make the action of the disinfectants difficult (Marin et al., 2009). Surprisingly, it has been reported that farms using cleaning protocols without disinfectants had lower *Salmonella* levels than those using disinfectants (Van der Wolf et al., 2001b). This fact indicates that disinfection protocols are sometimes not carried out properly and points towards the importance of performing adequate cleaning protocols if we want to achieve an effective disinfection. Moreover, particular attention should be paid not only to pens but also to corridors in order to prevent infections between batches and also to the instruments employed at farm level since they can constitute a source of *Salmonella* contamination. Gotter et al. (2011) found that elements such as driving boards, pig toys or boots, presented the higher contamination values, showing that the farm equipment can be a source of contamination that sometimes is underestimated. These results together with the risk factors studies in which not beneficial effects were found in AI/AO systems (Nollet et al., 2004; Rajic et al,. 2007; García-Feliz et al., 2009) show that cleaning protocols carried out routinely at farm sometimes do not reach their goal and so special attention should be paid in the cleaning and disinfection carried out between batches removing the

organic matter present, cleaning not only the surfaces visible to the naked eye but also equipments, corners, and other surfaces in which dust and contamination can be stored.

Regarding management and handling by farmers the main premises that should be taken into consideration are as follows; large facilities are usually supplied by several breeding origins and then *Salmonella*-free pigs can be mixed with infected pigs at the fattening unit. Thus, in order to avoid the risk of contamination by potentially infected pigs, the origin status of the piglets should be confirmed, above all in low *Salmonella* contaminated farms included in control programmes. Moreover, it is believed that mixing animals with different ages increases the risk of *Salmonella* transmission, so pig handling is also important in avoiding or minimizing *Salmonella* infections at the farm. Adequate handling and caring of the animals is also necessary to diminish the stress, which is related to an increase in pig susceptibility to *Salmonella* infection as well as to an increase in faecal shedding by carriers (Verbrugghe et al., 2011).

Biosecurity is essential at farm level to avoid the entrance of infectious diseases and most of the swine farms fulfil the basic biosecurity measures. General biosecurity measures such as double external fence, footbaths, changing rooms with showers and farm clothes to staff and visitors, external access to feed and dead animal trucks are essential in farms and several risk factor studies have associated them to a lower *Salmonella* prevalence (Amass et al., 2000; Lo Fo Wong et al., 2004).

Apart from infected animals, which constitute the main source of *Salmonella* infection, the indirect transmission of disease by feed or wild animals present at the farm can be also relevant. The importance of feed as *Salmonella* vehicle has been already discussed in the feeding strategies subheading. As it was pointed out if feed transport or storage in the farm are not carried out under strict isolation conditions, feed can be easily contaminated by *Salmonella*. Water supply can also be a significant vehicle to indirect *Salmonella* transmission. The ability of *Salmonella* to survive in water supply depends on the nature of the water and factors such as the presence of protozoa, the concentration of organic matter, toxins, heavy metals, and several physicochemical properties. Fish & Petiborne (1995) estimated that *Salmonella* can survive at least 56 days in water. Farmers should pay attention to water quality and also to guarantee a supply of potable water on their farms. At the same time, wild birds or rodents can also contaminate the feed if they can access to the places where it is stored. Feed and water are effective vehicles to *Salmonella* transmission because they are supplied to all the animals and bring *Salmonella* directly to the gastrointestinal tract. So appropriate production and feed handling as well as water treatment has to be done in order to avoid contamination by *Salmonella* from these two sources.

Probably one of the main factors implied in the spreading of *Salmonella* is its ability to colonize a wide range of animal species including warm or cold blooded animals; this fact implies that most of the animals, birds or insects present in an environment with

Salmonella will be infected or will carry *Salmonella.* This fact implies that all domestic and wild animals that get in touch with the farm can constitute a source of *Salmonella* for pigs. *Salmonella* has been isolated from rodents in several studies (Healing, 1991) and their faecal pellets can contain up to 10^5 CFU of *Salmonella* (Henzler & Opitz, 1992). Although wild birds have been recognised as carriers of *Salmonella,* evidence suggests that infected birds are rarely identified. It seems that birds are infected by their feeding environment with a short term carriage (Murray, 2000). *Salmonella* has been also isolated from insects including cockroaches, flies, and beetles (Benett, 1993; Davies & Wray 1996; Olsen & Hammack, 2000). Other wild animals are more related with the maintenance and perpetuation of the infection in the farm more than with the introduction of *Salmonella* thereof; finding positive mice or rats or cats for instance in the farm proves that *Salmonella* is distributed in the environment and the elimination of these animals is crucial if other efforts are taken at the same time to reduce the *Salmonella* prevalence.

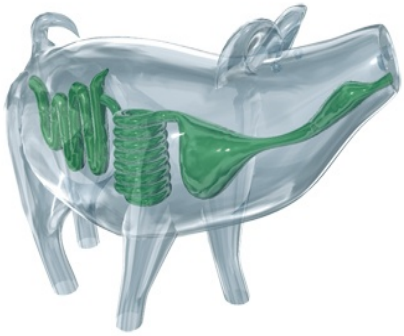
Hygiene, handling practices or biosecurity are not sometimes taken into account to battle against a pathogen but in facultative environmental pathogens such *Salmonella,* they can play a crucial role in its maintenance and perpetuation and must be included in the practices to reduce the prevalence at farm level if practitioners want to have success reducing *Salmonella.*

1.5. Conclusions

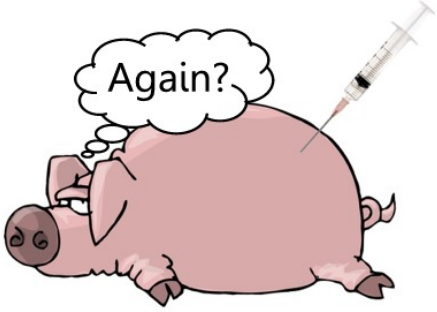
The main objective of this chapter was to identify the main potential control strategies applicable in swine production to reduce *Salmonella* prevalence. Fortunately there is enough background to discern which measures seem to be most efficient in general, but we must stress that *Salmonella* epidemiology is not completely understood and that there are many factors that can influence its presence at farm level. The achievement of success in a reduction programme will depend in which measures, of those described here, may be feasible applied taking into account the serotype involved in the infection, its prevalence, type of farm etc, and also factors such economical resources.

2. Publications included in chapter II

Publication 1. Effect of the addition of organic acids in drinking water or feed during part of the finishing period on the prevalence of *Salmonella* in finishing pigs.



Publication 2. Evaluation of protection conferred by a *Salmonella* Typhimuriuminactivated vaccine in *Salmonella*-infected finishing pig farms.



2.1. Publication 1

Running head: **Acids to control *Salmonella* in finishing pigs**
Status: **Accepted** in revised form (30/04/2013) for publication
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Effect of the addition of organic acids in drinking water or feed during part of the finishing period on the prevalence of *Salmonella* in finishing pigs

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ABSTRACT:

Pork is a major cause of foodborne salmonellosis. Consequently, effective measures which could reduce the prevalence of *Salmonella* at the farm are of interest. In the present study, three field trials were performed to evaluate the effect of strategic administration of organic acids, at concentrations estimated by economic criteria, on the *Salmonella* prevalence in finishing pigs. Pigs received either a mixture of acids (lactic, formic, propionic and acetic) added to their drinking water at a concentration of 0.035% (trial A), or a basal diet containing 0.5% potassium-diformate, $KH(COOH)_2$, (trials B and C), during the last 6-7 weeks of the finishing period. Fecal *Salmonella* shedding and seroprevalence were monitored in the animals at four time points during the study. Mesenteric lymph nodes and cecal contents were collected from a subset of animals at the slaughterhouse and cultured for *Salmonella*.

At the end of the finishing period in all three trials, the percentage of seropositive pigs was higher in the control group than in the experimental group, regardless of which cutoff value was used in the ELISA assay. The frequency of fecal shedding was lower also in the treated pig groups from the trials A and B at the end of the finishing period. The results from the samples collected at the slaughterhouse did not yield differences between groups in the trials A and B. However, *Salmonella* isolation was less frequent in the MLNs from the experimental pigs in trial C. The seroprevalence reductions together with some promising results in the reduction of shedding, support the idea that this intervention is a useful strategy to reduce *Salmonella* prevalence at the farm.

***Salmonella*, pig, control, farm, acid, seroprevalence**

INTRODUCTION

Salmonella is one of the major food-borne pathogens in industrialised countries. The European Union (EU) regulation 2160/2003 ensures that effective measures are going to be taken to detect and control *Salmonella* and other zoonoses during all stages of production, processing and distribution, with the inclusion of control programs at primary production of poultry, turkey and swine. Their implementation in the avian industry has yielded a significant reduction in the incidence of human salmonellosis (EFSA, 2013). Nevertheless, they are not compulsory at primary pig production and only several EU members have implemented them (Mousing et al., 1997; Osterkorn et al., 2001; Rowe et al., 2003). Consequently, the relative importance of pork and pork products as sources of human salmonellosis has increased in the last years and currently it is estimated to be the second most common source of infection for humans in the EU (Pires et al., 2011).

The necessity of identifying strategies to mitigate the risk of *Salmonella* infections has prompted the evaluation of different on farm control strategies for primary pig production (Dahl 1997; Denagamage et al., 2007; Garcia-Feliz et al., 2009; Mikkelsen et al., 2004; van Immerseel et al., 2006). Addition of acidic compounds to control *Salmonella* in finishing pigs have been tested in challenge studies (Anderson et al., 2005; Taube et al., 2009), but also in field trials, some with promising results (Creus et al., 2007; Visscher et al., 2009), and others without clear benefits (Dahl et al., 1996; De Busser

et al., 2009; Van der Wolf et al., 2001). More research about dosage and duration of treatments is needed to evaluate their efficacy.

The objective of this study was to determine if the strategic administration of organic acids in water or feed, at concentrations set by economic criteria, and during the last part of the finishing period had any effect on the prevalence of *Salmonella* in infected swine finishing herds.

MATERIALS AND METHODS

Experimental design

Three field trials were performed at three different finishing farms (trials A, B and C) where a *Salmonella* seroprevalence greater than 50% was reported in the previous batch of finishers.. The main characteristics of the facilities and treatments are described in Table 1. The three farms were integrated into multi-site production systems and followed a strict all-in/all-out management. Each selected farm consisted of two barns with harmonised characteristics and were filled at the same time with pigs that were approximately 70 days-old and had the same origin. Organic acids were administered by water or feed; therefore the treatments were set at the barn level. The acid treatment was administered to the pigs housed in one of the barns during the last 6-7 weeks of the finishing period (experimental group), while no treatment was administered to the animals housed in the other barn (control group). The veterinarians in charge of collecting the samples as well as the technicians who processed them at the laboratory were blinded.

In the first experiment (trial A), a commercial acid (Acidvall, MEVET) was administered by water to the experimental group animals (EG). Forty pigs from each group were randomly selected at the start of the finishing period; these animals were ear-tagged and tracked during their finishing. Another forty randomly selected pigs per group were sampled at the slaughterhouse as it was not possible to ensure the correct tracking of the previously selected animals within the slaughterhouse. The experimental and control pigs were allocated in different trucks and holding pens and were slaughtered separately to avoid mixing the pigs or samples between the groups. The feed was supplemented for 14 days at the start of the finishing with a combination of colistin (120 ppm), oxibendazole (40 ppm), lincomicin (44 ppm) and spectinomycin (44 ppm) in both barns. Moreover, during the third week of finishing, both barns were medicated with doxycycline (100 ppm) and lincomicin (50 ppm) for five days, due to a respiratory problem. No antimicrobial treatment was used during the administration of the acid, and none of the monitored pigs received individual antimicrobial treatments. Individual treated pigs were identified and marked to avoid sampling them at the slaughterhouse.

Table 1. Experimental design and main characteristics of the farms participating in three clinical trials (A, B and C) to evaluate the effect of the administration of organic acids, by water or feed, on the prevalence of *Salmonella* infection at the end of the fattening.

Clinical trial	Type of Farm	N° pigs per farm	N° barns (pigs per barn)	N° pigs per pen	Randomization	Age of vaccination (approx)	Vaccine boost (days later)	Fattening duration (final weight)	Outcome before vaccination	
									Seroprev.[1]	Serotype
A	Farrow to Finish	420	2 (180/240)	15	By pen	81 days of life	25	95 days (100 kg)	44%	Typhimurium
B	Farrow to Finish	400	1	15	By pen	68-75 days of life	21	90 days (100 kg)	60%	Typhimurium
C	Finishing Farm	2,500	2 (1,000/1,500)	20	By pen	70-85 days of life	23	129 days (100 kg)	50%	Typhimurium
D	Finishing Farm	2,960	2 (1,480/1,480)	18	By barn	70-85 days of life	21	102 days (115-120 kg)	92.5%	Typhimurium

[1]Treatment finished the day in which pigs were delivered to slaughterhouse.
[2] Percentage of seropositive pigs (50 sampled pigs per farm) by a commercial ELISA using a 40% OD cut-off.

Two finishing farms participated in a second experiment (trials B and C). During the last seven weeks of the finishing period, the pigs in the experimental groups received their basal diet (Table 2) containing 0.5% potassium-diformate, $KH(COOH)_2$, (Formi, BASF). Forty pigs were randomly selected from each group at each sampling. Transport and slaughter were performed under the same conditions as those described in trial A. Regarding the antimicrobial usage during these two trials, a prophylactic treatment with doxycycline-colistin (1 kg/100 l) was administered during 10 days at the beginning of the finishing in trial B, while initial feed in trial C was medicated with doxycycline (250 ppm) for 7 days. The same policy of antimicrobial restriction during the period of acid administration was followed in the trials B and C.

Table 2. Composition of the standard basal diets in Experiment 2.

Trial B[a]		Trial C[a]	
Ingredients	**Percentage**	**Ingredients**	**Percentage**
Barley	21	Rye	10
Corn	40	Corn	21.0
Wheat	10	Wheat	30.1
CaCO3	0.96	Biscuit Flour (Wheat)	13
NaH2PO4	0.23	Rape	6
NaCl	0.36	Sunflower	5
L-Lysine	0.43	Pea	4
L-Threonine	0.07	Fat	3
Methionine	0.01	Soy	1.3
Fat	2.80	CaCO3	0.8
Rape flour	9.00	L-Lysine	0.5
Soya flour	14.84	L-Threonine	0.1
Fitases	0.01	DL-Methionine	0.006
-	-	Szyme	0.1

[a]Both farms used non-pelleted feed.

Samplings performed and sample collection

Four samplings were carried out at the farm within each trial. A preliminary sampling (sampling 0), performed at the beginning of the finishing period, followed by three samplings at the beginning, half and last day of the treatment. In each of these samplings, blood and rectal feces (≥25 g) were directly recovered from the selected pigs. Pools of feces from pens, 10 of each group, were collected each sampling day during trials B and C (Table 1).

At the slaughterhouse, the gastrointestinal tracts from 40 randomly selected pigs per group were collected and processed in an adjoining facility to collect the mesenteric lymph nodes (MLNs) and caecal contents as described elsewhere (Arguello et al., 2012).

The sample collection was conducted aseptically, and instruments and gloves were changed between samples. All of the samples were transported directly to the laboratory under cooling conditions and were processed immediately after their arrival.

Sample processing

Sample processing and bacteriological analysis was performed as previously described (Arguello et al., 2012), following the current EN-ISO standard methodology 6579:2002/Amd 1:2007. A single confirmed *Salmonella* isolate from each positive sample was serotyped by slide agglutination using commercial antisera (BioRad) in accordance with the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007).

The serological analysis was performed with a commercially available indirect enzyme-linked immunosorbent assay (ELISA) for the detection of porcine-IgG anti-*Salmonella* LPS, according to the manufacturer's instructions (Herdchek Swine *Salmonella* Antibody Test Kit, Idexx laboratories). Three different cut-offs were fixed at optical densities (OD) of 10%, 20% and 40%. By the interpretation of the results obtained with these three cut-offs, it was tried to elucidate the effect of the treatments on *Salmonella* seroconversion and seroprevalence.

Statistical analysis

The number of sampled pigs within each sampling series (40 pigs per group) allowed for the detection of a reduction in prevalence of *Salmonella* from 40% to 15% with a confidence level of 95% and a

power of 80%. The data were stored and analysed using the EpiInfo™ for Windows software (CDC, USA). Within each trial, the Chi-square test at $\alpha = 0.05$ was used to detect significant differences between the proportion of *Salmonella*-positive samples in the treated and control groups ($p < 0.05$). The strength of the association was estimated using relative risk (RR).

RESULTS

Bacteriological results

The results of the bacteriological isolation of *Salmonella* from the samples collected at the farm and at the slaughterhouse are summarised in Table 3.

In trial A, the prevalence of *Salmonella* shedders was significantly lower in the CG (1 of 40) compared to the EG (10 of 40) at sampling 2 ($\chi^2=6.75$, $p<0.01$). However, at the end of the finishing period a lower prevalence was detected in the EG pigs (7 of 40) compared to the CG pigs (20 of 39) ($\chi^2=8.57$, $p<0.01$; RR=2.34, 95% CI 1.41-6.25). *Salmonella* isolation from the caecal content and the MLN samples was also lower among the EG pigs compared to the CG pigs although these differences did not reach statistical significance ($\chi^2=1.83$, $p=0.18$ and $\chi^2=1.33$, $p=0.24$, respectively). *S.* Typhimurium was the serotype detected of all the isolates obtained during the trial A.

The proportion of *Salmonella* shedders in the trials B and C was always low and did not exceed 22.5% in any of the samplings. In

trial B, statistically significant differences were reached in the sampling carried out at the end of the finishing period, when the prevalence of *Salmonella* shedders was significantly higher in the CG (9 of 40) compared to the EG (1 of 40) (χ^2=5.60, p=0.017; RR=9.0, 95% CI 1.20-67.78). No significant differences were detected in the frequency of shedding between the EG and the CG in any of the samplings performed during trial C.

No differences were detected in the prevalence of *Salmonella* from any of the samples collected at the slaughterhouse in trial B. *Salmonella* was present in 15 MLN samples from the CG but was not detected at any of the 40 MLN samples analysed from the EG pigs in trial C (χ^2=16.08, p<0.001).

S. Rissen was the serotype detected in the positive samples collected at the farm in trial B. It was also detected in the caecal content at the slaughterhouse although two additional serotypes, *S.* Derby and *S.* Typhimurium, were recovered from MLNs. All of the isolates recovered during trial C were *S.* Rissen

.

Table 3. Assessment of the effect of two acid treatments administered by water (clinical trial A) or feed (clinical trials B and C) in *Salmonella* infection by bacteriological analysis at farm (during treatment administration) and slaughterhouse (after its completion). *Salmonella* infection was evaluated by checking pooled-pen (10 samples) and individual faecal samples at the farm and caecal content and MLN at the slaughterhouse (40 pigs). Percentage of positive samples in each of the samplings performed.

	Farm								Slaughterhouse			
	Sampling series								Caecal Content		MLN[3]	
	Sampling 0		Sampling 1		Sampling 2		Sampling 3					
	CG[1]	EG	CG	EG	CG	EG	CG	EG	CG	EG	CG	EG
Trial A (40 days)												
Treatment Day	No treatment		Day 1		Day 22		Day 40[2]					
Positive Pens	0	0	-	-	-	-	-	-				
Positive Pigs	0	0	25%	20%	2.5%	25%	51.2%	17.5%	65%	47.5%	70%	55%
Trial B (52 days)												
Treatment Day	No treatment		Day 1		Day 34		Day 52					
Positive Pens	0	0	20%	10%	10%	0	10%	0				
Positive Pigs	7.5%	5%	12.5%	5%	0	5%	22.5%	2.5%	5%	2.5%	2.5%	2.5%
Trial C (49 days)												
Treatment Day	No treatment		Day 1		Day 26		Day 49					
Positive Pens	0	0	0	0	0	0	0	0				
Positive Pigs	0	0	0	5%	5%	2.5%	7.5%	0	12.5%	17.5%	37.5%	0

[1]Control group (CG) received no treatment. Experimental group (EG) received a treatment with acids during the last 6-7 weeks of the fattening period. Sampling series were distributed as follows: sampling 0 at the beginning of the fattening period, sampling 1 at the start of the treatment, sampling 2 at the middle of the treatment and sampling 3 at the end of the treatment in coincidence with the end of the fattening period EG. Experimental Group received a treatment with acids during the last 6-7 weeks of the fattening period.

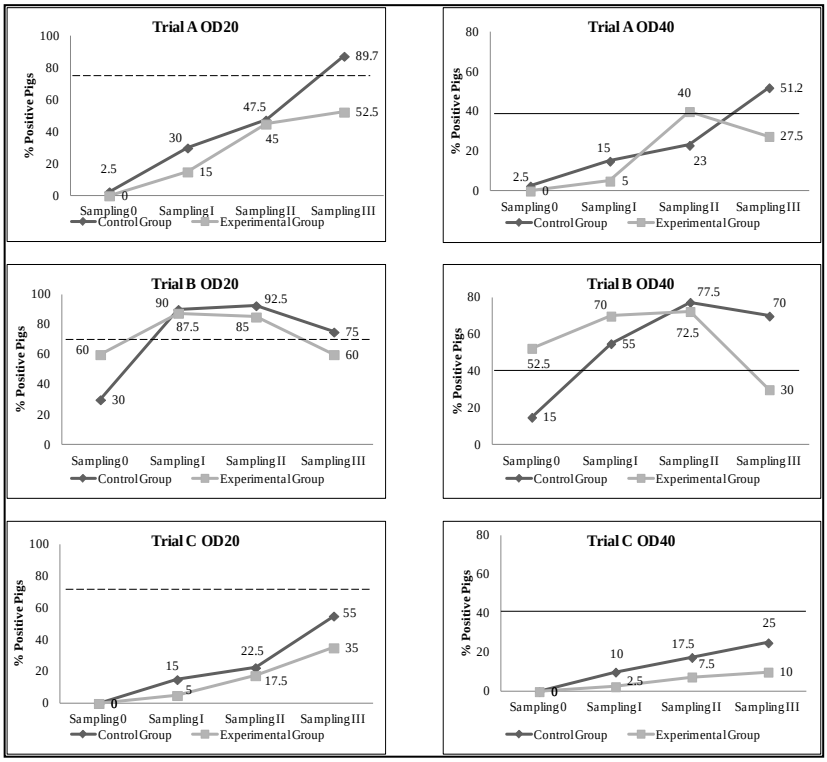
[2] 39 pigs were sampled in the control group at this sampling.

[3] MLN. Mesenteric lymph nodes.

Serological results

The serological results based on 10% OD, 20% OD and 40% OD cut-off points are summarised in Figure 1 and Table 4.

Figure 1. Assessment of the effect of two acid treatments administered by water (clinical trial A) or feed (clinical trials B and C) in *Salmonella* infection by serological analysis. Seroprevalence was estimated by ELISA using 20% OD and 40% OD cut-offs.



Control group received no treatment. Experimental group received a treatment with acids during the last 6-7 weeks of the fattening period. Sampling series were distributed as follows: sampling 0 at the beginning of the fattening period, sampling 1 at the start of the treatment, sampling 2 at the middle of the treatment and sampling 3 at the end of the treatment in coincidence with the end of the fattening period. Solid line (-) represents the index 40% of seropositive pigs while dotted line (- -) the index 70% of seropositive pigs which are the limits used in this study to establish high contaminated farms for the respective OD cut-offs.

In trial A, few pigs had seroconverted at the beginning of the finishing period (sampling 0). *Salmonella* seroprevalence continued to rise in both groups until the end of the experiment, regardless of the cut-off used. The proportion of seropositive pigs was significantly lower in the monitored pigs from the EG compared to those from the CG at the end of the treatment for the 10% OD cut-off (χ^2=4.04, p=0.044; RR=1.27, 95% CI 1.03-1.57) and the 20% OD cut-off (χ^2=11.53, p<0.001; RR=1.71, 95% CI 1.25-2.34). The difference was also close to statistical significance based on the 40% OD cut-off value (χ^2=3.37, p=0.053).

In trial B, at the beginning of the finishing period, significantly higher seroprevalence values were detected in pigs from the EG compared to pigs from the CG based on the results of the three cut-offs (χ^2=4.06, p=0.043; χ^2=6.11, p=0.013 and χ^2=10.96, p<0.001, respectively). However, no statistically significant differences were detected at the beginning of the treatment (sampling 1) or half-way through the treatment period (sampling 2). At the end of the finishing period (sampling 3), the number of seropositive pigs was higher in the CG pigs compared to the EG pigs regardless of the cut-off, although statistically significant differences were only achieved by the 40% OD cut-off (χ^2=11.25, p<0.001; RR=2.33, 95% CI 1.39-3.91).

The course of infection was different in trial C compared to the other two trials. Seroconversion mostly took place at the end of the finishing period, between samplings 2 and 3, (Figure 1). Significant differences in the proportion of seropositive animals

between groups were only achieved at the end of the treatment and based on the 10% OD cut-off. The percentage of seronegative pigs (less than 10% OD) was higher in the EG compared with the CG (χ^2=8.07, p=0.0045; RR=4.00, 95% CI 1.47-10.92).

Table 4. Assessment of the effect of two acid treatments administered by water (trial A) or feed (trials B and C) in *Salmonella* infection by serological analysis. Number and percentage of positive pigs. Results provided by a commercial indirect ELISA interpreted using three different cut-offs.

		Sampling 0		Sampling 1		Sampling 2		Sampling 3	
		CG[1]	EG	CG	EG	CG	EG	CG	EG
Trial A (40 days)	**Experiment Day**	Beginning of fattening		Day 1		Day 22		Day 40[2]	
	10% OD	6 (15%)	5 (12.5%)	19 (47.5%)	18 (45%)	26 (65%)	30 (75%)	36 (92.3%)	29 (72.5%)*
	20% OD	1 (2.5%)	0	12 (30%)	6 (15%)	23 (47.5%)	18 (45%)	35 (89.7%)	21 (52.5%)*
	40% OD	1 (2.5%)	0	6 (15%)	2 (5%)	10 (23%)	18 (40%)	20 (51.3%)	11 (27.5%)
Trial B (52 days)	**Experiment Day**	Beginning of fattening		Day 1		Day 34		Day 52	
	10% OD	14 (35%)	24 (60%)*	37 (92.5%)	38 (95%)	40 (100%)	37 (92.5%)	40 (100%)	36 (90%)
	20% OD	12 (30%)	24 (60%)*	36 (90%)	35 (87.5%)	35 (92.5%)	34 (85%)	30 (75%)	24 (60%)
	40% OD	6 (15%)	21 (52.5%)*	22 (55%)	28 (70%)	31 (77.5%)	29 (72.5%)	28 (70%)	12 (30%)*
Trial C (49 days)	**Experiment Day**	Beginning of fattening		Day 1		Day 26		Day 49	
	10% OD	3 (7.5%)	5 (12.5%)	9 (22.5%)	12 (30%)	17 (42.5%)	14 (35%)	36 (90%)	26 (65%)*
	20% OD	0	0	6 (15%)	2 (5%)	9 (22.5%)	7 (17.5%)	22 (55%)	14 (35%)
	40% OD	0	0	4 (10%)	1 (2.5%)	7 (17.5%)	3 (7.5%)	10 (25%)	4 (10%)

[1]Control group (CG) received no treatment. Experimental group (EG) received a treatment with acids during the last 6-7 weeks of the fattening period. Sampling series were distributed as follows: sampling 0 at the beginning of the fattening period, sampling 1 at the start of the treatment, sampling 2 at the middle of the treatment and sampling 3 at the end of the treatment in coincidence with the end of the fattening period.

[2]39 pigs were sampled in the control group at this sampling.

* Statistical significant differences were achieved between groups (p<0.05)

DISCUSION

Delivering pigs with reduced prevalence of *Salmonella* to slaughter would be expected to be beneficial for reducing the potential of horizontal spreading of the pathogen to non-infected animals and in reducing the overall *Salmonella* burden (Berends et al., 1996; Borch et al., 1996). With this premise, we aimed to determine the reduction in the prevalence of *Salmonella* at finishing farms that could be achieved by the addition of acids to water or feed. Considering that several studies have reported that acid administration is an expensive intervention (Goldbach and Alban, 2006; van der Gaag et al., 2004), the acid concentrations and treatment durations were estimated by economic criteria, together with the pig producer companies involved. Although the treatment cost were limited to 1.4 €[1] and 1.344 € per pig, for Acidvall and Formi respectively, the concentrations used were within the theoretically effectiveness margins according to the manufacturers' recommendations. The three pig producers involved considered these costs of the treatments affordable.

If well performed, field trials are the best tool to assess the efficacy of therapeutic or prophylactic procedures under field conditions. In contrast, the control of external factors which can affect or alter the experimental outcomes analysed is critical. In order to minimise these external factors, pigs from both groups were subject of the same husbandry practices at each of the studies performed. Prophylactic antimicrobial treatments in feed or water

[1] €1 = approx. US$1.18, UK£ 0.80 at 22 November 2012

are a common practice in pig production at the beginning of the finishing period. The effect of these antimicrobial treatments on the gut microflora and particularly in the establishment of *Salmonella,* were identical in the control and experimental groups and therefore they should affect both similarly. The use of antimicrobials in water or feed was avoided during the acid treatment.

The serological results confirmed that the herds were infected with *Salmonella* in each of the three field trials. The onset of the infection in trial B occurred before the beginning of the finishing period while according to serological data, most of the pigs from units A and C became infected during finishing since only a few of the sampled pigs at the beginning of this finishing period tested positive. Although no statistically significant differences between control and experimental groups were detected at the beginning of the acid treatment in any of the trials, differences in *Salmonella* seroprevalence between the groups were evident at the end of the finishing period regardless of the treatment used. This effect can be extrapolated to the classification of the groups in surveillance programs. While the experimental groups from trials A and B would be theoretically classified as moderately infected by the sero-surveillance programs used in Denmark (Alban et al., 2002) or Germany (Osterkorn et al., 2001), the control groups from these farms would be included in highly contaminated farms. According to serological data from trial C, the infection was established later and the probability of finding a seronegative pig (OD < 10%) at the end of trial was four times higher in treated pigs than in control pigs,

supporting that a protective effect was also achieved by the acidification treatment. By this finding, it is demonstrated that due to variations in the infection onset and intensity, it is strongly advisable to use several cut-offs to interpret these serological results. The results obtained are in accordance with previous studies (Creus et al. 2007;Visscher et al., 2009; van der Wolf et al., 2001), and support the idea that organic-acid preparations administered by feed or water to finishing pigs, can reduce the prevalence when they are used at effective concentrations and for a sufficient length of time. In contrast, other studies have failed in demonstrate any effect in the seroprevalence (Dahl et al., 1996; De Busser et al., 2009).

The use of organic acids has also been associated with decreased *Salmonella* shedding in challenge studies (Taube et al., 2009; Papenbrock et al., 2005), and a significant reduction in the number of *Salmonella* shedders was reported by Creus at al. (2007) in one of two herds that received an acidified feed. However, the intermittent fecal excretion of *Salmonella* from naturally infected pigs (Beloeil et al., 2003) makes difficult to assess acidification interventions under field conditions, hence many studies have failed to demonstrate any clear effect on the prevalence of *Salmonella* shedders (Dahl et al., 1996; De Busser et al., 2009). Based on the results of our study, no clear reduction in *Salmonella* shedding was achieved by the acidification, although part of the results provided some insights to this respect. Significant differences among pigs from the control groups compared with pigs receiving acid treatment were achieved at the end of the finishing in trials A and B. In contrast, it

must also be indicated that, a significantly lower prevalence of *Salmonella* shedders was detected in control pigs compared to acid-treated pigs in the sampling performed half-way through the treatment in trial A. This result was not supported by serological results from the same or subsequent sampling.

In previous field trials, limited impact of acid treatments was achieved in the samples collected at the slaughterhouse (Creus et al., 2007; Dahl et al., 1996; De Busser et al., 2009; Letellier et al., 2001). In the present research, the *Salmonella* prevalence in the caecal contents was similar in pigs from both groups in the three trials, and a similar result was obtained for the MLNs in trials A and B. It must also be considered that a number of studies have shown that carriers can become active shedders or new infections can occur (Arguello et al., 2012; Hurd et al., 2002), therefore complicating the interpretation of slaughter results when evaluating on-farm interventions. However, a reduction in the proportion of *Salmonella* carriers in the MLNs was demonstrated in trial C. Contrary to the other two trials, the infection in trial C occurred at the end of the finishing period when the acid treatment had already started. In agreement with this result, Creus et al. (2007) proposed that organic acids could limit the colonisation of MLNs if they are administered before the establishment of the infection.

CONCLUSIONS

In the present study the administration of organic acidic compounds via drinking water or feed during the last part of the

finishing was associated with a reduction in the seroprevalence and, to a lesser extent, a reduction in the prevalence of *Salmonella* shedders at the end of the finishing period. No benefits in the carriage of *Salmonella* in the gut were achieved at the slaughterhouse, although the acid administration could be linked to a decreased MLN colonization. The present study suggests that this intervention could be used at the farm to reduce *Salmonella* prevalence throughout the pork production chain.

AUTHORS DISCLOSURE

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

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2.2. Publication 2

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Journal: **Comparative, Immunology, Microbiology & Infectious Diseases**

Evaluation of protection conferred by a *Salmonella* Typhimuriuminactivated vaccine in *Salmonella*-infected finishing pig farms.

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ABSTRACT

The efficacy of an inactivated *S.* Typhimurium vaccine administered to pigs at the beginning of the fattening period was evaluated in four clinical trials (trials A, B, C and D).Faecal shedding and the systemic antibody response during fattening, as well as, the cecal contents and mesenteric lymph nodes collected after slaughtering were used to assess the outcome. *Salmonella* shedders prevalence in the control groups was six times higher than in the treated groups in trials A and D, both herds infected by *S.* Typhimurium. The risk of positive pens was also four or five times higher for the pens housing control pigs in trials A and C. Lower prevalence of *Salmonella* was observed in the slaughter samples from the vaccinated pigs in trial D and in the cecal content samples in trial A, when just the *S.* Typhimurium results were compared. The results suggest the effective homologous protection of the vaccinated pigs; however, the high humoral response elicited in the vaccinated pigs complicates their use in farms under serological surveillance programs.

KEYWORDS

Salmonella, vaccine, control, farm, Typhimurium, pig

1. INTRODUCTION

Bacterial foodborne pathogens are of increasing concern worldwide. The large number of human cases of *Salmonella* infection and outbreaks of the pathogen reported each year [1], together with the development of antimicrobial resistance [2], make *Salmonella* one of the most relevant of the foodborne pathogens. Pork and pork products are common sources of human salmonellosis [1,3], and in a number of countries, the occurrence of *Salmonella* is monitored in the pork production industry [4,5,6]. Currently, these control programs are not compulsory in the EU; however, Regulation 2160/2003 has established the need for developing appropriate and effective measures to detect and control *Salmonella* at all relevant stages of the pork production chain, particularly at the primary production level [7]. Although *Salmonella* infection can occur at all phases of swine production, the finishing stage is particularly relevant because finishing pigs usually become infected during fattening [8,9]. Moreover, these infected market-weight pigs continuously introduce *Salmonella* into the slaughterhouse environment [10,11].Therefore, it is believed that the reduction of *Salmonella* prevalence at this stage in the production chain will contribute significantly to protecting human health [12]. The reduction in *Salmonella* prevalence in swine farms can be achieved through several measures, including vaccination, sanitation, medication, and the management of known risk factors.

The pig-to-pig transmission of the pathogen usually occurs via the faecal-oral route, and self-limiting enterocolitis is the most

common clinical sign of infection in pigs (obviating *S.* Cholerasuis). However, *Salmonella* infections often pass subclinically in swine, seldom exhibit clinical symptoms, but intermittently shed the bacteriain the feces. Infected pigs may develop as carriers of *Salmonella* in several organs and tissues, particularly in the tonsils and mesenteric lymph nodes. These carrier pigs are a major source of contamination for other animals, the environment, or even the carcasses in the slaughterhouse [13].

Swine can be infected with a wide variety of *Salmonella enterica* serovars. *S.* Typhimurium is of particular interest because this serotypes the most prevalent in swine [14,15,16] and the second most frequent in human infections[1], frequently related to pork consumption in the EU [17]. Moreover, *S.* Typhimurium is often linked to human outbreaks caused by clones, such as the DT104 [18,19],which frequently harbours antimicrobial resistance genes [20], thus limiting the therapeutic alternatives [21].

Several studies have demonstrated that vaccination is an alternative for the control of *Salmonella* during swine production, although they used different vaccination protocols and evaluation parameters [22,23,24,25]. Despite the fact that live vaccines elicit better protection [26], several studies have used inactivated vaccines with promising results [23].The present study aimed at investigating the efficacy of immunising fattening pigs with an *S.* Typhimurium inactivated vaccine to prevent and/or reduce the infection at the farm level and, consequently, the risk of delivering infected pigs to the slaughterhouse.

2. MATERIALS AND METHODS

2.1. Experimental design

The control blinded clinical trials (trials A, B, C and D) were performed at four different fattening units with all-in/all-out management. Of the fattening units, two were part of farrow-to-finish farms (units A and B), and two were part of three-site vertical integration systems (units C and D). The pigs in units A, B and D came from a unique breeding farm, whereas the pigs in unit C were from two different origins. Information regarding the number of pigs bred per farm and housed per barn and pen is indicated in Table 1, which includes the data regarding the fattening duration and outcome reported.

Table 1. Main characteristics of the four *Salmonella* infected finishing herds selected for the evaluation of the protection conferred by a *Salmonella* Typhimurium-inactivated vaccine.

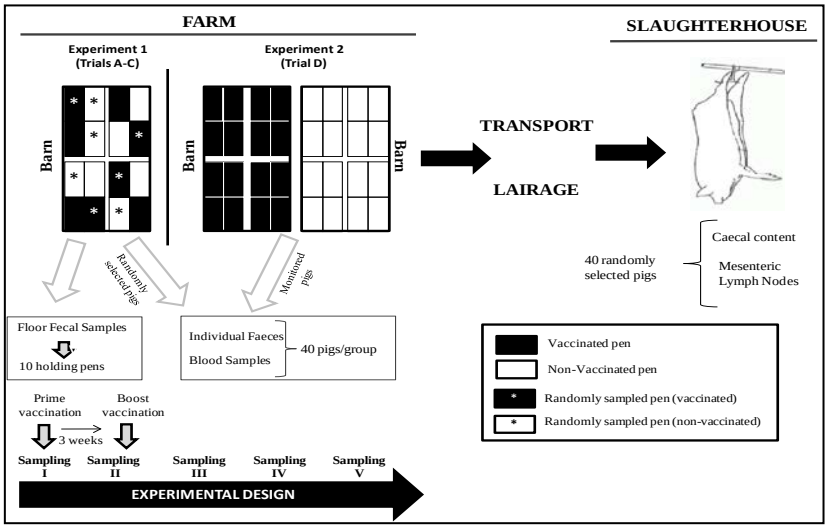
Clinical trial	Type of Farm	N° pigs per farm	N° barns (pigs per barn)	N° pigs per pen	Randomization	Age of vaccination (approx)	Vaccine boost (days later)	Fattening duration (final weight)	Outcome before vaccination	
									Seroprev.[1]	Serotype
A	Farrow to Finish	420	2 (180/240)	15	By pen	81 days of life	25	95 days (100 kg)	44%	Typhimurium
B	Farrow to Finish	400	1	15	By pen	68-75 days of life	21	90 days (100 kg)	60%	Typhimurium
C	Finishing Farm	2,500	2 (1,000/1,500)	20	By pen	70-85 days of life	23	129 days (100 kg)	50%	Typhimurium
D	Finishing Farm	2,960	2 (1,480/1,480)	18	By barn	70-85 days of life	21	102 days (115-120 kg)	92.5%	Typhimurium

[1]Percentage of positive pigs by a commercial ELISA (Herdcheck Salmonella®, Idexx laboratories) using a 40 OD% cut-off value.

The farms were selected to participate in this study based on historical data of persistent *Salmonella* infection and on the results of the previous fattening period, when the infection was corroborated by serology (50 pigs were tested) and bacteriology (10 faecal pooled pen samples were tested) (Table 1). The absence of other pathogens

that frequently cause gastrointestinal disorders at fattening (*Brachyspira hyodysenteriae*, *Brachyspira pilosicoli* and *Lawsonia intracellularis*) was also confirmed. Moreover, after routine cleaning and disinfection protocols and prior to the entrance of the pigs, samples from the pens and corridors and dust from the farm environment were checked for the presence of *Salmonella*.

Figure 1. Experimental design of the four clinical trials for the evaluation of the protection conferred by a *Salmonella* Typhimurium-inactivated vaccine.



Vaccination was performed at the beginning of the fattening and boosted three weeks later. Two approaches were followed at farm level. In Experiment 1 (trials A, B and C), half of the pens within each barn were randomly selected for vaccination (black squares) and the other half harboured control pigs (white squares). In Experiment 2 (trial D), all the pigs allocated in one barn were vaccinated while the other barn acted as control group. Three to five samplings were carried out within each farm. Forty randomly selected pigs per group (blood and feces) together with 10 faecal-pen samples were collected at each sampling during Experiment 1. On the other hand, forty randomly selected pigs were ear-tagged and monitored through Experiment 2.At slaughter caecal content and mesenteric lymph nodes from 40 randomly selected pigs within each group were sampled in each trial. Environmental samples from the transport and holding pens where the pigs rested were also evaluated.

As shown in Figure 1, two different approaches were followed for the group distribution and sample collection. Experiment 1 (trials A, B and C) was performed in two fattening units containing two barns that were filled simultaneously (units A and C) and one fattening unit containing just one facility (unit B). Once all the pigs were housed, half of the pens within each barn were randomly selected for vaccination (VG), whereas the other half constituted the control group (CG). The animals from the vaccinated group were injected intramuscularly with 2 ml of the inactivated vaccine ($5x10^9$ CFU/ml) at the beginning of the fattening period, several days after the units were filled completely. The boost was performed 21 to 25 days later (Table 1). The initial feed was supplemented with a combination of colistin and lincomycin in units A and B, whereas propionic acid (0.01%) was added to the water during the fattening period in unit A. During trial C, the initial feed was supplemented with propionic (0.8%) and formic (0.2%) acids, and a prophylactic treatment consisting of colistin and neomycin in water was administered during the first two weeks of the fattening period. The pigs receiving any individual intramuscular antimicrobial treatment (such as enrofloxacin) were identified and were not sampled. At the end of the fattening period, the vaccinated and control pigs were allocated into different trucks and holding pens. Moreover, the pigs were slaughtered at different times to avoid the mixing of the pigs or samples from the two groups.

Experiment 2 was performed in a fattening unit containing two barns (unit D). In this experiment, all the pigs in one barn were

vaccinated (VG), whereas the pigs in the second barn were not immunised (CG). The vaccination protocol was similar to that described for experiment 1 with an initial dose administered immediately after the unit was filled and a boost administered 21 days later (Table 1). During this trial, a combination of lincomycin and doxycycline was injected into the animals exhibiting respiratory disease during the first month of the fattening, although no treatment was administered to any of the monitored pigs. By the time of sampling performed on day 68, the pigs in both barns exhibited symptoms of swine-influenza (mainly fever) and were treated with doxycycline and paracetamol in water for five days, followed by amoxicillin and paracetamol in water for another five days. The feed was not supplemented with antimicrobial agents at any time. The same guidelines described for experiment 1 were followed for the transport, lairage and slaughter of the animals from the vaccinated and control groups.

2.2. Sample collection

The environmental contamination after the cleaning and disinfection was evaluated before the entrance of the pigs by collecting 10 surface samples in each barn participating in the study as follows: 4 samples from the holding pen floors, 4 samples from the holding pen walls, 1 sample from the corridor and 1 sample of the dust. The surface samples were collected using sterile gauzes previously moistened in buffered peptone water (BPW) (Merck®) to swab a surface measuring 100 cm^2 per sample. The gauzes were

immersed in 50 ml BPW and preserved at cooling temperatures until processing.

At the farm, three and five samplings rounds were performed during trials A and B and trials C and D, respectively. In each sampling, the following samples were collected: (i) 10 pooled-pen feces (≥25 g) samples recovered from ten randomly selected pens per group, (ii) 40 blood samples (approx. 1 ml) and (iii) 40 rectal feces (≥25 g) samples recovered directly from 40 pigs per group. These animals were selected within each sampling round during experiment 1 (trials A, B and C) by the random selection of eight pens per group and five pigs per pen. During experiment 2 (trial D), forty animals per group were randomly selected (2/3 animals per pen and 14 pens per barn) and ear-tagged during the first sampling round. The same animals were tracked throughout the fattening period.

At the slaughter level, the gastrointestinal tracts from 40 randomly selected pigs from each group were collected and processed in the contiguous facility. The mesenteric lymph nodes (MLN) (≥10 g), including the ileocecal lymph nodes, were removed from the intestinal packet and placed into 100-ml sterile flasks. The cecal contents (≥25 g) were collected by puncturing the cecum using a sterile disposable scalpel and placed into 100-ml sterile flasks. In addition, pooled faecal samples from the slaughter trucks and holding pens where the vaccinated and control pigs were allocated before the slaughter were also collected.

The sample collection was performed aseptically, and the implements and gloves were changed between samples to avoid cross-contamination. The samples were submitted directly to the laboratory under cooling conditions and were processed immediately after arrival.

2.3. Sample processing

The MLNs were processed as previously described [27] by removing the fat and the capsula, followed by immersion in 70% alcohol (v/v) and flaming to sterilise the surface. Finally, the MLNs were cut into small pieces using a sterile scissors, were weighed (10 g approx.) and processed.

