Streptococcus suis serotyping by a new multiplex PCR

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A multiplex PCR was developed to detect all true serotypes of Streptococcus suis. This multiplex PCR was composed of four reaction sets. The first set identified nine serotypes (serotypes 1/2, 1, 2, 3, 7, 9, 11, 14 and 16), the second set identified eight serotypes (serotypes 4, 5, 8, 12, 18, 19, 24 and 25), the third set identified seven serotypes (serotypes 6, 10, 13, 15, 17, 23 and 31), and the last set identified five serotypes (serotypes 21, 27, 28, 29 and 30). This assay correctly detected serotypes 2, 5, 14 and 24 in human isolates, and serotypes 1, 2, 1/2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 15, 16, 17, 19, 24, 28 and 31 in pig isolates from Thailand. No cross-reaction was observed with other bacterial species. Our multiplex PCR was able to simultaneously amplify a DNA mixture of reference Streptococcus suis serotypes. This assay should be useful for serotype surveillance of human and pig isolates of Streptococcus suis.

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, and this disease has received increasing attention worldwide (Gottschalk et al., 2010). Streptococcus suis serotype 2 is the most prevalent serotype in humans; however, human cases of serotypes 1, 4, 5, 14, 16 and 24 have also been reported (Gottschalk et al., 2010; Kerdsin et al., 2009, 2011a, b; Nghia et al., 2008).

Previously, Streptococcus suis had been classified into 35 serotypes (serotype 1/2, and 1–34) and then reduced to 33 serotypes because serotypes 32 and 34 were re-identified as Streptococcus orisratti (Gottschalk et al., 1989, 1991a, b; Higgins et al., 1995; Hill et al., 2005). More recently, it was proposed to remove serotypes 20, 22, 26 and 33 from the Streptococcus suis taxon (Tien et al., 2013; Okura et al., 2013). Hence, it is currently considered that there are 29 true Streptococcus suis serotypes. Identification of Streptococcus suis serotypes using the antiserum for each serotype is laborious, time-consuming and expensive. Therefore, PCR would be an attractive alternative for the identification of Streptococcus suis serotypes, due to its rapid analytical capacity and low cost.

The PCR-based identification of certain Streptococcus suis serotypes has been previously reported (Kerdsin et al., 2012; Liu et al., 2013; Marois et al., 2004; Okwumabua et al., 2003; Smith et al., 1999a, b; Wang et al., 2011; Wisselink et al., 2002). We have also previously described the use of multiplex PCR to identify 15 serotypes of Streptococcus suis (Kerdsin et al., 2012). However, the genetic sequences of all
**METHODS**

**Bacterial strains.** The current 29 serotypes of *Streptococcus suis* reference strains, including serotypes 1/2, 1–19, 21, 23–25, 27–31, and the former *Streptococcus suis* serotypes 20, 22, 26, 32, 33, 34 were used. In addition, a total of 184 human Thai isolates as well as 109 isolates from tonsils of clinically healthy pigs in Khon Kaen Province in 2008 (n = 47) and Phayao Province in 2010 (n = 62) were included in this study. The 184 human isolates had been previously serotyped using antisera, and these strains included 165 isolates of serotype 2, 12 isolates of serotype 14, and 3 and 4 isolates of serotypes 5 and 24, respectively (Kerdsin et al., 2009, 2011a, b).


**Primer design.** The primers for 13 serotypes had been previously designed and reported (Kerdsin et al., 2012). New primers were designed for 16 serotypes using the sequences of the capsule loci (cps) of *Streptococcus suis* serotypes 6, 10, 11, 12, 13, 15, 17, 18, 21, 23, 24, 27, 28, 29, 30 and 31 retrieved from GenBank under the accession numbers AB737818, BR001007, AB737819, AB737821, AB737823, AB737824, AB737825, AB737827, BR001010, AB737829 and AB737831–AB737835, respectively (Okura et al., 2013). The primers were designed as described in our earlier report (Kerdsin et al., 2012). The primer sequences used for the multiplex PCR are shown in Table 1.

