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1 **Genotypic versus phenotypic identification of staphylococcal species of canine**
2 **origin with special reference to *Staphylococcus schleiferi* subsp. *coagulans***

3
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1 Abstract

2 A comparative study was performed to examine the respective accuracy of 16S rDNA sequencing
3 and of the commercial biochemical assay ID32 STAPH (bioMérieux, Marcy l'Etoile, France) in the
4 identification of 232 staphylococcal samples representing 20 species and subspecies isolated from
5 367 dogs. Notable differences in species distribution were observed by comparing genotypic and
6 phenotypic data. Partial sequencing of 16S rDNA resulted in an unambiguous identification of 226
7 (97.4%) of the isolates, whereas the phenotypic approach resulted in a correct diagnosis of 162
8 (69.8%) of the isolates. Statistical agreement between genotypic and phenotypic identification of
9 staphylococci was substantial (Kappa coefficient of 0.6 to 0.8) for *S. aureus*, *S. hominis*, *S. warneri*,
10 *S. cohnii* subsp. *urealyticus*, and *S. simulans*, and “almost perfect” (Kappa coefficient of 0.8 to 1)
11 for *S. intermedius*, *S. epidermidis*, *S. equorum*, *S. haemolyticus*, *S. sciuri*, and *S. kloosi*. No
12 agreement above that expected by chance (Kappa coefficient = 0) was observed for *S. schleiferi*
13 subsp. *coagulans*, which was either confounded with *S. intermedius* and *S. capitis*, or categorized
14 as unacceptable by the biochemical assay. Given the growing importance of this pathogen in
15 veterinary medicine and its frequent misidentification with related staphylococci, a PCR-RFLP
16 approach producing a *S. schleiferi*-specific restriction profile was developed. This fast and reliable
17 assay represents a valuable tool in assisting in the monitoring of this pathogen.

18
19 **Keywords:** *Staphylococcus*, dogs, 16S rDNA, ID32 STAPH, statistical agreement, PCR-RFLP.

21 1. Introduction

22 Bacteria in the genus *Staphylococcus* serve as resident members of the normal cutaneous and
23 mucosal microflora of humans and animals. They are also opportunistic pathogens commonly found
24 in dogs with skin disorders, the most prevalent species being *S. intermedius* and *S. schleiferi*
25 (Morris et al., 2006). *Staphylococcus schleiferi* subsp. *coagulans* was identified from the external
26 auditory meatus of dogs with otitis externa by Igimi et al. (1990), and was also reported from dogs

1 with pyoderma (Frank et al., 2003). It is differentiated from *S. schleiferi* subsp. *schleiferi* in that it is
2 tube coagulase positive and urease positive. *Staphylococcus schleiferi* subsp. *coagulans*
3 phenotypically resembles other coagulase-positive staphylococci and may be easily misidentified as
4 *S. intermedius* or even as *S. aureus* (Calvo et al., 2000; Zdovc et al., 2004).

5 A variety of methods have been proposed for the phenotypical identification of staphylococcal
6 species, including conventional identification schemes (Kloos and Schleifer, 1975) and commercial
7 identification tests. These methods are in general hampered by the fact that they are dependent on
8 the expression of metabolic activities or morphological features and are generally not as accurate as
9 genotypic methods. In particular, 16S rDNA sequence analysis has emerged as a preferred
10 technique for bacterial identification (Clarridge, 2004). Genetic methods used in the identification
11 of staphylococci include 16S rDNA sequencing (Becker et al., 2004), PCR amplification of 16S-
12 23S rDNA intergenic spacer (Mendoza et al., 1998), real-time PCR of 16S rDNA (Skow et al.,
13 2005), and the development of species-specific PCR amplification primers targeting 16S rDNA
14 (Yamashita et al., 2005) or the *sodA* gene (Blaiotta et al., 2005; Voytenko et al., 2006).

