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Rapid and Economical Method for Species Identification of Clinically Significant Coagulase-Negative Staphylococci

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Four methods for the species identification of coagulase-negative staphylococci in the medical microbiology laboratory were compared with 444 consecutive isolates. The methods included (i) the reference method based on growth tests, (ii) API ID 32 Staph (bioMérieux), (iii) Staph-Zym (Rosco), and (iv) a rapid 4-h method developed in our laboratory (UZA method). The last method is based on the detection within 4 h of enzymatic activity of heavy bacterial suspensions in three substrate solutions (nongrowth tests). For 16.5% of the isolates some supplementary growth tests read after 24 h had to be added to the enzyme data for satisfactory identification. The reference method failed to identify four isolates. Of the 440 isolates identified by the reference method, API ID 32 Staph, Staph-Zym, and the UZA method correctly identified 419 (95.2%), 429 (97.5%), and 430 (97.7%) and misidentified 8 (1.8%), 4 (0.9%), and 1 (0.2%), respectively. *Staphylococcus epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. schleiferi*, and *S. capitis* were identified with an accuracy of 98 to 100% by all the systems tested. *S. capitis* subsp. *ureolyticus* was not recognized by the API ID 32 system because the biochemical profiles for it are not yet included in the corresponding database. Whereas API ID 32 identified all 13 *S. warneri* isolates, both Staph-Zym and the UZA method missed 2 of these. *S. hominis* was identified with the least accuracy by the API ID 32 system (26 of 39 isolates), whereas the UZA and Staph-Zym methods identified 36 of the isolates belonging to this species. The UZA method did not identify any of the *S. cohnii*, *S. xylosus*, *S. lentus*, and *S. sciuri* strains, since it included no discriminatory tests for these species, because they are extremely rarely found in humans. Of all 440 isolates tested, the UZA method failed to identify 9 and misidentified 1 other. Eighty-one percent of the isolates were identified within 4 h and 97.7% were identified after 24 h, at considerably less expense than by the API ID 32 Staph and Staph-Zym methods.

The genus *Staphylococcus* is currently divided into 31 species, of which about half are indigenous to humans. Eight subspecies have been described; four of them have been given names, and two of these are indigenous to humans (5). *Staphylococcus aureus* is one of the most common causes of nosocomial infections due to gram-positive organisms. However, in recent times, coagulase-negative staphylococci (CoNS) also emerged as significant pathogens, especially in medical-device-related infections and in immunocompromised patients (2, 3, 5, 7, 8, 12–16, 18, 19). In the hospital microbiology laboratory, identification of staphylococci is often limited to a rapid screening test for *S. aureus*, while non-*S. aureus* isolates are simply reported as CoNS. However, because of their increasing importance, clinically significant CoNS should be identified to the species level by a reliable, simple, rapid, and, preferably, inexpensive method (1, 4).

The objective of the present study was to compare four CoNS identification methods: the reference method (6), two commercial methods, and a rapid, economical method developed in our laboratory (UZA method), in which some growth tests of the reference method are replaced by nongrowth tests, resulting in a two-step procedure to be completed within 24 h.

MATERIALS AND METHODS

Origin of isolates. Consecutive isolates of CoNS were collected during the periods from October 1991 to March 1992 (n = 181) and October 1992 to March 1993 (n = 263) at the Department of Clinical Microbiology, University Hospital, Antwerpen, Belgium. The 444 CoNS strains were isolated from blood cultures (n = 268), central venous catheters (n = 54), skin (n = 19), wounds (n = 48), cerebrospinal fluids (n = 23), peritoneal dialysis fluids (n = 4), urine samples when present in pure culture in numbers $>10^5$/ml (n = 25), and other sources (n = 3).

The organisms were identified by Gram staining and catalase and coagulase tests (rabbit plasma tube test, read after 4 and 24 h). Bacitracin susceptibility was tested to exclude micrococci. CoNS were frozen at −20°C in 5% glycerol broth until they were tested.

Reference strains. *Staphylococcus lugdunensis*, *S. schleiferi*, and *S. hominis* were purchased from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. *S. haemolyticus* (CCM 2737), *S. saprophyticus*, *S. intermedius* (CCM 5739), *S. hyicus*, *S. capitis*, and *S. warneri* were from the Czechoslovak Collection of Microorganisms, J. E. Purkinje University, Brno, Czechoslovakia.

