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Comparative Study Using Phenotypic, Genotypic, and Proteomics Methods for Identification of Coagulase-Negative Staphylococci

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Five methods were compared to determine the most accurate method for identification of coagulase-negative staphylococci (CoNS) ($n = 142$ strains). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) showed the best results for rapid and accurate CoNS differentiation (99.3% of strains correctly identified). An alternative to this approach could be Vitek2 combined with partial *tuf* gene sequencing (100% of strains correctly identified when both methods are performed simultaneously).

Coagulase-negative staphylococci (CoNS) have emerged as significant pathogens and are mainly found in hospitalized immunocompromised patients, often with indwelling or implanted medical devices (10, 13, 15, 18, 22). Most infections are hospital acquired, and CoNS infections can result in several diseases, including bloodstream infection, endocarditis, mediastinitis, meningitis, urinary tract infections (*Staphylococcus saprophyticus*), and medical-device-related infections (1, 8, 12, 18, 19, 22). Accurate identification of CoNS is important when CoNS are isolated from multiple blood cultures of 1 patient. Many CoNS are described to be resistant to multiple antibiotics (11, 14, 17, 19). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is a relatively new technique that allows examination of protein profiles from bacteria, and this technique has been shown to work in a fast and accurate manner (4, 6, 7, 21).

In the present comparative study, five methods for differentiation of CoNS were compared, i.e., Vitek2 (Gram Positive card REF 21342; bioMérieux), the ID 32 Staph strip (bioMérieux), partial 16S rRNA gene sequencing (MicroSeq; Applied Biosystems), partial *tuf* gene sequencing (in-house), and MALDI-TOF MS (Bruker Daltonics), on the same set of CoNS strains in order to find the most suitable method available today for identification of staphylococci.

A total of 142 CoNS strains were included in this study. Isolates were derived from clinical cultures ($n = 117$), and 25 reference strains from the American Type Culture Collection (ATCC) and the National Institute for Health and Environment (RIVM; Bilthoven, The Netherlands) were included. The clinical isolates were selected from positive blood cultures (two or more positive cultures from 1 patient), urinary tract infections (*S. saprophyticus*), or deep-seated infections. The CoNS species name that was found with ≥3 of the 5 used methods was chosen as the true CoNS species name (referred to here as the true ID). When only 2 methods resulted in similar true IDs (4/142 cases [2.8%]), *tuf* sequencing was selected as the most reliable method, as the result for 16S rRNA gene sequencing was noninformative (*Staphylococcus* spp.).

CoNS strains were cultured overnight at 35°C on blood plates (made in-house). The phenotypic methods Vitek2 (bioMérieux, Marcy l'Etoile, France) and ID 32 Staph (bioMérieux, Marcy l'Etoile, France) were both performed as described by the manufacturer.

DNA for sequencing was isolated from 2 McFarland bacterial suspensions by heating them (for 10 min at 95°C). The 16S rRNA gene primers from the MicroSeq kit were used, and these primers amplify approximately the first 500 bp of the gene (Applied Biosystems, Foster City, CA). The *tuf* forward primer (tuf_32_FW) was described previously (16), and the *tuf* reverse primer (tuf_512_RV; 5′-CAGCTTCAGCGTAGTCTAATAATTTACG-3′) was designed for this study. The positions of the *tuf* primers were derived from the *Staphylococcus aureus tuf* gene sequence (GenBank accession no. AF298796), and these primers amplify a 480-bp fragment. The *tuf* primers were obtained from Eurogentec (Liège, Belgium). PCR was performed according to standard procedures. Sequencing was performed using BigDye XTerminator sequencing mix 3.1 (Applied Biosystems, Foster City, CA) and ran on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA). The 16S DNA sequences were analyzed by using MicroSeqID Analysis software (Applied Biosystems, Foster City, CA). The *tuf* DNA sequences were analyzed by using the Basic Local Assignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For MALDI-TOF MS (Bruker Daltonics) analysis, 1 colony of each CoNS strain was spotted with a sterile wooden stick, in duplicate, on the polished steel target plate. Samples that could not be identified directly by MALDI-TOF MS were retested after pretreatment ($n = 5$ clinical CoNS strains [1 strain each of *Staphylococcus hominis*, *S. epidermidis*, *S. cohnii*, *S. schleiferi*, and *S. saprophyticus*]) as described by van Veen et al. (21). However, in this study, formic acid (70%) and acetonitrile were added according to pellet size at a 1:1 ratio. BioTyper database version V3 1.1.0_3476-3740 was used as a reference.

