Development of a multiplex PCR assay to detect the major clonal complexes of *Streptococcus suis* relevant to human infection

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Multilocus sequence typing (MLST) is considered a reliable method for providing insight into the *Streptococcus suis* population structure, clonal relationships and the potential of particular clones to cause disease. Indeed, MLST has revealed the presence of several clonal complexes (CCs) within the *Streptococcus suis* population. However, the method is costly, time-consuming and difficult to use for screening large numbers of isolates. In this study, a multiplex PCR assay was developed to identify *Streptococcus suis* CCs that are relevant to human infections. The multiplex PCR assay was capable of simultaneously distinguishing CC1, CC25, CC28, CC104, CC221/234 and CC233/379, which are related to human infections in Thailand, in a single reaction. The multiplex PCR assay is useful for low-cost screening of large numbers of isolates with rapid analytical capacity and could be utilized in most laboratories.

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INTRODUCTION

*Streptococcus suis* is a zoonotic pathogen that causes invasive infections in humans who have been in close contact with infected pigs or contaminated pork-derived products, or who have consumed raw pork products; thus, *Streptococcus suis* infections have attracted increasing attention worldwide (Goyette-Desjardins et al., 2014). Multilocus sequence typing (MLST) can provide important insights into population structures. When applied to *Streptococcus suis*, this method revealed the presence of several clonal complexes (CCs) within the *Streptococcus suis* population. However, the method is costly, time-consuming and difficult to use for screening large numbers of isolates. In this study, a multiplex PCR assay was developed to identify *Streptococcus suis* CCs that are relevant to human infections. The multiplex PCR assay was capable of simultaneously distinguishing CC1, CC25, CC28, CC104, CC221/234 and CC233/379, which are related to human infections in Thailand, in a single reaction. The multiplex PCR assay is useful for low-cost screening of large numbers of isolates with rapid analytical capacity and could be utilized in most laboratories.

Abbreviations: CC, clonal complex; MLST, multilocus sequence typing; ST sequence type. The GenBank/EMBL/DDBJ accession numbers for the sequences derived in this paper are KT211632-KT211638.
CC28, CC221/234 and CC233/379, which are also related to human infections (Goyette-Desjardins et al., 2014; Kerdsin et al., 2011a; http://ssuis.mlst.net/).

METHODS

Bacterial strains. A total of 647 Streptococcus suis strains with known serotypes, sequence types (STs) and clonal complexes isolated from humans were included in this study (Table 1). Streptococcus suis serotype 2-ST104, strain DMST22713 isolated from a human with sepsis, and Streptococcus suis serotype 2-ST1, strain P1/7 isolated from pig meningitis, were used for a genomic subtraction experiment to identify the specific CC104 sequence.

Identification of specific sequence in Streptococcus suis CC104. Bacterial genome subtraction was performed using the PCR-Select Bacterial Genome Subtraction kit (Clontech Takara), according to the manufacturer’s instructions. Genomic DNA from representative CC1 serotypes, sequence types (STs) and clonal complexes isolated from CC28, CC221/234 and CC233/379, which are also related to human infections (Goyette-Desjardins et al., 2014; Kerdsin et al., 2011a; http://ssuis.mlst.net/).

DNA sequencing of the positive clones was performed by First BASE, Malaysia. DNA sequence similarity analyses were completed using the National Center for Biotechnology Information BLAST network service. According to the BLAST search results, seven assembled sequences (derived from 62 clones) shared very low similarity with known Streptococcus suis sequences deposited in GenBank. These sequences were assigned accession numbers, as shown in Table 2.

Distribution of CC104-specific sequences. Seven specific sequences were analysed by singleplex PCR for their distribution among different Streptococcus suis sequence types. The PCR primers used to amplify these seven sequences were designed using the Primer-BLAST program (accessed on 11 November 2014; http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 2). The PCR mixtures were assigned accession numbers, as shown in Table 2.

Table 1. Distribution of seven specific sequences of CC104 among Streptococcus suis strains isolated from humans and included in this study

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<th>Serotype</th>
<th>CC</th>
<th>ST</th>
<th>pep</th>
<th>ribG</th>
<th>col</th>
<th>hp1</th>
<th>hp2</th>
<th>mp</th>
<th>zot</th>
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RESULTS AND DISCUSSION

Identification of *Streptococcus suis* CC104-specific sequences

Initially, we used bacterial genome subtraction to identify specific sequences using CC104 (representative strain, ST104) and CC1 (representative strain, ST1), as described in Methods. Using this approach, we identified 62 CC104-specific cloned fragments, the analysis of which revealed seven significant assembled sequences having low similarity with known *Streptococcus suis* sequences deposited in the GenBank database. These seven sequences encoded two hypothetical proteins (hp1 and hp2), one RNase G (ribG), one peptidase (pep), one muramidase-like protein (mp), one zonula occludens toxin (zot) and one collagen adhesion (col), as shown in Table 2.