The primers targeted the following genes: (i) glycosyltransferase genes cps1J, cps14J, cps1/2J, cps2L, cps3L, cps7H, cps9H, cps16K, cps21N, cps23I and cps24L; (ii) capsular polysaccharide repeat unit transporter genes cps3K, cps4M and cps5N; (iii) UDP-glucose dehydrogenase gene cps4N; (iv) oligosaccharide repeat unit polymerase genes cps6L, cps10M, cps11N, cps12J, cps13L, cps15K, cps17O, cps18N, cps19L, cps25M, cps27K, cps28L, cps29L, cps30L and cps31L; (v) N-acetylmannosaminotransferase gene cps8H; and (vi) glycerophosphotransferase gene cps25N.

**Serotyping by multiplex PCR.** Multiplex PCR was carried out independently in four sets. The first set included the primers for serotypes 1/2, 1, 2, 3, 7, 9, 11, 14 and 16, the second set included the primers for serotypes 4, 5, 8, 12, 18, 19, 24 and 25, the third set included the primers for serotypes 6, 10, 13, 15, 17, 23 and 31, and the fourth set included the primers for serotypes 21, 27, 28, 29 and 30. The PCR mixture contained 1× KAPA2G Fast multiplex PCR mix (KAPA Biosystems) and 0.2 μM of each primer for each set of the PCR. The following PCR thermal profile of PCR was used: initial activation of DNA polymerase at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 20 s, primer annealing and extension at 62 °C for 90 s, and a final extension at 72 °C for 5 min.

The PCR products were analysed by gel electrophoresis for 30 min on 2% agarose gels in 0.5× Tris/borate/EDTA (TBE) buffer. The gels were stained with ethidium bromide and visualized under UV light (GeneGenius Bioimaging System; SynGene). The sizes of the PCR products were determined by comparison with a molecular size standard (GeneRuler 100 bp Plus DNA ladder; Thermo Fisher Scientific).

The multiplex PCR was evaluated for the limit of detection. *Streptococcus suis* reference strains were diluted in 10-fold serial dilutions from an original concentration of OD600=0.6, which was equivalent to 10⁸ c.f.u. ml⁻¹. Genomic DNA was prepared from 1 ml of each dilution using the QIAamp DNA Mini kit (Qiagen). Each dilution was plated out on sheep blood agar for c.f.u. quantification at the same time to determine the minimum c.f.u. required for four multiplex PCRs.

The multiplex PCR was evaluated for the efficiency of amplification of a DNA mixture of each serotype in each reaction. Reaction 1 contained a DNA mixture of serotypes 1/2, 1, 2, 3, 7, 9, 11, 14 and 16, reaction 2 consisted of a DNA mixture of serotypes 4, 5, 8, 12, 18, 19, 24 and 25, reaction 3 contained a DNA mixture of serotypes 6, 10, 13, 15, 17, 23 and 31, and reaction 4 contained a DNA mixture of serotypes 21, 27, 28, 29 and 30. Each reaction contained 15 ng DNA from each serotype.

**RESULTS AND DISCUSSION**

We expanded the multiplex PCR from a previous version that could identify only 15 serotypes to a new version that is capable of identifying 29 serotypes of *Streptococcus suis* (Kerdsin et al., 2012). We used reference strains of the 29 *Streptococcus suis* serotypes to verify the multiplex PCRs. As shown in Fig. 1, the 29 target serotypes were positive, and each amplified the 695 bp of the *gdh* gene and was serotype-specific for the *cps* gene (Fig. 1a) without any cross-reaction among different serotypes (Fig. 1b). The presence of this former *Streptococcus suis*-specific target gene (695 bp) could not distinguish among true *Streptococcus suis*, untypeable strains and the former *Streptococcus suis* serotypes 20, 22, 26, 32 and 34 produced only the 695 bp band (Fig. 1b). The presence of this former *Streptococcus suis*-specific target gene (695 bp) could not distinguish among true *Streptococcus suis*, untypeable strains and the former *Streptococcus suis* serotypes 20, 22, 26, 32, 33 and 34 (Hill et al., 2005; Tien et al., 2013). Therefore, confirmation via sequencing of the 16S rRNA gene, *cpn60*, *sodA* or *recN* may be necessary if a serotype-specific band is not identified. As expected, this PCR assay could not differentiate serotype 1 from 14, and serotype 2 from 1/2, because the *cps* loci of these
Table 1. Primers and target genes used in the multiplex PCRs