The bacteriological analysis was performed following the current EN-ISO standard methodology 6579:2002/Amd 1:2007. The pre-enrichment was performed by diluting the feces(25 g), cecal contents (25 g) or MLNs (10 g) 1:10 in BPW. The environmental samples (gauzes immersed in BPW) were processed directly. The samples were incubated at 37ºC for 18-24 h, and 0.1 ml of the pre-enrichment broth was transferred to modified semisolid Rappaport-Vassilliadis medium (MSRV) (Merck®). After incubation for 24-48 h at 41ºC, the MRSV plates exhibiting migration zones were investigated further by streaking the material from the edge of the zone onto Xylose Lysine Deoxycholate agar and Brilliant Green agar (Cultimed®). After incubation for 24 h, the suspected *Salmonella* colonies were identified, sub-cultured in tripticase soy agar (TSA) and biochemically tested using indol and the 4-methylumbelliferyl caprilate

fluorescence (Mucap test, Biolife®) tests. A single confirmed *Salmonella* isolate from each positive sample was serotyped by slide agglutination using commercial antisera (BioRad®) in accordance with the White-Kauffmann-Le Minor scheme [28]

The serological analysis was performed using a commercially available indirect enzyme-linked immunosorbent assay (ELISA) (Herdchek *Salmonella*®,Idexx Laboratories) todetect porcine-IgG anti-*Salmonella* LPS, in accordance with the manufacturer's instructions. The coating antigens in this ELISA included LPS of the serogroups B, C1 and D (O-antigens 1,4,5,6,7, and 12) [29]. The cut-offs were fixed at optical densities (OD) of 20% and 40%, in accordance with the manufacturer's recommendations and serological surveillance programs [4,30,31].

2.4-. Vaccine preparation

The vaccine was prepared using a *S.* Typhimurium DT104 strain of pig origin, previously isolated from the MLN in a separate research study performed by our group. Briefly, five colonies from a pure culture of the *S.* Typhimurium strain (grown in TSA plates containing 5% sheep blood) (Oxoid®) were inoculated into 2,000 ml of brain heart infusion broth (BHI) (Merck®) and incubated at 37°C aerobically for 8 h with continuous shaking (150 rpm). The optical density of the culture was measured, and the bacterial concentration was estimated using a standard curve previously prepared for this strain and corroborated by determining the number of colony forming units (CFU/ml) following serial dilutions in isotonic saline buffer (0.9% NaCl). Finally, 1 ml of the culture was used to check for

purity by culturing in TSA containing 5% sheep blood at 37ºC for 24 h. The culture was inactivated by adding 0.005 v/v 37% formaldehyde (Pancreac®) and maintaining the mixture at 4ºC for 48-72 hours under continuous shaking (200 rpm). To verify the inactivation, 1 ml of the formaldehyde-treated suspension was plated as previously described and incubated aerobically and anaerobically overnight at 37ºC. The inactivated culture was washed 3 times in sterile phosphate-buffered saline (PBS). The bacterial cells were suspended in a 0.6 v/v of adjuvant $Al(OH)_3$ and 0.4 v/v of buffer (PBS), adjusting the final bacterin concentration to $5x10^9$ CFU/ml, and the cell suspension was mixed by continuous and gentle shaking for 36-48 h at 4ºC. The vaccine preparation cost was estimated at 0.104 € per dose (under experimental conditions and without considering staff compensation and equipment expenses).

2.5. Statistical analysis

The total number of sampled pigs within each trial (40) allowed the detection of the reduction in prevalence from 40% to 15% with a confidence level of 95% and a power of 80%.

The data were were transferred to a database (Microsoft Excel spreadsheet). EpiInfo™ for Windows software (CDC, USA) and SPSS (IBM, USA) were used for statistical analysis. The prevalence of *Salmonella* in fecal samples was analysed in a multilevel mixed-effects model , using farm and vaccination status as fixed effects while a repeated measurement index was included as random effect. Moreover, within each trial and time-point, the Chi-square test at α=

0.05 was used to detect significant differences between the proportion of *Salmonella*-positive results in the vaccinated and control groups. The relative risk (RR) was used to measure the strength of the associations.

3. RESULTS

3.1. Bacteriological results

The presence of *Salmonella* before the introduction of the pigs was demonstrated in the facilities at unit A, in which *S.* Typhimurium was recovered from the floor and the walls in two different pens. *Salmonella* was not detected in the environmental samples collected in units B and C. During experiment 2, *S.* Typhimurium was recovered from the environment of the pens in the two barns in unit D and from the corridor of the barn where the vaccinated group was allocated (Table 2).

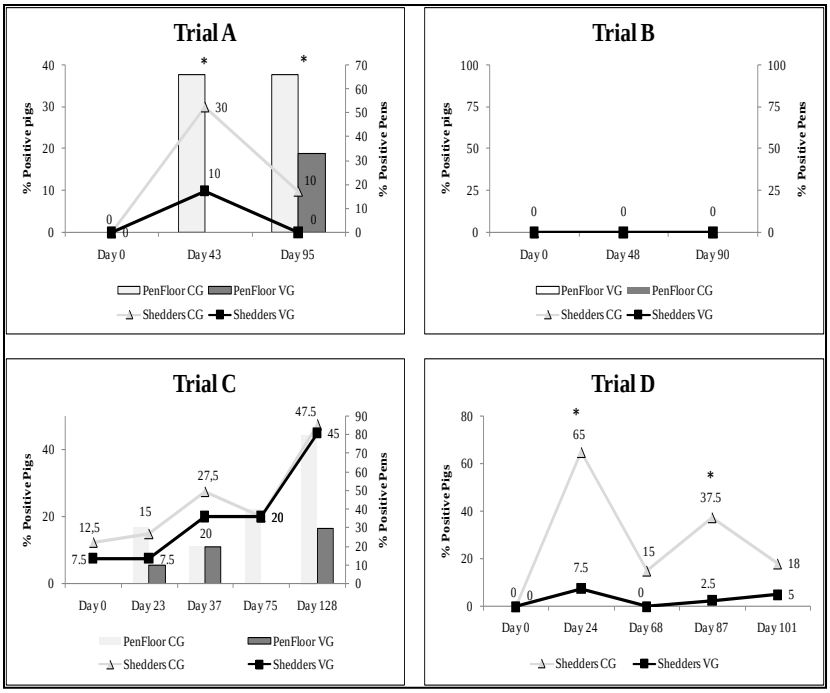
The bacteriological results for the samples collected at the farm from the animals and pens are summarised in Figure 2. Whereas *Salmonella* was not detected in the pens or faecal samples taken directly from the pigs in the first sampling round in trial A (day 0 post-vaccination), statistically significant differences were observed in the samplings performed half way through the fattening period (day 43 post-vaccination); 6 of the CG pens and none of the VG pens yielded positive results ($p<0.01$). In this sampling round, 30% of the control pigs shed *Salmonella* in their feces compared with 10% of the vaccinated pigs ($p=0.01$). At the end of the trial (day 95 post-vaccination), the proportion of shedders was significantly higher

among the control pigs (p=0.04), although no significant differences were observed in the proportion of positive pens (p=0.18). Overall, statistically significant differences were observed among the proportion of *Salmonella* shedders in the control (16 out of 120; 13%) and vaccinated groups (4 out of 120; 3%) (p=0.01; RR=4.0, 95% CI 1.38-11.62) as well as in the proportion of positive pens (12 out of 30, 40% and 3 out of 30, 10% for the control and vaccinated groups, respectively) (p=0.017; RR=4.0, 95% CI 1.25-12.75). The *S.* Typhimurium serotype was detected in all of the samples collected during trial A.

Salmonella was not detected in any of the 300 samples collected during trial B (240 individual samples and 60 pooled-pen faecal samples).

A similar proportion of *Salmonella* shedders were reported for both groups in the five sampling rounds performed during trial C (Figure 2). In total, 25.5% (51 out of 200) of the pigs from the CG and 21% (42 out of 200) of the vaccinated pigs had *Salmonella* in their feces. In contrast, the proportion of positive pens during the trial was significantly lower (p<0.01; RR=5.23, 95% CI 1.46-19.64) in the VG (6 out of 30;12%) compared with the CG (17 of 30;34%).In this trial, two different *Salmonella* serotypes were detected. The *S.* Rissen serotype was recovered in the samples from sampling rounds 1 to 4 and in a number of the samples from sampling round 5.*S.* Typhimurium was also detected in 2 pigs from the VG and in 5 pigs from the CG during sampling round 5 in unit C.

Figure 2. Bacteriological results from the individual fecal samples (lines) and pen fecal samples (bars) collected at farm in four clinical trials for the evaluation of the protection conferred by a *Salmonella* Typhimurium-inactivated vaccine.



Percentage of *Salmonella* positive pens from vaccinated (dark grey bars) or control group (light grey bars). Prevalence of shedders at each sampling in the vaccinated (■) or control (△) group and indicated by numbers. Significant differences are indicated at *(*P*-value <0.05).

Salmonella was not detected in the faecal samples collected during the first sampling round in trial D (Figure 2). The percentage of positive pigs in the VG was significantly lower than in the CG in the samplings performed at post-vaccination days 24 ($p<0.01$) and 87 ($p<0.01$).In total, 39 of the 40 ear-tagged pigs from the CG were monitored through the fattening period in unit D, and 34 of the pigs tested positive in at least one of the sampling rounds. In contrast, all 40 tracked pigs from the VG were monitored until the end of the

experiment, and only 5 of these pigs shed *Salmonella* in any of the five samplings performed. The proportion of shedders was significantly higher in the CG compared with the VG ($p<0.001$; RR=6.97, 95% CI 3.05-15.97).Moreover, whereas shedding in several samplings was common among pigs from the control group (14 yielded positive in two of the samplings, 8 in three of the samplings, and 3in four of the samplings), none of the pigs in the vaccinated group shed *Salmonella* in their feces more than once. The *S.* Typhimurium serotype was detected in all the positive samples from this trial.

The information gathered in trials A, C and D was analysed by a multilevel mixed-effects analysis. The prevalence of *Salmonella* fecal shedders was higher in the control group than in the vaccinated group ($p= 0.027$).

The slaughter samples were collected in trials A, C and D. Trial B was excluded because infection was not detected at the farm level. The results of the cecal content and MLN prevalence are reported in Table 2.

In trial A, significant differences were not observed between the CG and VG in the proportion of pigs positive for *Salmonella* in the cecal content ($p=0.81$) or MLN samples ($p=0.23$) or in the percentage of pigs that were positive for any of these two samples, 80% versus 67.5% ($p=0.16$). However, importantly, when only data from *S.* Typhimurium were considered, removing the positive samples of *S.* Rissen and *S*. Derby, only 4 of the26 positive cecal content samples

corresponded to the *S.* Typhimurium serotype in the VG, whereas 18 of the 27 isolates recovered from the CG were of this serotype ($p<0.01$; RR= 4.5, 95%CI 1.67-12.12). Most of the pigs in trial C were positive in at least one of the samples collected at the slaughter level (97.5% and 95% in the CG and VG, respectively). Slightly lower values were detected in the vaccinated pigs compared with the control pigs, although significant differences were not observed in the cecal content ($p=0.10$) or MLN samples ($p=0.24$). In contrast, statistically significant differences in *Salmonella* prevalence were observed in trial D for the cecal content samples ($p<0.01$; RR= 3.33, 95%CI 1.5-7.42), the mesenteric lymph nodes ($p<0.01$; RR= 2.44, 95%CI 1.29-4.64) or the pigs positive for any of these two samples (30% to 75%) ($p<0.01$; RR= 2.50, 95%CI 1.51-4.15).

Table 2. *Salmonella* serotypes detected in the samples collected during three clinical trials for the evaluation of the protection conferred by a *Salmonella* Typhimurium-inactivated vaccine. Data from trial B are not included since no *Salmonella* was detected along the finishing period and no samples were collected at the slaughterhouse.Prevalence of positive results at the slaughterhouse (cecal contents and mesenteric lymph nodes)are also showed.

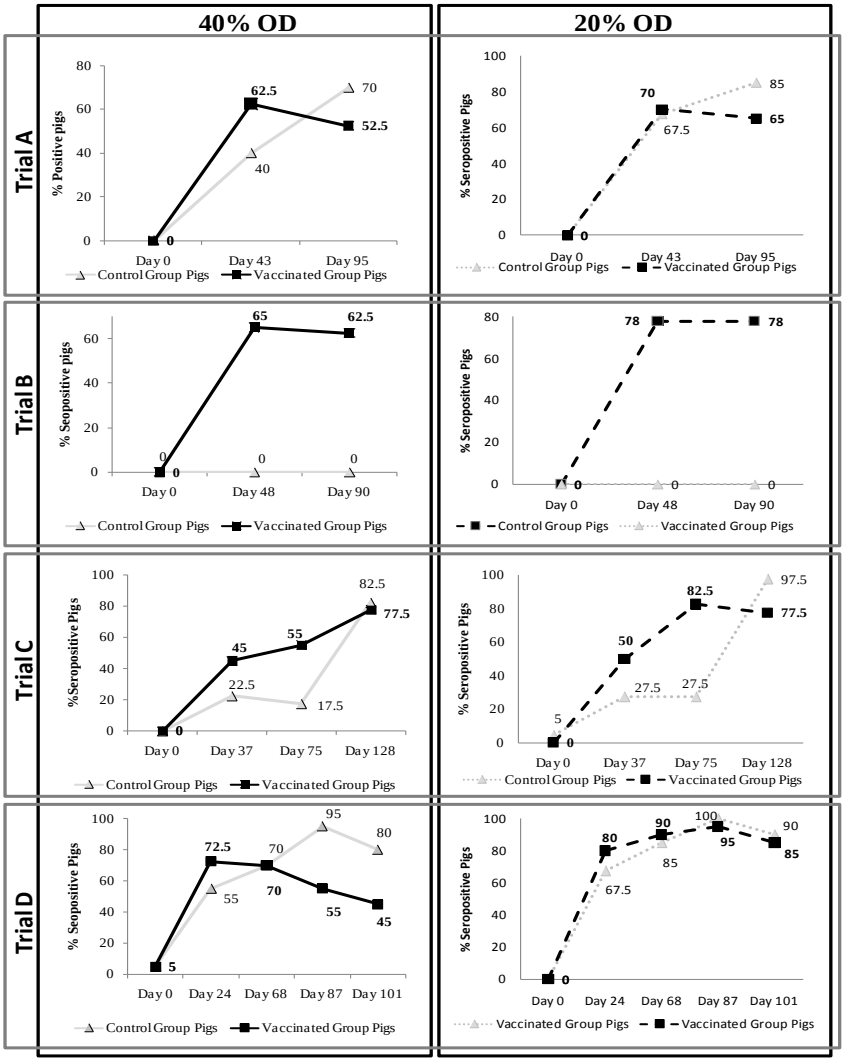
	Trial A		Trial C		Trial D	
	Control Group	*Vaccinated Group*	*Control Group*	*Vaccinated Group*	*Control Group*	*Vaccinated Group*
FARM						
Before pig introduction	-	Typhimurium	-	-	Typhimurium	Typhimurium
During the finishing	Typhimurium	Typhimurium	Rissen	Rissen	Typhimurium	Typhimurium
TRANSPORT	Typhimurium	Typhimurium	-	-	-	-
LAIRAGE	Rissen	Rissen	Rissen Typhimurium	Rissen Typhimurium	Typhimurium	Typhimurium
SLAUGHTERHOUSE						
Cecal Content	(67.5%) Typhimurium Rissen Derby	(65%) Typhimurium Rissen Derby	(72.5%) Rissen Typhimurium	(55%) Rissen Typhimurium	(50%) Typhimurium	(15%) Typhimurium
MLN[a]	(15%) Typhimurium	(10%) Typhimurium	(87.5%) Rissen	(77.5%) Rissen	(55%) Typhimurium	(22.5%) Typhimurium

[a]MLN Mesenteric lymph nodes

3.2. Serological results

No serocoversion was recorded at the beginning of the fattening period in trials A, B and D, whereas a low seroprevalence (only 2 seropositive pigs out of 40) was reported in the CG in trial C (Figure 3). High seroprevalence rates were reported in the control pigs sampled at the end of the fattening period in trials A, C and D. In contrast and consistent with the bacteriological results, seroreaction was not detected in the control pigs in trial B. More than half of the pigs tested exhibited values higher than 40 OD%3-5 weeks after vaccination, and the percentage of seropositive pigs detected using the 20 OD% cut-off at the end of the fattening period ranged from 65% to 85%. However, a slight decrease in the seroprevalence within the VG compared to previous samplings was reported using a 40 OD% cut-off value in trials A, C and D.

Figure 3. Seroprevalence (%) of *Salmonella* in vaccinated and control groups in four clinical trials for the evaluation of the protection conferred by a Salmonella Typhimurium-inactivated vaccine.



Seroprevalence of the vaccinated (■) and control pigs (△) with two different cut-offs: 40% and 20% optical density (OD) according to manufacturer's recommendations for the commercial ELISA. Vaccination was carried out once all the pigs were housed in the four units. At day 0, vaccinated group (VG) pigs were injected intramuscularly with a *Salmonella* Typhimurium-inactivated vaccine while no action was carried out in the control group pigs (CG).

4. DISCUSSION

The stimulation of the immune response by vaccines is a useful mechanism for fighting pathogens. A number of studies have tested vaccines against *Salmonella enteric* in challenge and clinical trials [32]. Based on this principle, most studies have focused on live attenuated *Salmonella* vaccines [25,33,34,35,36,37,38] because they elicit better cell-mediated immunity and therefore, they are more effective against facultative intracellular bacteria, such as *Salmonella* [13]. However, live vaccines exhibit several disadvantages [39,40], and there is still interest in *Salmonella* inactivated vaccines, which are easier to administer, cheaper to produce and even more secure than attenuated live vaccines. Therefore, several studies have tested the efficacy of inactivated vaccines with more-or-less promising results [22,23].In this study, we evaluated the usefulness of an inactivated *S*. Typhimurium vaccine to reduce *Salmonella* prevalence in fattening pigs. The trials, four in total, were performed in swine finishing farms with an endemic *Salmonella* infection as determined from the serological analysis of the last two fattened batches, which, according to current serological surveillance programs [30,31,41], were classified as highly contaminated.

Because pen-to-pen transmission occurs in approximately 90% of cases [8], separate pens were used as the experimental unit in clinical trials A, B and C. If the vaccinated and non-vaccinated animals were housed together, although the pen factor would be eliminated, the efficacy of the vaccination would be altered, considering that pigs

with theoretically different susceptibilities to *Salmonella* would be housed together, increasing the infectious pressure in the environment associated with faecal shedding by the pen mates to the vaccinated pigs while the opposite situation would occur to the control pigs. A markedly different design was used in clinical trial D, where the vaccination was performed at the barn level to simulate conditions close to those in the field, and 40 pigs were selected randomly at the beginning of the experiment and monitored throughout the fattening period in both the vaccinated and control groups. For this experiment, the selected farm had to fulfil several requirements; in addition to an endemic *Salmonella* infection reflected by a high serological score in previous batches, the infection had to be present in fatteners from both barns in the previous batch, and *Salmonella* had to be isolated from the environmental samples collected after the cleaning and disinfection procedures. These conditions allowed us to assume that the microorganism exposure and infection pressure at the start of the experiment in both groups was similar.

The vaccination strategies against *Salmonella* can be performed at several stages of swine production. For instance, by sows immunisation to protect their offspring [23] or vaccination early in life [24,35], during suckling [42] or after weaning [22]. However, several studies have reported that the majority of *Salmonella* infections in market-weight pigs occur during fattening [8,9,43]. In the present study, the vaccination was performed at the beginning of the fattening period with the idea of establishing proper measures at

the finishing farms which would reduce the number of *Salmonella*-infected pigs reaching the slaughterhouse and, consequently, would have an effect on consumer safety. The serotype selected to prepare the inactivated vaccine was *S.* Typhimurium because it is the most common serotype found in fattening pigs in Spain [15,16] and together with its monophasic variant, this serotype is relevant in human health [40,44,45].

Ideally, vaccines against *Salmonella* in pigs should prevent the colonisation of the host, shedding of the pathogen, development of the subclinical carrier state and development of the clinical disease [46]. Considering *Salmonella* infection is seldom associated with clinical disease in pigs, the efficacy of vaccination was evaluated by monitoring *Salmonella* shedding and colonisation of the host. The global analysis using multilevel mixed-effects model as previously described [22,47] demonstrated that vaccination was associated with a decreased *Salmonella* shedding. Nevertheless, consistent with other studies [49] a separate statistical analysis was also performed within each trial and time point. It must be considered that two different serotypes and two different experimental designs were performed. The analysis of the data within each farm revealed that the inactivated *S.* Typhimurium vaccine conferred protection against infection by the same serotype (trials A and D) whereas it failed to confer cross-protection, at least against the *S.* Rissen serotype and most likely against serotypes from *Salmonella* serogroup C_1 according to data from trial C. Consistent with our results, other studies have reported the low heterologous protection by *S.*

Typhimurium vaccines [22,49], especially against serogroup C_1, likely because of the disparity between the *S.* Typhimurium and the group C_1-typeantigens [49].

By the slaughterhouse results it can be deduced that protection was gained in vaccinated pigs against *S.* Typhimurium infections, with a lower number of *Salmonella* carriers in MLN and cecal content (trials A and D). In trial A this result was disguised by the presence of positive samples with serotypes not described previously at the farm, *S.* Derby and *S.* Rissen, which were probably acquired during the transport or lairage of the pigs. The appearance of new infections after leaving the farm are relatively common [10] and can modify the results of the clinical trials. According to farm results, no differences between the groups were observed in trial C where a heterologous infection was established.

Overall, our bacteriological data suggest that the inactivated *Salmonella* Typhimurium vaccine used in this study confers protective immunity to pigs reared in *S.* Typhimurium-infected farms. This concept is not new, and other studies have documented partial protection including the reduction in faecal shedding and intestinal colonisation after immunisation with an anti-*Salmonella* bacterin [50,51]. Much of the current knowledge regarding *Salmonella* infection and anti-*Salmonella* immune mechanisms are derived from the murine-typhoid model [52]; however, a number of gaps remain regarding the immune response elicited by non-typhoidal *Salmonella* serotypes in other species, such as swine [53,54]. Inactivated vaccines stimulate several potentially effective mechanisms.

However, the identification of effective antigens in *Salmonella* has proved elusive. Several antigens, such as LPS, flagella or fimbriae induce at least some degree of T-cell response [26,55]. Moreover, LPS could play an immunostimulatory role, as previously established, inducing the production of an enhanced number of antigen-specific T cells, increasing the migration into B-cell follicles and promoting antibody production [56]. Furthermore, the specific antibodies induced by inactivated vaccines can provide a degree of protection during the extracellular phases of the infection and IgA production, which contributes to mucosal protection [23].

The seroconversion of control pigs from trials A, C and D demonstrated the instauration of infection while the results from, trial B confirmed that no infection occurred during this trial. In contrast, the high seroprevalence reported in the vaccinated pigs, confirmed the strong immune response induced by the vaccine. This high anti-LPS response in the vaccinated animals, together with the failure in heterologous protection, is the main restricting factor for the bacterin used in this study to be applied in *Salmonella* control programs if surveillance is based on the detection of antibodies against LPS antigens [30,31,42]. The earlier vaccination of the pigs, at the growing/nursery period would be an option to confer protection and simultaneously allow time for a decrease in the anti-*Salmonella* titters in the serum. In addition, the development of DIVA-vaccines that do not interfere with *Salmonella* control programs has been proposed in other studies [25,36] or the use of bacteriology instead

of serology for the monitoring of those herds where the vaccine is applied would also avoid this problem.

In summary, the results of this study suggest that the vaccination of pigs with a *S.* Typhimurium bacterin reduces the shedding and horizontal transmission during the fattening period as well as the proportion of *Salmonella* shedders or carriers at the slaughterhouse when a homologous infection is established. In contrast, the high humoral response elicited complicates the use of these vaccines in farms that are under serological surveillance programs.

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CAPÍTULO III/CHAPTER III

El PAPEL DEL MATADERO

THE ROLE OF THE SLAUGHTERING

Calendario Agrícola Panteón de los Reyes de Léon. Siglo XII (San Isidoro, León, España).

Mes de noviembre. El matarife sujeta al cerdo por una oreja mientras se dispone a sacrificarle.

1. Introduction

Article review:

Arguello H., Álvarez-Ordoñez A., Carvajal A., Rubio P., Prieto M. 2013. Role of slaughtering in salmonella spreading and control in pork production. Journal of Food Protection, 76: 899-911.

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Review

Role of Slaughtering in *Salmonella* Spreading and Control in Pork Production

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ABSTRACT

Salmonella is one of the major foodborne pathogens worldwide. Pork products are among the main sources of *Salmonella* infection in humans, and several countries have established *Salmonella* surveillance and control programs. The role of slaughtering in carcass contamination has been indicated by studies focused on the slaughterhouse environment. In this review, we examine and discuss the information available regarding the influence that farm status, pig transport, and lairage have on the carriage of *Salmonella* by pigs entering the slaughter line. The evolution of carcass contamination throughout the slaughtering process, the main sources of contamination in the dirty and clean zones of the slaughter line, and previously reported prevalence of *Salmonella* on carcasses and factors affecting this prevalence also are discussed. The importance of implementing interventions at the slaughter level is discussed briefly. Consistent with the information available, pigs from infected farms and newly acquired or recrudescent infections in pigs at the subsequent stages of transport and lairage are important sources of *Salmonella* at the slaughtering plant. The continuous introduction of *Salmonella* into the slaughterhouse and the potential for resident flora constitute a risk for carcass contamination. At the slaughterhouse, some dressing activities can reduce carcass contamination, but others are critical control points that jeopardize carcass hygiene. This information indicates the importance of considering slaughter and previous stages in the pork production chain for controlling *Salmonella* in swine production.

Foodborne zoonoses control and prevention is a major concern in industrialized countries. Among these zoonoses, salmonellosis is cited as one of the most important. Currently, human nontyphoid salmonellosis is the most common bacteria-related foodborne illness in the United States *(26)* and has caused the highest number of outbreaks in the European Union (EU) *(40)*. Although the infection pressure has declined in Europe in the last years due to a decrease in *Salmonella* prevalence in the laying hen reservoir *(39),* the number of human salmonellosis cases has not declined significantly in more than a decade in the United States according to the Centers for Disease Control and Prevention *(27)*. In the United States, *Salmonella* was involved in 25% of foodborne outbreaks transmitted by pork and 36% of total human cases from 1990 to 2005 *(4)*. During the 1990s and early 2000s in the EU, pork products were estimated to be associated with 5 to 15% of total human salmonellosis cases *(13, 48)*; pork is usually categorized as the third most common source of human infections after eggs and poultry and turkey meat. Because of the implementation of control programs in avian production and the subsequent reduction of *Salmonella* prevalence in that industry *(40),* the relative importance of pork and pork products has increased and the pig reservoir has been reported as the second most common source of human salmonellosis, after laying hens, in the EU based on the microbial subtyping model for source attribution *(76)*. However, the fraction attributable can vary considerably among countries and depends on aspects such as the *Salmonella* prevalence in pork and pork products, consumption patterns, and the relative importance of other sources of *Salmonella*.

Two pieces of legislation have been implemented for the control and monitoring of *Salmonella* in the EU: Directive 2003/99/EC *(32)* on the monitoring of zoonoses and zoonotic agents and Regulation 2160/2003 *(33)* on the control of *Salmonella* and other specified foodborne zoonotic agents. Regulation 2160/2003 ensures that effective measures are taken to detect and control *Salmonella* and other zoonoses at all stages of production, processing, and distribution, particularly at primary production, to reduce the prevalence of these microorganisms and the concomitant public health risk. One of the measures foreseen is the adoption of targets for prevalence reduction in animal populations at the primary production level. With the idea of reducing the contamination of pork by *Salmonella,* national control programs and strategies are or will be getting underway *(33, 70, 73, 79)*. Finishing farms and slaughterhouses are the best targets for implementing successful

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control strategies in swine production. Being located at the end of the primary production chain, slaughtering interventions can play a decisive role in the control of dissemination of *Salmonella*. In the last 15 and especially the last 10 years, many research studies have been performed to understand the role of slaughtering in swine salmonellosis, mainly focused on *Salmonella* contamination of the carcass *(6, 15, 23, 29, 49, 56, 59, 75, 90, 94)*. The results obtained indicate that hygienic procedures during slaughtering are very important in the control of *Salmonella*. The present article, written from a European perspective but also considering information available from American studies, summarizes all this information, highlighting the most important findings regarding the impact of slaughtering in the control of *Salmonella* in the swine sector, analyzing the role that transport and lairage play in the establishment of new or reinfections, and discussing the spreading of *Salmonella* at the slaughter line level. Data on *Salmonella* prevalence in pig carcasses and the factors that can influence contamination of the carcass also are included. The importance of implementing interventions at slaughter level is discussed briefly. The potential for contamination after leaving the farm and the fact that proper implementation of slaughterhouse strategies can deliver the maximum risk reduction at minimal costs compared with previous points of the production chain *(45)* are considered. The slaughterhouse is the only point of the production chain, after leaving the farm, where the contamination can be removed.

SALMONELLA AT SLAUGHTER IN SWINE PRODUCTION: INFLUENCING FACTORS

Several authors have tracked pigs from the farm to the abattoir *(6, 31, 56, 62, 66)* to determine their *Salmonella* status throughout these stages. The results from these studies, supported by results of other specific studies on transport and lairage *(53, 68, 77, 89)*, draw a picture of what occurs in the stages preceding slaughtering in terms of the acquisition or recrudescence of *Salmonella* infections. Other authors have focused their efforts on the evaluation of *Salmonella* contamination throughout the slaughter line *(3, 23, 30, 49, 94, 98)*. This pattern of *Salmonella* contamination from farm to the slaughterhouse is schematically represented in Figure 1.

***Salmonella* infections at preharvest stages: influence of finishing pig status.** Finishing herds have been the target of *Salmonella* control programs *(5, 36, 70, 79)* and are the principal part of the production chain on which efforts to reduce *Salmonella* prevalence have been focused *(3)*. The complex epidemiology of this pathogen, its presence at all stages of the food chain, and the lack of consensus regarding the major sources of infection and contamination *(47)* lead to the following question: What role does the status of market weight pigs at the farm play in the final contamination of the same animal at the slaughterhouse?

Various studies have been conducted to establish an association between positive serology and *Salmonella* detection by culturing at slaughter, but conflicting results have been obtained. Some authors have found an association between herd or pig serological status and *Salmonella* in cecal contents or carcasses *(59, 66, 84)*. In contrast, other authors have not found such an association between herd or pig serological status and bacteriological results of individual pigs at slaughter *(23, 31, 97)*. When comparing the *Salmonella* serotypes and molecular types from farm and abattoir isolates, several surveys have revealed complex interrelationships. In many cases, the strains detected in samples collected at the slaughterhouse differed from those detected at the farm *(6, 23, 31, 53, 56, 62, 97)*. Hurd and colleagues *(53)* analyzed feces, cecal contents, and lymph nodes from pigs necropsied at the farm and compared the results with those from pigs from the same farm that were slaughtered at the slaughterhouse. The *Salmonella* prevalence was seven times higher at the slaughterhouse than at the farm. This finding is not an exception and agrees with results of other monitoring studies *(6, 12, 42)*. Results from these studies reveal that the final *Salmonella* status of pigs at slaughter is affected by more than the farm status. What is the actual contribution of finishing pig *Salmonella* status to final carcass contamination? Berends and colleagues *(13)* used a mathematical approach to determine the effects of six different scenarios for control of *Salmonella* in pork production. In their study, the percentage of *Salmonella*-positive pigs and carcasses was estimated from the end of the finishing period to the retail level. The results from their mathematical model were based on data extracted from the experiences of Berends and colleagues in The Netherlands during the 1990s *(12–15)*. These authors concluded that *Salmonella*-positive carcasses at slaughter and *Salmonella*-positive cuts at retail are clearly related to *Salmonella*-positive pigs at the farm. However, these results contrast with those of studies in which *Salmonella*-positive carcasses and/or cuts were equally distributed among pigs from *Salmonella*-positive and -negative farms *(6, 31)*.

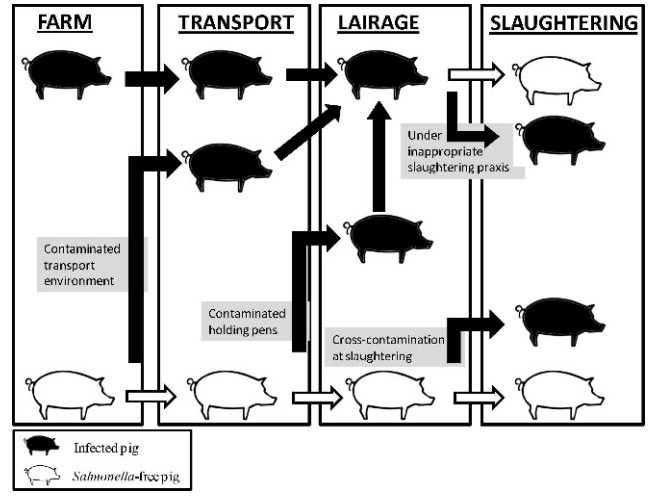

FIGURE 1. *Progress of contaminated pigs (black) and* Salmonella*-free pigs (white) from the farm to the slaughterhouse. Although infected pigs reach the slaughter line carrying* Salmonella *and the final status of each carcass depends on the slaughtering practices,* Salmonella*-free pigs can become contaminated during transport, in the holding pens, and by cross-contamination in the slaughter line.*

These conflicting results indicate that the effect of *Salmonella*-positive pigs at the farm level on carcass status at the slaughterhouse also depends on factors such as study design, slaughtering conditions, and global prevalence on those farms that supply the slaughterhouse. Infected pigs from *Salmonella*-positive farms constitute a risk and may be a vehicle for *Salmonella* spreading in later steps of the pork production chain (Fig. 1).

***Salmonella* infections at preharvest stages: the role of transport.** From farm to slaughter, an increase in *Salmonella* prevalence can be attributed to new contamination sources. Transport is undoubtedly one of these sources *(15, 42, 62, 63)*. Several factors favorable for *Salmonella* dispersion converge during transport, make this step one of the main risk areas in pig infection.

Stress and feed withdrawal are important factors related to transport that can promote *Salmonella* shedding by carrier pigs. Stress is associated with a recrudescence of the infection *(71, 95)*. Handling, loading, mixing with unfamiliar pigs, high stocking densities, and changes in environment, including temperature, are some of the associated factors that promote stress. Feed withdrawal during the 12 to 24 h before slaughtering is recommended to reduce the proportion of pale, soft, and exudative pork and also to facilitate evisceration *(69)*. Feed withdrawal can create stress and intestinal disorders, which can promote *Salmonella* shedding. However, published information indicates that the stress created by feed withdrawal does not clearly affect the shedding of *Salmonella (55, 65, 69)*. As concluded by Hurd and colleagues *(51),* more research is needed to identify all the factors associated with the increase of *Salmonella* shedding during transport.

The influence of transport time has been evaluated, and some researchers have assumed that the longer the haul the higher the level of *Salmonella* shedding *(56)*. However, in some studies no differences in *Salmonella* shedding have been found between short or large hauls *(77)*. However, an increase in *Salmonella* prevalence after transport has been detected in several studies *(8, 55, 63, 77)*. Because of this increase in shedding, which seems to be promoted by several factors, high levels of *Salmonella* can be found in trucks and bedding material after transport.

In most industrialized countries, washing and disinfection of slaughter trucks before they leave the abattoir is mandatory; nevertheless, *Salmonella* is frequently found in slaughter trucks after transport *(8, 63, 77, 88),* and several authors have reported high percentages of *Salmonella*-positive samples after truck washing *(8, 63),* which shows the inefficiency of the current washing protocols. Analyses of preloading samples from transport vehicles indicate that transport is also a source of infection for *Salmonella*-free pigs.

Considering the highly contaminated transport environment and the high stocking densities, surface-to-skin and skin-to-skin cross-contamination can occur with relative frequency. The presence of *Salmonella,* the rapid establishment of infection *(17, 37, 47, 52),* and the long trips from the farm to the slaughterhouse are the perfect combination for promoting new *Salmonella* infections.

***Salmonella* infections at preharvest stages: lairage as a critical control point.** After transport to the abattoir, pigs usually rest in holding pens before being slaughtered. Keeping pigs in lairage provides a buffer for the slaughter line, allows the pigs to recover from the stress of transport, and is essential for meat quality *(98)*. However, lairage has been cited by many authors as one of the foremost locations affecting the final *Salmonella* status of pigs at slaughter. The increase in *Salmonella* prevalence and the new serotypes detected at the slaughterhouse *(53)* were attributed to holding pen contamination. Other authors also have stressed the role of holding pens as a source of *Salmonella* contamination *(17, 31, 51, 78, 91)*. Several factors make lairage one of the most risky points for pig contamination through the soiling of skin or gastrointestinal infection by the oral route. These factors can be grouped into two categories: cross-contamination of pigs at lairage and the failure of lairage cleaning protocols.

Newly acquired infections or the recrudescence of those already present in carriers during transport can result in the shedding of *Salmonella* during the lairage period. During lairage, many of the same stress factors present during transport, such as variations in temperature, high stocking densities, and noises, also can occur *(68)*. As a result, high levels of *Salmonella* are shed during this time, and high *Salmonella* contamination levels in the holding pens during this time has been documented *(23, 24, 30, 31, 78, 80, 89)*. This highly contaminated environment increases the likelihood of introducing *Salmonella* into the slaughter line. Time spent in lairage is variable and depends on abattoir routines and on the slaughterhouse logistics and transport frequency. Lairage time can be up to 24 h, although shorter periods (e.g., 1 to 6 h) are most common but are long enough for *Salmonella* to colonize the gut. However, lack of rest or rest for short periods is detrimental to meat quality *(51)*. Even though some time in holding pens is necessary, prolonged resting times (including overnight stays) for infected pigs result in excretion of high levels of *Salmonella* in feces *(24),* and the placement of *Salmonella*-free pigs in contaminated holding pens may result in colonization of these pigs by these pathogenic bacteria *(31, 91),* with consequent increases in pig contamination *(3)*.

The other factor related to lairage contamination is the failure of the cleaning protocols. Insufficient cleaning protocols are common in abattoirs *(8, 24, 30, 80, 89)*. Cleansing often consists of the removal of gross fecal matter by high-pressure cold water washing. This practice does not remove *Salmonella* from the lairage environment *(8, 24, 80, 81, 91)*. Cleaning protocols could be improved by the addition of foaming and sanitizing solutions *(64),* and our personal experience supports this hypothesis (data not published). Nonetheless, several authors have reported the failure of these improved protocols *(24, 80, 89)*. Two factors are mainly responsible for the persistence of *Salmonella* in the holding pen environment: (i) the floor and wall surfaces are very uneven and contain many holes that hinder the action of cleaning solutions and accumulate organic matter where *Salmonella* can survive, and (ii) the organic matter can facilitate the survival of *Salmonella* within biofilms,

thereby decreasing the efficacy of common solutions used for cleaning and disinfection *(67, 87)*. Both factors diminish the efficiency of cleaning protocols and favor the persistence of *Salmonella* in the lairage environment. Despite the fact that cleaning and disinfection procedures described in the literature were not effective for completely removing *Salmonella* from the holding pens, some authors have reported lower *Salmonella* prevalence in those holding pens that were cleaned and disinfected compared with those in which only high-pressure water washes were applied *(80, 89)*. Swanenburg and colleagues *(89)* found lower levels of both *Salmonella* and *Enterobacteriaceae* in environmental samples after disinfection compared with those in samples collected after usual cleansing procedures. Boughton and colleagues *(24)* found that only 6% of the environmental samples were *Salmonella* positive after application of cleaning and disinfection protocols compared with 44% when holding pens were cleaned with high-pressure cold water. In a pilot field trial with *Salmonella*-contaminated bovine fecal matter, the combination of pressurized water and steam was the most effective cleaning protocol, followed by the application of a sanitizing agent at the greatest concentration recommended by the manufacturer, and then by pressure washing alone *(82)*. Pressure washing followed by a delayed steam application gave poor final results on the surface at that study. Although not reported in the literature, cleaning of the lairage area more frequently throughout the working day (i.e., between batches) should diminish the *Salmonella* burden at lairage. Proper cleaning is the most important activity at the lairage level, but other actions also are important, such as handling of pigs as gently and quietly as possible, avoiding comingling of unfamiliar pigs, keeping lairage time to an absolute minimum, processing *Salmonella*-free herds at the beginning of the slaughtering day, and designing premises with special attention to the presence of slatted floors and smooth surfaces in the holding pens. Because of the complex ecology and epidemiology of *Salmonella,* the success of interventions implemented at the lairage level does not guarantee the desired reduction in *Salmonella* prevalence unless other previous or subsequent points in the production chain also are under control *(80)*.

***Salmonella* at the slaughter line: factors affecting contamination.** *Salmonella* contamination at carcass level is also an important issue in the pork production chain. The changes in carcass contamination through the slaughtering process and the sources of contamination at the slaughter line are discussed here based on the literature available.

An understanding of the epidemiology of *Salmonella* at slaughtering level is needed when studying the various points of the slaughter line implicated in the final status of the carcass. Two sources of *Salmonella* have been identified within the slaughtering facility: (i) the environment harboring *Salmonella* already present in the slaughter line and (ii) the inputs of *Salmonella* introduced by contaminated or infected pigs.

Regarding the *Salmonella* strains already present in the environment, those isolates that survive in certain niches of the slaughterhouse can become part of the resident flora (house strains) *(7, 9, 23, 44, 49, 75, 88, 94),* thereby constituting a source of contamination for the incoming carcasses. Nevertheless, those strains introduced by pigs entering the slaughtering process are the strains responsible for the majority of carcass contamination. This assertion is based on the evaluation of typing results from slaughtering studies. These results indicate that *Salmonella* serotypes and their relative prevalence differ from one visit to another within the same slaughterhouse *(23, 37, 44, 88)* and that several serotypes can be involved in environmental and carcass contamination within the same sampling day *(6, 29, 31, 49, 75, 94)*. Molecular analysis of isolates of the same serotype has revealed that several differentiated strains enter the slaughterhouse continually *(7, 17, 75, 94)*. Molecular typing methods such as pulsed-field gel electrophoresis or multilocus variable tandem repeat analysis, provide the opportunity for clarifying the relatedness of *Salmonella* isolates from the same serotype *(57)*. Giovannacci and colleagues *(44)* found an association among profiles from different samples from the same sampling series, and in another study *(7) Salmonella* Typhimurium profiles in carcasses differed throughout the working day and sometimes matched profiles of those strains collected from workers and equipment at an adjacent time. The occurrence of a particular genotype was herd dependent in the study carried out by De Busser et al. *(30),* and Letellier et al. *(59)* found that strains from incoming animals were likely, within a limited period of time, to contaminate the slaughterhouse. Therefore, new strains of *Salmonella* are continuously entering the slaughterhouse and thus constitute a risk of carcass contamination. This continuous input can explain the increase in contamination throughout the working day reported by several authors *(6, 44, 49, 89)*. An increase in contamination across the week also has been described *(66)*. These results can be the consequence of the inefficiency of cleaning protocols used during the week, which would facilitate the accumulation of *Salmonella* in the environment, or of reduced observation of good manufacturing practices (GMPs) by the staff. Although machinery always works with a similar precision, the efficacy of the actions of human operators depends on aptitude and willingness. Thus, the attention paid to work activities may decrease through the day and the week because of factors such as fatigue and could result in an increase in carcass contamination. Training of staff is another important factor; the highest *Salmonella* prevalence detected at a slaughterhouse was linked to a staff change *(6)*.

The slaughter line can be divided into two zones: dirty and clean. The dirty zone is a mechanized area where the stunning, killing, bleeding, scalding, dehairing, singeing, polishing, and shaving processes are carried out. The clean zone comprises the carcass dressing activities, which are at least in part carried out manually by workers. The sources of contamination in both areas are very different. Figure 2 summarizes the effects of each point of the slaughtering process on *Salmonella* contamination of carcasses and the consequences for the final status of the carcass.