15 The purpose of the present study was primarily to compare the efficiency of genotypic (16S rDNA
16 sequencing) and phenotypic (biochemical assay ID32 STAPH) methods in the identification of
17 staphylococci isolated from the external ear and skin of dogs. Additionally, and because the
18 emergent pathogen *S. schleiferi* subsp. *coagulans* is frequently confounded with related
19 staphylococci, we evaluated a 16S rDNA-based PCR-RFLP approach for an inexpensive, rapid and
20 accurate identification of this species.

21

22 **2. Material and methods**

23 *2.1 Isolation and culture of staphylococci*

24 Staphylococcal isolates were obtained from clinical samples submitted to the Department of
25 Veterinary Clinics at the University of Pisa between January 2005 and June 2006. Samples were
26 isolated from external auditory meatus or skin of 367 dogs of both sexes (301 healthy individuals,

1 51 with pyoderma and 15 with otitis) using a sterile swab placed in transport medium (Transystem
2 Film-Film/Amies, Oxoid, Milano, Italy). In dogs with pyoderma, the swab specimen was obtained
3 from closed papule or pustule. Procedures for the care and management of animals were performed
4 in accordance with the provisions of the EC Council Directive 86/609 EEC, recognised and adopted
5 by the Italian Government (DL 27.01.1992, n° 116). Each swab sample was inoculated within 4 h
6 onto Mannitol Salt Agar (Oxoid, Milano, Italy) and incubated aerobically for 24 h at 37 °C.
7 Organisms obtained through bacterial culture were initially identified as staphylococci on the basis
8 of growth and colony characteristics on primary plating medium. One representative colony of each
9 colony type per specimen was picked and subcultured on blood agar (Oxoid, Milano, Italy)
10 aerobically for 24 h at 37 °C.

11 *2.2 Biochemical identification*

12 Strains were subjected to biochemical identification with the semi-automated MINI-API system
13 (bioMérieux, Marcy l'Etoile, France) using the API ID32 STAPH strip (bioMérieux) according to
14 the manufacturer's instructions. Briefly, the working bacterial suspension, prepared by suspending
15 3–5 isolated colonies in distilled water, was inoculated into the strip by a repeater pipette (ATB
16 Electronic Pipette, bioMérieux). Bacterial suspension had a turbidity equivalent to 0.5 McFarland,
17 adjusted photometrically with a densitometer (Densimat, bioMérieux). After 18–24 h incubation,
18 growth was read automatically by the mini-API system (bioMérieux). Analysis of the results was
19 based on the report provided by the APILAB Plus software according to the percent identification
20 accuracy (%ID), an estimate of how closely the profile corresponds to the taxon relative to all other
21 taxa in the database, and to the T index, an estimate of how closely the profile corresponds to the
22 most typical set of reactions for the stated taxon. Identifications were categorized into one of several
23 confidence levels: excellent (%ID \geq 99.9, $T \geq$ 0.75), very good (%ID \geq 99.0, $T \geq$ 0.5), good (%ID \geq
24 90.0, $T \geq$ 0.25), and acceptable (%ID \geq 80.0, $T \geq$ 0). Results below these levels were categorized as
25 unacceptable.

