**Staphylococcus equorum** and **Staphylococcus succinus** isolated from human clinical specimens

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A polyphasic identification approach was applied to a group of 11 novobiocin-resistant staphylococci isolated from human clinical materials. Phenotypic characteristics obtained by both commercial and conventional tests assigned eight strains as *Staphylococcus xylosus* and three strains as ambiguous *S. xylosus/Staphylococcus equorum*. In contrast to biotyping, ribotyping with EcoRI and *Hind*III restriction endonucleases and whole-cell protein fingerprinting assigned six analysed strains as *S. equorum*, and five strains as *Staphylococcus succinus*. Confirmation of the identification was done by partial 16S rRNA gene sequencing and *S. equorum* isolates were verified by a PCR assay targeting the sodA gene. From the data it has been implied that ribotyping and whole-cell protein analysis can be used to differentiate between the biochemically almost indistinguishable species *S. xylosus*, *S. equorum* and *S. succinus*. The present study confirms what is believed to be the first occurrence of *S. equorum* in a relevant human clinical material in the Czech Republic and describes what is believed to be the first-ever isolation of *S. succinus* from human clinical material.

**INTRODUCTION**

Coagulase-negative staphylococci (CoNS) are common inhabitants of the skin, skin glands and mucous membranes of various mammals and birds (Nagase et al., 2001; Hauschild, 2001). Although these organisms are part of the normal flora of animals and are generally considered to be of low virulence, they appeared to be implicated in the aetiology of a variety of human and animal infections, and they have established themselves as important pathogens showing increasing trends towards antibiotic resistance in the last decade (von Eiff et al., 2001). CoNS are also frequently isolated from bovine, caprine and ovine milk and dairy products (Bockelmann & Hoppe-Seyler, 2001; Meugnier et al., 1996; Vernozy-Rozand et al., 2000). *Staphylococcus xylosus*, *Staphylococcus equorum*, *Staphylococcus succinus*, *Staphylococcus nepalensis* and *Staphylococcus gallinarum* are phylogenetically and/or biochemically closely related novobiocin-resistant CoNS (Lambert et al., 1998; Spergser et al., 2003). *S. xylosus* is a common commensal inhabitant of animals. Nagase et al. (2001) studied the distribution of staphylococcal species on the skin of several animals and found *S. xylosus* to be predominant in chickens, laboratory mice and pigeons, and isolated this species very frequently from dogs, pigs, horses and cows. However, isolation of *S. xylosus* from human skin was rare in their study. In humans, *S. xylosus* may play a role in infections of the urinary tract and more rarely, it can be involved in endocarditis, pyelonephritis or pneumonia (Marsou et al., 2001; Siqueira & Lima, 2002). *S. equorum* subsp. *equorum* was originally isolated from healthy horses (Schleifer et al., 1984), and later isolates were obtained from the milk of a cow with mastitis and from healthy goats (Meugnier et al., 1996). Only a few *S. equorum* subsp. *equorum* strains were found in relevant human clinical materials (Alcaráz et al., 2003; Marsou et al., 2001; Pinna et al., 1999). The other subspecies, *S. equorum* subsp. *linens*, was isolated from the surface of ripening cheese (Place et al., 2003). *S. succinus* subsp. *succinus* was isolated from amber (Lambert et al., 1998), while *S. succinus* subsp. *casei* was obtained from the surface of ripened cheese (Place et al.,...
2002). *S. succinus* strains were also isolated as a part of the microflora in both fermented and fresh sausages (Blaiotta et al., 2004; Rantsiou et al., 2005). *S. nepalensis* was found as a pathogen of goats (Spergser et al., 2003) and *S. gallinarum* was isolated from the skin of poultry (Devirese et al., 1983).

The aim of this study was to characterize in detail 11 novobiocin-resistant CoNS strains isolated from human clinical materials and presumptively assigned as *S. xylosus* or ambiguous *S. xylosus/S. equorum*. Different phenotypical and molecular methods were applied to the analysed group to provide a reliable approach for the differentiation between these phylogenetically and phenotypically closely related staphylococcal species.

