Staphylococcus devriesei sp. nov., isolated from teat apices and milk of dairy cows

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Ten non-motile, Gram-stain-positive, coagulase-negative staphylococci were isolated from bovine milk and teat apices. All isolates were catalase-positive, with anteiso-C15 : 0, iso-C15 : 0, anteiso-C17 : 0, iso-C17 : 0 and C18 : 0 as predominant fatty acids and diphosphatidylglycerol and phosphatidylglycerol as major polar lipids. The results of sequence analysis of the 16S rRNA gene and four housekeeping genes (rpoB, hsp60, tuf and dnaJ) in combination with tRNA-intergenic spacer length analysis showed that the isolates form a separate branch within the genus Staphylococcus. Based on 16S rRNA gene sequencing, the phylogenetically most closely related species are Staphylococcus haemolyticus, S. hominis and S. lugdunensis, with 98.7 % sequence similarity. The DNA G+C content varies from 33.3 to 33.7 mol%, and DNA–DNA hybridization with the nearest neighbours, based on 16S rRNA gene sequences, confirmed that the isolates represent a novel Staphylococcus species. All isolates induced a small zone of complete haemolysis on Columbia agar with 5 % sheep blood and exhibited a homogeneous biochemical fingerprint that is discriminative from the phylogenetically most closely related species. Based on these results, it is proposed to classify the ten isolates as Staphylococcus devriesei sp. nov., with strain KS-SP 60T (=LMG 25332T =CCUG 58238T) as the type strain.

Abbreviations: CNS, coagulase-negative staphylococci; tDNA-PCR, tRNA-intergenic spacer PCR.


Details of strain isolation and amplification primers, phenotypic results for individual strains and dendrograms based on tDNA-PCR results and dnaJ, rpoB, hsp60 and tuf sequences are available as supplementary material with the online version of this paper.

In well-managed dairy farms in many parts of the world, coagulase-negative staphylococci (CNS) have become the predominant pathogens in milk samples from cows (Piepers et al., 2007; Schukken et al., 2009). Recent publications report on subclinical/clinical mastitis due to CNS (Gillespie et al., 2009), but beneficial effects have also been suggested (De Vliegher et al., 2003, 2004; Schukken et al., 2009). tRNA-intergenic spacer PCR (tDNA-PCR) has been shown to be useful for rapid, inexpensive and accurate identification of CNS at the species level (Supré et al., 2009). During a recent validation study applying the latter technique to 288 bovine field isolates from milk and teat apices, ten CNS isolates remained unidentified. The tDNA-PCR patterns of these isolates were highly similar to each other and closely related to those of Staphylococcus capitis, S. simulans, S. haemolyticus and S. xylosus, but they formed a well-defined, separate cluster (Supplementary
The cellular fatty acid patterns of the ten isolates were stain-positive cells, occurring in pairs and small clusters. Staphylococcal, non-spore-forming, non-motile, Gram-positive colonies were isolated from milk of a subclinical infection of the udder (KS-SP 11) and nine originated from teat apices (Supplementary Table S1). The isolates were preliminarily identified as CNS following the guidelines of the NMC (a global organization for mastitis control and milk quality; http://www.nnconline.org/), including determination of catalase and coagulase activity and detection of haemolysis (Hogan et al., 1999).

After aerobic incubation at 35 °C for 24 and 48 h on Colombia agar with 5 % sheep blood, all ten isolates showed homogeneous growth. After 48 h of incubation, colonies were circular with a diameter of 3–4 mm, smooth and glistening, and they showed a small zone (1 mm) of complete haemolysis. The majority of the colonies were grey–yellow, but KS-SP 27, KS-SP 66 and KS-SDV 16 were yellow and KS-SP 20 and KS-SP 65 showed a yellow to orange pigmentation. Incubation at 42 °C resulted in larger colonies, whereas incubation at 25 °C and under anaerobic conditions resulted in smaller colonies. All isolates were catalase-positive. Gram-staining and phase-contrast microscopy showed typical staphylococcal, non-spore-forming, non-motile, Gram-stain-positive cells, occurring in pairs and small clusters.

The cellular fatty acid patterns of the ten isolates were determined as described previously (Mergaert et al., 2001). Cells were harvested from cultures grown on trypticase soy agar (BBL 11768) at 28 °C for 24 h. All ten isolates possessed very similar fatty acid profiles, and the mean profile consisted of anteiso-C15:0 (48.2 %), anteiso-C17:0 (19.1 %), iso-C17:0 (6.1 %), C18:0 (3.6 %), anteiso-C19:0 (3.3 %), iso-C16:0 (2.5 %), iso-C19:0 (1.9 %), C16:0 (1.5 %), C14:0 (1.4 %) and iso-C15:0 (1.1 %), which corresponds to that of other CNS (Kotilainen et al., 1991).

