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Micromethod for Biochemical Identification of Coagulase-Negative Staphylococci

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We have endeavored to elaborate a suitable method for easy and rapid identification in clinical microbiology laboratories of the different species of infection-inducing, coagulase-negative staphylococci. Ten type strains described by Kloos and Schleifer and 269 strains isolated from 95 patients were tested; the classical tests were used for determination of *Staphylococcus* species. Strains were identified by using the Kloos-Schleifer reference method and the micromethod simultaneously. After preliminary tests on 77 substrates, 19 were retained, 15 for determination of species and 4 to reveal biotypes. The substrates were placed in wells in a rigid strip of inert plastic. Inoculation of wells was carried out with rich microbial suspensions in a special medium; reading of substrate reactions was done after incubation for 48 h at 35°C. The intrasystem reproducibility was excellent, from 91 to 100% for the 19 substrates. It was in excellent agreement with the reference method, 100% for type strains and 97.9% for hospital-isolated strains. Because it is simple and easy to reproduce, the micromethod will be most useful in clinical and ecological microbiology laboratories.

Because of the now well established pathogenic role of coagulase-negative staphylococci, it seems necessary that the clinical microbiologist be able to identify these species. Recent studies by Kloos and co-workers (9-12, 15, 16) have allowed the classification of 10 species of staphylococci. It is sometimes difficult to apply this classification, and in most cases the exact identification of bacterial species does not immediately follow initial isolation. In spite of a simplified scheme for identification (9), very few laboratories are able to carry out the routine tests recommended.

Buissière and Nardon (1) have designed a micromethod for the biochemical identification of bacteria which can routinely identify *Enterobacteriaceae*. In 1970, Peny and Buissière (14) proposed the same technique for the identification of staphylococci, using 63 different substrates. Although they had described distinctive characters to differentiate micrococci from staphylococci, the method was not generally applied. We therefore decided to investigate the method more thoroughly, using a strip with specific substrates to identify the 10 species recently described by Kloos and co-workers.

MATERIALS AND METHODS

Strains tested. The type strains described by Kloos and co-workers included *Staphylococcus aureus, S. capitis, S. cohnii, S. epidermidis, S. haemolyticus, S. hominis, S. saprophyticus, S. simulans, S. warneri,* and *S. xylosus.* A total of 269 infection-inducing strains were obtained from 95 patients and isolated at the Hôpital général Edouard Herriot and at the Hôpital Neuro-Cardiologique Louis-Pradel (Lyon, France) from blood cultures (46.1%), drain, catheter, or electrode cultures (10.6%), purulent wounds and abscesses (12.1%), cerebrospinal fluid and derivation valve cultures (17.3%), urine cultures (10.7%), and cultures of various fragments (bone, eye, etc.) (3.1%). Most of them had been recently isolated, and the others had been lyophilized for previous studies (6, 7). All the strains were staphylococci, as determined by the criteria described by Schleifer and co-workers (12, 15, 16): resistance to lysozyme, susceptibility to lysostaphin, and production of acid from glycerol in the presence of erythromycin.

Determination of species. The determination of species was carried out in parallel, using the reference method of Kloos and co-workers (5, 10, 15) and the micromethod described below. All the strains were incubated at 35°C for 48 h on P agar (12); the inocula used for the different reactions were obtained from colonies cultivated on this medium.

(i) Reference method. For the reference method, anaerobic growth was in fluid thioglycolate medium (Difco Laboratories, Detroit, Mich.) plus 0.30% agar (Difco); the procedure and reading were as described by Evans and Kloos (5).

Carbohydrate acidification was determined on Purple Agar Base medium (Difco) with 1% (wt/vol) of each of the following 13 substrates: D-(−)-ribose, D-mannitol, D-(+)-galactose, sucrose, D-(+)-xylose, D-(+)-mannose, xylitol, L-(+)-arabinose, maltose, D-(+)-trehalose, D-(+)-melezitose, β-D-(−)-fructose, and α-lactose. The inoculum was obtained from a brain

heart broth culture incubated at 35°C for 18 h and deposited on agar medium with a multiple replicator.

