JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1992, p. 2346–2352
0095-1137/92/092346-07$02.00/0

Vol. 30, No. 9

Usefulness of the ID32 Staph System and a Method Based on rRNA Gene Restriction Site Polymorphism Analysis for Species and Subspecies Identification of Staphylococcal Clinical Isolates

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Received 11 March 1992/Accepted 9 June 1992

The usefulness of the ID32 Staph System and a method based on rRNA gene restriction site polymorphism was evaluated by the study of 42 staphylococcal clinical isolates phenotypically difficult to identify. The ID32 Staph micromethod and the genomic method are adapted for recognition of 27 and 31 staphylococcal taxa, respectively. The genomic method is based on a Dice analysis of the hybridization patterns obtained by cutting the cellular DNA either with *Eco*RI or with *Hin*dIII and by probing with pBA2, containing the *Bacillus subtilis* gene encoding 16S rRNA, labeled either with [α-^{32}P]dCTP or with acetylaminofluorene. This study showed that the nonradioactive labeling provided a better resolution of the hybridizing bands than radioactive labeling. Of the 42 isolates selected, only 22 could be assigned to a staphylococcal species by the ID32 Staph System, whereas 35 could be identified by the genomic method. This latter method also enabled the screening of three unclassified isolates having hybridization patterns more closely related to each other than to any of the 31 staphylococcal taxa investigated. These three isolates could belong to a staphylococcal taxon not yet described.

Within the genus *Staphylococcus*, 29 species have been delineated by DNA-DNA hybridization. A simplified phenotypic scheme (26), which led to the commercialization of miniaturized methods (1, 2, 7, 14, 16, 28, 41), has been successively modified as new species have been described (12, 15, 20, 22, 27, 36a, 39). Since some species can be distinguished by only a limited number of stable biochemical tests, the precise assignment of a staphylococcal strain to a species may be difficult or even impossible if the strain has atypical characteristics. Recognizing the four free-coagulase-positive staphylococcal species, *S. aureus*, *S. hyicus*, *S. intermedius* and *S. delphini*, which are primary human and animal pathogens, is particularly important. Indeed, isolates belonging to these species, which may be present on the body surface of asymptomatic carriers, cause severe infections when they penetrate areas of trauma in the skin and the mucous membranes. In contrast, the free-coagulase-negative staphylococcal species (CoNS), with the exception of the urinary pathogen, *S. saprophyticus*, are secondary pathogens that cause infections mainly after implantation of foreign bodies or in immunocompromised hosts. Since the strains belonging to some CoNS are constituents of the normal skin flora, they often contaminate the clinical samples. For this reason, it is useful to be able to distinguish them from the infecting CoNS. Because of the polyclonal composition of species constituting the normal skin flora, repeated isolation of the same organism from a particular site in a patient would suggest that such cells are the progeny of a single clone. Therefore, the strains isolated from the same patient have to be fingerprinted and compared. Precise identification is the first step in fingerprinting and is important when the compared strains belong to one of the CoNS species which are not widespread in the skin flora, i.e., most CoNS except *S. epidermidis*. Indeed, the repeated isolation of such strains from the same patient suggests that they may be the cause of the clinical symptoms.

Among the numerous approaches proposed for species identification within the genus *Staphylococcus* (1–4, 6, 7, 9–12, 14, 16, 17, 21, 24, 27–30, 33–36, 38, 40–43), the two by which a great number of staphylococcal taxa thus far described can be recognized are the ID32 Staph System (6, 29), based on a miniaturized phenotypic characterization, and rRNA gene restriction site polymorphism (10, 11), in which the plasmid pBA2 (23), containing 16S rDNA of *B. subtilis*, is used as the probe.

The aim of this work was to evaluate the usefulness of these two methods. Forty-two staphylococcal isolates were tested by both methods. Either these strains could not be classified by the simplified phenotypic schemes (7, 12, 15, 27, 36a) proposed before the development of the ID32 Staph System or the results obtained with these methods did not accord with those obtained by serotyping with 11 CoNS-specific antisera (36).

