Reappraisal of the taxonomy of *Streptococcus suis* serotypes 20, 22 and 26: *Streptococcus parasuis* sp. nov.

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In order to clarify the taxonomic position of serotypes 20, 22 and 26 of *Streptococcus suis*, biochemical and molecular genetic studies were performed on isolates (SUT-7, SUT-286T, SUT-319, SUT-328 and SUT-380) reacted with specific antisera of serotypes 20, 22 or 26 from the saliva of healthy pigs as well as reference strains of serotypes 20, 22 and 26. Comparative recN gene sequencing showed high genetic relatedness among our isolates, but marked differences from the type strain *S. suis NCTC 10234T*, i.e. 74.8–75.7 % sequence similarity. The genomic relatedness between the isolates and other strains of species of the genus *Streptococcus*, including *S. suis*, was calculated using the average nucleotide identity values of whole genome sequences, which indicated that serotypes 20, 22 and 26 should be removed taxonomically from *S. suis* and treated as a novel genomic species. Comparative sequence analysis revealed 99.0–100 % sequence similarities for the 16S rRNA genes between the reference strains of serotypes 20, 22 and 26, and our isolates. Isolate STU-286T had relatively high 16S rRNA gene sequence similarity with *S. suis NCTC 10234T* (98.8 %). SUT-286T could be distinguished from *S. suis* and other closely related species of the genus *Streptococcus* using biochemical tests. Due to its phylogenetic and phenotypic similarities to *S. suis* we propose naming the novel species *Streptococcus parasuis* sp. nov., with SUT-286T (=JCM 30273T =DSM 29126T) as the type strain.

*Streptococcus suis* is a major pathogen of swine and has also been isolated from a variety of animals such as ruminants, cats, dogs and horses (Staats et al., 1997). Moreover, human *S. suis* infection is considered to be one of the most important emerging zoonotic diseases in Asian countries (Gottschalk et al., 2010). On the basis of their polysaccharide capsular antigens, a total of 33 serotypes (types 1–31, 33 and type 1/2) of *S. suis* have been described elsewhere (Gottschalk et al., 1989, 1991; Higgins et al., 1995), in which serotype 2 was most frequently associated with disease in both pigs and humans (Gottschalk et al., 2007). Genetic relatedness among *S. suis* serotypes has been described previously by several investigators. However, a phylogenetic analysis based on 16S rRNA gene and chaperonin-60 gene sequences comparison (Chatellier et al., 1998; Brousseau et al., 2001) indicated a marked genetic discrepancy between serotypes 20, 22, 26 and 33 and other serotypes of *S. suis*. Moreover, our previous study (Tien et al. 2013) suggested that the reference strains of serotypes 20, 22, 26 and 33 should be removed taxonomically from *S. suis*. This was based on DNA–DNA relatedness.
with the type strain \textit{S. suis} NCTC 10234\textsuperscript{T} and phylogenetic analyses of sequences of genes encoding manganese-dependent superoxide dismutase (sodA) and recombination/repair protein (recN). Additionally, the reference strains of serotypes 20, 22 and 26 were found to share more than 70\% DNA–DNA reassociation values with each other, suggesting that they represent a novel streptococcal species. In order to clarify their taxonomic position, here we examine isolates (SUT-7, SUT-286\textsuperscript{T}, SUT-319, SUT-328 and SUT-380) reacted with specific antisera of serotypes 20, 22 or 26 from the saliva of four clinically healthy pigs as well as reference strains of serotypes 20, 22 and 26. Using whole-genome sequence identities and sequences of the recN and 16S rRNA genes, we demonstrate that \textit{S. suis} serotypes 20, 22 and 26 belong to a single novel species. On the basis of the phenotypic and phylogenetic results, a novel species of the genus \textit{Streptococcus}, \textit{Streptococcus parasuis} sp. nov., is proposed.

A total of five isolates and reference strains of serotypes 20, 22 and 26 of \textit{S. suis} were used in this study and are listed in Table 1. Isolates SUT-7, SUT-286\textsuperscript{T}, SUT-319, SUT-328 and SUT-380 were isolated from the saliva of four clinically healthy pigs. Bacterial strains and isolates were cultured in Todd–Hewitt agar (Becton Dickinson) at 37 °C for 24 h and grown under 5\% CO\textsubscript{2}. They were stored in Luria–Bertani broth (Becton Dickinson) supplemented with 30\% (v/v) glycerol at −80 °C until use. Serotyping of these isolates was performed by a capsular reaction test, as described previously (Higgins & Gottschalk, 1990), using commercial antisera (Statens Serum Institute, Copenhagen, Denmark). Isolates SUT-286\textsuperscript{T} and SUT-380 were reacted with the antisera of serotypes 20 and 22, respectively, while isolates SUT-7, SUT-319 and SUT-328 were cross-reacted with antisera 22/26, 20/22 and 20/22, respectively (Table 1).