1 2.3 Molecular identification

2 DNA from *Staphylococcus* cultures was extracted in guanidine lysis buffer (Sambrook et al.,
3 1989), precipitated with isopropanol and dissolved in deionised water. A 451 bp-fragment
4 located at 5'-end of 16S rDNA was amplified by PCR using broad-range bacterial primers
5 SSU-bact-27f (5'-AGAGTTTGATCMTGGCTCAG-3') and SSU-bact-519r (5'-
6 GWATTACCGCGGCKGCTG) (Becker et al., 2004). The thermal cycling conditions
7 consisted in 30 cycles of denaturation at 94°C for 45 s (90 s for the first cycle), annealing at
8 53°C for 30 s, and elongation at 72°C for 90s (600 s for the last cycle). PCR products were
9 controlled by 1.2% agarose gel electrophoresis and were purified using High Pure PCR
10 Product Purification Kit (Roche Applied Science). The sequencing reactions were performed
11 in a total volume of 10 µl containing 0.5 µl of premix from the ABI prism BigDye
12 Terminator v3.0 ready-reaction cycle-sequencing kit (Applied Biosystems), 10 pmol of
13 sequencing primer SSU-bact-519r, and 2 µl of the purified PCR product. The sequencing
14 products were analyzed on an ABI 3730XL capillary sequencer. An alignment of all 16S
15 rDNA *Staphylococcus* sequences available in GenBank was performed using ClustalW, as
16 implemented in BioEdit version 7.0.1 (Hall, 1999). Most of the sequences were from the
17 RIDOM database (GenBank accession numbers AY688029-AY688109) (Harmsen et al.,
18 2002), which includes 81 type and reference strains. 16S rDNA sequences of the recently
19 described *S. pseudintermedius* (AJ780976-AJ780979) were also included in the alignment.
20 Sequences determined in this study have been deposited in the GenBank database under
21 accession numbers EF424582-EF424601. Sample identification was performed by analyzing a
22 pairwise distance matrix generated by BioEdit. Assuming a normal distribution for pairwise
23 distances of all unique staphylococcal sequences, a reporting criterion of $\geq 98.7\%$ identity was
24 selected for a "distinct species", which correlates with a statistical error probability of 1%.
25 The alignment of 16S rDNA sequences was analyzed to seek for restriction endonucleases
26 that give a unique restriction profile for *S. schleiferi* subsp. *coagulans*. Restriction fragment

1 length polymorphisms (RFLPs) of the amplicons were determined by digestion with the
2 frequent-cutter endonuclease *TaiI* (Fermentas, UK). Digestions were performed for 16 hours
3 at 65°C in a total volume of 50 µl including 1X Buffer R, 1 unit *TaiI*, and 20 µl of purified
4 PCR product. The sizes of the restriction DNA digests were estimated on a 3% high-
5 resolution agarose gel by comparison with a 100-bp molecular weight marker (DNA
6 Molecular Weight Marker XIV, Roche Applied Science).

7 2.4. Statistics

8 Agreement between genotypic (16S rDNA sequencing) and phenotypic (ID32 STAPH)
9 identification of staphylococci was assessed by kappa analysis, a chance-corrected statistical
10 measure of agreement between observers. Kappa coefficient values range from -1 (complete
11 disagreement) via 0 (no agreement above that expected by chance) to +1 (perfect agreement). Poor,
12 fair, moderate, substantial and “almost perfect” agreements are defined as a kappa coefficient of
13 <0.2, 0.2 to 0.4, 0.4 to 0.6, 0.6 to 0.8, and 0.8 to 1, respectively (Landis and Koch, 1977).

15 3. Results and discussion

16 A total of 232 *Staphylococcus* isolates were obtained from 367 dogs between January 2005 and
17 June 2006. Molecular characterization by 16S rDNA sequencing resulted in a reliable identification
18 (sequence identity with reference strain $\geq 98.7\%$) of 226 isolates (97.4%) representing 18
19 *Staphylococcus* species, among which 135 isolates (prevalence 58.2%) were of *S. intermedius*
20 (Table 1). The 6 unidentified 16S rDNA sequences were found to be closely related to *S. felis* (3
21 sequences, identity 98.2-98.4%), *S. simulans* (1 sequence, identity 98.0%), *S. kloosi* (1 sequence,
22 identity 97.1%), and *S. equorum* (1 sequence, identity 97.8%). A total of 16 *Staphylococcus* species,
23 among which *S. intermedius* was by far the dominant one (115 isolates, prevalence 61.8%), were
24 identified by ID32 STAPH among 186 isolates with acceptable identification values (Table 1).
25 Notable differences in species distribution were observed by comparing 16S rDNA and ID32
26 STAPH data (Table 1). A non-staphylococcal isolate, the alkalophilic bacterium *Exiguobacterium*

