**Streptococcus pharyngis** sp. nov., a novel streptococcal species isolated from the respiratory tract of wild rabbits

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Four isolates of an unknown Gram-stain-positive, catalase-negative coccus-shaped organism, isolated from the pharynx of four wild rabbits, were characterized by phenotypic and molecular genetic methods. The micro-organisms were tentatively assigned to the genus *Streptococcus* based on cellular morphological and biochemical criteria, although the organisms did not appear to correspond to any species with a validly published name. Comparative 16S rRNA gene sequencing confirmed their identification as members of the genus *Streptococcus*, being most closely related phylogenetically to *Streptococcus porcorum* 682-03T (96.9 % 16S rRNA gene sequence similarity). Analysis of *rpoB* and *sodA* gene sequences showed divergence values between the novel species and *S. porcorum* 682-03T (the closest phylogenetic relative determined from 16S rRNA gene sequences) of 18.1 and 23.9 %, respectively. The novel bacterial isolate could be distinguished from the type strain of *S. porcorum* by several biochemical characteristics, such as the production of glycyl-tryptophan arylamidase and α-chymotrypsin, and the non-acidification of different sugars. Based on both phenotypic and phylogenetic findings, it is proposed that the unknown bacterium be assigned to a novel species of the genus *Streptococcus*, and named *Streptococcus pharyngis* sp. nov. The type strain is DICM10-00796B\(^\top\) (=CECT 8754\(^\top\)=CCUG 66496\(^\top\)).

Wild European rabbits (*Oryctolagus cuniculus*) have ecological and economic importance in the Mediterranean area, as they represent an economically important hunting activity and sustain a large number of predator species, hence contributing to the preservation of the diversity of the Mediterranean ecosystem (Delibes-Mateos et al., 2008; Gálvez et al., 2009). However, knowledge of the bacterial species isolated from wild rabbits is very limited. In this study, we report the phenotypic and phylogenetic features of four *Streptococcus*-like organisms (DICM10-00793A, DICM10-00795A, DICM10-00796B\(^\top\) and DICM10-00803A) isolated from pharyngeal swab samples taken from four apparently healthy wild rabbits hunted on the same day and at the same location. Samples were collected, transported under refrigeration to the laboratory and processed for bacteriological analysis within 48 h. Rabbits were located in a plot inside an industrial park (429589.5 E 4593922.2 N). Samples were cultured on Columbia-CNA agar plates (bioMérieux) that were incubated at 37 °C for 24 h under aerobic and anaerobic [with 4–10 % CO\(_2\) using a GasPak Plus system (BBL)] conditions. Based on the phenotypic and phylogenetic results, a novel species of the genus *Streptococcus* is proposed.

Phylogenetic analysis was performed by comparative 16S rRNA gene sequence analysis as described previously (Vela et al., 2002). A large continuous fragment (approximately 1420 bases) of the 16S rRNA gene of the four isolates (DICM10-00793A, DICM10-00795A, DICM10-00796B\(^\top\) and DICM10-00803A) was determined from PCR-amplified products, derived from universal primers.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB* and *sodA* gene sequences of strain DICM10-00796B\(^\top\) are LN624399, LN624401 and LN624400, respectively. Those for the 16S rRNA gene sequences of strains DICM10-00793A, DICM10-00795A and DICM10-00803A are LN651169, LN651170 and LN651168, respectively.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.

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Fig. 1. Neighbour-joining phylogenetic tree inferred from comparison of partial 16S rRNA gene sequences of strains of Streptococcus pharyngis sp. nov. and related members of the genus Streptococcus. Enterococcus faecalis ATCC 19433T was used as an out-group (not shown). Filled circles indicate that the corresponding nodes (groupings) were also obtained in the maximum-parsimony tree. Open circles indicate that the corresponding nodes (groupings) were also obtained in the maximum-likelihood and maximum-parsimony trees. Bootstrap values (expressed as a percentage of 1000 replications) higher than 70 % are given at branch points. The different branches were supported by the results of the other two algorithms. Bar, 1 % sequence divergence.

The four novel isolates were Gram-stained and assessed for the presence of catalase. The haemolytic reaction was determined by the procedure described by Facklam & Elliott (1995). The isolates were biochemically characterized using the Rapid ID32 STREP and API ZYM systems (bioMérieux) according to the manufacturer's instructions. All these data support separate species status for the unidentitied streptococci from wild rabbits.

Additional gene sequence analyses were carried out to clarify the phylogenetic affinities of the novel isolates. Partial sequences of the rpoB (701 bp) and sodA (397 bp) genes were amplified using the primer pairs d1 and d2 (Poyart et al., 1998) and StreptoF and StreptoR (Drancourt et al., 2004), respectively, and sequenced as described previously (Glazunova et al., 2006). Comparative sequence analysis revealed 99.2–100 and 98.2–100 % sequence similarities between the isolates for the sodA and rpoB gene sequences, respectively. Isolate DICM10-00796BT exhibited highest sequence similarities with Streptococcus dysgalactiae CIP 102914T (85.3 %) and Streptococcus cuniciuli NED12-00049-6BT (76.3 %) based on the rpoB and sodA genes, respectively. Levels of rpoB and sodA gene sequence divergence between DICM10-00796BT and S. porcorum 682-03T were 18.1 and 23.9 %, respectively (sequence similarity values of 81.9 and 76.1 %, respectively). These divergence values are higher than the mean interspecies divergence values obtained with these genes between pairs of species of the genus Streptococcus (Glazunova et al., 2009). Isolate DICM10-00796BT formed a separate branch from other species of the genus Streptococcus in the phylogenetic trees inferred from the rpoB (Fig. S1, available in the online Supplementary Material) and sodA (Fig. S2) genes. All these data support separate species status for the unidentified streptococci from wild rabbits.

