NOTE

***Streptococcus entericus* sp. nov., isolated from cattle intestine**

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Biochemical, molecular chemical and molecular genetic studies were performed on an unknown Gram-positive, catalase-negative, coccus-shaped organism isolated from the intestine of a cow affected with catarrhal enteritis. The organism was tentatively identified as a streptococcal species based on results of cellular morphological and biochemical tests. 16S rRNA gene sequencing studies confirmed its provisional identification as a member of the genus *Streptococcus*, but the organism did not correspond to any recognized species of this genus. The nearest phylogenetic relatives of the unknown coccus from a calf were *Streptococcus acidominimus* and *Streptococcus suis*. The unknown bacterium, however, was distinguished from these species and other animal streptococci by biochemical tests and electrophoretic analysis of whole-cell proteins. 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 entericus* sp. nov. The type strain is CECT 5353^T (= CCUG 44616^T).

Keywords: *Streptococcus entericus* sp. nov., taxonomy, phylogeny, 16S rRNA

Molecular genetic methods, most notably 16S rRNA gene sequencing, have contributed greatly to improvements in the taxonomy of the genus *Streptococcus*. In particular, phylogenetic analysis based on comparative 16S rRNA analysis has helped to clarify the intra-generic relationships of the streptococci (Bentley *et al.*, 1991; Kawamura *et al.*, 1995; Hardie & Whiley, 1997) and has aided the recognition of a plethora of novel species from human (e.g. Kawamura *et al.*, 1998; Flint *et al.*, 1999; Collins *et al.*, 2000; Schlegel *et al.*, 2000), animal (e.g. Devriese *et al.*, 1997, 1999; Rurangirwa *et al.*, 2000) and food (e.g. Tsakalidou *et al.*, 1998; Schlegel *et al.*, 2000) sources. Although streptococci can be isolated as part of the normal flora of the alimentary, respiratory and genitourinary tracts, as well as the skin of man and different animals (Kilian, 1998), some species are established pathogens that cause a variety of diseases such as endometritis, respiratory infections, endocarditis, meningitis, arthritis and mastitis (Chanter, 1997). *Streptococcus bovis*,

Streptococcus intestinalis (junior synonym of *Streptococcus alactolyticus*) and *Streptococcus thoraltensis* have been isolated from or are members of the intestinal flora of different animals (Devriese *et al.*, 1997; Kilian, 1998; Vandamme *et al.*, 1999), but their association with pathological processes is not clear. Although there are some reports of intestinal disorders associated with streptococci (Bard *et al.*, 1987; Jergens *et al.*, 1991; Gelshorn *et al.*, 1994; Willard *et al.*, 1998; Svane, 2000), enteric disease associated with this group of organisms remains poorly defined. During the course of a study of a cow affected by catarrhal enteritis, we have isolated a Gram-positive, catalase-negative, coccus-shaped organism that resembles the streptococci. We report the results of a polyphasic taxonomic study on the isolated coccus. Based on the findings presented, a novel streptococcal species, *Streptococcus entericus* sp. nov., is described.

A diarrhoeic process affecting thirty, 7- to 10-day-old Holstein–Friesian calves of the same farm was investigated. Catarrhal enteritis was the clinical sign observed after post-mortem examination of one diseased animal. Gram stain of faeces and the mucous membrane of the jejunum revealed the presence of large numbers of Gram-positive, ovoid-shaped bacteria. A coccus–

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The GenBank accession number for the 16S rRNA gene sequence of strain CCUG 44616^T is AJ409287.