Determinations of nitrate reduction and acetylmethylcarbinol production were made by classical methods (4).

The Pennock and Huddy technique used by Kloos and co-workers for determination of phosphatase activity proved unreliable, so the Barber and Kuper method (4) was used.

The minimal inhibitory concentrations (MICs) for novobiocin were determined on Mueller-Hinton agar medium.

(ii) Micromethod. For preliminary tests, 191 strains were used (including type strains) to select substrates to be used to obtain reliable and discriminating results.

A list of carbohydrate substrates is given in Table 1; they were placed in 50 wells (300 μl each), in a rigid strip of inert plastic. Final concentration in each well was 1% (wt/vol) except for gentiobiose, with low solubility (0.4%, wt/vol). The strain was incubated for 48 h at 35°C on P agar; the inoculum (5×10^8 to 1×10^9 organisms per ml) was prepared in a growth medium made up of: $(NH_4)_2SO_4$, 2 g; NaCl, 5 g; yeast extract, 5 g; phenol red, 70 mg; phosphate buffer M/100, pH 7.8; mineral base (3), 10 ml; distilled water, 1,000 ml;

TABLE 1. *Substrates and reactions tested by micromethod (preliminary tests)*

Carbohydrate acidification	API 20E		APIZYM	
	Substrate	Enzyme	Substrate	Enzyme
Glycerol	*O*-Nitrophenyl-β-D-galactopyranoside	β-Galactosidase	2-Naphthylphosphate, pH 8.5	Alkaline phosphatase
Erythritol	Arginine monochlorhydrate	Arginine deiminase	2-Naphthylphosphate, pH 5.4	Acid phosphatase
D-Arabinose	Lysine monochlorhydrate	Lysine decarboxylase	2-Naphthylbutyrate	Esterase
L-Arabinose	Ornithine monochlorhydrate	Ornithine decarboxylase	2-Naphthylcaprylate	Esterase-lipase
Ribose	Sodium citrate	Citrate utilization	2-Naphthylmyristate	Lipase
D-Xylose	Iron–thiosulfate	H_2S production	L-Leucyl-2-naphthylamide	Aminopeptidase (cytosol)
L-Xylose	Urea	Urease	L-Valyl-2-naphthylamide	Valine aminopeptidase
Adonitol	Tryptophan	Tryptophan deaminase	L-Cystyl-2-naphthylamide	Cystyl-aminopeptidase
Methylxyloside	Tryptophan–peptone	Indole production	*N*-Benzoyl-DL-arginine-2-naphthylamide	Trypsin or related enzymes
Galactose	Sodium pyruvate	Acetoin production	*N*-Benzoyl-DL-phenylalanine-2-naphthylamide	Chymotrypsin or related enzymes
D-Glucose				
D-Fructose			6-Bromo-2-naphthyl-α-D-galactopyranoside	α-Galactosidase
D-Mannose			2-Naphthyl-β-D-galactopyranoside	β-Galactosidase
L-Sorbose			Naphthol AS-BI-β-D-glucuronic acid	β-Glucuronidase
Rhamnose			2-Naphthyl-α-D-glucopyranoside	α-Glucosidase
Dulcitol			6-Bromo-2-naphthyl-β-D-glucopyranoside	β-Glucosidase
Inositol			1-Naphthyl-*N*-acetyl-β-D-glucosaminide	β-Glucosaminidase
Mannitol			6-Bromo-2-naphthyl-α-D-mannopyranoside	α-Mannosidase
Sorbitol			2-Naphthyl-α-L-fucopyranoside	α-L-Fucosidase
Methyl-D-mannoside				
Methyl-D-glucoside				
N-Acetylglucosamine				
Amygdalin				
Arbutin				
Esculin				
Salicin				
D-Cellobiose				
Maltose				
D-Lactose				
D-Melibiose				
Sucrose				
Trehalose				
Inulin				
D-Melezitose				
D-Raffinose				
Amidone				
Glycogen				
Xylitol				
β-Gentiobiose				
D-Turanose				
D-Lyxose				
D-Tagatose				
D-Fucose				
L-Fucose				
D-Arabitol				
L-Arabitol				
Gluconate				
2-Ketogluconate				
5-Ketogluconate				

sterilized at 110°C for 40 min. Acidification was read after 24 and 48 h of incubation at 35°C; color changes were indicated by intensities from 0 to 5, and only reactions of 3 and over were considered positive.