Identification methods. (i) Reference method. The reference method consisted of 16 conventional growth tests as described in the *Manual of Clinical Microbiology* (6) (colony pigment, DNase, alkaline phosphatase, ornithine decarboxylase, urease, acetoin production, novobiocin resistance, polymyxin resistance, and acid production from D-trehalose, D-mannitol, D-mannose, D-turanose, D-xylose, D-cellobiose, maltose, and sucrose). The results of the pyrrolidonyl arylamidase and *o*-nitrophenyl-β-D-galactopyranoside tests were obtained in the API ID 32 Staph method (as mentioned in reference 6). The alkaline phosphatase test was carried out in 0.25 ml of physiological saline inoculated with a 2-mm loop of bacteria to produce a turbidity of 2 on the McFarland scale and an alkaline phosphatase disc (Rosco, Taastrup, Denmark) read after 4 h of incubation in a water bath at 37°C.

For novobiocin and polymyxin susceptibility tests, tablets (Rosco) were used according to the criteria proposed by the manufacturer: for both novobiocin and polymyxin susceptibility, zones of inhibition were ≥16 mm. Similar antibiotic discs are available from BBL. All biochemical tests were interpreted after 24 to 48 and 72 h of incubation at 37°C.

(ii) API ID 32 Staph. API ID 32 Staph (bioMérieux, La Balme les Grottes, Montalieu Vercieu, France) consists of a set of wells containing dried substrates for 26 colorimetric tests to which a bacterial suspension was added automatically. After 24 h of incubation at 37°C and addition of the necessary reagents to some wells, results were read automatically and interpreted by computer using the APILAB ID 32 software.

(iii) Staph-Zym. Staph-Zym (Rosco) consists of 10 metabolic or enzymatic tests in minitubes to which a bacterial suspension was added, results being read as color changes after incubation for 24 h, combined with tests for polymyxin and novobiocin susceptibility. For interpretation of the results, the instructions of the manufacturer were followed. Frequently fosfomycin susceptibility and sometimes other additional tests were required as well.

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TABLE 1. Identification of CoNS by a two-step procedure[a]

Species	Result[b] of:						
	1st step			2nd step			
	D-Trehalose	Urease	Alkaline phosphatase	Ornithine decarboxylase	Novobiocin susceptibility	Fosfomycin susceptibility	Anaerobic growth
S. epidermidis	−	+	±			S[c]	+[c]
S. haemolyticus	+	−	−				
S. saprophyticus	+	+	−	−	R		
S. lugdunensis	+	±	−	+			
S. schleiferi	±	−	+				
S. hominis	±	+	−		S		−
S. capitis	−	−	−				
S. capitis subsp. *ureolyticus*	−	+	−			R	
S. warneri	+	+	−		S		+

[a] For details, see Materials and Methods.
[b] +, positive result; −, negative result; ±, positive or negative result; R, resistant, S, susceptible.
[c] For *S. epidermidis* only if a −+− pattern is obtained in the first step.

(iv) UZA method. The rapid identification method developed in our laboratory (UZA method) aims at the identification of the species listed in Table 1. Since these species are the most prevalent clinical isolates (4, 5, 13), the minimum number of tests necessary and sufficient to discriminate between these was selected from the reference method (6) and fosfomycin susceptibility was added. The method was based mainly on the detection of enzyme activities of heavy bacterial suspensions in substrate solutions and involved a two-step procedure (Table 1). The first step consisted of three tests, read after 4 h at 37°C: urease (0.2 ml of urea broth [Oxoid, Basingstoke, United Kingdom] covered with liquid paraffin) and trehalose acidification (0.2 ml of 2% trehalose in phosphate buffer [pH 7.2] with phenol red), both heavily inoculated with a 2-mm loop of organisms, and alkaline phosphatase (as described for the reference method). The test tubes were incubated in a water bath and read after 4 h. Delayed reading potentially yields a false-positive result for the alkaline phosphatase test.

If supplementary tests were required after Table 1 was consulted, a maximum of two of the four possible supplementary tests were performed in a second step and read after 24 h at 37°C. The four possible supplementary tests were ornithine decarboxylase (0.2 ml of 1% ornithine HCl in Mueller-Hinton broth inoculated with a 2-mm loop of organisms and covered with liquid paraffin), anaerobic growth (lightly inoculated thioglycolate broth), and susceptibility to novobiocin (as in the reference method) and fosfomycin (using tablets [Rosco]; for susceptibility, inhibition zone diameter was ≥30 mm; for resistance, no zone of inhibition).

Interpretation of the results. Results obtained by the commercial identification systems API ID 32 Staph and Staph-Zym and by the UZA method were compared with those obtained by the reference identification method. Identical results were considered correct identifications. When discrepancies occurred, the isolates were retested in the four systems to exclude technical errors. When the identification of a strain in a given system was repeatedly different from the reference method, a misidentification was recorded for that system.

RESULTS

All reference strains were correctly identified by the four methods. Of 444 isolates of CoNS, 440 (99.1%) were identified by the reference method; 4 isolates could not be identified (0.9%). Two were isolates from blood cultures that could be *Micrococcus* spp., but they were bacitracin resistant. Another blood culture isolate was tentatively identified as *S. capitis* but was mannitol and mannose negative; a diabetic wound isolate could be considered as *S. caprae*, *S. simulans*, or *S. chromogenes* but had a variety of aberrant characters for each option. Neither of the three other methods identified any of these four isolates. They were therefore excluded from the analysis, which thus concerns 440 isolates.