1 *acetylicum*, was identified as *S. sciuri* by the biochemical assay. ID32 STAPH did not recognize *S.*
2 *schleiferi* subsp. *coagulans* (10 isolates identified by 16S rDNA sequencing), *S. succinus* (1 isolate)
3 and *S. pasteurii* (1 isolate). While the identification failure of a single isolate does not imply that the
4 biochemical assay would not correctly identify additional specimens, it appears that *S. schleiferi*
5 subsp. *coagulans* is either confounded with *S. intermedius* (5 isolates) and *S. capitis* (2 isolates), or
6 categorized as unacceptable (3 isolates). Apart from *S. schleiferi* subsp. *coagulans*, the majority of
7 the isolates from the most prevalent staphylococcal species (≥ 10 isolates identified by 16S rDNA
8 sequencing) were correctly identified by ID32 STAPH, including *S. equorum* (no misidentification),
9 *S. intermedius* (7% misidentification), *S. epidermidis* (8.3%), and *S. haemolyticus* (11.1%) (Table
10 1). Biochemical identifications using ID32 STAPH resulted in a correct diagnosis of 162 (69.8%) of
11 the isolates, while 46 isolates were categorized as unacceptable, and 24 isolates with acceptable
12 identification values ($\%ID \geq 80.0\%$) were misidentified (Table 2). Statistical agreement between
13 genotypic and phenotypic identification was substantial (Kappa coefficient of 0.6 to 0.8) for *S.*
14 *aureus*, *S. hominis*, *S. warneri*, *S. cohnii* subsp. *urealyticus*, and *S. simulans*, and “almost perfect”
15 (Kappa coefficient of 0.8 to 1) for *S. intermedius*, *S. epidermidis*, *S. equorum*, *S. haemolyticus*, *S.*
16 *sciuri*, and *S. kloosi* (Table 3). No agreement above that expected by chance (Kappa coefficient = 0)
17 was observed for *S. schleiferi* subsp. *coagulans*.

18 The characterization of staphylococci by 16S rDNA sequencing appears to be much more accurate
19 than identification using ID32 STAPH. However, the DNA sequencing approach is more expensive
20 and time-consuming than biochemical assays. For this reason, a number of inexpensive, rapid
21 molecular identification techniques have been used for diagnosis of staphylococci, including PCR
22 amplification of the polymorphic 16S-23S rDNA intergenic spacer (Mendoza et al., 1998), and the
23 development of species-specific PCR amplification primers (Yamashita et al., 2005; Voytenko et
24 al., 2006). The main failure of ID32 STAPH was the systematic misidentification of *S. schleiferi*
25 subsp. *coagulans* (Table 1). An identification test using specific PCR primers allowing to
26 distinguish *S. schleiferi* subsp. *coagulans* from *S. intermedius*, *S. aureus*, and *S. hyicus* has been

1 previously developed (Yamashita et al., 2005). However, the analysis of staphylococcal 16S rDNA
2 sequences indicated that the assay developed by Yamashita et al. would not distinguish *S. schleiferi*
3 subsp. *coagulans* from *S. equorum*, *S. xylosus*, and *S. succinus*. For this reason, we developed a
4 PCR-RFLP approach for a rapid and accurate identification of *S. schleiferi* subsp. *coagulans* and its
5 distinction from other staphylococci of the RIDOM database.