On API 20E strips, 10 substrates used for the identification of enterobacteria were tested (Table 1). The inoculum was prepared by emulsion of a number of colonies taken from P agar in saline solution; reading of biochemical reactions was done according to the manufacturer's instructions.

The APIZYM (API system) plate substrate technique, derived from that described by Buissière and co-workers (1, 14), was improved by Monget (13) and applied to bacterial taxonomy by Gauthier (8). Nineteen enzymatic substrates were used (Table 1). The inoculum (10^9 organisms per ml) was prepared in a saline solution and placed in wells containing a variety of substrates. After incubation for 4 h at 35°C, reading was done by adding into each well 1 drop of A reagent, composed of tris(hydroxymethyl)aminomethane (2.5 g), 37% HCl (1.1 ml), lauryl sulfate (1 g), distilled water (10 ml), pH 7.8, and 1 drop of B reagent (fast blue BB, FO 250, Sigma Chemical Co. [St. Louis, Mo.], 35 mg in 10 ml). A positive reaction was recorded when a blue, purple, or orange color (according to substrate) was obtained, of varying intensity (scale of 0 to 5); only readings of 3 and over were considered positive.

After a systematic study of 77 substrates (against 191 strains), 19 were selected whose reproducibility was satisfactory and whose discriminating capacities allowed identification of staphylococci according to the classification of Kloos and co-workers (10–15).

The substrates used in the definitive *Staphylococcus* identification strip are shown in Table 2; the colored indicator in the wells was phenol red (0.07% final concentration). All the wells were seeded with an inoculum containing 1×10^9 organisms per ml prepared from a 48-h culture at 35°C on P agar; the inoculum medium was as described above, omitting phenol red. The strip was incubated at 35°C for 48 h, acidification reactions were read directly (after 24 and 48 h), and a reagent was then used to reveal enzymatic reactions. Color reaction intensities of 3 and over were considered positive. Substrates 2 to 16 were used to identify the 10 type species described by Kloos and co-workers; substrates 17 to 20 were used to characterize biotypes.

Sources of substrates, growth media, and reagents. Lysozyme (muramidase, EC 3.2.1.17) was from Sigma, batch 75 C-8483, titer of 46,360 U/mg of protein; lysostaphin was from Schwarz/Mann (Orangeburg, N.Y.), batch AZ 1975, specific activity of 237 U/mg; erythromycin was from UCLAF (Paris, France), titer of 920 μg/mg; novobiocin was from The Upjohn Co. (Kalamazoo, Mich.), batch 0211, titer of 820 μg/mg; fluid thioglycolate medium was from Difco; rabbit plasma, oxalated and lyophilized, was from Biomerieux (Paris, France); Purple Agar Base was from Difco; Clark-Lubs medium was from Biomerieux; phenolphthalein phosphate, 1%, was from Oxoid Ltd. (London, England); agar was from Difco; Mueller-Hinton agar medium was from Biomerieux. D-Mannitol, α-lactose, and D-(+)-xylose were from Prolabo, (Paris, France); D-(−)-ribose, glycerol, D-(+)-mannose, xylitol, and β-D-(−)-fructose were from E. Merck AG (Darmstadt, West Germany); D-(+)-galactose, saccharose, L-(+)-arabinose, and maltose were from the Institut Pasteur (Paris, France); D-(+)-melezitose was from Calbiochem (La Jolla, Calif.).