As shown in Table 2, API ID 32 Staph, Staph-Zym, and the UZA method provided a correct species identification for 419 (95.2%), 429 (97.5%) and 430 (97.7%) of the 440 isolates, respectively.

TABLE 2. Comparison of species identification of CoNS by four methods

Species	No. of isolates identified by reference method (%)	No. of isolates UI or MIS[a] (misidentification) by:					
		API 32 Staph		Staph-Zym		UZA method	
		UI	MIS	UI	MIS	UI	MIS
S. epidermidis	304 (69.1)	2	0	5	1 (*S. hominis*)	0	0
S. haemolyticus	53 (12.0)	0	1 (*S. hominis*)	0	0	0	0
S. hominis	39 (8.9)	6	7 (*S. epidermidis*)	1	2 (*S. epidermidis*)	2	1 (*S. haemolyticus*)
S. warneri	13 (2.9)	0	0	1	1 (*S. kloosii*)	2	0
S. capitis	9 (2.0)	0	0	0	0	0	0
S. capitis subsp. *ureolyticus*	5 (1.1)	5	0	0	0	0	0
S. lugdunensis	6 (1.4)	0	0	0	0	0	0
S. schleiferi	5 (1.1)	0	0	0	0	0	0
S. saprophyticus	1 (0.2)	0	0	0	0	0	0
S. cohnii	2 (0.5)	0	0	0	0	2	0
S. xylosus	1 (0.2)	0	0	0	0	1	0
S. sciuri	1 (0.2)	0	0	0	0	1	0
S. lentus	1 (0.2)	0	0	0	0	1	0
Total no. (% [no. identified])[b]	440	13 (3)	8 (1.8 [427])	7 (1.6)	4 (0.9 [433])	9 (2)	1 (0.2 [431])

[a] UI, unidentified; MIS, misidentified.
[b] API 32 Staph, Staph-Zym, and the UZA method correctly identified 419 (95.2%), 429 (97.5%), and 430 (97.7%) of the 440 strains, respectively.

The results of the identification systems were very satisfactory for the species *S. haemolyticus*, *S. epidermidis*, *S. capitis* (except for *S. capitis* subsp. *ureolyticus*), *S. lugdunensis*, *S. schleiferi*, and *S. saprophyticus*, misidentifications occurring only for the first two of these species: API ID 32 Staph misidentified one *S. haemolyticus* isolate as *S. hominis* while two *S. epidermidis* isolates remained unidentified, and the Staph-Zym method did not identify five *S. epidermidis* isolates and misidentified one isolate as *S. hominis.*

Since a negative alkaline phosphatase test is a known aberrant character for some *S. epidermidis* strains, such isolates produced an identification pattern identical to that of *S. capitis* subsp. *ureolyticus* or *S. hominis* in the first step of the UZA method. For this reason, anaerobic growth and fosfomycin susceptibility were tested for all isolates producing a "−+−" pattern in the first identification step, since fosfomycin susceptibility differentiates *S. capitis* subsp. *ureolyticus* from alkaline phosphatase-negative *S. epidermidis* and trehalose-negative *S. hominis*, both of which are susceptible to fosfomycin. In our study, 2% of the *S. epidermidis* strains were alkaline phosphatase negative.

Whereas the API ID 32 Staph system identified all 13 *S. warneri* isolates, the UZA method failed to identify 2 of them while the Staph-Zym method misidentified 1 isolate as *Staphylococcus kloosii* and left 1 isolate unidentified.

S. hominis was identified with the least accuracy. API ID 32 Staph was particularly deficient for the identification of this species, with only 26 of 39 correct identifications. Of the 13 *S. hominis* isolates not recognized by API ID 32 Staph, 7 were misidentified as *S. epidermidis* and another 6 were identified at the genus level only. The Staph-Zym system did not identify 1 of the 39 *S. hominis* isolates and misidentified 2 of these as *S. epidermidis*. The UZA method also failed to identify two isolates, while it misidentified one isolate as *S. haemolyticus*. Both the Staph-Zym and the UZA methods were definitely superior for the identification of the species *S. hominis*, each identifying 36 of 39 isolates correctly.

All of 14 *S. capitis* isolates were correctly identified by both these systems. The five isolates unidentified in the API ID 32 Staph system all belonged to the subspecies *ureolyticus*. They were unidentified because the biochemical profile for this subspecies is not yet included in the manufacturer's database.

