Streptococcus rupicaprae sp. nov., isolated from a Pyrenean chamois (*Rupicapra pyrenaica*)

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Biochemical and molecular genetic studies were performed on an unknown Gram-stain-positive, catalase-negative, coccus-shaped organism isolated from clinical samples of a Pyrenean chamois. The micro-organism was identified as a streptococcal species based on its cellular morphological and biochemical tests. 16S rRNA gene sequence comparison studies confirmed its identification as a member of the genus *Streptococcus*, but the organism did not correspond to any species of this genus. The nearest phylogenetic relative of the unknown coccus from chamois was *Streptococcus ovis* (95.9 % 16S rRNA gene sequence similarity). The *rpoB* and *sodA* sequence analysis showed sequence similarity values of less than 85.7 % and 83.0 %, respectively, with the currently recognized species of the genus *Streptococcus*. The novel bacterial isolate was distinguished from *S. ovis* and other species of the genus *Streptococcus* using biochemical tests. Based on both phenotypic and phylogenetic findings, it is proposed that the unknown bacterium be classified as a novel species of the genus *Streptococcus*, *Streptococcus rupicaprae* sp. nov., with the type strain 2777-2-07^T (=CECT 7718^T =CCUG 59652^T).

Streptococci can be isolated as part of the normal flora of the alimentary, respiratory and genito-urinary tracts, as well as the skin of man and different domestic animals (Kilian, 1998), and some species are well-established pathogens causing a variety of diseases such as endometritis, respiratory infections, endocarditis, meningitis, arthritis or mastitis (Chanter, 1997). Members of the genus *Streptococcus* may also cause diseases in wild animals. Thus, *Streptococcus pneumoniae* has been associated with a lethal pneumonia in a captive juvenile chimpanzee (Szentiks *et al.*, 2009), *Streptococcus didelphis* has been isolated from opossums (*Didelphis virginiana*) with suppurative dermatitis and liver fibrosis (Rurangirwa *et al.*, 2000) and *Streptococcus equi* subsp. *ruminatorum* has been documented as an agent of a severe infection in spotted hyenas (Höner *et al.*, 2006; Speck *et al.*, 2008). However, in general, information on the nature of the streptococcal species and their host distribution is extremely poor and there are still many unknown or poorly understood aspects of the wildlife diseases. In this study, a septicaemic process affecting a Pyrenean chamois (*Rupicapra pyrenaica*) from the National Hunting Reserve of Freser-Setcases in the Pyrenees (NE Spain) was investigated. The animal was captured alive and transported to the facilities of the Wildlife Diseases Research Group at Universitat Autonoma of Barcelona. It arrived dying and was euthanized and necropsied. Macroscopically, multiple abscesses in the liver and subendocardic haemorrhages were observed. The main histological lesions were foci of suppurative necrosis in the spleen and liver. A coccus-shaped organism was isolated in pure culture from spleen (1382-7-09), liver abscess (1382-6-09) and liver (2777-2-07^T) on Columbia blood agar plates (bioMérieux) incubated for 24 h at 37 °C under both aerobic and anaerobic [with 4–10 % CO_2 using GasPak Plus (BBL) system] conditions. On the basis of the phenotypic and phylogenetic results, a novel species of the genus *Streptococcus*, *Streptococcus rupicaprae* sp. nov., is proposed.

A phylogenetic analysis was performed by comparative 16S rRNA gene sequence analysis as described previously (Vela *et al.*, 2002). A large, continuous fragment (approx. 1440

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB* and *sodA* gene sequences of strain 2777-2-07^T are FN813250, FN821809, FN821810, respectively.

A supplementary table and three supplementary figures are available with the online version of this paper.