**METHODS**

**Bacterial strains.** Eleven epidemiologically unrelated strains of presumptive *S. xylosus* and *S. xylosus/S. equorum* were isolated from human clinical materials in different clinical laboratories in the Czech Republic. The possibility that they were skin contaminants was excluded. All strains were collected by the National Reference Laboratory (NRL) for Staphylococci (Prague, Czech Republic) between 1999 and 2002 as clinically significant agents isolated from different clinical materials (Table 1). Strains CCM 7301, CCM 7302, CCM 7312, CCM 7313 and CCM 7314 were deposited in the Czech Collection of Microorganisms (CCM), Masaryk University, Brno (http://www.sci.muni.cz/ccm).

The reference strains of *S. xylosus* (CCM 2738T and CCM 2725), *S. equorum* subsp. *equorum* (CCM 3852T and CCM 3853), *S. equorum* subsp. *linens* (CCM 7278T), *S. gallinarum* (CCM 3572T and CCM 4506), *S. nepalensis* (CCM 7045T and CCM 7046), *S. succinus* subsp. *succinus* (CCM 7157T) and *S. succinus* subsp. *casei* (CCM 7194T) were obtained from the CCM.

**Cultivation and phenotypic identification.** Bacterial strains were routinely grown for 24 h at 37 and 30°C on sheep blood agar (HiMedia). Colony size and morphology on P agar were determined after 48 h of incubation at 37°C, and additionally after 72 h of incubation at laboratory temperature (Meugnier et al., 1996).

Biochemical properties were tested by API Staph, ID 32 Staph and API ZYM commercial kits (bioMérieux), and the STAPHyTest 16 system (PLIVA-Lachema), according to the manufacturers’ instructions.

When either a weak or a delayed reaction was observed the test was repeated by a conventional method (acid production from D-xylose and D-mannose, production of β-galactosidase and hydrolysis of ascinulin). Other tests, such as production of oxidase, pyrrolidonyl arylamidase (PLIVA-Lachema), catalase and acetoin, detection of clumping factor (Slixtex Staphy Plus; bioMérieux) and coagulase (ITTEST plus), acid production from D-cellobiose, L-arabinose, N-acetylglucosamine, D-raffinose, D-ribose, D-melezitose and D-melibiose, hydrolysis of Tween 80, gelatin and casein, were performed as described by Mannerová et al. (2003). Susceptibility to novobiocin (5 μg), bacitracin (0-04 IU), furazolidone (100 μg) and polymyxin B (300 IU) was tested by the disc diffusion method on Mueller–Hinton agar (Oxoid) according to Woods & Washington (1995). Evaluation of biochemical test results was carried out using TNW 6.0 software (PLIVA-Lachema) and published phenotype characteristics described by Lambert et al. (1998), Place et al. (2002) and Schleifer et al. (1984).

**Ribotyping.** Isolation of genomic DNAs and ribotyping with either EcoRI or HindIII restriction endonuclease, as well as hybridization with digoxigenin-labelled probes complementary to 16S and 23S rRNA, were done as described by Svec et al. (2001). The obtained ribotype profiles were digitised and analysed using GelComp II software (Applied Maths). Dendrogram construction was carried out using the Dice similarity coefficient and the UPGMA clustering method. A position tolerance of 1 % was allowed for the bands.

**Whole-cell protein analysis by SDS-PAGE.** Isolation of whole-cell protein extracts from bacterial cells, grown under standard conditions (24 h at 37°C) on nutrient agar (CM3; Oxoïd), and SDS-PAGE were performed as described by Pot et al. (1994). Wide range markers (Sigma) ranging from 6-5 to 205 kDa were used as the molecular mass markers. Densitometric analysis of the visualized protein bands was performed by GelCompar II software (Applied Maths). Pearson product-moment correlation coefficient converted for convenience to a percentage value and the UPGMA clustering method were used for the construction of the dendrogram.

**PCR identification of *S. equorum*.** A set of specific primers (SDaEqF and SDaEqR) targeting the manganese-dependent superoxide dismutase gene (sodA) was used for *S. equorum* identification (Blaiotta et al., 2004).