TABLE 2. *Substrates retained for biochemical identification of staphylococci by micromethod*

Substrate	Reaction
1. Control	
2. D-Glucose	Acidification
3. β-D-(−)-Fructose	Acidification
4. D-(+)-Mannose	Acidification
5. Maltose	Acidification
6. α-Lactose	Acidification
7. D-(+)-Trehalose	Acidification
8. D-Mannitol	Acidification
9. Xylitol	Acidification
10. D-Melibiose	Acidification
11. Nitrate	Nitrate reductase
12. 2-Naphtylphosphate	Alkaline phosphatase
13. Sodium pyruvate	Acetoin production
14. D-Raffinose	Acidification
15. D-(+)-Xylose	Acidification
16. Sucrose	Acidification
17. α-Methylglucoside	Acidification
18. *N*-Acetylglucosamine	Acidification
19. Arginine monochlorhydrate	Arginine deiminase
20. Urea	Urease

RESULTS

All the characters recommended by Kloos and co-workers were tested, but a number of them were not retained for the identification of our hospital-isolated strains. Size of colonies showed orientation only. Growth in thioglycolate medium was unreliable, since 7.3% of the strains failed to grow on this medium, 26.1% showed a gradient of growth, and 66.4% showed uniform growth. Novobiocin MICs were determined for 154 wild strains; many *S. saprophyticus* strains (14/19) had MICs of 1 μg/ml or less, and, inversely, 13 *S. epidermidis* strains were resistant (MICs of ≥2 μg/ml, 3 of which had MICs of ≥16 μg/ml) (Table 3).

Intrasystem reproducibility. The 10 type strains of Kloos and co-workers were each tested 10 times by the micromethod, to check reproducibility of results obtained for each substrate, using either the same inoculum (on the same day) or different inocula (on different days). Each substrate was therefore tested 100 times; results are shown in Table 4. There was not any difference in reproducibility among tests done the same day and those done on different days.

On the whole, reproducibility seemed to be excellent; it only reached 91% for mannitol, since certain species, such as *S. capitis*, produced little acid from this sugar.

TABLE 3. *MICs of novobiocin for 154 hospital-isolated, coagulase-negative strains of staphylococci*

Species	No. of strains with MIC[a] of:										
	≤0.06	0.125	0.25	0.5	1	2	4	8	16	32	64
S. epidermidis	21	34	31	12		1		9	2	1	
S. saprophyticus		3	2	3	6					1	4
S. hominis	1	1	1	2	1					1	
S. capitis	1	1	1	2							
S. warneri			3								1
S. haemolyticus		1									
S. simulans			2								
S. cohnii											1
Unclassifiable		1	2		1						
Percentage	14.9	26.6	27.3	12.3	5.2	0.6		5.8	1.3	1.9	3.9

[a] Novobiocin MIC (in micrograms per milliliter) inhibiting growth on Mueller-Hinton agar medium.

TABLE 4. *Intrasystem reproducibility for type strains*

Substrate	Reproducibility (%)
1. Control	
2. D-Glucose	100
3. β-D-(−)-Fructose	100
4. D-(+)-Mannose	99
5. Maltose	99
6. α-Lactose	99
7. D-(+)-Trehalose	99
8. D-Mannitol	91
9. Xylitol	100
10. D-Melibiose	100
11. Nitrate	100
12. 2-Naphtylphosphate	99
13. Sodium pyruvate	97
14. D-Raffinose	100
15. D-(+)-Xylose	100
16. Sucrose	100
17. α-Methylglucoside	100
18. *N*-Acetylglucosamine	97
19. Arginine monochlorhydrate	100
20. Urea	100

TABLE 5. *Diverging reactions[a] obtained with each method (190 wild strains)*

Substrate	No. of strains	
	+KS/−MM	−KS/+MM
1. Control		
2. D-Glucose	0	0
3. β-D-(−)-Fructose	0	3
4. D-(+)-Mannose	2	76
5. Maltose	1	1
6. α-Lactose	9	3
7. D-(+)-Trehalose	1	3
8. Mannitol	13	2
9. Xylitol	2	0
10. D-Melibiose	0	0
11. Nitrate	0	0
12. 2-Naphtylphosphate	0	0
13. Sodium pyruvate	0	0
14. D-Raffinose	0	0
15. D-(+)-Xylose	1	1
16. Sucrose	5	0

[a] +KS/−MM, Positive reaction by Kloos and Schleifer method and negative reaction by micromethod; −KS/+MM, negative reaction by Kloos and Schleifer method and positive reaction by micromethod.

Correspondence between reference method and micromethod. Analysis of reactions obtained with each substrate was carried out on 190 wild strains; the 15 substrates used for species determination were compared; results are given in Table 5.

