Staphylococcus jettensis sp. nov., a coagulase-negative staphylococcal species isolated from human clinical specimens

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Eight coagulase-negative, novobiocin-susceptible staphylococcal strains were isolated from human clinical specimens at two different Belgian medical facilities. All strains were non-motile, Gram-stain-positive, catalase-positive cocci. DNA G+C content, peptidoglycan type, menaquinone pattern, the presence of teichoic acid and cellular fatty acid composition were in agreement with the characteristics of species of the genus Staphylococcus. Sequencing of the 16S rRNA gene and four housekeeping genes (dnaJ, tuf, gap and rpoB) demonstrated that these strains constitute a separate taxon within the genus Staphylococcus. Less than 41 % DNA–DNA hybridization with the most closely related species of the genus Staphylococcus (Staphylococcus haemolyticus, Staphylococcus hominis and Staphlococcus lugdunensis) was observed. Key biochemical characteristics that allowed these bacteria to be distinguished from their nearest phylogenetic neighbours are arginine dihydrolase positivity, ornithine decarboxylase negativity and inability to produce acid aerobically from D-mannose, α-lactose and turanose. Acid is produced aerobically from trehalose. Based on these results, a novel species of the genus Staphylococcus is described and named Staphylococcus jettensis sp. nov. The type strain is SEQ110T (=LMG 26879T =CCUG 62657T =DSM 26618T).

Abbreviations: CoNS, coagulase-negative staphylococci; MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry.

The GenBank/EMBL/GenPept accession numbers for Staphylococcus jettensis isolates SEQ027, SEQ036, SEQ255, SEQ256, SEQ257, SEQ258, SEQ259 and SEQ110T are JN902111 to JN902118 (16S rRNA gene); JN902095 to JN902102 (tuf); JN907013 to JN907020 (rpoB); N0902103 to JN902110 (gap); HE687294 to HE687301 (dnaJ); and KC329839 to KC329847 (tarA).

Three supplementary figures and four supplementary tables are available with the online version of this paper.
validation of a MALDI-TOF MS instrument (Microflex, Bruker) for the identification of CoNS, we used, besides reference strains, a biochemically characterized strain collection (Leven et al., 1995). All strains for which MALDI-TOF MS-based identification results were discrepant using database version 3.0 had their 16S rRNA genes sequenced. Strain SEQ027, a clinical strain historically identified as *Staphylococcus warneri*, did not match any known CoNS species and was added to the reference database as a possible novel species. Between August 2009 and December 2010, seven clinical strains (see Table S1 available in IJSEM Online for details) with similar MS spectra (log score range: 2.119–1.830; see Fig. S1) were isolated from human clinical samples which were expected to be sterile (catheters, biopsies, cerebrospinal fluid, blood and deep swabs). Moreover, they were found almost always in mixtures with other CoNS, which made it difficult to assess their clinical significance. In the present study we fully characterized these strains.

Genomic DNA of the isolates was obtained after treatment of a bacterial suspension with 0.1 g lysostaphin 1\(^{-1}\) (Sigma-Aldrich) and 0.2 g proteinase K 1\(^{-1}\) (Sigma-Aldrich). The 16S rRNA gene sequencing was performed as described by Coenye et al. (2002). Determination of the closest relatives based on the 16S rRNA gene of the eight unknown isolates was carried out with the ARB software package using all type strains of species of the genus *Staphylococcus* present in the SILVA rRNA database (Pruesse et al., 2007). Type strain sequences of missing species were retrieved from the GenBank/EMBL/DDJB database and added. Phylogenetic trees were reconstructed using the built-in neighbour-joining method of the ARB software package implementing the Kimura-2 substitution model (Kimura, 1980). Near full-length 16S rRNA gene sequences (1443 bp) were used for the reconstruction of phylogenetic trees using the neighbor-joining method (Saitou & Nei, 1987). A 1000-replicates bootstrap analysis was performed to validate the reliability of the observed clustering (Felsenstein, 1985). In addition, maximum-likelihood clustering was performed to confirm the tree topology.

The topologies of the 16S rRNA derived neighbour-joining trees (Fig. 1 and Fig. S2) were equivalent to that of the maximum-likelihood tree (not shown) with the unknown strains grouping as an isolated cluster near *Staphylococcus hominis* subsp. *hominis*, *Staphylococcus hominis* subsp. *novobiosepticus*, *Staphylococcus devriesie*, *Staphylococcus haemolyticus* and *Staphylococcus lugdunensis*. 16S rRNA gene sequence similarity values with the nearest neighbours were 98.87 % (*S. lugdunensis*), 98.91 % (*S. haemolyticus*) and 99.15 % (both subspecies of *S. hominis*).

