Streptococcus suis is a bacterium commonly carried by pigs in the respiratory tract; thus the infections caused by virulent strains are considered a problem in the swine industry. A successful approach for the identification of virulent strains is the differentiation of capsular serotypes using specific antisera or the corresponding cps types by genotypic assessment, with the subsequent detection of virulence associated factors, namely the extracellular factor, the muramidase-released protein and the hemolysin suilysin. Data regarding serological and molecular identification of S. suis from pigs in Cuba are not available. This study was aimed at identifying the capsular types cps2, 7, and 9, as well as three genes related to virulence using PCR assays. According to the results, 31 isolates were evaluated and classified as cps2 (n = 21) or cps9 (n = 4), while six isolates were not typable. Considering the presence in these isolates of the genes mrp, epf and sly, six different genotypes were differentiated among the cps2 or cps9 strains and there were three non-typable isolates for the genes used in this study. The cps2 isolates were recovered from pigs between 6-12 and 14-17 weeks with pneumonia and systemic infection respectively, whereas the cps9 isolates were exclusively associated with pneumonia.

Keywords: Streptococcus suis, virulence markers, genotypes

RESUMEN

Tipificación molecular de Streptococcus suis aislado de cerdos en Cuba. Streptococcus suis es una bacteria común en el tracto respiratorio de los cerdos; sin embargo las infecciones causadas por cepas virulentas se consideran un problema en la industria porcina. Estas cepas se pueden identificar por la diferenciación de los serotipos capsulares con antiseros específicos o por sus correspondientes tipos cps, mediante ensayos genotípicos. También se pueden reconocer mediante la detección de factores asociados con la virulencia, como el factor extracelular, la proteína liberada por muramidasa y la hemolisina suilysina. Antes de esta investigación no había datos sobre la identificación serológica o molecular de los tipos capsulares de S. suis procedentes de cerdos en Cuba. El objetivo de esta investigación fue la detección de los tipos capsulares cps2, cps7 y cps9, y de los tres genes asociados con la virulencia, mediante ensayos de reacción en cadena de la polimerasa. De 31 aislamientos, 21 se clasificaron con el genotipo capsular cps2, 4 con el cps9. El genotipo capsular cps7 no se detectó y 6 aislados no se correspondieron con ninguno de los serotipos analizados. Considerando la presencia de los genes sly, epf y mrp, se identificaron seis genotipos en los aislados cps2 y cps9, y 3 en los aislados cuyo genotipo cps no se identificó. Los aislados cps2 se extrajeron de cerdos de 6 a 12 con neumonía y de 14 a 17 semanas con infección sistémica; mientras que los cps9 estaban asociados exclusivamente con neumonía.

Palabras clave: Streptococcus suis, marcadores de virulencia, genotipos
in a considerable number of farms (30 % of the swine population) [18]. However, data regarding S. suis isolates typing from pigs in Cuba are not available. In this work, the molecular identification of the capsule polysaccharide genes (cps) and the genotypic assessment of virulence markers in Cuban S. suis isolates were carried out for the first time.

**Materials and methods**

**Samples and classification**

A total of 31 isolates of S. suis from the collection of the Bacteriology Department of the National Center of Animal and Plant Health (Censa, Cuba) were included in this study. Most of these isolates were collected from diseased or dead animals during diagnostic procedures from farrow-to-finish farms in the Western region of Cuba from 2004 to 2011.

According to their source of isolation and clinical symptoms, the isolates were distributed in three groups as follows: i) Invasive group (n = 3), three isolates from absciss of pigs 10-17 weeks of age with arthritis and one isolate from aborted foetus; ii) pneumonia group (n = 21), isolates from the lung of animals between 10-12 weeks of age; and iii) carrier group (n = 6), comprising two isolates of tonsillar biopsies from healthy animals, one isolate from the vaginal secretion and three isolates of semen ejaculates from mature boars of a pig breeding farm.

