Staphylococcus nepalensis sp. nov., isolated from goats of the Himalayan region

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Four coagulase-negative, novobiocin-resistant cocci, designated CW1^T, PM34, MM3 and RW78, were isolated from the respiratory tract of goats kept in the Himalayan region. The four isolates were assigned to a single species on the basis of almost identical biochemical and physiological traits, protein profiles obtained after SDS-PAGE and identical genomic fingerprints generated after enterobacterial repetitive intergenic consensus (ERIC)-PCR. Strain CW1^T showed highest 16S rDNA sequence similarities to *Staphylococcus cohnii* subsp. *urealyticus* ATCC 49330^T, *Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305^T, *S. cohnii* subsp. *cohnii* ATCC 29974^T, *Staphylococcus arlettae* ATCC 43957^T, *Staphylococcus gallinarum* ATCC 35539^T, *Staphylococcus succinus* ATCC 700337^T and *Staphylococcus xylosus* ATCC 29971^T (99.0, 98.8, 98.8, 98.4, 98.2, 98.1 and 98.1 %, respectively), indicating its classification within the genus *Staphylococcus*. The polar lipid composition, fatty acid profiles, quinone systems and diagnostic cell-wall diamino acid were in agreement with the characteristics of the genus *Staphylococcus*. DNA–DNA hybridization with closely related *Staphylococcus* species suggested that strain CW1^T represents an as-yet unrecognized species. Based on these results, a novel species of the genus *Staphylococcus* is described, *Staphylococcus nepalensis* sp. nov. The type strain is CW1^T (= DSM 15150^T = CCM 7045^T) and the most dissimilar strain is PM34 (= DSM 15151 = CCM 7046).

Staphylococci are ubiquitous and diverse organisms widespread in nature. At the time of writing, the genus *Staphylococcus* consists of 38 species (Holt *et al.*, 1994; Lambert *et al.*, 1998; Vernozy-Rozand *et al.*, 2000). Several *Staphylococcus* species have been isolated from various mammals and birds. In the caprine host, *Staphylococcus* species have been recovered from skin and mucous membranes, as well as from goat products such as milk and cheese (Devriese *et al.*, 1985; Valle *et al.*, 1991; Ferrer *et al.*, 1997; Klinger & Rosenthal, 1997; Mahanta *et al.*, 1997; Bedidi-Madani *et al.*, 1998; Vernozy-Rozand *et al.*, 2000; Place *et al.*, 2002). In the course of a study to determine the prevalence and significance of bacteria in the respiratory

tract of goats with pneumonia from different areas of the Himalayan region, a small number of unidentified staphylococci were isolated. Preliminary routine investigations demonstrated that they were coagulase-negative and resistant towards novobiocin. The cluster of staphylococci that share these traits consists of *Staphylococcus arlettae*, *Staphylococcus cohnii*, *Staphylococcus equorum*, *Staphylococcus gallinarum*, *Staphylococcus kloosii*, *Staphylococcus saprophyticus*, *Staphylococcus succinus* and *Staphylococcus xylosus*. Phylogenetically, these species are located on a separate subline within the genus *Staphylococcus* (Takahashi *et al.*, 1999). Here, we report the characterization and classification of four isolates, for which we propose the name *Staphylococcus nepalensis* sp. nov.

The four strains were recovered from three nasal swabs (PM34, RW78, CW1^T) and one lung sample (MM3) collected from four goats with respiratory symptoms kept in different areas of Nepal (Pakhribas, Mangalbare, Rajarani, Chitwan). The organisms were isolated on blood agar base no. 2 (Oxoid) supplemented with 5 % (v/v) defibrinated sheep blood that was incubated aerobically at 37 °C for 24 h.

Published online ahead of print on 6 June 2003 as DOI 10.1099/ij.s.0.02646-0.

The EMBL accession number for the 16S rDNA sequence of strain CW1^T is AJ517414.