***Salmonella* at the slaughter line: contamination of carcasses in the dirty zone.** At the entrance of the slaughter

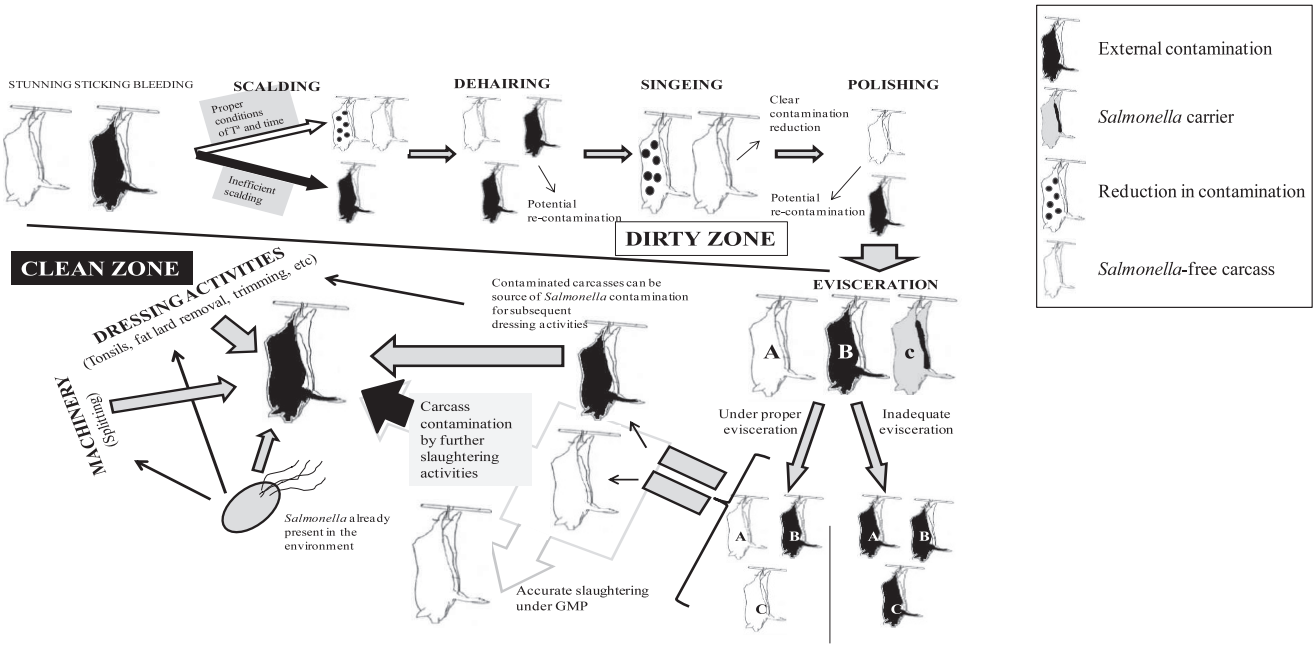

FIGURE 2. *Contaminated and* Salmonella*-free carcasses enter the dirty zone of the slaughterhouse. Proper scalding reduces contamination, but inadequate scalding can result in carcass contamination. Dehairing and to a lesser extent polishing are potential recontamination points for carcasses. Between these points, singeing reduces or removes external contamination. As a result, carcasses with and without* Salmonella *can enter the clean zone. At this stage, carrier pigs constitute a second source of* Salmonella *contamination. Under proper evisceration practice, only externally contaminated carcasses (B) carry* Salmonella. *When intestinal lacerations or ruptures occur,* Salmonella *can be spread directly to the carcass (C) or indirectly to carcasses of noncarrier pigs (A). After evisceration,* Salmonella*-contaminated carcasses are a potential source of recontamination for the environment and other carcasses. Under proper slaughtering practice, carcasses can reach the end of the slaughter line free of contamination, but a potential for recontamination is associated with further dressing activities or machinery that has become contaminated by other carcasses or by* Salmonella *already present in the environment.*

line, pigs are stunned, killed, and exsanguinated. A number of studies have revealed high levels of contamination of carcasses at the exsanguination point *(29, 74, 94)*. Skin contamination can occur frequently when the lairage environment is contaminated or penmates shed *Salmonella*. Workers in contact with carcasses can disperse *Salmonella* from contaminated to noncontaminated carcasses. Stunned pigs usually contact the same zones that may be contaminated by *Salmonella*. Stunning by carbon dioxide, as opposed to electrical stunning, may lead to increased shedding of *Salmonella* through voiding of feces after muscles relax *(38)*. After exsanguination, a variety of heating, washing, and abrasive activities, including scalding, dehairing, singeing, and polishing, are applied to reduce the visual and microbiological contamination of the hide *(18)*. Scalding occurs in water baths or steam showers at 55 to 70°C. Water usually is reused and becomes contaminated with dirt, feces, ingesta, and microorganisms carried by the pig *(18)*. When water temperature is less than 61 to 62°C or contains large amounts of organic matter, *Salmonella* can be isolated from scalding tank water *(6, 24, 49, 90)*. Some authors have suggested that scalding tank temperature is crucial because scalding water can be an important source of *Salmonella* cross-contamination *(44, 59),* and Hald and colleagues *(49)* described a link between scalding and carcass contamination at evisceration. Bolton and colleagues *(18)* recommended scalding temperatures of greater than 61°C. They found that a temperature decrease from 60 to 58°C resulted in a 2.2-fold increase in *D*-values for a cocktail of *Salmonella enterica* strains. In addition to temperature, treatment time must be included when developing thermal destruction models. Bolton and colleagues *(18)* found equivalence for time-temperature combinations of 1.4 min at 60°C, 0.94 min at 61°C, and 0.62 min at 62°C to ensure a *Salmonella* reduction of 1 log unit per 10 ml. However, higher target destruction levels should be established to include a satisfactory safety margin. Therefore, scalding must be included as a verifiable control point within improved GMPs and hazard analysis and critical control point (HACCPs) programs. After the reduction of bacterial populations expected at scalding, dehairing constitutes a point at which carcasses can be contaminated again. *Salmonella* can be spread to carcasses from the organic matter accumulated on the rotating dehairing flails. Evidence of dehairing contamination was reported by Davies and colleagues *(29)* and Pearce and colleagues *(74),* who described increases in *Salmonella* prevalence in carcasses from 1 and 5.7%, respectively, after scalding to 7 and 42%, respectively, after the subsequent dehairing. Singeing of carcasses after dehairing then reduces the presence of *Salmonella* to very low levels. According to Alban and Stärk *(3),* singeing is the only step in the production process at which *Salmonella* can actually be removed. However, reduced singeing efficacy related to

energy saving procedures or to the presence of large amounts of *Salmonella* in deeper layers of the skin increases carcass prevalence according to the model described by Alban and Stärk *(3)*. Berends et al. *(15)* estimated that when singeing is incorrectly performed a reduction in *Salmonella*-positive carcasses of only 5 to 30% is achieved. Some authors have concluded that polishing does not constitute an important point of recontamination *(3, 29, 74, 90)*. This step occurs after singeing, and the level of *Salmonella* at this point of the slaughter line is often negligible, as indicated by low *Salmonella* and *Enterobacteriaceae* counts usually found at this point *(15, 74)*. However, when singeing is not properly performed, as with dehairing equipment *(49)* polishing fingers and brushes can become contaminated, and contamination can be enhanced by the fact that cleansing of polishing equipment is difficult to achieve *(21),* thus allowing dispersal of *Salmonella* or other pathogens. Berends et al. *(15)* estimated that the polishing stage was directly responsible for 5 to 15% of all carcass contamination when singeing was not effective. In another study, the contamination of lairage was linked by logistic regression to the contamination of carcasses after polishing *(30)*. As suggested in that study, a second flaming step after polishing would be helpful for keeping contaminated carcasses from entering the clean zone of the slaughterhouse.

***Salmonella* at the slaughter line: contamination of carcasses in the clean zone.** External carcass contamination is not expected at entry to the clean zone. From there, the main source of carcass contamination is infected tissues from infected pigs. Tonsils, the gastrointestinal tract (especially the cecum but also the ileum or colon), and lymph nodes such as the mesenteric and ileocecal lymph nodes in the gastrointestinal tract and the mandibular or retropharyngeal lymph nodes in the head frequently harbor *Salmonella (41, 100, 101)*. Therefore, inappropriate dressing and inspection procedures can result in dispersal of *Salmonella* across the slaughter line, with consequent contamination of carcasses. Evisceration is one of the important activities evaluated in studies of *Salmonella* at slaughterhouses *(3, 6, 29, 46)*. The spillage of intestinal contents because of perforation during evisceration is one of the major sources of carcass contamination. The increase in carcass *Salmonella* prevalence reported after the evisceration process clearly reflects the major role that this activity plays in carcass contamination *(29, 31, 74)*. Berends and colleagues *(15)* estimated that 55 to 90% of all carcass contamination occurs during evisceration. Thus, evisceration must be taken into account when designating critical control points (CCPs) and GMPs within HACCP systems. Both Alban and Stärk *(3)* and Hald et al. *(49)* proposed several ideas for reducing the risk of spreading *Salmonella* at this point, such as sealing of the rectum with a plastic bag, automation of the process, or loosening the bung by lactic acid sanitation; some of these interventions could be easily included in routine evisceration processes. Analyses of other dressing activities, such as tonsil, kidney, fat, or lard removal, trimming, or meat inspection, have revealed that carcass contamination is not restricted to the evisceration step *(6, 23, 49, 96)*. Additional trimming of carcasses with obvious lesions has been associated with an increase in *Salmonella* prevalence *(54)*.

Contaminated carcass surfaces can carry and spread *Salmonella* to other points of the processing line, which subsequently constitute a source of contamination for *Salmonella*-free carcasses. Once the line is contaminated, *Salmonella* can be isolated from the machinery and the hands of workers up to the next break or possibly until the end of the working day, when the line is routinely cleaned and disinfected; thus, cross-contamination due to inappropriate procedures is likely to occur occasionally during working hours and cannot be avoided. Therefore, adequate cleaning and disinfection of the line is necessary to prevent the establishment of a permanent endemic flora. Workers and their tools are probably more important than the machinery *(15)*. Hands, knives, and knife sheaths are more commonly contaminated with *Salmonella* than is machinery. Frequent and systematic hygienic measures during carcass dressing, such as hand washing, changing of gloves, and two-utensil systems (while one knife is in use the other is being sanitized in a high-temperature bath at approximately 82°C), are necessary to avoid permanent contamination during these activities. The slaughter line equipment, principally the splitting saw, also has been analyzed in studies of contamination. The splitting saw is frequently contaminated with *Salmonella (6, 23, 49, 91)* and has been linked to cross-contamination and the establishment of an endemic house flora *(30, 83, 90, 94)*. Bung droppers and belly openers have also been cited as potential sources of *Salmonella* because these items are frequently contaminated, although adequate sanitizing temperatures can avoid problems *(6, 94)*. At the end of the slaughter line, carcasses are subjected to a chilling process. Although chilling temperatures are not bactericidal for most gram-negative bacteria, *Salmonella* prevalence has been reported to decrease after chilling procedures *(6, 11, 23, 30, 92)*.

In conclusion, adequate slaughtering activities cannot avoid the introduction of *Salmonella* into the slaughter line from infected pigs but can prevent or reduce part of the contamination from the slaughtering process. In another review of *Salmonella* prevalence in pork carcasses, the authors concluded that from slaughter to cooler there is a steady decrease in *Salmonella* prevalence *(72)*. Reductions in *Salmonella* prevalence can be achieved at some points of the slaughter line, whereas other points act as contamination promoters (Fig. 2). When the slaughtering process is carried out adequately, pigs carrying *Salmonella* in their intestines will theoretically not have these bacteria on their carcasses or in their meat after slaughtering *(90),* although this ideal is difficult to achieve in practice. HACCP programs are recommended by food safety agencies *(34, 93),* and slaughter hygiene and protocols conducted according to GMPs are important for preventing cross-contamination of carcasses during the slaughtering process. The previous determination of CCPs is essential for implementing additional hygienic measures in the slaughter process. Scalding, singeing, splitting, chilling, evisceration, and

other handling activities are all areas where CCPs can be designated and GMPs or standard manufacturing practices should be applied, but each slaughterhouse must revise its own CCPs and establish adequate GMPs.

SALMONELLA PREVALENCE IN CARCASSES

The carcass is the focus of all investigations of the slaughtering process. The status of the carcass at the end of the dressing steps is the consequence of all the factors previously discussed (farm, transport, lairage, and slaughtering) and reflects the actual risk of delivering contaminated meat to consumers.

Salmonella prevalence on carcasses is the result of all the factors discussed up to this point (Figs. 1 and 2). This question remains: What is the effect of each factor on the final level of carcass contamination? The data published by the European Food Safety Authority *(37)* in its slaughter pigs baseline study provides some answers to this question. In that study, the mean percentage of contaminated carcasses increased with the proportion of *Salmonella*-positive pigs (infected at farm, transport, or lairage) introduced to the slaughterhouse, but the proportion of contaminated carcasses differed significantly among slaughterhouses that had a similar proportion of infected pigs. These results indicate that *Salmonella*-positive animals supplied to the slaughterhouse represent a potential risk of contamination for their own carcasses and for other carcasses throughout the abattoir. Differences detected among slaughterhouses indicate that slaughtering activities also affect the final carcass status. Using a mathematical model including data from The Netherlands, Berends et al. *(15)* attributed 70% of carcass contaminations to infected pigs and the remaining 30% to cross-contamination during the slaughter process. This estimation has been confirmed by others *(68, 96)*. However, higher rates of cross-contamination rates have been reported by some authors. Cross-contamination accounted for half of the contaminated carcasses in two studies *(6, 99),* and Duggan and colleagues *(31)* estimated that 69% of all contaminated carcasses could be traced to environmental contamination from the slaughterhouse. In a study carried out in Belgian slaughterhouses *(23),* cross-contamination rates ranged from 16 to 100%. Molecular typing of isolates recovered in this study revealed complex cycles of origin of contamination from infected pigs and the contaminated environment *(22)*. In another study that included molecular typing *(94),* the authors estimated that at least 30% of cross-contaminated carcasses had different strain profiles at exsanguination and at rework stations. As previously suggested, to produce an accurate estimate of cross-contamination, studies should include molecular typing to corroborate the relatedness of isolates of the same serotype *(7, 22, 94)*. Overall results indicate that contamination rates differ among slaughter plants but cross-contamination levels are high, and improved slaughter practices are needed to reduce cross-contamination.

Carcass prevalence results from important studies carried out in the last decade are presented in Table 1 *(4, 6, 9, 20, 23, 28, 31, 49, 56, 66, 75, 84, 85, 90, 94, 96, 97)*. In these studies, prevalence values ranging from very low (1.4%) to very high (40%) have been described. Several authors have stated that comparison among study results is difficult because of differences in experiment design.

Carcass *Salmonella* prevalence may vary as a function of the point on the slaughter line where the carcass is tested; therefore, carcass sampling point has an influence on the final result *(29, 74, 94)*. Meat inspection or prechilling are the most common points selected for carcass sampling because they are located at the end of the slaughtering process. However, in some publications, the exact point where sampling was performed was not clearly stated; the authors refer to the ''slaughter line'' without making further specifications. In several studies, carcasses have been sampled after chilling and cooling procedures *(6, 9, 23, 31, 85, 94),* at which point *Salmonella* prevalence is expected to be reduced, making comparisons with some other studies difficult.

A second factor influencing the final carcass result is the sampling method used. Methods can be grouped in two classes: destructive (abrasion and excision) and nondestructive (usually swabbing). The majority of relevant studies indicate that excision followed by blending or stomaching of surface tissues is the most effective method for detecting bacteria because it provides more reliable and less variable bacterial counts than do other methods and also provides almost complete recovery of firmly attached bacteria *(25)*. However, in processing facilities, excision is not always practical or acceptable because of time constraints and the destructive nature of the procedure. Thus, most researchers prefer nondestructive methods for recovering bacteria from carcass surfaces. In studies of swine carcasses *(43, 60),* swabbing of a large surface can be at least as sensitive as the destructive excision method. However, the swabbing technique has inherent variability and the results obtained depend on the material used for swabbing *(64, 66),* the pressure exerted during application, the time span, and the sample collector's skills *(25)*.

Another important factor, especially for the swabbing technique, is the surface of the carcass sampled (Table 1). Variations in sampling area, number of points selected for sampling, and part of the carcass sampled (external, internal, or both) may influence the final result. The larger the surface sampled, the higher the sensitivity of the technique because it increases the detection of less prevalent microorganisms *(60, 85)*. The inclusion of areas close to the anus and the cutting edges at belly and chest or sampling of the inner part of the carcass in places that have been in contact with intestines can increase the chance of finding *Salmonella*. When external carcass surfaces are sampled, contamination occurring before slaughtering or cross-contamination during slaughtering should be detected. However, isolation of *Salmonella* from the inner part of the carcass is mostly informative of intestinal lacerations. Other factors such as pooling *(85)* can increase the prevalence results.

Large differences occur among studies regarding the sample processing procedure (Table 1), from studies in which the current EN-ISO 6579:2002/Amd 1:2007 has been

TABLE 1. Salmonella *prevalence results for carcasses in slaughterhouse studies*

Study	Year	Country	No. of abattoirs	Point of analysis	Sampling method	Surface sampled (cm^2)	Isolation method	No. of carcasses	Prevalence (%)
Algino et al. *(4)*	2009	Wisconsin	10	a) Slaughter line (after evisceration); b) after chilling	Swabbing external and internal belly, ham, and jowl	300	Enrichment and bacteriophages	181	a) 9.39; b) 18.78
Arguello et al. *(6)*	2012	Spain	2	a) Slaughter. line before chilling; b) postchilling (18 h)	Swabbing external and internal ham, chest, and jowl	1,350	ISO 6579/2002 adm:2007	a) 896; b) 445	a) 40; b) 10.8
Baptista et al. *(9)*	2010[a]	Denmark	23[b]	Postchilling (after 12 h)	Swabbing hind, chest, and cheek	300	Enrichment	20,196	3.7
Bonardi et al. *(20)*	2003	Italy	—[c]	—	Swabbing incision line from sternum to throat	—	ISO 6579/1993	150	6
Botteldoorn et al. *(23)*	2003	Belgium	5	a) Slaughter line before chilling; b) postchilling	Swabbing external and internal ham, sternum, and incision line		Enrichment (Diasalm)	a) 370; b) 75	a) 37; b) 16
Davies et al. *(28)*	2004	United Kingdom[b]	—	—	Swabbing chest and abdomen	100	Enrichment (Diasalm)	2,509	5.3
Duggan et al. *(31)*	2010	Ireland	1	End slaughter line	Swabbing ham, back, and belly	400	Enrichment and PCR	193	15.0
Hald et al. *(49)*	2003	Several	5	Before or after chilling	Swabbing. tarsus to neck and tarsus over belly	—	Close to ISO 6579/2002	1,623	3.8
Käsbohrer et al. *(56)*	1999	Germany	7	—	Swabbing inner thigh and external and internal chest		ISO 6579/1993	11,942	4.7
McDowell et al. *(66)*	2007	United Kingdom (Northern Ireland)	4	Meat inspection point	Swabbing external and internal belly, chest, and incision to neck	1,000	ISO 6579/2002	513	40
Piras et al. *(75)*	2011	Italy (Sardinia)	5	—	Swabbing hind abdomen and chest	1,400	ISO 6579/2002	85	14.1
Sørensen et al. *(84)*	2004	Denmark	1	Slaughter line	Swabbing ham, pelvis, and breast cut	1,400	—	159	9.6
Sørensen et al. *(85)*	2006	Denmark	9[a]	Postchilling (after 12 h)	Swabbing hind, chest, and cheek; individual sample or pool of five samples	300	Enrichment	a) individual: 10,099; b) pooled: 8,895	a) 4.1; b) 1.4
Swanenburg et al. *(90)*	2001	The Netherlands	2	After shock chiller	Swabbing tarsus to ear through the back and belly	400	ISO 6579/2002	213	1.4
Van Hoek et al. *(94)*	2012	The Netherlands	1	a) Exsanguination; b) rework station	a) Excision (5×4 cm^2); b) external: back, jowl, ham, and belly; internal: ham, back, sternum, and shoulder	200	Real-time PCR after enrichment	118	a) 96.6; b) external: 16.2; b) internal: 35.9
Vieira-Pinto et al. *(96)*	2005	Portugal	1	—	Swabbing tarsus to ear through the back and belly	—	ISO 6579/2002	101	2.9
Visscher et al. *(97)*	2011	Germany (Lower Saxony)	3	Slaughter line	Swabbing external and internal head to pelvis	—	ISO 6579/2002	501	4.6

[a] Data collected from 2002 to 2008.
[b] Data from a national survey.
[c] —, no information available.

applied *(6, 20, 56, 66, 75, 90)* to studies using microbiological methodologies similar to but not exactly those stipulated by the International Organization for Standardization (ISO) *(23, 28, 49)* or studies using alternative methods based on high-throughput techniques such as real-time PCR *(94)*. These variations in the microbiological methods used can result in differences in prevalence results. The selection of one or several culture colonies for further typing also can affect the number of *Salmonella* serotypes detected *(94, 99)*.

Apart from these technical factors, just a few slaughterhouses usually are included in a study (Table 1). The study design is influenced by such factors as the willingness of companies to take part in this sort of study and the economic resources needed. Therefore, most of the studies are pertinent to only a limited region or a single slaughter company, leading to some particularities that make it difficult to extrapolate results to an entire country from a global viewpoint. Additional factors that can affect *Salmonella* prevalence detected in carcasses include the season *(28, 37, 66)*, the day of the week *(6)*, the processing chain speed *(4)*, and the sampling frame of the study. In conclusion, several factors must be taken into account when carcass prevalence values are evaluated and comparisons are made among studies.

INTERVENTION STRATEGIES AT THE SLAUGHTER LEVEL

Years ago, European experts put more emphasis on control strategies implemented at the farm, and the roles of transport, lairage, and slaughtering were more important in the U.S. perspective *(86)*. Nevertheless, both U.S. and European researchers are now focusing on abattoir hygiene *(1)*. Although the purpose of this review was not to evaluate the efficacy of control strategies applied at the slaughterhouse level, we considered it necessary to describe some of the strategies that can be implemented to avoid *Salmonella* contamination throughout the slaughtering process.

The logistic slaughtering principle is based on the separation of pigs at slaughtering, taking into account their *Salmonella* status. This process is mandatory in Denmark, where farms are classified according to their serological *Salmonella* status. Those pigs from the most infected herds are slaughtered under conditions that include increased hygienic precautions (sanitary slaughter), i.e., pigs are slaughtered at the end of the day, they are not allowed to remain overnight at the lairage or to mix with noninfected pigs, the speed of the slaughter line is lowered, heads are not split, and lungs, heart, liver, and intestines are either condemned or heat treated. When swabs collected from carcasses reveal contamination exceeding the limits established by regulation, pork must be heat treated or cured before consumption *(84)*. However, according to Danish experts logistic slaughter can reduce the prevalence but cannot completely remove the *Salmonella* contamination from the carcass *(45)*. Swanenburg and colleagues *(91)* tested the efficiency of logistic slaughter in The Netherlands and found that *Salmonella* prevalence in samples from seronegative herds was lower than that from seropositive herds. These authors also linked the contamination of carcasses from *Salmonella*-free pigs to environmental and residential flora from trucks, holding pens, and the slaughter line and concluded that *Salmonella*-free pigs must be slaughtered in different slaughterhouses than those used for contaminated pigs; they also recommended improved cleaning protocols that can remove residential flora at various stages in the slaughtering process.

Apart from logistic slaughter, which would minimize the contact between infected and noninfected pigs, the slaughterhouse constitutes a perfect target for implementing *Salmonella* control measures. Effective actions taken at this level would diminish substantially the risk for human infection. Carcass decontamination is one of the most feasible actions that can be performed at slaughtering. According to Danish experts, further *Salmonella* reduction at the herd level may be difficult to achieve in countries with low *Salmonella* prevalence, which suggests that future strategies should focus on abattoir interventions *(1, 10)*.

A cost-benefit analysis comparing different control strategies in swine production *(45)* revealed that hot water decontamination of carcasses is a socioeconomically profitable option. The treatment characteristics were described by Alban and Sørensen *(2)*. In a Danish test study, an average *Escherichia coli* reduction of 2 log CFU/1,400 cm^2 (from 3 log CFU/1,400 cm^2) was reported; the intervention did not produce undesirable changes in smell or taste and the color changes were reversible. This method also was the most effective among those evaluated by Lawson et al. *(58)*, and its efficiency was confirmed by Hamilton et al. *(50)*. The disadvantage is that decontamination with hot water requires large investments in equipment acquisition and maintenance; therefore, this method is not cost-effective for small abattoirs *(58)*. The use of cold water is not effective for reducing *Salmonella* prevalence and produces only cosmetic effects *(19)*.

Apart from hot water, other physical decontamination technologies have been evaluated. Steam ultrasound is less effective than hot water treatment but is more cost-effective. Laboratory experiments revealed that two consecutive applications of this method may provide reductions in *Salmonella* of 2 and 1.2 log CFU/cm^2 on skin and meat sides, respectively *(58)*. Lower efficacy has been achieved with steam vacuuming. Experiments in a beef plant indicated that the use of a steam vacuum immediately before chilling may reduce the aerobic plate counts and *E. coli* counts by 1.31 to 1.74 log CFU/cm^2 after treatments of 10 and 30 s, respectively, from an initial value of 3.23 log CFU/cm^2. Similarly, *E. coli*–positive samples were reduced from 50.6% to 6.7 and 3.3% after treatments of 10 and 30 s, respectively *(58)*. UV light also can be used as a physical decontamination method. However, its restricted penetration depth and potential impact on fat oxidation must be considered. Depending on the UV intensity (20 to 1,000 mW/cm^2), *E. coli* and *Salmonella* Senftenberg reductions of 0.1 to 3.3 and 0.7 to 4.6 log CFU/cm^2, respectively, were achieved. *(61)*. Irradiation or pulsed electric fields also are potential decontamination treatments

(16). Another possibility is chemical decontamination, which involves the use of organic acids *(61),* hydrogen peroxide *(16),* acidified sodium chlorite *(50),* peroxyacids, or trisodium phosphate in water showers *(35)*. For pig carcasses, chemical decontamination is mainly restricted to organic acids *(61),* and the bactericidal activity of these acids may be counteracted by organic matter, some agents may be corrosive, and concentrated substances might constitute a health hazard *(61)*. In conclusion, although all carcass decontamination methods are cheaper than those recommended for the farm, such as home-mixed feeds or feed treatments based on acids *(45),* data about decontamination treatments for pig carcasses are limited and in most of the cases have been obtained under laboratory or experimental conditions, which would likely yield better results than those that would be obtained under field conditions.

Taking into account the wide range of opportunities to establish effective *Salmonella* control measures in the slaughterhouse, more research on these control strategies is needed. Research efforts should be focused on the search for a decontamination treatment that has no adverse effects on meat appearance or sensorial attributes, is safe for consumers and the environment, and has low costs.

CONCLUSIONS

Pork contamination by *Salmonella* is a complex issue affected by factors at all stages of the swine production chain. The studies carried out on pigs after they leave the farm, i.e., during transport, in lairage, or along the slaughtering chain, emphasize the role that all these points play in the final carcass status. Both finishing pig status and transport and lairage conditions determine the level of pig contamination before slaughter. Improvements in cleansing and disinfection procedures at both transport and lairage in addition to other control measures are necessary to decrease the likelihood of contamination before the pigs enter the slaughterhouse. At slaughtering, where *Salmonella* is continuously introduced, the establishment of CCPs and GMPs within HACCP systems at the slaughter level also is necessary to reduce the dispersion of and resulting cross-contamination by *Salmonella* along the slaughter line. The slaughterhouse is the point in the production chain where efficient control measures can be implemented with the least effort and highest level of cost-effectiveness.

More research is needed to decode the complex *Salmonella* epidemiology, paying special attention to activities surrounding slaughtering. The information available points to the research lines that can be followed, encouraging the cooperation of research groups and food agencies for standardizing surveys and making results comparable.

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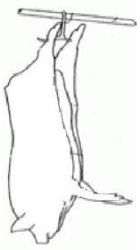
2-. Publications included in chapter III

Publication 1. Prevalence and serovars of *Salmonella enterica* on pig carcasses, slaughtered pigs and the environment of four Spanish slaughterhouses.



Publication 2 Sero- and Genotyping of *Salmonella* in slaughter pigs, from farm to cutting plant, with a focus on the slaughter process.

Publication 3. Effect of logistic slaughter on *Salmonella* contamination on pig carcasses and the slaughterhouse environment

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Prevalence and serovars of *Salmonella enterica* on pig carcasses, slaughtered pigs and the environment of four Spanish slaughterhouses

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ABSTRACT

The purpose of this study was to investigate the prevalence of *Salmonella* contamination and main serovars in pig slaughterhouses in Spain including carcasses, live animals and the environment. A total of 896 pig carcasses were randomly selected and swabbed before chilling in 3–5 visits to four pig slaughterhouses (A, B, C and D). *Salmonella* contamination was detected in 39.7% of the carcasses. The prevalence of positive carcasses was similar amongst slaughterhouses but significant differences were observed when taking sampling day into consideration within each of the slaughterhouses. Furthermore, a significant reduction in the prevalence of *Salmonella* contaminated carcasses (10.8%) was demonstrated in slaughterhouses C and D after chilling and cooling procedures.

Sixteen batches of 10 animals were tracked from farm-to-slaughterhouse in slaughterhouses A and B to investigate the relationship between carcass contamination and contamination in live animals entering the slaughterhouse. No difference was found between infected and uninfected animals with respect to *Salmonella* contamination of the carcass although an increase in *Salmonella* contamination during the processing of live pigs into pork carcasses was evident. Regarding contamination in the slaughterhouse environment, *Salmonella* was isolated from most of the evaluated points in the slaughter line of the four studied slaughterhouses. Holding pens were identified as highly contaminated and what is more the ineffectiveness of the routinely cleaning protocols at this level was demonstrated in slaughterhouses C and D.

The predominant *Salmonella* serovars found in carcasses, live pigs entering the slaughterhouse and the environment of the slaughterhouse were *S.* Typhimurium, *S.* Rissen, *S.* Derby and *S.* 4,[5],12:i:-. The same serovars were found in all the stages supporting the hypothesis that infected pigs are the main source of *Salmonella* contamination within slaughterhouses.

1. Introduction

Salmonella is the most common cause of food-borne outbreaks and the second most reported zoonotic disease in the EU (EFSA, 2010a). Although eggs (or products thereof) and poultry meat are the biggest contributors to these outbreaks, pig meat has also been recognised as an important source of human salmonellosis since the early 90s (Baggesen & Wegener, 1994; Mousing et al., 1997; Wegener, Baggesen, & Gaarslev, 1994). Due to this, many European countries have already implemented *Salmonella* control programmes for pigs and pork (Nielsen & Wegener, 1997; Rowe et al., 2003; Small et al., 2006). Such programmes should ensure that proper and effective measures to detect and control *Salmonella* are taken at all relevant stages of production, processing and distribution, particularly at the level of primary production, and they will soon be compulsory in the EU (Regulation EC no, 2160/2003).

Pigs are susceptible to a wide variety of serovars of *Salmonella*, and infected pigs usually remain healthy carriers. These sub-clinically infected pigs may excrete *Salmonella* spp. in their faeces or keep the bacteria in their digestive tract, the closely associated lymph nodes or the tonsils (Fedorka-Cray et al., 1999). *Salmonella*-carrier pigs are not identified during ante and post-mortem veterinary inspections, and they theoretically will not contain the bacteria on their carcass if the slaughtering process is carried out properly. However, it has been reported that these animals are the major contamination source for the slaughterhouse and the processing environment (Berends, Van Knapen, Snijders, & Mossel, 1997). Handling and hygiene practices are essential to prevent cross contamination during the slaughtering process, and several interventions at this level have been proposed in order to reduce the hazard to consumers (Alban & Stärk, 2005; Baptista, Halasa, Alban, & Nielsen, 2010; Goldbach & Alban, 2006; Lawson, Jensen, Christiansen, & Lund, 2009).

Spain is currently the second largest pig producer in the EU and pig meat exports have reached 1.25 t in 2008 (data from the Spanish Ministry of Agriculture and Livestock). Previous studies have reported a high prevalence of *Salmonella* infection in Spanish pigs (EFSA, 2008b,

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2009; García-Feliz et al., 2007; Mejía et al., 2006). However, there are no recent data available regarding the prevalence of *Salmonella* in pig carcasses. This study aimed at investigating the prevalence of *Salmonella* contamination and main serovars in pig slaughterhouses including carcasses, live animals and the environment. For this purpose, four pig slaughterhouses (A, B, C and D) were investigated in two different studies. *Salmonella* contamination in carcasses was investigated during both studies whilst the relationship between carcass contamination and contamination in live animals entering the slaughterhouse was specifically evaluated during study 1 in slaughterhouses A and B. A more intensive assessment of the slaughterhouse environment contamination was intended during study 2 in slaughterhouses C and D.

2. Material and methods

2.1. Experimental design

Two different studies were carried out in four Spanish commercial pig slaughterhouses (A, B, C and D) located in different regions of the country.

Study 1 was conducted between 2004 and 2006 in slaughterhouses A and B. Slaughterhouse A processed 300 pigs per hour and was visited five times (in five different days) whereas slaughterhouse B processed 800 pigs per hour and was investigated in four different days. Each sampling day, a total of 50 randomly selected carcasses before chilling as well as different points of the slaughter line (scalding tank water, bung dropper, evisceration and carcass splitting) were sampled. Moreover, a tracking study from the farm to the slaughterhouse was performed by following a total of eight different batches of slaughtered pigs in each slaughterhouse. Within each batch, the study started at the farm level by randomly selecting ten pigs allocated in two different pens within two-three days of slaughter (5 pigs in each pen). The selected pigs were identified using ear tags, which allowed the tracking of the animals throughout the study for recovering faecal samples at the farm as well as caecal contents, mesenteric lymph nodes (MLNs) and carcass swabs immediately before chilling at the slaughterhouse. Holding pens at the slaughterhouse were sampled by collecting faecal material and surface swabs from the floor before and after the entrance of the selected pigs.

Study 2 was carried out between 2009 and 2010 in slaughterhouses C and D that processed 400 and 375 pigs per hour, respectively. Each slaughterhouse was investigated in three different days distributed at the beginning, middle and end of the work week. Randomly selected carcasses before chilling, holding pens and several points along the slaughter line (scalding tank water, evisceration, renal extraction, carcass splitting, fat removal etc.) were sampled on Monday, Wednesday and Friday. Moreover, the same carcasses previously selected were swabbed after chilling/cooling procedures during the following working day (Tuesday for those sacrificed on Monday, Thursday for those sacrificed on Wednesday and Monday for those sacrificed on Friday). Within each sampling day, holding pens were evaluated in four sampling rounds. The first sampling round was done before the entrance of the pigs by collecting surface swabs in the cleaned floor. Second and third sampling rounds were done at half and end of the working day, respectively, by collecting faeces whilst the fourth round was done after the cleaning procedures at the end of the day by collecting surface swabs.

2.2. Sample collection and processing

Faecal samples ($\geq$25 g) were collected into 100 ml sterile flasks from individual animals at the farm level and directly from the floor at holding pens. Within each location, a pool of fresh faecal material was collected from five different points distributed all over the pen.

Surface samples were collected in holding pens and different points of the slaughter line (worker hands, implements and equipment surfaces) by using sterile gauzes moistened with buffered peptone water (BPW) (Merk®) as previously described (Giovannacci et al., 2001; Swanenburg, Urlings, Snijders, Keuzenkamp, & van Knapen, 2001). Immediately after collecting the sample, each gauze was stored in a 250 ml sterile flask containing 50 ml of BPW. Moreover, organic matter from the machinery in slaughterhouses C and D as well as water from the scalding tanks in slaughterhouses A, B and D was aseptically collected into 100 ml sterile flasks.

Mesenteric lymph nodes ($\geq$25 g) and caecal contents ($\geq$25 g) were recovered after slaughtering using 100 ml sterile flasks. Caecal contents were collected through puncture with a sterile disposable scalpel in the caecum, and MLNs were cut out of the intestine packet. Once in the lab, the MLNs were processed as previously described (Regulation EC 668/2006) by removing the fat and the capsula followed by immersion in alcohol 70% (v/v) and flaming in order to sterilise its surface. Finally, the MLNs were cut into small pieces using sterile scissors, weighed and processed.

Carcasses were swabbed on the external and internal surfaces on three different points: ham, rib cage and neck-upper-shoulder. Sampling was done using sterile gauzes moistened with BPW to swab a square of 15 cm $\times$ 15 cm within each point (approx. 1350 cm^2 in total). As described for surface samples, the gauzes were stored in 250 ml sterile flasks containing 50 ml of BPW. In study 2, carcasses were swabbed twice, before and after chilling, by sampling the right and left half-carcasses.

Sterile gloves were used during the sampling and were changed between each sample. Samples were sent to the laboratory in cooled containers within the same day for immediate analysis except for those collected on Fridays, which were held at 4 °C and dispatched on Mondays.

2.3. Diagnostic methodology

All samples were analysed by following the current ISO standard methodology at the time of the study. Therefore, EN-ISO 6579:2002 was used during study 1, whereas the modification of this method, EN-ISO 6579:2002/Amd 1:2007, was followed during study 2.

Pre-enrichment was performed by 1:10 dilution of faeces, caecal contents, MLNs, scalding tank water and organic matter (25 g when it was possible) in BPW followed by incubation at 37 °C for 18–24 h. Gauzes that were already immersed in 50 ml of BPW were shaken and directly incubated. During study 1, selective enrichment was performed by transferring 0.1 ml of the pre-enrichment broth to 9.9 ml of Rappaport-Vassiliadis (RV) enrichment broth (Merck®), whereas during study 2, RV broth was replaced by modified semisolid Rappaport-Vassiliadis medium (MSRV) (Merck®). After an incubation of 24–48 h at 41 °C, the RV broth was streaked onto Xylose lysine tergitol 4 agar (XLT4) (Merck®) and Xylose lysine deoxycholate agar (XLD) (Merck®). MRSV plates showing migration zones were further investigated by streaking material from the edge of the zone onto XLD and Brilliant green agar (Cultimed®). The plates were evaluated for colonies typical of *Salmonella* species after 24 h of incubation. Suspected *Salmonella* colonies were screened using the indol test and the 4-methylumbelliferyl caprilate fluorescence test (Mucap test, Biolife®). A single confirmed *Salmonella* isolate from each positive sample was serotyped according to Kauffmann–White scheme at the National Reference Laboratory for *Salmonella* and *Shigella* (NRLSS) (Instituto de Salud Carlos III, Madrid, Spain) or at our facilities.

2.4. Statistical analysis

All data were stored and analysed in EpiInfo for Windows (CDC, Atlanta, Georgia, USA). A univariate analysis using the chi-square test at $\alpha = 0.05$ was used to investigate any association between the

prevalence of *Salmonella*-contaminated carcasses amongst slaughterhouses and sampling days as well as before and after chilling. Moreover, associations between carcass contamination and the presence of *Salmonella* in faeces, caecal contents or MLNs were assessed using the chi-square test.

3. Results

3.1. Salmonella contamination in carcasses

A total of 896 carcasses were randomly sampled before chilling during the present survey that included three to five visits (visit 1 to visit 5) in four different slaughterhouses (A, B, C and D). *Salmonella* contamination was detected in 356 carcasses (39.7%; 95% CI: 36.5–43). The prevalence of positive carcasses was 38.8%, 40%, 37.9% and 45.2% in slaughterhouses A, B, C and D, respectively. No significant differences were found amongst the different slaughterhouses ($\chi^2 = 2.2$, $p = 0.534$). However, high variations were observed amongst different sampling days at the same slaughterhouse (Table 1). The prevalence of *Salmonella*-positive carcasses ranged from 10 to 68% in slaughterhouse A ($\chi^2 = 47.3$, $p < 0.001$), from 24 to 54% in slaughterhouse B ($\chi^2 = 9.8$, $p = 0.02$), from 27 to 57.6% in slaughterhouse C ($\chi^2 = 24$, $p < 0.001$) and from 20 to 72% in slaughterhouse D ($\chi^2 = 26.5$, $p < 0.001$).

In slaughterhouses C and D, where samplings were performed on three different visits distributed at the beginning, middle and end of the work week, a lower prevalence of *Salmonella*-contaminated carcasses was noticed at the beginning of the week in both slaughterhouses, whereas the highest values were observed when sampling was performed at the middle of the week. These differences reached statistical significance ($\chi^2 = 48.2$, $p < 0.001$). The risk of a carcass being *Salmonella*-positive was five times higher in the middle of the week as compared with the first working day (OR = 5.03, 95% CI: 3–8.6) and three times higher as compared with the end of the work week (OR = 3.39, 95% CI: 2.1–5.6).

Moreover, *Salmonella* was detected in 48 out of 445 carcasses that were swabbed after chilling in slaughterhouses C and D (10.8%, 95% CI: 7.9–13.7). *Salmonella* contamination in chilled carcasses (Table 1) did not differ between the two slaughterhouses ($\chi^2 = 0.6$, $p = 0.443$) but varied significantly amongst different sampling days within each slaughterhouse ($\chi^2 = 8$, $p = 0.018$ and $\chi^2 = 10.8$, $p = 0.004$, respectively, for slaughterhouses C and D). The prevalence of *Salmonella* contamination was compared before and after chilling in 323 carcasses that were sampled at both stages. Due to a problem with the carcass rail in the third visit to slaughterhouse C, as well as a few failures of identification, 122 chilled carcasses did not correspond to a previously sampled hot carcass. In those carcasses evaluated twice, *Salmonella* contamination was reduced from 45.8% to 10.8%, and a significant decrease in *Salmonella* contamination after chilling was detected ($\chi^2 = 95.64$, $p < 0.001$). However, 9 out of 35 *Salmonella*-positive chilled carcasses were not detected as positive before chilling.

The distribution of *Salmonella* serovars found in carcass swabs collected before and after chilling is shown in Table 2. A total of 18 different serovars were identified, although only 8 of them were represented by more than one isolate.

3.2. Salmonella contamination in live animals entering the slaughterhouse

A total of 160 animals from 16 batches were tracked during study 1 in slaughterhouses A and B. The *Salmonella* contamination frequencies were 5.6%, 33.8%, 18.2% and 57.5%, respectively, for faeces collected at the farm, caecal contents, MLNs and carcasses at the slaughterhouse. No statistically significant association was found between carcass contamination and either faecal shedding detected at the farm ($\chi^2 = 0.8$, $p = 0.358$), positively testing caecal contents ($\chi^2 = 0.02$, $p = 0.879$) or positively testing MLN samples ($\chi^2 = 1.4$, $p = 0.2421$). *Salmonella*-contaminated carcasses were equally distributed amongst animals that shed *Salmonella* in their faeces or animals that were carriers (48.9%) and animals that were not detected as positive in any of the tested samples (51.1%). Animal-related contamination, defined as the number of individuals that were identified as *Salmonella*-infected amongst those whose carcasses were contaminated, was 7.6% for faecal shedding at the farm level, 33.7% for caecal contents and 21.7% for MLNs.

Salmonella was isolated in 9 out of 11 faecal samples (81.8%) and 13 out of 16 surface samples (81.3%) collected in the holding pens before the entrance of the selected pigs. After the pigs stayed in these holding pens, *Salmonella* was recovered in 10 out of 16 faecal samples (62.5%) and 14 out of 16 surface samples (87.5%).

The *Salmonella* serovars identified in the samples from study 1 are shown in Table 3. All serovars identified in carcasses were also detected in at least one of the pig-related samples collected previously or in the holding pens. *S.* Typhimurium was the most frequently identified

Table 1
Prevalence of *Salmonella* contamination in randomly selected pig carcasses swabbed before (896) and after (445) chilling procedures in 3–5 visits to four commercial Spanish pig slaughterhouses (A, B, C and D).

	Before chilling									
	Visit 1		Visit 2		Visit 3		Visit 4		Visit 5	
Slaughterhouse	Nr. of samples	Positive samples	Nr. of samples	Positive samples	Nr. of samples	Positive samples	Nr. of samples	Positive samples	Nr. of samples	Positive samples
A[a]	50	68%	50	58%	50	28%	50	30%	50	10%
B[a]	50	38%	50	44%	50	24%	50	54%	–	–
C[a,b]	100	27%	99	57.6%	112	30.4%	–	–	–	–
D[a,b]	45	20%	50	72%	40	40%	–	–	–	–

	After chilling					
	Visit 1		Visit 2		Visit 3	
Slaughterhouse	Nr. of samples	Positive samples	Nr. of samples	Positive samples	Nr. of samples	Positive samples
A	–	–	–	–	–	–
B	–	–	–	–	–	–
C[b]	100	12%	98	14.3%	112	4.5%
D[b]	44	0%	49	22.4%	42	14.3%

In slaughterhouses C and D, visit 1 was done at the beginning of the work week (Monday for carcasses before chilling and Tuesday for carcasses after chilling), visit 2 was done at the middle of the work week (Wednesday for carcasses before chilling and Thursday for carcasses after chilling) and visit 3 was done at the end of the work week (Friday for carcasses before chilling and Monday for carcasses after chilling).

[a] Prevalence of contaminated carcasses was significantly different amongst visits to the same slaughterhouse ($p < 0.001$).

[b] Prevalence of contaminated carcasses was significantly lower after chilling as compared with the same carcasses before chilling ($p < 0.001$).

Table 2
Main *Salmonella* serovars recovered from 1341 randomly selected pig carcasses swabbed before and after chilling in four commercial Spanish pig slaughterhouses (A, B, C and D). Percentage of isolates from each serovar obtained in each slaughterhouse is indicated in brackets.

	Nr. of isolates[a] (%)					
	Before chilling				After chilling	
	Slaughterhouse				*Slaughterhouse*	
	A	B	C	D	C	D
Typhimurium	71 (73.2%)	103 (58.2%)	22 (18.6%)	8 (13.1%)	2 (6.1%)	3 (16.7%)
Rissen	4 (4.1%)	37 (20.2%)	53 (44.9%)	8 (13.1%)	20 (66.6%)	0
Derby	0	4 (2.2%)	10 (8.5%)	3 (4.9%)	2 (6.1%)	0
4,[5],12:i:-	0	1 (0.6%)	7 (5.9%)	9 (14.7%)	3 (9.1%)	2 (11.1%)
Brandemburg	1 (1%)	4 (2.2%)	0	0	0	0
Bredeney	2 (2.1%)	3 (1.7%)	0	23 (37.7%)	0	6 (33.3%)
Anatum	0	0	0	5 (8.2%)	0	0
Lille	13 (13.4%)	13 (7.3%)	0	0	0	0

[a] Serovars represented by a single isolate in each slaughterhouse have not been included.

serovar in carcasses as well as in caecal content, MLNs and holding pens before housing the pigs. On the other hand, *S.* Rissen was the most frequently identified serovar in faecal samples collected at the farm level and holding pens after housing the pigs. Overall, *S.* Typhimurium was recovered in 116 out of 230 *Salmonella*-positive samples (50.4%), followed by *S.* Rissen (26.5%), *S.* Branderburg (8.7%) and *S.* Derby (6.9%).

3.3. Salmonella contamination in the environment of the slaughterhouses

A total of 260 floor swabs or faecal samples were collected from 22 holding pens in slaughterhouses C and D. *Salmonella* was detected in 66.1% of these samples, and the number of positive samples increased throughout the working day. Contamination was detected in 26 out of 66 surface samples collected before the entrance of any animal (39.4%), in 55 out of 66 samples collected at midday (83.3%), in 49 out of 62 samples collected at the end of the working day (79%) and in 42 out of 66 samples (63.6%) collected after cleaning procedures. Four different serovars were identified amongst the 57 isolates recovered from holding pens in slaughterhouse C. The most prevalent was *S.* Rissen (39%), followed by *S.* Typhimurium (25%), *S.* Derby (24%) and *S.* 4,[5],12:i:- (14%). In addition, 7 different serovars were recognised amongst the 96 isolates recovered in the same location in slaughterhouse D, *S.* Derby being the most frequently identified (31%) followed by *S.* Bredeney (25%) and *S.* Rissen (24%).