6 The analysis of the 16S rDNA sequence alignment (comprising 81 type and reference strains of all
7 validly described staphylococci) indicated that the digestion of PCR products with restriction
8 endonuclease Tail would provide a unique RFLP pattern for *S. schleiferi* consisting in five
9 fragments of 20, 39, 116, 171, and 190 bp. The electrophoresis of Tail-digested PCR products of
10 the 19 *Staphylococcus* species isolated in the present study revealed a total of 7 distinct patterns
11 (Fig. 1). The restriction sites of some PCR products were partially cleaved, producing a higher
12 number of bands as predicted by sequence analysis. However, the restriction profile of *S. schleiferi*
13 subsp. *coagulans* (pattern B, see fig. 1) was unique and easily distinguishable from all other
14 electrophoresis patterns. The same profile was obtained for all *S. schleiferi* subsp. *coagulans*
15 isolates analyzed in the present work (data not shown). Additional unique restriction profiles were
16 obtained for *S. aureus* (pattern F) and for *S. cohnii* (pattern G).

17 In conclusion, our data indicates that 16S rDNA sequencing produce more consistent results than
18 biochemical assays in the identification of staphylococci. Interestingly, *S. schleiferi* subsp.
19 *coagulans* could not be identified using ID32 STAPH while it was among the most prevalent
20 staphylococcal species isolated from dogs. Previous reports (Calvo et al., 2000; Zdovc et al., 2004)
21 have demonstrated that this species is frequently confounded with related staphylococci. Given the
22 growing importance of *S. schleiferi* subsp. *coagulans* in veterinary medicine (May et al., 2005), and,
23 to a lesser extent, in human medicine (Vandenesch et al., 1994), the development of a fast and
24 reliable identification assay is essential to assist in the monitoring of this pathogen.

25

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3

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8

9

10 **Figure captions**

11 Fig. 1. PCR-amplified partial 16S rDNA of 19 staphylococcal species after enzymatic restriction
12 with *Tai*I. Open circles indicate restriction fragments predicted by sequence analysis; additional
13 fragments correspond to partial digestions. Small restriction fragments (20 bp and 39 bp) not critical
14 for species identification were difficult to visualize and are not shown. Capital letters A-G indicate
15 the different restriction patterns. Species are: lane 1, *S. intermedius*; lane 2, *S. schleiferi* subsp.
16 *coagulans*; lane 3, *S. epidermidis*; lane 4, *S. equorum*; lane 5, *S. haemolyticus*; lane 6, *S. aureus*;
17 lane 7, *S. hominis*; lane 8, *S. simulans*; lane 9, *S. warneri*; lane 10, *S. capitis*; lane 11, *S. xylosus*;
18 lane 12, *S. sciuri*; lane 13, *S. cohnii* subsp. *cohnii*; lane 14, *S. cohnii* subsp. *urealyticus*, lane 15, *S.*
19 *felis*; lane 16, *S. succinus*; lane 17, *S. lentus*; lane 18, *S. pasteurii*; lane 19, *S. kloosi*.