SDS-PAGE profiles of whole-cell lysates of the novel isolates and reference *Staphylococcus* species are available as supplementary material in IJSEM Online.

Subcultivation was done on trypticase soy agar (BBL) or trypticase soy broth (BBL). Type strains of *Staphylococcus* species were supplied by the American Type Culture Collection, Manassas, VA, USA (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ), and the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic (CCM).

16S rDNA sequence analyses

16S rDNA of strain CW1^T was amplified and sequenced as described by Wieser *et al.* (1999) and PCR products were purified using a GenElute purification kit (Sigma). The derived sequence was aligned and compared with other bacterial 16S rRNA sequences available in the EMBL database using the Wisconsin package (GCG, 1995). The 16S rDNA sequence of strain CW1^T consisted of 1470 nucleotides (positions 31–1492, *Escherichia coli* numbering; Brosius *et al.*, 1978). Sequence comparisons using FASTA (Pearson & Lipman, 1988) demonstrated that strain CW1^T is a member of the genus *Staphylococcus*. Highest sequence similarity values were obtained to *S. cohnii* subsp. *urealyticus* ATCC 49330^T, *S. saprophyticus* subsp. *saprophyticus* ATCC 15305^T, *S. cohnii* subsp. *cohnii* ATCC 29974^T, *S. arlettae* ATCC 43957^T, *S. gallinarum* ATCC 35539^T, *S. succinus* ATCC 700337^T and *S. xylosus* ATCC 29971^T (99.0, 98.8, 98.8, 98.4, 98.2, 98.1 and 98.1 %, respectively).

Physiological characterization and antibiotic susceptibilities

Morphological and physiological characteristics as well as susceptibility to lysostaphin, lysozyme, furazolidone, novobiocin, bacitracin and the vibriostatic compound O/129 were examined as described previously (Hájek *et al.*, 1992; Freney *et al.*, 1999). Susceptibility to other antibiotics was determined on Mueller–Hinton agar (Oxoid) by a standard diffusion technique using antibiotic-impregnated disks (Oxoid). Any sign of growth inhibition, after 24 h incubation at 37 °C, was scored. Other traits were examined using API ID 32 Staph and API 50 CH strips (bioMérieux). Characteristics of the four strains PM34, MM3, RW78 and CW1^T are summarized in the species description below and in Table 1.

Chemotaxonomic investigation

Menaquinones were analysed as described previously (Tindall, 1990; Altenburger *et al.*, 1996). Quinone systems consisted of the major menaquinone MK-7 (71–74 %) and minor amounts of MK-6 (10–16 %) and MK-8 (10–18 %). A similar quinone system with the predominant compound MK-7 has been reported for numerous *Staphylococcus* species, including strains of *S. cohnii*, *S. saprophyticus* and *S. xylosus* (Nahaie *et al.*, 1984). Polar lipids were extracted and analysed by two-dimensional TLC according to Ventosa *et al.* (1993). The polar lipid profiles of the three analysed strains were homogeneous. They consisted of the major

Table 1. Characteristics that differentiate *S. nepalensis* sp. nov. from other novobiocin-resistant, oxidase-negative *Staphylococcus* species

Species: 1, *S. nepalensis* sp. nov. (four strains); 2, *S. arlettae*; 3, *S. cohnii* subsp. *cohnii*; 4, *S. cohnii* subsp. *urealyticus*; 5, *S. equorum*; 6, *S. gallinarum*; 7, *S. kloosii*; 8, *S. saprophyticus* subsp. *saprophyticus*; 9, *S. saprophyticus* subsp. *bovis*; 10, *S. succinus* subsp. *succinus*; 11, *S. xylosus*. Data for reference species were taken from Behme *et al.* (1996) and Freney *et al.* (1999). Results are scored as: +, ≥90 % of strains positive; d, 11–89 % of strains positive; –, 0–10 % of strains positive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Urease activity	+	+	–	+	+	+	+	+	+	+	+
Nitrate reduction	+	–	–	–	+	+	–	–	+	–	+
Aesculin hydrolysis	+	+	–	d	d	+	d	–	–	+	d
Tween 80 hydrolysis	+	–	d	d	–	–	–	–	–	–	–
Pyrrrolidonyl arylamidase	+	–	–	d	–	–	d	d	–	–	d
Acid production from:											
L-Arabinose	+	+	–	–	d	+	d	–	d	+	d
D-Cellobiose	–	–	–	–	d	+	–	–	–	+	–
D-Galactose	+	–	d	d	d	–	–	–	+	+	d
Salicin	+	–	–	d	+	+	–	–	–	–	–