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Sequence (5’—3’)*</th>
<th>Gene</th>
<th>Multiplex PCR reaction set</th>
<th>PCR product sizes (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1 and 14 | F: AATCATGGAATAAAGCGGAGTACAG  
R: ACAATTGATACGTCGAATTCTCCACC | cps1J, cps14J | 1 | 550 | Kerdsin et al. (2012) |
| 2 and 1/2 | F: GATTTCGCGGAGGTTACTCTT  
R: TAAATAATGATCCGCATTGCTTGC | cps2L, cps2J | 1 | 450 | Kerdsin et al. (2012) |
| 3 | F: TGGGAGAAGGCAAAATGTCCAGA  
R: ACCCGGAGAAGGCCAGGAAGA | cps3J-cps3K | 1 | 1273 | Kerdsin et al. (2012) |
| 4 | F: ACTTGGAGGTTCGAGGGTATGCT  
R: ACCCGGATAGTACCGACACG | cps4M-cps4N | 2 | 783 | Kerdsin et al. (2012) |
| 5 | F: TGGGAGGAGATTGGTTGGACGC  
R: CGTAACACCGCCCACGCAC | cps5N | 2 | 166 | Kerdsin et al. (2012) |
| 6 | F: TACGGTCTCCCTTGCTCTGA  
R: AACTCAGCTAGTCGCTACG | cps6L | 3 | 325 | This study |
| 7 | F: GATGATCTATTTTGCCCAAGTAAACG  
R: AGTACATGTCGTCGTGCTGACCC | cps7H | 1 | 150 | Kerdsin et al. (2012) |
| 8 | F: ATGGGCGTTGCGGGGAGTTT  
R: TTACGGCCTCCATCAAGCTG | cps8H | 2 | 320 | Kerdsin et al. (2012) |
| 9 | F: GGGGATGTGTCGAATTTGGCTT  
R: CGGAAGTATCTGGGCTACTG | cps9H | 1 | 300 | Kerdsin et al. (2012) |
| 10 | F: TTACGGGAGATTTGGGGTGT  
R: CGGGACACAGATTGACACT | cps10M | 3 | 153 | This study |
| 11 | F: TACGTGTCTGCAGCCTCATTAC  
R: CGACTTCTGTCGGCTGAGT | cps11N | 1 | 896 | This study |
| 12 | F: TGGGCGATAGGCAACAAGGAG  
R: ACCAAAGATTTGGGCTCAG | cps12L | 2 | 209 | This study |
| 13 | F: CTGGGTCTGGCAATTTGGCAT  
R: GCAGATCTAGTCGCTACTG | cps13L | 3 | 1135 | This study |
| 15 | F: GCAGAAGAGCTTCCGGAGATGA  
R: CAAGAGAGTGGTCAACCCCA | cps15K | 3 | 274 | This study |
| 16 | F: TGCACTGCTAGCTCAGCCTGGAG  
R: TTTGTTTTGTGAATCACACGCCCAC | cps16K | 1 | 202 | Kerdsin et al. (2012) |
| 17 | F: ACCTGGGGTGGGAATGAGCAGA  
R: ACCCAGGAAACAGATCGAGTAC | cps17O | 3 | 906 | This study |
| 18 | F: CGGGCACTCATTCTACTCTG  
R: ATAGCAGCCGAAACGGGCAGA | cps18N | 2 | 432 | This study |
| 19 | F: AGCCGGGTGGTAGTGCTGGGAG  
R: ACAAGCACTCCAGCAAGCGCA | cps19L | 2 | 1024 | Kerdsin et al. (2012) |
| 21 | F: GGTGGCAGAAGGCAAGAAGGT  
R: ACATGGTAAGCATTGGCACGA | cps21N | 4 | 325 | This study |
| 23 | F: TGCTCAACAAAGCAAGCAGAAA  
R: TGACTGCTACATTGCGAGCC | cps23I | 3 | 454 | This study |
| 24 | F: ACCCGGAAAAAACAGGGAGTAT  
R: ACCAACTGAATTGCAAGCAGAC | cps24L | 2 | 500 | This study |
| 25 | F: GGAGGAGCTGGCGGGCTACTA  
R: TGCCGCAACCTGGATGCGCATTG | cps25M-cps25N | 2 | 1211 | Kerdsin et al. (2012) |
| 27 | F: CTACCAGCAACTGCAACGCAACAGA  
R: CCAGTAAAGAGCGGTCGCA | cps27K | 4 | 506 | This study |
| 28 | F: GGACGTCTGGAATACACGTCCCGA  
R: TCCCGACATCTCCCGTCACC | cps28L | 4 | 865 | This study |
| 29 | F: GTGCGGCGGGTTATTTGGGT  
R: AGCCTGTGACACCTCTTGGCTTAC | cps29L | 4 | 435 | This study |
| 30 | F: CTGTTAATTGGGCGGGGCTG  
R: ATCGGCGCTGCACCTTCGACG | cps30L | 4 | 170 | This study |
| 31 | F: GGAGTGCTCATAGCCGACCTTT  
R: GCATTGCCCCACACGCA | cps31L | 3 | 550 | This study |
serotypes are highly similar (Smith et al., 1999a; Okura et al., 2013).
Of 184 human isolates of *Streptococcus suis*, this assay confirmed 165 isolates of serotype 2 as serotypes 2 or 1/2, 12 isolates of serotype 14 as serotypes 1 or 14, 3 isolates of serotype 5 as serotype 5, and 4 isolates of serotype 24 as serotype 24.