A total of 486 environmental samples were analysed in the four studied slaughterhouses. The number of samples analysed at each point in each slaughterhouse and the occurrence of *Salmonella* contamination are shown in Table 4. The mean prevalence was 10% in slaughterhouse A, 56.2% in slaughterhouse B, 31% in slaughterhouse C and 48.7% in slaughterhouse D. Significant differences were detected amongst slaughterhouses ($\chi^2 = 36.99$, $p < 0.001$). In slaughterhouses C and D, where the number of environmental samples was higher, the prevalence varied from 24% to 36% in slaughterhouse C and from 36% to 67% in slaughterhouse D. Statistical differences were detected amongst the results obtained on different visits in slaughterhouse D ($\chi^2 = 8.74$, $p = 0.012$) but not in slaughterhouse C ($\chi^2 = 2.90$, $p = 0.23$).

A total of 11 different serovars were identified amongst *Salmonella* isolates recovered from slaughter line samples (Table 5). In slaughterhouse A, only *S.* Typhimurium and its monophasic variant, *S.* 4,[5],12:i:-, were found. In the other three slaughterhouses a greater variety of serovars was identified, and their prevalence fluctuated between visits. In slaughterhouses B and C the three most prevalent serovars were *S.* Typhimurium, *S.* Rissen and *S.* Derby. Despite the fact that *S.* Typhimurium was not found during the first visit to slaughterhouse B, it was the most frequent serovar on the other three sampling days. In the case of slaughterhouse C, *S.* Rissen was the predominant serovar (74%, 83% and 55%) at the three visits, whereas *S.* Typhimurium was identified just on one of the sampling days. Finally, eight different serovars were identified in the environmental samples in slaughterhouse D and moreover, it was in this slaughterhouse where the variations in serovar frequencies between samplings were more marked. *S.* Derby was the predominant serovar in the first visit (58%), *S.* Bredeney predominated in the second visit (74%), a serovar that was detected neither in the previous or subsequent visit nor in any of the other slaughterhouses; and finally *S.* Rissen in the third one (40%).

4. Discussion

The results obtained in this study reveal a high level of *Salmonella* contamination in several steps associated with the slaughtering process in the pork production chain in Spain. According to previous studies (Botteldoorn, Heyndrickx, Rijpens, Grijspeerdt, & Herman, 2003; Giovannacci et al., 2001; McDowell, Porter, Madden, Cooper, & Neill,

Table 3
Main *Salmonella* serovars recovered from pig-related samples and holding pens in the tracking of 160 animals from farm to slaughter in two commercial pig slaughterhouses (A and B) between 2004 and 2006 (Study 1). Percentage of isolates from each serovar recovered from each sample type is indicated in brackets.

	Nr. of isolates (%)							
	Faecal samples (farm level)	Caecal content	MLN[a]	Carcasses	Holding pens			
					Before housing the pigs		After housing the pigs	
					Faecal samples	Surface samples	Faecal samples	Surface samples
Typhimurium	1 (11.1%)	23 (42.6%)	13 (44.8%)	62 (67.4%)	5 (55.5%)	6 (46.1%)	3 (30%)	3 (21.4%)
Rissen	5 (55.5%)	15 (27.8%)	10 (34.5%)	13 (14.1%)	3 (33.3%)	6 (46.1%)	3 (30%)	6 (42.9%)
Derby	0	11 (20.4%)	0	1 (1.1%)	0	0	2 (20%)	2 (14.3%)
Brandemburg	0	2 (3.7%)	2 (6.9%)	15 (16.3%)	0	0	1 (10%)	0
Lille	1 (11.1%)	0	0	0	0	0	0	1 (7.1%)
Ohio	2 (22.2%)	0	0	0	0	0	0	0
Wien	0	0	0	0	0	0	0	2 (14.3%)

Serovars represented by a single isolate in a unique location have not been included.
[a] MLN: mesenteric lymph nodes.

Table 4
Prevalence of *Salmonella* contamination in different points of the slaughter line in four Spanish pig slaughterhouses (A, B, C and D).

	Slaughterhouse							
	A		B		C		D	
	No. of samples	% positive samples	No. of samples	% positive samples	No. of samples	% positive samples	No. of samples	% positive samples
Scalding tank	15	6.7%	12	42%	–	–	6	0
Bung dropper	15	20%	12	67%	15[a]–15[b]	33%[a]–0%[b]	12[a]–12[b]	33%[a]–25%[b]
Penis removal	–	–	–	–	–	–	11	36.4%
Abdominal open saw	15	13.3%	12	33%	–	–	–	–
Evisceration	–	–	–	–	45	33%	11	72.7%
Chest saw	–	–	–	–	–	–	6	83%
Kidney removal	–	–	–	–	15	27%	11	63.6%
Renal capsula removal	–	–	–	–	15	66.7%	–	–
Carcass splitting								
Surface saw	15	0%	12	83%	30	3.3%	–	–
Organic matter	–	–	–	–	6	50%	9	77.8%
Sewage water	–	–	–	–	–	–	9	55.6%
Lard extraction	–	–	–	–	45	31%	17	82.3%
Fat remover	–	–	–	–	30	40%	9	44.4%
Tonsils removal	–	–	–	–	–	–	9	55.6%

[a] Samples collected before sterilisation.
[b] Samples collected after sterilisation.

2007), *Salmonella* contamination is particularly high in the holding pens of slaughterhouses, in caecal contents and in MLNs of slaughtered pigs as well as at several points of the slaughter line and in carcasses.

Salmonella was isolated from 40% of the carcasses swabbed at the end of the slaughter line before chilling in the present study. Although only four different slaughterhouses were included, the fact that similar results were found in all of them together with their location in different regions of Spain (which ensured that the main pig producer areas were represented) allows us to hypothesise that our result could be extrapolated to large slaughterhouses throughout the country. Moreover, regardless of the differences in the time of sampling, with more than five years of delay between study 1 in slaughterhouses A and B and study 2 in slaughterhouses C and D, the prevalence of *Salmonella* contaminated carcasses was similar in the four studied slaughterhouses. This fact can be explained by the lack of a *Salmonella* control programme in pork in Spain. What is more, this high level of *Salmonella* contamination in pork carcasses correlates with the previously reported high prevalence of *Salmonella* infection in Spanish pig farms (EFSA, 2008a, 2008b, 2009; García-Feliz et al., 2007). The prevalence of *Salmonella* contaminated carcasses was higher than that previously reported in several studies (Bonardi et al., 2003; EFSA, 2008a; Hald, Wingstrand, Swanenburg, von Altrock, & Thorberg, 2003; Käsbohrer et al., 2000; Swanenburg, Urlings, Snijders, et al., 2001) but very similar to the 37% *Salmonella*-positive carcasses before chilling reported by Botteldoorn et al. (2003) in five commercial slaughterhouses in Belgium or the 40% positive swabs taken from the surface of carcasses post-evisceration in four slaughterhouses in Northern Ireland (McDowell et al., 2007). However, comparisons amongst different studies on *Salmonella* contamination of carcasses should be considered carefully because prevalence estimates are affected by the isolation procedures and particularly by the sampling strategy: the location and area of the carcass surface sampled or the point at which the swabs are taken within the slaughtering process. Thus, besides other factors, the swabbing of a large surface of 1350 cm^2 at the end of the slaughter line and before chilling may have accounted for the higher prevalence found in this study in comparison with previous ones.

Even though the prevalence of *Salmonella*-positive carcasses was similar amongst slaughterhouses, there were significant differences amongst different visits within each of the sampled slaughterhouses. This fact has also been reported in other studies (Botteldoorn et al., 2003; Hald et al., 2003; Käsbohrer et al., 2000; Swanenburg, Urlings, Snijders, et al., 2001), and it probably reflects the fact that internal factors of the slaughtering process (such as handling and hygienic standards), external factors (such as *Salmonella* infection in pigs entering the slaughterhouse) and transport conditions have an influence on *Salmonella* contamination at the end of the slaughter line. As an example of such internal factors, the highest prevalence of *Salmonella*-contaminated carcasses was detected in our study in samples taken one day after the staff of the slaughter line was

Table 5
Main *Salmonella* serovars recovered from different points of the slaughter line in four Spanish pig slaughterhouses (A, B, C and D). The percentage of each serovar in each slaughterhouse and the number of visits in which this serovar was detected are included.

	Slaughterhouse							
	A		B		C		D	
	% isolates	No. of visits (5)[a]	% isolates	No. of visits (4)[a]	% isolates	No. of visits (3)[a]	% isolates	No. of visits (3)[a]
Typhimurium	66.7	3	54.0	4	14.5	1	7.9	3
Rissen	–	–	19.2	3	68.1	3	13.7	2
Derby	–	–	19.2	3	7.2	2	19.6	1
Bredeney	–	–	–	–	–	–	33.3	1
4,5,12:i:-	11.1	1	–	–	7.2	2	11.8	3
Brandemburg	–	–	3.8	1	–	–	–	–
Anatum	–	–	–	–	–	–	7.9	1
Wien	–	–	3.8	1	–	–	–	–
Agama	–	–	–	–	1.5	1	3.9	1
Mkamba	–	–	–	–	1.5	1	1.9	1
Rough	22.2	1	–	–	–	–	–	–

[a] Total number of visits performed in each slaughterhouse.

completely changed, indicating the importance of being familiar with adequate standards of hygiene and handling. The identification of all these factors is essential to elucidating the microbiological cycles of *Salmonella* in the last steps of the pork production chain and to developing measures to reduce the risk to consumers. Moreover, in slaughterhouses C and D where sampling days were distributed at the start, middle and end of the work week, a lower prevalence was found at the beginning of the week probably due to improved cleaning and complete drying of all the instruments and the slaughterhouse facilities during the weekend. It might be expected that the highest prevalence would be found at the end of the week, but surprisingly it was found in both slaughterhouses in the sampling performed at the middle of the week.

The evaluation of chilled carcasses in slaughterhouses C and D revealed a significant reduction in the number of *Salmonella*-positive carcasses compared to the values obtained in the same carcasses before chilling and cooling. A total of 51 chilled carcasses (11.6%) were identified as contaminated during the study. When carcasses were stored in the cooling room for a period of 12–20 h, a similar result was obtained in both slaughterhouses, with a mean 3.8-fold reduction. When carcasses were kept in the cooling room for more than 50 h (sampling before chilling on Friday), the percentage of *Salmonella*-positive carcasses decreased by 7-fold in slaughterhouse C and 4-fold in slaughterhouse D. A similar result was previously reported by Tampling (Tamplin et al., 2001) and Botteldoorn et al. (2003) and can be attributed to two main factors that can decrease *Salmonella* concentration to non-detectable levels: (1) low temperature and (2) decline in water activity due to the air flow in the cooling room.

The transmission of *Salmonella* in the pork production chain was followed from farm-to-slaughter in study 1 by tracking the same pigs at the farm and slaughterhouse levels. In agreement with previous studies (Berends, Urlings, Snijders, & Van Knapen, 1996; Gebreyes et al., 2004; Hurd, McKean, Griffith, Wesley, & Rostagno, 2002), an increase in *Salmonella* contamination during the processing of live pigs into pork carcasses was demonstrated. Only 5.4% of the individuals were positive by processing faeces collected at the farm level, but *Salmonella* was detected in the caecal contents of 35.5% of the slaughtered pigs and 19.1% of these animals carried *Salmonella* in their MLNs. Altogether, 45.4% of the sampled pigs carried *Salmonella* in their faeces and/or MLNs. This result indicates that new infections might have emerged in at least a percentage of the animals. Taking into account that a resting period of two hours in a contaminated holding pen is enough for a pig to become infected (Hurd, Gailey, McKean, & Rostagno, 2001), the high contamination found in our study in holding pens before the arrival of the pigs could contribute to these new infections. We cannot make any conclusions about the transport process. An analysis of the lorries that were involved in the transport of the pigs was in the initial scheme of study 1, but unfortunately the number of samples collected by transport drivers immediately after the unloading of the pigs was very limited and these results were removed in order to avoid interpretation bias.

Despite the fact that highly contaminated intestines or lymph nodes can be a primary source of carcass contamination (Botteldoorn et al., 2003), we did not find any relationship among *Salmonella*-positive results in faeces, caecal contents or MLNs and carcass contamination of the same animal. According to our results, cross-contamination was estimated to account for 50% of all the contaminated carcasses, and no difference was found between infected and uninfected animals with respect to *Salmonella* contamination of the carcass. In contrast, Berends et al. (1997) and Borch, Nesbakken, and Christensen, (1996) estimated that 70% of pork carcass contamination was directly linked to the carrier pig itself. A similar result was described by Botteldoorn et al. (2003) in a study performed in five commercial slaughterhouses, although large variations amongst different facilities were noticed and cross-contamination accounted for 68.5% of the *Salmonella*-contaminated carcasses in one of the slaughterhouses and reached 100% in another one. On the other hand, in our study *Salmonella* was not identified in almost 40% of the carcasses from previously infected pigs, confirming that carrier pigs can lead to negative carcasses if the slaughtering process is carried out properly.

Holding pens have been identified as being highly contaminated in other studies (Botteldoorn et al., 2003; Boughton, Egan, Kelly, Markey, & Leonard, 2007; Swanenburg, Urlings, Keuzenkamp, & Snijders, 2001; Swanenburg, van der Wolf, Urlings, Snijders, & van Knapen, 2001). Accordingly, *Salmonella* was isolated from more than 80% of the faecal and surface samples collected in the holding pens before the entrance of the pigs in slaughterhouses A and B during study 1. An increase in the number of positive samples throughout the working day was demonstrated in slaughterhouses C and D. The highest level of *Salmonella* contamination was found at the middle of the working day, and a slight decrease in the percentage of contaminated pens was found at the end of the working day. A similar result was reported by Swanenburg, Urlings, Snijders, et al. (2001) in two Dutch pig slaughterhouses, and it was attributed to a dilution effect by the large amount of faeces that the pigs shed during their stay in the holding pens. The fact that more than 60% of the samples collected in holding pens after cleaning procedures and more than 30% of the samples collected at the start of the working day were positive confirms the ineffectiveness of the cleaning protocols used routinely, which are based on a combination of pressurised water and detergents. This situation has been described by other authors (Boughton et al., 2007; Small et al., 2006; Swanenburg, Urlings, Keuzenkamp, et al., 2001) and has been associated with the structural characteristics of the holding pens, which have many holes in the walls and floor where cleaning and disinfection solutions penetrate poorly. Therefore, special attention has to be paid to holding pens as highly contaminated points within slaughterhouses, and particularly to the cleaning and disinfection procedures used there in order to avoid their permanent contamination with *Salmonella*.

Several studies have evaluated *Salmonella* contamination in the slaughter lines of pig slaughterhouses (Botteldoorn et al., 2003; Giovannacci et al., 2001; Hald et al., 2003; Swanenburg, Urlings, Snijders, et al., 2001). Several points were evaluated during study 1 in slaughterhouses A and B, whereas a deeper evaluation was conducted during study 2 in slaughterhouses C and D. The prevalence of *Salmonella* contamination was high where actions are performed by the hands of operators and their implements: the evisceration point; kidney, lard and tonsil withdrawal points; and also renal capsula and fat removal points. At some of these points, a tendency towards an increase in contamination throughout the working day was observed, probably due to an accumulation of *Salmonella*. Evisceration is a critical point within the slaughtering process, and its importance in the spread and dissemination of *Salmonella* is well documented (Berends et al., 1997). A similar result has been described for the tonsil withdrawal point (Vieira-Pinto, Temudo, & Martins, 2005). We also found elevated contamination at the renal capsula removal point. The proximity between intestines and kidneys and also the proximity between the evisceration and kidney extraction points in the slaughter line make the surface contamination of the kidneys a possibility, which could explain our results. On the other hand, lard extraction and fat removal, activities that were done manually, also presented a high prevalence of *Salmonella* contamination. These points should be taken into consideration because they can be easily contaminated by positive carcasses and contribute to cross contamination due to the large surface of each carcass manipulated at this level.

Salmonella contamination was less frequently observed at the abdominal opening saw (mechanical) and at the carcass splitting and penis removal points. The bung dropper was analysed before (dirty) and after sterilisation (clean). It is remarkable that positive samples were found after sterilisation in three of the four slaughterhouses, showing an inadequate sterilisation protocol. *Salmonella* was also detected in the scalding tank water in slaughterhouses A and B. This

fact has been previously described by other authors and has been associated with scalding water of inadequate temperature (Hald et al., 2003; Swanenburg, Urlings, Snijders, et al., 2001). As previously proposed (Giovannacci et al., 2001; Letellier et al., 2009), the temperature of the scalding water seems to be of crucial importance, and *Salmonella* was not identified in the scalding tank in slaughterhouse D where the water was always around 62 °C.

The most prevalent serovars found in our study of pig carcasses and slaughter lines correspond to those previously reported in Spanish swine farms (EFSA, 2008b; García-Feliz et al., 2007; Mejía et al., 2006), supporting the hypothesis that infected pigs are the main source of *Salmonella* within slaughterhouses. In general, *S.* Typhimurium was the most frequently isolated serovar followed by *S.* Rissen, a relevant serovar in Spanish pig farms (EFSA, 2008a; García-Feliz et al., 2007; Vieira-Pinto et al., 2005). *S.* Derby, a common serovar in other slaughterhouse studies (Beloeil et al., 2004; Botteldoorn et al., 2003; EFSA, 2008a; McDowell et al., 2007; Vieira-Pinto et al., 2005), and the monophasic variant of *S.* Typhimurium *S.* 4,[5],12:i:-, which has increased in prevalence in the latest decade (EFSA, 2010b; Hopkins et al., 2010), were also identified in a relevant number of samples. *S.* Bredeney was the fourth serovar identified in our study, although it should be pointed out that the 72 *S.* Bredeney isolates were all found in the same visit to slaughterhouse D (more than 70% of the *Salmonella* isolates recovered in that visit). Consistent with previous studies (Botteldoorn et al., 2003; EFSA, 2008a; Giovannacci et al., 2001; Swanenburg, Urlings, Snijders, et al., 2001), strong variation between different visits was observed in both *Salmonella* prevalence and *Salmonella* serovars, showing that the *Salmonella* population in slaughterhouses is dynamic and suggesting the importance of live pigs as the main source of *Salmonella* in slaughterhouses. At the moment, we are involved in the molecular typing of the isolates in order to study the relationships between isolates from different sources and points.

5. Conclusion

This is the first report regarding *Salmonella* contamination in Spanish pig slaughterhouses. It provides relevant information to understand the dynamics of *Salmonella* in the last steps of the pork production chain and to develop the forthcoming National Control Program. Our results clearly indicate that in countries like Spain, with a high prevalence of the infection in swine farms, *Salmonella* control programmes should include both primary production and slaughterhouses. Although live pigs are the main source of *Salmonella* contamination in the slaughterhouses, new infections and cross contamination are very common during slaughtering and strict control measures should be taken at holding pens and the slaughter line to avoid them.

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Sero- and genotyping of *Salmonella* in slaughter pigs, from farm to cutting plant, with a focus on the slaughter process

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ABSTRACT

The objective of this study was to investigate the role of the slaughtering process in *Salmonella* carcass contamination by typing isolates recovered previously in a double study of the following: (1) a tracking survey from the farm to the slaughterhouse and (2) a survey of the slaughterhouse environment (i.e., lairage area, slaughter line, cutting plant and carcasses).

The *Salmonella* serotypes identified on the carcasses of the 16 tracked batches were frequently linked to lairage, whereas the serotypes detected at the farm, transport or pig-related samples (i.e., caecum content and lymph nodes) were only occasionally detected at the carcass level. Multi-locus variable-number tandem repeats (MLVA) of 77 *Salmonella enterica* ser. Typhimurium isolates from seven of these batches confirmed the link between the isolates recovered from carcasses and holding pens. Only four of the 16 positive carcasses had profiles previously isolated from lymph nodes or caecal content.

In the second part of the study, a total of 131 *S. enterica* ser. Typhimurium and 74 *S. enterica* ser. Derby isolates were further characterised by MLVA and Pulsed Field Gel Electrophoresis (PFGE), respectively. The MLVA profiles identified in carcasses varied throughout the working day and were frequently linked to those identified in samples from the slaughter line points collected close in time. PFGE and MLVA profiles identified at lairage were also detected in later processing facilities (i.e., slaughter line and cutting plant) as well as in carcasses. Finally, most of the profiles found at the cutting plants were previously identified in the slaughter line or carcass samples. The results from this study show that *Salmonella* contamination in pigs entering the slaughterhouse can be attributed to several sources. Typing of isolates by MLVA and PFGE clarified the sources of carcass contamination and improved the accuracy of cross-contamination attributable values. Without obviating the relevant role of infected pigs entering the slaughterhouse, the present study highlights the lairage and slaughtering as important sources of carcass contamination.

1. Introduction

During the last two decades, the concern about the role that pork plays in human salmonellosis has gradually increased. During the 1990s and the first years of the present century, it was estimated that 10% of human salmonellosis cases could be attributed to pork (Hald et al., 2004). In the last EU summary report on zoonoses, zoonotic agents and food-borne outbreaks published by the European Food Safety Authority (EFSA), pork was the third most frequently contaminated meat, after fresh chicken and turkey meat (EFSA, 2012). Nevertheless, with the implementation of control programmes in avian production in the European Union (EU) and the consequent reduction in the prevalence of poultry-related salmonellosis, the relative role of pork products has been enhanced (Pires et al., 2011). According to EU regulation 2160/2003, in the near future, control programmes for swine production will also be compulsory in the EU. In those countries where these programmes have not yet been implemented, such as Spain, accurate information regarding the status of *Salmonella* infection or *Salmonella* contamination at different stages of pork production is essential to elaborate programmes designed to reduce its prevalence (Arguello et al., 2012; EFSA, 2008a, 2009; García-Feliz et al., 2007, 2009; Gómez-Laguna et al., 2011; Torres et al., 2011).

Several studies have implicated the slaughtering process in the spreading of *Salmonella* in pork (Botteldoorn et al., 2003; Käsbohrer et al., 2000; McDowell et al., 2007). Two studies were performed in Spain to investigate the prevalence and main serovars in four Spanish commercial pig slaughterhouses (Arguello et al., 2012). The relationship

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between carcass contamination and contamination in live animals entering the slaughterhouse was specifically evaluated in the first study, whereas a more intensive assessment of slaughterhouse environmental contamination was the goal of the second study. Results from Arguello et al. (2012) described an increase in *Salmonella* contamination during the processing of live animals into pork carcasses, together with an unsuccessful linkage of *Salmonella* positive results in pig caecum samples or lymph nodes and positive carcasses. Moreover, cross-contamination was estimated to account for 50% of all contaminated carcasses and several critical points in the slaughter line were identified.

Molecular genotyping methods give us the chance to establish the relatedness among *Salmonella* isolates grouped by serotyping (Foley et al., 2007). Several authors have further investigated by molecular typing techniques the relationships among isolates obtained at slaughterhouses to elucidate the sources of contamination and the contamination cycles of the slaughter process (Botteldoorn et al., 2004; De Busser et al., 2011; Duggan et al., 2010; Giovannacci et al., 2000; Swanenburg et al., 2001c; Van Hoek et al., 2012; Wonderling et al., 2003).

The aim of the present study was to further investigate the sources of *Salmonella* carcass contamination in four Spanish commercial pig slaughterhouses. For this purpose, serotype results from the tracking study, described in Arguello et al. (2012), were analysed by batch and a selection of *S. enterica* ser. Typhimurium and *S. enterica* ser. Derby isolates from both studies were further characterised using molecular genotyping methods to more deeply examine the relationships among isolates obtained at different levels of the slaughtering process.

2. Materials and methods

2.1. Study design

The *Salmonella* isolates included in this study were obtained in the course of a previous investigation of the prevalence of *Salmonella* contamination and the main serovars found in Spanish pig slaughterhouses, including on carcasses, live animals and in the slaughterhouse environment (Arguello et al., 2012). The survey included two studies, a tracking from farm to slaughterhouse study and an environmental contamination study, performed in four Spanish commercial pig slaughterhouses (A, B, C and D). The tracking study from the farm to the slaughterhouse was performed by following a total of sixteen different batches of slaughtered pigs in slaughterhouses A and B, with eight batches per slaughterhouse. Briefly, 10 selected pigs within each batch were sampled at the farm by collecting faecal samples. After slaughtering, the caecal contents, mesenteric lymph nodes (MLN) and carcass swabs immediately before chilling were collected from the same selected pigs. Moreover, swabs from the surfaces of trucks and holding pens involved in the transport and resting of the selected animals as well as random holding pens, different points along the slaughter line (e.g., scalding tank water, bung dropper, evisceration and carcass splitting) and 50 randomly selected carcasses before chilling were sampled at each sampling day.

A more intensive assessment of the slaughterhouse environment was conducted during the environmental contamination study in slaughterhouses C and D, which were investigated on three different days distributed at the beginning, middle and end of the work week. Holding pens, several points along the slaughter line (e.g., scalding tank water, evisceration, renal extraction, carcass splitting and fat removal) and carcasses before and after chilling were sampled on Monday, Wednesday and Friday in each slaughterhouse. More information about the slaughterhouses' characteristics, the sampling design and the samples collected is available in the previous article (Arguello et al., 2012). Moreover, the adjacent cutting plant of slaughterhouses C and D was evaluated the following working day by collecting samples from machinery, belt surfaces and the hands of the personnel. Despite the fact that the results obtained in cutting plants were not included in the previous publication, the isolates collected from the meat processing activities of this facility (e.g., belt surfaces, personnel hands and their implements) were also included in the present study.

2.2. Salmonella isolation and serotyping

All of the samples were analysed according to the ISO standard methodology (EN-ISO 6579:2002 and EN-ISO 6579:2002/Amd 1:2007). Suspected *Salmonella* colonies were screened using the indole test and the 4-methylumbelliferyl caprilate fluorescence test (Mucap test, Biolife). A single confirmed *Salmonella* isolate from each positive sample was serotyped according to the Kauffman–White scheme using commercial antisera (Grimont and Weill, 2007).

2.3. Salmonella isolates selection

Seventy-seven *S. enterica* ser. Typhimurium isolates (henceforth named *S.* Typhimirium) were selected for further molecular typing. These isolates were obtained in tracking from farm to slaughterhouse study, during the tracking of three batches of slaughtered pigs (IV, V and VI) in two different visits (3 and 4) to slaughterhouse A and four batches of slaughtered pigs (XI, XII, XIII and XIV) in another two visits (2 and 3) to slaughterhouse B. All *S.* Typhimurium isolates recovered from samples collected at the farm, trucks, holding pens, caecal content, MLN, carcasses and slaughter environment during each sampling day were included. Batches were selected on the basis of the presence of isolates at several tracking stages to clarify relationships among *S.* Typhimurium isolates from different production steps.

All of the *S.* Typhimurium and *S. enterica* ser. Derby isolates (henceforth named *S.* Derby) recovered from the samples collected during the three visits to slaughterhouses C (74 *S.* Typhimurium and 22 *S.* Derby isolates) and D (57 *S.* Typhimurium and 52 *S.* Derby isolates) during the environmental contamination study were also selected for molecular typing.

2.4. Pulsed-field gel electrophoresis (PFGE)

PFGE using *Xba*I (Fermentas, Lifesciences, Spain) for restriction enzyme digestion was used to characterise *S.* Derby isolates. The technique was performed using the Centers for Disease Control and Prevention (CDC) PulseNet protocol (Ribot et al., 2006) with minor changes. The plugs of DNA were washed twice with double-distilled water and another four times with TE 10:1 buffer (10 mM Tris-1 mM EDTA) at 54 °C. Electrophoresis was performed in a Chef-DR®-III (Bio-Rad Laboratories, S.A., Madrid, Spain) using the following settings: initial switch time 2.2 sec, final switch time 63.8 sec, a gradient of 6 V/cm, 120º angle and 22 h of electrophoresis in 0.5×TBE buffer (Sigma-Aldrich). Two lambda markers (lambda DNA *cI*857 *ind* 1 *Sam*7, GelSyringe™, New England Biolabs, Ipswich, UK) and two size standard strains *Salmonella* Braenderup H9812 were included on each gel.

2.5. Multi-locus variable-number tandem repeats (MLVA)

Four of the VNTR loci described by Lindstedt et al. (2004), STTR5, STTR6, STTR9 and STTR10, as well as two new tandem repeat loci identified as STTR11 and STTR12 were used to characterise the selected *S.* Typhimurium isolates. The new loci were identified with the Tandem Repeat Finder program (Benson, 1999) available at http://tandem.bu.edu. STTR11 and STTR12 are situated in genes *tol*A, a membrane spanning protein, and *yoh*M, a nickel-cobalt efflux protein, at positions 814617 and 3184177 of the *S.* Typhimurium LT2 chromosome, respectively (McClelland et al., 2001). The main characteristics of the VNTR loci used are described in Table 1.

Briefly, one or two colonies of a pure culture of each selected *S.* Typhimurium isolate were suspended in 100 µl molecular grade water contained in a 1.5 ml screw cap microcentrifuge tube. The

samples were boiled in water at 100 °C and then centrifuged at 14,000 rpm for 8 min. The supernatant was used for PCR amplification. The primers were multiplexed in two solutions using the Maxima™ Hot Start PCR Master Mix (Fermentas, Lifesciences, Spain) in a total volume of 50 μl and a final primer concentration of 0.1 μM. Multiplex 1 was performed with primers STTR12 and STTR6, whereas multiplex 2 included the STTR5, STTR9, STTR10 and STTR11 primers. PCR parameters were set as indicated by Lindstedt et al. (2004). PCR products were pooled as follows: 5 μl of multiplex PCR1 and 2 μl multiplex PCR2 were mixed and diluted with 43 μl of molecular grade water to obtain a total volume of 50 μl. Two μl of the pooled products were combined with 0.25 μl of Rox-labelled MegaBACE ET550-R standard (GE Healthcare, Madrid, Spain) and 7.75 μl of 0.1% Tween 20 for a final volume of 10 μl. The ET size standard was previously diluted 1:20 in MegaBACE loading solution containing 70% formamide, 1 mM EDTA (GE Healthcare, Madrid, Spain). The samples were then denatured at 94 °C for 2 min and immediately subjected to capillary electrophoresis. The electrophoresis was run for 75 min using MegaBACE matrix (GE Healthcare, Madrid, Spain) with an injection voltage of 3 kV for 45 s and a running voltage of 10 kV. The amplification for each locus was identified using the MegaBACE Genetic Profiler software v1.2 (GE Healthcare, Madrid, Spain) taking into account the colour and size range. Internal control strains (SM 492, SM 353, SM 349) were used to corroborate the accuracy and repeatability of the technique. The fragment sizes were transferred to an Excel file. The amplicon sizes were extracted and converted to allele numbers following Lindstedt et al. (2004). The smallest amplicon found was named 01 for each locus and successive allele sizes were named 02, 03, *etcetera*. When no amplification was found, the allele was named 0. The allele strings were introduced in the following order: STTR12-STTR9-STTR5-STTR6-STTR10-STTR11.

2.6. Bioinformatics tools

The banding patterns from PFGE and repeat units from MLVA analysis were analysed with BioNumerics® version 6.5 (Applied-Maths, Ghent, Belgium). The PFGE results were compared by cluster analysis using the Dice coefficient and unweighted pair group method with arithmetic mean (UPMGA) with a position tolerance of 1.5% and optimisation of 1.0%. The Hunter-Gaston diversity index was used to calculate the polymorphism of individual loci (Hunter and Gaston, 1988) by the free software provided by the Health Protection Agency's Bioinformatics Unit (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl).

3. Results

3.1. Tracking from farm to slaughterhouse study results

3.1.1. Serotyping results

Fifteen *Salmonella* serotypes were recovered during the tracking of sixteen batches of 10 animals each from farm to slaughterhouse in abattoirs A and B (Table 2). Only in batch III, all of the samples yielded negative results. Four batches were positive at the farm and in three of them, at least one of the serotypes identified at the farm was also detected in animal-related samples collected at the slaughterhouse (i.e., caecum content, MLN or carcass). In contrast, *Salmonella* Derby was identified in faecal samples collected at the farm in one of the batches (batch VIII) but could not be isolated in any of the animal-related samples collected at the slaughterhouse. *Salmonella* Typhimurium was found in two of the three positive trucks and the same serotype was also found in animal-related samples at slaughter, whereas *S.* Rissen, which was found on the other slaughter truck, was isolated from the caecal content of the transported pigs but not from their MLN or carcasses (Table 2).

The widest diversity of serotypes was found at lairage, which was highly contaminated both before and after the stay of the tracked pigs. *S.* Typhimurium was identified on holding pens harbouring pigs from 14 of the 16 batches. This serotype was also identified in the caecal content or MLN samples in 11 of these batches. In contrast, from 11 batches in which serotypes other than *S.* Typhimurium were detected at the holding pens, only 6 were also contaminated by with the same serotype in the caecal content or MLN samples. Moreover, serotypes found at holding pens were also frequently identified on carcasses. In fact, only the serotype *S.* Brandenburg was identified in carcasses (batch IX in visit 1 to slaughterhouse B), without being previously isolated from the lairage area during the same working day.

In 11 of the 16 batches, the serotypes detected on the carcasses were also found in caecal contents or MLN samples. All of the sampled carcasses were negative in three of the selected batches. One of them (batch III) was also negative in the other animal-related samples (i.e., MLN and caecum content), whereas the other two were positive in the caecal content samples. Carcass contamination with *Salmonella* serotypes not previously detected in caecal contents or MLN was observed in 5 batches. In batches IX and XII, the serotypes detected in carcasses were completely different from those recovered from the caecum content and MLN (Table 2).

3.1.2. MLVA results

MLVA typing divided *S.* Typhimurium isolates into 21 different profiles in total, with five among the *Salmonella* isolates recovered in visits 3 and 4 to slaughterhouse A and seven and six different profiles detected in visits 2 and 3 to slaughterhouse B, respectively. One profile was shared by isolates from both visits to slaughterhouse A and another one by isolates from visit 4 to slaughterhouse A and visit 2 to slaughterhouse B. The MLVA profiles detected in tracked pigs are shown in Table 3.

The profile 11-12-5-3-0-2 was the most common in animal-related samples from both visits to abattoir A. This profile was identified in 10 of 21 and 16 of 27 *S.* Typhimurium isolates recovered during visits 3 and 4, respectively and could be detected in the lairage, the slaughter line and the pig-related samples (e.g., caecal content, MLN and carcass) from batches IV, V and VI as well as in the truck involved in the transport of the pigs from batch IV (Table 3). In contrast, there was no predominant molecular type among *S.* Typhimurium isolates from slaughterhouse B.

Table 1
Main information and results of loci employed in *Salmonella* Typhimurium MLVA analysis.

Loci	Dye and primers sequence (5′-3′)	Repeat (bp)	Smallest product size (bp)	No. alleles	Max (pi)[a]	Missing amplicon (%)	D. Index[b]
STTR 5	Lindstedt et al., 2004	6	212	12	0.434	0	0.761
STTR 6	Lindstedt et al., 2004	6	300	12	0.332	12.7	0.810
STTR 9	Lindstedt et al., 2004	9	153	5	0.790	7.3	0.362
STTR 10	Lindstedt et al., 2004	6	329	8	0.732	73.2	0.444
STTR 11	HEX-GGCGCAACAGCAGCAGCAACAGG/ GTTTTCGGCGCATTCTTACCC	45	434	2	0.956	0	0.085
STTR 12	TET-GCAGTGGCTGGCGGGAAACC/ GGGCATCCTGATACGCTTTTGACG	6	114	10	0.307	0	0.813

[a] Max (pi). Fraction of samples that have the most frequent repeat number in this locus (range 0.0 to 1.0).
[b] Diversity Index based on Hunter-Gaston index.

Table 2
Salmonella serotypes identified in the tracking of 16 batches of slaughtered pigs from farm to slaughterhouse in the farm to slaughterhouse tracking study.

Abattoir visit	Batch	Samples from tracked batches							Random samples		
		Farm	Transport	Holding pens[1]		Caecal content	MLN[2]	Carcasses	Holding pens	Slaughter environment	Carcasses
				Before pigs	*After pigs*						
A-1	*I*	–	–	–	Typ	Typ	Ent/Ris/ Bran/Lon	Typ	Typ/Bra	Typ	Typ/Bra
A-2	*II*			Typ/ Ris	Typ/ Lil	Typ	–	Typ'	Typ/ Ris/Lil/ Der/Ana	–	Typ/Lil/ Had
	III	–	–	–	–	–	–	–			
A-3*	*IV*	–	Typ	Typ	Typ	Typ	Typ/New	Typ	Typ/Ris/Der	Typ	Typ/Ris
A-4*	*V*	–	–	Typ	Typ	Typ	Typ	Typ	Typ	Typ	Typ/Bre
	VI	–	–	Typ/Bre	Typ/Der	Typ	–	–			
A-5	*VII*	–	–	Ris/Der/Lon	Typ/Ris	Lon/Ris	–	Ris	Ris/Typ / Der/Bra	Ris/Typ	Ris/Typ
	VIII	Derby	–	Typ/Ris	Ris	Typ/Ris	Typ	Typ/Ris			
B-1	*IX*	–	–	Typ/Ris	Gol/Der/Ris	Der/Typ	Typ	Bran	Typ/Ris/Der/Wie	Wie Der	Typ/Bra/Ris
	X	–	–	Typ	Typ/Bra	–	Typ	Ris/Typ/Bra			
B-2*	*XI*	–	–	Ris/Typ	Ris/Typ	Typ	Typ	Typ	Ris/Typ/Sen/Der	Ris/Typ	Ris/Typ/ Der
	XII	Typ/Lil/Ohi	Ris	Typ	Ris/Typ	Ris	–	Typ			
B-3*	*XIII*	–	Typ	Typ	Ris/Wie	Ris/Typ	Ris/Bra	Ris/Der/Typ	Typ/Der/Ris	Typ	Typ/Ris/ Bre/Der
	XIV	–	–	Typ/Wie	Typ/Wie	Typ	Ris/Typ	Ris/Typ			
B-4	*XV*	Ris	–	–	Der/Ris/Typ	Der/Inf/Bra	Ris	Ris/Typ	Ris/Gol/Typ/ Wie/Der	Bra/Der/ Ris/Typ	Typ/Ris/ Gol
	XVI	Ris	–	–	–	Typ/Ris/Der	–	–			

Ana = Anatum, Bra = Bradenburg, Bre = Bredeney, Der = Derby, Ent = Enteritidis, Gol = Goldcoast, Had = Hadar, Inf = Infantis, Lil = Lille, Lon = London, Ohi = Ohio, Ris = Rissen, Sen = Senftemberg, Typ = Typhimurium, Wie = Wien.
*Visit selected for further molecular typing analysis (MLVA) of *S.* Typhimurium isolates (in grey).
[1]Holding pens, where tracked pigs were housed, were sampled before and after the staying of the selected pigs.
[2]MLN. Mesenteric lymph nodes.

The MLVA profile identified at the farm (Batch XII, visit 2) was also detected at the holding pens but not in isolates recovered from other samples. It is noteworthy that at visit 2 to slaughterhouse B, none of the three profiles found in monitored or randomly sampled carcasses were related to those identified in any of the other samples collected. In visit 3 to slaughterhouse B, the *S.* Typhimurium profile 8-9-0-3-7-2 was identified in the samples from the truck, in holding pens and in caecal contents but not on carcasses.

In total, six MLVA profiles found at the carcass level could not be identified at any of the other pig-related, holding pen or slaughterline samples and another three MLVA profiles were restricted to isolates recovered from caecal contents or MLN. MLVA analysis showed that among 16 *S.* Typhimurium isolates recovered from carcasses, only four could be linked to caecal contents or MLN isolates, whereas the other 12 matched isolates recovered from the slaughterhouse environment or did not fit with any other sample.

3.2. Environmental contamination study

3.2.1. Serotyping results

Nine serotypes were found among 546 *Salmonella* isolates recovered during the environmental contamination study, considering also the cutting plant isolates (not included in the previous publication by Arguello et al., 2012). *S.* Typhimurium, *S.* Rissen and *S.* Derby were detected on different sampling days within each slaughterhouse. In total, they constituted more than 80% of the isolates recovered from slaughterhouses C and D (data not shown).

3.2.2. MLVA characterisation of S. Typhimurium isolates

Seventy-four *S.* Typhimurium isolates obtained from slaughterhouse C and 57 *S.* Typhimurium isolates from slaughterhouse D were further typed with MLVA. The profiles detected are shown in Table 4. The number of profiles identified on each sampling day ranged from 3 (visit 2 to slaughterhouse C) to 10 (visit 1 to slaughterhouse C). Three MLVA profiles were shared among isolates from different visits to slaughterhouse C and another three were also shared by isolates from visits 2 and 3 in slaughterhouse D. At least one of the profiles detected at each visit could be tracked in different facilities. Moreover, samples collected together in time (at the same sampling round) from different slaughter line activities and from carcasses frequently shared the same MLVA profile, whereas profiles from samples of the same slaughter line activity but collected at different times had different profiles.

In slaughterhouse C, the MLVA profiles identified in 19 *S.* Typhimurium-contaminated carcasses (57.6%) did not match those identified in any of the isolates from holding pens or the slaughter line. In slaughterhouse D, 14 of the *S.* Typhimurium-positive carcasses (70%) shared profiles with the slaughterhouse environment, whereas only six of them (30%) were not linked to other samples. *S.* Typhimurium MLVA profiles identified among isolates recovered from the slaughter line were frequently linked to those detected on carcasses in both slaughterhouses and all of the MLVA profiles except for one detected at the cutting plant facilities were previously detected on carcasses or at earlier points from the slaughter line or lairage.

3.2.3. PFGE characterisation of S. Derby isolates

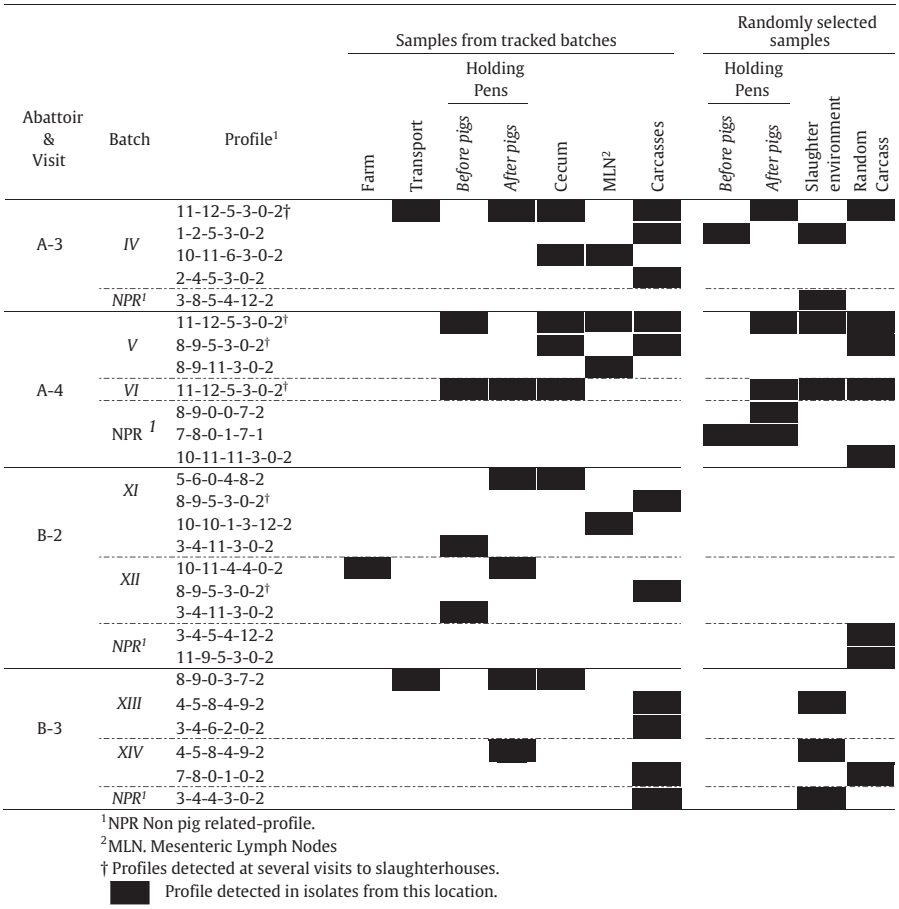
A total of 74 isolates of *S.* Derby recovered from slaughterhouses C and D were further analysed. PFGE using *Xba*I yielded 24 different profiles (Fig. 1). The number of profiles identified ranged from 2 (visit 3 to slaughterhouse D) to 15 (visit 1 to slaughterhouse D). Profile *XD23* was the most prevalent, comprising 38 isolates identified on carcasses and in environmental samples in slaughterhouse D as well as on carcasses in slaughterhouse C. Profile *XD21* was only detected on two of the sampling days in slaughterhouse C and was identified in holding pens, along the slaughter line and in cutting plant, although it was not detected on carcasses. The other profiles were represented by a lower number of isolates and 18 of them were identified in a single isolate.

Twelve of 15 *S.* Derby-contaminated carcasses (80%) shared their PFGE profile with those detected at the slaughterhouse on the same sampling day, whereas 3 of them (20%) presented a single PFGE profile unique among the *S.* Derby isolates.

3.3. MLVA loci results

Loci STTR5, STTR11 and STTR12 were amplified in the 205 isolates of *S.* Typhimurium, whereas locus STTR10, located on a plasmid, was

Table 3
MLVA profiles detected in *S.* Typhimurium isolates recovered in seven batches tracked from farm to slaughterhouse during the farm to slaughterhouse tracking study.



[1]NPR Non pig related-profile.
[2]MLN. Mesenteric Lymph Nodes
† Profiles detected at several visits to slaughterhouses.
Profile detected in isolates from this location.

only present in 16.8% of these isolates. The highest diversity index was found for STTR12, followed by STTR6 and STTR5 (Table 2). In contrast, only two different profiles were found for the most stable locus, STTR11.

