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Development and Evaluation of a Quality-Controlled Ribosomal Sequence Database for 16S Ribosomal DNA-Based Identification of *Staphylococcus* Species

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To establish an improved ribosomal gene sequence database as part of the Ribosomal Differentiation of Microorganisms (RIDOM) project and to overcome the drawbacks of phenotypic identification systems and publicly accessible sequence databases, both strands of the 5′ end of the 16S ribosomal DNA (rDNA) of 81 type and reference strains comprising all validly described staphylococcal (sub)species were sequenced. Assuming a normal distribution for pairwise distances of all unique staphylococcal sequences and choosing a reporting criterion of ≥98.7% similarity for a "distinct species," a statistical error probability of 1.0% was calculated. To evaluate this database, a 16S rDNA fragment (corresponding to *Escherichia coli* positions 54 to 510) of 55 clinical *Staphylococcus* isolates (including those of the small-colony variant phenotype) were sequenced and analyzed by the RIDOM approach. Of these isolates, 54 (98.2%) had a similarity score above the proposed threshold using RIDOM; 48 (87.3%) of the sequences gave a perfect match, whereas 83.6% were found by searching National Center for Biotechnology Information (NCBI) database entries. In contrast to RIDOM, which showed four ambiguities at the species level (mainly concerning *Staphylococcus intermedius* versus *Staphylococcus delphini*), the NCBI database search yielded 18 taxon-related ambiguities and showed numerous matches exhibiting redundant or unspecified entries. Comparing molecular results with those of biochemical procedures, ID 32 Staph (bioMérieux, Marcy l'Etoile, France) and VITEK 2 (bioMérieux) failed to identify 13 (23.6%) and 19 (34.5%) isolates, respectively, due to incorrect identification and/or categorization below acceptable values. In contrast to phenotypic methods and the NCBI database, the novel high-quality RIDOM sequence database provides excellent identification of staphylococci, including rarely isolated species and phenotypic variants.

The emerging role of coagulase-negative staphylococci (CoNS), along with *Staphylococcus aureus*, in connection with the expanding number of staphylococcal (sub)species described necessitates their comprehensive and accurate identification. A variety of methods have been proposed for the identification of staphylococcal species, including conventional identification schemes based on publications of Kloos and Schleifer (18) and commercial identification tests. In general, methods based on phenotypic characteristics are hampered by the fact that they are dependent on the expression of metabolic activities and/or morphological features. Furthermore, commercial systems often offer two or more suggestions for identification with a comparable safety level.

Nowadays, with the advent of molecular biology-based techniques, investigations based on comparative DNA sequence analysis of the genes of conserved macromolecules have become commonplace in microbiology as a tool for classification of microbial organisms. The most useful and extensively investigated taxonomic marker molecules are the larger rRNAs and their corresponding genes, respectively, especially 16S rRNAs and to a lesser extent 23S rRNAs (14). While molecular identification based on sequence analysis of universal targets offers several advantages, such as improved accuracy and short turnaround time, some drawbacks in currently available databases should be noted, such as the presence of faulty and/or redundant sequence entries, ragged sequence ends, outdated nomenclature, and unavailable quality assurance.

Since the 5′ end of the gene encoding 16S rRNA (16S rDNA) contains enough information for the identification of almost all staphylococcal species, we established a new quality-controlled part of the Ribosomal Differentiation of Microorganisms (RIDOM) sequence database based on 81 type and reference strains encompassing all species and subspecies of the genus *Staphylococcus* recognized as valid. In addition, the recently proposed candidate species *"S. pettenkoferi"* was included (36). Using a broad spectrum of clinical human and veterinary staphylococcal isolates, the 16S rDNA-derived results were analyzed by using the RIDOM database in comparison to the entries of the sequence database of the National Center for Biotechnology Information (NCBI). Furthermore, the molecular biology-based results were compared to those obtained biochemically using the ID 32 Staph gallery and the VITEK 2 system. Since phenotypic variants are known to cause diagnostic difficulties, isolates exhibiting the small-colony variant (SCV) phenotype were also included.

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MATERIALS AND METHODS

Bacterial strains. Altogether, 81 type and other culture collection reference strains encompassing all hitherto validly described species and subspecies of the genus *Staphylococcus* and the recently proposed candidate species *"S. pettenkoferi"* (36) (Table 1) were used to establish a quality-controlled ribosomal sequence database for 16S rDNA-based differentiation of staphylococci. Using this database, 55 clinical staphylococcal isolates collected from human (n = 52) and veterinary (*S. intermedius,* n = 2; *S. felis,* n = 1) specimens during the course of several German multicenter studies (5, 6, 38, 41) were analyzed and the molecular biology-based results were compared to those obtained by conventional biochemical methods. Six of the human isolates exhibit the SCV phenotype (39, 42).

Biochemical identification. Gallery API ID 32 Staph (bioMérieux, Marcy l'Etoile, France) and the ID-GBP card of the VITEK 2 system (bioMérieux) were used for biochemical identification as recommended by the manufacturer. Analysis of the results was based on the report provided by the APILAB Plus software (version 3.3.3) and the computerized software of VITEK 2 (version 3.01) according to the percent identification accuracy (%ID), an estimate of how closely the profile corresponds to the taxon relative to all other taxa in the database, and the T index, an estimate of how closely the profile corresponds to the most typical set of reactions for the stated taxon. Identifications were categorized into one of several confidence levels: excellent (%ID $\geq$ 99.9, $T \geq 0.75$), very good (%ID $\geq$ 99.0, $T \geq 0.5$), good (%ID $\geq$ 90.0, $T \geq 0.25$), and acceptable (%ID $\geq$ 80.0, $T \geq 0$). Results below these levels were categorized as unacceptable.

