**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.

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..
Fig. S1, available in IJSEM Online), indicating that the ten isolates could represent an undefined *Staphylococcus* species. The aim of this study was to characterize these isolates further and to determine whether they constitute a hitherto-unknown CNS species.

The ten isolates originated from six animals from five herds in Belgium and the Netherlands. One isolate originated 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.nmconline.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 (5.9 %), iso-C15 : 0 (5.1 %), C20:0 (3.6 %), anteiso-C19 : 0 (3.3 %), iso-C16 : 0 (2.5 %), iso-C19 : 0 (1.9 %), iso-C14 : 0 (1.5 %), C16 : 0 (1.4 %) and iso-C18 : 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 molydbdophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum 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 diphosphatidylglycerol 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 to be the closest relatives, namely *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. pasteuri*, *S. warneri*, *S. caprae* and *S. piscifermentans* (not shown). Partial phylogenetic trees for the *rpoB*, *hsp60* and *tuf* genes, in which the unknown cluster and only these closest relatives were included, are shown in Supplementary Fig. S3. Because *S. piscifermentans* was distant from 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 60 and KS-SDV 19), taken from the subclusters and covering the total branch of the isolates in

![Phylogenetic tree](http://ijs.sgmjournals.org)

**Fig. 1.** Phylogenetic tree, constructed using the neighbour-joining method, based on the 16S rRNA gene sequences of strains of *Staphylococcus devriesei* sp. nov. and reference sequences of *Staphylococcus* strains available online. *Macrococcus caseolyticus* ATCC 13548 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.*
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 13349\(^T\), S. hominis LMG 13348\(^T\) and S. lugdunensis LMG 13346\(^T\)). DNA was extracted as described by Gevers et al. (2001). DNA–DNA hybridizations were carried out according to a modification of the microplate 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 60\(^T\) and KS-SDV 19. Although KS-SDV 19 belonged to a different cluster based on 16S rRNA, dnaJ and hsp60 gene sequences, it showed similarly high hybridization with KS-SP 11 and KS-SP 60\(^T\) 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 60\(^T\) and the type strains S. haemolyticus LMG 13349\(^T\) (17%), S. hominis LMG 13348\(^T\) (23%) and S. lugdunensis LMG 13346\(^T\) (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 60\(^T\) 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 60\(^T\) 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; Difco 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 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 based on 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>
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<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>S. haemolyticus LMG 13349(^T)</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>S. hominis LMG 13348(^T)</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>S. lugdunensis LMG 13346(^T)</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>

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

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>Arginine dihydrolase</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</td>
<td>b [-]</td>
<td>c</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>– D-Mannose</td>
<td>a [-]</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>– Melezitose</td>
<td>b [-]</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>c</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>– Urease</td>
<td>b [-]</td>
<td>+</td>
<td>c</td>
<td>+</td>
</tr>
</tbody>
</table>
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‘se.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 alkaline phosphatase, β-glucuronidase and β-galactosidase activities. Acid is produced from D-galactose, lactose, D-ribose, sucrose, turanose, D-fructose, maltose, trehalose and L-sorbos. No acid production from melibiose, L-fucose, L-rhamnose, amygdalin, D-adonitol, arabinitol, cellobiose, D-fucose, D-lyxose, raffinose, D-sorbitol, D-tagatose, xylose, erythritol, gentiobiose, salicin or xylitol. Utilization of glycerogen, dulcitol, inositol, inulin, potassium 2-ketogluconate, potassium 5-ketogluconate, pyrrolidonyl aminopeptidase, methyl β-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, melezitose and glycerol, acid is not produced from D-mannose, the asesuccin ferric citrate reaction is weakly positive, utilization of potassium gluconate, N-acetylgalcosamine 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


