Staphylococcus simiae sp. nov., isolated from South American squirrel monkeys

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Eight coagulase-negative, oxidase-negative and novobiocin-susceptible staphylococcal strains were isolated from the gastrointestinal tracts of South American squirrel monkeys (*Saimiri sciureus* L.). These strains were differentiated from known staphylococcal species on the basis of 16S rRNA gene and *hsp60* gene sequencing, and from the most closely related species by using DNA–DNA hybridization, ribotyping, whole-cell protein profiles and biotyping. Phylogenetic analysis based on 16S rRNA gene sequences confirmed that these strains are members of the *Staphylococcus aureus* species group (99 % similarity) but are biochemically similar to *Staphylococcus piscifermentans*, from which they can be phenotypically distinguished by resistance to polymyxin B, acid production from D-mannitol, the inability to hydrolyse aesculin and DNA and the absence of α -glucosidase. On the basis of these analyses, a novel species of the genus *Staphylococcus* is described, for which the name *Staphylococcus simiae* sp. nov. is proposed, with CCM 7213^T (=LMG 22723^T) as the type strain.

Staphylococci are among the most widespread of the pathogenic and opportunistic pathogenic bacteria (Kloos *et al.*, 1992). At present, 36 staphylococcal species are known: nine of them include two subspecies, and one of them includes three subspecies (Garrity *et al.*, 2004; Spengler *et al.*, 2003;

Place *et al.*, 2003; Švec *et al.*, 2004). Several *Staphylococcus* species have been isolated from non-human primates and in some cases were associated with illness and mortality, particularly in newly imported animals (Padovan & Cantrell, 1983; Crouch *et al.*, 1984; Stasilevich *et al.*, 1986). An unnamed coagulase-negative and novobiocin-resistant '*Staphylococcus* sp. 5' isolated from skin of squirrel monkeys has been reported previously by Kloos *et al.* (1976). During an outbreak of diarrhoeal disease in squirrel monkeys in a zoo in Olomouc, Czech Republic, staphylococcal strains with atypical features were isolated from both ill and healthy animals. The South American squirrel monkey (*Saimiri sciureus* L.) is an arboreal, small-sized, non-human primate from equatorial South America; it has always been a common sight in zoos, and laboratory-bred squirrel monkeys have long been used as an animal model in various fields of biomedical research (Rosenblum & Coe, 1985).

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Abbreviation: ERIC, enterobacterial repetitive intergenic consensus sequence.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and the partial *hsp60* gene sequences of strain CCM 7213^T are AY727530 and AY727531.

SDS-PAGE whole-cell protein profiles, ribotype patterns, internal transcribed ribosomal spacer-PCR patterns and PFGE macrorestriction patterns of the novel isolates and reference *Staphylococcus* species are available as supplementary figures in IJSEM Online.

Eight *Staphylococcus* strains were isolated from rectal swabs or faeces obtained from different specimens of squirrel monkeys kept in Olomouc Zoo. Five strains (CCM 7213^T, CCM 7214, CCM 7215, CCM 7216 and P 908) were collected in April 2000: one of them (CCM 7214) was isolated from a monkey with diarrhoea and rectal haemorrhage that died after 5 days, despite therapy with gentamicin, netilmicin, Ringer's solution and glucose. The causative agent remained unidentified. Three additional strains (CCM 7229, CCM 7230 and P 1040) were isolated in June 2004 from quarantined healthy monkeys that had just been imported from Guyanese forests; they had not come into contact with zoo monkeys. Other species of the genus *Staphylococcus* detected among zoo-bred squirrel monkeys were *Staphylococcus aureus* subsp. *aureus*, *Staphylococcus cohnii* subsp. *urealyticum* and *Staphylococcus haemolyticus*. The organisms were isolated on trypticase soy II agar plates with 5% sheep blood (Becton-Dickinson International) and subcultured on nutrient agar (Oxoid) in the presence or absence of 5% (v/v) defibrinated sheep blood at 37 °C for 24 h. Six representative strains have been deposited in the Czech Collection of Microorganisms (Brno, Czech Republic) as CCM 7213^T (= LMG 22723^T), CCM 7214 (= LMG 22724), CCM 7215, CCM 7216, CCM 7229 and CCM 7230.