Due to the limited discriminatory power of the 16S rRNA gene (Shah et al., 2007), the phylogenetic position of the unknown isolates was also assessed using sequences of the protein-coding regions of the housekeeping genes *dnaJ*, *rpoB*, *gap* and *tuf*. An overview of the primers used in this study for amplification and sequencing can be found in Table S2. Partial sequences of *dnaJ* (429 bp), *rpoB* (474 bp), *gap* (811 bp) and *tuf* (612 bp) were used for the reconstruction of phylogenetic trees using the neighbour-joining method (Saitou & Nei, 1987). Again, a 1000-replicate bootstrap analysis was performed to validate the reliability of the observed clustering (Felsenstein, 1985) and maximum-likelihood clustering was performed to confirm the tree topology.

Neighbour-joining and maximum-likelihood phylogenetic trees for the housekeeping genes, only including the closest relatives based on the position of the unknown isolates in the 16S rRNA gene phylogenetic tree, were reconstructed using MEGA5 (Tamura et al., 2011). The best fit substitution model parameters to infer phylogenies were determined using MEGA5: Jukes–Cantor (Jukes & Cantor, 1969) for *dnaJ* and *rpoB* and Kimura-2 for *gap* and *tuf*. In all models the rate variation among sites modelled with a gamma distribution (shape parameter = 1) was taken into account.

For each of the housekeeping genes, neighbour-joining (Fig. S3) and maximum-likelihood trees (not shown) were very similar; *dnaJ*, *rpoB* and *gap*-based analyses confirmed that the unknown isolates represented a well delineated phylogenetic lineage within the genus *Staphylococcus*, separate from its nearest neighbours; yet, in the *tuf*-based analysis strain SEQ027 occupied a more aberrant position in the tree.

To perform DNA–DNA hybridizations, DNA was isolated using the method described by Marmur (1961). Biomass (± 0.8 g) was harvested from Columbia agar (bioMérieux). Lysozyme (Serva; 8 g 1\(^{-1}\)) treatment was performed overnight and for more lysis-resistant strains a combination of lysostaphin (0.05 g 1\(^{-1}\)) and lysozyme (1 g 1\(^{-1}\)) was used. Based on their 16S rRNA sequence similarity, the following type strains were included for DNA–DNA hybridization and DNA base composition experiments: *S. haemolyticus* ATCC 29970\(^T\), *S. hominis* subsp. *hominis* ATCC 27844\(^T\), *S. hominis* subsp. *novobiosepticus* ATCC 700236\(^T\) and *S. lugdunensis* ATCC 43809\(^T\). Three representative strains (SEQ027, SEQ110\(^T\) and SEQ256) were selected for these experiments. Microplate DNA–DNA hybridization was performed according to the method of Ezaki et al. (1989). The hybridization temperature was 32 °C.

The DNA–DNA hybridization experiments revealed high hybridization values (70 % or more) among strains SEQ027, SEQ110\(^T\) and SEQ256, confirming that they represent a single genomic species. In addition, 41 % or less DNA–DNA hybridization was measured with the most closely related species of the genus *Staphylococcus* (Table S3). Reciprocal reactions were within the limits of the method, i.e. mean ± 7 % (Goris et al., 1998) (our mean ±SD: 5.9 %).