To differentiate between *S. aureus* and other staphylococcal species, a PCR amplification targeting the *nuc* gene (8) was additionally applied. The SCV phenotype was identified as described elsewhere (37).

Chemotaxonomic characterization. Cell wall analysis was performed as previously described (12). For gas chromatographic analysis of cellular fatty acids, 40-mg samples of cells were saponified, methylated, extracted, and analyzed using the microbial identification system described by Miller (25). The peptidoglycan types were classified by the system proposed by Schleifer and Kandler (33) using abbreviations as listed in the *DSMZ Catalogue of Strains*, 7th ed. (http://www.dsmz.de/species/murein.htm).

Riboprinting. Riboprinting analysis was done as described elsewhere, using the restriction enzyme EcoRI (1).

DNA preparation and amplification. DNA isolation and amplification procedures were performed as previously described (4). Briefly, the thermal cycling conditions were 30 cycles of denaturation at 94°C for 45 s (300 s for the first cycle), annealing at 53°C for 60 s, and polymerization at 72°C for 90 s (600 s for the last cycle). For amplification, the broad-range primers SSU-bact-27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and SSU-bact-907r (5′-CCG TCA ATT CMT TTR AGT TT-3′) reported by Lane (21) were applied.

DNA sequencing. As described elsewhere (16), the broad-range primers SSU-bact-27f and SSU-bact-519r (5′-GWA TTA CCG CGG CKG CTG-3′) were used for sequencing of both strands of the 5′ end of the 16S rDNA genes. Briefly, sequencing was performed with a total volume of 10 μl containing 0.5 μl of premix from the ABI Prism BigDye Terminator v3.0 ready-reaction cycle-sequencing kit (Applied Biosystems), 1.8 μl of Tris-HCl/$MgCl_2$ buffer (400 mM Tris-HCl, 10 mM $MgCl_2$), 10 pmol of sequencing primer, and 2 μl of the cleaned PCR product. The sequencing products were purified using Centri-Sep spin columns (Princeton Separations, Adelphia, N.J.) and analyzed on an ABI Prism 310 or Avant Genetic Analyzer 3100 as specified by the manufacturer (Applied Biosystems).

Analysis of the rDNA sequences. The region from base position 54 to 510 (corresponding to *E. coli* 16S rDNA positions) of the 16S rDNA was used for further analysis. The pairwise distance matrix was calculated using the CLUSTAL W program. A Kolmogorov-Smirnov normality test was used to test the goodness of fit to a normal distribution of all pairwise distances. QAlign program version 1.10 was used for multiple simultaneous alignments (32). To evaluate the quality and accuracy of reference sequence services, we queried the RIDOM (version 1.2; Ridom GmbH, Würzburg, Germany) (15) and GenBank (search dated 12 January 2004) databases (7) with the sequences of the 55 clinical staphylococcal isolates.

Nucleotide sequence accession numbers. All staphylococcal partial rDNA reference sequences determined in this study were deposited in GenBank under accession numbers AY688029 to AY688109.

TABLE 1. List of 5′ 16S rDNA-sequenced staphylococcal culture collection strains (n = 81)

Species or subspecies	Strain(s)[a]
S. arlettae	DSM 20672^T
S. aureus subsp. *anaerobius*	DSM 20714^T
S. aureus subsp. *aureus*	DSM 20231^T, ATCC 25904, ATCC 31890, ATCC 29247
S. auricularis	DSM 20609^T
S. capitis subsp. *capitis*	DSM 20326^T, DSM 6180, DSM 20325
S. capitis subsp. *urealyticus*	DSM 6717^T
S. caprae	DSM 20608^T
S. carnosus subsp. *carnosus*	DSM 20501^T
S. carnosus subsp. *utilis*	DSM 11676^T, DSM 11677
S. chromogenes	DSM 20454^T
S. cohnii subsp. *cohnii*	DSM 20260^T, DSM 20261
S. cohnii subsp. *urealyticus*	DSM 6718^T
S. condimenti	DSM 11674^T, DSM 11675
S. delphini	DSM 20771^T
S. epidermidis	DSM 20044^T, ATCC 35984, DSM 1798
S. equorum subsp. *equorum*	DSM 20674^T, DSM 20675
S. equorum subsp. *linens*	DSM 15097^T
S. felis	DSM 7377^T
S. fleurettii	DSM 13212^T
S. gallinarum	DSM 20610^T
S. haemolyticus	DSM 20263^T, DSM 20264, DSM 20265
S. hominis subsp. *hominis*	DSM 20328^T, DSM 20329
S. hominis subsp. *novobiosepticus*	ATCC 700236^T
S. hyicus	DSM 20459^T
S. intermedius	DSM 20373^T, ATCC 49051, ATCC 49052, ATCC 51874
S. kloosii	DSM 20676^T, DSM 20677
S. lentus	DSM 20352^T
S. lugdunensis	DSM 4804^T, DSM 4805, DSM 6670
S. lutrae	DSM 10244^T, DSM 10245
S. muscae	DSM 7068^T
S. nepalensis	DSM 15150^T, DSM 15151
S. pasteuri	DSM 10656^T
"S. pettenkoferi"	B3117, A6664[b]
S. piscifermentans	DSM 7373^T
S. pulvereri[c]	DSM 9930^T
S. saccharolyticus	DSM 20359^T
S. saprophyticus subsp. *bovis*	CCM 4410^T
S. saprophyticus subsp. *saprophyticus*	DSM 20229^T, DSM 20038
S. schleiferi subsp. *coagulans*	DSM 6628^T
S. schleiferi subsp. *schleiferi*	DSM 4807^T, DSM 4808, DSM 4809
S. sciuri subsp. *carnaticus*	ATCC 700058^T
S. sciuri subsp. *rodentium*	ATCC 700061^T
S. sciuri subsp. *sciuri*	DSM 20345^T, DSM 6671
S. simulans	DSM 20322^T, DSM 20323, DSM 20723[d]
S. succinus subsp. *casei*	DSM 15096^T
S. succinus subsp. *succinus*	DSM 14617^T
S. vitulinus[c]	ATCC 51145^T
S. warneri	DSM 20316^T, DSM 20036
S. xylosus	DSM 20266^T, DSM 20267, DSM 6179