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TABLE 1 Summary of the performance of methods used for differentiation of CoNS[a]

True organism	No. of strains	No. of strains identified by indicated method														
		ID 32 Staph			Vitek2			16S sequencing			*tuf* sequencing			MALDI-TOF MS		
		Correctly	Incorrectly	ND	Correctly	Incorrectly	ND	Correctly	Incorrectly	ND	Correctly	Incorrectly	ND	Correctly	Incorrectly	ND
S. capitis	13	12	1		12	1		11	2		13			13		
S. caprae	1		1		1				1		1			1		
S. cohnii	1	1			1				1		1			1		
S. epidermidis	29	24	5		26	3		24	5		29			29		
S. haemolyticus	18	18			16	2		13	5		18			18		
S. hominis	22	19	3		21	1		17	5		22			21	1	
S. lentus	1	1			1				1		1			1		
S. lugdunensis	11	11			11			9	2		11			11		
S. pasteuri	1		1			1		1			1			1		
S. pettenfoferi	2		2			2			2		2			2		
S. saprophyticus	12	12			12			8	4		12			12		
S. schleiferi	10	4	6		10			4	6		10			10		
S. sciuri	5	5			5				5		5			5		
S. simulans	6	6			5	1		6			6			6		
S. warneri	10	9	1		10			7	3			10		10		
Total (%)	142 (100)	122 (85.9)	20 (14.1)	0 (0)	131 (92.3)	11 (7.7)	0 (0)	100 (70.4)	42 (29.6)	0 (0)	132 (93)	10 (7)	0 (0)	141 (99.3)	1 (0.7)	0 (0)

[a] The acceptance criteria for the results were as follows: for ID 32 Staph, a score of ≥70%; for Vitek2, a score of ≥70% (in-house validation); for *tuf* sequencing, a species name provided by BLAST showing a ≥99% sequence match (identity) and a mismatch of at least a 1 nucleotide with the second hit; for 16S sequencing, a first hit showing a ≥99% sequence match (identity) and a second hit having a mismatch of at least 1 nucleotide (MicroSeq software); for MALDI-TOF MS, a hit rate of ≥1.7 (however, <2 was considered reliable to the species level if the first and second database matches resulted in the same strain name; ideally, ≥2 reference spectra need to be available in the database, and scores of ≥2 were considered reliable to the species level). ND, not determined.

The results (Table 1) for the phenotypic identification methods used in this study indicate that Vitek2 performs best, with a correct-identification rate of 92.3%, compared to 85.9% for ID 32 Staph. The acceptance criteria for all results are described in the footnote to Table 1.