The UZA method identified 358 isolates within 4 h and 72 isolates at 24 h; it did not identify any of the *Staphylococcus cohnii*, *S. xylosus*, *S. lentus*, and *S. sciuri* isolates. Discriminatory tests for these species were not selected in the system, because these four species are extremely rarely found in humans (0.2 to 0.4% in the present study).

The most common clinical isolates were *S. epidermidis* (69% of all isolates), *S. haemolyticus* (12%), and *S. hominis* (8.8%), a distribution identical to that found by others (4, 12).

DISCUSSION

Several reviews have emphasized the need for species identification of CoNS (4, 5, 15).

The correct identifications obtained by the two commercial systems API ID 32 Staph and Staph-Zym in 95.2 and 97.5% of the isolates, respectively, were higher than previously found for the Staph-Zym system by Refsahl and Andersen (13).

The most prevalent species in clinical isolates, *S. epidermidis* and *S. haemolyticus*, as well as the less prevalent, more recently described species *S. lugdunensis* and *S. schleiferi*, could be identified accurately by all the identification systems tested. Both *S. lugdunensis* and *S. schleiferi* produce fibrinogen affinity factor. Some laboratories use rapid slide tests based on the detection of clumping factor or fibrinogen affinity factor, protein A, or both for the identification of *S. aureus*. Consequently the slide agglutination test may result in the misidentification of *S. schleiferi* or *S. lugdunensis* as *S. aureus*, a reason why the coagulase test is to be preferred.

S. hominis was identified with the least accuracy, especially by the API ID 32 Staph system. This species has also been identified with a comparably low accuracy in other reports in which the Gram-Positive Identification test card (bioMérieux, Vitek) was used (1). API ID 32 Staph was also deficient in identifying *S. capitis* subsp. *ureolyticus*. Biochemical profiles for this subspecies should be included in the API database.

Both API ID 32 Staph and Staph-Zym are user-friendly. For the latter, supplementary tests are frequently required, thus delaying the results. The susceptibility tests could be added at the start of testing but at increased cost. The interpretation of the API ID 32 Staph requires a computer and accompanying program. Although conventional and commercial identification methods including automated systems (12, 17) can provide accurate identifications, it would be uneconomical to use them for all isolates of CoNS. Thus, there remains a need for rapid and inexpensive systems.

The UZA method is based on nongrowth tests detecting enzymatic activity of heavy suspensions of fresh cultures in solutions of substrates, analogous to those previously described for neisseriae (20), members of the family *Enterobacteriaceae* (10, 11), and anaerobic bacteria (11). The advantage of the proposed system is that up to 81.3% of the CoNS isolates could be identified within 4 h on the basis of three tests and another 16.5% could be identified by the addition of one or two tests, to be read after 24 h. Only 2% of isolates remained to be identified by the reference method. Sealed minitubes with the trehalose solution and urea and ornithine decarboxylase broths in 0.2-ml volumes can be stored for at least 1 year, tablets for alkaline phosphatase and susceptibility tests are stored at room temperature, and thioglycolate broth is always freely available. The rapid method is user-friendly, produces the highest percentage of correct identifications compared with the reference method, and is eight times less expensive than the two other methods: the mean costs for one identification are $0.50 (inclusive of salaries) for the UZA method and $4 for each of the commercial systems. In addition, because only a small number of selected characters need to be tested, the UZA method fits well in the daily work schedule of a routine clinical laboratory.

Nosocomial infections caused by CoNS are becoming increasingly important in most countries. Because of their ubiquitous nature and relatively low virulence, CoNS other than *S. epidermidis* and *S. saprophyticus* have long been considered clinically insignificant. However, *S. hominis*, *S. haemolyticus*, and *S. warneri* are increasingly reported as causes of nosocomial infections (5, 12). Isolates from normally sterile sites therefore require identification to the species level. Many CoNS isolates are true contaminants and do not require identification to the species level. However, with an economical, user-friendly methodology, rapid species identification of virtually all clinically significant CoNS becomes possible, leading ultimately to improved, clinically relevant predictions concerning CoNS isolates (4, 5, 15). Examples are *S. capitis* subsp. *ureolyticus*, shown in a recent study to be a cause of abscess formation (18); *S. warneri* as a cause of bacteremia (3); and *S. lugdunensis* and *S. capitis* subsp. *capitis* as causes of endocarditis (8, 16). Repeat CoNS isolates from patients with invasive disease for whom no other organisms are isolated should be identified to allow comparison of isolates. Identification of CoNS also currently leads to some predictions about their methicillin susceptibility: in another study (submitted for pub-

lication) we did not find any methicillin-resistant isolates among *S. warneri*, *S. capitis*, *S. lugdunensis*, and *S. schleiferi*. Furthermore, species identification is a prerequisite before typing procedures for epidemiologic studies are undertaken. The decision for species identification will frequently depend on a clinico-microbiological discussion and will be facilitated by the availability of a rapid and economical procedure.

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