The DNA G + C content of strains SEQ027, SEQ110\(^T\) and SEQ256 was determined with nuclease P1-mediated DNA degradation and separation by HPLC in triplicate (Mesbah & Whitman, 1989) and was 33.8 ± 0.1 mol%. This range of DNA base composition is lower than 2 mol%, as generally
Fig. 1. Phylogenetic position of the eight unknown isolates in the neighbour-joining tree based on 16S rRNA gene sequences of the 15 closest relatives. Bootstrap values (%) are based on 1000 replicates, only percentages equal to or above 70% shown at the nodes. The bar represents 1% sequence divergence. GenBank accession numbers are indicated in parentheses.
accepted within a single species and is consistent with results observed for members of the genus Staphylococcus (Kocur et al., 1971). The phenotypes of the eight strains were examined as follows. Smears for Gram staining were prepared from overnight cultures in tryptic soy broth (Lab M). Inoculated tryptic soy agar (Lab M) with 5% horse blood (E&O Laboratories) were incubated at 35° C in 5% CO2. Colony morphology, colour formation and haemolysis were examined after 24 and 48 h. The same media were incubated at 35°C aerobically and at room temperature to compare pigment production. Fastidious anaerobe agar (Lab M) supplemented with 5% horse blood was inoculated and incubated anaerobically to test facultative anaerobic growth. Mannitol salt agar (MSA; Lab M) was incubated at 35° C in ambient air. Catalase was tested using the standard procedure (Freney et al., 1999). Cytochrome oxidase was determined using DrySlide (BBL). DNase agar (Lab M) was used to assess DNase activity; clumping factor and tube coagulase were determined using rabbit plasma (Bio-Rad). Biochemical characteristics were obtained using the ID 32 STAPH gallery (bioMérieux), with tests performed according to the manufacturer’s instructions. Aerobic acid production from D-glucose, D-mannitol, L-arabinose, D-xylose, cellobiose, raffinose, salicin, sucrose, maltose, D-mannose, trehalose, α-lactose, D-galactose, β-D-fructose, melezitose, turanose, D-ribose and xylitol was examined using purple agar base medium (Atlas, 1993; Freney et al., 1999) and interpreted as described by Freney et al. (1999). Susceptibility to 11 antimicrobial agents (penicillin, ceftoxitin, erythromycin, clindamycin, amikacin, gentamicin, doxycycline, ciprofloxacin, rifampicin, trimethoprim with sulfamethoxazole and linezolid) was determined by disc diffusion (Neosensitabs; Rosco) according to Clinical and Laboratory Standards Institute approved standards M2-A10 (Clinical and Laboratory Standards Institute, 2009) and M100-S21 (Clinical and Laboratory Standards Institute, 2011). Vancomycin susceptibility was determined with E-test (bioMérieux).

An overview of phenotypic characteristics and susceptibility patterns is given in the species description below and in Table S4. Biochemical analysis with ID 32 STAPH gallery failed to identify these strains. Discriminatory reactions that differentiate the new taxon from the phylogenetically most closely related species of the genus Staphylococcus, selected on the basis of the 16S rRNA phylogenetic tree – S. warneri –, are arginine dihydrolase positivity, ornithine decarboxylase negativity, ability to produce acid aerobically from D-mannose, α-lactose or turanose. These differentiating reactions are presented in Table 1. Strain SEQ027 had a biochemical profile that was aberrant from that of the other seven strains by its pigmentation (the colonies were yellow pigmented after overnight incubation), urease positivity and pyrrolidonaryl arylamidase negativity. These two last aberrant biochemical characteristics made it impossible to distinguish SEQ027 biochemically from S. warneri.

The chemical structure of the cell wall was characterized by analysis of the peptidoglycan and the presence of teichoic acid.

The peptidoglycan structure was analysed according to published protocols (Schumann, 2011). Purified cell walls were isolated after disruption of the cells by shaking with glass beads and subsequent trypsin digestion. The amino acids and peptides in the cell wall hydrolysates were analysed by 2D-TLC on cellulose plates. The molar ratios of the amino acids were determined by GC–MS (320-MS Quadrupole GC/MS; Varian) of N-heptafluorobutyryl amino acid isobutyl esters. The total hydrolysate of the

Table 1. Phenotypic characteristics that differentiate S. jettensis sp. nov. from the phylogenetically most closely related members of the genus Staphylococcus and S. warneri

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<td>Arginine dihydrolase</td>
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The peptidoglycan (4 M HCl, 16 h at 100 °C) of strain SEQ110T contained the amino acids lysine, alanine, glycine, serine and glutamic acid in molar ratios of 0.8:2.7:3.6:0.5:1.0, respectively. The peptides L-Ala-D-Glu, D-Ala-Gly, L-Lys-Gly, D-Ala-L-Lys-Gly and oligo-Gly were detected by 2D-TC of the partial hydrolysatse of the peptidoglycan (4 M HCl, 0.75 h at 100 °C). Due to the occurrence of serine and the reduced amount of glycine it is most likely that glycine is partially substituted by serine in the interpeptide bridge like in the peptidoglycan structure A11.3 (http://www.peptidoglycan-types.info).

From these data it was concluded that the peptidoglycan type of strain SEQ110T is A3x L-Lys-Gly3-4(Ser).

The presence of teichoic acid was demonstrated by sequencing the N-acetyl-β-d-mannosaminyltransferase tarA gene (Riesen & Perreten, 2010).