As shown in Table 2, the multiplex PCR assay also confirmed the serotypes of the 109 pig isolates that had been

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**Table 1. cont.**

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Sequence (5'–3')*</th>
<th>Gene</th>
<th>Multiplex PCR reaction set</th>
<th>PCR product sizes (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>F: TTCTGCAGGTTATCTGCAACG</td>
<td>gdh</td>
<td>All reactions</td>
<td>695</td>
<td>Kerdin et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>R: TGTCCATGGAGATAAGATGG</td>
<td></td>
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*F, forward; R, reverse.

![Fig. 1. (a) Agarose gel electrophoresis of PCR-amplified products from 29 serotypes of *Streptococcus suis* using the four reaction sets of the multiplex PCR. Top left, PCR product bands produced by *Streptococcus suis* serotypes 1, 14, 1/2, 2, 3, 4, 7, 9, 11 and 16 using the first set of multiplex PCR; top right, PCR product bands produced by *Streptococcus suis* serotypes 4, 5, 8, 12, 18, 19, 24 and 25 using the second set of multiplex PCR; bottom left, PCR product bands produced by *Streptococcus suis* serotypes 6, 10, 13, 15, 17, 23 and 31 using the third set of multiplex PCR; bottom right, PCR product bands produced by *Streptococcus suis* serotypes 21, 27, 28, 29 and 30 using the fourth set of multiplex PCR. M, molecular mass marker. (b) PCR-amplified products of the former *Streptococcus suis* serotype 20, 22, 26, 32, 33 and 34 (lanes 1–6), respectively. M, molecular mass marker.](http://jmm.sgmjournals.org)
originally serotyped using the co-agglutination test with antisera. The multiplex PCR assay was able to identify the serotypes of two isolates as serotypes 3 (n=1) and 5 (n=1) even though the antiserum results revealed these isolates as untypable.

As shown in Fig. 2, our multiplex PCR allowed amplification of serotype-specific bands simultaneously from a DNA mixture of each serotype in each reaction. It seems to be advantageous to detect various serotypes in a single sample. In contrast, a similar experiment using primers previously reported by Liu et al. (2013) was conducted and it was found that several serotypes (serotypes 3, 9, 16, 19 and 33) were not detected in the DNA mixture containing each serotype in each reaction (data not shown); however, the PCR was carried out under our optimal conditions except for the primers used.

The specificity of the four multiplex PCR for the Streptococcus suis serotypes was examined using 29 Streptococcus suis serotype strains and reference strains of other bacterial species as described in Methods. No cross-reactivity was detected with these samples (Fig. S2). The limit of detection in this assay was $10^3$ c.f.u. for serotypes 1/2, 2, 5, 7, 10, 15, 21 and 30, $10^4$ c.f.u. for serotypes 1, 3, 4, 6, 9, 11, 12, 13, 14, 16, 17, 18, 23, 24, 27, 28, 29 and 31 and $10^5$ c.f.u. for serotypes 8, 19 and 25 (Fig. S3).

