**Streptococcus ovuberis** sp. nov., isolated from a subcutaneous abscess in the udder of a sheep

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**Abstract**

One unidentified, Gram-stain-positive, catalase-negative coccus-shaped organism was recovered from a subcutaneous abscess of the udder of a sheep and subjected to a polyphasic taxonomic analysis. Based on cellular morphology and biochemical criteria, the isolate was tentatively assigned to the genus *Streptococcus*, although the organism did not appear to match any recognized species. 16S rRNA gene sequence comparison studies confirmed its identification as a member of the genus *Streptococcus* and showed that the nearest phylogenetic relatives of the unknown coccus corresponded to *Streptococcus morocccensis* and *Streptococcus camelii* (95.9% 16S rRNA gene sequence similarity). The *sodA* sequence analysis showed less than 89.3% sequence similarity with the currently recognized species of the genus *Streptococcus*. The novel bacterial isolate was distinguished from close relatives of the genus *Streptococcus* by using biochemical tests. A mass spectrometry profile was also obtained for the novel isolate using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Based on both phenotypic and phylogenetic findings, it is proposed that the unknown bacterium be classified as a representative of a novel species of the genus *Streptococcus*, *Streptococcus ovuberis* sp. nov. The type strain of *Streptococcus ovuberissp. nov. is VB15-00779¹ (=CECT 9179¹=CCUG 69612¹).

Streptococci are responsible for a wide range of infections in domestic animals [1, 2]. In sheep, the major clinical relevance of streptococci is their role in mastitis [3–5], but they have also been associated with other pathologies such as pneumonia, arthritis, lameness and abscesses [6–9].

*Streptococcus pyogenes*, *Streptococcus uberis* and *Streptococcus ovis* are streptococcal species that have previously been associated with abscesses in sheep [10, 11]. In many cases, the streptococci are merely identified as *Streptococcus* spp. [10, 12–14], which makes it difficult to determine the precise diversity of streptococcal species associated with abscesses in sheep. This study reports on the phenotypic and phylogenetic features of a novel species of streptococci, isolated in pure culture from a subcutaneous abscess in the udder of a sheep. Its isolation in pure culture is clinically relevant, suggesting it was likely associated with the animal infection. The novel species description and differential tests available will help clinical laboratories to identify it accurately and increase knowledge of its clinical relevance as the aetiological agent involved in abscesses in sheep or other domestic animals.

Pus sample was collected from the udder abscess using a sterile syringe. Isolation of the novel isolate (designated VB15-00779¹) from the udder abscess was carried out on Columbia blood agar plates (bioMérieux) that were incubated at 37°C for 24 h under aerobic and anaerobic (with 4–10% CO₂ using a GasPak Plus (BBL) system) conditions. The taxonomic position of the clinical isolate was first determined by 16S rRNA gene sequence analysis. DNA was isolated using the method proposed by Lawson et al. [15]. A large continuous fragment (approximately 1430 bases) of the 16S rRNA gene of the isolate was amplified by PCR [16] using the universal primers pÅ (5′-AGAGTTTGAT CCTGGCTCAG; positions 8–28, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCGCA; positions 1542–1522) [17], and additional primers for the determination of the 16S rRNA gene sequence, which were described by Pascual [18]. The amplified products were purified by using a QIAquick PCR purification kit (Qiagen) and sequenced bidirectionally using an automatic DNA sequencer. The identification of the phylogenetic neighbours and calculations of pairwise 16S rRNA gene sequence...
similarities were achieved using the EzTaxon server ([19], https://www.ezbiocloud.net/). Sequence searches revealed that the unknown coccus was a member of the genus *Streptococcus*, being most closely related to *Streptococcus morocensis* and *Streptococcus cameli* (95.9 % 16S rRNA gene sequence similarity). Sequence similarity of the isolate VB15-00779 with other species of the genus *Streptococcus* was less than 95.7 %. 16S rRNA sequence divergence values ≥4.0 % between the novel clinical isolate and all other streptococcal species suggest that it represents a distinct species [20]. Sequences of the type strains of all species of the genus *Streptococcus* with validly published names were retrieved from GenBank and aligned with the newly determined sequence using the program SeqTools [21]. Phylogenetic trees were reconstructed according to three different algorithms: neighbour-joining [22] using the programs SeqTools and TREEVIEW [21, 23], maximum-parsimony and maximum-likelihood using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5 [24]. Genetic distances for the neighbouring and maximum-likelihood algorithms were calculated using Kimura's two-parameter method [25], and close-neighbour-interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. Phylogenetic trees obtained by using the neighbour-joining method (Fig. 1) and the other two methods (Fig. S1, available in the online Supplementary Material) revealed a clear affiliation of the unknown bacterium with the genus *Streptococcus*. It is evident from the phylogenetic tree based on the neighbour-joining algorithm (Fig. 1) that isolate VB15-00779 formed a distinct subline, clustering with three species (*S. morocensis*, *Streptococcus rifensis* and *Streptococcus merionis*). Although bootstrap resampling analysis did not demonstrate a significant association between the novel isolate and the aforementioned species, a similar tree topology was observed by maximum-likelihood analysis (Fig. S1a).