Menaquinones were extracted according to the method of Collins et al. (1977) and analysed by HPLC as described by Groth et al. (1997). The menaquinone profile for strain SEQ110T was MK-7 (92%), MK-6 (2%), MK-8 (1%).

Cellular fatty acids were measured by a 6890N gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector and Microbial Identification System (MIDI), according to the manufacturer’s instructions. For this purpose, bacteria were harvested from tryptic soy broth (Becton Dickinson) with agar (Becton Dickinson) after incubation for 48 h at 28 °C. The predominant fatty acids were ai-C15:0 and i-C15:0, whilst ai-C17:0, i-C17:0, C18:0, C16:0 and C20:0 were present in moderate amounts. These results are in concordance with previous reported patterns for CoNS (Freney et al., 1999; Kotilainen et al. 1991).

In summary, data from the present study demonstrate that the eight clinical isolates represent a single novel genomic species within the genus Staphylococcus that can be distinguished from its nearest neighbours by both genotypic and phenotypic characteristics. We therefore propose to classify these strains as representatives of the novel typic and phenotypic characteristics. We therefore propose
distinguished from its nearest neighbours by both geno-

**Description of Staphylococcus jettensis sp. nov.**

*Staphylococcus jettensis* (jet.ten’sis, N.L. masc. adj. jettensis from Jette, the municipality of Brussels-Capital region where most of these strains were isolated and where the medical campus of the Vrije Universiteit Brussel is housed).

Gram-stain-positive cocci occurring singly, in pairs and in irregular clusters, non-motile and non-spore-forming. Cell diameters range from 0.5 to 1.5 μm. After 2 days of growth, colonies are 3-4 mm in diameter, circular, smooth, raised to umbonate, glistering and opaque with entire edges. Isolates typically become yellow pigmented after prolonged incubation (except for strain SEQ027 which is yellow after overnight incubation). Colour production is clearer at room temperature. Narrow (about 1 mm), diffuse zone of β-haemolysis on sheep and horse blood. Capnophilic and facultatively anaerobic. Growth on MSA is red. Catalase-positive, cytochrome oxidase-negative, novobiocin-susceptible, urease activity is variable (negative, in the majority of strains). Tube coagulase, clumping factor and DNase-negative. All strains produce acid aerobically from D-glucose, sucrose, maltose, trehalose and β-D-fructose. Acid is not produced from D-mannitol, L-arabinose, D-xylene, cellobiose, D-fucose, raffinose, salicin, D-mannose, α-lactose, melezitose, turanose, D-ribose and xylitol. As determined with the API 32 STAPH system, glucose fermentation, nitrate reduction, Voges–Proskauer and arginine dihydrolase-positive. Pyrrolidonyl arylamidase is variable (positive in the majority of strains). The following substrates are not metabolized: L-ornithine, aesculin, mannotol, 2-naphthyl-β-D-galactopyranoside, L-arginine-β-naphthylamide, 2-naphthyl phosphate and N-acetylglucosamine. Acid production from D-galactose and β-glucuronidase is variable. Three susceptibility patterns are recognized. One pattern is omni susceptible (n=1), a second pattern is penicillin-resistant (n=2) and most strains (n=5) are penicillin-, cefoxitin-, erythromycin- and clindamycin- (inducible, positive D-zone test) resistant. One isolate is resistant to rifampicin. All strains are vancomycin-susceptible (MIC range 1.0-2.0 μg ml⁻¹). The peptidoglycan is of the type A3x L-Lys-Gly3-4(Ser), MK-7 is the major menaquinone and the predominant fatty acids are ai-C15:0 and i-C15:0, whilst ai-C17:0, i-C17:0, C18:0, C16:0, C20:0 are present in moderate amounts. The G+C content of the DNA is 33.8 mol%, derived from the analysis of three representative strains (SEQ027, SEQ110 and SEQ256). The type strain is SEQ110T (=LMG 26879T=CCUG 62657T=DSM 26618T) and was isolated from a blood culture in Jette, Belgium.

**Acknowledgements**

The authors thank Danny Cnudde, Elly Roebben, Patrick Geens, Ina Van Cauwenbergh and Cindy Snaauwaert for skilled technical assistance. Professor Sabine Lauwers and Paul Rosset are acknowledged for their important contribution to the implementation of the MALDI-TOF MS technology at the laboratory of Universiteit Ziekenhuis Brussel, leading to the observations forming the basis of this publication.

**References**