shaped organism was isolated from the jejunum mucous membrane and faeces on Columbia blood-agar plates (bioMérieux) after 24 h incubation at 37 °C under aerobic and anaerobic conditions. The isolate has been deposited in the Spanish Type Culture collection (CECT) and in the Culture Collection of the University of Göteborg (CCUG), Sweden, under accession numbers CECT 5353^T and CCUG 44616^T. The isolate was characterized biochemically using the API Rapid ID32 Strep and API 20 Strep systems (bioMérieux) according to the manufacturer's instructions. Conventional physiological tests were also performed (Facklam & Elliott, 1995). The Lancefield serological group reaction was determined with the commercial Slidex Streptokit (bioMérieux) following the manufacturer's instructions. Haemolytic reaction was determined on Columbia blood-agar plates incubated aerobically at 37 °C for 24 and 48 h. PAGE analysis of whole-cell proteins was performed as described by Pot *et al.* (1994). For densitometric analysis, normalization and interpretation of protein patterns, the GCW 4.0 software package (Applied Maths) was used. The similarity between all pairs of traces was expressed by Pearson's product-moment correlation coefficient converted, for convenience, to a percentage similarity. Phylogenetic analysis was performed by comparative 16S rRNA gene sequence analysis. A large fragment of the 16S rRNA gene was amplified from DNA by PCR and sequenced directly using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the new isolate were determined by performing a database search. These sequences and those of other known related strains were retrieved from GenBank and aligned with the newly determined sequence by using the program DNATools version 5.01.696 (Rasmussen, 1993). The resulting multiple sequence alignment was corrected manually using the program GeneDoc (Nicholas *et al.*, 1997) and a distance matrix was calculated using the program DNADIST (using the Kimura-2 correction parameter). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) with the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

Two Gram-positive, coccus-shaped isolates, which formed short chains, were isolated from faeces and jejunum mucous membrane. The two isolates exhibited identical physiological, serological and biochemical characteristics and were assumed to represent a single clinical strain, as they were isolated from the same animal. The unidentified organisms were facultatively anaerobic, catalase-negative bacteria producing unpigmented, circular colonies, 1 mm in diameter after 24 h incubation at 37 °C on blood agar. They were α -haemolytic. The isolates grew at pH 9.6 and at 30 and 37 °C, but not in 6.5% (w/v) NaCl broth or at 10 or

45 °C. They were bile-/aesculin-negative. Using commercial API systems, they produced acid from starch, cyclodextrin, glycogen, lactose, maltose, trehalose, sucrose and methyl β -D-glucopyranoside, but failed to produce acid from D-arabitol, L-arabinose, inulin, mannitol, melibiose, melezitose, pullulan, ribose, raffinose, sorbitol or tagatose. Activity was observed for β -glucosidase, leucine arylamidase, β -galactosidase, alanine-phenylalanine-proline arylamidase and glycyl-tryptophan arylamidase, but not for alkaline phosphatase, β -glucuronidase, α -galactosidase, pyroglutamic acid arylamidase, *N*-acetyl- β -glucoaminidase or β -mannosidase. The isolates hydrolysed aesculin but not arginine, hippurate or urea. They were Voges-Proskauer-negative and vancomycin-sensitive. The morphological and biochemical characteristics of the unknown clinical isolates were consistent with those of the genus *Streptococcus*, but they did not correspond to any of the currently defined species of this genus. The numerical profile with API 20 Strep was 4040413, corresponding to a doubtful discrimination between *S. bovis* biotype I and *Streptococcus suis* biotype I. API Rapid ID32 Strep gave a profile 60036421111, corresponding to an unacceptable identification.

To investigate the phylogenetic affinities of the two unknown isolates, comparative 16S rRNA gene sequencing was performed. Approximately 600 bases (including variable regions V1, V2 and V3) were compared for the two isolates and were found to be identical, thereby strengthening the view that they represent a single strain. The almost complete (> 1400 bases) 16S rRNA gene sequence of the bacterium (as exemplified by CCUG 44616^T) was determined and subjected to a comparative phylogenetic analysis. Sequence searches of GenBank revealed that the unknown coccus was phylogenetically most closely related to the genus *Streptococcus* (data not shown). Treeing analysis confirmed this affinity and a dendrogram depicting the phylogenetic relationships of the unidentified coccus within the genus *Streptococcus* is shown in Fig. 1. The coccus formed a distinct subline with *Streptococcus acidominimus* (94.8% sequence similarity) and *S. suis* (95% sequence similarity) as its closest phylogenetic relatives. Bootstrap resampling, however, showed that the unknown coccus did not share a statistically significant association with either of these taxa. In order to investigate further the affinity of the unidentified coccus with the aforementioned species and other streptococci, PAGE analysis of whole-cell proteins was performed. The protein analysis showed that the unknown organism was different from all described streptococcal species (data not shown). The coccus formed a long line and displayed a loose affinity (correlation level < 70%) with *Streptococcus hyovaginalis* and *Streptococcus pluranimalium*. *S. acidominimus* and *S. suis* displayed lower similarities to the unknown organism (correlation level of 50%).