4. Discussion

Several studies have investigated *Salmonella* contamination of pigs at the time of slaughter and the role that the harvest plays in the final carcass contamination (Berends et al., 1997; Botteldoorn et al., 2003; McDowell et al., 2007; Swanenburg et al., 2001a). Consistently, the environment is contaminated to a greater or lesser extent in commercial pig slaughterhouses and these authors have stressed the importance of the slaughter process in the final carcass contamination by *Salmonella*. In accordance with these assertions, a previous study in four Spanish pig slaughterhouses demonstrated an increase in *Salmonella* contamination during the processing of live animals into pork as well as the importance of slaughtering practices; cross-contamination accounted for an estimated 50% of all of the contaminated carcasses (Arguello et al., 2012). *Salmonella* contamination was reported in most of the evaluated points along the slaughter line and *Salmonella* was frequently present in the lairage area, even after cleaning procedures.

Although phenotypic typing methods such as serotyping or phage typing are useful as preliminary tools for *Salmonella* classification, molecular methods such as PFGE or MLVA are needed to clarify the relationship among isolates from the same serotype (Larsson et al., 2009). The accuracy, simplicity and lower cost of MLVA compared to other typing methods enhances its usefulness for *S.* Typhimurium analysis (Lindstedt et al., 2004) and it has been successfully used with isolates recovered from the slaughterhouse (Prendergast et al., 2011; Van Hoek et al., 2012). In contrast, PFGE remains the gold standard for *Salmonella* typing and *Salmonella* Derby isolates from the slaughterhouse environment have been successfully restricted using *Xba*I (Botteldoorn et al., 2004; De Busser et al., 2011; Giovannacci et al., 2000; Gomes-Neves et al., 2012; Piras et al., 2011; Wonderling et al., 2003). The present study is a further investigation of contamination sources and pathways in these Spanish pig slaughterhouses by using serotyping and molecular typing methods in *Salmonella* isolates obtained in the course of the previous survey.

Two hundred and eight *S.* Typhimurium and 74 *S.* Derby isolates could be further separated into profiles by MLVA and PFGE, respectively. Loci STTR5 and STTR6 showed frequent polymorphism according to previous studies (Dyet et al., 2010; Lindstedt et al., 2004; Prendergast et al., 2011). One of the two new loci included in this study, STTR12, also had high variability. On the other hand, the other three loci used, STTR10, STTR9 and STTR 11, were less polymorphic. Only two different alleles were found in STTR11, which seems to be highly preserved among *S.* Typhimurium isolates. In our study, the *S.* Derby isolates were divided

Table 4
MLVA profiles detected in 74 and 57 *Salmonella* Typhimurium isolates recovered in slaughterhouses C and D during the environmental contamination study.

Abattoir-visit	Genotype	N° Isolates	Holding Pens	Slaughter Line	Carcasses	Cutting plant
C-1	10-9-5-3-0-2	8	■		■	
	10-9-6-3-0-2	4	■		■	
	8-9-6-3-0-2†	6		■		■
	10-9-8-3-0-2†	4			■	
	12-11-7-4-6-2	1			■	
	11-10-11-3-0-2	2			■	
	9-10-11-3-0-2	1			■	
	9-10-6-3-6-2	9		■	■	
	8-9-5-3-0-2	4		■		■
	8-9-0-3-1-2	1			■	
C-2	12-11-8-3-0-2	8	■	■	■	
	10-9-11-3-15-2†	4				
	8-9-6-3-0-2†	1		■		
C-3	9-8-5-3-0-2	3	■			
	11-10-0-0-6-2	4	■			■
	10-9-4-3-0-2	2	■			
	10-9-2-3-0-2	2	■			
	10-9-11-3-15-2†	2			■	
	10-9-8-3-0-2†	4			■	
	9-8-5-3-6-2	4	■		■	
D-1	7-8-4-4-6-2	3	■	■	■	
	8-1-0-1-0-1	2	■			
	10-11-8-4-6-2	4		■	■	
	9-10-4-3-0-2	1				■
	12-5-4-1-6-2	1		■		
	10-13-11-4-15-2	1		■		
D-2	8-9-6-3-0-2†	2	■	■		
	8-9-7-3-0-2†	2		■		
	8-9-8-3-0-2†	5			■	■
	11-12-0-0-0-1	2			■	
	3-11-9-4-6-2	1	■			
	3-5-8-3-0-2	1	■			
	8-5-0-1-0-1	1			■	
	3-11-9-4-6-2	1	■			
	3-5-8-3-0-2	1	■			
	8-5-0-1-0-1	1			■	
D-3	8-9-8-3-0-2†	2	■	■		
	8-9-7-3-0-2†	3	■		■	
	8-9-6-3-0-2†	13		■	■	■
	8-9-5-3-0-2	1	■			
	9-10-4-3-0-2	2			■	■
	9-10-4-3-0-2	1	■			
	7-8-5-3-0-2	4	■		■	■
	9-10-3-3-8-2	2			■	■

†Profiles detected at several visits to the same slaughterhouse.
■ Profile detected in isolates from this location.

into 24 profiles, including 18 single profiles. *S.* Derby is a highly variable serovar which complicates the establishment of relationships among isolates (Hauser et al., 2011; Valdezate et al., 2005). Although Wonderling et al. (2003) tried to overcome this problem by grouping related profiles, in the present study a decision was made to avoid grouping similar profiles based on the assumption that those *S.* Derby isolates which share a common PFGE profile would definitely have a common origin.

Several profiles were found at each visit to the four monitored slaughterhouses. This result is in agreement with previous surveys, which by slaughter isolates genotyping, have indicated that the slaughter environment is contaminated by several strains at the same time (Botteldoorn et al., 2004; Piras et al., 2011; Prendergast et al., 2011; van Hoek et al., 2012; Wonderling et al., 2003). Furthermore, most profiles differed among visits to the same slaughterhouse, a fact previously reported (Giovannacci et al., 2000; Letellier et al., 2009; Piras et al., 2011; Wonderling et al., 2003). The fact that a few MLVA and PFGE profiles were shared at different visits to the same slaughterhouse could be ascribed to permanent contamination of the slaughter environment, as has been asserted by other authors (Baptista et al., 2010; Piras et al., 2011; Swanenburg et al., 2001c; van Hoek et al., 2012). Another reasonable hypothesis is that pigs entering the slaughterhouse might share the same or very similar profiles. The same farms or the same suppliers usually slaughter their pigs at the same slaughterhouses and furthermore, even without common farm of origin, *S.* Typhimurium or *S.* Derby isolates could share the same MLVA or PFGE profiles.

The role that transport plays in carcass contamination has been previously highlighted by Magistrali et al. (2008). In our tracking study, some of the carcasses from batch IV in slaughterhouse A were contaminated with the same *S.* Typhimurium MLVA profile found on the trucks used for the transport of the pigs, thus confirming that *Salmonella* isolates present at transport can reach the slaughtering process and contaminate carcasses. In contrast, the MLVA profile detected in the truck of batch XIII at slaughterhouse B was also detected in holding pens and caecal contents but not in the monitored carcasses. Likewise, *S.* Rissen was detected in the truck and caecal content of pigs from batch XII but not in other samples from the same batch.

Serotypes recovered from carcasses were related to those identified in holding pens in almost all of the positive batches. The lairage has been linked to carcass contamination in several studies and it is fairly clear that this constitutes an important source of contamination for the incoming pigs (Hurd et al., 2002; Swanenburg et al., 2001b). Molecular typing of *S.* Typhimurium isolates confirmed the link among isolates detected on carcasses and in the holding pens where the pigs were kept in the three batches from slaughterhouse A and in some of the batches from slaughterhouse B. The link between isolates from carcasses and lairage has been previously demonstrated (De Busser et al., 2011; Duggan et al., 2010). In the environmental study (abattoirs C and D), MLVA profiles detected at holding pens were frequently identified among isolates from the slaughter line or cutting plant facilities and from carcasses. Interestingly, MLVA and PFGE profiles found in holding pens before the entrance of the pigs were afterwards found at other sampling points on the same day such as the slaughter line, carcasses and even the cutting plant. This result confirms that the lairage is an important risk point for incoming *Salmonella*-free pigs, as has been proposed previously (Arguello et al., 2012; Letellier et al., 2009) and that it constitutes a source of contamination for further processing at harvest. Another interesting finding was that one *S.* Derby PFGE profile, *XD23,* was detected at several holding pens after cleaning procedures. This fact could be a consequence of spreading by the staff in charge of the lairage cleaning by their boots or by washing activities. Moreover, this profile was detected in holding pens at visits 1 and 3 to slaughterhouse D, performed over a six-week period. Although it is reasonable to think that the origin of the isolate detected at visit 3 was different from the one detected at visit 1, the possibility that it persisted during this time in the environment, as previously proposed (Duggan et al., 2010; Hald et al., 2003), cannot be excluded. These results highlight, once more, the importance of proper cleaning and disinfection procedures in the control of *Salmonella* within the slaughterhouse.

In some individuals, the serotype found on the carcass differed from the serotypes previously detected in caecum contents or MLN. Moreover, several MLVA profiles detected at the farm, transport and in caecal content or MLN from monitored pigs did not match the MLVA profiles from carcasses. These interesting results have been reported previously (Botteldoorn et al., 2004; De Busser et al., 2011) and yield two lessons: on the one hand, they show that carcass contamination can be minimised by good slaughtering practices, even when contaminated pigs enter the slaughter line. On the other hand, they reveal that the *Salmonella* isolates carried by pigs are not always the source of contamination for their carcasses. The same authors (Botteldoorn et al., 2004) reported significant differences in the fraction attributable to cross-contamination between slaughterhouses. Likewise, in the EFSA baseline study on *Salmonella* prevalence in slaughter pigs, the proportion of contaminated carcasses differed significantly among slaughterhouses supplied with similar proportions of initially contaminated pigs (EFSA, 2008b). Both studies highlight the importance of slaughtering practices. In the present study, the MLVA results also revealed clear differences in the sources of carcass contamination between slaughterhouses C and D. While most of the profiles from carcasses were linked to those detected at dressing

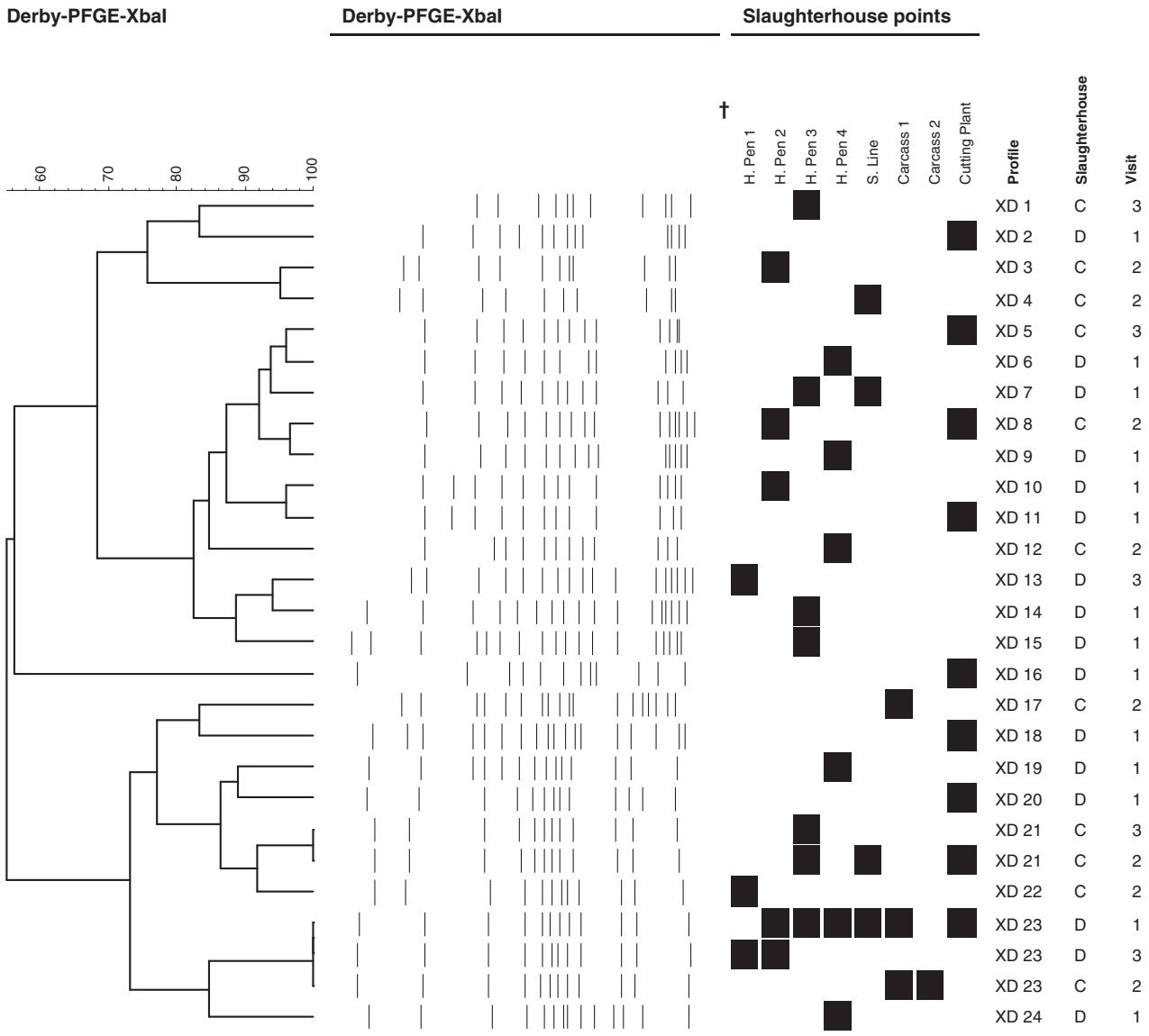


Fig. 1. Profiles of 74 *S.* Derby, restricted with *XbaI*, detected in the different visits to abattoirs C and D. Similarity analysis was done using Dice coefficient (position tolerance of 1.5% and optimisation 1.0%) and clustering was done by the unweighted pair group method with arithmetic mean (UPGMA). Rectangles show profiles detected at different visits and/or abattoirs.

activities from the slaughter line in slaughterhouse D, only some of them matched the profiles found at the slaughter line in slaughterhouse C, mainly among isolates obtained during visit 1 to this abattoir. Despite the small number of carcasses that were contaminated with *S.* Derby, these isolates usually shared a PFGE profile with those recovered from the environment, although a few of them displayed single profiles. Both slaughterhouses had similar slaughter line structure, slaughtered similar number of pigs per hour and shared pig suppliers (Arguello et al., 2012). Therefore, this result demonstrates that part of the contamination can be attributed to internal factors of the slaughtering process, such as handling and hygienic standards.

In the preliminary analysis based on prevalence results, it was estimated that half of the positive carcasses were cross-contaminated in slaughterhouses A and B (Arguello et al., 2012). Further analysis using MLVA for the characterisation of a selection of *S.* Typhimurium isolates from these slaughterhouses showed that only 25% of the isolates obtained from carcasses matched those recovered from the caecal contents or MLN of the same animals. Therefore, the proportion of cross-contaminated carcasses should be higher than what was previously assumed. As has been formerly suggested, to have an accurate value of cross-contamination, studies should include molecular typing methods to corroborate the relatedness of isolates of the same serotype (Botteldoorn et al., 2004; Wonderling et al., 2003).

At slaughterhouses C and D, the same MLVA profile was detected in carcasses sampled close in time, a fact which allows us to suggest a common source of contamination for these carcasses. In addition, some of the profiles from dressing activities isolates matched those recovered from carcasses sampled near the same time. In relatively few cases, MLVA profiles were shared by isolates recovered from carcasses and the slaughter line at different sampling rounds on the same sampling day. Giovannacci et al. (2000) also reported a high concordance among profiles identified among isolates recovered from different samples collected at the same sampling series and De Busser et al. (2011) grouped samples by herd-dependent genotypes. According to these results, it seems that there are continuous *Salmonella* inputs, most likely from infected pigs, into the slaughter line and that the strains involved in contamination vary through the working day. Taking into account that a new batch of pigs was slaughtered every half hour at both slaughterhouses, this variability in *Salmonella* profiles can be explained by the introduction of new profiles by infected pigs. As described by Botteldoorn et al. (2004), these profiles can, in some cases,

persist along the slaughter line during slaughtering activities and contaminate carcasses later in time.

Of the nine MLVA profiles detected at the cutting plant facilities, eight had already been detected at other facilities or carcasses on the previous day. The two main PFGE profiles (*XD21* and *XD23*) were also identified in carcasses and samples collected from the three facilities (e.g., holding pens, slaughter line and cutting plant). Other authors have also reported identical profiles in the cutting plant and other plant facilities (Duggan et al., 2010; Giovannacci et al., 2000; Gomes-Neves et al., 2012). The present data are further evidence that *Salmonella* isolates introduced by *Salmonella*-positive pigs in the slaughterhouse can reach the processing plant and are possibly introduced by carcasses. The cutting plant is the last processing stage before the meat is delivered to retail or butcher's shops. The presence of *Salmonella* at this facility represents a risk for meat contamination and thus for human health.

5. Conclusions

In summary, the results from this study show that *Salmonella* contamination in pigs entering the slaughterhouse can be attributed to several sources, including the farm, transport and especially the lairage. These contaminated pigs are constantly introducing *Salmonella* into the slaughter process and together with inappropriate slaughter practices, contribute significantly to carcass contamination. The molecular typing methods MLVA and PFGE have proven to be useful tools that should be considered in slaughter studies to investigate the sources of carcass contamination. Both methods have been able to distinguish among isolates of the same serotype and have demonstrated a continuous flow of *Salmonella* within the slaughterhouse, from the farm level, transport or lairage to the slaughter line, carcasses and finally to the cutting plant. They have also been used to increase the accuracy of cross-contamination attributable values. According to the results obtained in four Spanish commercial pig slaughterhouses, carcass contamination can be related to several sources. Without obviating the relevant role of infected pigs entering the slaughterhouse, the lairage and slaughtering are indicated as important sources of the final carcass contamination.

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Effect of logistic slaughter on *Salmonella* contamination on pig carcasses and the slaughterhouse environment

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HIGHLIGHTS:

- Study to evaluate the logistic slaughter survey in pork Salmonella contamination
- Low, high, moderate risk batches slaughtered in three consecutive days
- No clear effect of logistic slaughter on carcass contamination
- Low risk batches contaminated at the lairage and slaughtering activities
- Proper slaughter practices prevented the carcass contamination in high risk batches

ABSTRACT

A relevant proportion of the finishing pigs carry *Salmonella* in Spain. Previous studies have shown that infected pigs are the source of carcass and slaughterhouse environment contamination by *Salmonella.* The strategic separation of pigs according to their *Salmonella* status would reduce the cross-contamination by decreasing the inputs of *Salmonella* by infected pigs. This study tried to evaluate the effect of a logistic slaughter, organized according to *Salmonella* serological data, on *Salmonella* contamination on carcasses and the environment at the slaughterhouse. Nineteen batches of pigs were analysed at the beginning of slaughtering during three consecutive days. Low risk herds (8 batches) were slaughtered on Monday, high risk herds (6 batches) on Tuesday and, finally, moderate risk herds (5 batches) were slaughtered on Wednesday. Each slaughtering day, holding pens, five points of the slaughter line, and 80 carcasses were sampled. The results evidenced no clear effect of logistic slaughter on carcass contamination. On day III, when moderate *Salmonella* risk batches were slaughtered, the risk of finding a positive carcass was three times higher than that for the other two days. In contrast, the proportion of *Salmonella* contaminated carcasses was similar between low and high risk herds. Carcass contamination in low risk herds was linked to the contamination of holding pens and slaughter line activities. *Salmonella* was not detected in any of the sampled carcasses in three out of six high risk *Salmonella* batches, showing that proper slaughtering practices can prevent carcass contamination. The experience reported here demonstrates that to be effective logistic slaughter should be performed with an accurate batch separation according to seroprevalence levels and together with strict measures of cleaning and disinfection in the lairage and the slaughterhouse facilities.

KEYWORDS

Salmonella, slaughterhouse, carcass, logistic slaughter

1. INTRODUCTION

Human salmonellosis is a major concern food-borne illness in industrialized countries. The last report on trends and sources of zoonoses of the European Food Safety Authority (EFSA) accounted for 95,548 human cases of salmonellosis in 2011in the European Union (EU) and identified pork and pork products as the third most frequently contaminated food after chicken and turkey meat (EFSA, 2013). While the number of human cases of salmonellosis has dropped during the last 6 years (EFSA, 2013), linked to poultry prevalence reduction, the role of pork has been enhanced as a consequence of the lack of mandatory control programs or reduction targets in swine production (Pires et al. 2011).

Salmonella infected pigs constitute a potential source of contamination for the slaughterhouse environment and consequently for carcasses during slaughtering (Duggan et al. 2010). A high *Salmonella* prevalence in Spanish swine production has been reported by several studies (García-Feliz et al. 2007; Gomez-Laguna et al. 2011; Mejía et al. 2006;) as well as by the two mandatory baseline studies conducted across the EU in slaughter pigs and breeding herds (EFSA, 2008; EFSA, 2009). Accordingly, high prevalence on carcasses as well as frequent contamination of the slaughterhouse environment and dressing activities has also been reported (Arguello et al. 2012; Arguello et al. 2013a; Hernandez et al. 2013).

Logistic or sanitary slaughter (i.e. the separation of pigs at slaughtering considering their *Salmonella* burden) would overcome, at least partially, the cross-contamination by decreasing the inputs of *Salmonella* by infected pigs meanwhile non-infected animals are being slaughtered. Indeed, sanitary or logistic slaughter is mandatory in Denmark (Alban et al. 2012). Fattening farms are classified by their serological status and those pigs from highly infected herds are slaughtered separately and under increased hygienic precautions. Pigs from these herds are compulsorily slaughtered in specific slaughterhouses and at the end of the working day, avoiding overnight lairage housing and mixing with non-infected pigs. Furthermore, the slaughter line speed is lowered, heads are not spitted and pluck and intestines are either condemned or heat-treated. Finally, carcass swabs are randomly taken and, if *Salmonella* prevalence exceeds a certain limit, pork is heat-treated or cured before consumption (Sorensen et al. 2004). The Irish National *Salmonella* Monitoring Program also recommends the slaughtering of high *Salmonella* risk farms at the end of the work day or at the end of the work week (Duggan et al. 2010).

Nevertheless, as far as we know only one field survey has been carried out to evaluate the effect of logistic slaughter of pigs on *Salmonella* contamination (Swanenburg et al. 2001a). The aim of

this study was to evaluate the effect of a logistic slaughter, organized according to *Salmonella* serological data, on *Salmonella* contamination on carcasses and the environment at the slaughterhouse.

2. MATERIALS AND METHODS

2.1. Sample collection at the farms

Forty-two supplier herds that would potentially slaughter their pigs at the dates fixed for the study took part in the initial farm analysis. Between 18 to 12 days before the scheduled slaughter, 40 blood individual samples from randomly selected pigs close to market-weight and 10 faecal samples from ten randomly selected pens (one sample consisted of five portions of fresh pinches of faeces) were collected in each finishing herd and were immediately submitted to the laboratory for their processing. The information provided by the laboratorial analysis of the sera samples was used to classify the batches as low, moderate or high *Salmonella* risk, by the percentiles 35^{th} and 65^{th}, described in the results section.

2.2. Study design and sample collection at the slaughterhouse

The study was performed in a commercial slaughterhouse that slaughtered approximately 380 pigs per hour. Holding pens with concrete-floor were washed daily according to the slaughterhouse procedures and most of the dressing activities were performed manually.

Each of 19 monitored batches consisted approximately on 190 pigs from a single farm. These pigs were transported to the slaughterhouse in a separate truck that was previously thoroughly cleaned and disinfected. Mingling at lairage was avoided and no pigs were housed at the holding pens before the entrance of the monitored batches to minimize cross-contamination. Resting time varied between two and four hours.

The study was conducted throughout three consecutive days (Monday, Tuesday and Wednesday). The slaughtering of pigs from monitored batches was performed at the start of each working day and was organized as follows: low *Salmonella* risk batches were slaughtered on day I (Monday), high *Salmonella* risk batches were slaughtered on day II (Tuesday) and moderate *Salmonella* risk batches on day III (Wednesday).The lairage area, the slaughter line and carcasses were sampled within each sampling day. Holding pens housing the pigs from the monitored batches were sampled twice. Before the entrance of the pigs, the surface of these holding pens was sampled in five points($25cm^2$ each) by using sterile gauzes previously moistened in buffered peptone water (BPW), while after the stay of the animals, a pool of faeces was collected from each pen, as described

for faecal samples collected at the farm. Five points of the slaughter line in which contamination was frequently detected in a previous study in the same slaughterhouse (Arguello et al. 2012), were sampled five times during slaughtering of the selected batches. The selected points were evisceration, renal-capsula removal, lard removal, kidney extraction and fat removal. Except from the fat removal point where two different samples were collected, one from the personnel and the other from the tool used, the hands of personnel together with their instruments (knives basically) were sampled in the selected points using sterile gauzes previously moistened in BPW. The first sampling of each point was programmed before the onset of the activity, while the other four were distributed uniformly along the slaughtering of the monitored batches.

The scheduled number of carcasses sampled per sampling day was 75. All of them were randomly selected among those of the monitored batches and were sampled in the slaughter line, after the meat inspection point. Sampling was performed by swabbing on the external and internal surface of the right-half carcass using an sterile gauze previously moistened in BPW, on three different points: ham, rib-cage and neck-upper-shoulder. Within each point, a square of 15 $cm^2$was swabbed, encompassing an area of approximately 1,350 cm^2.

All these samples were collected using sterile gloves which were changed between each collected sample. Samples collected by gauzes were stored in 250 ml sterile flasks containing 50 ml of BPW. Samples were sent to the laboratory in cooled containers within the same day for their immediate analysis.

2.3. Sample analysis

2.3.1. *Salmonella* isolation and serotyping

All the samples were analyzed according to the current EN-ISO standard methodology 6579:2002/Amd 1:2007, following the protocol previously described (Arguello et al. 2012). Suspected *Salmonella* colonies were confirmed using the indol test and the 4-methylumbelliferyl caprilate fluorescence test (Mucap test, Biolife). A single definite *Salmonella* isolate from each positive sample was serotyped according to the Kauffman-White scheme using commercial antisera (Grimont and Weill, 2007).

2.3.2. Genotyping of *S.*Typhimurium isolates

Molecular typing of *S.*Typhimurium isolates was carried out by Multilocus Variable number of tandem repeat Analysis (MLVA) as previously described (Arguello et al. 2013a) using six VNTR loci: STTR5, STTR6, STTR9, STTR10 (Linstedt et al. 2004), STTR11 and STTR12 (Arguello et al. 2013a).

Fragment sizes were transferred to our MLVA database (Microsoft Excel spreadsheet) and amplicon sizes were extracted and turned into allele numbers following the premises cited by Lindstedt et al. (2004). The allele strings were introduced in the following order STTR12-STTR9-STTR5-STTR6-STTR10-STTR11.

2.3.3. Serological analysis

Serological analysis was carried out using a commercially available indirect enzyme-linked immunosorbent assay (ELISA) (Herdchek *Salmonella*®, Idexx laboratories) for the detection of porcine-IgG anti-*Salmonella* LPS, according to manufacturer's instructions. Coating antigens in this ELISA included LPS of serogroups B, C1 and D (O-antigens 1,4,5,6,7, and 12) (Farzan et al. 2007). The cut-off was fixed at 20% of the optical density (OD%=20).

2.4. Statistical analysis

All data were stored and analysed in EpiInfo for Windows (CDC, Atlanta, Georgia, USA). A univariate analysis using the chi-square test at $\alpha = 0.05$ was used to investigate any association between the prevalence of *Salmonella*-contaminated carcasses and the proportion of positive samples from the slaughter line among sampling days.

3. RESULTS

3.1. Farm results

A total of 19 batches from the 42 initially tested farms were finally slaughtered during the three sampling days of the present study. The selected batches were classified according to the percentage of *Salmonella* seropositive pigs (Table 1). By the percentiles 35^{th} and 65^{th}, batches were separated in low *Salmonella* risk batches (8 batches, seroprevalence ≤20%), moderate *Salmonella* risk batches (6 batches, seroprevalence 21-49%) and high *Salmonella* risk batches (5 batches, seroprevalence≥50%) that were slaughtered on day I (Monday), day III (Wednesday) and day II (Tuesday), respectively. *Salmonella* was not detected in any of the pooled-faecal samples collected at the farm from the 19 selected batches.

Table 1. *Salmonella* contamination on pigs at farm level and carcasses and the lairage at slaughterhouse level after the logistic slaughter of 19 batches of pigs separated according to their seroprevalence.

Day & Company	Batch	FARM RESULTS		SLAUGHTER RESULTS			
		Pens1	Seroprevalence2	Lairage		Carcasses	
				H. Pen before	H. Pen after	Total sampled	Positive Carcass
Day I							
C	B1.1	Negative	12.5 [2.3-22.7]	Positive	Negative	8	2
C	B1.2	Negative	20 [7.6-32.4]	Positive	Negative	10	4
C	B1.3	Negative	18.75 [6.7-30.8]	Positive	Negative	14	0
A	B1.4	Negative	0	Positive	Negative	10	1
C	B1.5	Negative	15 [3.9-26.1]	Positive	Negative	9	0
A	B1.6	Negative	0	Positive	Negative	10	0
A	B1.7	Negative	15 [3.9-26.1]	Negative	Positive	10	0
B	B1.8	Negative	0	Negative	Negative	9	0
Total	-	-	-	6	1	80	7
Day II							
C	B2.1	Negative	52.5 [37.0-68.8]	Negative	Negative	11	0
A	B2.2	Negative	62.5 [47.5-77.5]	Negative	Negative	11	0
A	B2.3	Negative	65 [50.2-79.8]	Negative	Negative	12	3
A	B2.4	Negative	67.5 [7.6-32.4]	Negative	Positive	14	1
B	B2.5	Negative	65 [50.2-79.8]	Negative	Negative	16	0
A	B2.6	Negative	70 [55.8-84.2]	Negative	Negative	15	1
Total	-	-	-	0	1	79	5
Day III							
B	B3.1	Negative	42.5 [27.2-57.8]	Negative	Positive	15	7
B	B3.2	Negative	47.5 [31.2-63.8]	Positive	Negative	15	5
A	B3.3	Negative	45 [29.6-60.4]	Positive	Negative	15	2
A	B3.4	Negative	47.5 [31.2-63.8]	Negative	Positive	15	0
C	B3.5	Negative	35 [20.2-49.8]	Positive	Negative	18	5
Total	-	-	-	3	2	78	19

Low Salmonella Risk [0-≤20]

High Salmonella Risk [≥50]

Moderate Salmonella Risk [>21-<50]

[1] Results of *Salmonella* isolation from ten samples of pooled faeces collected from ten pens within each farm.

[2]Seroprevalence obtained from 40 sera using a 20% OD cut-off. 95% of interval confidence in brackets.

3.2. *Salmonella* contamination at the slaughterhouse

Summarized results from samples collected at the farms, holding pens and carcasses at the slaughterhouse are shown in Table 1, while results obtained in the slaughter line during the three sampling days are shown in Table 2.

Eight low *Salmonella* risk batches were slaughtered on day I and from the 80 carcasses analysed seven yielded positive (8.75%). *Salmonella* was isolated before the entrance of the pigs in six of the eight holding pens where the monitored batches were housed. On this day, the first sampling round in the slaughter line was performed once 22 pigs from the first batch had already been eviscerated and not before the beginning of the slaughtering as was foreseen. At this moment, three of the selected points in the slaughter line were already contaminated by *Salmonella.* Four of the five sampled points yielded positive to *Salmonella* at least at one of the samplings performed during the slaughtering of the pigs and only the lard removal point resulted negative at all the samplings performed. Overall, six out of 24 samples collected in the slaughter line during the slaughtering of the monitored batches (25%) were contaminated by *Salmonella*.

Six high *Salmonella* risk batches were slaughtered on day II and *Salmonella* was detected in five out of 79 sampled carcasses (6.3%). No *Salmonella* was detected in holding pens before the housing of the monitored pigs neither in any of the samples collected in the slaughter line before the beginning of the activity on day II. Only two samples from the slaughter line yielded positive (8.3%) in the samplings performed along the slaughtering of the monitored batches during day II, both in the third sampling round.

On day III, five batches of moderate *Salmonella* risk were slaughtered. Nineteen of the 78 sampled carcasses were contaminated by *Salmonella* (24.4%). *Salmonella* was isolated from three of the five monitored holding pens before the entrance of the pigs. Similarly to day II, no *Salmonella* was detected in any of the samples collected from the slaughter line before the beginning of the work. However, three samples (12.5%) from the slaughter line were positive during the slaughtering of the monitored batches.

The risk of finding a positive carcass was three times higher on day III compared to the other two days (χ^2=11.57, p<0.001; RR=3.22, 95% CI 1.63-6.25). In contrast, the proportion of *Salmonella* contaminated carcasses was similar on days I and II. No differences among days were detected in the proportion of positive samples of the slaughter line points evaluated during the slaughtering of the selected batches (χ^2=3.66, p=0.16).

Table 2. *Salmonella* contamination in the slaughter line before and during the logistic slaughter of 19 batches of pigs separated according to their seroprevalence. Samples collected before the beginning of the slaughtering (round 1) and during slaughtering of selected batches (rounds 2 to 5).

	Evisceration	Renal Capsula	Kidney Extraction	Lard Removal	Fat removal (tool)	Fat Removal (hand)
Prior the beginning of the day(Round 1)						
Day I*	POS	POS	POS	NEG	NEG	NEG
Day II	NEG	NEG	NEG	NEG	NEG	NEG
Day III	NEG	NEG	NEG	NEG	NEG	NEG
During the slaughtering**						
Day I						
Round 2	NEG	NEG	NEG	POS	NEG	POS
Round 3	NEG	NEG	NEG	NEG	NEG	NEG
Round 4	NEG	NEG	NEG	NEG	NEG	NEG
Round 5	POS	POS	NEG	POS	POS	NEG
Day II						
Round 2	NEG	NEG	NEG	NEG	NEG	NEG
Round 3	POS	NEG	NEG	NEG	NEG	POS
Round 4	NEG	NEG	NEG	NEG	NEG	NEG
Round 5	NEG	NEG	NEG	NEG	NEG	NEG
Day III						
Round 2	POS	NEG	POS	NEG	NEG	NEG
Round 3	NEG	NEG	NEG	NEG	NEG	NEG
Round 4	NEG	NEG	NEG	NEG	NEG	NEG
Round 5	NEG	NEG	NEG	POS	NEG	NEG

* Round 1 of day I was performed when 20 pigs had already entered in the slaughter line.
** Low risk herds where slaughtered on day I; high risk herds were slaughtered on day II and moderate risk herds were slaughtered on day III.

3.3 Typing results

A total of 64 *Salmonella* isolates were recovered throughout the study: 58 were identified as *S.*Typhimurium, three as *S.* Derby and the other three were rough isolates. *S.* Derby was only identified on day I from two samples collected at the holding pens before the pig entrance (batches B1.5 and B1.6) and one faecal sample collected from the holding pen where batch B1.7 was allocated (Table 3).

*S.*Typhimurium isolates were recovered from the lairage area, slaughter line and carcasses. Fifty-four of these *S.*Typhimurium isolates were genotyped by MLVA and further divided into six different MLVA profiles (Table 3). The profile 9-9-5-3-0-2, detected in 43 isolates from samples collected along the three days of the study, was the predominant genotype. It was identified in part

of the positive samples from the lairage before the pigs'entrance on day I, in the faecal sample from the holding pen where pigs from batch B3.4were housed on day III and in one positive sample from the slaughter line on this day. Furthermore, all the positive carcasses presented this MLVA genotype. Genotype 9-9-11-4-12-2 was detected in three points of the slaughter line in the last sampling round on day I and also in holding pens before the pigs stay on day III. Genotype 9-9-6-3-10-2 was detected in holding pens and slaughter line points, 9-9-9-3-0-2 to slaughter line points on day III, while genotypes 9-9-6-3-8-2 and 9-9-15-4-6-2 were restricted to single isolates from holding pens.

Table 3. Characterization by serotyping (*S.* Derby) and MLVA (*S.* Typhimurium) of the isolates obtained from the slaughterhouse after the logistic slaughter of 19 batches of pigs.

			Lairage		Slaughter line points						
Day		Batch	*H. Pen before*	*H. Pen after*	Carcass	Evisceration	Renal Capsula	Kidney Extraction	Lard Removal	Fat removal (tool)	Fat Removal (hand)
Day I	Low *Salmonella* *Risk*[0-≤20]	B1.1	9-9-5-3-0-2		9-9-5-3-0-2	9-9-5-3-0-2	9-9-5-3-0-2	9-9-5-3-0-2			
Day I		B1.2	9-9-5-3-0-2		9-9-5-3-0-2						9-9-5-3-0-2
Day I		B1.3	9-9-6-3-8-2								
Day I		B1.4	9-9-5-3-0-2		9-9-5-3-0-2						
Day I		B1.5	*S.*Derby								
Day I		B1.6	*S.*Derby								
Day I		B1.7		*S.*Derby							
Day I		B1.8				9-9-11-4-0-2	9-9-11-4-0-2			9-9-11-4-0-2	9-9-11-4-0-2
Day II	High *Salmonella* *Risk*[≥50]	B2.1									
Day II		B2.2									
Day II		B2.3			9-9-5-3-0-2	9-9-5-3-0-2				9-9-5-3-0-2	
Day II		B2.4		9-9-15-4-6-2	9-9-5-3-0-2						
Day II		B2.5									
Day II		B2.6			9-9-5-3-0-2						
Day III	Moderate *Salmonella* *Risk*[>21-<50]	B3.1		9-9-5-3-0-2	9-9-5-3-0-2	9-9-9-3-0-2		9-9-5-3-0-2	9-9-9-3-0-2		
Day III		B3.2	9-9-11-4-0-2		9-9-5-3-0-2						
Day III		B3.3	9-9-9-3-0-2		9-9-5-3-0-2						
Day III		B3.4		9-9-5-3-0-2							
Day III		B3.5	9-9-11-4-0-2		9-9-5-3-0-2						

4-. DISCUSSION

The purpose of *Salmonella* surveillance and control programmes in pigs is to reduce the public health risk arising from the consumption of contaminated pork, reducing human disease and maintaining consumers' confidence (Boyen et al. 2008). Despite most of the *Salmonella* infections are subclinical in pigs they induce an immune response which is used in surveillance programmes to classify pig farms according to their *Salmonella* risk (Sorensen et al. 2004). Those farms classified as high *Salmonella* risk by their serological results are the target of different control measures to avoid the entrance of the bacteria in the food chain (Alban et al. 2002; Davies and Cook 2003; Osterkorn et al. 2001; Quirque et al. 2001) including, in some cases, the separate slaughtering under special sanitary conditions of highly contaminated herds (Quirke et al.2001; Sorensen et al. 2004).

An accurate classification of the farms or batches is needed in order to obtain the presumable benefits of a logistic slaughter procedure. According to this premise, in the present research study, blood was collected from the monitored farms close in time to the harvest, avoiding the use of information from previous batches that could not be precise enough, as previously reported (Swanenburg et al. 2001a). Bacteriological analyses were performed to complement the information given by the serology. In spite of using pooled-pen faecal samples, which increases the sensitivity of bacteriological diagnosis (Arnold and Cook, 2009; EFSA, 2011), no *Salmonella* could be isolated from the faeces at any of the farms that were finally included in the study. Apart from economic reasons, serology has replace bacteriology in *Salmonella* surveillance because *Salmonella* faecal shedding in pigs is characterized by variable durations and intermittent shedding (Gray et al. 1995; Nielsen et al. 1995). Moreover, it has been described that *Salmonella* shedding usually occurs during the first half of the fattening, when infection is frequently established, while the seroconversion usually occurs in the last third (Beloeil et al. 2003; Pires et al. 2012). Most of the infected pigs develop a carrier state in which the bacteria remain viable in several organs and tissues, particularly tonsils and mesenteric lymph nodes (Fedorka-Cray et al. 1995). Stressing factors such as transport or lairage can reactivate the *Salmonella* shedding of these carriers and, thus, non-shedding seropositive pigs at the farm can become an important source of *Salmonella* contamination at the slaughterhouse (Arguello et al. 2012; Hurd et al. 2002).

In the present study, the cut-off selected to analyze the sera was the 20% OD. A higher cut-off value (e.g., 40% OD) would be recommended to initiate a surveillance programme (Sorensen et al. 2004). Nonetheless, the use of high cut-offs implies that part of the infected

farms can be misclassified as low *Salmonella* risk farms. In contrast, lower cut-offs (e.g, 11% OD) could facilitate false positive reactions (Alban et al. 2012). An intermediate value such as 20% OD offers the best correlation between bacteriological and serological results (Alban et al. 2002). The distribution of batches according to the proportion of seropositive pigs was similar to the current classification of the Danish National *Salmonella* monitoring programme (Alban et al. 2002). When farms are classified by serology, it must be kept in mind that the antibody response against *Salmonella* LPS is affected by two main factors: the serotype involved and the pig exposure to the pathogen (Ivanek et al. 2012). Underestimation of the actual farm infection-level can occur in those herds infected by *Salmonella* serotypes that do not elicit high immune response, or in which the pigs are exposed to low doses of the pathogen. Furthermore, the delay in the immune response (Ivanek et al. 2012; Nielsen et al. 1995) can also lead to misclassification of the farms if the infection occurs at the end of the fattening period. In all these cases, batches should be erroneously classified as low risk *Salmonella* regardless of a high risk of *Salmonella* shedding at harvest.

The previous research on the effect of logistic slaughter concluded that pork from sero-negative herds was less contaminated with *Salmonella* than pork from sero-positive herds. Nevertheless, *Salmonella* contamination on pig carcasses from sero-negative herds has also been reported (Swanenburg et al. 2001a). In the present study, there was no clear effect of logistic slaughter on carcass contamination. On day III, when moderate *Salmonella* risk batches were slaughtered, the risk of obtaining a positive carcass was three times higher than that calculated for the other two days, when low and high *Salmonella* risk batches were slaughtered. The prevalence of *Salmonella* contamination on carcasses was similar on day I, when low risk batches were slaughtered, and on day II, when high risk *Salmonella* batches were processed. Surprisingly, the lowest percentage of *Salmonella* contaminated carcasses was observed on day II. Similarly to our study, Duggan et al. (2010) reported the highest carriage of *Salmonella* in gastrointestinal contents and lymph nodes from moderately infected herds. Both results highlight the risk of those farms classified as moderately contaminated according to serological results.

Agreement between serology and carcass contamination has been reported by several authors (Lettellier et al. 2009; McDowell et al. 2007; Sorensen et al. 2004). In the study performed by Sorensen et al. (2004), *Salmonella* was only detected in carcasses from one of fifteen sero-negative batches. In contrast, half of the low seroprevalence batches slaughtered on day I in our study yielded positive carcasses. Discrepancy among serological status and *Salmonella* isolation from pig samples has been demonstrated in other studies (Botteldoorn et

al. 2003; Duggan et al. 2010; Visscher et al. 2011). This unexpected carcass contamination in low *Salmonella* risk batches can be attributed to the contamination of the holding pens and the slaughter line by *Salmonella*. In our study, six of the eight holding pens where pigs were allocated were contaminated by *Salmonella* before the entrance of the animals. Moreover, the *S.* Typhimurium MLVA genotype identified in isolates from four of these holding pens was also found on positive carcasses from the first four batches slaughtered on that day. Pigs can become infected by *Salmonella* within three hours after the contact with contaminated environments (Fedorka-Cray et al. 1995; Rostagno et al. 2003) and, for this reason, a number of studies have put lairage forward as a source of infection for *Salmonella*-free pigs (Arguello et al. 2013b; Duggan et al. 2010; Hurd et al. 2002; Hurd et al. 2005; Rostagno et al. 2003; Swanenburg et al. 2001b). In agreement with our results, positive carcasses from low seroprevalence herds were also linked to holding pens in the study performed by Duggan et al. (2010) and lairage was stated as the most important source of contamination for low risk herds as well. Swanenburg et al. (2001a) also reported contamination in carcasses from low seroprevalence herds slaughtered at the beginning of the day in their logistic slaughter study. It is worth noting that, when just 20 carcasses had entered in the clean zone of the slaughter line, samples from the evisceration point together with renal-capsula and kidney removal points yielded positive for *S.* Typhimurium with the same MLVA genotype. No *Salmonella* was detected in the slaughter line before the onset of slaughtering the two following days, so presumably contamination detected at the beginning of the slaughtering on day I was introduced by pigs contaminated in the lairage. The spillage of intestinal content during evisceration, due to intestinal perforation, has been highlighted as one of the major sources of carcass contamination (Berends et al. 1997; Davies et al. 1999; Pearce et al. 2004).

Salmonella was not detected in any of the sampled carcasses in three out of six high risk *Salmonella* batches that were slaughtered on day II. This fact corroborates that pork from seropositive farms can be *Salmonella*-free and confirms the importance of the slaughterhouse and the slaughtering process in the control of *Salmonella* within the food chain (Arguello al., et al. 2013a; Arguello et al. 2013b; EFSA, 2008). Adequate slaughter procedures during the slaughtering of *Salmonella*-seropositive pigs can avoid contamination of carcass surfaces (Swanenburg et al. 2001b). Another interesting data is that the prevalence obtained in the slaughter line points checked did not varied among days, despite different risk herds were slaughtered and different carcass prevalences were detected.

MLVA has been successfully used to determine the interrelationships among isolates recovered from slaughterhouse studies (Arguello et al. 2013a; Prendergast et al. 2011; Van

Hoek et al. 2012). In the present study, all *S.* Typhimurium isolates recovered from positive carcasses were grouped in the profile 9-9-5-3-0-2, the most prevalent through the three sampling days. In earlier studies performed within the same slaughterhouse (Arguello et al. 2013a), a broader number of serotypes and genotypes were detected at each visit. Moreover, *S.*Typhimurium genotypes differed from one visit to another and even at different moments through the same sampling day, linked to new batches introduced. A possible explanation is that coming from a few suppliers, batches could be contaminated with the same strain. Another possibility is that the strain was established as slaughterhouse resident-flora as it has been reported by previous studies (Baptista et al. 2010; Botteldoorn et al. 2003; Duggan et al. 2010; Giovanacci et al. 2001). Contamination was not evaluated in points such as the polishing machine or the splitting saw, which could be the source of contamination, spreading this strain to carcasses through the three days.