[a] T, type strain; ATCC, American Type Culture Collection, Manassas, Va.; CCM, Česká sbirka mikroorganismů (Czech Collection of Microorganisms); DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany.

[b] Courtesy of the authors, both strains were used to acknowledge the recently proposed but not yet validly described staphylococcal species "*S. pettenkoferi*" (36).

[c] It was recently proposed that strains (including the type strain) of *S. pulvereri* actually belong to the species *S. vitulinus* (26).

[d] Strain NRRL B-2628 (DSM 20723), originally described as "*S. staphylolyticus*," was classified as a biovar of *S. simulans* (34).

RESULTS

Establishing the staphylococcal part of the RIDOM database service. A total of 81 staphylococcal type and reference strains including all validly described and recently proposed

members of the *Staphylococcus* genus were analyzed by 5′- 16S rDNA sequencing and used to establish a quality-controlled ribosomal sequence database. Most staphylococcal sequences exhibited a length of 464 bp, analyzing the nucleotides at positions 54 to 510 (corresponding to the *Escherichia coli* 16S rDNA positions), except those of the *S. sciuri* cluster (*S. sciuri* subspecies, *S. pulvereri*, *S. vitulinus*, *S. fleurettii*, and *S. lentus*), which contained one additional guanine (*E. coli* position 214). The mean pairwise distance of 52 sequences (all 37 type strains and other *Staphylococcus* culture collection strains with unique sequences) was 4.7%, and the standard deviation was 1.5%. Assuming a normal distribution for the distances and choosing a reporting criterion of ≥98.70% similarity for a "distinct species" correlates with a statistical error probability of 1.0%.

Analyses of these sequences revealed that all of the type and reference strains were distinguishable at the species level, with the exception of *S. intermedius* and *S. delphini*. In addition, *S. pulvereri* and *S. vitulinus*, recently proposed to be identical species (26), showed the same nucleotide sequences. At the subspecies level, the established subspecies of *S. carnosus*, *S. cohnii*, *S. hominis*, *S. schleiferi*, and *S. succinus* could be distinguished. The type strains of *S. aureus* subsp. *aureus* versus *S. aureus* subsp. *anaerobius*, *S. capitis* subsp. *capitis* versus *S. capitis* subsp. *urealyticus*, *S. equorum* subsp. *equorum* versus *S. equorum* subsp. *linens*, *S. saprophyticus* subsp. *saprophyticus* versus *S. saprophyticus* subsp. *bovis*, and *S. sciuri* subsp. *carnaticus* versus *S. sciuri* subsp. *rodentium* were indistinguishable in the range of the 5′ 16S rDNA fragment used. In contrast, *S. sciuri* subsp. *sciuri* was distinguishable from both other subspecies of this species.

Comparison of RIDOM and NCBI database queries. A total of 55 clinical staphylococcal isolates were analyzed by 16S rDNA sequencing. Using the newly established RIDOM database, the isolates were assigned to 20 different species and subspecies (Tables 2 and 3). Except for isolate M08, with 98.49% similarity, all other isolates (98.2%) had a similarity score above the proposed threshold in the RIDOM database. Of the 55 isolates, 48 (87.3%) had sequences which gave a perfect match. Performing a similarity search against the NCBI database, a perfect match was yielded in 83.6% of cases.

At the species level, the RIDOM database contained a total of four ambiguities (Tables 3 and 4). In three of these cases, ambiguous results were caused by the nucleotide sequence identity of *S. intermedius* and *S. delphini* in the fragment analyzed. In addition, isolate M55 yielded an ambiguous result in the RIDOM analysis, showing an identical percent similarity to two species (*S. xylosus* and *S. saprophyticus*; 99.35%). Minor ambiguities were found at the subspecies level for *S. aureus*, *S. capitis*, *S. equorum*, and *S. saprophyticus*. Analyzing the sequences of the clinical isolates tested by the GenBank nucleotide database at NCBI, a total of 18 ambiguities were observed: (i) *S. aureus* versus *S. haemolyticus* ($n = 7$), (ii) *S. saprophyticus* versus *S. cohnii* ($n = 2$), (iii) *S. cohnii* versus *S. succinus* ($n = 2$), (iv) *S. intermedius* versus *S. delphini* ($n = 3$), (v) *S. capitis* versus *S. caprae* versus *S. epidermidis* ($n = 2$), and (vi) *S. capitis* versus *S. caprae* versus *S. arlettae* ($n = 2$). The NCBI results were not analyzed at the subspecies level because of a frequent lack of appropriate taxonomic indications in the database entries.