bases) of the 16S rRNA gene of one isolate (2777-2-07^T) and 1000 nt from the other two isolates (1382-6-09 and 1382-7-09) was obtained bidirectionally using universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′; positions 8–27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA-3′; positions 1541–1522, *E. coli* numbering). Comparative sequence analysis revealed 100 % sequence similarity between the isolates, thereby demonstrating their high genealogical relatedness. Sequence searches of GenBank using the program FASTA (Pearson, 1994) revealed that the unknown cocci were member of the genus *Streptococcus*, being most closely related to *Streptococcus ovis* S369/98/1^T (95.9 % 16S rRNA gene sequence similarity). Sequence similarity of isolate 2777-2-07^T with other species of the genus *Streptococcus* was less than 95.3 %. This sequence and those of other representative species with validly published names within the genus *Streptococcus* were retrieved from GenBank 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 the PHYML software (Guindon & Gascuel, 2003). Genetic distances for the neighbour-joining algorithm were calculated by the Kimura two-parameter model (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). The maximum-likelihood tree was calculated using the GTR model (Lanave *et al.*, 1984), based on the hill-climbing principle and estimated proportion of invariable sites, as well as the Gamma distribution parameter. The parameters in the program PHYML were as follows: input sequences are interleaved, with 500 non-parametric bootstrap analysis, GTR model of nucleotide substitution, 4 substitution rate categories, fixed Gamma distribution parameter (alpha=2.00). Phylogenetic trees obtained by using the neighbour-joining (Fig. 1; an extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online) and the other two methods (data not shown) revealed a clear affiliation of the unknown cocci (as exemplified by strain 2777-2-07^T) to the genus *Streptococcus*. It is evident from the phylogenetic tree based on the neighbour-joining algorithm (Fig. 1) that isolate 2777-2-07^T formed a distinct subline, clustering within a small subgroup of species (embracing *S. ovis*, *Streptococcus minor*, *Streptococcus merionis* and *Streptococcus gallinaceus*). Although bootstrap resampling analysis did not demonstrate a significant association between isolate 2777-2-07^T and the aforementioned species, the different branches were supported by the results of the other two algorithms. 16S rRNA sequence divergence values of >4 % between the novel isolate and *S. ovis* suggests that it represents a distinct species (Stackebrandt & Goebel, 1994).

Sequence analysis of *sodA* and *rpoB* genes has been demonstrated to be a useful tool for differentiating streptococci on phylogenetic grounds (Poyart *et al.*, 1998, 2002; Drancourt *et al.*, 2004). Partial sequences of *rpoB*

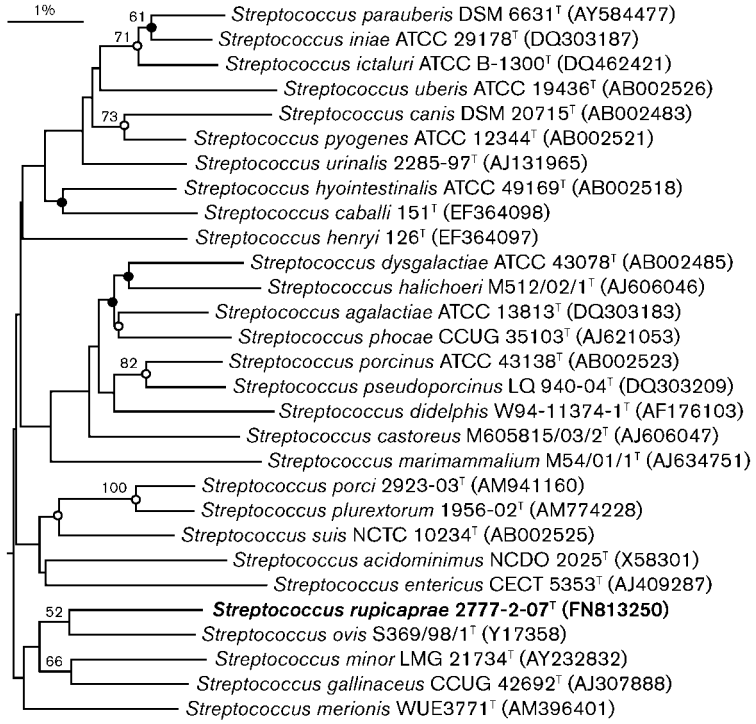

Fig. 1. Partial neighbour-joining phylogenetic tree inferred from comparison of 16S rRNA gene sequences showing the position of strain 2777-2-07^T (*Streptococcus rupicaprae* sp. nov.) and its closest phylogenetic neighbours. *Enterococcus faecalis* ATCC 19433^T was used as the out-group. Filled circles indicate that the corresponding nodes (groupings) are also obtained in the parsimony tree. Open circles indicate that the corresponding nodes (groupings) are also obtained in the maximum-likelihood and parsimony trees. Bootstrap values (expressed as a percentage of 1000 replications) higher than 50 % are given at the branching points. The different branches were supported by the results of the other two algorithms. Bar, 1 % sequence divergence.