The following reference strains were used: *S. aureus* subsp. *aureus* CCM 885^T, *S. aureus* subsp. *aureus* 30KR (a co-isolate of the novel staphylococcal strains), *S. aureus* subsp. *aureus* NCTC 8325, *Staphylococcus epidermidis* CCM 2124^T, *Staphylococcus capitis* subsp. *capitis* CCM 2734^T, *Staphylococcus caprae* CCM 3573^T, *Staphylococcus piscifermentans* CCM 4345^T, CCM 4346, CCM 4347, CCM 4789 and CCM 4790 and *Staphylococcus saccharolyticus* LMG 22204^T. Morphological and physiological characteristics of the tested cultures, as well as susceptibility to furazolidone (100 µg), novobiocin (1.6 µg), bacitracin (0.04 U), polymyxin B (300 U) and lysostaphin (200 mg l⁻¹) were examined by using conventional key tube or plate tests as described previously (Freney *et al.*, 1999; Pantůček *et al.*, 1999; Mannerová *et al.*, 2003). Additional biochemical profile data were obtained by using the ID32 Staph, API Staph and API ZYM systems (bioMérieux). Susceptibility to other antibiotics was determined on Mueller–Hinton agar (Oxoid) by using a standard diffusion technique with a standard set of antibiotic discs for staphylococci (Oxoid).

The characteristics of the novel isolates are summarized in the species description below. The squirrel monkey strains are phenotypically close to *S. piscifermentans* (Tanasupawat *et al.*, 1992; Pantůček *et al.*, 1999) but differ from the latter in terms of the following biochemical characteristics: aesculin hydrolysis, DNA hydrolysis, α- and β-glucosidase production, acid production from D-mannitol and resistance to polymyxin B. Differentiating phenotypic features among the squirrel monkey isolates and phylogenetically and phenotypically related neighbours are indicated in Table 1. Although strains CCM 7229, CCM 7230 and P 1040 were resistant to tetracycline, the strains studied showed

Table 1. Biochemical characteristics that differentiate *S. simiae* sp. nov. (eight strains) from phylogenetically and phenotypically related *Staphylococcus* species

Taxa: 1, *S. simiae* sp. nov.; 2, *S. piscifermentans*; 3, *S. aureus* subsp. *aureus*; 4, *S. epidermidis*; 5, *S. capitis* subsp. *capitis*; 6, *S. caprae*. Data for related species are from Devriese *et al.* (1983), Kloos & Wolfshohl (1991), Tanasupawat *et al.* (1991, 1992), Kloos *et al.* (1992), Kawamura *et al.* (1998), Kloos & Bannerman (1999) and Pantůček *et al.* (1999). Symbols: +, 90% or more strains positive; −, 90% or more strains negative; d, 11–89% of strains positive; w, weak reaction; (), delayed reaction.

Characteristic	1	2	3	4	5	6
Coagulase	−	−	+	−	−	−
Clumping factor	−	−*	+	−	−	−
Acid production (aerobically) from:						
D-Maltose	+	d	+	+	−	(+)
D-Mannitol	+	d, w	+	−	+	d
D-Mannose	−	−	+	(d)	+	+
D-Melezitose	(+)	d	−	(d)	−	−
D-Trehalose	+	+	+	−	−	(+)
N-Acetylglucosamine	(+)	d	+	−	−	−
Production of:						
Acetoin	−	−	+	+	d	+
α-Glucosidase†	−	+	−‡	‡	−‡	‡
β-Glucosidase	−†	†	+	(d)	−	−
Hydrolysis of DNA	−	+, w	+	−	d	+
Hydrolysis of aesculin	−	+	−	−	−	−
Resistance to polymyxin B	+	−	+	+\$	−	−

*Weakly positive using latex agglutination test (Murex Diagnostica).

†Tested using API ZYM (bioMérieux).

‡Result for type strain.

\$*S. epidermidis* strains were reported as resistant by Kloos & Bannerman (1999); our results suggest that only 70% of strains are resistant (85 clinical isolates tested).

susceptibility to the other antibiotics tested, i.e. penicillin, oxacillin, erythromycin, trimethoprim/sulfamethoxazole, chloramphenicol, clindamycin, gentamicin, amoxicillin/clavulanate, teicoplanin, vancomycin, ciprofloxacin and rifampicin.

The 16S rRNA genes of strains CCM 7213^T and CCM 7229 were amplified using primers 16Sfw (5'-AGAGTTTGA-TCTGGCTCAG-3') and 16Srev (5'-GGTTACCTTGTTA-CGACTT-3'). A PCR was performed according to Švec *et al.* (2001). Both strands of amplified DNA were sequenced by dideoxynucleotide chain termination sequencing with PCR primers; this was followed by primer walking with forward primers 271U (5'-CGGCTTACCAAGGCAACGA-3') and 1148U (5'-GTTGGGCACTCTAAGTTGA-3') and reverse primers 352L (5'-TCCCTACTGCTGCCTCCCG-3') and 1218L (5'-TGTAGCCCAAATCATAAGG-3'). Primer synthesis, DNA sequencing and phylogenetic analyses were performed as previously described (Mannerová *et al.*, 2003).