Isolates yielding RIDOM results ranging between the threshold (98.7%) and a 100% match ($n = 6$) were classified by the RIDOM database search as *S. capitis* subsp. *capitis* (isolate M13), *S. caprae* (isolate M14), *S. warneri* (isolate M52), and *S. xylosus* (isolates M54 and M56) (each with 99.78% similarity). In contrast, the NCBI database search resulted in ambiguous results for isolates M13 and M14 (Table 4). Regarding isolates M52, M54, and M56, the NCBI database search reached 99% similarity, exhibiting the same species affiliation as found by RIDOM.

Analyzing ambiguous or below-the-threshold RIDOM results by chemotaxonomy and riboprinting. Concerning isolate M55, two results with the same similarity (99.35%) were offered by RIDOM, i.e., *S. xylosus* and *S. saprophyticus* subsp. *saprophyticus* (Table 4). The NCBI database showed a perfect match with *S. xylosus*. Major fatty acids were ai-$C_{15:0}$ (12-methyltetradecanoic acid) followed by i-$C_{15:0}$ (13-methyl tetradecanoic acid), i-$C_{17:0}$ (15-methylhexadecanoic acid), and ai-$C_{17:0}$ (14-methylhexadecanoic acid), indicating *S. xylosus* (similarity index, 0.779) or, with lower likelihood, *S. gallinarum* (similarity index, 0.538). The peptidoglycan type of this isolate was A3α L-Lys-$Gly_{5–6}$ (A11.2), which is in accordance with *S. xylosus*. Also, riboprinting analysis confirmed the species to be *S. xylosus* (data not shown).

Isolate M08, found to be marginally below the threshold, showed the highest similarity to *S. haemolyticus* when the RIDOM database was used (Table 4). Also, the NCBI database search yielded this species with 98.5% similarity. Furthermore, both phenotypic test systems used resulted in *S. haemolyticus*. However, when the fatty acid profile was analyzed (main fatty acid components, ai-$C_{15:0}$, $C_{20:0}$, $C_{18:0}$, and i-$C_{15:0}$), an ambiguous result indicating either *S. epidermidis* (similarity index, 0.696) or *S. haemolyticus* (similarity index, 0.536) was found. In addition, the riboprinting pattern revealed grouping of this isolate into the *S. aureus-S. epidermidis-S. warneri* cluster (data not shown). The peptidoglycan of this isolate was based on the diamino acid lysine and contained glycine and serine residues in its interpeptide bridge, which corresponds to the type A3α L-Lys–$Gly_{5–6}$–L-$Ser_{1–2}$–Gly (A11.3), which is known to be associated with *S. haemolyticus* but also with *S. epidermidis* and *S. warneri* (not with *S. aureus*).

Comparison of RIDOM results with biochemical identification results. In a second assessment, the results of the 16S rDNA sequencing were compared to two phenotypic approaches comprising the commercially available differentiation systems ID 32 Staph and VITEK 2 (Table 3).

Analyzing results obtained by the ID 32 Staph system, 39 (70.9%) of the isolates were identified at confidence levels from excellent ($n = 12$; 21.8%) to very good ($n = 13$; 23.6%) to good ($n = 14$; 25.4%). Another six (10.9%) were identified but at a low level of discrimination categorized as acceptable. The ID 32 Staph system could not identify 10 (18.2%) of the 55 isolates tested because the %ID and/or *T* was below acceptable levels. In comparison with the RIDOM results, three (5.4%) of the isolates categorized as good to acceptable by ID 32 Staph were incorrectly identified (Table 3). Overall, ID 32 Staph failed in the case of 13 (23.6%) of the isolates due to incorrect identification and/or categorization below acceptable values, although 11 results were at least in the correct genus (Table 3). However, three of them categorized as questionable by the software represented *S. felis* isolates, which are not part of the

TABLE 2. Species and subspecies of the clinical staphylococcal isolates (n = 55) as determined by the RIDOM database

Species or subspecies tested (no. of isolates)	Species or subspecies indistinguishable (no. of isolates)
S. arlettae (1)	
S. aureus subsp. *aureus* (7)	*S. aureus* subsp. *anaerobius* (7)[a]
S. capitis subsp. *capitis* (2)	*S. capitis* subsp. *urealyticus* (2)[b]
S. caprae (2)	
S. chromogenes (1)	
S. cohnii subsp. *cohnii* (2)	
S. epidermidis (10)	
S. equorum subsp. *equorum* (2)	*S. equorum* subsp. *linens* (2)[c]
S. felis (3)	
S. haemolyticus (3)	
S. hominis subsp. *novobiosepticus* (1)	
S. hyicus (1)	
S. intermedius (3)	*S. delphini* (3)[d]
S. lugdunensis (2)	
S. saprophyticus subsp. *bovis* (2)	
S. schleiferi subsp. *schleiferi* (2)	
S. sciuri subsp. *sciuri* (3)	
S. simulans (1)	
S. warneri (3)	
S. xylosus (4)	*S. saprophyticus* subsp. *saprophyticus* (1)[e]

[a] Since the isolates tested were collected from human specimens, showing a positive catalase reaction and growing aerobically without exception, the presence of isolates of the sheep-adapted, catalase-negative, anaerobic subspecies *S. aureus* subsp. *anaerobius* could be excluded.

[b] Since isolates tested showed negative urease activity and were unable to produce acid aerobically from maltose, the presence of *S. capitis* subsp. *urealyticus* isolates is improbable.

[c] Since isolates tested produced acid aerobically from maltose, D-lactose, D-trehalose, and D-ribose, the presence of *S. equorum* subsp. *linens* isolates is improbable.