(701 bp) and *sodA* (356 bp) genes were amplified using primer pair d1 and d2 (Poyart *et al.*, 1998) and primers StreptoF and StreptoR (Drancourt *et al.*, 2004), respectively, and sequenced as described previously (Glazunova *et al.*, 2006). The three isolates shared 100 % similarity for the *rpoB* and *sodA* gene sequences, respectively. When comparing the *rpoB* and *sodA* gene sequences of strain 2777-2-07^T with those from the type strains of species of the genus *Streptococcus* available in GenBank, the highest sequence similarity for isolate 2777-2-07^T was with *S. ovis* CIP 107097^T (82.8 %) based on the *sodA* gene, while it shared the highest sequence similarity with *S. minor* CIP 108314^T and *S. ovis* CIP 107097^T (85.7 % and 85.6 %, respectively) based on the *rpoB* gene. Evolutionary distances, the resulting trees and bootstrap values were determined as described above. Isolate 2777-2-07^T formed a separate branch from other species of the genus *Streptococcus* in the phylogenetic trees inferred from *rpoB* and *sodA* gene sequence comparisons (Supplementary Figs S2 and S3, respectively).

The determination of the G+C content of the DNA of one representative isolate (strain 2777-2-07^T) was performed at the DSMZ (Braunschweig, Germany) by using the HPLC method of Mesbah *et al.* (1989). The DNA G+C content of the type strain was 43.8 mol%.

The three new isolates were Gram-stained and assessed for the presence of catalase. 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 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, 4.5 and 6.5 % NaCl was assessed as recommended by Facklam & Elliott (1995). Growth at pH 9.6 was assessed in brain heart infusion broth (Facklam & Elliott, 1995). Lancefield serological group reaction was determined with the commercial Slidex Strepto kit (bioMérieux) by using specific group A, B, C, D, F and G streptococcal latex agglutinating antisera. The isolates were biochemically characterized using the Rapid ID32 Strep, API 50 CH and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The API 50 CH strips using the CHB suspension medium were read at up to 7 days of incubation at 37 °C. Isolates exhibited almost identical biochemical characteristics, except for the production of glycyl-tryptophan arylamidase (isolate 1382-6-09 was positive).

Strain 2777-2-07^T is phylogenetically related to streptococcal species, such as *S. ovis*, *S. minor* and *S. gallinaceus*, not assigned to any of the major recognized species groups (Wheiley & Hardie, 2009). Phenotypic characteristics that differentiate the proposed species from closely related species are shown in Table 1. Determination of the Lancefield group antigen is still an important routine identification technique (Quinn *et al.*, 1999). *S. rupicaprae* sp. nov. reacted with Lancefield group D antisera and, in addition to the closely related species *S. gallinaceus* (see Table 1), it can be differentiated from other streptococci of the Lancefield group D by several biochemical characteristics (Supplementary Table S1).

Overall, the results of the present study show that the unidentified catalase-negative cocci merit classification as a novel species of the genus *Streptococcus*, for which the name *Streptococcus rupicaprae* sp. nov. is proposed. Although it was isolated from the liver and spleen of a chamois with a septicaemic process, it is not possible to draw conclusions about the clinical significance of the novel species.

Description of *Streptococcus rupicaprae* sp. nov.

Streptococcus rupicaprae (ru.pi.cap′rae. L. fem. n. *rupicapra* chamois; L. fem. gen. n. *rupicaprae* of a chamois).

