Staphylococcus pseudintermedius sp. nov., a coagulase-positive species from animals

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Four staphylococcal isolates from clinical and necropsy specimens from a cat, a dog, a horse and a parrot (*Psittacus erithacus timneh*) were found to constitute a distinct taxon. 16S rRNA gene sequence analysis revealed that its closest phylogenetic relatives are *Staphylococcus intermedius* and *Staphylococcus delphini*. Growth characteristics, biochemical features and DNA–DNA hybridizations demonstrated that the strains differ from these and other known species and that they represent a single, novel *Staphylococcus* species for which the name *Staphylococcus pseudintermedius* sp. nov. is proposed. The novel species is commonly confused with *S. intermedius* in routine diagnostic veterinary bacteriology. Although the strains described were isolated from lesions and show several characteristics typical of pathogenic staphylococci, such as coagulase, DNase and β -haemolysin production, the pathogenic significance of the novel species remains unclear. The type strain, LMG 22219^T (= ON 86^T = CCUG 49543^T), was isolated from lung tissue of a cat.

In routine diagnostic bacteriology, the major pathogenic staphylococcal species *Staphylococcus aureus* has long been differentiated from other species by means of a single test, most often coagulase or clumping-factor production. In veterinary bacteriology, the taxonomic situation is more complex. In the 1970s, the animal pathogens *Staphylococcus intermedius* (Hajek, 1976) and *Staphylococcus hyicus* (Devriese *et al.*, 1978) were described as having certain characteristics that, until then, had been considered as typical for *S. aureus*. Other features were provided that allowed these taxa to be differentiated from others (Table 1). Since then, several novel (sub)species presenting similar features have been described, e.g. *S. aureus* subsp. *anaerobius* from sheep (De La Fuente *et al.*, 1985), *Staphylococcus delphini* from dolphins (Varaldo *et al.*, 1988), *Staphylococcus schleiferi* subsp. *coagulans* from dogs (Igimi *et al.*, 1990) and *Staphylococcus lutrae* from otters (Foster *et al.*, 1997). With the exception of the non-haemolytic

S. hyicus, strains from all these species are easily mistaken in routine diagnostic bacteriology as *S. aureus* or *S. intermedius*. Extensive phenotypic testing or molecular identification methods are needed in order to identify these strains adequately.

During routine veterinary diagnostic work, one of these molecular identification techniques – tRNA intergenic length polymorphism analysis – was applied and revealed a group of four strains with similar electrophoretic patterns which differed from those of the other staphylococcal species known to date (data not shown). However, their profiles were similar to those of *S. intermedius* and *S. delphini*. The tRNA intergenic length polymorphism analysis was performed as described previously (Baele *et al.*, 2000, 2001). The group was peculiar in that each of the strains originated from a different animal species: strain LMG 22219^T (= ON 86^T) was isolated in 1999 from the lung tissue of a cat, LMG 22220 (= ON 88) was isolated in 1999 from a skin lesion in a horse, LMG 22221 (= ON 252) was isolated in 2001 from an ear lesion (otitis externa) in a dog and LMG 22222 (= ON 576) was isolated in 2003 from the liver of a parrot (*Psittacus erithacus timneh*).

The phylogenetic position of all four strains (LMG 22219^T to

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 22219^T, LMG 22220, LMG 22221 and LMG 22222 are AJ780976, AJ780977, AJ780978 and AJ780979, respectively.

Table 1. Characteristics that differentiate *S. pseudintermedius* sp. nov. from other coagulase-positive and related *Staphylococcus* species

Taxa: 1, *S. pseudintermedius* sp. nov.; 2, *S. aureus* subsp. *aureus*; 3, *S. aureus* subsp. *anaerobius*; 4, *S. intermedius*; 5, *S. hyicus*; 6, *S. schleiferi* subsp. *schleiferi*; 7, *S. schleiferi* subsp. *coagulans*; 8, *S. delphini*; 9, *S. lutrae*. Data for reference taxa are based on Calvo *et al.* (2000), De La Fuente *et al.* (1985), Devriese (1981), Devriese & Hajek (1980), Foster *et al.* (1997), Freney *et al.* (1999), Hébert *et al.* (1988), Igimi *et al.* (1990), Kloos & Bannerman (1999) and Varaldo *et al.* (1988). Traits are scored as follows: D, strain-dependent; D+, usually positive; D−, usually negative; ND, not determined; W, weak; results shown in parentheses were tested with the type strain in the present study.