In this study, our multiplex PCR distinguished 29 serotypes of Streptococcus suis isolates from humans and pigs. This single assay correctly identified Streptococcus suis at the species level and differentiated between its serotypes using the same system. Thus, this multiplex PCR has advantages over previously reported PCR systems, which detected only a limited range of serotypes (Kerdsin et al., 2009; Marois et al., 2004; Okwumaba et al., 2003; Smith et al., 1999a, b; Wang et al., 2011; Wisselink et al., 2002). Our multiplex PCR assay was developed to identify the 29 currently known Streptococcus suis serotypes. A multiplex PCR identifying 33 serotypes of Streptococcus suis was recently developed; however, the former Streptococcus suis serotypes 20, 22, 26 and 33 were still included in that assay (Liu et al., 2013). Our multiplex PCR assay targets true Streptococcus suis serotypes only. It also combines the annealing and extension reactions into a single step that can shorten the whole PCR time to about 50 min; therefore, the multiplex PCR reported in this study is less time-consuming than previously reported PCRs.

### Table 2. Serotyping of 109 Streptococcus suis isolates from pig tonsils using the multiplex PCR and with antisera

<table>
<thead>
<tr>
<th>Serotype using antiserum</th>
<th>No.</th>
<th>Serotype using multiplex PCR</th>
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<tbody>
<tr>
<td></td>
<td>1/2</td>
<td>1 and 14</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>1/2</td>
<td>1</td>
<td>1</td>
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<tr>
<td>3</td>
<td>10</td>
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<tr>
<td>14</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>NT*</td>
<td>15</td>
<td>1</td>
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</tbody>
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<thead>
<tr>
<th>Serotype using antiserum</th>
<th>No.</th>
<th>Serotype using multiplex PCR</th>
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<tbody>
<tr>
<td></td>
<td>15</td>
<td>16</td>
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<tr>
<td>15</td>
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<td>16</td>
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<tr>
<td>24</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>31</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

*Untypable serotypes determined with antisera were included (n=15).

Notably, our multiplex PCR was able to identify the serotype in some isolates even though serological tests showed that these isolates were untypable. Loss of capsule among *Streptococcus suis* isolates due to a mutation in the *cps* locus has been reported (Lakkitjaroen *et al.*, 2011). Isolates with mutations in the *cps* loci that result in the loss of the ability to synthesize capsule are untypable by the traditional co-agglutination test.

Our multiplex PCR did not differentiate serotype 2 from serotype 1/2, or serotype 14 from serotype 1; therefore serological testing remains necessary to distinguish between these serotypes. However, this multiplex PCR can reduce the cost and labour required to type the other 25 serotypes without using antisera. Our method could confirm serotypes 2, 4, 5, 14, 16 and 24 in human isolates and detect serotypes 3, 4, 5, 7, 8, 9 and 16, which are frequently isolated from diseased pigs (Gottschalk *et al.*, 2010; Kerdsin *et al.*, 2011b; Nghia *et al.*, 2008; Schultsz *et al.*, 2012). In conclusion, this assay will be useful for serotype surveillance of human and pig isolates of *Streptococcus suis*.

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**REFERENCES**


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**Fig. 2.** Multiplex PCR amplified a DNA mixture of each serotype in reaction 1 (serotypes 1/2, 1, 2, 3, 7, 9, 11, 14 and 16), reaction 2 (serotypes 4, 5, 8, 12, 18, 19, 24 and 25), reaction 3 (serotypes 6, 10, 13, 15, 17, 23 and 31) and reaction 4 (serotypes 21, 27, 29 and 30). The amount of DNA mixture in each reaction is 15 ng for each serotype. Each DNA mixture for multiplex PCR was analysed in triplicate (lanes A–L).


Hill, J. E., Gottschalk, M., Brousseau, R., Harel, J., Hemmingsen, S. M. & Goh, S. H. (2005). Biochemical analysis, cpn60 and 16S rDNA sequence data indicate that Streptococcus suis serotypes 32 and 34, isolated from pigs, are Streptococcus orisratti. Vet Microbiol 107, 63–69.