The phylogenetic relationships between isolate VB15-00779 and the other species of the genus *Streptococcus* were also inferred from the comparison of the sodA nucleotide sequences. A partial sequence (384 bp) was amplified using primer pair d1 and d2 [26] and sequenced as described by Glazunova et al. [27]. Evolutionary distances, the resulting trees and bootstrap values were determined as described above. When comparing the sodA gene sequence of isolate VB15-00779 with those from the type strains of the species of the genus *Streptococcus* available in GenBank, the new isolate exhibited the highest sequence similarity with *Streptococcus rupicaprae* 2777-2-07 (89.3 %). Isolate VB15-00779 formed a separate branch from other species of the genus *Streptococcus* in the phylogenetic trees inferred from the sodA gene sequence comparison (Fig. S2). The sodA sequence similarities of VB15-00779 and the type strains of its closest phylogenetic relatives based on the 16S rRNA gene sequence were below 83 % (divergence values of 17.3 % with regard to *S. morocensis* and 15.4 % with regard to *S. cameli*). These divergence values are higher than the mean interspecies divergence values obtained for this gene between pair species of the genus *Streptococcus* [28]. These data support a separate species status for the unidentified catalase-negative cocci.

The determination of the G+C content of the DNA of strain VB15-00779 was performed at the Spanish Type Culture Collection (CECT; Valencia, Spain) by using the real-time PCR System described by Gonzalez and Saiz-Jimenez [29]. Calibration curves were obtained from melting temperatures (Tm) of total genomic DNA and the DNA G+C content (mol%). The DNA G+C content (mol%) was obtained from the whole-genome sequence of the microorganism used in this study, including every genetic element, as [(G+C)/(A+T+C+G)]×100. Genome sequences were obtained from Entrez Genomes (https://www.ncbi.nlm.nih.gov/genome/browse/) at NCBI. Least-square linear regression analyses and slope comparisons were performed according to the methods of Sokal and Rohlf [30]. The DNA G+C content of the type strain VB15-00779 was 41.3 mol%.

The newly isolated strain was Gram-stained and assessed for the presence of catalase. A haemolytic reaction was determined on Columbia agar containing 5 % defibrinated sheep blood (bioMérieux) and incubated aerobically at 37 °C for 24 and 48 h [31]. Determination of the growth at 15, 25, 30, 37 and 42 °C was performed in brain heart infusion broth (Difco) with the pH adjusted to pH 7.5 [31]. The ability of the isolate to tolerate the presence of 3.5, 4.5 and 6.5 % NaCl was assessed as recommended by Facklam and Elliot [31]. Lancefield serological group reaction was
Table 1. Characteristics useful in differentiating *Streptococcus ovuberis* sp. nov. from closely phylogenetically related species

<table>
<thead>
<tr>
<th>Strains: 1, <em>S. ovuberis</em> sp. nov. VB15-00779(^1); 2, <em>S. moroccensis</em> LMG 27682(^2); 3, <em>S. cameli</em> LMG 27685(^1). Data are taken from this study.</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tr>
<td><strong>API Rapid ID 32 Strep</strong></td>
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<tr>
<td>Hydrolysis of:</td>
<td>Arginine</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Urea</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Production of:</td>
<td>Glycyl-tryptophan arylamidase</td>
<td>–</td>
<td>+</td>
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<tr>
<td>β-Mannosidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td><strong>Production of acid from:</strong></td>
<td>Methyl β-D-glucopyranoside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pullulan</td>
<td>+</td>
<td>–</td>
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<td><strong>API ZYM</strong></td>
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<tr>
<td>Production of:</td>
<td>β-Glucuronidase</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Valine arylamidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Naphthol-AS-BI-phophohydrolase</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td><strong>API 50 CH</strong></td>
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<tr>
<td>Production of acid from:</td>
<td>D-Xylose</td>
<td>–</td>
<td>+</td>
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<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>D-Mannitol</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Amygdalin</td>
<td>+</td>
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<td>Turanose</td>
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<td>Gentiobiose</td>
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<td>Melezitose</td>
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<td>Raffinose</td>
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<td>Starch</td>
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<tr>
<td>Glycogen</td>
<td>–</td>
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</table>