It is clear from the polyphasic taxonomic study that the unidentified catalase-negative coccus represents a

Fig. 1. Unrooted tree showing the phylogenetic relationships of *Streptococcus entericus* sp. nov. and some other reference streptococcal species. The tree, constructed using the neighbour-joining method, was based on a comparison of approx. 1320 nucleotides. Bootstrap values, expressed as percentages of 500 replications, are given at branching points. Bar, 1% sequence divergence.

Table 1. Characteristics useful in differentiating *Streptococcus entericus* sp. nov. from genotypically related species and other Lancefield group D streptococci

Taxa are identified as: 1, *S. entericus* sp. nov.; 2, *S. acidominimus*; 3, *S. suis* type I; 4, *S. suis* type II; 5, *S. bovis* biotype II.1; 6, *S. bovis* biotype II.2; 7, *S. equinus*; 8, *S. infantarius* subsp. *infantarius* (data from Schlegel *et al.*, 2000); 9, *S. infantarius* subsp. *coli* (data from Schlegel *et al.*, 2000); 10, *Streptococcus gallolyticus*; 11, *S. alactolyticus*; 12, *Streptococcus macedonicus* (data from Tsakalidou *et al.*, 1998); 13, *S. pluranimalium* (data from Devriese *et al.*, 1999). Data for species other than *S. entericus*, *S. acidominimus*, *S. infantarius*, *S. pluranimalium* and *S. macedonicus* were obtained from the Rapid ID32 Strep database, except for hydrolysis of hippurate and production of acid from starch, which were obtained from the API 20 Strep database. Characters are scored as: +, positive; –, negative; v, variable; v+, variable (most strains positive); v–, variable (most strains negative).

Characteristic	1	2	3	4	5	6	7	8	9	10*	11	12	13
Hydrolysis of:													
Hippurate	–	–	–	–	–	–	–	–	–	–	–	–	+
Arginine	–	–	+	+	–	–	–	–	–	–	–	–	–
Production of:													
α-Galactosidase	–	+	+	+	+	+	(v)†	–	+	+	v– (+)	+	v
β-Galactosidase	+	+	v–	v–	–	+	–	–	–	–	–	–	–
β-Glucuronidase	–	v–	+	+	–	v+	(+)	–	–	–	–	–	v
β-Mannosidase	–	–	–	–	–	+	–	–	–	v–	–	–	–
Production of acid from:													
Glycogen	+	–	+	+	v+	(+)	–	–	+	–	–	–	–
Lactose	+	v+	+	+	v–	(+)	+	–	+	+	–	+	v
Mannitol	–	+	–	–	–	–	–	–	–	+	v+‡	–	v
Melibiose	–	–	–	–	v	(+)	–	–	+	–	–	–	v
Methyl β-D-glucopyranoside	+	–	v+	+	+	+	+	v	+	+	+	–	v
Pullulan	+	–	+	+	v	(–)	–	–	+	–	–	–	–
Trehalose	+	v+	+	+	v–	+	v–	–	–	+	–	–	+
Starch	+	–	+	+	+	v+	–	+	v	+	v–	–	–

* *S. gallolyticus* includes the strains formerly identified as *S. bovis* biotype I (Osawa *et al.*, 1995).

† Different results were reported by Schlegel *et al.* (2000), as given in parentheses.

‡ Given as variable (most strains negative) by Vandamme *et al.* (1999).

previously unrecognized streptococcal species. Phylogenetically, the bacterium from a calf forms a distinct subline within the genus and exhibits a loose association with *S. acidominimus* and *S. suis*. 16S rRNA

sequence divergence values of > 4% between the novel isolate and *S. acidominimus* and *S. suis*, however, demonstrate unequivocally that it represents a distinct species (Stackebrandt & Goebel, 1994). PAGE analysis

of whole-cell proteins and biochemical profiling also showed that the isolate was distinct from all recognized streptococcal species. It is pertinent to note that the novel coccus-shaped organism gave a positive reaction for Lancefield group D. No other Lancefield group was detected. The unknown isolate is phenotypically distinct from *S. bovis*/*Streptococcus equinus* and *S. suis*, however, as well as from other Lancefield group D streptococci (Table 1). The Lancefield group D antigen can also be found in enterococci, but the novel isolate could be readily distinguished from enterococci by its lack of resistance to bile, its negative pyrrolidonylarylamidase reaction and its failure to grow at 10 °C and 6.5% NaCl, enterococci being positive for these tests (Facklam & Elliott, 1995). Therefore, based on both phylogenetic and phenotypic criteria, it is evident that the unidentified coccus merits classification as a novel species of the genus *Streptococcus*, for which the name *Streptococcus entericus* sp. nov. is proposed.