In conclusion, in the present study the effectiveness of a logistic slaughter strategy was measured by the evaluation of carcass and slaughterhouse contamination by *Salmonella*. Our results highlight the role of the slaughterhouse and all the slaughtering procedures in *Salmonella* contamination of pork. Cleaning and disinfection procedures starting during lairage and extended throughout the slaughter line are crucial to avoid new infections and cross-contaminations. The experience reported here exhibits that to be effective logistic slaughter should be performed with an accurate batch separation according to seroprevalence levels and together with strict cleaning and disinfection measures in the lairage and slaughterhouse facilities.

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DISCUSIÓN GENERAL

Calendario Agrícola Panteón de los Reyes de León. Siglo XII (San Isidoro, León, España).

Meses de junio a septiembre. Representan la recogida de la cosecha (trigo y uva) en los meses de verano.

Las toxi-infecciones alimentarias son, sin lugar a dudas, las zoonosis más frecuentes en los países desarrollados. Los actuales sistemas de producción animal intensiva, el incremento de consumo de productos de origen animal y los cambios en los hábitos alimenticios, entre otros factores, han facilitado que en las últimas décadas se mantenga una elevada incidencia de estas toxi-infecciones alimentarias, que además, en ocasiones, están asociadas a tratamientos arduos y costosos, consecuencia del desarrollo de resistencias a los antimicrobianos. La preocupación y exigencia crecientes por parte de las administraciones públicas y del consumidor para recibir productos seguros desde un punto de vista microbiológico, se ha traducido en que en los países europeos, uno de los retos de las dos últimas décadas en salud pública haya sido el control y reducción de las toxi-infecciones alimentarias asociadas, directa o indirectamente, al consumo de productos de origen animal bajo el concepto "de la granja a la mesa". En la última década, en la UE, *Salmonella* ha sido el segundo agente más frecuentemente identificado en toxi-infecciones alimentarias y el primero en cuanto a número de brotes se refiere (EFSA, 2013).

En el año 2003, la UE elaboró una normativa, reglamento CE 2160/2003, con las bases para la reducción de *Salmonella* en la producción primaria y en otras etapas de la cadena alimentaria. Según este reglamento, los países miembros deben elaborar programas de monitorización y control de *Salmonella* en producción avícola y porcina. Estos programas ya son preceptivos en avicultura pero por el momento no son obligatorios en el ganado porcino. Algunos países disponen de programas de control de salmonelosis porcina en marcha (Mousing et al., 1997; Osterkorn et al., 2001; Rowe et al., 2003) mientras que otros permanecen a la espera de que la UE fije los objetivos de reducción de prevalencia para esta especie animal. Con el fin de disponer de información adecuada para fijar estos objetivos, la UE ha financiado estudios de prevalencia basal en cerdos de cebo y en granjas de cerdos reproductores (EFSA, 2008a; EFSA, 2009a).

En lo que respecta a España, desde mediados de la pasada década se han realizado diversos estudios para mejorar el conocimiento sobre la infección por *Salmonella* en explotaciones porcinas. Los estudios de Mejía et al. (2006) en Cataluña y de García-Feliz et al. (2007) a nivel nacional fueron los primeros trabajos que alertaron sobre la elevada prevalencia de esta infección en las granjas españolas. Este dato fue refrendado con posterioridad por los estudios basales en cerdos de cebo y en granjas de reproductores (EFSA, 2008a; EFSA 2009a) que situaron a España a la cabeza en lo que respecta a la prevalencia de *Salmonella* en el ganado porcino de la UE. Además se han realizado estudios de factores de riesgo (García-Feliz et al., 2009), de resistencia a antimicrobianos (Astorga et al., 2007; García-Feliz et al., 2008), de

contaminación del pienso (Torres et al., 2011) o de empleo de ácidos para la reducción de la prevalencia en granja (Creus et al., 2007). Finalmente, se han llevado a cabo diversos estudios centrados en la infección por *Salmonella* en la producción de cerdo ibérico (Gómez-Laguna et al., 2011; Hernández et al., 2012; 2013).

De acuerdo a Davies et al. (2004), la cadena alimentaria puede ser considerada como un conjunto de fases lineales dedicadas a la producción, sacrificio, distribución y consumo. *Salmonella* ha sido detectada en todas las etapas (Lo Fo Wong et al., 2004) y por ello, en cada una de estas fases se pueden aplicar medidas de control para reducir el riesgo. Los programas de control de *Salmonella* deben definir la combinación de actuaciones que permita alcanzar la mayor reducción del riesgo con el mínimo coste (Davies et al., 2004). En la presente tesis doctoral se han abordado estudios que comprenden las fases de reproducción, de cebo y el matadero, con el fin de aportar nuevas nociones en el control de la infección por *Salmonella* en cerdos.

1. *Salmonella* en reproductores

La mayoría de los estudios de prevalencia de *Salmonella* en cerdos se han centrado en animales en la fase de cebo (Baggesen et al., 1996; Fedorka-Cray et al., 1996; Van der Wolf et al., 1999; Christensen et al., 2002; Stege et al., 2002; Garcia-Feliz et al., 2007; Bonardi et al., 2013). Por el contrario, pocos estudios han analizado la prevalencia en granjas reproductoras. Únicamente en Dinamarca, como parte del estudio de evolución de la prevalencia después de la instauración del programa de control, Christensen et al. (2002) realizaron un estudio transversal con el objetivo de determinar esta prevalencia en cerdos reproductores.

Durante el año 2008, se llevó a cabo un estudio transversal en todos los países miembros de la UE con el fin de determinar la frecuencia de la infección en granjas de reproductoras y los factores de riesgo asociados a la presencia de *Salmonella* (EFSA, 2009a). En España se recogieron dos tipos de muestras en este estudio: las muestras de heces obligatorias y comunes a todos los países miembros y, además, muestras de sangre de diez cerdas reproductoras en cada granja. Los resultados del análisis bacteriológico sobre las muestras de heces mostraron que, al igual que en cerdos de cebo (Mejia et al., 2006; García-Feliz et al., 2007; EFSA, 2008a; Gómez-Laguna et al., 2011), la prevalencia de *Salmonella* era muy elevada, detectándose al menos una muestra positiva en dos de cada tres granjas analizadas. Como cabía esperar, la prevalencia serológica fue aun superior y dependiendo del punto de corte empleado en el Mix-LPS-ELISA hasta nueve de cada 10 granjas fueron seropositivas. El número

de muestras positivas identificadas en cada granja positiva o prevalencia dentro de granja también fue superior en los análisis serológicos en comparación con la bacteriología. Este hecho se debe a que mientras que la eliminación por *Salmonella* es intermitente (Fedroka-Cray et al., 1994), los anticuerpos y particularmente las inmunoglobulinas de la clase IgG permanecen detectables en suero durante largos periodos de tiempo. En concordancia con los datos globales para el conjunto de la UE (EFSA, 2011b) y con el análisis más particular realizado en el caso de Dinamarca (Arguello et al., 2013), la prevalencia tanto bacteriológica como serológica de *Salmonella* fue similar en las granjas de multiplicación y de producción a pesar de que, probablemente, existen diferencias en el manejo, la bioseguridad y la sanidad entre estos dos tipos de granjas.

S. Rissen fue el serotipo detectado con mayor frecuencia en granjas de reproductoras en España. Este serotipo es muy prevalente en la península ibérica; fue el segundo serotipo más frecuente en granjas de cebo españolas (Garcia-Feliz et al., 2007) y ha sido aislado con frecuencia en estudios portugueses (Vieira-Pinto et al., 2005). Aunque la mayoría de los aislados de *S.* Rissen obtenidos en el estudio basal de granjas de cerdas reproductoras de la UE estaban asociados a estos dos países, de acuerdo con publicaciones recientes (Van Hoek et al., 2012; Bonardi et al., 2013; Thai et al., 2013) podríamos estar ante un serotipo en expansión y con posibles ventajas adaptativas para la infección de cerdos. Además, *S.* Rissen ha sido asociado con brotes de salmonelosis en personas. En el año 2007 fue la causa de un brote de *Salmonella* en Italia (Boschi et al., 2010), mientras que recientemente, en nuestro país, se ha identificado en un brote en Asturias asociado al consumo de productos crudo-curados de cerdo (Rodicio, comunicación personal). El segundo serotipo más prevalente fue *S.* Typhimurium, que es el serotipo más frecuentemente aislado en cerdos de cebo en Europa (EFSA, 2008a). En el estudio llevado a cabo por García-Feliz et al. (2007), *S.* Typhimurium fue detectado en el 40% de las granjas de cebo españolas, que sumadas al 15% de granjas infectadas por su variante monofásica (*S.* 4,[5],12:i-), permitió concluir que más de la mitad de las granjas de cebo españolas estaban infectadas por "*Salmonella* Typhimurium-like strains". Teniendo en cuenta este dato, la prevalencia detectada en granjas de reproductoras es aproximadamente la mitad para este serotipo. Esta variabilidad en la prevalencia de *S.* Typhimurium se extendió a otros serotipos, siendo evidentes las diferencias entre los serotipos detectados en las dos poblaciones. Así, de los diez serotipos más frecuentemente aislados en granjas de cerdos reproductores seis coincidieron con los identificados en las granjas de cebo (aunque no en el mismo orden de prevalencia). Por el contrario, serotipos muy prevalentes en el estudio de reproductoras como *S.* Anatum o *S.* London solo fueron moderadamente

frecuentes en cerdos de cebo (García-Feliz et al., 2007). Un resultado similar se observó al comparar los serotipos presentes en cerdos de cebo y en reproductores en Dinamarca, empleando igualmente los datos de los estudios basales de la EFSA (Arguello et al., 2013). Este dato puede ser un indicio de una limitada relevancia de la transmisión vertical en esta infección. Finalmente, también existieron diferencias en lo que respecta a los serotipos más prevalentes en España en comparación con los datos globales descritos para el total de la UE. Así, *S.* Derby que fue el serotipo más frecuente en granjas de reproductoras europeas ocupó el cuarto lugar en importancia en España mientras que serotipos como *S.* Infantis o *S.* Livingstone, situados entre los seis más frecuentes en la UE, no aparecieron entre los diez más relevantes en nuestro país.

Además de este análisis descriptivo de los datos obtenidos en el estudio basal de granjas con cerdos reproductores, se llevó a cabo un estudio de factores de riesgo. Este tipo de estudios son fundamentales para la identificación de intervenciones que puedan emplearse en las granjas infectadas en el marco de los programas de control (Van der Wolf et al., 2001b). En el año 2009, se publicó el primer trabajo sobre factores de riesgo en explotaciones con cerdos de cebo en España (García-Feliz et al., 2009) y mediante la aportación incluida en esta tesis hemos tratado de completar la información en granjas de reproductores. Conjuntamente a la recogida de muestras en las explotaciones de cerdos reproductores se cumplimentó un cuestionario elaborado de acuerdo a los dictámenes de la Comisión Europea CE 55/2008 y que incluía preguntas referidas a las características de la granja, del tipo de producción, estructura, manejo, alimentación o el empleo de antimicrobianos. Existen pocos trabajos sobre factores de riesgo en cerdos reproductores. El primero fue llevado a cabo por investigadores daneses en 2003 (Kranker et al., 2003) y los otros dos trabajos publicados hasta el momento actual corresponden al análisis de los datos del estudio basal de cerdos reproductores de Portugal (Correia-Gomes et al., 2012; 2013).

Para la identificación de factores de riesgo mediante el análisis de datos de bacteriología empleamos una regresión logística binomial, siendo este el abordaje más frecuentemente empleado para el análisis de este tipo de datos (van der Wolf et al., 1999; Bahnson et al., 2006; Rajic et al., 2007; García-Feliz et al., 2009). La variable dependiente y dicotómica fue el estatus frente a *Salmonella*, granja positiva o negativa, definiendo granja positiva como aquella en la que se identificó *Salmonella* en al menos una de sus muestras. Dos variables demostraron estar relacionadas con *Salmonella* en el modelo final: el uso de piensos granulados y la reposición de los verracos. La forma de presentación del pienso, granulado o en "pellet" frente a las harinas ha sido uno de los factores más comúnmente señalados por los

estudios de factores de riesgo en cerdos de cebo, habiéndose encontrado una mayor prevalencia de *Salmonella* en granjas alimentadas con estos piensos granulados (Beloeil et al., 2003; Lo Fo Wong et al., 2004; Rajic et al., 2007; Hautekiet et al., 2008; García-Feliz et al., 2009). Tanto nuestros datos, como los generales de la UE (EFSA, 2011b) o los obtenidos por Kranker et al. (2003) avalan la misma relación en el caso de las granjas con cerdos reproductores. Aunque el proceso de granulación implica la obtención de un alimento más higiénico desde el punto de vista microbiológico, la causa por la que este tipo de dieta se asocia a una mayor prevalencia de *Salmonella* está relacionada con la molturación y el tamaño del grano (Mikelsen et al, 2004). Los piensos granulados tienen una molturación más fina, lo que se traduce en una mayor digestibilidad y una menor proporción de nutrientes disponibles en el intestino grueso. Por el contrario los piensos en harina, con molturación grosera, permiten la llegada de mayor cantidad de nutrientes al intestino grueso que son aprovechados por la microbiota beneficiosa creando un ambiente hostil para *Salmonella* y una mejora de la salud intestinal. En lo que respecta al origen de la reposición de los verracos, la compra de la reposición en otras explotaciones se asoció a la presencia de *Salmonella* en cerdos reproductores. Este no es el primer trabajo que resalta la importancia de la reposición de los verracos. Previamente, Correia-Gomes et al. (2013) establecieron la relación entre la infección por *Salmonella* y el origen del semen empleado en las explotaciones. En el estudio general de los datos de reproductores en la UE, la reposición de cerdas desde un origen externo fue también asociada a la presencia de *Salmonella* en la explotación (EFSA, 2011b). Igualmente, en un estudio de factores de riesgo en cerdos de cebo, la presencia de *Salmonella* estuvo asociada a la compra de la reposición a tres o más proveedores (Lo Fo Wong et al., 2004). Teniendo en cuenta estos resultados, parece evidente que la reposición puede jugar un papel relevante en la introducción de *Salmonella* en las explotaciones y, consecuentemente, el chequeo de enfermedades infecciosas en estos animales de reposición es de gran importancia para evitar la entrada de este y de otros muchos patógenos.

En lo que respecta a los datos de serología, las granjas fueron clasificadas, teniendo en cuenta el número de sueros positivos, en tres categorías de contaminación. Se emplearon dos sistemas de clasificación, coincidentes en el punto de corte empleado y en el porcentaje de sueros positivos con las empleadas por los programas de control de salmonelosis porcina danés inicial y actual (Alban et al., 2012). El análisis de los datos se llevó a cabo empleando una regresión logística ordinal, con tres niveles de contaminación, bajo, moderado y elevado, como variable dependiente. Cabe destacar que los resultados obtenidos empleando ambos sistemas de clasificación fueron coincidentes, detectándose el alojamiento individual, el trimestre y el

tipo de suelo como factores asociados a la prevalencia serológica de *Salmonella. Salmonella* es un patógeno de transmisión fecha-oral y, consecuentemente, el contacto entre animales favorece la dispersión de la enfermedad. Sin embargo en el presente trabajo el alojamiento individual estuvo asociado a una mayor contaminación por *Salmonella*. Este resultado podría estar asociado al estrés que sufren los animales alojados individualmente y a contaminaciones permanentes de comederos y bebederos. Debido a la regulación 2001/88/CE, desde el 1 de enero de 2013, los cerdos reproductores no pueden ser alojados de forma individual salvo durante la lactación y hasta el día 28 post-cubrición, por lo que la relación detectada carece de importancia práctica. Igualmente, la existencia de suelos enrejillados "tipo slatt", que reducen el contacto de los cerdos con las heces, se asoció a una menor prevalencia serológica en las explotaciones. Este dato coincide con lo descrito anteriormente por otros trabajos (Nollet et al., 2004; Hotes et al., 2010) así como con los resultados obtenidos para el conjunto de granjas de cerdos reproductores de la UE (EFSA, 2011b). Finalmente, se comprobó que la prevalencia serológica era más elevada durante los meses comprendidos entre octubre y marzo. En general, los resultados de los estudios respecto a la presentación estacional de la salmonelosis porcina son dispares (Christensen & Rudemo, 1998; Funk et al., 2001b). Hautekiet et al. (2008) encontraron una mayor seroprevalencia en granjas porcinas de cebo durante los meses de verano mientras que otros trabajos sugieren que los cambios en la prevalencia podrían estar asociados a cambios de temperatura en el interior de la granja y no tanto con la estacionalidad (Hald & Andersen, 2001; Funk et al., 2001b; Hautekiet et al., 2008). En nuestra opinión, un incremento en el número de casos durante los meses de verano, podría explicar el incremento retardado de la serología durante los meses posteriores.

2. Control de *Salmonella* en la fase de cebo

Siguiendo la filosofía anteriormente expuesta de identificar medidas que consigan la mayor reducción de prevalencia con el mínimo coste, numerosos trabajos han señalado la fase de cebo como uno de los puntos de la cadena donde mayor repercusión puede tener la aplicación de medidas de control de *Salmonella* (Berends et al., 1996; Borch et al., 1996). De acuerdo con diversos autores, la mayoría de las infecciones por *Salmonella* en animales destinados al engorde se produce durante la fase de cebo (Berends et al., 1996; Kranker et al., 2003; Merialdi et al., 2008). Si además tenemos en cuenta que el engorde es la etapa previa al sacrificio de los cerdos y a su procesado en carne, la instauración de estrategias de control en esta fase de la producción, reduciría teóricamente el riesgo potencial de de contaminación de la carne. Apoyados en este concepto, los países europeos, liderados por Dinamarca, han

centrado sus esfuerzos en el control de *Salmonella* en esta etapa de la producción porcina hasta hace pocos años (Stark et al., 2002).

Teniendo en cuenta la información aportada por estudios previos del grupo de investigación DIGESPOR y referida a la prevalencia en granjas de cebo (Vidal, 2005; García-Feliz et al., 2007; Collazos, 2008) y a la evaluación en condiciones experimentales de algunas medidas para el control de *Salmonella* (Collazos et al., 2008a; 2008b), nos planteamos avanzar en la evaluación de estrategias de control de *Salmonella* empleando pruebas de campo. Los ensayos clínicos o pruebas de campo permiten evaluar la eficacia de las actuaciones realizadas en condiciones "reales", de modo que sus resultados pueden ser directamente extrapolables. Por el contrario, en estas pruebas de campo el control de los posibles sesgos que puedan afectar a las variables en estudio es mucho más complejo y requiere de un mayor esfuerzo. Además, en el caso de las infecciones por *Salmonella* las pruebas de campo se ven afectadas por la complejidad de esta infección, cuya dinámica varía en función de numerosos factores como el momento de infección, el serotipo implicado o la intensidad de exposición, entre otros muchos (Berends et al., 1996; Beloeil et al., 2003; Funk et al., 2001a).

La eficacia de las dos estrategias valoradas en esta etapa del trabajo se determinó en función de la reducción de la prevalencia de *Salmonella* al final de la fase de cebo, para disminuir el riesgo de contaminación en el matadero, y este hecho se tuvo en cuenta en el diseño de los estudios. Una característica esencial de las medidas de control utilizadas fue el hecho de que su administración no debía suponer un gasto inasumible económicamente, de forma que su aplicación pudiera ser extrapolada a las condiciones reales de la producción porcina. Considerando las potenciales estrategias de control de *Salmonella* en cerdos (Boyen et al., 2008a), se seleccionaron dos actuaciones: la administración de ácidos en pienso o agua de bebida al final del periodo de cebo y la aplicación de una vacuna inactivada al comienzo del periodo de cebo.

La selección de las explotaciones donde se llevaron a cabo las pruebas de campo se basó, en una primera etapa, en la detección de una seroprevalencia elevada de *Salmonella* en el lote de cebo previo al del estudio. Además, un requisito importante fue que, independientemente de si los cebaderos pertenecían a granjas de ciclo cerrado o a sistemas de producción en puntos múltiples, el llenado de las instalaciones debía ser simultáneo y con cerdos de la misma edad y origen común. Con el fin de reducir al máximo posibles factores externos que pudiesen alterar los resultados, las granjas elegidas disponían de naves

comparables, con similares características de tamaño, estructura, número de corrales, etc., y los grupos control y experimental recibieron los mismos tratamientos antimicrobianos.

La valoración del efecto de la adición de acidificantes se llevó a cabo mediante tres pruebas de campo con dos productos diferentes, administrados en el agua de bebida o en el pienso, durante un periodo que varió entre 40 y 52 días. Los ácidos empleados fueron seleccionados en base a experiencias previas con resultados positivos (Canibe et al., 2001; van der Wolf et al., 2001c; Creus et al., 2007; Boyen et al., 2008b; Taube et al., 2009; Visscher et al., 2009). Independientemente del ácido empleado, de la duración del tratamiento, del serotipo implicado en la infección o del momento en que esta se produjo (anterior o posterior al comienzo del tratamiento), la administración de acidificantes redujo la seroprevalencia en los animales del grupo experimental en comparación con los del grupo control. De forma similar a nuestros resultados, Creus et al. (2007) empleando un punto de corte del 40% DO demostraron una menor seroprevalencia en los cerdos tratados con dos concentraciones diferentes de una combinación de ácido láctico y fórmico (1,2%-0,8%). Otro estudio en el que se empleó diformiato-potásico (1,2%) adicionado a pienso en harina demostró una reducción de seroprevalencia empleando el mismo punto de corte (Visscher et al., 2009) y en un estudio realizado en Holanda, el empleo de una mezcla de ácidos al 0,002% fue también efectivo con un punto de corte del 10% (van der Wolf et al., 2001c). En nuestro estudio, resulta destacable que en dos de las tres pruebas de campo realizadas, si las granjas hubieran estado incluidas en un plan de control como los empleados en la actualidad en algunos países europeos (Osterkorn et al., 2001; Quirke et al., 2001; Alban et al., 2002), los grupos experimentales se habrían clasificado como de riesgo moderado mientras que los grupos control habrían sido clasificados como de alto riesgo de *Salmonella*. Este resultado es muy interesante de cara a la implementación de medidas correctoras en aquellas granjas identificadas como altamente contaminadas por *Salmonella* en los programas de control basados en resultados de serología. Del mismo modo, pudimos comprobar que, empleando un punto de corte inferior (10% DO), el porcentaje de cerdos que habían seroconvertido era inferior en el grupo experimental en comparación con el grupo control en el tercero de los ensayos. Es importante destacar que nuestro estudio es el primero que emplea varios puntos de corte para el análisis de los resultados de serología y que de acuerdo a los hallazgos obtenidos, el empleo de diferentes puntos de corte resulta esencial para interpretar adecuadamente los resultados.

En coincidencia con los resultados de serología, la administración de ácidos se tradujo en una menor eliminación de *Salmonella* por los cerdos tratados al final del periodo de estudio en dos de las explotaciones. Sin embargo, dichos resultados no pudieron ser refrendados por

los proporcionados por las muestras recogidas al día siguiente en el matadero. La aparición de nuevas infecciones durante el transporte y la espera en el matadero (Hurd et al., 2002) puede enmascarar el efecto de las intervenciones en granja en las muestras recogidas tras el sacrificio, como ha ocurrido en experiencias de campo previas (Dahl et al., 1996; Letellier et al., 2001; Creus et al., 2007; De Busser et al., 2009). Por otra parte, en la tercera de las pruebas de campo la infección por *Salmonella* se instauró después del comienzo del tratamiento, observándose en este caso un efecto protector frente a la colonización por *Salmonella,* similar al propuesto por Creus et al. (2007), que se tradujo en un menor porcentaje de cerdos infectados en el tejido linfoide asociado al intestino entre los que recibieron el tratamiento acidificante en comparación con los controles.

La estimulación del sistema inmunológico es una de las mejores herramientas para proteger a los animales frente a los patógenos. Con esta premisa diversos estudios han probado la eficacia de vacunas frente a *Salmonella* en desafíos experimentales o en pruebas de campo (Denagamage et al., 2007). *Salmonella* es un microorganismo intracelular facultativo y por ello la inmunidad de base celular desempeña un papel relevante en la defensa del organismo frente a la infección. Debido a que las vacunas vivas proporcionan una mejor respuesta celular, la mayoría de los estudios que han desarrollado vacunas se han centrado en este tipo de vacunas (Lumsden et al., 1991; Springer et al., 2001; Selke et al., 2007; Hur et al., 2010; Haneda et al., 2011; Leyman et al., 2011). Sin embargo, las vacunas vivas también tienen aspectos negativos como la administración por vía oral, la mayor complejidad en su desarrollo, necesidades durante el almacenamiento para asegurar la viabilidad así como ciertos signos clínicos como pirexia, diarrea o reducción de la ganancia media diaria en los días posteriores a su administración (Husa et al., 2008). Por el contrario, las vacunas inactivadas son fáciles de producir, con una administración sencilla, más seguras y más económicas. Por estos motivos, decidimos elaborar una vacuna inactivada de *S.* Typhimurium, el serotipo más frecuente en las granjas de cebo españolas (García-Feliz et al., 2007) y europeas (EFSA, 2008a) y comprobar la eficacia de su aplicación a cerdos de comienzo de cebo en granjas con alta seroprevalencia de *Salmonella*. Mientras que la eficacia de la administración de ácidos pudo ser evaluada empleando análisis serológicos y bacteriológicos, en este estudio únicamente los datos bacteriológicos fueron útiles para determinar la eficacia. Los resultados de serología permitieron confirmar la instauración de la infección en los animales de los grupos control y evaluar la respuesta humoral asociada a la administración de la vacuna en los animales de los grupos experimentales.

El estudio de eficacia de una bacterina incluyó cuatro pruebas de campo en cuatro explotaciones diferentes. En tres de ellas (A, B y C) la unidad de estudio fue el corral, con corrales experimentales o vacunados y corrales control distribuidos por cada una de las naves de cebo (dos en cada granja). En la cuarta prueba de campo (D) la vacunación se realizó a nivel de nave, nave experimental o vacunada y nave control, para simular las condiciones de uso más habituales para una vacuna, con todos los cerdos alojados en una misma unidad con un semejante estatus inmunitario. De acuerdo a los resultados bacteriológicos y a la seroconversión de los grupos control, la infección se estableció de forma satisfactoria en tres de las cuatro explotaciones (A, C y D). Según Rostagno (2011) la vacuna ideal frente a *Salmonella* debe prevenir la colonización del hospedador, la eliminación del patógeno, el desarrollo del estado de portador y el desarrollo de enfermedad clínica. Considerando que en condiciones de campo la infección rara vez se asocia a signos clínicos, la eficacia de la vacunación fue evaluada mediante la comparación de la eliminación en heces y la presencia de la bacteria en el contenido de ciego y ganglios linfáticos mesentéricos tras el sacrificio. En las pruebas de campo A y D, en las que los cerdos fueron infectados por una cepa del serotipo *S.* Typhimurium, la eliminación fecal de *Salmonella* en el grupo control fue seis veces superior a la del grupo experimental. Sin embargo, no se observaron diferencias en la proporción de cerdos eliminadores, aunque si en la proporción de corrales positivos, en la prueba C, en la que los cerdos se infectaron por el serotipo *S.* Rissen. Estos resultados nos permiten concluir la existencia de una protección serotipo o serogrupo dependiente. Una menor protección frente a las infecciones heterólogas ha sido descrita previamente por otros estudios (Hassan et al., 1994; Farzan & Friendship, 2010). En concordancia con nuestros resultados, otros estudios han señalado la eficacia de bacterinas frente a *Salmonella* (Gast et al., 1992; Barbour et al., 1993). Aunque diversos autores han desestimado las vacunas inactivadas por no producir inmunidad celular, existen todavía muchos interrogantes sobre el desarrollo de inmunidad en las infecciones por serotipos no tifoideos y en especies diferentes al ratón, frecuentemente empleado como modelo de inmunidad para *Salmonella*. Se ha propuesto que otros mecanismos potenciales de inmunidad como el LPS, las fimbrias o los flagelos pueden inducir cierta respuesta de células T (Mastroeni et al., 2001; McSorley et al., 2009). Además, otros mecanismos como la inducción de inmunoglobulinas de la clase IgA (Roesler et al., 2006) o de anticuerpos específicos capaces de actuar en la fase extracelular de la infección podrían explicar el efecto protector de la vacuna.

Es importante destacar que la elevada respuesta humoral de inmunoglobulinas del tipo IgG frente al LPS inducida por la bacterina dificulta su aplicación en programas de control

que empleen la serología para la determinación del estatus de las explotaciones. En cualquiera de las pruebas de campo realizadas, el grupo vacunado hubiese sido clasificado como altamente contaminado por los actuales programas de control (Osterkorn et al., 2001; Quirke et al., 2001; Alban et al., 2002).

3. *Salmonella* en el matadero

La presente tesis dedica un capítulo al estudio de la infección o contaminación por *Salmonella* en las fases posteriores a la granja. Al igual que se ha hecho en otros países como Francia, Bélgica, Holanda o Dinamarca, además de evaluar la prevalencia existente en granja, es necesario comprobar que ocurre en las fases posteriores de transporte, espera y sacrificio. Hasta la realización de esta tesis no existía ninguna publicación sobre lo que acontecía en estas etapas en el sector porcino de España. Teniendo en cuenta la elevada contaminación demostrada en las explotaciones de cerdos se hacía necesario estudiar lo que ocurre cuando los cerdos salen de estas granjas para ser sacrificados y transformados en diferentes productos cárnicos.

En una primera etapa tratamos de determinar la prevalencia de la contaminación por *Salmonella* en canales porcinas y los principales serotipos implicados y para ello se realizaron dos estudios en cuatro mataderos. El primero de estos estudios consistió en la monitorización en 16 lotes de cerdos de engorde procedentes de 16 explotaciones diferentes, desde el momento en que abandonaron la granja y hasta su transformación en canales en dos mataderos diferentes. En el segundo de los estudios, se realizó un análisis exhaustivo de las tres fases en que pueden dividirse las actividades en el matadero: los corrales de espera, el sacrificio y el despiece. Al igual que en estudios previos (Berends et al., 1997;1998; Swanenburg et al., 2001a; Botteldoorn et al., 2003; McDowell et al., 2007; van Hoek et al. 2012), se emplearon técnicas de caracterización molecular para analizar las relaciones entre los aislados obtenidos, con el fin de comprender mejor la dinámica de *Salmonella* en los mataderos así como la importancia relativa de las diferentes fuentes de contaminación de las canales.

Los estudios de monitorización de cerdos desde la granja al matadero han demostrado que existe un aumento de la eliminación de *Salmonella* asociado a la reactivación de infecciones latentes y a las nuevas infecciones adquiridas en los camiones de transporte o en los corrales de espera del matadero (Berends et al., 1996; Hurd et al., 2002; Gebreyes et al., 2004). En nuestro estudio de monitorización, el porcentaje de cerdos eliminadores en granja

se multiplicó por nueve en las muestras recogidas en el matadero, contenido de ciego y ganglios linfáticos mesentéricos. La contaminación de los camiones así como la alta prevalencia encontrada en los corrales de espera contribuyeron a este aumento de prevalencia. Así, las técnicas de caracterización molecular demostraron que las canales de uno de los lotes se contaminaron con la misma cepa que se detectó en un camión de transporte. Además, la tipificación molecular de los aislados reveló que en casi todos los lotes evaluados, los perfiles de MLVA de *S.* Typhimurium encontrados en los corrales de espera se detectaban en alguna de las canales contaminadas. Dichos datos corroboran la importancia de los corrales de espera en la contaminación de las canales porcinas, como se ha propuesto en otros estudios (Duggan et al., 2010; De Busser et al., 2011).

La monitorización de canales reveló que al menos el 50% de los resultados positivos obtenidos eran consecuencia de contaminaciones cruzadas, reflejando la gran relevancia del matadero en la contaminación por *Salmonella* de las canales. El porcentaje de canales positivas atribuidas a contaminación cruzada varía entre estudios. Por ejemplo, un estudio holandés basado en modelos matemáticos, lo estimó en el 30% (Berends et al., 1996). Hasta un 69% de canales positivas a consecuencia de contaminación cruzada fueron detectadas en un estudio irlandés (Duggan et al., 2010) mientras que un estudio belga comprobó que la contaminación cruzada en cinco mataderos porcinos afectaba a un porcentaje variable de canales, entre el 16% y el 100% (Botteldoorn et al., 2003). En nuestro caso, la tipificación posterior de los aislados de *S.* Typhimurium reveló que tan solo en el 25% de las canales contaminadas por este serotipo, el perfil de MLVA encontrado coincidía con el detectado en muestras de contenido de ciego o de ganglios linfáticos mesentéricos. Los resultados obtenidos nos permiten concluir que el empleo de técnicas de tipificación molecular resulta imprescindible cuando se quiere estimar, con una mínima precisión, la proporción de canales positivas atribuidas a la contaminación cruzada en el matadero, tal y como se había propuesto anteriormente (Wonderling et al., 2003; Botteldoorn et al., 2004).

Entre los cuatro mataderos se analizaron un total de 896 canales al final de la línea de sacrificio obteniéndose una prevalencia del 39,7%, significativamente superior a la reportada por la mayoría de los estudios de prevalencia de *Salmonella* en canales porcinas (Kasbohrer et al., 1999; Swanenburg et al., 2001a; Hald et al., 2003; Sorensen, 2004, 2007; Vieira-Pinto et al., 2005; McDowel et al., 2007; Baptista et al., 2010; Piras et al., 2011; Van Hoek, 2012; Visscher et al., 2011; Bonardi et al., 2013). Un resumen de los resultados obtenidos por diferentes estudios se muestra en la Tabla 1 del artículo de revisión de mataderos. Resulta muy complicado establecer comparativas entre los valores proporcionados por diferentes estudios

debido a que diversos factores como el momento del muestreo, la superficie muestreada, los métodos para la recogida de la muestra o los protocolos de aislamiento, entre otros, influyen considerablemente en el resultado obtenido. En cualquier caso, la prevalencia obtenida en nuestro estudio es un reflejo más del serio problema que la producción porcina de España tiene en lo que respecta a la infección por *Salmonella* y de la necesidad acuciante de establecer medidas para su vigilancia y control.

En dos de los mataderos evaluados, mataderos C y D, las canales fueron de nuevo analizadas después del enfriamiento y oreo. Tras estos dos procesos, la prevalencia se redujo notablemente (3,5 veces de media) en comparación con la obtenida en la línea de sacrificio. Antes de la realización de este trabajo, pocos estudios habían reportado esta reducción en la detección de *Salmonella* tras el proceso del oreo (Booteldoorn et al., 2003), aunque un reciente meta-análisis ha corroborado su efecto reductor en la prevalencia de *Salmonella* en canales (Gonzales-Barron et al., 2013).

Los serotipos identificados en canales coinciden con los detectados en los estudios de prevalencia en granja (García-Feliz et al., 2007), demostrando que existe un vínculo entre las cepas de *Salmonella* identificadas en la granja y en el matadero. Tanto la prevalencia de *Salmonella* como los serotipos detectados variaron entre diferentes visitas realizadas a un mismo matadero. Estas variaciones de la prevalencia pueden ser atribuidas a diferencias en el estatus de los cerdos sacrificados así como en las prácticas del matadero. Las variaciones observadas en los serotipos detectados así como en los perfiles de los aislados de un mismo serotipo, tanto entre diferentes visitas al mismo matadero como en diferentes momentos dentro de un mismo día de estudio, nos indican que la población de *Salmonella* en los mataderos es dinámica y que existe un flujo continuo de entrada asociado, con gran probabilidad a los cerdos contaminados. Otro dato relevante obtenido del análisis genotípico fue el hecho de que las canales muestreadas en un periodo corto de tiempo estuvieron contaminadas, en muchas ocasiones, por un mismo genotipo y que dicho genotipo también fue encontrado en puntos de la línea muestreados en un momento cercano, poniendo de manifiesto la relación entre la contaminación de las actividades de la línea y las canales.

En este estudio inicial, también fue evaluada la contaminación de las instalaciones de dos de los mataderos. Los resultados evidenciaron, en coincidencia con estudios previos (Swanenburg et al., 2001b; Botteldoorn et al., 2003; Duggan et al., 2010), la elevada contaminación de los corrales de espera antes, durante y después de la estancia de los cerdos, así como la ineficiencia de los protocolos de limpieza y desinfección empleados. La tipificación

de aislados empleando técnicas moleculares reveló que una elevada proporción de la contaminación en las canales, en la línea de sacrificio y en la sala de despiece se producía por los mismos perfiles detectados en las muestras recogidas en los corrales de espera, algunas de ellas antes de la entrada de los cerdos, subrayando la importancia de la adecuada aplicación de protocolos eficaces de limpieza y desinfección (Lettellier et al., 2009).

Salmonella también fue detectada con frecuencia en las muestras recogidas en la línea de sacrificio. Las practicas en las que se detectó contaminación con mayor reiteración, al igual que en estudios previos, fueron aquellas realizadas manualmente por los operarios tales como la evisceración (Berends et al., 1996; Hald et al., 2003; Pearce et al., 2004), la extracción de grasa y mantecas (Botteldoorn et al., 2003; Hald et al., 2003; Vieira-Pinto et al., 2005) o la retirada de los riñones. La proporción de muestras positivas a *Salmonella* en la línea de sacrificio aumentó según avanzaba la jornada de trabajo y, en uno de los mataderos, los genotipos identificados en la línea estuvieron asociados a los perfiles obtenidos en las canales, lo que permitió concluir que, en este matadero, las actividades de la línea tenían relevancia en la contaminación de canales. Aunque en menor proporción, también se detectó contaminación en la sala de despiece, con muestras positivas en la superficie de las cintas de trabajo, manos y cuchillos de operarios así como en la materia orgánica acumulada en las máquinas. Los perfiles de los aislados recuperados de la sala de despiece coincidieron con otros previamente detectados en corrales, línea de sacrificio o canales.

En conjunto, los resultados obtenidos demuestran la importancia de las etapas posteriores a la granja, el transporte y particularmente la espera en corrales, en la contaminación de los cerdos así como la relevancia de las actividades del matadero en la contaminación de las canales y de la carne despiezada. Por ello es necesaria la mejora de los protocolos de limpieza y desinfección de los camiones y corrales, así como de los procedimientos en la línea de sacrificio, sobre todo en puntos críticos donde se puede producir contacto con el contenido intestinal o en actividades que impliquen la manipulación de una gran superficie de la canal. Finalmente, es importante que la carga de *Salmonella* en la sala de despiece sea la mínima posible, tanto mediante la implementación de medidas eficaces en fases anteriores como por la aplicación de un correcto oreo de las canales y de una estricta higiene durante el despiece de las mismas. El trabajo de tipificación molecular de las cepas obtenidas en cuatro mataderos nos permite concluir que la técnica de MLVA es adecuada para la investigación de las fuentes de infección y de las relaciones entre las cepas de *S.* Typhimurium, como había sido propuesto previamente (Prendergast et al., 2011; Van Hoek et al., 2012) Pese a que existe un protocolo de MLVA para *S.* Typhimurium estandarizado

(Lindstedt et al., 2004; Torpdahl et al., 2007), los problemas derivados de la detección del loci STTR13 en el secuenciador de la Universidad de León, nos llevó a desarrollar dos nuevos loci, uno de los cuales ha demostrado estar en todas las cepas analizadas con una elevada variabilidad y poder discriminatorio (STTR12). Dicho loci, puede ser candidato, junto con otros descritos (Kruy et al., 2011), a ser incorporado en el análisis por MLVA en futuros estudios que requieran una elevada capacidad de discriminación.

Una vez identificado el problema asociado a la contaminación por *Salmonella* en el matadero, decidimos completar esta parte del trabajo con la evaluación de algunas medidas de control en esta etapa. Se valoró el efecto de mejoras en los protocolos de limpieza y desinfección en alguno de los mataderos que habían colaborado en la etapa anterior. Estos nuevos protocolos que incluían detergentes espumantes y desinfectantes lograron reducir la contaminación de camiones y eliminarla en los corrales de espera. Además, valoramos la eficacia en canales de la aplicación de una ducha de agua tratada con peróxido de hidrógeno al final de la línea de sacrificio de uno de estos mataderos Dichos estudios no han sido incluidos en la presente tesis doctoral por ser trabajos muy específicos de colaboración con los mataderos implicados, sin una perspectiva de estudio científico para publicaciones.

Por último, decidimos evaluar el efecto que el sacrificio sanitario o sacrificio logístico tiene en la contaminación de las canales y del matadero. El sacrificio logístico se basa en la separación de lotes de cerdos para su sacrificio en función de su contaminación por *Salmonella* y se emplea en los programas de control de salmonelosis porcina danés e irlandés, utilizando datos de serología para la clasificación de las explotaciones. En el programa danés, los lotes de cerdos provenientes de granjas altamente contaminadas son sacrificados bajo medidas higiénicas más estrictas; los cerdos se sacrifican al final del día, no pueden permanecer en los corrales por la noche ni ser mezclados con otros lotes de cerdos, la velocidad de la línea de sacrificio se ralentiza para su procesado y el material de riesgo (intestinos y cabeza) se desecha o se trata con agua caliente. Finalmente, si la prevalencia de canales contaminadas por *Salmonella* supera cierto límite, estas deben ser duchadas con agua caliente (Sorensen et al., 2004). A pesar de su aplicación en programas de control, tan solo hay publicado un estudio experimental, realizado en Holanda, que valore la eficacia del sacrificio logístico en la prevención de la contaminación de canales porcinas por *Salmonella* (Swanenburg et al., 2001c).

En nuestro estudio se evaluaron lotes de cerdos, sacrificados al comienzo de la jornada de trabajo y durante tres días consecutivos, que fueron organizados en función de su

seroprevalencia del siguiente modo: lotes de baja seroprevalencia sacrificados en el día 1, lotes de elevada seroprevalencia sacrificados en el día 2 y lotes de seroprevalencia moderada en el día 3. El sacrificio de lotes en función de su seroprevalencia, siguiendo una clasificación muy similar a la empleada por el actual sistema de clasificación de granjas de cebo danés (Alban et al., 2002), no ofreció los resultados esperados. La prevalencia de canales contaminadas por *Salmonella* fue similar en el día 1, cuando se sacrificaron lotes de baja prevalencia serológica, y en el día 2, cuando se sacrificaron lotes con elevada prevalencia serológica. La contaminación de canales en los lotes de baja seroprevalencia, de acorde a los resultados de tipificación, fue debida a la contaminación existente en los corrales de espera donde se alojaron los cerdos antes del sacrificio o a la contaminación en la línea de matanza, donde puntos de alto riesgo como la evisceración estaban contaminados a primera hora del día. La contaminación de canales procedentes de granjas de baja seroprevalencia asociada a la contaminación de los corrales de espera ha sido descrita en otras publicaciones (Swanenburg et al., 2001c; Duggan et al., 2010). Por el contrario, el sacrificio de lotes con elevada seroprevalencia no se correlacionó con contaminación de canales, hecho que ha sido igualmente descrito por estudios previos (Botteldoorn et al., 2003; Duggan et al., 2010; Visscher et al., 2011). Este resultado demuestra que aunque entren en la línea de sacrificio cerdos infectados por *Salmonella*, una adecuada praxis puede evitar o al menos reducir la contaminación de las canales y de la línea.

Finalmente, podemos destacar que la mayor contaminación de canales se detectó en el día 3, cuando se sacrificaron lotes clasificados como moderadamente contaminados. El riesgo de obtener una canal positiva fue tres veces superior en ese día que en cualquiera de los dos días precedentes. Debido al retraso que se produce desde el momento de infección hasta el desarrollo de anticuerpos (Kranker et al., 2003), las granjas infectadas recientemente y con un elevado porcentaje de cerdos eliminadores pueden clasificarse como moderadamente infectadas al emplear sistemas basados en la serología. En concordancia con nuestros resultados, Duggan et al. (2010) detectaron una mayor prevalencia de *Salmonella* en ganglios linfáticos mesentéricos en las explotaciones con una prevalencia serológica moderada.

De forma global, nuestros resultados señalan que, en los mataderos porcinos de España, la organización del sacrificio de los lotes de cerdos en función de su prevalencia serológica no es eficiente si otras medidas como la mejora de los protocolos de limpieza de los corrales y de la línea de sacrificio no son mejorados.

CONCLUSIONES/CONCLUSIONS

Calendario Agrícola Panteón de los Reyes de León. Siglo XII (San Isidoro, León, España).

Meses de octubre y noviembre, relacionados con el mundo animal.

1. La prevalencia de la infección por *Salmonella enterica* en cerdos reproductores en España es elevada. Se detectó eliminación fecal en 3 de cada 4 granjas estudiadas y anticuerpos específicos en 2 de cada 3 explotaciones. *Salmonella* Rissen y *Salmonella* Typhimurium fueron los dos serotipos más frecuentemente identificados aunque la limitada coincidencia entre los serotipos detectados en reproductores y los previamente descritos en explotaciones de cerdos de engorde de nuestro país sugiere que la transmisión vertical tiene una importancia limitada. El análisis multivariado demostró que el empleo de pienso granulado y la reposición externa de los verracos estaban asociados a un mayor riesgo de eliminación fecal. Los suelos total o parcialmente enrejillados se asociaron a una menor seroprevalencia de la infección.

2. La administración de ácidos orgánicos en el agua de bebida o en el pienso durante las últimas 6-7 semanas del cebo y a dosis limitadas es una estrategia viable desde un punto de vista económico y eficaz para el control de *Salmonella* en explotaciones de engorde. Con independencia del ácido empleado, esta intervención se asoció a una reducción de la seroprevalencia y, en menor medida, de la eliminación fecal de *Salmonella* al final del cebo. Sin embargo, este efecto no fue evidente tras el sacrificio, probablemente a consecuencia de nuevas infecciones ocurridas durante las fases de transporte y espera en los corrales del matadero.

3. El empleo de una vacuna inactivada de *Salmonella* Typhimurium administrada al comienzo del periodo de engorde reduce la eliminación fecal de *Salmonella* y la proporción de cerdos portadores en el matadero en aquellas explotaciones infectadas por este serotipo. Sin embargo, la elevada respuesta de anticuerpos séricos inducida por la vacuna limita su empleo en aquellas granjas incluidas en programas de vigilancia basados en la monitorización serológica.