The new isolates exhibited almost identical biochemical and physiological properties, being similar to Enterococcus faecalis (ATCC 19433) and being unable to grow at 42 °C.

The new isolates exhibited high homology to the type strain of the species Strep S. porcorum, being related most closely to S. porcorum 682-03T (96.9 % 16S rRNA sequence similarity). Sequence similarities among the novel isolates and other Streptococcus species were in the range of 99.2–100 %, with the highest value obtained between DICM10-00796BT and S. porcorum 682-03T, followed by the similarity between DICM10-00796BT and S. porcorum 682-03T (76.3 %). These results support the conclusion that the novel isolates belong to a new species of the genus Streptococcus.
related species based on 16S rRNA gene similarities) by several phenotypic characteristics such as acid production from lactose, methyl β-D-glucopyranoside, maltose and sucrose (S. porcorum is positive), and the production of glycy1-tryptophan arylamidase and α-chymotrypsin (S. porcorum is negative). The phenotypic characteristics that differentiate the novel strains from S. cuniculi, which was isolated from nasal and tonsil samples of rabbits (Vela et al., 2014), are shown in Table 1. Because determination of the Lancefield group antigen is still an important routine identification technique, the serological group reaction of the isolates was determined with a commercial Slidex Strepto kit (bioMérieux) by using specific group A, B, C, D, F and G streptococcal latex agglutinating antisera. The novel strains reacted with Lancefield group G antisera. Characteristics differentiating the proposed novel species from other streptococcal ‘species groups’ (Whiley & Hardie, 2009) with respect to the Lancefield group G antigen are indicated in Table S1.

The four strains from wild rabbits were characterized by pulsed-field gel electrophoresis profiling of their genomic DNAs, after digestion with the restriction enzyme Bsp120I, according to previous specifications (Vela et al., 2003). Similarities between restriction endonuclease digestion profiles were based on visual comparisons of the band patterns of strains run in the same gel. Isolates displayed indistinguishable pulsed-field gel electrophoresis restriction profiles (data not shown), which suggests a clonal relationship (although isolates were derived from different animals).

Based on the phylogenetic, genotypic and phenotypic data presented, we consider DICM10-00796B\textsuperscript{T} to be the type strain of a novel species of the genus Streptococcus, for which the name Streptococcus pharyngis sp. nov. is proposed. Given that four isolates were obtained from apparently healthy animals, it is not possible to reach any conclusions about possible non-pathogenic roles for this novel species of Streptococcus in wild rabbits.

**Description of Streptococcus pharyngis sp. nov.**

Streptococcus pharyngis (pha.ryn’gis. Gr. n. pharynx throat; Gr. gen. n. pharyngis of the throat).

Cells are Gram-stain-positive, non-spore-forming cocci, 0.5–0.75 mm in diameter, occurring in pairs or chains commonly over 7–12 cells long. Colonies on blood agar are small, circular and non-pigmented, 0.25–0.5 mm in diameter and α-haemolytic at 37 °C after 24 h. Cells are facultatively anaerobic, catalase-negative and non-motile. Reacts with Lancefield group G antisera. Growth occurs at 30, 37 and 42 °C, but not at 4, 15 or 22 °C. Growth does not occur in broth containing 3.5, 4.5 or 6.5 % (w/v) NaCl. Cells are not able to produce acid from ribose, mannitol, sorbitol, trehalose, raffinose, sucrose, L-arabinose, D-arabitol, cyclodextrin, glycogen, pullulan, maltose, lactose, melibiose, melezitose, methyl β-D-glucopyranoside or tagatose. Leucine arylamidase, esterase C4, lipase C14 (weak reaction), naphthol-AS-BI-phosphohydrolase (weak reaction), acid phosphatase, α-chymotrypsin and glyceryl-tрypthphan arylamidase activities are detected. No activity is detected for alanine-phenylalanine-proline arylamidase, α-mannosidase, α-fucosidase, esterase lipase C8, valine arylamidase, cystine arylamidase, trypsin, β-glucuronidase, β-N-acetyl-α-glucosaminidase, β-galactosidase, α-galactosidase, pyrogglutactic acid arylamidase or β-mannosidase. Arginine, hippurate and urea are not hydrolysed and acetoin is not produced. The type strain, DICM10-00796B\textsuperscript{T} (=CECT 8754\textsuperscript{T} =CCUG 66496\textsuperscript{T}), was isolated from the pharynx of a wild rabbit. Three additional strains of the species (DICM10-00793A, DICM10-00795A and DICM10-00803A) were also isolated from the pharynx of wild rabbits.

**Acknowledgements**

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**References**


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**Table 1. Characteristics that are useful in differentiating Streptococcus pharyngis from S. cuniculi**

Data are taken from this study. +, Positive; –, negative; NG, non-groupable against Lancefield grouping antisera.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DICM10-00796B\textsuperscript{T}</th>
<th>S. cuniculi NED12-00049-6B\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lancefield antigen*</td>
<td>G</td>
<td>NG</td>
</tr>
<tr>
<td>Production of acid from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pullulan</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Sucrose</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Production of:</td>
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<tr>
<td>Alanine-phenylalanine-proline arylamidase</td>
<td>–</td>
<td>+</td>
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<tr>
<td>α-Galactosidase</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>β-Galactosidase</td>
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<td>+</td>
</tr>
<tr>
<td>Pyroglutamic acid arylamidase</td>
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<td>+</td>
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<tr>
<td>Production of:</td>
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</tr>
<tr>
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<td>–</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
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