Multiplex PCR for *Streptococcus suis* CCs

To develop a multiplex PCR assay covering the major CCs of *Streptococcus suis* relevant to human infections, including CC1, CC25, CC28, CC104, CC221/234 and CC233/379 (Feil et al., 2008; Kerdsin et al., 2011a; http://ssuis.mlst.net/), we selected hp1, mp, pep and col of the CC104-specific sequences as targets for primer design, including the srtBCD primers described by Takamatsu et al. (2009). This assay allows simultaneous differentiation of CC1, CC25, CC28, CC104, CC221/234 and CC233/379 in a single reaction.

### Table 2. Primers and target genes used in the multiplex PCRs

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5’ to 3’)</th>
<th>PCR product sizes (bp)</th>
<th>Protein and/or gene</th>
<th>Accession number/ reference</th>
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<tr>
<td>Pep-F</td>
<td>AGACCTCCGGACATACCCAC</td>
<td>1420</td>
<td>Peptidase (pep)</td>
<td>KT211632</td>
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<td>Pep-R</td>
<td>GCGGCGCAAGCGCTGTTATG</td>
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<td>Hp2-R</td>
<td>AAGCTGACGAGACGCGGGA</td>
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<td>Hypothetical protein (hp2)</td>
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<td>Hp2-F</td>
<td>GGAAGCGGCTCTGCTGCTG</td>
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<tr>
<td>RibG-F</td>
<td>CCCAGGGCAAAGCTGATGTA</td>
<td>2463</td>
<td>RNase G (ribG)</td>
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<tr>
<td>RibG-R</td>
<td>AGTACAGCCCTCTGGAGTC</td>
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<tr>
<td>Col-F</td>
<td>AGAAAAATGGGCTGCTGGAATA</td>
<td>1586</td>
<td>Collagen adhesion (col)</td>
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<tr>
<td>Col-R</td>
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<td>Hp1-F</td>
<td>TGAATTACCTCGTATTGCTGGAAGGT</td>
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<td>Hypothetical protein (hp1)</td>
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<td>Hp1-R</td>
<td>TCCTATGACTTACATAAGCAGG</td>
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<td>Mp-R</td>
<td>GTATGTCGCTAGCGTTGGTCG</td>
<td>901</td>
<td>Muramidase-like protein (mp)</td>
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<tr>
<td>Mp-F</td>
<td>TCTCCCAAACGCCACTCTGAGC</td>
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<td>Zot-R</td>
<td>AGACCTCGAAGAGTTGCGGACT</td>
<td>561</td>
<td>Zonular occludens toxin (zot)</td>
<td>KT211638</td>
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<td>Zot-F</td>
<td>GGAAGCTCGAGCTGCCGTTGAG</td>
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<tr>
<td>srtBCD-F</td>
<td>TGGAGGGTAGATGATGAC</td>
<td>2065</td>
<td>srtBCD</td>
<td>Takamatsu et al. (2009)</td>
</tr>
<tr>
<td>srtBCD-R</td>
<td>AGGAGGAACCTAGTGATGAC</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 2. Primers and target genes used in the multiplex PCRs

In a single reaction, the primer pairs for the target genes pep, ribG, col and mp were also present in CC221/234 and CC233/379. Analysis using eBURST (http://ssuis.mlst.net/eburst/) demonstrated that CC233/379 shared a double locus variant (gki and thrA) with CC104 (data not shown). Therefore, some specific sequences found in CC104 may also be present in CC221/234 and CC233/379. However, most of the CC104-specific sequences were not found in CC1, except for ribG (10 %) and zot (44 % in ST1 and 40 % in ST105; Table 1). CC28 contained the pep and col genes (100 %), while CC25 was pep-positive (100 %) and only ST25 was zot-positive (100 %) (Table 1). In addition, hp1 was present in CC221/234 as well as in CC104.