[d] Since isolates tested were collected from human and canine specimens, the presence of isolates of the strictly dolphin-adapted subspecies *S. delphini* is most improbable.

[e] One isolate (M55), which gave the same similarity (99.35%) for *S. xylosus* and *S. saprophyticus* subsp. *saprophyticus*, was chemotaxonomically and by riboprinting confirmed as *S. xylosus*.

TABLE 3. Results of identification of clinical staphylococcal isolates (n = 55) by 16S rDNA differentiation-based systems compared to biochemical assays

Result[a]	No. (%) of isolates tested by:			
	16S rDNA-based identification		Biochemical identification	
	RIDOM	GenBank	ID 32 Staph	VITEK 2
Correctly identified[b]	50 (90.9)	36 (65.5)	42 (76.4)	36 (65.5)
Failure due to:				
Correct identification, but below acceptable values[c]	1 (1.8)	1 (1.8)	6 (10.9)	4 (7.3)
Ambiguous results in the case of the highest match	4 (7.3)	18 (32.7)	NA[d]	NA
Misidentification	0	0	7 (12.7)	15 (27.3)
Total failures	5 (9.1)	19 (34.5)	13 (23.6)	19 (34.5)

[a] Based on species level.

[b] For biochemical assays, classification is based on a %ID that is acceptable or better (%ID $\geq$ 80.0) and on a T index value that is acceptable or better ($T \geq 0$); for 16S rDNA-based identification, classification is based on percent similarity (%S $\geq$ 98.5) to the 16S rDNA sequence of the type and reference strains.

[c] For biochemical assays, classification is based on a %ID that is below acceptable (%ID $<$ 80.0) and on a T value that is below acceptable ($T = 0$); for 16S rDNA-based identification, classification is based on a %S of $<$98.5%.

[d] NA, not applicable.

ID 32 Staph identification table. One *S. cohnii* isolate and one *S. capitis* (SCV phenotype) isolate were misidentified at the genus level as *Micrococcus lylae* and *Kocuria rosea*, respectively.

VITEK 2 identified 40 (72.7%) of the isolates at confidence levels of excellent (n = 30; 54.5%), very good (n = 5; 9.1%), and good (n = 5; 9.1%). Two isolates (3.6%) were categorized as acceptable. Because of the %ID and/or T being below acceptable levels, the VITEK 2 system could not identify 13 (23.6%) of the isolates. Although categorized as excellent to acceptable by VITEK 2, seven (12.7%) of the isolates were incorrectly identified compared with the RIDOM results (Table 3). Due to the lack of appropriate species data in the VITEK 2 database, all *S. arlettae*, *S. caprae*, *S. equorum*, and *S. felis* isolates (n = 8) were misidentified. Overall, VITEK 2 failed for 19 (34.5%) of the isolates due to incorrect identification and/or categorization below acceptable value, but 17 of the misidentifications were placed at least into the correct genus. Two *S. epidermidis* isolates both exhibiting the SCV phenotype were misidentified at the genus level as *Kocuria varians*.

Six SCV isolates included were found to belong to *S. aureus* (n = 2), *S. epidermidis* (n = 3), and *S. capitis* (n = 1) by RIDOM analyses. Compared with the RIDOM results, both ID 32 Staph and VITEK 2 failed to identify two (*S. aureus* and *S. capitis*) and four (*S. aureus*, *S. capitis*, and *S. epidermidis* [n = 2]) SCVs, respectively. The slow growth of SCVs and their reduced biochemical activity were responsible for three of the four misidentifications at the genus level when the commercial kits were used. The remaining case of genus misidentification using ID 32 Staph was caused by an *S. cohnii* isolate exhibiting a normal morphotype.

DISCUSSION

Since the first taxonomic description of the genus *Staphylococcus* by Rosenbach in 1884 (31), the division of this genus—based on colony pigment—into the two species *S.* (*pyogenes*) *aureus* and *S. albus* (now referred to as *S. epidermidis*) primarily has been the basis to differentiate between the opportunistic pathogen *S. aureus*, which causes high morbidity and mortality, and other staphylococci generally considered to be harmless commensals or saprophytic bacteria. Using a variety of morphological criteria and physiological and biochemical tests, Baird-Parker created several schemes to differentiate between *S. epidermidis* and *S. saprophyticus*, subdivided into subgroups or biotypes (2, 3). Despite the subsequent introduction of some more species considered to be part of the family *Micrococcaceae* (19) and their differentiation in particular by Kloos and Schleifer (18), it soon became obvious that more staphylococcal species are associated with humans and animal species than had been recognized so far. Therefore, the genus *Staphylococcus* was enlarged to 37 validly described species, including 21 subspecies, according to the current *List of Bacterial Names with Standing in Nomenclature*, updated 24 January 2004 (9). To take into account the recent results of molecular phylogenetic classification, it was proposed to reclassify the genus *Staphylococcus* into a family *"Staphylococcaceae"* (order *Bacillales*, class *"Bacilli,"* phylum *Firmicutes*) (10).