MATERIALS AND METHODS

Bacterial isolates and plasmids. The 42 epidemiologically unrelated isolates studied came from human (34 isolates), animal (5 isolates), food (1 isolate), and unknown (2 isolates) sources (Table 1). All the isolates were gram-positive cocci forming pairs, tetrads, or irregular clusters and were producing catalase. Staphylococci were differentiated from micrococci and stomatococci by the following tests: production of acid from glycerol under aerobic conditions and sensitivity to lysostaphin, nitrofurantoin, vibriostatic agent 0/129, and bacitracin (5, 25).

Plasmid pBA2 (23) was used as the probe in the hybridization experiments. It contained a 2.3-kb *B. subtilis* DNA fragment, encoding 16S rRNA, inserted in the *Hin*dIII site of pBR322.

Phenotypic identification at the species and subspecies levels. All the isolates were sent to the API BioMérieux

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TABLE 1. Sources and species assignments of 42 staphylococcal isolates

Isolate	Source	Species assignment according to:					
		ID32 Staph System			rRNA gene polymorphism		
		Code[a]	Species	Atypical characteristic(s)[b]	*Eco*RI HP[c]	*Hin*dIII HP[c]	Species
BM9357	Vomit	3623202⅘	*S. warneri*	None	E850*	H850*	Unidentified
BM9359	Mixed vegetables	36033024	*S. warneri*	Mal^-	E851*	H851*	Unidentified
BM9363	Urine	⅑62130⅖5	Unidentified		E851*	H852*	Unidentified
BM9364	Prosthetic valve	06613461	Unidentified		E842*	H840*	*S. haemolyticus*
BM9366	Blood	⅑66½242⅑	Unidentified		E842*	H840*	*S. haemolyticus*
BM9368	Bronchial mucus	26603461	*S. haemolyticus*	Tre^-	E843*	H843*	*S. haemolyticus*
BM9369	Pus	26633431 26633471	*S. haemolyticus* *S. haemolyticus*	Nv^r Nag^+ Nv^r	E29	H841*	*S. haemolyticus*
BM9371	Goat's milk	⅓451⅓260	Unidentified		E880*	H880*	*S. chromogenes*
BM9372	Unknown	06233024	*S. warneri*	Ure^-	E22	H24	*S. warneri*
BM9376	Skin	12603040	*S. hominis*	Fru^- Sac^- Tur^-	E830*	H830*	*S. hominis*
BM9377	Blood	16702021	*S. epidermidis*	Nit^-	E21	H48*	*S. epidermidis*
BM9380	Unknown	36722⅑20	Unidentified		E131	H131	*S. capitis*
BM9382	Blood	36232025	*S. warneri*	None	E22	H24	*S. warneri*
BM9384	Bronchial mucus	26303260	Unidentified		E9	H7	*S. aureus*
BM9385	Blood	16333661	Unidentified		E9	H64*	*S. aureus*
BM9388	Urine	06333⅑20	*S. aureus*	Lac^- Nag^-	E70*	H1	*S. aureus*
BM9389	Urine	0663⅓⅐⅑21	Unidentified		E830*	H830*	*S. hominis*
BM9396	Pus from hen	07771270 07775670	*S. lentus* *S. lentus*	None $\beta\text{-}Gal^+$ Pyr^+	E820	H820	*S. lentus*
BM9399	Open cut	⅔6613461	Unidentified		E844*	H844*	*S. haemolyticus*
BM9400	Open cut	16602220	*S. epidermidis*	None	E21	H51	*S. epidermidis*
BM9401	Blood	46313461	*S. lugdunensis*	Clu^- Tur^+	E27	H31	*S. lugdunensis*
BM9402	Synovial fluid	46713460	*S. lugdunensis*	Clu^-	E27	H31	*S. lugdunensis*
BM9405	Pus from rat	26713261	*S. aureus*	Man^-	E70*	H7	*S. aureus*
BM9407	Blood	36633425	Unidentified		E902*	H902*	Unidentified
BM9408	Prosthetic valve	36212024	*S. warneri*	None	E22	H24	*S. warneri*
BM9409	Ear	56603220	*S. epidermidis*	Alp^-	E47	H50	*S. epidermidis*
BM9410	Nose	26303⅔60	Unidentified		E4	H66*	*S. aureus*
BM9412	Urine	0662646⅑	Unidentified		E842*	H841*	*S. haemolyticus*
BM9413	Toe	062⅑32½	Unidentified		E901*	H901*	Unidentified
BM9414	Bone	06212024	*S. warneri*	Ure^-	E22	H25	*S. warneri*
BM9415	Blood	16313421	*Stomatococcus mucilaginosus*	Ure^+ Arg^-	E24	H27	*S. hominis*