**Multiplex PCR for *Streptococcus suis* CCs**

To evaluate the limit of detection of the multiplex PCR test, *Streptococcus suis* strains P1/7 and DMST22713 were diluted using 10-fold serial dilutions, starting from an original concentration of OD$_{600}$=0.6 (equivalent to 10$^6$ c.f.u. ml$^{-1}$). Genomic DNA was prepared from 1 ml of each dilution using the NucleoSpin Tissue kit (Macherey-Nagel). Each dilution was also plated onto sheep blood agar (0.5 % peptone, 0.3 % yeast extract, 0.5 % NaCl, 1.5 % agar, 5 % sheep blood, pH 7.4) for c.f.u. quantification to determine the minimum c.f.u. required for detection with the multiplex PCR.
As shown in Fig. 1, our multiplex PCR assay accurately differentiated *Streptococcus suis* strains of each CC, in agreement with previous MLST results. Multiplex reactions with CC104 strains generated four bands of 408, 901, 1420 and 1586 bp for the *hp1*, *mp*, *pep* and *col* genes, respectively, whereas reactions with CC233/379 strains resulted in three bands of 901 bp (*mp*), 1420 bp (*pep*) and 1586 bp (*col*). Multiplex reactions with CC25 strains revealed only a single band (1420 bp) for *pep*, while CC28 strains showed two bands (1420 bp and 1586 bp), corresponding to *pep* and *col*, respectively. CC1 and CC221/234 strains showed only a single band: 2065 bp, which corresponded to *srtBCD* in CC1, and 408 bp for *hp1* in CC221/234. These results demonstrated that multiplex PCR can be used to distinguish different CCs related to human infections. The limit of detection in the multiplex assay was $10^3$ c.f.u. for both strains P1/7 and DMST22713, which are representative of CC1 and CC104, respectively (data not shown).

MLST is a powerful method for typing *Streptococcus suis* isolates, providing insight into population structures; however, development of other methods such as PCR to identify *Streptococcus suis* CCs would be useful for the rapid screening of numerous isolates at relatively low cost, and could be applied in most laboratories. To the best of our knowledge, only two reports have demonstrated the application of PCR in determining CCs in *Streptococcus suis* isolates. The first report revealed that a variant of serum opacity factor (*ofs*) can serve as a genetic marker to determine CCs of *Streptococcus suis* (Takamatsu et al., 2008b). The report showed that type-1 *ofs* was strongly associated with CC1, while type-3 and type-4 *ofs* were associated with CC27. Importantly, CC25, CC28 and CC104 were included in CC27 with the less-stringent group definition used in that study (Takamatsu et al., 2008b). However, type-3 *ofs* was present in both CC94 and CC27, making it difficult to distinguish one from another. Similarly, CC25 and CC104 could not be differentiated from CC27 (Takamatsu et al., 2008b).

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**Fig. 1.** Agarose gel electrophoresis of PCR-amplified products from the representative STs of *Streptococcus suis* strains isolated from humans. Lanes: 1, ST104; 2, ST391; 3, ST392; 4, ST393; 5, ST512; 6, ST513; 7, ST514; 8, ST233; 9, ST379; 10, ST28; 11, ST382; 12, ST25; 13, ST102; 14, ST103; 15, ST380; 16, ST381; 17, 395; 18, ST515; 19, ST516; 20, ST221; 21, ST234; 22, ST1; 23, ST126; 24, ST144; 25, ST298; 26, ST337; 27, ST394; 28, ST11; 29, ST105; 30, ST127; 31, ST237; 32, ST16; N, negative control; M, 100 bp DNA ladder.
A second study described a PCR assay to determine how pilus-associated gene profiles correlated with CCs of *Streptococcus suis* (Takamatsu et al., 2009). Results showed that such gene profiling could be used as a screening method for potentially hazardous *Streptococcus suis* groups. For example, genotype A of the pilus-associated gene profile was strongly associated with CC1, genotype B was associated with CC25 and CC27, and genotype C was related to CC104. Unfortunately, this assay does not distinguish between CC25 and CC27, and multiple PCRs are necessary to reveal the gene profiles for each genotype.

Our multiplex PCR is capable of identifying *Streptococcus suis* CCs that are relevant to human infections in a single reaction. The test developed in this study differentiates between CC1, CC25, CC28, CC104, CC221/234 and CC233/379, which are commonly associated with human infections (Goyette-Desjardins et al., 2014; Kerdsin et al., 2011a; http://ssuis.mlst.net/). Although we recently reported the emergence of *Streptococcus suis* serotype 9 ST16 (CC16) in humans (Kerdsin et al., 2015), our multiplex PCR assay could not be used to amplify this isolate (Table 1, Fig. 1). Therefore, a whole-genome sequencing or allele-specific sequencing of this isolate is required to design specific primers for CC16 amplification, which is also important, although mainly for studying disease in pigs. Moreover, the multiplex PCR should be extended to validate the human CC strains isolated from different regions such as Europe and North America as well as the CC strains isolated from pigs. In conclusion, the assay developed in this study will be useful for screening or determining major CCs relevant to human isolates of *Streptococcus suis*. To investigate the STs in each CC, MLST should be performed.

**REFERENCES**