While conventional differentiation schemes based on physiological and biochemical tests are relatively cumbersome and time-consuming and require various approaches, the commer-

TABLE 4. Clinical staphylococcal isolates with dissenting or ambiguous results in identification

Isolate	16S rDNA-based identification		Biochemical identification	
	RIDOM (% similarity)[a]	GenBank (% similarity)[b]	ID 32 Staph (*T* index; %ID)[c]	VITEK 2 (*T* index; selectivity)[c]
M01	*S. arlettae* (100.0)	*S. arlettae* (100.0)	*S. klosii* (0.65; 63.7), *S. saprophyticus* (0.27; 33.4), *S. xylosus* (0.29; 2.6), *S. warneri* (0.00; 0.2)	*S. kloosii* (0.37; good)
M02	*S. aureus* subsp. *aureus* (100.0), *S. aureus* subsp. *anaerobius* (100.0)	*S. aureus* subsp. *aureus* (100.0), *S. aureus* (100.0), *S. haemolyticus* (100.0)	*S. aureus* (0.96; 99.9)	*S. aureus* (0.97; excellent)
M03	*S. aureus* subsp. *aureus* (100.0), *S. aureus* subsp. *anaerobius* (100.0)	*S. aureus* subsp. *aureus* (100.0), *S. aureus* (100.0), *S. haemolyticus* (100.0)	*S. aureus* (0.63; 99.9)	*S. aureus* (1.0; excellent)
M04	*S. aureus* subsp. *aureus* (100.0), *S. aureus* subsp. *anaerobius* (100.0)	*S. aureus* subsp. *aureus* (100.0), *S. aureus* (100.0), *S. haemolyticus* (100.0)	*S. aureus* (0.52; 93.0)	*S. aureus* (0.65; very good)
M05[d]	*S. aureus* subsp. *aureus* (100.0), *S. aureus* subsp. *anaerobius* (100.0)	*S. aureus* subsp. *aureus* (100.0), *S. aureus* (100.0), *S. haemolyticus* (100.0)	*S. cohnii* subsp. *cohnii* (0.23; 64.7), *S. caprae* (0.21; 30.6), *S. hominis* 2[e] (0.0; 3.2)	*S. hyicus* (0.42; poor), *S. chromogenes* (0.33; poor)
M06	*S. aureus* subsp. *aureus* (100.0), *S. aureus* subsp. *anaerobius* (100.0)	*S. aureus* subsp. *aureus* (100.0), *S. aureus* (100.0), *S. haemolyticus* (100.0)	*S. aureus* (0.72; 99.9)	*S. aureus* (0.86; excellent)
M07[d]	*S. aureus* subsp. *aureus* (100.0), *S. aureus* subsp. *anaerobius* (100.0)	*S. aureus* subsp. *aureus* (100.0), *S. aureus* (100.0), *S. haemolyticus* (100.0)	*S. aureus* (0.72; 99.9)	*S. aureus* (0.25; good)
M08	*S. haemolyticus* (98.5)	*S. haemolyticus* (98.5)	*S. haemolyticus* (0.45; 76.9), *S. warneri* (0.37; 22.4), *S. hominis* (0.04; 0.6)	*S. haemolyticus* (0.89; excellent)
M10	*S. capitis* subsp. *S. capitis* (100.0)	*S. capitis* (100.0), *S. epidermidis* (100.0), *S. caprae* (100.0)	*S. capitis* (0.82; 99.9)	*S. capitis* (0.59; poor), *Kocuria rosea* (0.50; poor), *S. cohnii* subsp. *cohnii* (0.49; poor)
M12[d]	*S. epidermidis* (100.0)	*S. epidermidis* (100.00)	*S. epidermidis* 1[e] (0.54; 83.3)	*Kocuria varians* (0.44; good)
M13[d]	*S. capitis* subsp. *capitis* (99.8)	*S. capitis* (99.8), *S. epidermidis* (99.8), *S. caprae* (99.8)	*Kocuria rosea* (0.95; 81.5)	*S. chromogenes* (0.49; poor), *S. auricularis* (0.43; poor)
M14	*S. caprae* (99.8)	*S. caprae* (99.8), *S. capitis* (99.8), *S. arlettae* (99.8)	*S. caprae* (0.58; 99.6)	*S. chromogenes* (0.44; good)
M15	*S. caprae* (100.0)	*S. caprae* (100.0), *S. capitis* (100.0), *S. arlettae* (100.0)	*S. caprae* (0.22; 97.4)	*S. chromogenes* (0.36; poor), *S. aureus* (0.35; poor), *S. warneri* (0.29; poor)
M17	*S. cohnii* subsp. *cohnii* (100.0)	*S. cohnii* (100.0), *S. saprophyticus* (100.0)	*S. cohnii* subsp. *cohnii* (0.33; 99.3)	*S. cohnii* subsp. *cohnii* (0.56; poor), *S. saprophyticus* (0.54; poor)
M18	*S. cohnii* subsp. *cohnii* (100.0)	*S. cohnii* (100.0), *S. saprophyticus* (100.0)	*Micrococcus lylae* (0.52; 96.9)	*S. cohnii* subsp. *urealyticus* (0.77; excellent)
M20	*S. saprophyticus* subsp. *bovis* (100.0)	*S. cohnii* (99.8), *S. succinus* (99.8)	*S. saprophyticus* (1.00; 99.9)	*S. saprophyticus* (1.00; excellent)
M25[d]	*S. epidermidis* (100.0)	*S. epidermidis* (100.0)	*S. epidermidis* 2[e] (0.62; 86.1)	*Kocuria varians* (0.24; poor)
M26	*S. equorum* subsp. *equorum* (100.0), *S. equorum* subsp. *linens* (100.0)	*S. equorum* (100.0), *S. equorum* subsp. *linens* (100.0)	*S. equorum* (0.31; 97.6)	*S. xylosus* (0.44; good)
M27	*S. felis* (100.0)	*S. felis* (100.0)	*S. intermedius* (0.31; 48.6), *S. chromogenes* (0.32; 25.9), *S. xylosus* (0.43; 15.4), *S. simulans* (0.29; 10.1), *S. aureus* (0.00; 0.1)	*S. chromogenes* (0.78; poor), *S. schleiferi* (0.70; poor)
M33	*S. intermedius* (100.0), *S. delphini* (100.0)	*S. intermedius* (100.0), *S. delphini* (100.0)	*S. intermedius* (0.99; 99.7)	*S. intermedius* (0.66; poor), *S. chromogenes* (0.61; poor)
M34	*S. intermedius* (100.0), *S. delphini* (100.0)	*S. intermedius* (100.0), *S. delphini* (100.0)	*S. intermedius* (0.46; 95.4)	*S. intermedius* (0.66; poor), *S. chromogenes* (0.61; poor)