Continued on following page

TABLE 1—*Continued*

Isolate	Source	Species assignment according to:					
		ID32 Staph System			rRNA gene polymorphism		
		Code[a]	Species	Atypical characteristic(s)[b]	*Eco*RI HP[c]	*Hin*dIII HP[c]	Species
BM9417	Blood	26631461	*S. haemolyticus*	Vp^-	E29	H32	*S. haemolyticus*
BM9418	Bone	16203024	*S. warneri*	Tre^-	E22	H24	*S. warneri*
BM9419	Blood	9/16%03220	Unidentified		E47	H21	*S. epidermidis*
BM9421	Blood	06%03%%21	Unidentified		E830*	H830*	*S. hominis*
BM9424	Urine	12%06%%0	Unidentified		E903*	H903*	Unidentified
BM9425	Blood	0%001600	Unidentified		E904*	H904*	Unidentified
BM9428	Blood	26613460	*S. haemolyticus*	None	E840*	H842*	*S. haemolyticus*
BM9429	Blood	1661%%%0%	Unidentified		E24	H830*	*S. hominis*
BM9431	Pubis	42613060	*S. lugdunensis*	Clu^- Fru^- Mne^- Pyr^-	E27	H31	*S. lugdunensis*
CH21	Goat's milk	2671%%2%0	Unidentified		E801	H800	*S. caprae*
CH41	Goat's milk	9/6737600	Unidentified		E800	H800	*S. caprae*

[a] The two values reported for some isolates resulted either from uncertainty or from the lack of reproducibility in the reading of the colorimetric tests.
[b] −, negative reaction, +, positive reaction; Mal, maltose; Tre, trehalose; Nv^r, novobiocin resistance; Nag, *N*-acetylglucosaminidase; Ure, urease; Fru, fructose; Sac, saccharose; Tur, turanose; Nit, nitrate reduction; β-Gal, β-galactosidase; Lac, lactose; Pyr, pyrrolydonyl arylamidase; Clu, clumping factor; Mne, mannose; Man, mannitol; Alp, alkaline phosphatase; Arg, arginine arylamidase.
[c] The HP marked with an asterisk are those which were not detected previously (10, 11).

research laboratory (La Balme les Grottes, Montalieu-Vercieu, France). The phenotypes were characterized with the ID32 Staph strip, which consists of a set of wells containing dried biochemical media for 26 colorimetric tests (6). Tests results were read automatically. The eight-digit numerical code obtained was checked against those in the computer data base by APILAB ID32 software. The following additional tests were used: detection of clumping factor (8), free-coagulase production (8), and thermonuclease activity before and after sero-inhibition (19) with a polyclonal serum directed against *S. aureus* thermonuclease (BioMérieux, Marcy-l'Etoile, France).

Genomic identification at the species and subspecies levels. Cellular DNA was extracted, cleaved with *Hin*dIII or *Eco*RI (Amersham), and electrophoresed as previously described (10, 11). Bidirectional DNA transfer onto nitrocellulose membranes (Schleicher & Schuell BA85) and hybridization under stringent conditions with pBA2 radiolabeled with [α-^{32}P]dCTP (110 TBq/mmol) were carried out as described elsewhere (32).

Plasmid pBA2 was also labeled with acetylaminofluorene (AAF) (37). Hybridization was carried out according to the technique described by Grimont et al. (18), except that the hybridization temperature was raised from 57 to 68°C. Immunodetection of the restriction fragments hybridizing with the nonradioactive probe was performed in a two-step procedure (31) with a monoclonal anti-AAF antibody (Eurogentec, Liège, Belgium) and goat anti-mouse immunoglobulin G antibodies labeled with peroxidase (Biosys, Compiègne, France). Since the enzymatic staining faded after drying, it was necessary to rewet the membranes before taking a photograph.