Polar lipids were extracted and separated by using two-dimensional TLC according to Tindall (1990a, b). The total lipid profile was visualized by spraying with molybdo-phosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdeneum blue (specific for phosphates) and 2-naphthol (specific for sugars). Biomass used for the extraction was grown on trypticase soy agar (BBL) at 28 °C for 24 h. The polar lipid profiles of the three representative isolates tested, KS-SP 11, KS-SP 60T and KS-SDV 19, were very similar. They consisted of the major lipids diphasphatidylglycerol and phosphatidylglycerol. Moderate amounts of three unknown glycolipids, of which two corresponded to GL1 and GL2 as described by Nahaie et al. (1984), were detected. Also, minor to trace amounts of unknown aminolipids were observed. These profiles are in agreement with other Staphylococcus species profiles (Nahaie et al., 1984; Nováková et al., 2010).

An almost-complete fragment of the 16S rRNA gene and partial fragments of the housekeeping genes coding for the β-subunit of the RNA polymerase (rpoB), the heat-shock protein 60 (hsp60), the elongation factor Tu (tuf) and the heat-shock protein DnaJ (dnaJ), were amplified and sequenced according to previously described procedures (for rpoB, hsp60 and tuf; Supré et al., 2009; for dnaJ; Shah et al., 2007), except that other primers were used for 16S rRNA gene sequencing (Mergaert et al., 2001; Coenye et al., 1999) (Supplementary Table S2). Forward and reverse sequences were aligned with the Vector NTI Advance 10 software (Invitrogen Life Technologies) and compared to GenBank sequences via the nucleotide–nucleotide BLAST algorithm (http://ncbi.nlm.nih.gov/). For alignment of nucleotide sequences, the CLUSTAL W program (Thompson et al., 1994) was used. Neighbour-joining and maximum-parsimony (Saitou & Nei, 1987) trees were obtained with the PHYLIP program (Felsenstein, 1989) and drawn with the software TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). DNADIST was utilized for distance analysis according to Kimura’s two-parameter model (Kimura, 1980). Bootstrap values were determined based on 1000 replications.

The discriminatory power of the 16S rRNA gene is limited in the genus Staphylococcus (CLSI, 2007; Shah et al., 2007), whereas the housekeeping genes rpoB, hsp60, tuf and dnaJ show more variation and are preferred over 16S rRNA gene sequencing for species identification (Zadoks & Watts, 2009). The cut-off values previously reported for species identification were 98.7 % for 16S rRNA (Jousson et al., 2007), 94 % for rpoB (Mellmann et al., 2006) and 97 % for hsp60, tuf and dnaJ (CLSI, 2007). For each of the genes, the sequences of the ten isolates were compared against each other. Within each gene, the sequence similarities were high, namely 99.6–100 % for the 16S rRNA gene sequence, 99.1–100 % for dnaJ, 99.7–100 % for rpoB, 98.6–100 % for hsp60 and 99.6–100 % for tuf. The overall mean intraspecies divergence per housekeeping gene was 0.7 %, which is within the variability noted for Staphylococcus species (Shah et al., 2007). The high similarities therefore indicate that the isolates probably belong to a single species. Neighbour-joining phylogenetic trees based on 16S rRNA (Fig. 1) and dnaJ (Supplementary Fig. S2) gene sequences were constructed. The phylogenetic position of the ten isolates was in the genus Staphylococcus, confirming the previous data. The topology of the maximum-parsimony tree was comparable (not shown). Sequencing of all five genes revealed seven different species, of which the unknown cluster in the 16S rRNA gene tree (Fig. 1), this species was not included in the...
partial trees. Within each gene, sequences of the unknowns were compared to those of the three closest relatives based on the 16S rRNA gene (S. haemolyticus, S. hominis and S. lugdunensis) and similarities were calculated (Table 1). The similarities obtained were below the cut-off values used previously for species identification, which suggests that the isolates represent a novel Staphylococcus species.

In some phylogenetic trees, namely in those based on 16S rRNA, dnaJ and hsp60 gene sequences, the isolates formed a cluster divided into subclusters, with KS-SDV 19 belonging to the smallest subcluster (Fig. 1 and Supplementary Figs S2 and S3b). Therefore, three of the unknown isolates (KS-SP 11, KS-SP 60T and KS-SDV 19), taken from the subclusters and covering the total branch of the isolates in

Fig. 1. Phylogenetic tree, constructed using the neighbour-joining method, based on the 16S rRNA gene sequences of strains of Staphylococcus devriesiei sp. nov. and reference sequences of Staphylococcus strains available online. Macroccocus caseolyticus ATCC 13548T was chosen as the outgroup. Bootstrap values, calculated from 1000 resamplings, are given at nodes if higher than 70 %. Bar, 1 % sequence divergence. *, Type strain of Staphylococcus pulvereri.
Table 1. Intraspecies and interspecies sequence similarity of the ten strains of *Staphylococcus devriesei* sp. nov.