Tests that are useful in differentiating *S. entericus* from its nearest phylogenetic relatives and from some other animal streptococci with which it may be confused are shown in Table 1. Although it is not possible to conclude that *S. entericus* was involved in the pathological process, its isolation from faeces and intestinal mucosa of a calf with catarrhal enteritis indicates that it is possibly clinically significant. The formal description of *S. entericus* and the availability of tests to facilitate its identification will aid clinical laboratories in recognizing this species in the future and improve knowledge of its distribution and possible association with disease.

Description of *Streptococcus entericus* sp. nov.

Streptococcus entericus (en.te'ri.cus. N.L. adj. *entericus* from Gr. n. *enteron* gut, pertaining to the gut).

Cells are Gram-positive cocci arranged in short chains. Non-motile. Facultatively anaerobic, catalase- and oxidase-negative. Colonies are non-pigmented, circular and 1 mm in diameter after 24 h on blood agar and produce an α -haemolytic reaction. Reacts with Lancefield group D antisera. Growth occurs at 37 °C but not at 10 or 45 °C. Growth occurs at pH 9.6 but not in broth containing 6.5% NaCl. Bile/aesculin test is negative. Sensitive to vancomycin. Using the commercial API Rapid ID32 Strep and API 20 Strep systems, acid is produced from starch, cyclodextrin, glycogen, lactose, maltose, trehalose, sucrose and methyl β -D-glucopyranoside but not from D-arabitol, L-arabinose, inulin, mannitol, melibiose, melezitose, pullulan, ribose, raffinose, sorbitol or tagatose. β -Glucosidase, leucine arylamidase, β -galactosidase, alanine-phenylalanine-proline arylamidase and glycyl-tryptophan arylamidase are detected. No activity is detected for β -glucuronidase, α -galactosidase, alkaline phosphatase, pyroglutamic acid arylamidase, *N*-acetyl- β -glucoaminidase or β -mannosidase. Aesculin is hydrolysed but arginine, hippurate and urea are not. Acetoin is not

produced. Isolated from faeces and jejunum of a calf with enteritis. Habitat is not known.

The type strain of *Streptococcus entericus* is CECT 5353^T (= CCUG 44616^T).