The sizes of the bands constituting the hybridization patterns (HP) were introduced into our data base (10) to compare each of these HP with each of the 81 *Eco*RI HP and 74 *Hin*dIII HP previously detected for 271 validly classified strains belonging to 31 staphylococcal taxa (10, 11). The similarity was evaluated according to the Dice coefficient (13). The percentage of similarity (%S) was calculated as follows: %S = [(number of matching bands × 2)/total number of bands] × 100.

RESULTS

As shown in Table 1, 22 of the 42 isolates tested were assigned to a staphylococcal species by the use of the ID32 Staph System. Seventeen of these 22 isolates exhibited uncommon characteristics, i.e., characteristics detected in fewer than 10% of independent strains belonging to a given taxon.

The HP of the 42 isolates were obtained by cutting the cellular DNA either with *Eco*RI or with *Hin*dIII and by probing with pBA2 either radiolabeled or chemically modified by AAF labeling. The bands hybridizing with the radioactive probe after an exposure of 24 h were also detected with the nonradioactive probe if at least 3 μg of cellular DNA had been deposited on the gel (Fig. 1A and B, respectively). As shown in Fig. 1, the hybridizing bands were better resolved with the nonradioactive probe.

The *Eco*RI HP and the *Hin*dIII HP of the 42 isolates are reported in Table 1. Eighteen of these isolates had *Eco*RI HP and *Hin*dIII HP indistinguishable from those previously detected among validly classified strains, including type strains (10, 11), and could, therefore, be immediately assigned to a species. New HP, indicated with an asterisk in Table 1, were detected for the remaining 24 isolates. The %S