Values are mean (range) percentage similarity. Sequences of the ten novel strains were compared with sequences from the type strains of the three phylogenetically most closely related species on the basis of the 16S rRNA gene sequence. Gene-specific cut-off percentages for each gene are given as cited in literature.

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>16S rRNA</th>
<th>dnaJ</th>
<th>rpoB</th>
<th>hsp60</th>
<th>tuf</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. devriesei</em> sp. nov. (n=10)</td>
<td>99.9 (99.6–100)</td>
<td>99.6 (99.1–100)</td>
<td>99.9 (99.7–100)</td>
<td>99.6 (98.6–100)</td>
<td>99.9 (99.6–100)</td>
</tr>
<tr>
<td><em>S. haemolyticus</em> LMG 13349T</td>
<td>99.2 (98.6–99.3)</td>
<td>83.4 (82.8–84.0)</td>
<td>92.4 (92.0–93.3)</td>
<td>90.0 (88.3–91.6)</td>
<td>95.5 (94.6–98.4)</td>
</tr>
<tr>
<td><em>S. hominis</em> LMG 13348T</td>
<td>98.9 (98.8–98.9)</td>
<td>82.6 (82.2–82.9)</td>
<td>90.7 (90.4–91.1)</td>
<td>89.5 (88.2–91.2)</td>
<td>94.5 (93.3–96.9)</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> LMG 13346T</td>
<td>98.8 (98.8–98.9)</td>
<td>80.2 (79.8–80.9)</td>
<td>89.4 (89.0–90.4)</td>
<td>87.4 (86.0–89.3)</td>
<td>94.1 (93.0–97.6)</td>
</tr>
</tbody>
</table>

Gene-specific cut-off (%)b

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*a* Obtained from: a, Jousson et al. (2007); b, CLSI (2007); c, Mellmann et al. (2006).

all sequencing trees, were selected for DNA–DNA hybridizations. Hybridization was performed with the type strains of the three phylogenetically most closely related species based on 16S rRNA gene sequence analysis (*S. haemolyticus* LMG 13349T, *S. hominis* LMG 13348T and *S. lugdunensis* LMG 13346T). DNA was extracted as described by Gevers et al. (2001). DNA–DNA hybridizations were carried out according to a modification of the micropalte method (Ezaki et al., 1989) as detailed by Goris et al. (1998) and Cleenwerck et al. (2002). The hybridization temperature was 34 °C. Reciprocal reactions (e.g. A × B and B × A) showed variations within the limits of the method, i.e. mean SD of 7% (Goris et al., 1998). DNA–DNA hybridizations revealed 97–99% DNA binding between isolates KS-SP 11, KS-SP 60T and KS-SDV 19. Although KS-SDV 19 belonged to a different subcluster based on 16S rRNA, dnaJ and hsp60 gene sequences, it showed similarly high hybridization with KS-SP 11 and KS-SP 60T and, therefore, hybridization of this strain with type strains of other species was not deemed necessary. There was a low level of DNA binding between isolates KS-SP 11 and KS-SP 60T and the type strains *S. haemolyticus* LMG 13349T (17%), *S. hominis* LMG 13348T (23%) and *S. lugdunensis* LMG 13346T (27%). These data indicate that the ten isolates belong to a single novel species within the genus *Staphylococcus* (Wayne et al., 1987).

The DNA base composition (mol%) of KS-SDV 16, KS-SP 11, KS-SP 16, KS-SP 20 and KS-SP 60T was determined in triplicate by HPLC, according to Mesbah et al. (1989), using DNA extraction procedures described by Gevers et al. (2001). The DNA G+C content of KS-SDV 16, KS-SP 11, KS-SP 16 and KS-SP 20 was 33.7 mol% and that of KS-SP 60T was 33.3 mol%. The range of DNA base composition is lower than 2 mol%, as generally accepted within a single species, and is consistent with that of members of the genus *Staphylococcus* (Kocur et al., 1971).