From the results obtained by DNA sequencing of the 16S rRNA genes, it is clear that the squirrel monkey isolates CCM 7213^T and CCM 7229 belong to the *S. aureus* species group (Takahashi *et al.*, 1999), which includes two subspecies, *S. aureus* subsp. *aureus* and *S. aureus* subsp. *anaerobius* (Takahashi *et al.*, 1999; Probst *et al.*, 1998; Trülsch *et al.*, 2002; Sperser *et al.*, 2003). The sequences of strains CCM 7213^T and CCM 7229 were identical and showed a similarity of 99 % with respect to that of *S. aureus*. The phylogenetic positions on the 16S rRNA gene tree are indicated in Fig. 1(a). The novel isolates and *S. aureus* differ from one another by six point mutations in the region 73–226.

The anticipated fragment for the *hsp60* gene of the type strain CCM 7213^T was amplified with degenerate primers H279 (5'-GAATTCGAIIGCIGGIGAYGGIACIAC-3') and H280 (5'-CGCGGGATCCYKIYKITCICCAAICGIGGICGTT-3'), using thermal cycler Tgradient (Biotetra), *Taq* DNA polymerase (Invitrogen Life Technologies) and PCR thermal cycling conditions, as described previously (Goh *et al.*, 1996). The amplicon obtained was purified by using the QIAquick PCR purification kit (Qiagen), digested with *Eco*RI and *Bam*HI restriction endonucleases (Roche Diagnostics) and cloned in the pBluescript II SK(+) vector (Stratagene). Both strands of DNA were sequenced with M13 forward and reverse sequencing primers. Estimation of the phylogenetic relationships of the squirrel monkey isolates and previously described *Staphylococcus* species (Kwok & Chow, 2003) by means of comparison of partial *hsp60* DNA sequences (Fig. 1b) showed that the highest similarity was with *S. aureus* (88 % similarity). Likewise, Hsp60 protein sequence similarity grouped the strains close to *S. aureus* (98 % identity). The next nearest related species were *Staphylococcus warneri* and *Staphylococcus pasteurii* (both sharing 96 % identity), *S. epidermidis*, *S. saccharolyticus*, *S. capitis*, *Staphylococcus muscae* and *Staphylococcus hominis* (all sharing 95 % identity). The other species exhibited Hsp60 protein sequence identity below 94 %.

SDS-PAGE profile analysis, including preparation of whole-cell protein extracts, densitometric analysis and reading of protein profiles as well as their numerical analysis were performed in accordance with the procedures described by Pot *et al.* (1994) and Švec *et al.* (2004). The novel isolates constituted a single cluster distinct from the type strains of phylogenetically related taxa (see Supplementary Fig. S1 available in IJSEM Online).

Various methods were used to detect intra-species genotypic relationships (Table 2). Ribotyping was performed using *Eco*RI (Roche Diagnostics) according to Pantůček *et al.* (1999). DNA was isolated by using guanidine hydrochloride extraction with the High Pure PCR template preparation kit (Roche Diagnostics). The 16S plus 23S rRNA gene hybridization profile analysis as well as cluster analysis were done using GelCompar II software (Applied Maths). The dendrogram was calculated by means of UPGMA clustering using the Dice coefficients. Three different ribotypes were

Fig. 1. Unrooted phylogenetic trees constructed on the basis of (a) 16S rRNA gene sequences and (b) *hsp60* gene sequences, showing the relationship between *S. simiae* sp. nov. and previously described staphylococci. In species that include two or more subspecies, the nominal one was used for the comparison. The maximum-likelihood method for DNA sequences, which correspond to region 55–1495 of *Bacillus subtilis* *rrn0*-16S (Kunst *et al.*, 1997) and to a 554 nt segment of the *hsp60* gene, respectively, was used for the analysis. The GenBank accession numbers of the sequences of the organisms used are shown in parentheses.

recognized at 82 % similarity level (see Supplementary Fig. S2). This cluster comprising all of the novel isolates was clearly distinct from other reference *Staphylococcus* species at a level of similarity below 62 %, as used by Chesneau *et al.* (2000) for separating different species of the genus *Staphylococcus*.

Table 2. Genotype variability of eight *S. simiae* sp. nov. strains

ITS, Internal transcribed ribosomal spacer; RAPD, random amplification of polymorphic DNA; ND, none detected.