lipids diphosphatidylglycerol, phosphatidylglycerol and a glycolipid. The chromatographic behaviour of this glycolipid corresponded to a glycolipid G₁, which was assumed to be β-gentiobiosyl diacylglycerol (Nahaie *et al.*, 1984). Additionally, two unknown aminophospholipids, three unknown glycolipids and one unknown polar lipid were detected. Concerning the major components, this polar lipid profile was in good agreement with the profiles of *S. cohnii* NCTC 11041^T, *S. saprophyticus* SCH94^T and *S. xylosus* NCTC 11043^T (Nahaie *et al.*, 1984). Diagnostic cell-wall diamino acids were extracted from cell walls as described by Schleifer (1985) and identified by comparison with the chromatographic behaviour of references. The characteristic cell-wall diamino acid of strains CW1^T, PM34 and MM3 was L-lysine, which was reported to be characteristic of staphylococci (Kloos *et al.*, 1992).

Fatty acids were extracted from biomass grown at 28 °C for 24 h on trypticase soy agar according to Osterhout *et al.* (1991) and analysed using GLC (Wieser *et al.*, 1999). In the fatty acid profiles, the predominant fatty acids were ai-C_{15:0}, i-C_{15:0} and ai-C_{17:0}, whilst i-C_{17:0}, C_{18:0}, C_{16:0} and C_{20:0} were present in moderate amounts (Table 2). Similar fatty acid profiles were reported for several *Staphylococcus* species by Kotilainen *et al.* (1991). The combination of these chemotaxonomic characteristics supports the assignment of the isolates to the genus *Staphylococcus*.

For analysis of protein patterns, SDS-PAGE of the four isolates and their nearest related *Staphylococcus* species was performed as described previously (Laemmli, 1970; Rosengarten *et al.*, 1994). Each of the 11 species and subspecies displayed a unique band profile, allowing their

Table 2. Fatty acid composition of *S. nepalensis* CW1^T

Values in parentheses indicate the range detected for the four strains.

Fatty acid	Content (range) (%)
C _{12:0}	0.4 (0.0–0.4)
i-C _{14:0}	0.3 (0.3–0.5)
C _{14:0}	0.7 (0.7)
i-C _{15:0}	20.7 (18.0–20.7)
ai-C _{15:0}	42.9 (41.8–43.9)
C _{15:0}	0.2 (0.0–0.2)
i-C _{16:0}	1.3 (1.0–1.4)
C _{16:0}	3.8 (3.3–3.8)
i-C _{17:0}	7.3 (6.6–7.3)
ai-C _{17:0}	13.1 (13.1–14.8)
C _{18:0}	4.6 (4.5–4.7)
i-C _{19:0}	1.5 (1.5–2.0)
ai-C _{19:0}	0.0 (0.0–1.3)
C _{20:0}	3.0 (3.0–4.4)

differentiation from each other (see supplementary material available in IJSEM Online). Partial similarities were observed between *S. succinus* subsp. *succinus* ATCC 700337^T and *S. kloosii* CCM 3834^T, *S. arlettae* CCM 3830^T, *S. cohnii* subsp. *cohnii* CCM 2736^T and *S. cohnii* subsp. *urealyticus* CCM 4294^T, and *S. saprophyticus* subsp. *saprophyticus* CCM 883^T and CW1^T, but almost identical protein profiles were only detected for isolates PM34, MM3, RW78 and CW1^T. These results support assignment of the four isolates to a single species that has not yet been recognized.