Characteristic	1	2	3	4	5	6	7	8	9
Aerobiosis	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Colony diameter >5 mm	+	+	+	+	+	+	+	+	+
Pigment	+	+	+	+	+	+	+	+	+
Coagulase	+	+	+	+	D−	−*	+	+	+
Clumping factor	+	+	+	D	+	+	+	+	+
DNase	+	+	+	+	+	+	+	+	+
Haemolysis	+	+	+	+	+	+	+	+	+
β-Haemolysin	+	D	+	+	+	+	ND	ND	+
Acetoin (Voges-Proskauer test)	+	+	+	W	+	+	+	+	+
Pyrrolidonyl arylamidase	+	+	+	+	+	+	D	ND	ND
Acid production from:									
Maltose	+	+	+	+/W	+	+	D	+	+
Sucrose	+	+	+	+	+	+	+	+	ND
D-Trehalose	+	+	+	+	D+	D	+	+	+
D-Xylose	+	+	+	+	+	+	+	+	+
Sensitivity to:									
Acriflavine	+	+	ND	+	+	(+)	(+)	(+)	(+)
Polymyxin	+	+	ND	+	+	(+)	(+)	(+)	(+)

*A pseudocoagulase may be produced by certain *S. schleiferi* subsp. *schleiferi* strains (Vandenesch *et al.*, 1994).

†*S. lutrae* and *S. intermedius* biotypes E and F isolated from pigeons and mink do not produce a strong DNase effect. The other species and the canine and equine biotypes of *S. intermedius* are strongly DNase-positive.

LMG 22222) was determined by 16S rRNA sequence analysis. DNA was extracted as indicated for tRNA intergenic length polymorphism analysis. To amplify the 16S rRNA gene, a PCR was performed using the conserved primers $\alpha\beta$ -NOT (5'-TCAAACCTAGGACCGAGTC-3') and ω_{MB} (5'-TACCTTGTTACTTCACCCCA-3') and the *Taq* Mastermix (Qiagen). After PCR product purification using the Qiaquick PCR purification kit (Qiagen), sequencing reactions were performed using the BigDye Terminator sequencing kit (Applied Biosystems) and primers *, *O, PD and 3 described by Coenye *et al.* (1999). The sequences were determined using an ABI PRISM 310 Genetic Analyzer

(Applied Biosystems). Phylogenetic analysis was done using the program BIONUMERICS (version 3.5; Applied Maths). Pairwise alignment similarities were calculated and a dendrogram was constructed using the neighbour-joining method (Fig. 1). No correction factor was used. The strains showed sequence similarities of 100% among each other and were classified within the *S. intermedius* phylogenetic species group as defined by Takahashi *et al.* (1999). Sequence similarities above 99% were obtained with the species *S. delphini*, *S. intermedius* and *S. schleiferi*.

DNA G+C contents were determined for the cat strain (LMG 22219^T), the horse strain (LMG 22220) and the type strains of *S. delphini* (DSM 20771^T), *S. intermedius* (LMG 13351^T) and *S. schleiferi* subsp. *schleiferi* (LMG 13347^T). Strains were cultivated on trypticase soy agar (BBL) for 24 h at 37 °C. DNA was extracted from 0.75–1.25 g cells (wet weight) using the protocol described by Pitcher *et al.* (1989), with the following modifications: the washed cell pellet was resuspended and lysed in a buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) containing RNase (200 µg ml^{−1}; Sigma), lysostaphin (100 U ml^{−1}; Sigma) and lysozyme (25 mg ml^{−1}; SERVA) for 1 h at 37 °C. Before the addition of GES reagent (Pitcher *et al.*, 1989), proteinase K (200 µg ml^{−1}; Merck) was added to the mixture for 15 min. For determination of the DNA G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters SymmetryShield C8 column maintained at a temperature of 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. The DNA G+C contents were 38 mol% for the novel isolates (LMG 22219^T and LMG 22220) and 38, 37 and 36 mol% for the type strains of *S. delphini* (DSM 20771^T), *S. intermedius* (LMG 13351^T) and *S. schleiferi* subsp. *schleiferi* (LMG 13347^T), respectively.

DNA–DNA hybridizations were performed between strains LMG 22219^T and LMG 22220, and the type strains of *S. delphini* (DSM 20771^T), *S. intermedius* (LMG 13351^T) and *S. schleiferi* subsp. *schleiferi* (LMG 13347^T). DNA was prepared as described above. The microplate method was used as described by Ezaki *et al.* (1989) and Goris *et al.* (1998), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with single-stranded unlabelled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 35 °C in a hybridization mixture containing 2 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate), 5 × Denhardt's solution (Quantum), 2.5% dextran sulphate, 50% formamide, 100 µg denatured salmon sperm DNA ml^{−1} and 1250 ng biotinylated probe DNA ml^{−1}. Hybridization levels of 96% were found between strains LMG 22219^T and LMG 22220, which indicates that the strains constitute a single species. Both strains had binding values of 54% with *S. delphini* DSM 20771^T, 38–46% with *S. intermedius* LMG 13351^T and 16–18% with

Fig. 1. Distance matrix tree showing the phylogenetic relationships of *S. pseudintermedius* sp. nov. and other staphylococcal reference species, based on 16S rRNA gene sequence comparisons. *Bacillus subtilis* was used as the outgroup. Bootstrap probability values above 80 % (percentages of 500 tree replications) are indicated at branch-points.