20

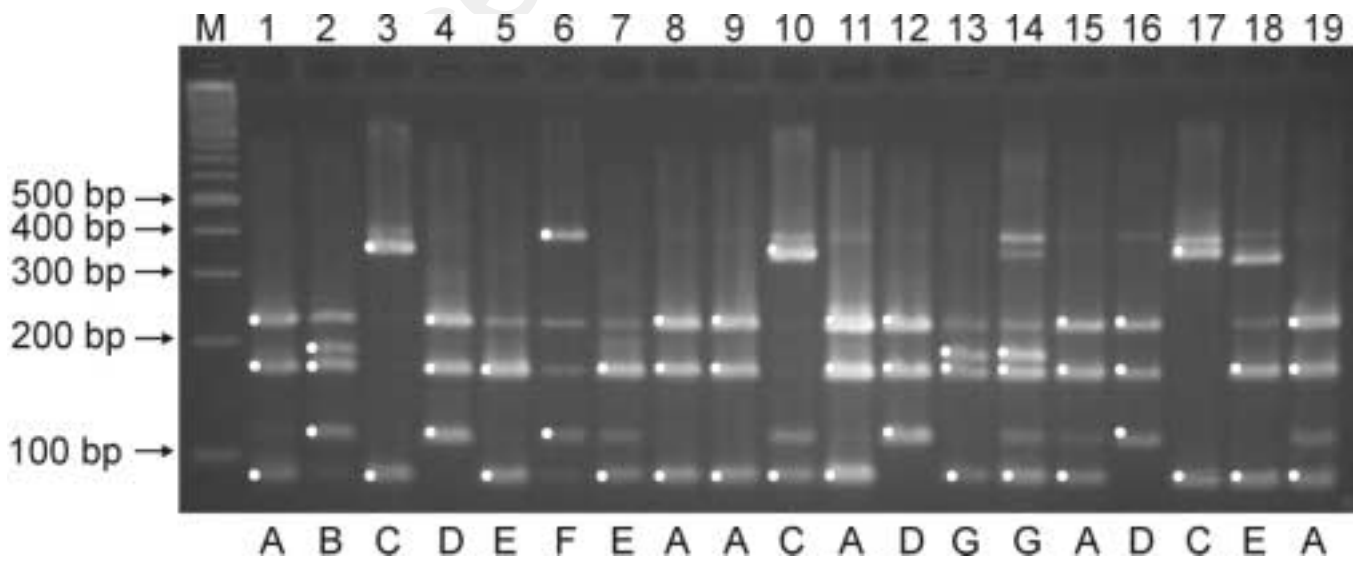


Table 1. Identification of staphylococcal isolates by 16S rDNA sequencing compared to ID32 STAPH.

Species	16S rDNA sequence data (n = 226) ^a			ID32 STAPH data (n = 186) ^b	Isolates wrongly identified by ID32 STAPH (%)	Misidentification with ^c
	Ref. strain accession number	Seq. identity with ref. strain (%)	No isolates (% prevalence)	No isolates (% prevalence)		
<i>S. intermedius</i>	AY688070	99.6-100	135 (58.2)	115 (61.8)	8/115 (7.0)	<i>S. schleiferi</i> (5), <i>S. aureus</i> (1), <i>S. felis</i> (1), <i>S. simulans</i> (1)
<i>S. epidermidis</i>	AY688051	99.6-100	16 (6.9)	12 (6.5)	1/12 (8.3)	<i>S. lentus</i> (1)
<i>S. equorum</i>	AY688054	98.7-100	13 (5.6)	8 (4.3)	0/8	NM
<i>S. schleiferi</i> subsp. <i>coagulans</i>	AY688091	100	10 (4.3)	0	NA	NA
<i>S. haemolyticus</i>	AY688060	100	10 (4.3)	9 (4.8)	1/9 (11.1)	<i>S. felis</i> (1)
<i>S. aureus</i>	AY688031	99.6-100	6 (2.6)	5 (2.7)	1/5 (20.0)	<i>S. intermedius</i> (1)
<i>S. hominis</i>	AY688063	99.6-100	6 (2.6)	6 (3.2)	2/6 (33.3)	<i>S. haemolyticus</i> (1), <i>S. epidermidis</i> (1)
<i>S. warneri</i>	AY688105	99.8-100	5 (2.2)	6 (3.2)	2/6 (33.3)	<i>S. hominis</i> (1), <i>S. cohnii</i> subsp. <i>cohnii</i> (1)
<i>S. simulans</i>	AY688100	99.3-99.8	4 (1.7)	3 (1.6)	0/3	NM
<i>S. capitis</i>	AY688037	100	4 (1.7)	5 (2.7)	3/5 (60.0)	<i>S. schleiferi</i> (2), <i>S. hominis</i> (1)
<i>S. xylosus</i>	DQ361017	99.3-100	4 (1.7)	4 (2.2)	2/4 (50.0)	<i>S. equorum</i> (1), <i>S. succinus</i> (1)
<i>S. sciuri</i>	AY688097	99.8-100	4 (1.7)	5 (2.7)	1/5 (20.0)	<i>E. acetylicum</i> (1)
<i>S. cohnii</i> subsp. <i>cohnii</i>	AY688045	100	3 (1.3)	3 (1.6)	1/3 (33.3)	<i>S. capitis</i> (1)
<i>S. cohnii</i> subsp. <i>urealyticus</i>	AY688047	100	2 (0.9)	2 (1.1)	0/2	NM
<i>S. succinus</i>	AY688103	99.6	1 (0.4)	0	NA	NA
<i>S. lentus</i>	AY688073	99.8	1 (0.4)	1 (0.5)	1/1 (100)	<i>S. epidermidis</i> (1)
<i>S. pasteurii</i>	AY688082	99.8	1 (0.4)	0	NA	NA
<i>S. kloosi</i>	AY688071	NA	0	1 (0.5)	NA	NA
<i>S. chromogenes</i>	AY688044	NA	0	1 (0.5)	1/1 (100)	<i>S. equorum</i> (1)
<i>Exiguobacterium acetylicum</i>	AY297792	99.8	1 (0.4)	0	NA	NA