**16S rDNA sequencing.** The 16S rRNA gene was amplified using primers 16Sfw (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16Srev (5′-GGTTACCTTGACAGCTT-3′). The S′ portion of the ampli

Table 1. Origin of strains phenotypically assigned to *S. xylosus* or ambiguous *S. xylosus/S. equorum*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Male/female</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRL 00738</td>
<td>Pus</td>
<td>Gallstone</td>
<td>55 years</td>
<td>Male</td>
</tr>
<tr>
<td>NRL 02216</td>
<td>Blood</td>
<td>Fever of unknown origin</td>
<td>79 years</td>
<td>Female</td>
</tr>
<tr>
<td>CCM 7302</td>
<td>Blood</td>
<td>Chronic ischaemic heart disease</td>
<td>81 years</td>
<td>Male</td>
</tr>
<tr>
<td>NRC 99524</td>
<td>Exudate</td>
<td>Appendicitis</td>
<td>57 years</td>
<td>Male</td>
</tr>
<tr>
<td>CCM 7301</td>
<td>Blood</td>
<td>Unknown</td>
<td>61 years</td>
<td>Male</td>
</tr>
<tr>
<td>NRC 99698</td>
<td>Blood</td>
<td>Ulcerative colitis</td>
<td>17 years</td>
<td>Female</td>
</tr>
<tr>
<td>CCM 7312</td>
<td>Blood</td>
<td>Femoral fracture</td>
<td>93 years</td>
<td>Male</td>
</tr>
<tr>
<td>CCM 7313</td>
<td>Eye swab</td>
<td>Conjunctivitis</td>
<td>2 months</td>
<td>Male</td>
</tr>
<tr>
<td>CCM 7314</td>
<td>Wound swab</td>
<td>Acute pulmonary insufficiency</td>
<td>81 years</td>
<td>Male</td>
</tr>
<tr>
<td>NRL 02262</td>
<td>Eye swab</td>
<td>Cataract</td>
<td>70 years</td>
<td>Female</td>
</tr>
</tbody>
</table>
S. equorum and S. succinus human clinical isolates

dideoxynucleotide chain termination sequencing using internal sequencing primer 16S553L (5′-CGCTTACGGCCATAATTCCG-3′). The primer synthesis, amplification conditions, DNA sequencing and phylogenetic analysis were performed as described previously (Mannerová et al., 2003). The following nucleotide sequences of close evolutionary relatives of the analysed isolates were retrieved from the GenBank database: S. xylosus, accession no. D83374; S. equorum subsp. equorum, accession no. AB009939; S. equorum subsp. linens, accession no. AF527483; S. succinus subsp. succinus, accession no. AF004220; S. succinus subsp. casei, accession no. AJ32072; S. gallinarum, accession no. D83366; S. nepalensis, accession no. AJ317414. Multiple alignments were performed by using the program ClustalW available at the EMBL-EBI server (http://www.ebi.ac.uk/clustalw/).

RESULTS AND DISCUSSION

Novobiocin-resistant CoNS species are phylogenetically and phenotypically very close, and their biochemical identification is often unclear and unreliable (Marsou et al., 2001; Meugnier et al., 1996; Pennington et al., 1991). Evaluation of the obtained phenotypical results assigned strains CCM 7301, NRL 99/524 and NRL 99/760 as ambiguous S. xylosus/S. equorum strains, strain CCM 7302 as atypical S. xylosus and the remaining strains as S. succinus. The tested biochemical properties corresponded with the S. xylosus species description. Some atypical and differential reactions occurred (Table 2). All tested strains assigned by molecular approaches as S. equorum (and the S. equorum subsp. equorum type strain) showed strong production of β-glucosidase, while strains assigned as S. succinus did not produce this enzyme. Differentiation between S. xylosus, S. equorum and S. succinus is generally very difficult and ambiguous, because no clear key biochemical tests are available. S. xylosus can be distinguished from S. equorum by colony size and morphology on P agar. Growth at 37 and 30°C can also be a useful indicator. S. equorum forms small colonies (<6 mm) without pigmentation and grows preferably at 30°C. In contrast, S. xylosus strains form larger colonies (>6 mm) often with yellowish pigment and have more abundant growth at 37°C (Meugnier et al., 1996). In this study, strains NRL 00/738, NRL 02/216, CCM 7302 and CCM 7301 grew better at 30°C than at 37°C, and formed small, grey, circular colonies on P agar. Strains CCM 7313, CCM 7314 and NRL 02/262 formed small, dry, flat colonies. Because the identification based on biotyping was unclear, the ribotyping and whole-cell protein analysis by SDS-PAGE were chosen for more detailed characterization and identification.