As for the streptococcal species, phylogenetic analysis based on a recN gene was found to be a useful taxonomic tool for identification at the species level (Glazunova \textit{et al.}, 2010). Therefore, in order to determine the phylogenetic relationships of the partial sequence of recN (1056 bp) genes of our isolates they were amplified and sequenced following subjected comparative analysis as described previously (Tien \textit{et al.}, 2013). The recN sequences obtained were aligned using \textsc{clustal} \textsc{w} using the MEGA5 software package (Tamura \textit{et al.}, 2011). Phylogenetic trees were reconstructed using the neighbour-joining method (Saitou & Nei, 1987) with MEGA5. Other phylogenetically related recN sequences of members of the genus \textit{Streptococcus} retrieved from GenBank were also included. The stability of the groupings was estimated by bootstrap analysis with 1000 replications. The percentage similarity between nucleotide sequences was calculated using BioEdit software (Hall, 1999). Comparative sequence analysis revealed 97.7–100\% sequence similarities for recN between the reference strains of serotypes 20, 22 and 26, which were previously determined by Tien \textit{et al.} (2013), and our isolates, thereby demonstrating their high genetic relatedness, but marked differences from the recN sequence of the type strain NCTC.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>86-5192</th>
<th>88-1861</th>
<th>SUT-7</th>
<th>SUT-286\textsuperscript{T}</th>
<th>SUT-319</th>
<th>SUT-328</th>
<th>SUT-380</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotype</td>
<td>20</td>
<td>22</td>
<td>26</td>
<td>22/26</td>
<td>22</td>
<td>20/22</td>
<td>20/22</td>
</tr>
<tr>
<td>Source</td>
<td>Diseased pig</td>
<td>Diseased pig</td>
<td>Diseased pig</td>
<td>Healthy porcine saliva</td>
<td>Healthy porcine saliva</td>
<td>Healthy porcine saliva</td>
<td>Healthy porcine saliva</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>40</td>
<td>39.4</td>
<td>39.8</td>
<td>39.8</td>
<td>39.3</td>
<td>38.3</td>
<td>38.3</td>
</tr>
<tr>
<td>NGS contig. size (bases)</td>
<td>89,116</td>
<td>36,856</td>
<td>75,931</td>
<td>74,855</td>
<td>75,921</td>
<td>74,855</td>
<td>74,855</td>
</tr>
<tr>
<td>Contig count</td>
<td>40</td>
<td>34</td>
<td>319</td>
<td>319</td>
<td>319</td>
<td>319</td>
<td>319</td>
</tr>
<tr>
<td>Total length (bases)</td>
<td>89,4109 1</td>
<td>2,552,497</td>
<td>2,186,636</td>
<td>2,122,164</td>
<td>2,154,045</td>
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<tr>
<td>Accession no.</td>
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<td>DRX016761</td>
<td>DRX016762</td>
</tr>
</tbody>
</table>
10234T, i.e. 74.8–75.7 % sequence similarity. Moreover, these isolates and strains formed a branch separate from other species of the genus *Streptococcus* in phylogenetic trees inferred from *recN* gene sequence comparisons (Fig. S1, available with the online Supplementary Material); the highest sequence similarity was between strain SUT-286T and *Streptococcus acidominimus* CIP 82.4 (74.1 % similarity).

To further differentiate serotypes 20, 22 and 26 from recognized members of the *S. suis* taxon, draft genome sequences were generated. Whole genome sequences of reference strains 86-5192, 88-1861 and 89-4109-1, and of isolates SUT-7, SUT-286T, SUT-319, SUT-328 and SUT-380 were determined using Illumina/Solexa technology. A mean of 0.75–3.38 million paired-end reads of length 262.1 bp were generated per strain by a MiSeq system (Illumina). All the reads generated were assembled into contigs using CLC Genomics Workbench v. 6.0 (CLCbio), which was also used to determine the nucleotide sequence statistics such as the approximate draft genome size and DNA G+C content. The resulting draft genomes of the three reference strains and five isolates had 54–319 contigs with 82–479 fold coverage and the genome size ranged from 2.12–2.55 Mb with DNA G+C contents of 38.3–40.0 mol% (Table 1). The DNA G+C contents of our isolates and the reference strains were very similar to the DNA G+C contents reported for the whole sequenced genome of strain P1/7 of *S. suis* (40.0 mol%) (Holden et al., 2009). The degree of pairwise genome-based relatedness was calculated as an average nucleotide identity (ANI) value following the BLAST-based ANI calculation method by using JSpecies software (Richter & Rossello-Móra, 2009). ANI values for the strains of species of the genus *Streptococcus*, including those designated to *S. suis*, whose genome sequences were available in the GenBank database were calculated and used to generate a dendrogram based on the UPGMA algorithm by using the TreeView program (Page, 1996) (Fig. 1). The ANI values among reference strains 86-5192, 88-1861 and 89-4109-1, and our isolates ranged from 95.3–99.9 % (Table S1); these ANI values are higher than the 95 % cut-off ANI value for bacterial species proposed by Goris et al. (2007). However, the ANI values between our isolates and strains belonging to the species *S. suis* were well below the proposed cut-off ANI value for