With the use of 16S rRNA gene and *tuf* sequencing, 70.4% and 93% of the CoNS strains, respectively, were correctly identified. When the first hit with the MicroSeqID database was selected as the true finding (disregarding analysis rules), then 137 out of 142 strains were correctly identified (96.5%). Application of the CLSI guidelines (23), which are stricter than those used in this study, will lead to even more results that are noninformative (*Staphylococcus* spp.) and thus incorrect identifications with 16S sequencing in this study. Whole-16S-gene sequencing will improve the *Staphylococcus* species differentiation ability of this specific gene but will also increase the number of required sequence reactions and consequently slightly increase costs and, in some cases, time-to results. Becker et al. (2) showed that with the use of the quality-controlled Ribosomal Sequence Database (RIDOM), identification of staphylococci improved from 83.6% to 98.2%, compared to the results obtained with the NCBI database. With the use of the RIDOM database, the results obtained in this study might positively change as well. However, *tuf* gene sequencing resulted in more informative data (species name) and was also in concordance with MALDI-TOF MS results. Although all *Staphylococcus warneri* strains ($n = 10$) resulted in incorrect identification with *tuf* gene sequencing, due to high homology to *S. pasteuri*, extensive analysis of *S. pasteuri* and *S. warneri* strains might solve the current problems. Therefore, the *tuf* gene seems more suitable for identification of staphylococci, as described by others (5, 9).

The MALDI-TOF MS data obtained in this evaluation indicate the superiority in identification of CoNS, as 99.3% of the strains were correctly identified with this method. Strains were spotted in duplicate and resulted in the same identification (strain name). The database (V3 1.1.0_3476-3740) used for this study contained the following numbers of reference spectra per tested CoNS species: *Staphylococcus capitis*, 6; *S. caprae*, 1; *S. cohnii*, 5; *S. epidermidis*, 9; *S. haemolyticus*, 8; *S. hominis*, 6; *S. lentus*, 2; *S. lugdunensis*, 5; *S. pasteuri*, 2; *S. pettenkoferi*, 4; *S. saprophyticus*, 8; *S. schleiferi*, 6; *S. sciuri*, 4; *S. simulans*, 5; and *S. warneri*, 4. For *S. caprae*, only 1 reference spectrum is available, and duplicate measurement indicated *S. caprae* (hit 1; score, 1.853). The second hit for this CoNS species indicated *S. epidermidis* twice (<1.5). For the proposed result acceptance criteria for MALDI-TOF MS to be used, the number of reference spectra per CoNS strain ideally needs to be ≥2. However, as hit 2 provided an unreliable score for *S. epidermidis*, we considered the *S. caprae* result the correct identification. On average, there was a 4.82% difference in score value between hits 1 and 2. The combination of MALDI-TOF MS with *tuf* gene sequencing resulted in a correct-identification rate of 100%.

The 25 reference strains used in this study showed that different misidentifications occurred with the investigated methods. With the 16S sequencing protocol, 9/25 reference strains could not be identified to the species level (36%). With Vitek2, ID 32 Staph, and *tuf* sequencing, 2/25 reference strains were misidentified (8%). MALDI-TOF MS resulted in correct identification of all reference strains. MALDI-TOF MS has been investigated by others in relation to *Staphylococcus* species identification directly from agar plates (3, 6, 7, 20), and the correct-identification rates range from 74.2% to 99.3%. The data obtained in this study are comparable with data reported by Spanu et al. (20) and Dubois et al. (6), in which a sensitivity of 99.3% was found. In this study, only one *S. hominis* strain could not be identified with MALDI-TOF MS (Table 1), for unknown reasons.

Sequencing of the partial 16S gene resulted in the most clinically relevant misidentifications, as both *S. lugdunensis* and *S. saprophyticus* were not correctly identified.

In conclusion, this is one of the most extensive comparative studies for CoNS identification. The results obtained in this study demonstrate the good performance of MALDI-TOF MS for iden-

tification of CoNS, as a correct-identification rate of 99.3% was achieved with this method. *tuf* gene sequencing is the most suitable substitute for MALDI-TOF MS. When cheap, fast, and accurate identification of CoNS is needed, the usage of MALDI-TOF MS is the method of choice and, when necessary, the *tuf* gene can be sequenced. As an alternative method, due to the fact that not every diagnostic microbiology laboratory has access to a MALDI-TOF MS system, Vitek2 combined with *tuf* sequencing is suggested as an accurate approach for CoNS differentiation.

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We declare that we have no conflict of interest.

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