Genomic analysis

The DNA extraction protocol was based on a previously described procedure (Lambert *et al.*, 1998). Determination of DNA G + C content was performed as described by Wieser *et al.* (1999). The G + C content of the genomic DNA of CW1^T was 33 mol%, which is in the range reported for other *Staphylococcus* species (Kloos & Schleifer, 1986).

Enterobacterial repetitive intergenic consensus (ERIC)-PCR has been shown to be a useful approach for identification of *Staphylococcus epidermidis* and its differentiation from related species. In order to examine this approach for identification of our four isolates, their ERIC-fingerprints, generated as described by Wieser & Busse (2000) using a Gene Amp PCR System 2400 thermal cycler (Perkin Elmer Applied Biosystems), were analysed. Each isolate displayed a single band consisting of approximately 640 bp (Fig. 1). Other *Staphylococcus* species that were considered to be the nearest relatives of CW1^T based on 16S rDNA sequence similarity values exhibited clearly distinguishable banding patterns. These observations indicated that the four isolates are members of a single novel species.

DNA–DNA hybridization was performed according to Ziemke *et al.* (1998) under optimal conditions at 58 °C (30° below the melting temperature of the homoduplex DNA; $T_m = 88$ °C). The results (Table 3) demonstrated that strain CW1^T represents a novel species of the genus *Staphylococcus*.

RAPD (randomly amplified polymorphic DNA) PCRs were performed by using RAPD primers 1 to 6 of the RAPD analysis primer set (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Genomic fingerprints were distinct for each strain, indicating that isolates PM34, MM3, RW78 and CW1^T are not clonally related (results not shown).

Based on the results presented here, it is demonstrated that strains PM34, MM3, RW78 and CW1^T are members of a novel species of the genus *Staphylococcus*, for which we propose the name *Staphylococcus nepalensis* sp. nov.

Description of *Staphylococcus nepalensis* sp. nov.

Staphylococcus nepalensis (ne.pa.len'sis. N.L. masc. adj. *nepalensis* pertaining to the kingdom of Nepal, where clinical samples for bacteriological examination were collected from local goats).

Fig. 1. ERIC-PCR-generated DNA fingerprints of *Staphylococcus* species. Lanes: 1 and 15, 100 bp ladder; 2 and 16, *S. nepalensis* CW1^T; 3, *S. cohnii* subsp. *urealyticus* CCM 4294^T; 4, *S. cohnii* subsp. *cohnii* CCM 2736^T; 5, *S. saprophyticus* subsp. *saprophyticus* CCM 883^T; 6, *S. xylosus* DSM 20231^T; 7, *S. equorum* CCM 3832^T; 8, *S. succinus* subsp. *succinus* ATCC 700337^T; 9, *S. kloosii* CCM 3834^T; 10, *S. lugdunensis* CCM 4064^T; 11, *S. arlettae* CCM 3830^T; 12, *S. haemolyticus* DSM 20263^T; 13, *S. gallinarum* DSM 20610^T; 14, negative control; 17–19, *S. nepalensis* strains PM34, MM3 and RW78.

Table 3. DNA–DNA relatedness between *S. nepalensis* CW1^T and *S. xylosus* DSM 20231^T and other species in the genus *Staphylococcus*

Strain	DNA-DNA relatedness (%) to:	
	<i>S. nepalensis</i> CW1 ^T	<i>S. xylosus</i> DSM 20231 ^T
<i>S. nepalensis</i> CW1 ^T	100	52
<i>S. xylosus</i> DSM 20231 ^T	68	100
<i>S. lugdunensis</i> CCM 4064 ^T	65	34
<i>S. gallinarum</i> DSM 20610 ^T	64	44
<i>S. saprophyticus</i> subsp. <i>saprophyticus</i> CCM 883 ^T	63	63
<i>S. arlettae</i> CCM 3830 ^T	58	ND
<i>S. cohnii</i> subsp. <i>cohnii</i> CCM 2736 ^T	56	43
<i>S. equorum</i> CCM 3832 ^T	53	47
<i>S. succinus</i> subsp. <i>succinus</i> ATCC 700337 ^T	51	51
<i>S. kloosii</i> CCM 3834 ^T	47	37
<i>S. haemolyticus</i> DSM 20263 ^T	43	42
<i>S. cohnii</i> subsp. <i>urealyticus</i> CCM 4294 ^T	42	ND

ND, Not determined.

