**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 DICM10-00796Bᵀ (=CECT 8754ᵀ=CCUG 66496ᵀ).

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; Galvez 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, DICM10-00796Bᵀ 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 (42°58’59.5E 45°39’22.2N). 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 % CO₂ using a GasPak Plus system (BBL)] conditions. Based on the phenotypic and phylogenetic findings, it is proposed that the unknown bacterium be assigned to 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, DICM10-00796Bᵀ and DICM10-00803A) was determined from PCR-amplified products, derived from universal primers.
pA (59-AGAGTTTGATCCTGCTGTCAG-39; positions 8–27, Escherichia coli numbering) and pH+ (59-AAGGAG-GTGGATCCAGCCGC-39; positions 1541–1522) and sequenced bidirectionally. Primers pA, pH+ and antiKK (59-GTGCCAGCAGCGCAGGTAAT-39; positions 517–537, E. coli numbering) and 3 (59- GTTGCCGTCGT-TGCCGGA-39; positions 1109–1090, E. coli numbering) were used in the gene sequencing reaction. Comparative sequence analysis revealed 100 % sequence similarity between the isolates, thereby demonstrating their high genealogical relatedness. The identification of phylogenetic neighbours and calculations of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (http://ezbiocloud.net/eztaxon; Kim et al., 2012). Sequence searches revealed that the unknown cocci were members of the genus Streptococcus, being related most closely to Streptococcus porcorum 682-03T (96.9 % 16S rRNA gene sequence similarity). Sequence similarities between isolate DICM10-00796B T and other species of the genus Streptococcus were less than 96.5 %. Sequences of the type strains of all species of the genus Streptococcus with validly published names were retrieved from the GenBank database and aligned with the newly determined sequence using the program SeqTools (Rasmussen, 2002). Phylogenetic trees were reconstructed according to three different algorithms: neighbour-joining (Saitou & Nei, 1987) using the programs SeqTools and TreeView (Page, 1996; Rasmussen, 2002), maximum-parsimony using the software package MEGA version 4 (Kumar et al., 2004) and maximum-likelihood using PHYML software (Guindon & Gascuel, 2003). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated using Kimura’s two-parameter method (Kimura, 1980), and close-neighbour-interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications). Phylogenetic trees obtained by using the neighbour-joining algorithm (Fig. 1) and the other two methods revealed a clear affiliation of the unknown cocci to the genus Streptococcus. It is evident from the phylogenetic tree based on the neighbour-joining algorithm (Fig. 1) that the unknown isolates formed a separate subline, not clustering with any species of the genus Streptococcus, which was not supported by bootstrap resampling. A similar result was obtained with maximum-parsimony analysis. However, strains were recovered with Streptococcus danielliae in the phylogenetic tree based on the maximum-parsimony method, although this relationship was not supported by bootstrap resampling (bootstrap support 10 %; data not shown).

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 DICM10-00796B T exhibited highest sequence similarities with Streptococcus dysgalactiae CIP 102914T (85.3 %) and Streptococcus cuniculi NED12-0049-6B T (76.3 %) based on the rpoB and sodA genes, respectively. Levels of rpoB and sodA gene sequence divergence between DICM10-00796B T and S. porcorum 682-03T were 18.1 and 23.9 %, respectively (sequence similarities 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 DICM10-00796B T 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 four novel isolates were Gram-stained and assessed for the presence of catalase. The haemolytic reaction was determined on Columbia agar containing 5 % defibrinated sheep blood (bioMérieux) incubated aerobically at 37 °C for 24 and 48 h (Facklam & Elliott, 1995). Determination of growth at 4, 15, 22, 30, 37 and 42 °C was performed in brain heart infusion broth (Difco) with the pH adjusted to 7.5 (Facklam & Elliott, 1995). The ability of the isolates to tolerate the presence of 3.5, 4.5 and 6.5 % (w/v) NaCl was assessed as recommended 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. The new isolates exhibited almost identical biochemical characteristics, except for the acidification of trehalose (isolate DICM10-00803A was positive). The novel isolates could be distinguished from S. porcorum (the closest
related species based on 16S rRNA gene similarities) by several phenotypic characteristics such as acid production from lactose, methyl $\beta$-d-glucopyranoside, maltose and sucrose (S. porcorum is positive), and the production of glycyl-tryptophan arylamidase and $\alpha$-chymotrypsin (S. porcorum is negative). The phenotypic characteristics that differentiate the novel strains from S. cuniculi, which was isolated from nasal and tonsill 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 Strept 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 antiserum. 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).

### 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$^T$</th>
<th>S. cuniculi</th>
<th>NED12-00049-6B$^T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lancefield antigen*</td>
<td>G</td>
<td>NG</td>
<td></td>
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<tr>
<td>Production of acid from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pullulan</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Maltose</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Production of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine–phenylalanine–proline arylamidase</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>$\alpha$-Galactosidase</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>$\beta$-Galactosidase</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Pyroglutamic acid arylamidase</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Production of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

$Lancifield serological group reaction using group A-, B-, C-, D-, F- and G-specific streptococcal latex agglutinating antisera.

Based on the phylogenetic, genotypic and phenotypic data presented, we consider DICM10-00796B$^T$ 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 $\alpha$-haemolytic at 37 $^\circ$C after 24 h. Cells are facultatively anaerobic, catalase-negative and non-motile. Reacts with Lancefield group G antiserum. Growth occurs at 30, 37 and 42 $^\circ$C, but not at 4, 15 or 22 $^\circ$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 $\beta$-d-glucopyranoside or tagatose. Leucine arylamidase, esterase C4, lipase C14 (weak reaction), naphthol-AS-BI-phosphohydrolase (weak reaction), acid phosphatase, $\alpha$-chymotrypsin and glycolytic-tryptophan arylamidase activities are detected. No activity is detected for alanine–phenylalanine–proline arylamidase, $\alpha$-mannosidase, $\alpha$-fucosidase, esterase lipase C8, valine arylamidase, cystine arylamidase, trypsin, $\beta$-glucuronidase, N-acetyl-$\beta$-glucosaminidase, $\beta$-galactosidase, $\beta$-galactosidase, pyroglutamic acid arylamidase or $\beta$-mannosidase. Arginine, hippurate and urea are not hydrolysed and acetoin is not produced.

The type strain, DICM10-00796B$^T$ (=CECT 8754$^T$ =CCUG 66496$^T$), 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.

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### References