**PCR**

The isolates were identified by conventional biochemical procedures following the methods reported by Berthelot-Herault et al. [19]. The isolates were cultured in Todd Hewitt medium (5 mL) at 37 °C for 24 h and the DNA was extracted as described previously [20]. The S. suis-like strains were identified by amplification of a 294-bp-long 16S rDNA gene fragment by PCR using S. suis species-specific primers [13]. The capsular genotype (cps2) was determined by PCR as previously described by Smith et al. [12]. Capsular genotypes cps7 and cps9, and the mrp (188 bp) gene were analyzed by monoplex PCR assays using the sequence of primers and conditions described previously by Silva et al. [14]. Other two conventional PCR assays were used to screen the presence of sly (1492 bp) [21] and epf (626 bp) genes [17]. Details of all oligonucleotide primers used and PCR conditions applied for the detection are listed in table 1. Each isolate was tested under the same conditions twice.

The reaction was carried out in a 25-μL PCR mixture containing 20 pmol of each primer, 200 μM of dNTPs, 1× PCR buffer, 3 mM MgCl2, and 1× Taq DNA polymerase (Invitrogen). The amplified DNA was visualized in 2 % agarose gels in TBE buffer (90 mM Tris, 90 mM borate, 2.5 mM EDTA; pH 8) for 1 h at a constant voltage of 125 V. Amplified products were stained with ethidium bromide (0.5 μg/mL) and detected by UV transillumination. The 50 bp ladder (Promega) was used as standard.

**DNA sequencing**

Partial sequences corresponding to cps2 and 16S rDNA genes were amplified by using chromosomal DNA obtained from sample 13 (designated as isolate SS13) collected in October 2010 from the lung of a pig with pneumonia. The sequences of both genes were determined by an automated sequence analyzer (CEQ® 8800, Beckman Coulter, USA) using the same primers as for PCR amplification and Genome LabTM DTCS- Quick start kit. Prior to the sequencing reaction, PCR products were purified with the Wizard PCR purification kit (Promega) according to the manufacturer's instructions.

**Sequence analysis**

Sequences of both fragments corresponding to cps2 and 16S rDNA genes were aligned and assembled using the Vector NTI® software (Invitrogen, USA) to obtain two sequences of 260 and 637 bp, which were deposited in GenBank under accession numbers SS13) collected in October 2010 from the lung of a pig with pneumonia. The sequences of both genes were determined by an automated sequence analyzer (CEQ® 8800, Beckman Coulter, USA) using the same primers as for PCR amplification and Genome LabTM DTCS- Quick start kit. Prior to the sequencing reaction, PCR products were purified with the Wizard PCR purification kit (Promega) according to the manufacturer's instructions.


**Table 1. Oligonucleotide primer sequences and PCR conditions applied for the detection of virulence associated genes in S. suis strains used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>PCR product (bp)</th>
<th>PCR conditions (°C)</th>
<th>Annealing Elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rDNA</td>
<td>cagttattacgcagtggtagatc aagtcgacatgagaaaaagttcgcac</td>
<td>294</td>
<td>94/30 60/30 72/60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gtaagatcgaccgtaagagagga</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aaaccgccgagccagatggtccgcaggtcgatctga</td>
<td>652</td>
<td>59/60 56/120 72/120</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aatgccctcgtggaatacag</td>
<td>379</td>
<td>94/60 56/60 72/120</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ggcacatataatggaagccc</td>
<td>303</td>
<td>59/60 56/120 72/120</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gatgccacgctgccgaagcc</td>
<td>188</td>
<td>94/60 56/60 72/120</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gttgatccctcggtaagaggt</td>
<td>626</td>
<td>94/60 56/60 72/60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aagtcgacagtggaaagtgcacc aactgcaggattactctatcacctca</td>
<td>1492</td>
<td>94/60 56/60 72/60</td>
<td></td>
</tr>
</tbody>
</table>

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Table 2. Distribution of S. suis genotypes among different sources of isolation (n = 31)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Genotype</th>
<th>Vaginal discharge</th>
<th>Lung</th>
<th>Joint</th>
<th>Aborted fetus</th>
<th>Semen</th>
<th>Tonsil</th>
</tr>
</thead>
<tbody>
<tr>
<td>cps2</td>
<td>cps2+mrp+epf+sly+</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cps2+mrp+epf+sly-</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cps2+mrp+epf+sly-</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cps2+mrp+epf+sly-</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cps9</td>
<td>1</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Non-type</td>
<td>mrp+epf+sly+</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cps2+ cps7+ cps9)</td>
<td>mrp+epf+sly-</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mrp+epf+sly-</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple alignment of the nucleotide sequences of the cps2 fragment (JF266697) revealed that the sequence corresponded to strains of S. suis (Figure 2), only differing in one nucleotide change at position 836 (A→T), this mutation resulting in a possible amino acid variation from Lys279 by Ile279 at the C-terminus. The 1492 bp PCR product corresponding to the suilysin gene was identified in three cps2 isolates from animals with pneumonia and only in one non-typable isolate from tonsils of an asymptomatic animal (Table 2). Suilysin is a pore-forming cholesterol-dependent cytotoxin. Though suilysin is not essential for virulence of S. suis cps2 isolates in pigs, in vitro experiments suggest that it may have important functions for the interactions with host cells [5]. Tarradas et al. [33] detected suilysin production by S. suis isolated from diseased and healthy carrier pigs in Spain.