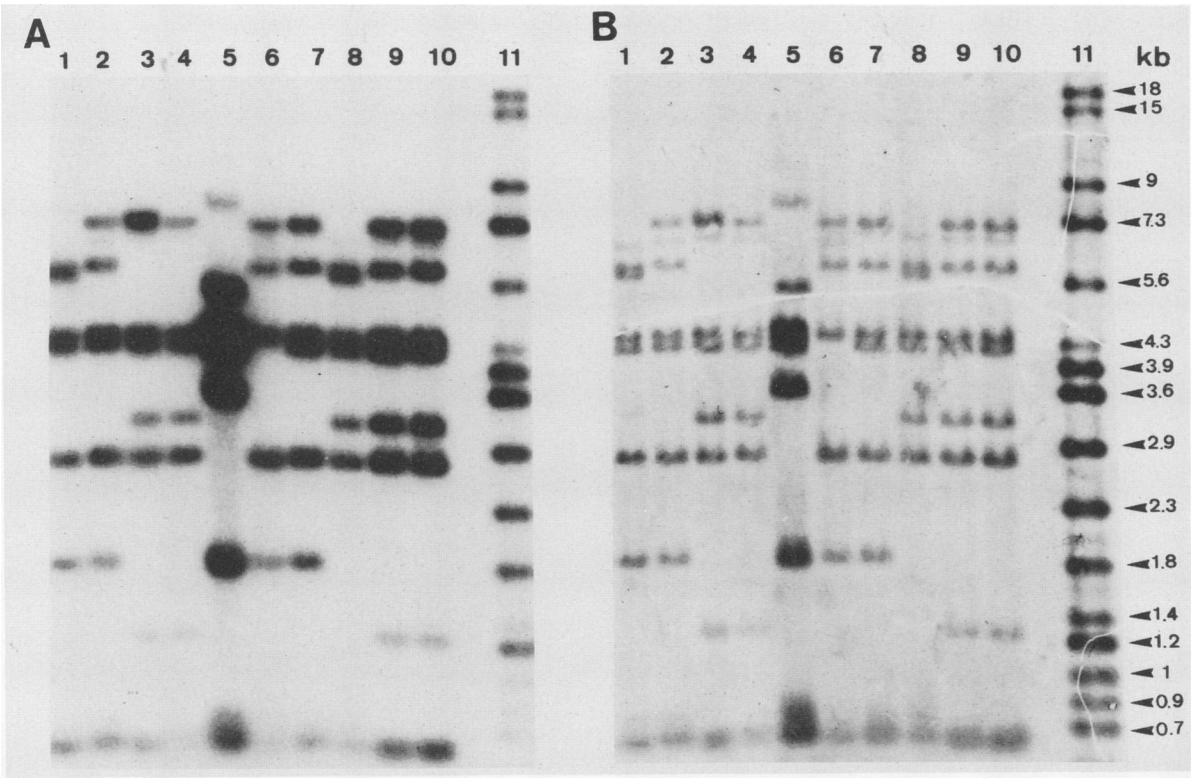

FIG. 1. *Hin*dIII restriction patterns of nine *S. haemolyticus* strains and one unclassified strain probed with pBA2 labeled with [α-^{32}P]dCTP (A) or AAF (B). Lanes: 1, BM9428; 2, BM9417; 3, BM9412; 4, BM9369; 5, BM9407; 6, BM9399; 7, ATCC 29970 (type strain); 8, BM9368; 9, BM9366; 10, BM9364; 11, Raoul I DNA ladder (Appligene, Strasbourg, France).

with each of the 81 *Eco*RI HP or 74 *Hin*dIII HP previously described (10, 11) was calculated. An isolate exhibiting a new HP could be assigned to a known taxon if the highest %S values obtained were clustered within a single staphylococcal taxon. The extreme %S values detected within the assigned taxa as well as the highest intertaxon values are reported in Table 2. The ability to identify an isolate to a taxon increases when the difference between the lowest intrataxon %S and the highest intertaxon %S is great. By this type of analysis, 17 of the 24 isolates exhibiting new HP were identified, whereas 7 isolates remained unclassified.

Three of the seven unclassified isolates that could not be identified by the method based on rRNA gene restriction site polymorphism (BM9357, BM9359, and BM9363) had highly similar HP. Indeed, the two *Eco*RI HP obtained with these three isolates (E850 and E851) exhibited 90% similarity. This value is above the highest values observed for the *Eco*RI HP previously found for the 31 staphylococcal taxa investigated (66.6% for E850 and 63.1% for E851). Similar results were observed by analysis of the *Hin*dIII HP of these three isolates. Indeed, the %S values for the three *Hin*dIII HP (H850, H851, and H852), which ranged from 62.5 to 87.5%, are above the highest values detected for the *Hin*dIII HP previously described for the 31 staphylococcal taxa (37.5% for H850, 40% for H851, and 35.2% for H852). Discrepancies were observed between these results and those obtained with the ID32 Staph System, with which BM9357 and BM9359 were identified as *S. warneri*. BM9363, on the other hand, could not be identified by either of the two methods. A discrepancy was also observed for BM9415. This isolate was identified as *Stomatococcus mucilaginosus* by the ID32 Staph System, whereas its *Eco*RI and *Hin*dIII HP (E24 and H27) were indistinguishable from those of the five *S. hominis* isolates, including the type strain, examined previously (11).

DISCUSSION

rRNA gene restriction site polymorphism has been recently proposed for identifying species and subspecies within the genus *Staphylococcus* (3, 10, 11, 34, 38). The probes used, as well as the enzymes proposed for cleaving the cellular DNA, varied among the studies. The probes consist of either 16S and 23S rRNA from *Escherichia coli* (3, 34, 38) or plasmid pBA2 (10, 11) containing a cloned-2.3 kb DNA fragment encoding 16S rRNA from *B. subtilis* (23). For this study, we chose pBA2 as the probe and *Eco*RI and *Hin*dIII as the restriction enzymes. These experimental conditions were used in our previous studies (10, 11), the only studies thus far to include 28 of the 29 species currently described for the genus *Staphylococcus*. Furthermore, we have introduced a computerized system to compare pairs of HP (10). With this method, 35 of the 42 staphylococcal isolates tested in the present study could be identified, whereas only 22 of the same 42 isolates could be assigned to a staphylococcal species by the phenotypic micromethod ID32 Staph. The isolates were phenotypically characterized under optimal experimental conditions (including performance of the tests at least twice by the same technician); nevertheless, the proportion of the isolates that could be identified in our study with the ID32 Staph System is much lower than that reported by other investigators (6). The poor performance of the ID32 Staph System in our study may be

