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Molecular typing of *Streptococcus suis* isolates from Iberian pigs: A comparison with isolates from common intensively-reared commercial pig breeds

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ABSTRACT

The Iberian pig (IP) is a traditional Spanish breed variety of the domestic pig (*Sus scrofa domesticus*) with high economic importance because of the value of the dry-cured products in national and international markets. The genetic characteristics of tonsillar and clinical *Streptococcus suis* isolates from the IP maintained under extensive or intensive management conditions were investigated. *S. suis* isolates from IP pigs were compared with *S. suis* isolates from intensively-farmed pigs of common breeds (CBP). *S. suis* was isolated from 48.4% of the IP tonsils examined, indicating wide distribution among IP pigs.

Serotypes 1 (9.4%), 2 (8.6%) and 9 (7%) were the most commonly found, although a high percentage of *S. suis* isolates were not typeable by coagglutination testing. No significant differences in carrier rates or serotype diversity were observed between management systems, indicating that intensive farming does not influence *S. suis* colonisation. Both pulsed-field gel electrophoresis and multiple-locus variable number tandem repeat analysis showed a serotype-based distribution of *S. suis* IP isolates. Serotypes 1 and 2 *S. suis* isolates were grouped in the same cluster, whereas isolates of serotypes 9 and 7 were assigned to another cluster. All clinical and most tonsillar serotype 2 IP isolates were assigned to sequence type 1 (ST1) and exhibited the virulence genotype *mrp+/epf+/sly+*, indicating a high distribution of this genetic lineage among IP as well as a population of serotype 2 common to IPs and CBPs. The only clinical isolate of serotype 9 from IP was assigned to ST123, a sequence type associated with clinical isolates in CBPs in Spain.

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Introduction

Streptococcus suis is one of the most important pathogens in the swine industry worldwide, causing meningitis and a wide range of diseases such as arthritis, endocarditis, pneumonia and septicaemia (Gottschalk, 2012). *S. suis* has also been recognised as an emerging human pathogen over the past few years, affecting people in close contact with pigs or pork-derived products (Gottschalk et al., 2007).

Many studies have concentrated on intensively-farmed pigs of various common breeds (common breed pigs, CBPs) (Wisselink et al., 2000; Vela et al., 2003; Rehm et al., 2007; Blume et al., 2009; Gottschalk et al., 2013). However, none of these studies focussed on pigs reared outdoors. The Iberian pig (IP) is a traditional Spanish breed variety of the domestic pig (*Sus scrofa domesticus*), and its production is highly adapted to the Mediterranean ecosystem. In traditional management systems, IPs are reared outdoor in sparse oak forests (‘*dehesa*’) where they feed exclusively on acorns and grass and share natural resources with other wild and domestic animals. This particular production system is referred to as ‘*montanera*’. IPs are highly important economically because of the growing demand for their high quality meat products, especially dry-cured products (Cava et al., 1997). In response to this high demand, the IP is now also being produced indoors. This production system is termed ‘*cebo*’ and the pigs are primarily fed with commercial feed.

Different farming practices and interaction with other animals can alter the epidemiology of pathogenic microorganisms (Mennerat et al., 2010). In view of the differences in farming practices between

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the IP and CBP breeds we decided to investigate the population of *S. suis* carried by the IP. Specifically, the aim of this study was to determine the *S. suis* carriage rates and serotypes in the IP, as well as the virulence factor profiles and genetic diversity of IP isolates and compare them with those from the CBP.

Materials and methods

S. suis *isolates*

A total of 128 *S. suis* isolates from slaughterhouse IP pigs were analysed in this study. Isolates were recovered from the tonsils of 182 finishing IPs collected at different slaughterhouses from 11 provinces in Spain and one province in Portugal in 2010 and 2011. Isolates were classified according to the production system used in their host as either '*montanera*' (MIP; $n = 111$) or '*cebo*' (CIP; $n = 71$). Another 15 *S. suis* clinical isolates were obtained from the same number of diseased CIP with pneumonia ($n = 4$), meningitis ($n = 5$), arthritis ($n = 2$) and septicaemia ($n = 4$).

Tonsils from swine carcasses and clinical samples were removed aseptically and submitted to our laboratory in sterile containers under refrigeration for processing within 24 h of sampling. Samples were cultured on Columbia-CNA agar and incubated at 37 °C for 24–48 h under aerobic conditions. From each tonsillar sample, a maximum of six colonies presumptively considered as *S. suis* by colony morphology (small slightly mucoid grey–white colonies with α-haemolysis) were subcultured on blood agar for further identification. A representative colony from each clinical sample was also identified.

Colonies of catalase negative, Gram positive cocci exhibiting α-haemolysis were identified as *S. suis* by the Rapid ID 32 STREP system (bioMérieux) according to the manufacturer's instructions. Biochemical identification was further confirmed based on the sequence of the gene encoding glutamate dehydrogenase (*gdh*) by polymerase chain reaction (PCR) (Okwumabua et al., 2003).

For comparison purposes, 50 *S. suis* isolates from CBPs available in the Centro de Vigilancia Sanitaria Veterinaria culture collection were included in the study. These isolates, from pigs on farms located in different geographical areas of Spain, included 41 clinical isolates of serotype 2 ($n = 15$) and serotype 9 ($n = 26$) and nine tonsillar isolates of serotype 2.

Identification of capsular types and serotyping

A multiplex PCR was used to identify capsular types 1 and 14, 1/2 and 2, 7 and 9 as described by Silva et al. (2006). All isolates that were negative for these capsular types were serotyped using a coagglutination test (Gottschalk et al., 1993). Non-typeable isolates were further examined for the presence of capsules using cell surface hydrophobicity (Rosenberg, 1980). Isolates were classified into those with low hydrophobicity (<30%, isolates classified as capsulated) and high hydrophobicity (>70%, isolates most probably not capsulated) (Gottschalk et al., 2013).