Strain	Ribotype (<i>EcoRI</i>)	ITS-PCR	PFGE type (<i>SmaI</i>)	Plasmid content (kb)	RAPD		
					ERIC2	ERIC1R	1026
CCM 7213 ^T	E1	RS-1	A	ND	2-a	1R-a	AP-a
CCM 7214	E1	RS-1	B	ND	2-b	1R-a	AP-b
CCM 7215	E1	RS-1	B	ND	2-a	1R-a	AP-c
CCM 7216	E1	RS-1	A	ND	2-a	1R-a	AP-c
P 908	E1	RS-1	A	ND	2-a	1R-a	AP-c
CCM 7229	E2	RS-2	C2	1.6 + 6.1	2-d	1R-b	AP-d
P 1040	E3	RS-2	C1	1.6	2-c	1R-b	AP-e
CCM 7230	E3	RS-2	C1	1.6	2-c	1R-b	AP-f

Internal transcribed ribosomal spacer-PCR was performed as previously described (Jensen *et al.*, 1993), using identical amplification conditions and primers G1 (5'-GAAGTC-GTAACAAGG-3') and L1 (5'-CAAGGCATCCACCGT-3'). Amplification products were separated in high-resolution 3.5 % gels (Eliphore agarose; Elisabeth Pharmaton) in 1 × TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA; pH 8.2) for 6 h, using a 100 bp ladder (New England Biolabs) as a molecular size marker. Two internal transcribed ribosomal spacer-PCR patterns were obtained. Although these patterns are to a certain extent related to that of *S. capitis* (see Supplementary Fig. S3), they are unique, share six common fragments and can be used after visual comparison with other profiles of control strains obtained by Mendoza *et al.* (1998) and Couto *et al.* (2001) for characterization of the novel isolates.

PFGE using *SmaI* macrorestriction pattern analysis was performed as described by Pantůček *et al.* (1996). The novel isolates showed four distinct *SmaI* digest patterns containing 19–20 restriction fragments sized from 13 kb to 550 kb (see Supplementary Fig. S4). Patterns C1 and C2 differ from each another by the presence of 6.1 kb plasmid DNA. The mean genome size (2.404 ± 81 kb) is comparable to that of the *S. epidermidis* species group (George & Kloos, 1994) but lower than that of *S. aureus* (Pantůček *et al.*, 1996).

Isolation of plasmid DNA was performed by alkaline lysis and extraction using the High Pure plasmid isolation kit (Roche Diagnostics) according to the manufacturer's instructions but with minor modifications, i.e. the addition of lysostaphin (Sigma) to the lysis buffer to a final concentration of $50 \mu\text{g ml}^{-1}$, and prolonged lysis for 2 h. Plasmid DNA was subjected to slab gel electrophoresis on a 0.8 % agarose horizontal gel in 1 × TAE buffer at 1.5 V cm^{-1} for 6 h at room temperature. A supercoiled DNA ladder (Sigma) was used to estimate plasmid size. Two types of small plasmids were detected in three strains (Table 2).

Random amplification of polymorphic DNA was performed

using enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR as described previously (Pantůček *et al.*, 1999). The amplification program consisted of 2 min predenaturation at 95 °C followed by 40 cycles of 1 min at 94 °C, 2 min at 25 °C and 2 min at 74 °C. Primers ERIC1R and ERIC2 (Versalovic *et al.*, 1991) and primer 1026, the complement of ERIC1R (van Belkum *et al.*, 1993), were used. Primer 1026 showed the highest discriminatory power and allowed the differentiation of all but two strains, CCM 7216 and P 908, which were also identical when typed by other DNA-based techniques (Table 2). On the other hand, the banding patterns obtained with primer ERIC1R or primer ERIC2 allowed differentiation of the monkey strains from the most closely related staphylococcal species.

The eight monkey strains did not produce enterotoxins SEA, SEB, SEC₁, SEC₂, SED and SEE or toxin TSST-1 in investigations using the Reverse passive latex agglutination kit (Denka Seiken), and they gave negative results in PCRs performed according to Johnson *et al.* (1991).

As the novel isolates are closely related to *S. aureus*, we screened the former using different PCR assays for the detection of *S. aureus*-specific DNA sequences. All of these assays, e.g. amplification of the thermonuclease (*nuc*) gene (Chesneau *et al.*, 1993), the Sa826 bp fragment (Štěpán *et al.*, 2001), the coagulase (*coa*) gene (Schmitz *et al.*, 1997), the *femA* regulator gene (Vannuffel *et al.*, 1995) and the accessory gene regulator (*agr_{sa}*) locus (Dufour *et al.*, 2002), gave negative results.