4. La monitorización de lotes desde la granja hasta su sacrificio demuestra que la contaminación por *Salmonella* de los cerdos y de sus canales en el matadero puede atribuirse a diferentes orígenes, incluyendo las infecciones en granja así como nuevas infecciones o contaminaciones durante el transporte, la espera en los corrales del matadero y las actividades de la línea de sacrificio. Estos resultados permiten concluir que las etapas posteriores a la granja son muy relevantes en la contaminación final de las canales y que deben ser incluidas en un programa de control de la salmonelosis porcina en España.

5. El estudio realizado en cuatro mataderos de nuestro país demostró que la contaminación por *Salmonella enterica* en canales porcinas al final de la línea de sacrificio es elevada, siendo positivas 4 de cada 10 canales analizadas, aunque la detección de *Salmonella* se reduce considerablemente tras la refrigeración. Los serotipos detectados en las canales coinciden, en gran medida, con los identificados en las explotaciones proveedoras de los mataderos estudiados. Sin embargo, la tipificación de los aislados de *Salmonella* reveló que en 3 de cada 4 canales la contaminación no era atribuible a la infección previa de los cerdos en la granja.

6. El sacrificio logístico, organizado en función de la proporción de seropositivos a *Salmonella,* no se asoció a variaciones en la prevalencia de la contaminación por *Salmonella* en las canales porcinas. Nuestros resultados indican que para que esta estrategia de control sea eficaz debe aplicarse simultáneamente a estrictas medidas de limpieza y desinfección en los corrales de espera y en la línea de sacrificio así como a unas adecuadas prácticas en el matadero.

1. The prevalence of *Salmonella enterica* in Spanish breeding pigs is high. *Salmonella* was detected in faecal samples from three out of four herds and specific antibodies for *Salmonella* were detected in two out of three evaluated herds. *Salmonella* Rissen and *Salmonella* Typhimurium were the two most prevalent serotypes. However the limited agreement between the serotypes detected in breeding pigs and those previously reported in finishing pigs in Spain suggests a limited impact of the vertical transmission. Two variables were associated to *Salmonella* shedding using a multivariate analysis: the use of pelleted feed and the purchase of the boar replacement. Contrarily, the presence of slatted or partially slatted floor was associated to a lower *Salmonella* seroprevalence.

2. The administration of organic acids supplied by water or feed throughout the last 6-7 weeks of the finishing period and at doses determined by economic criteria is a useful strategy in the control of *Salmonella* in swine finishing farms. This intervention was associated with a lower seroprevalence and to a lesser extent with a lower shedding at the end of the finishing period, regardless of the acids used. Nonetheless, this effect was not evidenced after the slaughtering, probably as a consequence of the emergence of new infections occurring during the transport and lairage.

3. The vaccination of finishing pigs at the beginning of the finishing period with an inactivated bacterin of *Salmonella* Typhimurium reduces the faecal shedding and the horizontal spread of *Salmonella* during the finishing period as well as the proportion of *Salmonella* shedders or carriers at the slaughterhouse in those farms with a homologous infection. In contrast, the high humoral response elicited complicates the use of this vaccine in farms that are under serological surveillance programs.

4. The monitoring of pigs from the farm to the slaughterhouse demonstrates that carcass contamination at the slaughterhouse can be attributed to a number of sources including infections occurred at the farm as well as new infections or contamination of the pigs throughout the transport, the lairage or the slaughter line activities. Therefore, post-harvest stages significantly contribute to pig carcass contamination and should be included in a presumptive *Salmonella* control programme in swine in Spain.

5. The research performed in four Spanish pig slaughterhouses reveals that the prevalence of *Salmonella enterica* contamination of carcasses at the end of the slaughter line is high. Four out of ten carcasses yielded positive although the detection of *Salmonella* was significantly reduced after chilling and cooling procedures. *Salmonella* serotypes detected in carcasses matched with those identified in the pigs of the main supplier farms. Nonetheless, further typing of *Salmonella* isolates revealed that three out of four carcasses were cross-contaminated.

6. Logistic slaughter organised according to the proportion of *Salmonella* seropositive pigs was not associated to any variation on the prevalence of *Salmonella* contamination on pig carcasses. Our results point out that to be effective, logistic slaughter should be performed together with strict cleaning and disinfection measures in the lairage and slaughterhouse facilities as well as proper slaughtering practices.

PERSPECTIVAS

Los datos arrojados por los diferentes estudios, entre los que se incluyen los realizados en la presente tesis, demuestran la importancia de las infecciones por *Salmonella* en el sector porcino español y la necesidad de elaborar un programa de vigilancia y control acorde a la importancia de este sector en la producción agraria nacional. Dicho programa debería de desarrollarse en base a la experiencia aportada por los programas de control que ya están en marcha en otros países y teniendo en cuenta las singularidades de esta infección y de la producción porcina en nuestro país.

Los resultados obtenidos en la presente tesis demuestran que el matadero y las actividades previas deben ser tenidos en cuenta si se desea que las medidas de control sean efectivas. Además el matadero constituye un punto de la cadena donde, si la legislación se flexibiliza, se pueden aplicar múltiples medidas de control cuya repercusión en la contaminación de canales sería inmediata. Una de esas posibles medidas de control en el matadero es el uso de duchas de agua tratada que reduzcan la contaminación de la superficie de las canales.

En cuanto a las granjas, es necesario encontrar medidas que reduzcan la prevalencia de la infección sin suponer un elevado coste. Entre ellas, el empleo de nuevos antimicrobianos de origen natural como por ejemplo extractos de plantas así como el uso de estrategias que mejoren la salud intestinal del cerdo como los probióticos, prebióticos o simbióticos. Las vacunas pueden ser igualmente una estrategia eficaz de control que es necesario desarrollar y que además puede ser muy útil en la reducción de la prevalencia de la infección en reproductoras. En este sentido, es importante que se lleve a cabo un estudio exhaustivo y profundo con el fin de determinar el verdadero papel que las reproductoras juegan en la infección de los cerdos de engorde y, consecuentemente, la necesidad de incluir o no esta etapa de la producción porcina en los programas de monitorización y control de la salmonelosis porcina.

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ANEXOS

Durante la realización de esta tesis el doctorando realizó una estancia en el National Food Institute de la Danish Technical University (Copenhage, Denmark) bajo la tutela de los doctores Karl Pedersen y Dorte L. Bagessen.

En dicha estancia se elaboraron dos trabajos científicos que aparecen como anexos de la tesis doctoral.

El primero de los trabajos es un estudio de caracterización de cepas de origen animal de la emergente variedad monofásica de *Salmonella* Typhimurium (*S.* 4,5,12:i:-) que en los últimos años ha estado relacionada con brotes de salmonelosis humana, principalmente asociada al consumo de carne de cerdo.

El segundo de los trabajos es un análisis de los datos obtenidos en el último estudio transversal en cerdos de engorde y reproductores realizado en Dinamarca como parte de los estudios basales en cerdos de la Unión Europea. Los datos de este estudio fueron cotejados con los obtenidos en previos estudios transversales para conocer la evolución del control de *Salmonella* en cerdos en Dinamarca.

As part of the doctoral thesis, the PhD student performed a staying in the National Food Institute from the Danish Technical University (Copenhagen, Denmark), supervised by PhDs Karl Pedersen and Dorte L. Bagessen.

In the present annex the two research studies performed during this staying are included.

The first of the studies is focused on the characterization of isolates from animal origin of the emergent monophasic variant of *Salmonella* Typhimurium (*S.* 4,5,12:i:-), which in the last years has prompted as one of the most relevant serotypes in human salmonellosis, associated to pork consumption.

The second study was focused on the analysis of the last cross-sectional study performed in Danish pig populations as part of the baseline study in finishing and breeding pigs in the European Union. Data from these studies were compared to previous cross-sectional studies performed in Denmark to know the trends in *Salmonella* prevalence in pigs from the control programme implementation till the present.

Publication 1:

Characterization of the emerging *Salmonella* 4,[5],12:i:- in Danish animal production

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ABSTRACT:

The monophasic *Salmonella* variant with the antigenic formula *S.* 4,[5],12:i.- has emerged in the last decade as one of the main serotypes related to human salmonellosis. In the present study a collection of 94 isolates of the *S.* 4,12:i:- and *S.* 4,5,12:i:- coming from Danish farm animals, swine (86), cattle (7), and poultry (1), with well defined identification was further typed by PCR serotyping, phage typing, and molecular typing, (PFGE and MLVA). Moreover, the antimicrobial resistance pattern of each isolate was tested. In 68 of the isolates the *fljB* gene was absent, i.e. they were true monophasic strains, whereas in 26 isolates, the gene was present. The results clustered the isolates in three main pulse-types. The dominant cluster, which was compatible to a previously described pattern, STYMXB.0131, was composed mainly of isolates in which the *fljB* gene was absent. It had a DT 193 phage type with the AMP-STR-SMX-TET resistance pattern and also included several of the main MLVA types described in other studies.

1. INTRODUCTION:

Food-borne salmonellosis has been the cause of concern and research for decades in Denmark, in particular in the last few decades, e.g. with the implementation of the Danish Salmonella Control Programme (DSCP) in production animals and their feed suppliers. The laying out of the National Control Programme in animals and their linkage to the human surveillance, allows the authorities to detect outbreaks and determine the origin of the strains involved and their characteristics by phenotypic, i.e. phage type or antimicrobial resistance profile, and molecular markers, like PFGE or MLVA.

The emergence and spreading of multiple resistant clones has been reported in the last decades: The spread of *S.* Typhimurium DT 193, DT 204 and DT 204c phage types during the seventies and eighties linked to calves and cattle (Wray, 2000) and above all the emergence and spreading of *S.* Typhimurium DT 104 are examples of clone propagation. The DT 104 clone, which appeared in the late eighties and was propagated throughout the nineties and first years of the last decade is characterised principally by the chromosomal resistance profile AMP-CHL-STR-SMX-TET. The reason why these clones have success in the propagation and colonisation of different species is unknown but seems to be a combination of several factors, such as improved abilities to survive in the host and the environment, antimicrobial resistance, or acquisition of bacteriophages encoding factors of importance for fitness and virulence (Müller 2012).

Serotyping is commonly used as a first step to differentiate *Salmonella* isolates. The O-chain of the lipopolysaccharide joined to flagellar proteins encoded by two structural flagellin genes (*fliC* and *fljB*) determines the antigenic formula. *Salmonella* 4,[5],12:i:- has evolved from *S.* Typhimurium strains (Echeita *et al.,* 2001) which have lost the second phase flagellin or lost the ability to express it due to a problem in the flagellar switching mechanism (*fljA* gene or Hin recombinase) or in the expression of *fljB*. Despite the fact that *S.* 4,[5],12:i:- was rarely identified before de mid-1990s, the isolation of this serovar in surveillance, diagnostic samples and human cases/outbreaks has increased in the last decade (Echeita *et al.,* 1999; CDC, 2008; Mossong *et al.,* 2007; Dionisi *et al.,* 2009; Switt *et al.,* 2009; Soyer *et al.,* 2009; Trüpschuch *et al.,* 2010; Hauser *et al.,* 2010), and become one of the major problems related to *Salmonella* in humans. It is accepted that *S.* 4,[5],12:i:- has evolved from several clones (Soyer *et al.,* 2009) of *S.* Typhimurium, an assertion based on phenotypic and genotypic relationships (Echeita *et al.,* 2001; Guerra *et al.,* 2000; Agasan *et al.,* 2002; Garaizar *et al.,* 2002; Amavisit *et al.,* 2005; Zamperini *et al.,* 2010). Despite the diversity of phage types, resistance profiles and molecular types found in the *S.* 4,[5],12:i:- strains, it is noteworthy that many of the

isolates reported in studies carried out to improve the knowledge about this serotype or outbreak publications share several characteristics: (1) DT 193 as predominant phage type, (2) a common tetra-resistance pattern (AMP-STR-SXT-TET) (3) a few predominant PFGE patterns STYMXB.0131and STYMXB.0083 and MLVA profiles (3-11-9-NA-211, 3-12-9-NA-211, 3-13-10-NA-211, 3-13-15-NA-211).

Due to the reasons described above various studies have been carried out in Europe and other parts of the world in order to improve the knowledge about the circulating populations of this serotype in different countries. Until now, there are no official reports about the situation concerning animals and food in Denmark so, the purpose of this study was to investigate Danish isolates of the *S.* 4,12:i:- and *S.* 4,5,12:i:- coming from Danish farm animals and with well defined identification of origin, i.e. DSCP isolates, fresh meat surveillance programme or EU baseline studies and make an evaluation of their clonality by phage typing, MLVA and PFGE, together with their antimicrobial susceptibility profiles.

2. MATERIALS AND METHODS:

2.1 Study design:

A total of 94 isolates from the Danish animal production system were selected from the laboratory database for further studies. To be chosen each strain had to fulfill two conditions, being serotyped as 4,[5],12:i:- and being derived from well defined sources, registered with its herd identification number or slaughterhouse authorization number. The origin of the isolates was samples obtained from the DSCP, from clinical submissions, the fresh meat surveillance programme, and the EU baseline studies. The isolate set was obtained from three animal species: swine (86), cattle (7), and poultry (1) and different material sources including faecal samples (rectal faeces and floor faecal samples), surface samples (floor gauzes and socks), environmental samples, lymph nodes, animals (without a specific origin), and carcasses.

2.2 Serotyping:

Serotyping of *Salmonella* isolates was performed by slide agglutination (Statens Serum Institut antisera) according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). Strains that did not display the second phase were tested again and it was attempted to make them migrate through an U-shaped glass tube containing agar with factor H:i antiserum incorporated. Strains which still did not display the second phase were considered phenotypical monophasic.

All the isolates included in our study were then tested by PCR according to the PCR identification guidelines published by EFSA (EFSA, 2010), adapted to our laboratory conditions. A multiplex PCR was performed using the Qiagen Multiplex PCR kit with two pairs of primers to amplify the *fliB-fliA* intergenic region (0.2 µM primers FFLIB/RFLIA) and the *fljB* allele (1 µM sense-59/antisense-83). Cycling parameters were denaturation at 95 °C for 15 min, amplification during 30 cycles (95 °C for 30 sec, 64 °C for 30 sec, 72 °C for 90 sec) and a final step of post-extension at 72 °C for 10 min. PCR products were separated on 2 % (w/v) agarose gels, stained with ethidium bromide and photographed under UV light. An amplification of a 1 kb fragment of the *fliB-fliA* intergenic region is specific for *S*. Typhimurium, and the absence of a *fljB* PCR fragment defines *S*. 4,[5],12:i:- (EFSA 2010).

2.3 Phage typing:

Susceptibility to *S.* Typhimurium phages was investigated according to the International Federation for Enteric Phage Typing scheme (IFEP, Laboratory of Enteric Pathogens, Health Protection Agency, Colindale, UK)(Anderson *et al.*, 1977). One to three colonies of each isolate were suspended in nutrient broth and incubated at 37 ºC under continuous shaking for 1 h, whereafter nutrient agar plates were flooded with the culture. Once the plates were properly dried, the phages were spotted onto the surface and the plates were incubated and read after approximately 18 h.

2.4 Antimicrobial resistance profile:

Antimicrobial susceptibility testing was performed to establish MIC values by a broth microdilution procedure in accordance with CLSI and DANMAP guidelines, using the semiautomatic SensiTitre system (Trek Diagnostic Systems Ltd., UK). The following antimicrobials were tested: amoxicillin-clavulanic acid (AUG), ampicillin (AMP), apramycin (APR), cefotaxime (FOT), ceftiofur (XNL), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), florfenicol (FFN), gentamicin (GEN), nalidixic acid (NAL), neomycin (NEO), spectinomycin (SPE), streptomycin (STR), sulphamethoxazole (SMX), tetracycline (TET) and trimethoprim (TMP). Data were interpreted using EUCAST epidemiological cut-off values (www.eucast.org).

2.5 Pulsed-Field Gel Electrophoresis (PFGE):

PFGE using *Xba*I (Fermentas, Lifesciences) as restriction enzyme, was carried out under the Centers for Disease Control and Prevention (CDC) PulseNet protocol (Ribot *et al.,* 2006) with minor changes. Electrophoresis was carried out in a Chef-DR®-III (Bio-Rad®) using the following settings: initial switch time 2.2 sec, final switch time 63.8 sec, a gradient of 6 V/cm, 120º angle and 22 h of

electrophoresis in 0.5×TBE buffer (Sigma®). Apart from the four size standard strains included in the PulseNet protocol in each gel (*Salmonella* Braenderup H9812) we also added an internal *S.* Senftenberg control which let us corroborate the repeatability and correct management of the technique.

2.6 Multiple Locus Variable number tandem repeats Analysis (MLVA):

MLVA was performed using Lindstedt primers (Lindstedt *et al.,* 2004) under the protocol described by Torpdahl *et al.* (2007) with modifications in the equipment used to separate the final PCR products (CEQ™ 8000 Genetic Analysis System (Beckman Coulter). Fragment sizes were imported to an Excel file and the number of repeat units was calculated for each allele. The allelic combinations were recorded in the following order: STTR9-STTR5-STTR6-STTR10-STTR3.

2.7 Bioinformatics tools and statistical methods:

Banding patterns from PFGE and repeat units from MLVA analysis respectively were analyzed using BioNumerics® (Applied Maths, Sint-Martens-Latem, Belgium). PFGE results were compared by cluster analysis using the Dice coefficient and unweighted pair group method with arithmetic mean (UPMGA) with a position tolerance of 1.5 % and optimization of 1.0 %.

3. RESULTS:

3.1 Serotyping results:

From the overall 94 isolates included in our study, 60 (63.8 %) had the antigenic formula *S.* 4,5,12:i:- while 34 (36.2 %) were *S.* 4,12:i:-.

All the isolates tested amplified a 1-kb product corresponding to the *fliB-fliA* intergenic region, proving that they were all derivatives of *S.* Typhimurium or derivatives hereof. Sixty-eight of the isolates (72.3 %) were negative for the *fljB* allele amplification, which means that they were true *S* 4,[5],12:i:-. The remaining 26 (27.7 %) presented the *fljB* allele (1389 bp fragment), so according to the EFSA guidelines, these were genotypically *S.* Typhimurium. The *fljB* allele results were also compared in several strain groups as it is indicated in Table 1. *fljB*-negative isolates were more frequently observed in *S.* 4,5,12:i:- strains than *S.* 4,12:i:- strains (χ^2= 10.02, p=0.0016) and in DT 193 compared to other phage types (χ^2= 17.03; p<0.0001).

Table 1. Prevalence of *fljB* gene encoding the second phase flagellum in Danish phenotypic monophasic *S.* Typhimurium-like isolates.

	4,5,12:i:-	4,12:i:-	Total	DT 193	Other phage types*
***fljB* -**	50	18	68	49	11
***fljB* +**	10	16	26	7	14
Total	60	34	94	56	25

* *rdnc and non-typable not included*

3.2 Phage typing results:

The isolates belonged to several different phage types. The most prevalent phage type was DT 193, 56 isolates (59.6 %); 11 isolates (11.7 %) were DT 120 and another eight (8.5 %) were U302. Other phage types found were U311 (4), DT 104b (1), and DT 41 (1); four isolates showed a pattern which did not conform (RDNC) with any defined pattern, and finally, nine could not be phage typed (NT).

3.3 Antimicrobial resistance results:

The antimicrobial susceptibility analysis showed that 95.7 % of the isolates were resistant to at least one compound with only four strains being pan-susceptible. The resistance profiles and phage types are shown in Table 2. Of the 94 isolates, 67 presented multi-resistance profiles (71.3 %), defining multi-resistance as resistance to four or more antimicrobials. Fifty-nine isolates had the antimicrobial pattern AMP-STR-SMX-TET, which is 88.1 % of the multidrug-resistant isolates and 62.8 % of the total collection. Furthermore, 64 strains presented this pattern alone or in combination with other antimicrobial resistances, which means a 95.5 % of the multidrug resistant strains.

Table 2. Resistance profiles among monophasic isolates and their distribution on phage types

Resistance profile	No. of resistances	No. of isolates	Phage types (no. of strains)
Susceptible	0	4	DT 193 (3), NT (1)
AMP	1	6	DT 193 (3), DT 120 (1), U302 (1), NT (1)
TET	1	7	DT 193 (4), DT 120 (2), U302 (1)
AMP-TET	2	2	DT 193 (1), DT 120 (1)
AMP-SMX	2	1	DT 193 (1)
AMP-STR-TET	3	1	U302 (1)
AMP-GEN-TET	3	1	Rdnc
AMP-SMX-TET	3	1	DT 120 (1)
AMP-STR-SMX	3	4	DT 193 (1), U302 (2), DT 41 (1)
AMP-STR-SMX-TET	4	59	DT 193 (40), DT 120 (5), U302 (3), U311 (3), rdnc (3), NT (5)
AMP-SPE-SMX-TET	4	1	NT (1)
AMP-SPE-STR-SMX-TET	5	2	DT 193 (2)
AMP-SPE-STR-SMX-TMP	5	1	DT 120 (1)
AMP-CHL-FFN-STR-SMX-TET	6	1	U311 (1)
AMP-CHL-SPE-STR-SMX-TET	6	1	DT 104b (1)
AMP-NEO-SPE-STR-SMX-TET	6	1	DT 193 (1)
AMP-SPE-STR-SMX-TET-TMP	6	1	DT 120 (1)

The highest resistance was found against ampicillin and tetracycline with 83 and 76 isolates, respectively, corresponding to 88.3 % and 80.9 %, respectively of the isolates closely followed by streptomycin (72) and sulphamethoxazol (73). In contrast, resistance to other antimicrobials was low. All isolates were susceptible to colistin, nalidixic acid, ceftiofur, apramycin, and amoxicillin-clavulanic acid, while 2 and 1 isolates, respectively, were resistant to chloramphenicol and florfenicol, 1 to gentamicin, 1 to neomycin and 2 to trimethoprim. Notably, resistance, to the critical substances, used to treat human salmonellosis, ciprofloxacin (fluoroquinolones) or ceftiofur and cefotaxime (cephalosporins) was not found.

The AMP-STR-SMX-TET pattern with or without other resistances was the most commonly found in the different groups (Table 2). On the basis of phage types, 40/56 (71.4 %) of DT 193, presented this antimicrobial profile, together with 3/4 of the U311, 3/8 of the U302 isolates, and

5/11 of the DT 120 isolates. However, in DT 104b, the typical multiresistant DT 104 resistance profile AMP-CHL-STR-SMX-TET was found in combination with SPE. Regarding the main MLVA profiles or PFGE profile (cluster C) there was also a higher number of isolates that presented this R-type.

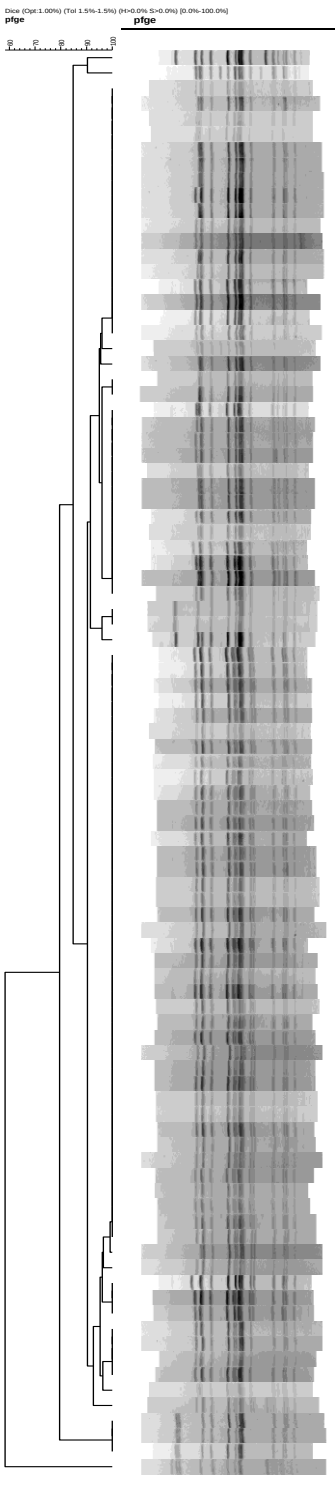
3.4 Molecular characterization results:

3.4.1 PFGE results:

Seventeen different *Xba*I banding profiles were found by PFGE, and the results indicated both some diversity among the monophasic isolates and the presence of specific clones. In the dendrogram obtained (Figure 1) three major clusters, A, B, and C, were formed comprising 17, 13 and 39 of the isolates, respectively. Cluster A was composed of DT 120 (7 strains) but also DT 193 (5), U311 (3) and a single strain of DT 104b and U302. The majority of isolates in this cluster were without the O:5 factor (11/16) and in 9 of the 17 isolates the *fljB* gene was present. Cluster B was composed of U302 and DT 193 predominantly, 5 isolates of each, but also 2 rdnc and one non-typeable strain. Also in this cluster was the majority of isolates without the O:5 factor (8/11). In the largest cluster C, 38 of the 39 isolates were phage type DT 193 while one was non-typeable. Thirty-six of the 39 isolates had the AMP-STR-SMX-TET resistance profile, while one isolates was only resistant to tetracyclines, one was fully susceptible, and one isolate hade the AMP-STR-SMX-TET profile with an additional resistance to spectinomycin. Some PFGE profiles were closely related to this cluster C profile, indicating a high similarity. In all cluster C isolates the *fljB* allele was absent i.e. they were all true monophasic isolates. This cluster contained isolates from both swine (n=34), cattle (n=4) and poultry (n=1). The remaining PFGE patterns were either unique (n=10) or shared by two (n=2), three (n=2) or four (n=1) isolates.

Most PFGE profiles shared several bands irrespective of clustering, but some patterns deviated clearly from the others. Thus, a DT 41 strains and two non-typeable isolates had indistinguishable profiles, but clearly different from the others, and one non-typeable isolate had a deviating profile (Figure 1).

Figure 1. Dendrogram of Danish monophasic *S.* Typhimurium-like Salmonella isolates based on PFGE. Phage types, antimicrobial resistance profile, MLVA type, and presence or absence of the *fljB* gene is indicated.



Strain number	antigen	phage type	*fljB* gene	MLVA loci repeats					Resistance to
2007-60-3615	4,12:i:-	DT 193	Absent	3	15	8	-2	0211	AMP-NEO-SPE-STR-SMX-TET
2007-60-684	4,12:i:-	DT 120	Present	3	12	9	-2	0211	AMP-SPE-STR-SMX-TET-TMP
2005-60-1524	4,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-SPE-STR-SMX-TET
2005-60-1681-1	4,12:i:-	U311	Present	3	14	10	-2	0211	AMP-CHL-FFN-STR-SMX-TET
2006-60-1012	4,12:i:-	U311	Present	3	14	10	-2	0211	AMP-STR-SMX-TET
2006-60-1243	4,12:i:-	U311	Present	3	14	10	-2	0211	AMP-STR-SMX-TET
2006-60-1622-1	4,12:i:-	DT 193	Absent	3	15	10	-2	0211	AMP
2006-60-1788-1	4,12:i:-	DT 193	Present	3	15	10	-2	0211	AMP
2006-60-1909-1	4,12:i:-	DT 120	Present	3	15	10	-2	0211	AMP
2006-60-1910-5	4,12:i:-	DT104b	Present	3	14	10	-2	0211	AMP-CHL-SPE-STR-SMX-TMP
2006-60-1910-7	4,12:i:-	U302	Present	3	14	10	-2	0211	AMP-STR-SMX-TET
2006-60-1924-1	4,5,12:i:-	DT 120	Absent	3	12	10	-2	0211	AMP-STR-SMX-TET
2006-60-2075-1	4,12:i:-	DT 193	Absent	3	13	9	-2	0111	TET
2006-60-2147-1	4,5,12:i:-	DT 193	Present	3	15	10	-2	0211	AMP
2006-60-2600-1	4,5,12:i:-	DT 120	Absent	3	12	8	-2	0211	TET
2007-10-2068	4,12:i:-	DT 120	Present	3	13	9	-2	0211	AMP-STR-SMX-TET
2007-60-2705-1	4,5,12:i:-	DT 120	Absent	3	12	10	-2	0211	AMP-SMX-TET
2008-10-2575	4,5,12:i:-	DT 120	Absent	3	13	9	-2	0211	AMP-TET
2008-60-2993-9	4,5,12:i:-	DT 120	Absent	3	15	8	-2	0211	AMP-STR-SMX-TET
2008-60-3485	4,5,12:i:-	NT	Absent	3	58	11	-2	0211	AMP-STR-SMX-TET
2010-10-1554-1	4,5,12:i:-	DT 120	Absent	3	15	10	-2	0211	TET
2010-60-1167	4,5,12:i:-	DT 193	Absent	3	13	10	-2	0211	TET
2010-60-2662-1	4,12:i:-	U302	Absent	3	13	10	-2	0211	AMP-STR-TET
2006-60-1910-1	4,12:i:-	U302	Present	3	14	11	-2	0211	AMP-STR-SMX-TET
2007-60-225-1	4,5,12:i:-	DT 193	Present	3	14	10	-2	0211	SUSCEPTIBLE
2007-60-2603-1	4,5,12:i:-	DT 193	Present	3	14	11	-2	0211	AMP-SMX
2008-60-3440-1	4,12:i:-	RDNC	Absent	3	12	12	-2	0211	AMP-STR-SMX-TET
2009-60-1190	4,12:i:-	U302	Present	3	13	10	-2	0211	AMP-STR-SMX-TET
2009-60-2677-1	4,12:i:-	U302	Absent	3	15	10	-2	0211	AMP-STR-SMX
2009-60-2679-1	4,12:i:-	U302	Absent	3	15	9	-2	0211	AMP-STR-SMX
2009-60-899	4,5,12:i:-	DT 193	Present	3	12	9	-2	0211	AMP-TET
2009-60-915	4,12:i:-	NT	Present	3	15	9	-2	0211	AMP-STR-SMX-TET
2009-60-93	4,12:i:-	U302	Absent	3	12	12	-2	0211	TET
2010-60-1374	4,5,12:i:-	DT 193	Absent	3	14	11	-2	0211	AMP-STR-SMX
2010-60-2256-1	4,12:i:-	DT 193	Present	3	14	10	-2	0211	AMP-STR-SMX-TET
2010-60-8	4,5,12:i:-	RDNC	Absent	3	12	9	-2	0211	AMP-GEN-TET
2008-60-2277	4,5,12:i:-	RDNC	Absent	3	12	13	-2	0112	AMP-STR-SMX-TET
2008-60-2284	4,5,12:i:-	RDNC	Absent	3	12	12	-2	0211	AMP-STR-SMX-TET
2006-60-1669	4,12:i:-	U311	Present	3	13	10	-2	0211	AMP-STR-SMX-TET
2007-60-2722	4,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2007-60-2723	4,12:i:-	DT 193	Absent	3	13	10	-2	0211	AMP-STR-SMX-TET
2008-10-2111	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2008-60-1447	4,12:i:-	DT 193	Absent	3	13	7	-2	0211	TET
2008-60-2335	4,5,12:i:-	DT 193	Absent	3	13	7	-2	0211	AMP-STR-SMX-TET
2008-60-2562	4,5,12:i:-	NT	Absent	3	16	13	-2	0211	AMP-STR-SMX-TET
2009-10-1972	4,5,12:i:-	DT 193	Absent	3	13	10	-2	0211	AMP-STR-SMX-TET
2009-60-1200	4,5,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2009-60-1456	4,5,12:i:-	DT 193	Absent	3	11	9	-2	0211	AMP-STR-SMX-TET
2009-60-1830	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2009-60-2038	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2009-60-2187	4,5,12:i:-	DT 193	Absent	3	12	9	-2	0211	AMP-STR-SMX-TET
2009-60-226	4,5,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2009-60-2289	4,5,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2009-60-2316	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2009-60-2325	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2009-60-2579	4,5,12:i:-	DT 193	Absent	3	13	8	-2	0211	SUSCEPTIBLE
2010-10-285	4,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2010-10-947-1	4,5,12:i:-	DT 193	Absent	3	14	10	-2	0211	AMP-STR-SMX-TET
2010-60-1375	4,12:i:-	DT 193	Absent	3	14	10	-2	0211	AMP-STR-SMX-TET
2010-60-1583	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2010-60-1612	4,5,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2010-60-1725	4,5,12:i:-	DT 193	Absent	3	11	9	-2	0211	AMP-STR-SMX-TET
2010-60-190	4,5,12:i:-	DT 193	Absent	3	12	9	-2	0211	AMP-STR-SMX-TET
2010-60-200	4,5,12:i:-	DT 193	Absent	3	12	9	-2	0211	AMP-STR-SMX-TET
2010-60-2253	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2010-60-2254-1	4,12:i:-	DT 193	Absent	3	12	9	-2	0211	AMP-STR-SMX-TET
2010-60-2421	4,5,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2010-60-2426	4,5,12:i:-	DT 193	Absent	3	12	9	-2	0211	AMP-STR-SMX-TET
2010-60-2975	4,5,12:i:-	DT 193	Absent	3	11	9	-2	0211	AMP-STR-SMX-TET
2010-60-3081	4,5,12:i:-	DT 193	Absent	3	13	10	-2	0211	AMP-STR-SMX-TET
2010-60-323	4,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2010-60-3863	4,5,12:i:-	DT 193	Absent	ND	ND	ND	ND	ND	AMP-SPE-STR-SMX-TET
2010-60-431-1	4,5,12:i:-	DT 193	Absent	3	12	10	-2	0211	AMP-STR-SMX-TET
2010-60-4455	4,5,12:i:-	DT 193	Absent	3	12	12	-2	0211	AMP-STR-SMX-TET
2010-60-54	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2010-60-55	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2010-60-744	4,5,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2010-60-751	4,5,12:i:-	DT 193	Absent	3	14	9	-2	0211	AMP-STR-SMX-TET
2008-60-494-1	4,5,12:i:-	DT 193	Absent	3	13	10	-2	0211	SUSCEPTIBLE
2010-60-87-1	4,5,12:i:-	DT 193	Absent	3	12	9	-2	0211	AMP-STR-SMX-TET
2008-60-1700	4,12:i:-	DT 193	Absent	3	15	8	-2	0211	AMP-STR-SMX-TET
2010-60-2257	4,5,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2010-60-4162	4,12:i:-	NT	Absent	3	31	9	-2	0211	AMP-STR-SMX-TET
2006-60-2159-1	4,5,12:i:-	DT 120	Present	3	12	9	-2	0211	AMP-STR-SMX-TET
2008-60-2688-6	4,5,12:i:-	DT 120	Present	3	15	13	-2	0311	AMP-STR-SMX-TET
2010-60-20	4,5,12:i:-	DT 193	Present	3	13	13	-2	0211	TET
2010-60-3696	4,5,12:i:-	NT	Absent	3	12	10	-2	0211	AMP-SPE-STR-SMX
2006-60-276	4,12:i:-	NT	Present	3	14	9	-2	0211	AMP-STR-SMX-TET
2008-60-1606	4,5,12:i:-	DT 193	Absent	3	13	9	-2	0211	AMP-STR-SMX-TET
2008-60-1871-2	4,5,12:i:-	DT 41	Absent	3	12	10	-2	0211	AMP-STR-SMX
2008-60-2861-1	4,5,12:i:-	NT	Present	3	15	13	-2	0311	AMP
2009-60-1232	4,5,12:i:-	NT	Present	3	11	11	-2	0211	SUSCEPTIBLE
2010-60-4279-1	4,5,12:i:-	NT	Present	2	17	6	7	0312	AMP-SPE-STR-SMX-TMP

Cluster A

N=17

Cluster B

N=13

Cluster C

N=39

3.4.2 MLVA results:

MLVA typing divided the isolates collection in 24 different profiles. The most common (Table 4) were 3-13-9-NA-211 (14 isolates); 3-14-9-NA-211 (12 isolates); 3-14-10-NA-211 (9 isolates); 3-12-9-NA-211 (10 isolates); 3-13-10-NA-211 (8 isolates) and 3-15-10-NA-211 (7 profiles). These six types grouped a total number of 60 isolates (63.8 % of the overall). Many isolates with MLVA profiles 3-13-9-NA-211 and 3-14-9-NA-211 belonged to PFGE cluster C, but some had other PFGE profiles.. The other MLVA profiles were more scattered among the PFGE patterns obtained, with the 3-14-10-NA-211 pattern principally linked to PFGE cluster A. The most variable loci were STTR5 and STTR6 with nine different profiles. Most of the STTR3 and STTR9 isolates had a common tandem repeats number but variation with both fewer and additional repeats in both were found, resulting in five and three different profiles, respectively. One isolate amplified the Typhimurium-specific virulence plasmid pSLT-bound at locus STTR10 and one could not be MLVA typed.

4-. DISCUSION:

This study is the first to characterize monophasic *S.* Typhimurium-like isolates and *S.* 4,[5],12:i:- isolates of food animal origin in Denmark. A total number of 94 isolates that had been identified as monophasic variants of *S.* Typhimurium by the classical slide agglutination method were included in the study. Eighty-six (91.5%) of these isolates were of swine origin, seven were from cattle and one isolate was from poultry. The dominant porcine origin may be attributed to the fact that the prevalence of *Salmonella* in Danish pigs is relatively high, whereas the prevalence of *Salmonella* in poultry is very low in Denmark and cattle is not included in the surveillance programmes (Wegener *et al.,* 2003), and consequently, the number of isolates available from these two species is low. In a European perspective, *S.* 4,[5],12:i:- isolates are widespread in many species, but it seems that they are most related to the swine sector (Hopkins *et al.,* 2010, 2012). Pork products have been the sources of infection in several outbreaks (Mossong *et al.,* 2007; Barone *et al.,* 2008; Dionisi *et al.,* 2009*;* Switt *et al.,* 2009; Bone *et al.,* 2010, Gossner *et al.* 2012), and in addition *Salmonella* 4,[5],12:i:- was one of the 10 most prevalent serotypes isolated from pigs and pork products in 2006 (EFSA, 2006), and in Germany, between 2006 and 2008, 48 % of the *S.* 4,[5],12:i:- isolates came from pigs, followed by cattle 13 % and poultry 5 % (Hauser *et al.,* 2010).

The absence/presence of the *fljB* gene is the criterium for distinction between true monophasic *S*. 4,[5],12:i:- and *S*. Typhimurium according to the EFSA guideline (EFSA, 2010). Sixty-

eight of the isolates selected for this investigation were confirmed as monophasic *S.* Typhimurium by lacking *fljB* gene and the other twenty-six should be classified as *S.* Typhimurium according to this guideline. It means that 27.7 % of the isolates were phenotypically categorized as monophasic variants of *S.* Typhimurium by slide agglutination while genotypically they were true *S*. Typhimurium. This information is in accordance with other studies although the proportion is higher than reported elsewhere (Hopkins *et al.,* 2010, 2012) and shows that even when serotyping is repeated several times or include other tools like migration through U-tubes, for some strains it is not possible to demonstrate the second phase flagella. There may be several explanations for this apparent discrepancy. There are "inconsistent serotype strains", which express the *fljB* gene intermittently (Switt *et al.,* 2009), or strains in which the *fljB* gene is present and can be detected by PCR but is not expressed due to point mutations (Zamperini *et al.,* 2007). In addition also other elements that take part in the switching mechanism, like the recombinase *Hin* or the first phase repressor *fljA,* may be damaged (Soyer *et al.,* 2009) and thereby inhibit the expression of flagella (Hopkins *et al.,* 2010, 2012). Thus, strains may be monophasic for other reasons than merely absence of the *fljB* gene.

In accordance with data published by Hauser and colleagues (2010), the majority of the isolates in this investigation (72.9 %) presented the factor O:5 in their antigenic formula. Those in which it was absent seemed to be less prevalent. It is also worth noting that the absence of the *fljB* gene was more associated with 4,5,12:i:- isolates (77 %) than 4,12:i.- isolates (46 %). Thus, the 4,5,12:i:- strains were predominantly true monophasic, lacking the *fljB* gene, whereas the 4,12:i:- strains were almost evenly distributed between true monophasic strains without the *fljB* gene and phenotypically monophasic strains possessing the *fljB* gene but seemingly unable to express it. This was mainly due to the presence of a dominant 4,5,12:i:- DT 193 clone (Fig. 1).

Due to its relatedness to *S.* Typhimurium, *S.*4,[5],12:i:- has the ability to be lysed by *S.* Typhimurium phages and so, isolates can be classified by definitive types (Anderson *et al.,* 1977). The results obtained in our study showed that the most common phage types described in other studies (EFSA, 2010) were also present in ours. The predominant phage type DT 193, found in almost 60 % of the isolates included in our study is also the most representative definitive type found in other studies like the one carried out in Germany (Hauser *et al.,* 2010). Like reported from other countries, most of the Danish DT 193 isolates obtained presented the R-type AMP-STR-SMX-TET. It seems that DT 193 isolates are related to this antimicrobial resistance profile more than other phage types, not only in *S.* 4,[5],12:i:- strains but also in true *S.* Typhimurium DT 193 strains (Hampton *et al.,* 1995, Garcia-Feliz *et al.,* 2009; Hauser *et al.,* 2010). Most of the DT193 isolates, 39 of 56 isolates clustered

together by PFGE, in a similar way as reported by Dionisi and colleagues (2009), sharing a common pattern compatible with STYMXB.0131 and with the predominant MLVA types 3-13-9-NA-211 and 3-14-9-NA-211. In this cluster 36 strains shared the R-type AMP-STR-SMX-TET described above and all isolates were lacking the *fljB* gene. It is interesting that these isolates originated from all three animal species included, 34 from swine, four from cattle and one from poultry. It is noteworthy that the single isolate collected from poultry had these characteristics. This result shows that this clone is widely spread in the animal production system, even though the production systems for the three animal species are quite separated. They have also been reported in turkeys (Wasyl and Hoszowski 2012). As in other studies DT 120 was the second most common phage type found (Hauser *et al.,* 2010; Hopkins *et al.,* 2010), but from the 11 isolates included in our study five could not be categorized as true monophasic *S.* Typhimurium because they contained the *fljB* gene. None of the four U302 isolates which were *fljB*(-) had the antimicrobial resistance profile reported for U302 isolates from Spain (Echeita *et al.,* 2001; De la Torre *et al.,* 2003) but were closer to other S. Typhimurium U302 (Ethelberg *et al.,* 2004) indicating that the origin of these U302 is different from the Spanish clone. Finally, a single isolate was classified as DT 41, a phage type which has not been described previously in the studies related to monophasic *S.* Typhimurium showing that new lineages are appearing from other *S.* Typhimurium sources. The PFGE profile of the DT 41 isolate deviated somewhat from most other isolates, but interestingly, two other isolates, both nontypeable by phage typing, had the same PFGE profile, which indicates that these three isolated were genetically closely related, although they had different resistance profiles.

The fact that just four strains were pan-susceptible to the antimicrobials tested shows the high level of resistance among monophasic *S.* Typhimurium. Resistance to classical antimicrobials like ampicillin, tetracyclines, streptomycin or sulphonamides was common and what is more, the association of resistances in multiresistant types was seen in 71 % of the strains selected. The most common R-type found was AMP-STR-SMX-TET with or without other resistances in 64 isolates, which was 62.8 % of the isolates and 88.1 % of the multiresistant isolates. This R-type seems to be common among the European *S.* 4,[5],12:i:- isolates (Barone *et al.,* 2008; Dionisi *et al.,* 2009; Hauser *et al.,* 2010; Hopkins *et al.,* 2010a; Lucarelli *et al.,* 2010). It was indicated by Lucarelli and colleagues (2010), that these resistance factors were harbored on chromosomal genes *bla*TEM-1, *strA-strB*, *sul2*, and *tet*(B) and could be related to the acquisition of a new genomic island which encloses antimicrobial resistance genes (Trüpschuch *et al.,* 2010). A notable fact is that the high level of phenotypic resistance profiles found in European studies (Guerra *et al.,* 2000; Barone *et al.,* 2008; Hauser *et al.,* 2010; Hopkins *et al.,* 2010) contrasts to the low level of resistance found in American countries (Switt

et *al.* 2009). In spite of the high degree of resistance observed to several antimicrobials, a high susceptibility to cephalosporins, fluoroquinolones, amphenicols and aminoglycosides other than streptomycin remained, like in *S.* Typhimurium.

Molecular typing methods clustered the 94 isolates. PFGE analysis using *Xba*I as restriction enzyme divided the overall collection in 17 profiles that had more than a 75 % similarity, except for one isolate, which seemed more separated. Three main clusters were obtained by PFGE. Clusters A and B showed a wide range of variability including 4,[5],12:i:-/ 4,12:i:-, isolates or isolates in which the *fljB* gene was present or not and several phage types, MLVA types and resistance profiles. However, isolates grouped in cluster C (39 isolates) shared characteristics like, just four presented the *fljB* allele, 38 were DT 193 and the last one was non-typable. Finally as was described above most of the isolates (36) presented the R-type AMP-STR-SMX-TET. It is also noteworthy that some isolates with different phage types or different antigenic formula could still have indistinguishable PFGE profiles.

MLVA typing using the primers designed by Lindstedt and colleagues (2005) divided the isolates in 24 profiles. The six most common profiles just differed in one or two repetitions in loci STTR5 or STTR6. The fact that 63.8 % of the isolates were grouped in these six profiles confirms the high level of similarity among the strains, like for the PFGE results. Two of these five main profiles 3-12- 9-NA-211 and 3-13-10-NA-211 that represented 10.6 % and 8.5 % of the isolates, respectively, are the most commonly found in other European studies, but several of the Dansih MLVA profiles have also been reported by others (EFSA, 2010; Hopkins *et al.* 2010, 2012). The Danish main profiles, 3-13-9-NA-211 and 3-14-9-NA-211, were only separated by a single repetition in locus STTR5 and most of these were also *fljB* (-), R-type AMP-STR-SMX-TET and clustered in PFGE cluster C. The profile that caused the epidemic associated with the consumption of pork sausages in France in 2010 (Bone *et al.,* 2010), (3-13-15-NA-211) was not found among the Danish isolates tested.

