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Species distribution of staphylococci from small wild mammals

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ABSTRACT

A total of 197 isolates of *Staphylococcus* from small wild animals (insectivores and rodents) were identified by partial sequencing of the *rpoB* and *dnaJ* genes. Among the identified isolates the predominant species was *S. succinus* (28%), followed by *S. xylosus* (20.8%) and *S. stepanovicii* (18.3%). The other 14 *Staphylococcus* species were occasionally isolated. PCR-RFLP of the *rpoB* gene digested by *Hpy*8I was a fast and simple method to distinguish the two subspecies of *S. succinus*. More than 90% of the 55 *S. succinus* strains isolated belonged to *S. succinus* subsp. *casei* and only 9% to *S. succinus* subsp. *succinus*. Moreover, the present study describes the first ever isolation of *S. fleurettii* from healthy animals.

1. Introduction

Staphylococci are widely distributed in various environments. Natural populations are mainly associated with skin, skin glands and mucous membranes of humans and many animals alike. They have also been isolated from animal products and other sources, such as soil, sand, seawater, freshwater, dust, and air [10]. To date, 41 species of the genus *Staphylococcus* have been identified (excluding *Staphylococcus pulvereri* as a separate species and *Macrococcus caseolyticus* that was formerly *Staphylococcus caseolyticus*), 10 of which contain a subdivision with subspecies designations [5].

From the epidemiological point of view, it is important to determine the origin of organisms involved in the aetiology of disease. Therefore, an exact identification of bacterial pathogens is an essential prerequisite for the detection of reservoirs and sources of infection. It is also a prerequisite for monitoring the spread of bacterial pathogens within, between and beyond animal populations. However, there are only a few ecological studies on staphylococci inhabiting animals [11,16]. In a previous study on the distribution of staphylococci on small wild mammals, we found mostly novobiocin-resistant species. In this study, we used only phenotypic and biochemical properties, and 16S–23S rDNA intergenic spacer region polymorphism (ITS-PCR) as identification tools [7]. However, some novobiocin-resistant staphylococcal species (*Staphylococcus xylosus*, *Staphylococcus succinus*, *Staphylococcus equorum* and *Staphylococcus saprophyticus*) are difficult to distinguish on the basis of phenotypic properties and ITS-PCR analysis [4,14,17,18]. A better approach may be the application of PCR amplicon sequencing-based methods and/or restriction fragment length polymorphism (RFLP) analysis of PCR products [1,9,12,13,15,21,25].

Therefore, in this study, the distribution of *Staphylococcus* species from small mammals was re-examined using partial sequencing of the *rpoB* and *dnaJ* genes for species identification.

2. Materials and methods

2.1. Bacterial isolates

A total of 197 *Staphylococcus* isolates were included in this study. These isolates were recovered from individual free living insectivores: common shrew (*Sorex araneus*) and lesser shrew (*Sorex minutus*), and rodents: bank vole (*Clethrionomys glareolus*), root vole (*Microtus oeconomus*) and field mouse (*Apodemus agrarius*). Isolation and identification of staphylococci to the genus level was carried out as described previously [8]. In addition, two type strains of *S. succinus* were included in the study: *S. succinus* subsp. *succinus* CCM 7157 and *S. succinus* subsp. *casei* CCM 7194.

2.2. Species identification

Identification of staphylococcal isolates to the species level by partial sequencing of the *rpoB* and *dnaJ* genes was carried out as described previously with minor modifications [15,21]. Briefly, sequencing was performed with a total volume of 10 μL containing 0.5 μL of premix from the ABI Prism BigDye Terminator (version 3.0) ready reaction cycle sequencing kit (Applied Biosystems), 1.8 μL of buffer (400 mM Tris–HCl, 10 mM $MgCl_2$), 10 pmol

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Table 1
Molecular identification of the *Staphylococcus* spp. isolates used in this study.

Species identification (no. of strains) based on sequencing of gene				Definitive identification
SV[a]	*rpoB*	SV	*dnaJ*	
98–99%	*S. succinus* (55)	98–99%	*S. succinus* (55)	*S. succinus*
97–98%	*S. xylosus* (41)	94%	*S. xylosus* (41)	*S. xylosus*[b]
100%	*S. stepanovicii* (36)	99–100%	*S. stepanovicii* (36)	*S. stepanovicii*
99%	*S. sciuri* (15)	99%	*S. sciuri* (15)	*S. sciuri*
98%	*S. warneri* (11)	98%	*S. warneri* (11)	*S. warneri*
97%	*S. epidermidis* (8)	97%	*S. epidermidis* (8)	*S. epidermidis*
99%	*S. vitulinus* (5)	99%	*S. vitulinus* (5)	*S. vitulinus*
98%	*S. equorum* (4)	98%	*S. equorum* (4)	*S. equorum*
100%	*S. pasteuri* (4)	99%	*S. pasteuri* (4)	*S. pasteuri*
99%	*S. aureus* (3)	99%	*S. aureus* (3)	*S. aureus*
99%	*S. hominis* (2)	98%	*S. hominis* (2)	*S. hominis*
98%	*S. haemolyticus* (2)	98%	*S. haemolyticus* (2)	*S. haemolyticus*
99%	*S. lentus* (2)	97%	*S. lentus* (2)	*S. lentus*
100%	*S. saprophyticus* (1)	98%	*S. saprophyticus* (1)	*S. saprophyticus*
98%	*S. fleurettii* (1)	98%	*S. fleurettii* (1)	*S. fleurettii*
98%	*S. microti* (1)	98%	*S. microti* (1)	*S. microti*
91–93%	*S. saprophyticus* (6)	88–89%	*S. saprophyticus* (6)	*Staphylococcus* sp.[c]