^a Number of isolates with sequence identity $\geq 98.7\%$ to the 16S rDNA sequence of the reference strain

^b Number of isolates with identification accuracy values that are acceptable or better (%ID ≥ 80.0)

^c Values in parentheses represent the number of misidentified isolates

NA: not applicable; NM: no misidentification

Table 2. Identification accuracy of staphylococcal isolates ($n = 232$) by 16S rDNA sequencing and ID32 STAPH.

Result	Number (%) of isolates tested by:	
	16S rDNA	ID32 STAPH
Correctly identified ^a	226 (97.4)	162 (69.8)
Failure due to:		
Unacceptable identification values	6 (2.6)	46 (19.8)
Misidentification	0	24 (10.3)
Total failures	6 (2.6)	70 (30.2)

^a For 16 rDNA, classification is based on sequence identity $\geq 98.7\%$ to the sequence of the reference strain; for ID32 STAPH, classification is based on identification accuracy values that are acceptable or better (%ID ≥ 80.0), excluding misidentified isolates.

Table 3. Statistical agreement between genotypic and phenotypic identification of staphylococci.

Species	Kappa coefficient ^a	Standard error	95% confidence interval ^b
<i>S. intermedius</i>	0.813	0.039	0.736 – 0.889
<i>S. epidermidis</i>	0.857	0.07	0.72 – 0.995
<i>S. equorum</i>	0.837	0.08	0.681 – 0.994
<i>S. schleiferi</i> subsp. <i>coagulans</i>	0	0	0
<i>S. haemolyticus</i>	0.835	0.093	0.653 – 1
<i>S. aureus</i>	0.604	0.162	0.287 – 0.922
<i>S. hominis</i>	0.604	0.162	0.287 – 0.922
<i>S. warneri</i>	0.658	0.16	0.345 – 0.972
<i>S. simulans</i>	0.746	0.173	0.407 – 1
<i>S. capitis</i>	0.488	0.179	0.138 – 0.838
<i>S. xylosum</i>	0.414	0.167	0.088 – 0.741
<i>S. sciuri</i>	0.887	0.112	0.667 – 1
<i>S. cohnii</i> subsp. <i>cohnii</i>	0.325	0.25	-0.166 – 0.815
<i>S. cohnii</i> subsp. <i>urealyticus</i>	0.798	0.197	0.411 – 1
<i>S. succinus</i>	0	-	-
<i>S. lentus</i>	-0.004	0.003	-0.01 – 0.002
<i>S. pasteurii</i>	0	-	-
<i>S. kloosii</i>	1	0	1
<i>S. chromogenes</i>	0	0	0
<i>S. felis</i>	0	0.987	0

^a Kappa coefficient values ranges from -1 (complete disagreement) via 0 (no agreement above that expected by chance) to +1 (perfect agreement)

^b The confidence interval gives an estimated range of values which is likely to include kappa coefficient