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References

- Bard, G., Visconti, E. B., Curran, J. P. & de Caprariis, P. J. (1987). Diarrhea caused by group G *Streptococcus*. *N Y State J Med* **87**, 366–367.
- Bentley, R. W., Leigh, J. A. & Collins, M. D. (1991). Intrageneric structure of *Streptococcus* based on comparative analysis of small-subunit rRNA sequences. *Int J Syst Bacteriol* **41**, 487–494.
- Chanter, N. (1997). Streptococci and enterococci as animal pathogens. *Soc Appl Bacteriol Symp Ser* **26**, 100S–109S.
- Collins, M. D., Hutson, R. A., Falsen, E., Nikolaitchouk, N., LaClaire, L. & Facklam, R. R. (2000). An unusual *Streptococcus* from human urine, *Streptococcus urinalis* sp. nov. *Int J Syst Evol Microbiol* **50**, 1173–1178.
- Devriese, L. A., Pot, B., Vandamme, P., Kersters, K., Collins, M. D., Alvarez, N., Haesebrouck, F. & Hommez, J. (1997). *Streptococcus hyovaginalis* sp. nov. and *Streptococcus thoralensis* sp. nov., from the genital tract of sows. *Int J Syst Bacteriol* **47**, 1073–1077.
- Devriese, L. A., Vandamme, P., Collins, M. D., Alvarez, N., Pot, B., Hommez, J., Butaye, P. & Haesebrouck, F. (1999). *Streptococcus pluranimalium* sp. nov., from cattle and other animals. *Int J Syst Bacteriol* **49**, 1221–1226.
- Facklam, R. R. & Elliott, J. A. (1995). Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin Microbiol Rev* **8**, 479–495.
- Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.
- Flint, S. H., Ward, L. J. H. & Brooks, J. D. (1999). *Streptococcus waius* sp. nov., a thermophilic streptococcus from a biofilm. *Int J Syst Bacteriol* **49**, 759–767.
- Gelshorn, C., Piffaretti, J. C., Haldimann, B. & Martinoli, S. (1994). Diarrhoea and peritonitis in infection caused by type A beta haemolytic *Streptococcus*. *Helv Chir Acta* **60**, 931–934 (in German).
- Hardie, J. M. & Whiley, R. A. (1997). Classification and overview of the genera *Streptococcus* and *Enterococcus*. *Soc Appl Bacteriol Symp Ser* **26**, 1S–11S.
- Jergens, A. E., Moore, F. M., Prueter, J. C. & Yankauskas, P. J. (1991). Adherent gram-positive cocci on the intestinal villi of two dogs with gastrointestinal disease. *J Am Vet Med Assoc* **198**, 1950–1952.
- Kawamura, Y., Hou, X.-G., Sultana, F., Miura, H. & Ezaki, T. (1995). Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. *Int J Syst Bacteriol* **45**, 406–408.
- Kawamura, Y., Hou, X.-G., Todome, Y., Sultana, F., Hirose, K., Shu, S.-E., Ezaki, T. & Ohkuni, H. (1998). *Streptococcus peroris* sp. nov. and *Streptococcus infantis* sp. nov., new members of the *Streptococcus mitis* group, isolated from human clinical specimens. *Int J Syst Bacteriol* **48**, 921–927.
- Kilian, M. (1998). *Streptococcus* and *Lactococcus*. In *Topley & Wilson's Microbiology and Microbial Infections*, vol. 2, *Systematic Bacteriology*, pp. 633–667. Edited by A. Balows & B. I. Duerden. London: Arnold.
- Nicholas, K. B., Nicholas, H. B., Jr & Deerfield, D. W., II (1997). GeneDoc: analysis and visualization of genetic variation. *EMBNEW News* **4**, 14.

- Osawa, R., Fujisawa, T. & Sly, L. I. (1995). *Streptococcus gallolyticus* sp. nov.; gallate degrading organisms formerly assigned to *Streptococcus bovis*. *Syst Appl Microbiol* **18**, 74–78.
- Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Modern Microbial Methods. Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.
- Rasmussen, S. W. (1993). DNA Tools, a software package for DNA sequence analysis. Carlsberg Laboratory, Copenhagen.
- Rurangirwa, F. R., Teitzel, C. A., Cui, J., French, D. M., McDonough, P. L. & Besser, T. (2000). *Streptococcus didelphis* sp. nov., a streptococcus with marked catalase activity isolated from opossums (*Didelphis virginiana*) with suppurative dermatitis and liver fibrosis. *Int J Syst Evol Microbiol* **50**, 759–765.
- Schlegel, L., Grimont, F., Collins, M. D., Régnault, B., Grimont, P. A. D. & Bouvet, A. (2000). *Streptococcus infantarius* sp. nov., *Streptococcus infantarius* subsp. *infantarius* subsp. nov. and *Streptococcus infantarius* subsp. *coli* subsp. nov., isolated from humans and food. *Int J Syst Evol Microbiol* **50**, 1425–1434.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Svane, S. (2000). Acute phlegmonous jejunitis and viridans streptococcal peritonitis associated with bronchial carcinoma. *Scand J Infect Dis* **32**, 421–422.
- Tsakalidou, E., Zoidou, E., Pot, B., Wassill, L., Ludwig, W., Devriese, L. A., Kalantzopoulos, G., Schleifer, K. H. & Kersters, K. (1998). Identification of streptococci from Greek Kasseri cheese and description of *Streptococcus macedonicus* sp. nov. *Int J Syst Bacteriol* **48**, 519–527.
- Vandamme, P., Devriese, L. A., Haesebrouck, F. & Kersters, K. (1999). *Streptococcus intestinalis* Robinson *et al.* 1988 and *Streptococcus alactolyticus* Farrow *et al.* 1984 are phenotypically indistinguishable. *Int J Syst Bacteriol* **49**, 737–741.
- Willard, M. D., Berridge, B., Braniecki, A. & Bouley, D. (1998). Possible antibiotic-associated colitis in a dog. *J Am Vet Med Assoc* **213**, 1753–1754.