S. schleiferi subsp. *schleiferi* LMG 13347^T. The latter data indicate that the novel isolates constitute a single species that is distinct from staphylococcal species with validly published names.

Phenotypic tests were applied to the novel isolates. Haemolysis and growth characteristics were tested on Columbia agar base (Oxoid) supplemented with 5 % sheep blood. Coagulase and clumping-factor tests were carried out with rabbit plasma for the detection of staphylocoagulase (bioMérieux). For DNase testing, a standardized plate method was used which discriminates between the strong reactions typical of most coagulase-positive species and the weak reactions often seen with other staphylococci (Devriese & Van de Kerckhove, 1979). Acid production from carbohydrates was tested in API 50 CH galleries (bioMérieux) under paraffin cover, and other biochemical reactions were tested in API STAPH (bioMérieux) and STAPH-ZYM galleries (Rosco) with the additional susceptibility tests included in this system and acetoin diagnostic test tablets of the same origin. It was observed that alkaline phosphatase reactions were positive in the STAPH-ZYM tests, but negative in the API STAPH tests. Acriflavine MICs were determined as described by Devriese (1981). The strains differed from *S. aureus* subsp. *aureus* by their lack of pigment, by the absence of clumping-factor activity, by their positive reactions in tests for pyrrolidonyl arylamidase and ONPG (β -galactosidase) and by their sensitivity to 8 μ g acriflavine ml⁻¹. The acriflavine MICs were in the range 0.8–1.6 μ g ml⁻¹ for the four novel isolates, whereas they amounted to 12.5 and 25 μ g ml⁻¹ for *S. aureus* strains (Devriese, 1981). *S. aureus* strains are unique in being the only staphylococci described,

to date, that are resistant to more than 8 μ g acriflavine ml⁻¹ – a level used in the selective isolation of this species in food microbiology (Isigidi *et al.*, 1989).

The strains are less easy to differentiate from *S. intermedius*. Many *S. intermedius* strains have clumping-factor activity and that latter species shows either weak or non-existent acid production from maltose. Erroneous identifications as *S. schleiferi* subsp. *coagulans* can be avoided by testing for acidification of trehalose. In addition, *S. delphini* differs from the novel species in its negative DNase reaction. Methods of differentiation from other coagulase-positive and related species are shown in Table 1.

Overall, the results of the present study allowed us to assign the four strains LMG 22219^T to LMG 22222 to a novel species, for which we propose the name *Staphylococcus pseudintermedius* sp. nov. A detailed description is given below. The identification of this novel species offers a real challenge to the veterinary diagnostic bacteriologist. These strains are most likely to be confused with *S. intermedius*. Confirmation of *S. pseudintermedius* identifications by molecular methods is recommended.

Despite the fact that all four strains upon which the species description is based were isolated from lesions, internal as well as external, in none of the cases was a clear-cut indication of a pathogenic role present.

Description of *Staphylococcus pseudintermedius* sp. nov.

Staphylococcus pseudintermedius [Gr. adj. *pseudēs* or *pseudos* false; L. masc. adj. *intermedius* intermediate, and also a

specific epithet; N.L. masc. adj. *pseudintermedius* a false (*Staphylococcus*) *intermedius*, because of the high phenotypic similarity to *S. intermedius*].

Consists of Gram-positive cocci predominantly arranged in groups. Colonies on Columbia sheep blood agar are non-pigmented and surrounded by double zone haemolysis. The outer band, which is incompletely haemolytic, turns into complete haemolysis after being put at 4 °C (hot-cold haemolysis), and is typical of staphylococcal β -haemolysin (a sphingomyelinase). The strains are catalase-positive and coagulate rabbit plasma, but they are clumping-factor-negative in the so-called slide or rapid coagulase test. A strong DNase is produced. Positive in tests for acetoin, β -glucosidase, arginine dihydrolase, urease, nitrate reduction, pyrrolidonyl arylamidase and ONPG (β -galactosidase). Does not produce β -glucuronidase. Susceptible to 8 μ g acriflavine ml⁻¹ and to novobiocin. Resistant to deferroxamine. Acid is produced from glycerol (weakly and delayed), ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol (weakly and delayed), N-acetylglucosamine, maltose, lactose, sucrose, trehalose and D-turanose (weakly and delayed). No acid is produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β -D-xyloside, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α -D-glucoside, methyl α -D-xyloside, amygdalin, arbutin, aesculin, salicin, cellobiose, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The G+C content of the DNA is 38 mol%.

The type strain is LMG 22219^T (=ON 86^T=CCUG 49543^T). Strains have been isolated from lesions in different animal host species, but the habitat and pathogenic activity are unknown.

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