Continued on following page

TABLE 4—*Continued*

Isolate	16S rDNA-based identification		Biochemical identification	
	RIDOM (% similarity)[a]	GenBank (% similarity)[b]	ID 32 Staph (*T* index; %ID)[c]	VITEK 2 (*T* index; selectivity)[c]
M35	*S. intermedius* (100.0), *S. delphini* (100.0)	*S. intermedius* (100.0), *S. delphini* (100.0)	*S. intermedius* (0.67; 98.0)	*S. intermedius* (0.75; excellent)
M39	*S. saprophyticus* subsp. *bovis* (100.0)	*S. cohnii* (99.8), *S. succinus* (99.8)	*S. saprophyticus* (0.83; 99.9)	*S. saprophyticus* (0.91; excellent)
M40	*S. aureus* subsp. *aureus* (100.0), *S. aureus* subsp. *anaerobius* (100.0)	*S. aureus* subsp. *aureus* (100.0), *S. aureus* (100.0), *S. haemolyticus* (100.0)	*S. aureus* (0.69; 99.3)	*S. aureus* (0.50; very good)
M46	*S. simulans* (100.0)	*S. simulans* (100.0)	*S. simulans* (0.73; 98.6)	*S. haemolyticus* (0.48; poor), *S. simulans* (0.46; poor)
M47	*S. hominis* subsp. *novobiosepticus* (100.0)	*S. hominis* (100.0)	*S. hominis* 1[e] (0.21; 65.9), *S. warneri* (0.18; 17.0), *S. epidermidis* 2 (0.15; 10.9)	*S. capitis* (0.86; excellent)
M48	*S. felis* (100.0)	*S. felis* (100.0)	*S. xylosus* (0.58; 60.8), *S. intermedius* (0.30; 22.6), *S. simulans* (0.36; 14.8), *S. chromogenes* (0.16; 1.7), *S. aureus* (0.00; 0.1)	*S. simulans* (0.60; poor), *S. chromogenes* (0.53; poor)
M49	*S. felis* (100.0)	*S. felis* (100.0)	*S. simulans* (0.65; 83.3)	*S. chromogenes* (0.53; poor), *S. schleiferi* (0.45; poor)
M51	*S. warneri* (100.0)	*S. warneri* (100.0)	*S. warneri* (0.76; 99.9)	*S. warneri* (0.46; poor), *S. haemolyticus* (0.46; poor), *S. kloosii* (0.39; poor)
M53	*S. equorum* subsp. *equorum* (100.0), *S. equorum* subsp. *linens* (100.0)	*S. equorum* (100.0), *S. equorum* subsp. *linens* (100.0)	*S. equorum* (0.68; 66.0), *S. xylosus* (0.74; 34.0), *S. sciuri* (0.03; 0.1)	*S. xylosus* (0.88; excellent)
M55	*S. xylosus* (99.35), *S. saprophyticus* subsp. *saprophyticus* (99.35)	*S. xylosus* (100.0)	*S. xylosus* (0.89; 99.2)	*S. xylosus* (0.90; excellent)

[a] Percent similarity compared to the 16S rDNA sequence of the type strain of the staphylococcal taxon.
[b] Results of redundant database entries as well as of entries without taxonomic classification were not included.
[c] In the case of identification with low discrimination below acceptable values as given by the biochemical identification kits, i.e., a percent identification accuracy (%ID) of <80.0 (as specified by ID 32 Staph) and poor selectivity (as specified by VITEK 2), all results are listed; otherwise, only results with the highest %ID and best selectivity, respectively, are shown.
[d] Isolate exhibiting the SCV phenotype.
[e] The numeral identifies the biotype.

cial "rapid" identification systems share the problems of failure to identify commonly encountered bacteria, uselessness in identifying uncommon isolates, and lack of adequate (reference) strains in the accompanying databases (30, 35). Furthermore, commercial systems may provide ambiguous results, presenting two or more suggestions for identification with a comparable safety level.

With the advent of molecular biology techniques, investigations based on comparative DNA sequence analysis of the genes of conserved macromolecules have become commonplace in microbiology as a tool for classification of microbial organisms. Presently, the most useful and extensively investigated taxonomic marker molecules are the larger rRNAs and their corresponding genes, respectively, especially 16S rRNAs and to a lesser extent 23S rRNAs. However, analysis of rDNA sequences does not necessarily show agreement with characterizations based on classical taxonomic methods. While molecular identification based on sequence analysis of universal targets offers several advantages, such as improved accuracy and short turnaround time, some drawbacks in currently available databases should be noted. The deficiencies of publicly accessible databases without controlled input in the contents include the presence of faulty and/or redundant sequence entries, ragged sequence ends, outdated nomenclature, and unavailable quality assurance. Since "gold standard" sequences are lacking, we established a quality-controlled sequence database based on 81 type and reference strains encompassing all species and subspecies of the genus *Staphylococcus* recognized as valid and the recently proposed candidate species *"S. pettenkoferi"* (36).