The DNA–DNA hybridization experiments and analysis of the DNA G + C content were performed as described by Mannerová *et al.* (2003). Comparison between strains CCM 7213^T and CCM 7214 revealed 100 % hybridization, but all of the DNA–DNA hybridization values between squirrel monkey isolates and the type strains of phylogenetically related species were lower than 28 %. Both strains had binding values of 11–13 % with *S. aureus* subsp. *aureus* CCM 885^T, 20–22 % with *S. epidermidis* CCM 2124^T, 20 % with *S. capitis* subsp. *capitis* CCM 2734^T, 19–20 % with *S. caprae*

CCM 3573^T and 27–28 % with *S. saccharolyticus* LMG 22204^T. These data clearly indicate that the staphylococcal strains from the squirrel monkeys represent a novel species. The DNA G + C contents of strains CCM 7213^T and CCM 7214 were 33.8 and 31.6 mol%, respectively, which is within the range (30–39 mol%) reported for other staphylococcal species (Kloos *et al.*, 1992).

On the basis of the results obtained, the novel strains could not be assigned to any of the known staphylococcal species and therefore a polyphasic taxonomic approach was used for their analysis. From this study, we conclude that these strains represent a hitherto undescribed staphylococcal species, for which we propose the name *Staphylococcus simiae* sp. nov.

Description of *Staphylococcus simiae* sp. nov.

Staphylococcus simiae (si'mi.æ. L. gen. fem. n. *simiae* off from a monkey).

Cells are Gram-positive cocci of classical staphylococcal appearance, i.e. they occur singly, in pairs and in irregular clusters. Cells are non-motile, non-spore-forming and 0.7–0.8 µm in diameter. Colonies reach 1–1.5 mm in diameter on glucose/yeast extract/peptone agar after 24 h at 36.5 °C and are circular, smooth, flat with low-convex centres, glistening, white and have continuous margins. The strains grow anaerobically in thioglycolate medium. Growth occurs at 15 and 45 °C and in 12 % NaCl. All strains are susceptible to lysostaphin, novobiocin and nitrofurantoin and are resistant to bacitracin and polymyxin B. They are catalase-positive, oxidase-negative, coagulase-negative and clumping-factor-negative. All strains produce alkaline phosphatase, acid phosphatase, urease, leucine arylamidase, esterase and esterase-lipase and reduce nitrate to nitrite. They exhibit weak δ -haemolysis activity. None of them produce acetoin, heat-stable or heat-labile nuclease, arginine arylamidase, ornithine decarboxylase, valine arylamidase, cystine arylamidase, pyrrolidonyl arylamidase, lipase, *N*-acetyl- β -glucosaminidase, α -fucosidase, α -galactosidase, α -glucosidase, α -mannosidase or β -glucuronidase. Test-dependent results are obtained for the production of β -galactosidase, i.e. weakly positive when ONPG is used as a substrate and variable when 2-naphthyl β -D-galactopyranoside is used; the type strain is β -galactosidase-negative. Similarly, cells are β -glucosidase-positive when 2-nitrophenyl β -D-glucopyranoside is used as a substrate and negative when 6-bromo-2-naphthyl β -D-glucopyranoside is used. All strains are positive for aerobic acid production from D-fructose, D-glucose, galactose, lactose, D-maltose, D-mannitol, sucrose, D-trehalose and D-turanose. A delayed positive test result (2 days) is obtained for acidification of melezitose and *N*-acetylglucosamine. Negative results are obtained for aerobic production of acid from D-cellobiose, D-raffinose, D-mannose, D-melibiose, D-ribose, xylitol, methyl α -D-glucoside and for anaerobic fermentation of D-mannitol. Variable reactions are obtained for acid production from L-arabinose (3/8 positive, type strain

negative), sorbitol (3/8 positive, type strain negative) and D-xylose (3/8 positive, type strain negative), for production of arginine dihydrolase (5/8 positive, type strain positive) and lecitinase (4/8 positive, type strain weakly positive) and for hydrolysis of casein (6/8 positive, type strain weakly positive), gelatin (6/8 positive, type strain positive) and Tween 80 (5/8 positive, type strain positive). No hydrolysis of elastin, aesculin, starch and tyrosine is observed.

The type strain, CCM 7213^T (= LMG 22723^T), was isolated from the faeces of a squirrel monkey. Its characteristics are in full agreement with those given in the species description. The DNA G + C content of the type strain is 33.8 mol% (by HPLC).

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