The 626 bp fragment of the gene encoding EF was detected only in two cps2 isolates. The EF was identified as an extracellular protein associated with virulence in serotypes 1, 2, 1/2, 14 and 15 [17]. The function of EF is still unknown, since isogenic epf mutants were as virulent as the wild-type in experimental infections, indicating that EF is associated but not essential for virulence [34].

The gene fragment encoding the MRP protein was amplified in cps2 isolates from cases of pneumonia and asymptomatic animals; this fragment was detected in the four cps9 isolates. The function of the 136 kDa MRP protein is unknown. It was discovered as a factor released from virulent cps2 strains after muramidase treatment but expression of the MRP was found among strains of serotypes 1, 2, 1/2, 14 and 15 [17]. Different studies have shown that the presence of the gene did not always correlate with actual expression of the respective protein. Fittipaldi et al. [35] detected the mrp gene in 92 isolates of S. suis but the expression of MRP by Western blotting was only positive in 46 isolates, due, in most cases, to frame-shift mutations in the 5’ end of the gene, resulting in premature stop codons. The effective production of the virulence markers by the tested isolates specifically in the genotype cps2’ mrp’ epf’ sly’ would also be verified.

As mrp seems to be a silent gene in some strains, it might also be discussed that phenotyping rather than genotyping should be performed in diagnostic laboratories [4]. Although not ideal, typing methods based on protein expression of these markers confirm the

...
phenotype because the PCR assays only detect a partial sequence of a gene. However, protein expression levels may be affected by many factors, as growth conditions, or even induced at specific stages of the infection process as it is known for many important, highly regulated virulence factors [36, 37]. The most promising candidate for improvement of S. suis diagnostics is the use of probes as the first study on the molecular identification of Streptococcus suis from pigs in Cuba. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

Figure 1. Phylogenetic tree of S. suis strains (GenBank accession number JF266696), fourteen known serotypes of S. suis strains and tenth other streptococcal species with the genomic DNA fragment of the 16S rRNA. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.19159970 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA5.

Conclusions

So far, this is the first study on the molecular identification of S. suis isolates in Cuba from disease cases in pigs. S. suis isolates belonging to the capsular serotypes cps2 and cps9 were detected by genotyping that the virulence of the Cuban S. suis strains associated with invasive processes is similar to that reported for North American strains. Further studies with a larger number of Cuban isolates are required to definitively confirm this hypothesis.

Serotype determination remains a valuable tool used by veterinary practitioners and diagnosticians to understand the epidemiology of a particular outbreak and/or to increase the possibility for success of a vaccination program for a given herd [35]. The PCR assays used in this study can differentiate the three cps genotypes (2, 7 and 9) and additionally detect the important virulence associated factors genes mrp, epf and sly (Table 2). Taking into account methods for serotyping, the PCR assays are rapid, reliable, and sensitive. Moreover, these PCR assays could be used directly with tonsillar specimens from infected or carrier animals so that isolation of single colonies could be omitted. Therefore, these tests will undoubtedly contribute to a more rapid and reliable diagnosis of S. suis and may facilitate control and eradication programs [12].

Figure 2. Alignment of the nucleotide sequences of the cps2 fragment genes from S. suis. Sequence of the Cuban isolate (GenBank accession number JF266697) was taken as consensus. Identical bases are indicated by points and gaps inserted into the sequences are indicated by horizontal dots.
of the cps2 and cps9 genes, respectively. This is also the first report indicating the occurrence of at least two capsular types and nine different genotypes among the *S. suis* isolates tested. A larger scale sampling among pig farms from different Cuban regions could enable us to design a strategy to minimize the risk of exposure to *S. suis*.

**Acknowledgments**

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