The comparison between molecular typing methods shows that MLVA created more profiles than PFGE but the clustering for the two methods were not congruent. MLVA has been reported to be more discriminatory than PFGE for typing of *S.* Typhimurium and the difference in discriminatory power was most marked for DT 104, whereas the difference was much less for DT 193 (Torpdahl *et al.,* 2007). In any case, the DT 193 PFGE cluster C in our study was further divided into several MLVA profiles. These results indicate that both molecular typing methods are useful to discriminate among *S.* 4,[5],12:i:- isolates and the different methods yield different results. Probably PFGE is more interesting for comparison of results from studies carried out in different countries

while MLVA profiles seem to change faster over time suggesting its usefulness in outbreaks. MLVA profiles 3-13-9-NA-211 and 3-14-9-NA-211 shared the main characteristics including their main PFGE pattern, but the different numbers of repeats in locus STTR5 let us to divide them in two different patterns.

5-. CONCLUSIONS:

The present study demonstrates the presence of monophasic *S.* Typhimurium-like strains in Danish food animal production. Both the antigenic formulae *S*. 4,12,i:- and *S.* 4,5,12:i:- were present, although the latter dominated. Several different clones were demonstrated using molecular typing methods, but from the PFGE results three different clonal lineages were dominant, one of them being a major clone of DT 193 isolates, which is also known from other countries. The isolates were characterized by a high level of antimicrobial resistance, most notably to ampicillin, streptomycin, sulphonamides, and tetracyclines, whereas no isolates were resistant to the critical compounds fluoroquinolones and cephalosporins. A PCR test for the presence of the *fljB* gene showed that some strains, especially in the 4,12:i:- group, carried the gene, but were seemingly unable to express it. This result demonstrates that both phenotypical and genotypical monophasic strains exist. This is an important issue when reporting prevalence of monophasic strains, whether the diagnosis is made on the basis of slide agglutination or PCR. The PCR is a useful tool complementary to the classical slide agglutination method, to distinguish between phenotypical and true monophasic strains

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Prevalence, serotypes and resistance patterns of *Salmonella* in Danish pig production

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Antimicrobial resistance

ABSTRACT

The objective of this paper is to analyse in further detail the Danish results of the EFSA baseline studies in slaughter pigs and breeding herds, and compare them with the results obtained in (1) the pre-implementation study that was carried out to establish the initial prevalence values in fattening herds as part of the Danish *Salmonella* control programme, and (2) the study performed four years later in breeding and finishing herds to obtain information about the prevalence in breeding farms and the status of the finishers after the first years of the National *Salmonella* Control Programme. In the slaughter pigs *Salmonella* was detected in a 7.4% of 1218 ileocaecal lymph nodes and on 3.2% of 438 carcasses examined. Among the breeding herds examined by floor faecal or swab samples 122 of 298 (40.9%) were positive in at least one of the ten samples collected. The most prevalent serotypes were *Salmonella* Typhimurium in finishers and *Salmonella Derby* in breeding herds while the most prevalent phage types of the *S.* Typhimurium isolates were DT 12 and DT 120. The antimicrobial resistance analysis yielded a 35.2% of the isolates from the slaughter pigs resistant to one or more antimicrobials while 19.3% were resistant to four or more antimicrobials. A significantly higher percentage of resistance to antimicrobials was found in the *S.* Typhimurium isolates ($\chi^2 = 4.72$, $p = 0.029$), where 42.9% presented resistance to one or more compounds. In breeding herds, just *S.* Typhimurium and *S.* 4,5],12:i: – isolates were tested. As many as 56.8% of the *S.* Typhimurium-like strains positive breeding farms had resistant strains, while 27% had multidrug resistant strains. The distribution of the isolates in regions showed that *S. Derby* is at present the predominant serotype in breeding farms from most of the regions of the country.

1. Introduction

A *Salmonella* control programme in pigs and pork was implemented in Denmark in 1994 (Stege, 1994; Mousing et al., 1997) after a *Salmonella* Infantis epidemic related to pork, during the summer of 1993. The surveillance in the Danish Salmonella Control Programme (DSCP) consists of two major parts: one part is a herd-monitoring programme based on serological and bacteriological follow-up (Alban et al., 2002). Every month, herds are assigned to one of three levels on the basis of the proportion of sero-positive meat-juice samples taken over the previous three months. The other part is a post-chilling monitoring of carcasses on the basis of bacteriology (Nielsen et al., 2001). Apart from the continuous information obtained throughout the national control programme, large-scale prevalence screenings for *Salmonella* have been carried out in Denmark. The first one was executed in 1993/1994 as part of the establishment of the national programme (Baggesen et al., 1996), here cited as the pre-implementation study. In that first microbiological survey, a total of 13,468 slaughter pigs from 1363 herds were examined by the bacteriological analysis of caecal contents collected at the slaughterhouse. Here a first *Salmonella* prevalence estimation was obtained together with the serotypes present. A second bacteriological survey was performed in 1998 (Christensen et al., 2002). In that survey a broader swine population was analyzed, including 1962 slaughter pig herds, 305 farrow-to-grower herds (sows) and all breeding herds (multiplying herds). The survey embraced two objectives, the evaluation of the prevalence of *Salmonella* spp., and determines the specific prevalence of the multiresistant *S.* Typhimurium DT104. Similarly, in 1999 a subclinical prevalence study was performed (Stege et al., 2000). That study comprised 96 farms selected randomly and 36 high-seroprevalence herds which were evaluated by both bacteriology and serology. Finally, between 2006 and 2008 the European Food Safety Authority (EFSA) baseline studies were carried out in all EU Member States including Denmark. The objective of these surveys was to determine the prevalence of pigs infected with *Salmonella* as a starting point in a European Union programme

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attempting to reduce the prevalence of *Salmonella* in food animal populations in order to decrease the incidence of human salmonellosis. Between 2006 and 2007 the baseline survey on the prevalence of *Salmonella* was carried out in slaughter pigs (EFSA, 2008) and in 2008 the baseline survey in breeding herds (EFSA, 2009).

The present study reports the prevalence of *Salmonella* obtained from the data collected from the EU baseline investigations from 2006 to 2008 in slaughter pigs and breeding herds cross-sectional surveys, together with the occurring serotypes and their antimicrobial susceptibility patterns by the analysis of the data collected. In addition, these data were compared to those previously reported in Denmark, the 1993/1994 study (Baggesen et al., 1996) and the 1998/1999 study (Christensen et al., 2002), with the aiming of describing changes of *Salmonella* prevalence in pigs from the control programme implementation till the last cross-sectional survey.

2. Materials and methods

2.1. Study design and sample collection

The present study analyzed the data from the isolates recovered from the baseline surveys on the prevalence of *Salmonella* in slaughter and breeding pigs in Denmark. Two cross-sectional studies were performed: the EFSA baseline study in slaughter pigs and the EFSA baseline study in breeding pigs, both under the guidelines established by the EC (2006, 2008). The survey on the prevalence of *Salmonella* in slaughter pigs was carried out between October 2006 and September 2007 and included ileocaecal lymph nodes (ILN) ($n = 1218$) and carcass swabs ($n = 438$) collected in different slaughterhouses which made a total of 1656 samples. The baseline survey on the prevalence of *Salmonella* in breeding pigs was performed throughout 2008 in breeding (multiplication) and production holdings (farrow-to-weaners/growers/finishers) and included a total of 298 Danish pig farms. Within each farm, 10 pooled samples of faeces or floor swabs were collected and processed according to EC guidelines. The target population, samples collected and sampling protocol is described elsewhere (EC, 2006, 2008). Herd apparent prevalence (HAP) in the breeding herds baseline study was based on a herd-test cut-off equal to one isolate detected, criteria that were also followed in the previous cross-sectional survey (Christensen et al., 2002).

2.2. Salmonella isolation

All samples were analysed by the ISO 6579:2002/Amd 1:2007 standard methodology. Samples were diluted 1:10 in Buffered Peptone Water (BPW) (Oxoid) and incubated during 18–24 h at 37 °C. One hundred µl of the incubated BPW were transferred to Modified Semisolid Rappaport-Vassilliadis (MSRV) medium (Merck) and incubated 24/48 h at 41.5 °C. Plates with swarming growth were subcultured in two selective and differentiating media, Xylose-Lysine-Deoxycholate agar and Brilliant Green agar (Oxoid), and incubated at 37 °C for 18–20 h. Presumptive *Salmonella* colonies were subcultured on non-selective media and confirmed by serotyping.

2.3. Serotyping and phage typing

Serotyping of *Salmonella* spp. isolates was performed by slide agglutination using commercial antisera (Statens Serum Institut, Copenhagen, Denmark) according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). Phenotypically monophasic isolates of *Salmonella* Typhimurium were checked by molecular serotyping according to the PCR identification guidelines published by EFSA (EFSA, 2010) adapted to our laboratory conditions. A multiplex PCR was performed using the Qiagen Multiplex PCR kit for the amplification of the *fljB-fljA* intergenic region (0.2 µM primers FFLIB/RFLIA) and the verification of the absence of the *flj*B allele (1 µM sense-59/antisense-83). Cycling parameters were denaturation at 95 °C for 15 min, amplification during 30 cycles (95 °C for 30 s, 64 °C for 30 s, 72 °C for 90 s) and a final step of post-extension at 72 °C for 10 min. PCR products were separated on 2% (w/v) agarose gels, stained with ethidium bromide and observed and photographed under UV light.

Phage susceptibility of *S.* Enteritidis and *S.* Typhimurium isolates was investigated according to the International Federation for Enteric Phage Typing (IFEP, Laboratory of Enteric Pathogens, Health Protection Agency, Colindale, London, UK). One to three colonies of each *S.* Enteritidis, *S.* Typhimurium, or *S.* 4, [5],12:i:- isolate were suspended in nutrient broth and incubated at 37 °C under continuous shaking for 1 h. After that, nutrient agar plates were flooded with the culture obtained. Once the plates were properly dried, the phages were spotted onto the surface and the plates were incubated and read after approx. 18 h.

2.4. Antimicrobial susceptibility analysis

In the slaughter pig study, all the isolates available (88), without serotype specifications were tested for antimicrobial susceptibility. On the contrary in the breeder study, only the *S.* Typhimurium and monophasic *S.* Typhimurium-like isolates were included for antimicrobial testing. Antimicrobial susceptibility testing was performed by means of a broth microdilution procedure in accordance with the CLSI guidelines and using the semiautomatic Sensititre system (Trek Diagnostic Systems Ltd., UK). Antimicrobials tested included amoxicillin-clavulanic acid (AUG), ampicillin (AMP), apramycin (APR), cephotaxime (FOT), ceftiofur (XNL), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), florfenicol (FFN), gentamicin (GEN), nalidixic acid (NAL), neomycin (NEO), spectinomycin (SPE), streptomycin (STR), sulfonamide (SMX), tetracycline (TET) and trimethoprim (TMP). Data were interpreted using EUCAST epidemiological cut-off values (www.EUCAST.org).

2.5. Data storage and statistical analysis

All data was entered into a local LIMS software from where it could be exported as an Excel file for further analysis. Statistical analyses were carried out using EpiInfo™ for Windows (CDC, Atlanta). A univariate analysis using the chi-squared test at $\alpha = 0.05$ was used to detect any association between the herd-apparent prevalence among the breeding farm categories and the different regions. This test was also used to compare the prevalence of antimicrobial resistance and multiresistance among breeding and slaughter pigs as well as among the farm categories and the different serotypes.

3. Results

3.1. Prevalence results

3.1.1. Baseline survey in slaughter pigs

Salmonella was detected in 90 out of 1218 (7.4%) ileocaecal lymph node samples from slaughter pigs and in 14 of the 438 carcass swabs (3.2%) (Table 1).

3.1.2. Baseline survey in breeding herds

Salmonella herd apparent prevalence in pooled pen-floor samples in breeding farms was 40.9% (122 of 298 herds) (Table 1). Moreover, *Salmonella* was identified in 375 out of 2,980 pen samples (12.6%). Within each positive farm, the number of positive samples varied from 1 (43 farms, 35.2%) to 10 (1 farm, 0.8%) with

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Table 1
Apparent bacteriological prevalence (HAP) of *Salmonella* in different surveys carried out in pig farms in Denmark.

Target population	1993/1994 study Baggesen et al. (1996)			1998/1999 study Christensen et al. (2002)			2007–2009 EFSA studies		
	Individuals	HAP[a] (%)	[95% CI]	No. samples	HAP[a] (%)	[95% CI]	No. samples	HAP[a] (%)	[95% CI]
Slaughter herds	1362	22.2	20.2–24.2	1962	11.4	10.0–12.8			
Slaughter pigs									
Carcasses	–	–		–	–	–	438	3.2	1.7–5.1
Lymph nodes	–	–		–	–	–	1218	7.4	6.2–9.2
Breeding herds									
Global results	–	–		671	14.0	11.4–16.6	298	40.9	35.3–46.4
Breeding herds[b]	–	–		305	16.7	12.5–20.9	94	41.5	31.5–51.5
Production herds	–	–		366	11.7	8.4–15.0	166	40.4	32.9–47.9

[a] HAP Apparent prevalence.
[b] Breeding farms for multiplication purposes.

a mean of 3.23 positive samples/farm. The results are shown in Fig. 1.

Out of the 298 breeding farms, 94 were classified as pure breeding farms (also named through the manuscript as multiplication herds or genetic herds), while 166 were classified as both breeding and production herds. The remaining 38 were not further classified. Among the pure breeding farms, 39 were positive for *Salmonella* (41.5%), while among the breeding and production holdings, 67 were *Salmonella* positive (40.4%). Thus, there was no difference in *Salmonella* infection levels between the two breeding farm categories.

3.2. Typing results

3.2.1. Baseline survey in slaughter pigs

A total of 14 different serotypes of *Salmonella* were identified in the ileocaecal lymph node samples (Table 2). *S.* Typhimurium was by far the most prevalent serotype, identified in 54.4% of the positive individuals, followed by *Salmonella Derby* (17.8%) and *S. Infantis* (11.1%). One of the isolates tested was rough. From seven phenotypically monophasic *S.* Typhimurium-like isolates, four were classified as true *S.* 4,[5],12:i:-after the PCR confirmation of absence of the *fljB* allele. Results of phage typing of the isolates of *S.* Typhimurium and *S.* 4,[5],12:i:- are shown in Table 2. Phage type DT 12 was the most common followed by DT 120 and DT 193. Four isolates reacted but did not conform to any known pattern (rdnc) while another four could not be phage typed. Three of the four *S.* 4,[5],12:i:- isolates were identified as DT 120 while one of these isolates could not be phage typed. The *S.* Enteritidis isolate belonged to phage type PT 11.

S. Typhimurium and *S. Infantis* were the most frequent serotypes among isolates recovered from carcasses (Table 2). Results of phage typing for these isolates are shown in Table 2.

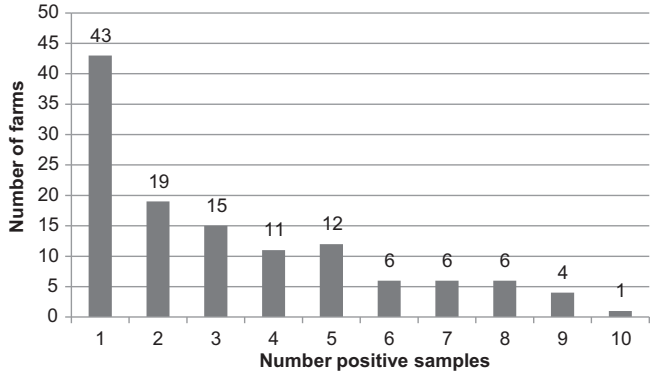

Fig. 1. Number of positive samples within a *Salmonella* positive farm.

3.2.2. Baseline survey in breeding herds

The serotyping of the isolates from the baseline survey in breeding farms yielded in the identification of 19 *Salmonella* serotypes (Table 2). *Salmonella Derby* was the most prevalent serotype, detected in 34.4% of the herds, followed by *S.* Typhimurium (31.1%) and *S. Infantis* (18.9%). Two isolates from this survey could not be serotyped (rough strains). The nine phenotypically monophasic *S.* Typhimurium isolates were verified by PCR and five of them were confirmed as *S.* 4,[5],12:i:- lacking the *flj*B gene. Phage typing of *S.* Typhimurium and *S.* 4,[5],12:i:- isolates revealed 16 different phage types with DT 120 as the most prevalent followed by DT 12, DT 193, DT 170, U302, DT 66, and U292 (Table 2). Six isolates recovered from five different farms reacted but did not conform to any defined phage type pattern and another three could not be phage typed. The phage types of *S.* Enteritidis isolates were PT 8 and PT 33.

Only one *Salmonella* serotype was identified in 104 of the 122 positive farms (85.2%). However, two different serotypes were found in 16 farms (13.1%), three different serotypes in two farms and four serotypes in another two farms. In a similar way, just one phage type was identified in 36 out of a total of 40 farms infected by *S.* Typhimurium or *S.* 4,[5],12:i:-, while in four of them two different phage types were found.

3.3. Antimicrobial resistance

3.3.1. Baseline survey in slaughter pigs

Among 88 isolates analysed from ILN samples (Table 3), 31 showed resistance to one or more antimicrobials (35.2%). Multiresistance, defined as the resistance to four or more antimicrobials, was identified in 17 isolates (19.3%). The resistance pattern AMP-STR-SMX-TET was identified in 8 multidrug resistant (MDR) isolates while the penta-resistance profile AMP-CHL-STR-SMX-TET with additional resistance to FFN and SPE was found in four isolates, all of them *S.* Typhimurium isolates from the phage type DT 104. There were differences in the presence of resistance to antimicrobials among serotypes. From the 49 *S.* Typhimurium isolates tested, 21 (42.9%) were resistant while 12 (24.5%) were MDR. Moreover, three out of four *S.* 4,[5],12:i:- isolates were MDR and shared the antimicrobial resistance profile AMP-STR-SMX-TET. In contrast, antimicrobial resistance was less common among non-*S.* Typhimurium or *S.* 4,[5],12:i:- isolates. Thirty-four of these isolates were tested and 28 of them yielded susceptible to all tested antimicrobials (82.4%). Three of them presented MDR profiles, AMP-SPE-STR-SMX-TET-TMP found in one *S. Derby* isolate, SPE-STR-SMX-TET found in one *S. Agona* isolate and CIP-NAL-SMX-TET-TMP found in an isolate that could not be typed (rough isolate). The statistical analysis showed significant differences in the frequency of antimicrobial resistance among *S.* Typhimurium or *S.* 4,[5],12:i:- and other *Salmonella* serotypes ($\chi^2 = 4.72$, $p = 0.029$), but not in the pro-

Table 2
Distribution of *Salmonella* serotypes and *S.* Typhimurium and *S.* 4,[5],12:i:- phage types in the 1993/1994 prevalence study and in the EFSA baseline studies in Denmark.

Serotype	1993/1994 study Baggesen et al. (1996)				2007–2009 EFSA studies					
					Slaughter pigs				Breeding pigs	
	No. isolates	% Isolates	No. positive herds	% Positive herds	No. positive ILN[a] (individuals)	% Positive ILN (individuals)	No. positive carcasses (individuals)	% Positive carcasses (individuals)	No. positive herds[b]	% Positive herds
Typhimurium	536	64.4	182	61.1	49	54.4	5	35.7	38	31.1
Derby	41	4.9	12	4.0	16	17.8	2	14.3	42	34.4
Infantis	60	7.2	32	10.6	10	11.1	5	35.7	23	18.9
Livingstone	21	2.5	7	2.3	0	0	1	7.1	17	13.9
4,[5],12:i:-	0	–	0	–	4	4.4	0	–	6	4.9
4,12:b:-	52	6.3	25	8.3	1	1.1	0	–	0	–
Panama	32	3.9	15	5.0	0	–	0	–	2	1.6
Worthington	12	1.4	5	1.7	0	–	0	–	0	–
Mbandaka	11	1.3	6	2.0	0	–	0	–	1	0.8
Enteritidis	5	0.6	3	1.0	0	–	1	7.1	2	1.6
Other serotypes	62	7.5	33	10.9	10[e]	11.1	–	–	19[f]	15.6
Total	832		302		90		14		122	
Phage types			No. herds	% Positive herds[c]	No. ILN (individuals)	% Positive ILN (individuals)[c]	No. Positive carcasses (individuals)	% Positive carcasses (individuals)	No. herds[d]	% Positive herds
DT 12			84	51.5	11	20.3	–	–	4	9.1
DT 66			18	11.0	0	0	–	–	2	4.5
DT 120			3	1.8	9	16.7	3	60	15	34.1
DT 193			14	8.6	6	13.0	1	20	3	6.8
DT 17			11	6.7	5	9.2	–	–	1	2.3
DT 10			9	5.5	0	–	–	–	1	2.3
DT 170			0	–	4	7.4	–	–	3	6.8
DT 104			1	0.6	4	7.4	–	–	2	4.6
RDNC			9	5.5	4	7.4	0		6	13.6
Other phage types			36	22.1	9[g]	18.6	1[h]	20	9[i]	20.5
Total			184		52		5		44	

[a] ILN ileocaecal lymph nodes.
[b] 20 herds were infected with more than one serotype.
[c] Percentage of *S.* Typhimurium positive herds. More than one serotype can be found in one herd.
[d] *S.* Typhimurium and *S.* 4,[5],12:i:- herds are included.
[e] Other serovars detected were *S.* 9,12:l,v:-(1), *S. Goettingen* (1), *S. Adelaide* (1), *S. Agona* (1), *S. Anatum* (1), *S. Braenderup* (1), *S. Newport* (2), *S. Ohio* (1), *S.* 4,12:-:1,2 (1), and rough (1).
[f] Other serotypes detected were *S. Agona* (3), *S. London* (3), *S. Schwarzengrund* (1), *S. Rissen* (2), *S. Maleagridis* (1), *S. Idikan* (1), *S. Uganda* (1), *S. Muenchen* (2), *S. Newport* (1), S. 6,7:-:- (1), *S. Kedougou* (1), and rough (2).
[g] Other phage types detected were DT 41 (1), U302 (1), DT 40 (2), U292 (1), and non-typeable (4).
[h] Other phage types detected: non-typeable (1).
[i] Other phage types detected: DT7 (4), DT 208 (2) DT U292 (2), DT U302 (2), DT41 (1), DT 104B (1), DT 135 (1), non-typeable 1.

Table 3
Antimicrobial resistance in the *Salmonella* isolates recovered during the EFSA baselines studies in Denmark.

Antimicrobial	Slaughter pigs (ilecocecal lymph nodes)										Breeding herds[d]			
	All serovars % (No. isolates)		*S. Typhimurium* % (No. isolates)		*S. Derby* % (No. isolates)		*S. Infantis* % (No. isolates)		*S.* 4,[5],12:i:- % (No. isolates)		Production % (No. isolates)		Breeding % (No. isolates)	
Apramycin	0%		0%		0%		0		0%		0%		8.3% (2)	
Amphenicols[a]	4.4% (4)		7.4% (4)		0%		0		0%		7.5%(4)		0%	
Ampicillin	25.3% (23)		31.5% (17)		28.6% (4)		0		75% (3)		32.1% (17)		29.2% (7)	
Cephalosporins[b]	0%		0%		0%		0		0%		0%		0%	
Colistin	0%		0%		0%		0		0%		0%		0%	
Nalidixic acid	2.2% (2)		1.9% (1)		0%		0		0%		0%		0%	
Ciprofloxacin	1.1% (1)		1.9% (1)		0%		0		0%		0%		0%	
Gentamicin	0%		0%		0%		0		0%		0%		8.3% (2)	
Neomicin	1.1% (1)		1.9% (1)		0%		0		0%		1.9%(1)		0%	
Spectinomycin	6.6% (6)		7.4% (4)		7.1% (1)		0		0%		11.3% (6)		8.3% (2)	
Streptomycin	19.8% (18)		31.5% (17)		7.1% (1)		0		75% (3)		37.7% (20)		45.8% (11)	
Sulfonamide	23.1% (21)		27.8% (15)		7.1% (1)		0		75% (3)		32.1% (17)		50% (12)	
Tetracycline	30.8% (28)		35.2% (19)		21.4% (3)		0		100% (4)		26.4% (14)		25% (6)	
Trimetoprim	4.4% (4)		3.7% (2)		7.1% (1)		0		0%		1.9%(1)		0%	
Global data	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Resistance isolates	31	35.2	21	42.9	5	35.7	0	0	4	100	23	43.4	12	50
MDR[c] isolates	17	19.3	12	24.5	1	7.1	0	0	3	75	8	15.1	7	29.2

[a] Chloramphenicol and Florfenicol.
[b] Ceftiofur and Cephalothin (the last one just in slaughter pig isolates).
[c] MDR: multidrug resistant.
[d] Farms not classified as production or breeding herds were not included. Only *S.* Typhimurium included.

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portion of MDR isolates ($\chi^2 = 2.35$, $p = 0.125$). Regarding the results of the other two main serotypes found, no antimicrobial resistance was found in *S. Infantis* isolates while five of the *S. Derby* isolates (31.25%) presented any antimicrobial resistance, principally to ampicillin or tetracycline, and the aforementioned multidrug resistant isolate (7.1%).

Only five *S.* Typhimurium isolates recovered from carcasses were tested. Four strains were susceptible to all antimicrobials and one had the AMP-STR-SMX resistance pattern.

3.3.2. Baseline survey in breeding herds

Antimicrobial resistance was only tested for *S.* Typhimurium and *S.* 4,[5],12:i:- isolates in the breeding farm study (Table 3). From the 82 *S.* Typhimurium isolates, 40 (48.8%) yielded resistant to any of the antimicrobials tested. MDR patterns were present in 18 (22%) of these 82 *S.* Typhimurium isolates. Among the resistance and MDR patterns observed the AMP-STR-SMX-TET pattern yielded the most frequently observed, detected in 12 *S.* Typhimurium isolates (30% of the resistant isolates). It is worth noting that the five *S.* 4,[5],12:i:- isolates tested presented antimicrobial resistances, and three of them shared the MDR pattern AMP-STR-SMX-TET. No significant differences were found among *S.* Typhimurium and *S.* 4,[5],12:i:- isolates between slaughter or breeding pigs ($\chi^2 = 0.74$, $p = 0.39$) and ($\chi^2 = 1.75$, $p = 0.18$), respectively for antimicrobial resistance or MDR.

From the 38 herds infected by *S.* Typhimurium, isolates with any antimicrobial resistance were present in 21 (55.2%) and MDR isolates were detected in 10 of these 38 infected herds (26.3%). The predominant resistance pattern AMP-STR-SMX-TET was present in seven herds, (33% of herds with resistant isolates). Two of the herds presented isolate with different resistance patterns and in another three farms, isolates with resistance patterns and with pan-susceptible patterns were identified.

Thirteen of 25 production herds (52%) had resistant isolates while five herds (20%) had a MDR profile. Ten of the 15 breeding herds evaluated (66.7%), had resistance to at least one antimicrobial, whereas seven of these breeding herds (46.7%) presented isolates with MDR profiles. No statistical differences in number of herds with antimicrobial resistant *S.* Typhimurium and *S.* 4,5,12:i:-isolates between groups were found ($\chi^2 = 0.33$, $p = 0.56$), although MDR differences between breeding and production herds almost reached statistical significant difference values ($\chi^2 = 2.03$, $p = 0.15$).

3.4. Regional results

3.4.1. Baseline survey in slaughter pigs

According to Veterinary and Food Administration district, each herd referred to, slaughter pigs were classified into three different regions: Northern region, Southern region and South-East region. The number of positive pigs varied from 15 in the South-east Region to 43 in the Northern Region. Stereotyping and phage typing results for these regions are shown in Table 4. *S.* Typhimurium, *S. Derby* and *S. Infantis* were the three main serotypes detected in the three regions and despite that the proportions varied from one to another, there were no statistical differences ($p > 0.05$).

3.4.2. Baseline survey in breeding herds

Breeding herds were classified according to their location into four different regions: Zealand and Eastern islands, Funen, Northern Jutland and Southern Jutland. The number of positive farms and of serotypes found was higher in the Northern Jutland region followed by Southern Jutland, Zealand and Funen. Data regarding the number of positive farms as well as stereotyping and phage typing results are shown in Table 4. *S. Derby* was the most prevalent serotype in Northern Jutland, Funen and Zealand while in Southern Jutland *S.* Typhimurium was the most prevalent serotype. No statistically significant differences between regions were found regarding the number of positive farms in which *S.* Typhimurium, *S. Derby* or *S. Infantis* were found ($\chi^2 = 2.48$, $p = 0.48$, $\chi^2 = 5.53$, $p = 0.14$ and $\chi^2 = 0.71$, $p = 0.87$).

4. Discussion

Between 2006 and 2008, EFSA designed two surveys according to the regulation (EC) no. 2160/2003 on the control for *Salmonella* and other zoonotic agents which aims to reduce the incidence of food-borne diseases in the EU. These baseline surveys comprised the two populations involved in the swine production system: slaughter pigs (finishers) and breeding pigs. Just before initiation

Table 4
Distribution of *Salmonella* serotypes and phage types of *S.* Typhimurium and *S.* 4,[5],12:i:- recovered in the slaughter pigs and breeding farms baseline studies performed in Denmark in relation to the division of the country in regions.

Serotypes	Slaughter pig study						Breeding Pig Study[a]							
	Northern region		Southern region		South-east region		Northern Jutland		Southern Jutland		Funen		Zealand	
	No. isolates	% Isolates	No. isolates	% Isolate	No. isolates	% Isolates	No. Farms	% Farms	No. Farms	% Farms	No. Farms	% Farms	No. Farms	% Farms
Typhimurium	27	62.8	17	47.3	5	33.3	16	21.1	14	32.6	2	16.7	3	21.4
Derby	8	18.6	7	19.4	1	6.7	20	26.2	11	25.6	7	58.3	4	28.6
Infantis	2	4.7	5	13.9	5	33.3	12	15.8	7	16.3	3	25.0	2	14.3
Livingstone	1	2.3	0	–	0	–	12	15.8	2	4.6	0	–	1	7.1
Other Serotypes	5	11.6	7	19.4	4	26.7	16	21.1	9	20.9	0	–	4	28.6
Total	43		36		15		76		43		12		14	
S. Typhimurium and S. 4,[5],12:i:- phage types													Zealand	
DT 12	7	25.9	2	11.1	2	28.6	3	17.6	1	6.3	0	–	0	–
DT 104	3	11.1	1	5.6	0	–	0	–	0	–	0	–	1	20
DT 120	2	7.4	5	27.7	2	28.6	6	35.3	6	37.5	0	–	1	20
DT 170	1	3.7	3	16.7	0	–	2	11.8	1	6.3	0	–	0	–
DT 193	6	22.2	1	5.6	0	–	1	5.9	1	6.3	1	50	1	20
DT U302	0	–	1	5.6	0	–	2	11.8	0	–		–	0	–
Other DTs/ Rough	8	29.7	5	27.7	3	42.8	6	35.3	9	56.3	1	50	2	40

[a] Several serotypes or phage types could be found at the same farm.

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of the Danish *Salmonella* control programme (DSCP) a cross-sectional study was carried out in 1993/1994 (Baggesen et al., 1996) in order to estimate the prevalence and the serotypes involved at that time in finishing farms. Four years later (1998/1999) a similar study was performed (Christensen et al., 2002) but in this case, the populations included were finishers and also breeding pigs. The implementation of the *Salmonella* control programme seemed to work and a reduction in the human cases attributed to pork consumption was achieved in this period of time (Wegener et al., 2003). Apart from the global objectives designed by EFSA, the data are also interesting in a pure Danish perspective as they were obtained ten years after the last cross-sectional bacteriological survey carried out in Denmark, and thus, the information allows comparison with the situation ten years earlier. When a number of studies are going to be compared, differences in factors such as matrix and quantity of sample analyzed, methodology used or population frame included in the study have to be considered to determine which data can be compared, what biases might be present and what effect these biases may have on the interpretation of the results (Christensen et al., 2002). Differences in sampling design and potential biases between the pre-implementation study (1993/1994) and the next cross-sectional survey (1998/1999) were analyzed by Christensen et al. (2002). Table 5 reports the main characteristics of the three cross-sectional surveys. These modifications are considered in the subsequent paragraphs to provide some insight about the prevalence changes, while the other data, serotypes, phage types or antimicrobial resistance, presented here are more suitable to be compared.

Thirteen years after the pre-implementation study, the baseline study of EFSA in finishers was carried out. To perform this survey, samples from ILN and carcass surface were chosen to detect *Salmonella* in contrast to the caecal contents evaluated in the pre-implementation survey. Moreover the herd was not selected as epidemiological unit and pigs were sampled according to the number of pigs slaughtered in each slaughterhouse participating in the study. The choice of the ILN implies that not only shedders can be detected but also pigs in a carrier state. Furthermore, the collection of samples at the slaughterhouse implies that new infections occurring at transport or lairage can be also detected, despite the fact that transport and lairage impact in Denmark is limited (Alban, 2012), compared to other countries (Arguello et al., 2012); Hurd et al., 2002) so the apparent prevalence results offer an accurate capture of the pig status at the harvest. However, the difference in the nature of the samples collected; prevent any comparison between the pre-implementation results obtained with caecal contents and the ILN prevalence results. In any case, the 7.4% prevalence found in ILN is on its own important information for future studies. This prevalence reported here is not exactly the one included by EFSA, but for this study all the samples collected and properly processed were included, obviating other requirements that exclude them for the EFSA report. However no significant differences between both data were found ($\chi^2 = 0.19$, $p = 0.66$). The carcass prevalence detected, 3.2%, was higher than in previous Danish reports. In an investigation performed in 2000 on 18,984 carcasses in nine abattoirs, the individual prevalence found was 1.4%: similar results were obtained in another Danish study, which estimated a prevalence on carcasses between 0.9–1.2% (Nielsen et al., 2001) and finally data reported by the surveillance for Danish pork shows that mean prevalence has not varied significantly in the first decade of this century (Alban et al., 2012). In the EFSA baseline study carcasses were evaluated before the chilling process, while the results of previous Danish studies (Nielsen et al., 2001; Sørensen et al., 2007; Alban et al., 2012) refer to carcasses analyzed after chilling. The higher percentage of positive carcasses found in the EFSA survey can be in part explained by the differences in sampling conditions, and the lower prevalence reported after chilling process (Botteldoorn et al., 2003; Arguello et al., 2012), but even then the final result seems to be higher than in previous Danish studies. A temporary slight increase of prevalence in the period 2007–2008 was reported by the carcass surveillance (Alban et al., 2012). It could be an explanation for this prevalence detected at the EFSA survey together with factors related to study design and variable abattoir conditions (Alban et al., 2012).

The apparent prevalence detected in breeding farms, including multiplication and production farms, was 40.9%, which is more than doubled (almost tripled) compared to the 14.0% prevalence found in the 1998 study (Christensen et al., 2002) when breeding farms were evaluated for the first time. Between both studies there were minor changes in size of the farms selected (farms with less than 100 sows were discarded in the 1998 study), amount of faeces collected for each faecal pool and the pigs targeted for the sampling (gilts or any stage of production), or the enrichment media used for the isolation. Although surely all these factors produce biases, it seems that they do not hinder the conclusions extracted and the increase in prevalence cannot be only attributed to these factors. This result supports the idea that restrictions imposed in high *Salmonella* index herds (measure by blood samples) taken at the beginning of the Danish programme turned out to be insufficient to limit the *Salmonella* spreading among herds (Alban et al., 2012). Together with *Salmonella* dissemination, the fact that the three main serotypes detected in breeding herds, *S.* Typhimurium, *S. Derby* and *S. Infantis*, can persist in infected herds up to several years (Baptista et al., 2009) could contribute to such dramatic increase of the number of infected breeding farms, which is of serious concern as infected animals from the breeding farms can be transferred and bring the infection with them to other farms.

Table 5
Study design differences among the pre-implementation study (1993/1994), the next cross-sectional survey (1998/1999) and the EFSA baseline studies.

	1993/1994 Study	1998/1999 Study Christensen et al. (2002)		2007–2009 EFSA baseline studies	
		Slaughter herds	Breeding herds	Slaughter pigs	*Breeding herds*
Sampling size	1363 herds 13,468 pigs	1,962 herds (>500pigs/herd) 19,614 pigs	305 sow herds (>100 sows herd) 366 genetic herds (gilts 3–9 months)	1,218 pigs	166 sow herds 98 genetic herds 38 not identified
Target population	Market-weight pigs 10 pigs/herd	Market-weight pigs 10 pigs/herd		Market-weight pigs. Sample size subjected to slaughterhouse	Pooled sample from 10 individuals (>6 months) and representing different stages of the production
Kind of sample collected and weight	Caecal content 5 gr.	Caecal content 25 gr.	Faeces 25 gr (5 × 5gr.)	Ileocaecal lymph nodes 25 gr	Faeces 25 gr (10 × 2.5gr.)
Isolation Methodology (enrichment media)	MSRV	RVB		MSRV	

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Unfortunately, the results do not allow us to conclude on the development of the prevalence of *Salmonella* in Danish slaughter pig production since 1999. However, due to the large increase in prevalence in breeding herds it is most likely that it finally affects finishing herds population, rising their prevalence. Finally, such as in the EFSA report (EFSA, 2011) there were no differences in prevalence between breeding and production holdings. Although the median of positive samples recovered in an infected farm was 2 out of 10, just one positive sample was the most frequent result. Similar values in the within-herd prevalence were found in 1998, with only one positive result in each positive farm as the most common result found in 40–45% of the sows and genetic herds (Christensen et al., 2002) This result was also the most common in the other participants of the EFSA baseline study, therefore it could be concluded that despite *Salmonella* can be found in a high number of farms, it seems that only a few sows shed the pathogen. In future prevalence studies, the expected low within-herd prevalence must also be considered to calculate the number of samples required and avoid prevalence underestimations (Arnold and Cook, 2009).

S. Typhimurium remains the most prevalent serotype among finishers as previously reported in the 1993/1994 pre-implementation study. The other two most prevalent serotypes in the EFSA survey, *S.* Infantis and *S. Derby*, were also present as some of the most important serotypes in the 1993/1994 study but the results indicate that, in particular, *S. Derby* has become more abundant in slaughter herds. *S.* Typhimurium and *S. Derby* were also the most prevalent serotypes detected in the global results in the EU. *S.* Typhimurium was found in all 25 countries that participated in the survey and *S. Derby* in 20. *S. Infantis* was the 8th most prevalent serotype and it was found in 16 countries. The total number of serotypes recovered was reduced from 30 in the pre-implementation study to only 14 in the baseline slaughter pig's survey. This may be a consequence of the DSCP and farm interventions, of an increase in the feedstuff control, or just as result of to the matrix and number of samples examined. It may also be taken into consideration that new infections may occur during transport and/or lairage (Arguello et al., 2012). This can increase the serotypes detected when sampling the caecal contents but hardly when sampling ILN. Although the number of insolates from carcasses was low, the main serotypes detected on carcasses were also *S.* Typhimurium, *S. Infantis* and *S. Derby*, demonstrating a potential link between ILN and carcass serotypes.

S. Derby was the most prevalent serotype in breeding farms likewise in the global results for the EU members. *S.* Typhimurium and *S. Infantis* were again among the three main serotypes. Although the three main serotypes were shared between finishing and breeding pigs, the fact that seven of the serotypes found in slaughter pigs could not be detected at breeding level and eleven serotypes found in the breeding farms were not found in the slaughter pigs neither, indicates a certain diversity between pig populations. Only a single serotype was found in more than 85% of the infected breeding farms. Similarly, 89.2% of the farms infected by *S.* Typhimurium were infected by only a single phage type and presumably by the same clone. So it seems that the population of *Salmonella* at farm level is well established with one strain dominant in the farm environment.

As regards the results of *S.* Typhimurium phage typing, *S.* Typhimurium DT 12, which was the main type in pigs in Denmark in the nineties (Baggesen and Wegener, 1994; Baggesen et al., 1996) was also the most prevalent among the *S.* Typhimurium isolates from ILN. It seems that even though more than one decade has passed since the pre-implementation study, it is still the predominant phage type detected in Danish finishing farms. It is worth noting that there also exists a marked increase of the percentage of strains belonging to DT 104, DT 120 and DT 170; and a slight increase of the DT 193 strains. The last two were in the nineties linked to poultry and were not common in humans (Baggesen and Wegener, 1994; Wegener et al., 1994). At present, these phage types are common in pork and are also related to human outbreaks. DT 104 is especially relevant due to its antimicrobial penta-resistance and hence part of the 1998 cross-sectional survey was focused on evaluating the prevalence of this phage type. At this survey, only one finishing herd (0.05%) was infected by this phage type (Christensen et al., 2002). In the EFSA baseline studies four *S.* Typhimurium (7.4%) were DT 104 in slaughter pigs isolates, while one of the sow herds evaluated was also infected with this phage type (no breeding herd presented this phage type in 1998). From ultimo 1996 to medio 2000 an eradication policy was followed with respect to DT 104, nevertheless, herds re-infection and the expenses spent, caused the Danish surveillance to change to a reduction strategy (Møgelmose, 2001). Its relative significance has decreased; further research has demonstrated that *S.* Typhimurium DT104 did not cause any more serious disease than a sensitive type of *S.* Typhimurium (Anonymous, 2003). In contrast to the phage types with increasing prevalence there was a decrease of isolates belonging to the phage type DT 66. This is in accordance with a recent investigation which described a shift of phage types in Danish pigs in the direction of more resistant types, such as DT 120 and DT 193, whereas the proportion of susceptible types, such as DT 12 and DT 66 was decreasing (Emborg et al., 2007). Similar to the serotyping results, the most prevalent phage types were shared by slaughter and breeding pigs. The role of the sow to piglet *Salmonella* transmission is unclear or at least it could be asserted that the pig *Salmonella* status at the end of the production chain is also affected by factors other than the breeding herd status. Further research is needed to elucidate the impact of the establishment of control measures at breeding herd level in order to reduce the prevalence at finishing farms.

Antimicrobial resistance was common among *Salmonella enterica* isolates, particularly in those *S.* Typhimurium tested. The low number of resistant isolates among other serotypes precludes the detection of any statistical difference. One out of each three isolates from slaughter pigs was resistant to one or more compounds and 54.8% of these resistant isolates showed a MDR pattern. Most of these resistant and MDR isolates were *S.* Typhimurium or *S.* 4,5,12:i:- whereas only 17.6% of the non-Typhimurium isolates carried any antimicrobial resistance and only three of them had a MDR pattern. Although antimicrobial resistance is a matter of serious concern, the results described here are lower than the levels described in the antimicrobial resistance report prepared from the baseline surveys (EFSA, 2007), indicating that the presence of antimicrobial resistance in slaughter pigs is lower in Denmark compared to the median European levels. Danish policy is thoroughly restrictive with respect to the antimicrobial usage in animal production. The lower antimicrobial resistance levels detected in Danish isolates could be the result of this restrictive policy and limited use of antimicrobials in Denmark (Bager et al., 1997; Jensen et al., 2006; Emborg et al., 2007; Vieira et al., 2009). Among the breeding farm isolates only *S.* Typhimurium isolates and *S.* 4,[5],12:i:- isolates were tested and here the levels of resistance were similar among the slaughter pigs, 48.8% and 47.3%, respectively. A 56.8% of the farms, infected by *S.* Typhimurium or its monophasic variant, had a strain with antimicrobial resistance and 27% a MDR *S.* Typhimurium strain showing that in breeding herds there are strains that carry one or several antimicrobial resistances and that they can be a starting point in the transmission of resistant strains to the food chain. MDR isolates were more common in breeding than in production herds, although the difference was not statistically significant, this result should be revised in future studies to know the antimicrobial resistance evolution at both populations. The tetra-resistance pattern AMP-STR-SPE-TET was the

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most common found in both groups with or without other resistances. The penta-resistance pattern AMP-CHL-STR-SMX-TET was associated with DT 104 as previously described (Skov et al., 2008). No isolates showed resistance to cephalosporins and only two isolates were resistant to quinolones. This is comparable to previous findings in Denmark (Wiuff et al., 2000). These two groups of antimicrobials are of critical concern for treatment of humans. All isolates in both groups were susceptible to colistin and highest frequency of resistance was found against ampicillin, streptomycin, sulfamethoxazole and tetracycline.

The breeding farms were classified in four regions in order to establish the geographical *Salmonella* distribution in the Danish breeding farms. *S. Derby* was the predominant serotype in isolates and herds from Northern Jutland, Funen and Zealand-Eastern islands regions but not in the Southern Jutland where *S.* Typhimurium was the predominant serotype. A high prevalence of *S. Derby* in finishers from Northern Jutland was also described in the pre-implementation study (Baggesen et al., 1996). By the results given in both EFSA surveys, finishing pigs and breeding herds, it seems that now *S. Derby* is distributed more or less uniformly in all countries in both pig populations. Indirect spreading by vehicles such as feed and transport systems or by animal movement are the major responsible implied in its spreading (Sandvang et al., 2000). However, differences between regions were not statistically significant so we cannot draw definitive conclusions about the distribution of the most predominant serotypes. In a similar way there were differences in the number of serotypes found between regions. This might be ascribed to a broader diversity of *Salmonella* in Northern Jutland compared to the rest of the country or simply those farms and samples from this region were numerically dominant and therefore, the higher the number of samples, the wider the spectrum of serotypes expected.

5. Conclusions

The present investigation has analyzed more deeply the results extracted from the EU baseline studies in finishers and breeding pigs carried out in Denmark. Slaughter pigs data gave us new information about Danish prevalence in ileocecal lymph nodes and carcasses before chilling process. Breeding herd results showed an increase of the prevalence values compared to those obtained in previous investigations, with as many as 40.9% of the herds infected. *S.* Typhimurium was still the main serotype found in finishers while *S. Derby* was the main serotype in breeding herds and had also increased its prevalence in finishers. DT 12 and DT 120 were the main phage types found in finishers and breeding herds respectively. Resistant and multi-drug resistant patterns were more common in *S.* Typhimurium isolates compared to other serotypes but a decrease in resistance levels was found compared to previous investigations.

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