In the present study, ribotyping done separately with EcoRI and HindIII restriction enzymes grouped, in contrast to biotyping, all analysed strains into two clearly distinguished clusters. The same results were obtained by whole-cell protein fingerprinting. Fig. 1 shows combined cluster analysis containing ribotypes obtained by both restriction enzymes, as well as whole-cell protein patterns. The cluster analysis grouped six of the tested strains with S. equorum reference strains, and five remaining clinical strains with those of S. succinus. The remaining reference strains of S. xylosus, S. nepalensis and S. gallinarum formed clearly separated clusters by ribotyping, as well as by whole-cell protein analysis. Any relationship to other species of the genus *Staphylococcus* included in the in-house CCM bacteria collection database based on ribotype similarity was not observed (data not shown). Because of a relatively high similarity of ribotype profiles and protein patterns between the studied and the reference S. equorum and S. succinus strains, these methods did not allow classification of the tested strains to the subspecies level. The percentage of similarity between the tested and reference strains was more than 75% for S. equorum and 69% at least for the S. succinus cluster (Fig. 1). Analogous results were reported by other authors who applied ribotyping and whole-cell protein analysis for characterization of novobiocin-resistant CoNS. The S. xylosus and S. equorum isolates from cheese and goat’s milk were correctly identified by ribotyping, while the ID 32 Staph system classified them into the S. xylosus/S. equorum group (Meugnier et al., 1996). Marsou et al. (2001) identified several S. equorum clinical isolates by ribotyping and amplification of 16S–23S rDNA intergenic spacers. Similarly, ribotyping appeared to be a suitable tool for species classification and identification of certain CoNS in other studies (Izard et al., 1992; Švec et al., 2004; Villard et al., 2000). The results of whole-cell protein analysis by separated cluster analysis were in full agreement with those obtained by ribotyping, and confirmed this method as a useful means for identification of CoNS, as also shown by other studies (Pennington et al., 1991; Pot et al., 1994; Švec et al., 2004).

The sequencing of a 5′ 527 bp fragment of the 16S rRNA gene, which was described as a useful tool able to distinguish most staphylococcal species (Becker et al., 2004), proved to be useful in unambiguous classification of the tested isolates into S. equorum and S. succinus species, and confirmed the results obtained by other methods. The best 16S rRNA sequence matches and similarities obtained using the RDP-II Seqmatch tool (http://rdp.cme.msu.edu/) are shown in Table 2. Analysis of the sequences determined for strains NRL 99/698, CCM 7312 and CCM 7313 showed their identity with the type strain S. succinus subsp. *casei* DSM 15096T (Place et al., 2002); however, these identification results were not clearly confirmed by further identification methods used in this study. Partial 16S rRNA sequencing did not allow identification of the S. equorum strains to the subspecies level (Table 2). The isolates CCM 7314 and NRL 02/262 exhibited high similarity to the 16S rRNA gene sequence for S. succinus subsp. succinus strain AMG-D11 (Lambert et al., 1998), with the only exception at nucleotide position 264 (E. coli coordinate), the A residue being found in the two clinical isolates instead of the T residue in the amber isolate.

In this study, a polyphasic identification approach based on ribotyping, whole-cell protein analysis by SDS-PAGE and sequencing of the 16S rRNA gene assigned six strains to S. equorum and five strains to S. succinus. In addition, a 193 bp
Table 2. Variable biochemical reactions and results of polyphasic identification