![Fig. 1. A dendrogram describing the UPGMA clustering of genome-sequenced strains of species of the genus *Streptococcus* based on their ANI values. The accession numbers of draft genome sequences of our five isolates (SUT-7, SUT-286T, SUT-319, SUT-328 and SUT-380) and reference strains of serotypes 20, 22 and 26 were DRX016751–DRX016758, respectively.](image-url)
bacterial species (88.1–89.0 %) (Table S1). By combining recN gene phylogeny and genome sequence comparisons, it is concluded that reference strains serotype 20, 22 and 26, and our isolates are representatives of a novel genomic species.

The 16S rRNA gene sequences of our isolates and reference strains 86-5192, 88-1861 and 89-4109-1 were obtained from draft genome sequences. Alignment of the 16S rRNA gene sequences obtained with 16S rRNA gene sequences of the type strains of S. suis NCTC 10234\(^T\) and other streptococcal species, was used to reconstruct a neighbour-joining tree using MEGA5 software (Tamura et al., 2011) (Fig. 2). Statistical reliability of the phylogenetic tree was evaluated by bootstrap analysis of 1000 replicates. Accession numbers of the reference sequences used in the phylogenetic analysis are shown in Fig. 2. Comparative sequence analysis revealed 99.0–100 % sequence similarities for 16S rRNA genes between the

![Fig. 2. 16S rRNA gene sequence-based phylogenetic tree of strain SUT-286\(^T\) and selected type strains of species of the genus Streptococcus. The neighbour-joining tree is shown here with bootstrap support values; only values \>50 % are shown. Bar, 5 % sequence divergence.](http://ijs.sgmjournals.org)
reference strains of serotypes 20, 22 and 26, and our isolates. Isolate SUT-286T had relatively high 16S rRNA gene sequence similarities with S. suis NCTC 10234T (98.8 %). The next most closely related type strain was Streptococcus porcorum 682-03T (97.9 % similarity).

The five new isolates were Gram-stained and assessed for the presence of catalase. The haemolytic reaction was determined on Columbia agar (Oxoid) containing 5 % (v/v) defibrinated sheep’s blood incubated aerobically at 37 °C for 24 and 48 h. Determination of growth at 10, 22, 30, 37 and 42 °C and with 6.5 % (w/v) NaCl added in brain heart infusion broth (Difco) at pH 7.5 was performed as recommended by Facklam & Elliott (1995). The isolates were characterized biochemically using the Rapid ID32 Strep kits, glycyl-tryptophan arylamidase, pullulan, glycogen and sucrose, but not from melibiose, L-arabinose, D-ribose, D-arabitol, D-mannitol, D-sorbitol, melezitose, cyclodextrin or tagatose. Most strains produce acid from raffinose and lactose (Rapid ID32 Strep; type strain positive). Leucine arylamidase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase (API ZYM) are detected. Most strains do not produce β-galactosidase (Rapid ID32 Strep; type strain negative). No activity is detected for N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, lipase (C14), acid phosphatase, valine arylamidase, α-galactosidase, cystine arylamidase, trypsin, α-chymotrypsin (API ZYM), alkaline phosphatase, α-galactosidase, β-glucosidase, β-galactosidase (API ZYM and Rapid ID32 Strep), glycyrl-tryptophan arylamidase, β-mannosidase or pyroglycamic acid arylamidase (Rapid ID32 Strep). Voges–Proskauer test is negative (Rapid ID32 Strep). Arginine, hippurate and urea are not hydrolysed (Rapid ID32 Strep).

The type strain, SUT-286T (=JCM 30273T=DSM 29126T), was isolated from the saliva of a clinically healthy pig. The DNA G+C content of the type strain is 39.8 mol%.

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**References**