TABLE 2. Relevant %S of new HP detected in this study and used for taxon assignment[a]

HP	Isolate	Intrataxon analysis		Intertaxon analysis	
		%S (extremes)	Taxon assigned	Highest %S	HP (taxon)
H48	BM9377	73.6–90.0	*S. epidermidis*	58.8	H17 (*S. hyicus*)
H64	BM9385	61.5–90.9	*S. aureus*	50	H99 (*S. aureus* subsp. *anaerobius*)
H830	BM9376 BM9421 BM9429	88.8	*S. hominis*	52.6	H31 (*S. lugdunensis*)
H840	BM9364 BM9366	77.7–88.8	*S. haemolyticus*	42.1	H23 (*S. epidermidis*)
H841	BM9369 BM9412	70.5–82.3	*S. haemolyticus*	44.4	H23 (*S. epidermidis*)
H842	BM9428	70.5–88.8	*S. haemolyticus*	52.6	H50 (*S. epidermidis*)
H843	BM9368	70.5–88.8	*S. haemolyticus*	44.4	H56 (*S. epidermidis*)
H844	BM9399	58.8–94.1	*S. haemolyticus*	44.4	H50 (*S. epidermidis*)
H880	BM9371	83.3–90.9	*S. chromogenes*	60	H12 (*S. intermedius* subsp. *carnivora*)
E70	BM9388	70.5–93.3	*S. aureus*	66.6	E99 (*S. aureus* subsp. *anaerobius*)
E830	BM9376 BM9421	93.3	*S. hominis*	66.6	E29 (*S. haemolyticus*) E30 (*S. haemolyticus*)
E840	BM9428	80	*S. haemolyticus*	66.6	E24 (*S. hominis*)
E842	BM9364 BM9366 BM9412	75	*S. haemolyticus*	62.5	E24 (*S. hominis*)
E843	BM9368	62.5–87.5	*S. haemolyticus*	62.5	E24 (*S. hominis*)
E844	BM9399	71.4–85.7	*S. haemolyticus*	57.1	E24 (*S. hominis*)
E880	BM9371	93.3	*S. chromogenes*	47	E28 (*S. lugdunensis*)

[a] Each of the new *Eco*RI HP or *Hin*dIII HP (Table 1) was compared with each of the 81 *Eco*RI HP or 74 *Hin*dIII HP, respectively, previously detected (10, 11) within the 31 staphylococcal taxa investigated. The %S values reported are the extreme values observed within the taxon assigned to the isolates and the highest intertaxon value.

explained by the fact that the 42 isolates analyzed were selected precisely because they could not be classified by simplified phenotypic schemes (7, 12, 15, 27, 36a) or by serotyping with CoNS-specific antisera (36).

A discrepancy in species identification between the two methods used in this study was observed for BM9415. The assignment of this isolate to *Stomatococcus mucilaginosus* by ID32 Staph was erroneous, since this isolate was found to belong to the genus *Staphylococcus* by culture and antimicrobial testing (25). In contrast, the assignment given by rRNA gene restriction site polymorphism was more accurate, because the *Eco*RI HP and *Hin*dIII HP of this isolate were identical to those of the *S. hominis* type strain (11). In addition, the three isolates BM9357, BM9359, and BM9363, phenotypically close to *S. warneri*, were shown to be more closely related to each other (90% for *Eco*RI HP and 62.5 to 87.5% for *Hin*dIII) than to any of the 31 staphylococcal taxa, including *S. warneri* (10, 11). It seems likely, therefore, that these three isolates belong to a new staphylococcal taxon.

As stated previously, the method based on rRNA gene restriction site polymorphism is limited by the lack of precision with which the sizes of the hybridizing bands are evaluated (10). Nonradioactive labeling of the probe with AAF, which was shown in this study to provide a better resolution of DNA bands than that obtained with radioactive labeling, improved the accuracy of measurement. Therefore, this nonradioactive labeling, which also has the advantage of being safer than isotopic labeling, has been adopted by our Reference Center for the routine identification of clinical isolates.

ACKNOWLEDGMENTS

We thank D. Chevrier, who performed labeling of plasmid pBA2 with AAF, and O. Rouelland for secretarial assistance.

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