Analyses of the newly determined sequences of the 5′ end of the 16S rDNA revealed that all type and reference strains were distinguishable at the species level, with two exceptions: (i) *S. intermedius* versus *S. delphini* and (ii) *S. pulvereri* versus *S. vitulinus*. Whereas it has been recently proposed that the strains assigned to the last two species belong to the same species (26), the coagulase-positive species *S. intermedius* and *S. delphini* represent well-accepted different species. Comparing their 16S rDNA regions deposited in the EMBL database (accession numbers AB009938 and D83369, respectively), downstream of the fragment analyzed here, five base exchanges between both sequences were noted, which may be useful for differentiation between these species if needed. Whereas the chaperonin 60 gene (*hsp60*) as sequencing target does not allow discrimination between *S. intermedius* and *S. delphini*, the sequencing of a manganese-dependent superoxide dismutase gene (*sodA*) fragment was able to differentiate between them (11, 29).

At the subspecies level, sequencing of the 5′ 16S rDNA fragment was discriminative enough to differ specifically between the type strains of the established subspecies of *S. carnosus*, *S. cohnii*, *S. hominis*, *S. schleiferi*, and *S. succinus*. Our

results confirm that 16S rDNA might be a more discriminative target sequence than other ribosomal regions or target genes to differentiate among staphylococcal subspecies. Neither the 16S-23S rDNA intergenic spacer region nor genes such as *hsp60* or *sodA* allow discrimination at the subspecies level (20, 24, 29).

Using 55 clinical staphylococcal isolates, the 16S rDNA-derived results obtained by using the RIDOM database were queried with NCBI database entries and compared to results obtained by using commercial "rapid" identification systems. The range of clinical isolates was chosen in such a manner that a broad diversity of different human and animal species was covered and examples of diagnostically challenging isolates such as phenotypic variants were acknowledged. However, this selection does not reflect the typical distribution of staphylococcal (sub)species in a human or veterinary routine microbiological laboratory.

Overall, the RIDOM database was proven to be particularly suitable for differentiation of staphylococcal isolates belonging to common as well as rare species and different phenotypes. When analyzing the sequence of isolate M08, both RIDOM and NCBI database queries resulted in the same percent similarity below the threshold with a best match indicating *S. haemolyticus*. Whereas the VITEK 2 system displayed *S. haemolyticus*, the ID 32 Staph result was ambiguous, as were the results of chemotaxonomic and riboprinting analyses. Thus, the diversity of the 16S rDNA sequence of this species might be broader than is currently appreciated or the proposal of new subspecies to accommodate this isolate should be considered.

Searching the NCBI database gave numerous ambiguous results not only for CoNS but also for *S. aureus*, which displayed a perfect match with an *S. haemolyticus* entry (accession no. Z26896). This obviously incorrect database entry highlights the common presence of faulty sequence entries.

Apart from staphylococcal isolates with the SCV phenotype, phenotypically normal clinical isolates were also misidentified or showed ambiguous results if the commercial kits were used. This was shown for species seldom associated with infections and was also shown for commonly encountered staphylococcal species. This is of particular importance, as shown here, if the kits suggested good or better identification accuracy but indeed failed in species (and in one case also in genus) identification. Comparing the results of the two commercial kits used, the VITEK 2 kit showed more misidentifications, mainly due to the limited database of gram-positive cocci (19 staphylococcal species) included in the ID-GBP card, whereas ID 32 Staph covers 24 *Staphylococcus* species. Beside the utmost importance of *S. aureus* identification, assured identification results, at least at the species level, are vital for differentiation of the expanding number of CoNS (sub)species increasingly characterized as emerging pathogens (22, 40) and also recently recovered as food-fermenting microorganisms, which may have to be considered relevant to food safety and quality (27, 28). Differentiation up to the species level may also have substantial consequences for the management of patients in the case of CoNS. Since infectious endocarditis caused by *S. lugdunensis* is usually associated with left-side valvular disruption and life-threatening embolic complications, correct identification of this aggressive species is critical to distinguish it from other CoNS (17).

In general, the RIDOM approach (http://www.ridom-rdna.de) based on quality-controlled sequence entries offers a comprehensive and validated sequence database (15) which has already been shown to be useful for species belonging to the *Neisseriaceae* and *Moraxellaceae* as well as for *Mycobacterium* and *Nocardia* species (13, 16, 23). The RIDOM system has a *Neisseriaceae* and *Mycobacterium* data set for demonstration purposes, and an increase in the number of entries for other microorganisms is in progress.

In summary, the 5′ 16S rDNA fragment used to establish the staphylococcal part of the RIDOM database contains enough information suitable for the identification of almost all staphylococci at the species level and, with some exceptions, also at the subspecies level. Furthermore, recognition of hitherto undescribed species may be possible. The increasing feasibility of high-throughput sequencing, accompanied by falling costs, suggests that 16S rDNA sequencing holds promise as a rapid alternative to biochemical and other phenotypic procedures for differentiation of pathogens. In contrast to public databases, which are characterized by a policy of unchecked sequence input (especially disadvantageous for diagnostic purposes), quality-controlled sequence databases such as RIDOM, based only on type and well-characterized reference strains, may prove to be of maximum benefit for identification purposes. A substantial increase in the number of RIDOM entries and their continuing updates will further improve the value of this database and will broaden its possible fields of application.

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