[a] SV, similarity values with type strain.
[b] Identification was confirmed by *gap* gene sequencing.
[c] Identification was confirmed by *gap* and 16S rRNA gene sequencing.

of sequencing primer, and 3 μL of the cleaned PCR product. DNA sequencing was carried out in two directions. The sequencing products were purified using ExTerminator kits (A&A Biotechnology, Poland) and were analyzed on an ABI PRISM 3130 genetic analyzer, as specified by the manufacturer (Applied Biosystems). For identification, the sequence data were compared with sequence data in GenBank using the nucleotide–nucleotide BLAST program (http://www.ncbi.nlm.nih.gov/). Additional identification was performed by partial sequencing of the *gap* and 16S rRNA genes, as described previously [6,9], to determine the identity of isolates with ≤94% (*dnaJ*) and ≤93% (*rpoB*) similarity to the reference data, respectively.

2.3. PCR-RFLP analysis of the rpoB gene of S. succinus subspecies

The previously described [15] *rpoB* primers rpoB-(F) 5′-CCA TTC ATG GAC CAA GC-3′ and rpoB-(R) 5′-CCG TCC CAA GTC ATG AAA C-3′ were used to amplify the fragment of the *rpoB* gene. PCR was performed in a Biometra Tpersonal thermal cycler at 94 °C for 5 min, then 35 cycles at 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s. A final extension cycle of 72 °C for 10 min was then applied. The presence of a PCR product was confirmed by 1% (w/v) agarose gel electrophoresis and visualization with ethidium bromide.

On the basis of computational restriction fragment analysis of the partial *rpoB* gene sequences (GenBank database accession numbers HM159266 and HM159267) with the Restriction Mapper program (version 3 [http://www.restrictionmapper.org]), the *Hpy*8I restriction enzyme was chosen, because it provided subspecies-specific restriction profiles for the two *S. succinus* type strains and for all S. *succinus* isolates under study. Digestion was performed with 10 μL of the PCR products in a total volume of 15 μL with 1× reaction buffer and 10 U of *Hpy*8I endonuclease (Fermentas, Lithuania) for 1.5 h at 37 °C. The resulting fragments were separated by electrophoresis on a 2% (w/v) TopVision agarose gel (Fermentas, Lithuania) and visualized under UV light after ethidium bromide staining.

2.4. Nucleotide sequence accession number

The GenBank accession numbers for the *rpoB* gene sequences of *S. succinus* subsp. *succinus* and *S. succinus* subsp. *casei* type strains are HM159266 and HM159267, respectively, and for isolated *S. xylosus* strains are HM159269 and FJ906727. The GenBank accession numbers for the *dnaJ* gene sequences of isolated *S. xylosus* strains are HM159271 and GQ222250.

3. Results and discussion

3.1. Species distribution of staphylococci

The occurrence of different staphylococcal species on small wild mammals was already previously reported [7], but the identification of *Staphylococcus* isolates was performed primarily on the basis of three methods: biochemical tests, API numerical profiles and ITS-PCR profiling. At present, it is known that the identification of staphylococci using phenotypic methods, as well as ITS-PCR profiling, may often not provide reliable results, especially for strains isolated from environmental sources [2,4]. Moreover, it has been shown that more than one third of isolates (34.5%) may not be identified [7]. Therefore, in this study, the species distribution of staphylococci from small wild mammals was re-examined with a new set of isolates using genotypic methods in order to provide a more reliable identification. These methods allow correct and reliable identification of staphylococcal strains that cannot be assigned to a species level or that are misidentified by phenotypic tests as related to staphylococci [20,23,26].

Recently, partial sequencing of the highly conserved and ubiquitous *rpoB* and *dnaJ* genes has been considered useful for the identification and taxonomic classification of all known *Staphylococcus* (sub)species [15,21]. Based on these methods it was possible to identify as many as 191 (97%) out of 197 staphylococcal isolates at the species level. Among the identified isolates the predominant species was *S. succinus* (27.9% of 197 isolates), followed by *S. xylosus* (20.8%), *S. stepanovicii* (18.3%), *S. sciuri* (7.6%), *S. warneri* (5.6%), *S. epidermidis* (4.1%), and *S. vitulinus* (2.5%). *S. equorum*, *S. pasteuri*, *S. aureus*, *S hominis*, *S. haemolyticus*, *S. lentus*, *S. saprophyticus*, *S. fleurettii* and *S. microti* were only occasionally isolated from small mammals (Table 1). Although *S. xylosus* isolates showed a relatively low similarity value for *dnaJ* gene sequences (94%) compared to the reference data, the *gap* gene sequences (98–99% similarity to those of the type strain of *S. xylosus*) validated their correct identification.

Since 94% and 91% DNA-sequence similarity is considered sufficient for identification of staphylococcal isolates to the species level using *rpoB* and *dnaJ* genes, respectively [15,21], the remaining six isolates could be identified at the genus level but not at the species level (Table 1). They were also unsatisfactorily identified at