Phenotypic characteristics of all ten isolates were obtained using API 50 CH with CHB/E medium (bioMérieux), API Staph Id 32 (bioMérieux) and Staph Zym (Rosco) test kits. Tube coagulase (coagulase plasma; Diféo Laboratories) and clumping factor (Devriese, 1979) were also examined. The DNase reaction was assessed using DNase agar (Oxoid). The phenotypic features of the ten isolates were compared with those of the three phylogenetically most closely related species based on 16S rRNA gene sequences (*S. haemolyticus*, *S. hominis* and *S. lugdunensis*). Differentiating biochemical characteristics are listed in Table 2 and individual phenotypes of each isolate are given in

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<tbody>
<tr>
<td>Arginine dihydrodrolase</td>
<td>+</td>
<td>+</td>
<td>c</td>
<td>−</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>c</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Mannitol b</td>
<td>[+]</td>
<td>c</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D-Mannose a</td>
<td>[−]</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Melezitose b</td>
<td>[+]</td>
<td>−</td>
<td>c</td>
<td>ND</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>+</td>
<td>c</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>a</td>
<td>−</td>
<td>c</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>b</td>
<td>+</td>
<td>c</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 2. Phenotypic characteristics of *S. devriesei* sp. nov. compared with the phylogenetically most closely related *Staphylococcus* species

Species: 1, *S. devriesei* sp. nov. (for variable tests, reaction of strain KS-SP 60T in brackets); 2, *S. haemolyticus*; 3, *S. hominis*; 4, *S. lugdunensis*. Closely related species were selected on the basis of the 16S rRNA gene sequence. Data for reference taxa were obtained from Freneny et al. (1999). +, 90% or more isolates positive; −, 90% or more isolates negative; a, 11–50% of isolates positive; b, 51–89% of isolates positive; c, 11–89% of isolates positive; ND, no data available. All tested strains were positive for acetyl methyl carbinol utilization and negative for starch utilization, alkaline phosphatase and β-galactosidase. Oxidation and fermentation of D-glucose was positive. Acid was produced from D-fructose, maltose, trehalose and L-sorbose. There was no acid production from amygdalin, D-adonitol, arabinose, arabitol, cellobiose, D-fucose, D-xylose, raffinose, D-sorbitol, D-tartarose, xylose, erythritol, gentiobiose, salicin or xylitol. Utilization of glycoce, inositol, inulin, potassium 2-ketogluconate, methyl x-D-mannopyranoside, methyl β-D-xylopyranoside and methyl α-D-glucopyranoside was negative.
Supplementary Table S3. Tube coagulase, clumping and the DNase reaction were negative for all isolates. All isolates were considered sensitive to novobiocin.

In conclusion, the data from this study demonstrate that the ten isolates are members of a novel *Staphylococcus* species, for which the name *Staphylococcus devriesei* sp. nov. is proposed. In ongoing field studies in Belgium, additional isolates belonging to this species have been detected in milk and on teat apices of dairy heifers and cows.

**Description of *Staphylococcus devriesei* sp. nov.**

*Staphylococcus devriesei* (de.vrie’s.e.i. N.L. masc. gen. n. devriesei of Devriese, named in honour of the Belgian microbiologist Dr Luc A. Devriese for his contribution to the taxonomy of staphylococci).

This description is based on the characteristics of nine isolates originating from teat apices and one isolate originating from milk of dairy cows. Cells are Gram-stain-positive, non-spore-forming cocci that occur in pairs or small clusters. Colonies are 3–4 mm in diameter after 48 h of growth on Colombia blood agar with 5% sheep blood at 35°C, have a zone of complete haemolysis of 1 mm and are grey–yellow, yellow or yellow-orange, smooth and glistening. Coagulase- and oxidase-negative and catalase-positive. Negative for starch utilization and production is seen from D-mannitol (eight strains positive). Variable acid activities. Acid is produced from D-galactose, lactose, D-glucose, sucrose, turanose, D-fructose, maltose, trehalose and L-sorbos. No acid production from melibiose, L-fucose, L-rhamnose, amygdalin, D-adonitol, arabinose, arbutol, cellobiose, D-fucose, D-lxysos, raffinos, D-sorbitol, D-tagatose, xylose, erythritol, gentiobiose, salicin or xylitol. Utilization of glycogen, dulcitol, inositol, inulin, potassium 2-ketogluconate, potassium 5-ketogluconate, pyrrolidonyl aminopeptidase, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside and methyl α-D-glucopyranoside are negative. Acid is not produced from D-mannose for eight of the tested strains. Variable acid production is seen from D-mannitol (eight strains positive), melezitose (seven positive) and glycerol (nine positive). Acetic acid is produced from D-mannitol, melezitose and glycerol, acid is not produced from D-mannose, the aesculin ferric citrate reaction is weakly positive, utilization of potassium gluconate, N-acetylglucosamine and arbutin is negative, urease activity is positive and nitrate is not reduced. The DNA G+C content of the type strain is 33.3 mol%. Details of the ten isolates studied are included in Supplementary Table S1.

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**References**


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