Cells are Gram-stain-positive, non-spore-forming cocci, 0.7–1 μm in diameter, occurring in pairs or chains commonly over 7–10 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 and non-motile. Reacts with Lancefield group D antisera. Cells are able to grow at 22, 30, 37 and 42 °C but do not grow at 15 °C. Growth occurs at pH 9.6 and in the presence of 3 % NaCl but not in broth containing 4.5 or 6.5 % NaCl. With the API 50 CH and Rapid ID32 Strep kits, cells are able to produce acid from D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, inulin, raffinose, starch, glycogen, melibiose, pullulan, L-arabinose, gentiobiose and methyl β-D-glucopyranoside but not from glycerol, erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-arabitol, D-arabitol, D-mannitol, D-sorbitol, inositol, dulcitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, melezitose, turanose, D-lyxose, xylitol, D-fucose, L-fucose, 2-ketogluconate, 5-ketogluconate, cyclodextrin or tagatose. Leucine arylamidase, β-galactosidase (API ZYM), β-glucuronidase (API ZYM and Rapid ID32 Strep), β-mannosidase and alanine-phenylalanine-proline arylamidase (Rapid ID32 Strep) are detected. No activity is detected for acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-mannosidase, α-fucosidase, esterase C4, ester lipase C8, lipase C14, valine arylamidase, α-glucosidase, cystine arylamidase, trypsin, α-chymotrypsin (API ZYM), *N*-acetyl-β-glucosaminidase, alkaline phosphatase (API ZYM and Rapid ID32 Strep) or pyroglutamic acid arylamidase (Rapid ID32 Strep). Cells produce β-glucosidase and α-galactosidase using the Rapid ID32 Strep system. Arginine is hydrolysed but not hippurate or urea (Rapid ID32 Strep). Type strain (2777-2-07^T) does not produce glycyl-tryptophan arylamidase (Rapid ID 32 Strep). Acetoin is not produced (Rapid ID 32 Strep).

The type strain, 2777-2-07^T (=CECT 7718^T =CCUG 59652^T), was isolated from the liver of a chamois with

Table 1. Characteristics useful in differentiating strain 2777-2-07^T from closely related species of the genus *Streptococcus*

Strains: 1, *S. rupicaprae* sp. nov. 2777-2-07^T; 2, *S. ovis* CCUG 39485^T; 3, *S. minor* CCUG 47487^T; 4, *S. merionis* CCUG 54871^T; 5, *S. gallinaceus* CCUG 42692^T; 6, *Streptococcus pyogenes* CCUG 4207^T. *S. pyogenes* was included as representative type species of the genus *Streptococcus*. Lancefield serological group reaction using group A-, B-, C-, D-, F- and G-specific streptococcal latex agglutinating antisera. Phenotypic data for all strains from this study. +, Positive reaction; −, negative reaction; NG, non-groupable; PA, polyagglutination reaction with A, B, C, D, F and G antisera; ^{W}, weak reaction.

Characteristic	1	2	3	4	5	6
Lancefield group antigen	D	NG	NG	D^W	PA	A
API Rapid ID 32 Strep						
Hydrolysis of arginine	+	−	+	−	+	+
Production of:						
β-Glucosidase	+	+	+	−	+	−
β-Glucuronidase	+	−	−	−	−	−
α-Galactosidase	+	+	+	−	+	−
Alkaline phosphatase	−	−	−	+	−	+
β-Mannosidase	+	−	+	−	−	−
Production of acid from:						
D-Mannitol	−	+	+	−	+	−
Raffinose	+	+	−	−	+	−
Sorbitol	−	+	+	−	−	−
Methyl β-D-glucopyranoside	+	−	+	−	+	+
Lactose	+	+	+	−	+	+
Pullulan	+	−	+	+	+	+
D-Ribose	−	−	−	−	+	−
API ZYM						
Production of:						
α-Glucosidase	−	+	−	+	−	+
Esterase C4	−	+	−	−	−	+
Acid phosphatase	−	+	−	+	−	+
Naphthol-AS-BI-phosphohydrolase	−	+	−	−	−	+
β-Galactosidase	+	+	−	+	−	−
Valine arylamidase	−	+	−	−	−	+
Cystine arylamidase	−	+	−	−	−	+
API 50 CH						
Production of acid from:						
D-Xylose	+	−	−	−	−	−
L-Arabinose	+	−	−	−	−	−
D-Galactose	+	+	−	−	+	+
N-Acetylglucosamine	+	+	−	−	+	+
Amygdalin	+	−	−	−	−	+
Salicin	+	+	−	−	+	+
Cellobiose	+	+	−	−	+	+
Melibiose	+	−	−	−	−	−
Inulin	+	−	−	−	−	−
Starch	+	+	−	−	−	+
Glycogen	+	+	−	−	−	+
Gentiobiose	+	−	−	−	−	−

septicaemia. Full range of habitat is not known. The DNA G+C content is 43.8 mol%.

Acknowledgements

This work was funded by the Spanish Ministry of Education and Science (MEC), project AGL2009-14303-C02-01. The authors thank N. Carmona for technical assistance and Juncal Fernández-Garayzábal for her assistance with the English reviewing of the manuscript.

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