Virulence factor profiling

The presence of genes encoding for the putative virulence associated factors muramidase released protein (*mrp*), extracellular protein factor (*epf*) and suilysin (*sly*) was determined for all strains belonging to serotypes 1, 2, 7 and 9 by a multiplex-PCR as described previously (Silva et al., 2006). Differentiation of *mrp* and *epf* variants was carried out by monoplex PCR assays (Silva et al., 2006).

Molecular typing

Only isolates of prevalent European serotypes 1, 2, 7 or 9 were further characterised by multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and multiple-locus variable number tandem repeat analysis (MLVA).

Multilocus sequence typing

MLST analysis was performed on seven housekeeping genes *aroA* (5-enolpyruvylshikimate3-phosphate synthase), *cpn60* (60-KDchappcronin), *dpr* (a putative peroxide resistance protein), *gki* (glucose kinase), *mutS* (a DNA mismatch repair enzyme), *recA* (homologous recombination factor) and *thrA* (aspartokinase/homoserine dehydrogenase) as described by King et al. (2002). MLST alleles and sequence types (STs) were assigned through the submission of the respective data to the *S. suis* MLST database.[1]

Pulsed-field gel electrophoresis

Molecular typing was performed by PFGE as previously described (Vela et al., 2003). The restriction enzyme *Bsp*120I was used for digestion of genomic DNA. Restriction patterns were analysed and the dendrogram was constructed based on unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis using BioNumerics software 4.0 (Applied Maths).

Multiple-locus variable number tandem repeat analysis

Investigation of variable number tandem repeat loci was performed as described previously (Li et al., 2010) using single PCR, with the exception of tandem repeat 9, which was excessively polymorphic and therefore was replaced by a new locus (SSTR1862_46pb_361_3.9U) designed in the present study. Search for this new locus was based on the analysis of the genome sequences of *S. suis* 98HAH33 and 05ZYH33 using the tandem repeats database (Denoeud and Vergnaud, 2004) with the following parameters: total length (between 100 and 100,000), unit length (between 20 and 5000) and percent matches (>70%).[2] Only repeat units at least 10 bp long were evaluated.

The new locus was designated using the nomenclature described previously (Sawires and Songer, 2005): SSTR locus position, size of repeat unit, PCR product length repeat number. The primer sequences were SSTR18-F (5′-ATCACACGGTTAGCCAAAGG-3′) and SSTR18-R (5′-GCAGGTGTAGCCGTTATGGT-3′). The PCR amplification conditions for this locus were: an initial denaturing at 95 °C for 10 min followed by 35 cycles of amplification: 95 °C for 1 min, annealing temperature at 65 °C for 1 min, and extension at 72 °C for 1 min; with a final extension at 72 °C for 10 min. The amplified products were resolved using electrophoresis on horizontal 3% agarose gel (Bio-Rad) at a voltage of 90 V/cm for approximately 3 h using TAE 1 X (Bio-Rad). A 100 bp DNA Ladder (Biotools) was included in all gels to determine the size of the amplified DNA fragments.

The genotyping data obtained were used to construct a phylogenetic tree based upon the UPGMA method.[3] The discriminatory power of the technique was calculated using the Simpson's index (Hunter, 1990). This index was also used to estimate the discriminatory power of the other two methods, PFGE and MLST.

Statistical analysis

The chi-square test was performed to analyse the differences between production systems and virulence genotypes, sequence types, PFGE patterns and MLVA profiles, using the WinPepi program version 11.25.[4] Differences were considered significant at $P < 0.05$.

Results

One hundred twenty-eight isolates of *S. suis* were recovered from 88 tonsils of the 182 IPs examined (carrier rate of 48.4%). Carrier rate differences between MIP (51.4%) and CIP (45.1%) were not significant ($P > 0.05$). The distribution of the serotypes in tonsils from IPs according to the production system is shown in Table 1. Serotype 1 (12 isolates, 9.4%) and serotype 2 (11 isolates, 8.6%) were the most common, followed by serotype 9 (9 isolates, 7%). Only one isolate of serotype 7 (1.2%) was found. Other serotypes identified included serotypes 3, 4, and 31 (Table 1). Eighteen isolates (14.1%) reacted with more than one antiserum and 37 (28.9%) were non-typeable isolates.

Most clinical isolates recovered from diseased CIPs belonged to serotype 2 ($n = 13$, 86.7%) and only one isolate of serotype 7 and another of serotype 9 were identified. All 33 *S. suis* tonsillar IP isolates of serotypes 1, 2, 7 and 9 (all Spanish isolates) were further characterised by PFGE, MLST, MVLA analysis and virulence factor profiling. For comparison, 15 and 41 clinical isolates from IPs and CBPs, respectively, and nine tonsillar isolates from CBPs were also included in molecular typing analysis.

On PFGE analysis, a total of 38 different pulsotypes were identified among the 98 *S. suis* isolates that could be assigned to two main clusters (Fig. 1), corresponding to the serotype distribution. Cluster A (PFGE pulsotypes 1–25) included all *S. suis* serotype 2 isolates from IP and CBP, and also included all *S. suis* serotype 1 isolates from IPs. Cluster B (PFGE 26–37) included all *S. suis* serotype 9 clinical isolates from CBPs and the only clinical strain of this serotype

[1] See: http://ssuis.mlst.net (Accessed 11 October 2014).

[2] See: http://minisatellites.u-psud.fr (Accessed 11 October 2014).

[3] See: http://pubmlst.org/software/ (Accessed 11 October 2014).

[4] See: http://www.brixtonhealth.com/pepi4windows.html.

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