For each method, 2,850 reactions (190 strains × 15 substrates) were read, and for the evaluation of correspondence between the two methods, there were 5,700 reactions; only 123 (2.16%) diverging reactions were noted.

Mannose, the most diverging substrate, was more often positive by the micromethod (76 of 78 differences [Table 5]). However, valid results were obtained with the micromethod, since the acidification intensity was always ≥4. On the other hand, mannose was totally negative for 35 of 190 strains. This would seem to rule out the hypothesis of a nonspecific acidification. Results obtained with mannose were not in contradiction with the classification of Kloos and co-workers, since 12 of 142 *S. epidermidis* strains were mannose negative against 23 of the non-*S. epidermidis* strains. Consequently, the substrate was retained.

Strain determination analysis by each method revealed a 100% agreement for type strains and 97.9% for the 190 wild strains; identical identifications were not obtained in 4 cases owing to differing reactions obtained with trehalose, xylose, mannitol, or sucrose.

Identification of hospital-isolated strains. The Kloos and Schleifer classification (10, 15) indicated that infections were induced more frequently by *S. epidermidis* (71.4%), followed by *S. saprophyticus* (7.4%) (Table 6). The other species were seldom found. Of all strains,

TABLE 6. *Identification of hospital-isolated strains by Schleifer and Kloos classification (10, 15)*

Species	Strains identified	
	No.	%
S. epidermidis	192	71.4
S. saprophyticus	20	7.4
S. cohnii	1	0.4
S. hominis	2	0.7
S. haemolyticus	2	0.7
S. capitis	5	1.9
S. simulans	1	0.4
Unclassifiable	46	17.1

17% could not be classified. The percentage was reduced to 1.9% when the Kloos and Schleifer simplified identification scheme (9) was applied; a large number of *S. epidermidis* strains were identified, as well as other species (Table 7); however, identification was not so precise.

DISCUSSION

It is essential that routine identification of coagulase-negative strains of staphylococci isolated in human pathology be carried out easily and rapidly. The need was felt for an improved and reliable biochemical micromethod. We have always made use also of the morphological and physiological characters described (5, 12), but, as they have sometimes proved to be unreliable, they have been used mainly for orientation.

We are of the opinion that the tests using susceptibility to antibiotics (novobiocin) are not adapted to medical microbiology conditions owing to the wide distribution of various antibiotics, to cross-resistance, and to the highly localized ecological character of resistance phenomena.

The biochemical identification micromethod is reproducible, the range for each substrate being 91 to 100%; the lowest reproducibility is for mannitol, but it corresponds to species weakly acidifying this sugar. Our results are in agreement with those published by Butler et al. (2), who, using the API 20E system for the identification of *Enterobacteriaceae,* obtained 86 to 99% reproducibility with regard to substrates and 97.3% for species determination. The Kloos and Schleifer method used as a reference method is in excellent agreement with the micromethod: 100% for type strains, 97.9% for hospital-isolated strains.

The method is simple: the coagulase-negative staphylococci of interest in pathology are grown on P agar for 48 h and then inoculated on a "staphylococci" strip; identification of species is obtained after 48 h, often after 24 h.

The predominance of *S. epidermidis* strains will incite the search for specific type markers.

TABLE 7. *Identification of 46 unclassifiable strains, using the Kloos and Schleifer simplified scheme (9) and total frequency of species*

Species	Unclassifiable strains	Total frequency	
		No.	%
S. epidermidis	20	212	78.8
S. saprophyticus	0	20	7.4
S. hominis	9	11	4
S. capitis	2	7	2.6
S. warneri	7	7	2.6
S. haemolyticus	1	3	1.1
S. simulans	1	2	0.7
S. cohnii		1	0.4
S. xylosus	1	1	0.4
Not classified	5	5	1.9

At present, only antibiotyping can be used routinely; we are pursuing our studies on the discriminating capacity obtained when using four substrates (17 through 20) contained on the present plate.

The micromethod also allows identification on a large scale of strains of various origins; it is well adapted to ecological research. Last, it is easily amended to suit possible taxonomic changes.

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