Cells are Gram-positive cocci, 1.1–1.6 µm in diameter, that occur singly, in pairs and in irregular clusters. Motility is not observed. Colonies after 2 days on P agar are circular, low-convex, smooth, glossy, opaque white and 2–6 mm in diameter. Growth occurs aerobically and anaerobically in the presence of 0–7.5 % NaCl (w/v). The type strain grows well in the presence of 10 % NaCl (w/v), but growth is variable for other strains. No growth is observed in the presence of 15 % NaCl. Growth occurs between 20 and 40 °C, best growth at 30 °C. No growth at 15 or 45 °C. Oxidase-negative. Catalase-positive. Produces urease, alkaline phosphatase, pyrrolidonyl arylamidase, β-galactosidase and β-glucuronidase. Hydrolyses aesculin and Tween 80. Reduces nitrate to nitrite. Positive for aerobic production of acid from D-glucose, D-fructose, D-mannose, maltose, lactose, trehalose, mannitol, sucrose, L-arabinose, N-acetylglucosamine, galactose, glycerol, erythritol, D-xylose, arbutin and salicin. Negative for oxidase activity, clumping factor, coagulase, hyaluronidase, arginine dihydrolase, ornithine decarboxylase, acetoin, arginine arylamidase, α- and β-haemolysins, heat-stable and heat-labile nucleases, indole, hydrogen sulphide and lecithinase. Acid is not formed aerobically from D-raffinose, ribose, D-cellobiose, D-arabinose, L-xylose, adonitol, methyl β-D-xyloside, rhamnose, L-sorbose, dulcitol, starch, inositol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, melibiose, melezitose, gentiobiose, glycogen, inulin, D-tagatose, D-lyxose, cellobiose, D-fucose, L-fucose, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Acid production from D-arabitol, sorbitol, turanose and xylitol is variable; the type strain is positive. Resistant to novobiocin, bacitracin, vibriostatic agent O/129, lysozyme, metronidazole and optochin. Susceptible to lysostaphin, furazolidone, ampicillin, amoxicillin, amoxicillin/clavulanic acid, ceftiofur, cephalexin, cephalothin, chloramphenicol, clindamycin,

colistin sulphate, enrofloxacin, erythromycin, florfenicol, fosfomycin, fusidic acid, gentamicin, kanamycin, lincomycin, neomycin, nitrofurantoin, oxacillin, penicillin G, polymyxin B, sulfamethoxazole/trimethoprim, tetracycline and vancomycin. The quinone system consists of the major menaquinone MK-7 and minor amounts of MK-6 and MK-8. Predominant fatty acids are ai-C_{15:0}, i-C_{15:0} and ai-C_{17:0}, whilst i-C_{17:0}, C_{18:0}, C_{16:0}, C_{20:0} and i-C_{19:0} are present in moderate amounts. Polar lipid profile consists of the major lipids diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid (assumed to be β-gentiobiosyl diacylglycerol). Additionally, minor amounts of two unknown aminophospholipids and three unknown glycolipids are detected. The G+C content of the type strain is 33 mol% (HPLC).

The type strain is CW1^T (=DSM 15150^T=CCM 7045^T) and the most dissimilar strain is PM34 (=DSM 15151=CCM 7046).

Acknowledgements

The authors thank T. Beier for kindly providing clinical samples and S. Wildmann and M. Valens for skilled technical assistance.

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