PHS, phosphatase production; NIT, nitrate reduction; TW 80, hydrolysis of Tween 80; XYL, acid production from xylose; ESC, hydrolyse of aesculin; CEL, acid production of celllobiose; β-GLU, production of β-glucosidase; HEM, α-haemolysis.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PHS</th>
<th>NIT</th>
<th>TW 80</th>
<th>XYL</th>
<th>ESC</th>
<th>CEL</th>
<th>HEM</th>
<th>β-GLU</th>
<th>Biotyping</th>
<th>Ribotype and protein profile cluster analysis</th>
<th>S. equorum-specific PCR</th>
<th>16S rRNA sequence match and similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRL 00/738</td>
<td>−*</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>S. xylosus</td>
<td>S. equorum</td>
<td>+</td>
<td>S. equorum† ATCC 43958T and DSM 15097T (100)</td>
</tr>
<tr>
<td>NRL 02/216</td>
<td>−*</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>S. xylosus</td>
<td>S. equorum</td>
<td>+</td>
<td>S. equorum† ATCC 43958T and DSM 15097T (100)</td>
<td></td>
</tr>
<tr>
<td>CCM 7302</td>
<td>−*</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>S. xylosus</td>
<td>S. equorum</td>
<td>+</td>
<td>S. equorum† ATCC 43958T and DSM 15097T (100)</td>
<td></td>
</tr>
<tr>
<td>NRL 99/524</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>S. xylosus/S. equorum</td>
<td>S. equorum</td>
<td>+</td>
<td>S. equorum† ATCC 43958T and DSM 15097T (100)</td>
</tr>
<tr>
<td>NRL 99/760</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>S. xylosus/S. equorum</td>
<td>S. equorum</td>
<td>+</td>
<td>S. equorum† ATCC 43958T and DSM 15097T (100)</td>
<td></td>
</tr>
<tr>
<td>CCM 7301</td>
<td>−*</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>+</td>
<td>S. xylosus/S. equorum</td>
<td>S. equorum</td>
<td>+</td>
<td>S. equorum† ATCC 43958T and DSM 15097T (100)</td>
<td></td>
</tr>
<tr>
<td>NRL 99/698</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>S. xylosus</td>
<td>S. succinus</td>
<td>−</td>
<td>S. succinus subsp. casei DSM 15096T (100)</td>
</tr>
<tr>
<td>CCM 7312</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>S. xylosus</td>
<td>S. succinus</td>
<td>−</td>
<td>S. succinus subsp. casei DSM 15096T (100)</td>
</tr>
<tr>
<td>CCM 7313</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>S. xylosus</td>
<td>S. succinus</td>
<td>−</td>
<td>S. succinus subsp. casei DSM 15096T (100)</td>
<td></td>
</tr>
<tr>
<td>CCM 7314</td>
<td>−*</td>
<td>−*</td>
<td>+*</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>S. xylosus</td>
<td>S. succinus</td>
<td>−</td>
<td>S. succinus subsp. succinus AMG-D1T (98-6)</td>
</tr>
<tr>
<td>NRL 02/262</td>
<td>−*</td>
<td>−*</td>
<td>+*</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>S. xylosus</td>
<td>S. succinus</td>
<td>−</td>
<td>S. succinus subsp. succinus AMG-D1T (98-6)</td>
</tr>
</tbody>
</table>

*Atypical reaction for S. xylosus species.
†S. equorum subsp. equorum ATCC 43958T (Takahashi et al., 1999) and S. equorum subsp. linens DSM 15097T (Place et al., 2003).
sodA gene amplicon specific for *S. equorum* species was obtained from six of the tested strains, as well as from the *S. equorum* reference strains (Table 2). Our results imply that these methods are suitable for the identification of biochemically similar novobiocin-resistant CoNS species and exhibit higher reliability than biotyping. However, identification of analysed taxa to the subspecies level using these methods was revealed to be uncertain.

The strains under study were isolated from relevant clinical specimens such as blood culture, pus, cerebrospinal fluid, exudate, eye swabs and wound swab. The results obtained indicate that *S. equorum* and *S. succinus* clinical isolates may be misidentified as *S. xylosus* strains in routine clinical laboratory analysis. The incidence of the two species in clinical specimens may not be as sporadic as believed until recently and this fact must be taken into account in microbiological laboratories. This is believed to be the first report of an occurrence of *S. equorum* in relevant human clinical specimens in the Czech Republic and the first isolation of *S. succinus* from human clinical material.

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