strains were grown on Columbia sheep agar plates at 37 °C under aerobic conditions for 24 h, and single colonies were selected for MALDI-TOF MS analysis. A loop of cells was harvested, and protein extraction for MALDI-TOF MS analysis was performed according to the method of Vela et al. [32]. Mass spectra acquisition was performed on a Bruker UltraFlextrem platform (Bruker Daltonics) using a mass range of 2–20 kDa. The Bruker Bacterial Test Standard (E. coli DH5) was used as an external protein calibration mixture [33]. Mass spectra were smoothed, the baseline was corrected and peaks were detected using the FlexAnalysis software (version 3.4; Bruker Daltonics). Three biological replicates from each sample were created and analysed to test the reproducibility of the procedure. The spectrum of the strain VB15-00779\(^1\) generated always gave score values of <1.6, indicating an unreliable identification and therefore that strain VB15-00779\(^3\) was not misidentified as any of the streptococcal species included in the MALDI Biotyper database. The spectrum of strain VB15-00779\(^1\) showed differential mass peaks compared with the other two type strains (e.g. m/z 4044, 4877, 7390 and 8088; Fig. 2).

Based on 16S rRNA gene sequences, phylogenetic analysis, phenotypic analysis and MALDI-TOF MS data, it may be concluded that strain VB15-00779\(^1\) represents a novel species of the genus *Streptococcus* for which we propose the name *Streptococcus ovuberis* sp. nov.

**DESCRIPTION OF *STREPTOCOCCUS OVUBERIS* SP. NOV.**

*Streptococcus ovuberis* (ov.u’be.ris. L. fem. n. ovis sheep; L. neut. n. uber udder; N.L. gen. n. ovuberis of a sheep udder).

Cells are Gram-stain-positive, non-spore-forming cocci, 0.5 μm in diameter, occurring in pairs or chains commonly over three cells long. Colonies on blood agar are small, circular and non-pigmented, 0.75–1.0 mm in diameter and α-haemolytic at 37 °C. Cells are facultatively anaerobic, catalase-negative, non-motile and do not react with Lancefield group A, B, C, D, F or G antiserum. Cells are able to grow at 30, 37 and 42 °C but not at 15 or 25 °C. Growth occurs in the presence of 3.5 and 4.5% NaCl, not however with 6.5% NaCl. Cells are able to produce acid from L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, N-acetylgulcosamine, methyl β-D-glucopyranoside, amygdalin, arbutin, ascelin, salicin, cellobiose, maltose, lactose, sucrose, starch, trehalose, inulin, glycogen, pullulan and gentiobiose but not from L-rhamnose, methyl α-D-glucopyranoside, raffinose, glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylpyranoside, L-sorbitose, L-arabitol, D-arabitol, D-mannitol, D-ribose, melibiose, melezitose, D-sorbitol, inositol, dulcitol, methyl α-D-mannopyranoside, turanose, D-lyxose, xylitol, D-fucose, L-fucrose, 2-keto-gluconate, 5-keto-gluconate, cyclodextrin or tagatose. Alanine-phenylalanine-proline arylamidase, β-mannosidase, β-glucuronidase, leucine arylamidase and naphthol-AS-BI-phophohydrolase are detected. No activity is detected for alkaline phosphatase, esterase C4, ester lipase C8, glycyll...
tryptophan arylamidase, lipase C14, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucosaminidase, pyroglytamic acid arylamidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, valine arylamidase, β-galactosidase or α-glucosidase. Arginine is hydrolysed but not hippurate or urea. Acetoin is not produced.

The type strain, VB15-00779T (=CECT 9179T =CCUG 69612T), was isolated from a subcutaneous abscess in the udder of a sheep. Full range of habitat is not known. The DNA G+C content of the type strain is 41.3 mol%.

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Conflicts of interest
The authors of this paper deny any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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