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Characterization of Coagulase-Negative Staphylococci by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblot Analyses

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Coagulase-negative staphylococci are important nosocomial pathogens. At present, no wholly satisfactory typing scheme exists for these organisms. Therefore, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were assessed as characterization methods. A total of 100 type strains and nontyped isolates representing nine species of coagulase-negative staphylococci were analyzed. Each species had a reproducible, characteristic whole-cell banding pattern when analyzed by either method. These species-specific profiles were obtained for all isolates despite disparate geographical origins and clinical isolation sites. Intraspecies similarities, calculated by using the Dice coefficient, were significantly higher than interspecies similarities. Although some species were more heterogeneous than others, the allocation of isolates to any particular species was reinforced by the high degree of interspecies dissimilarity. Application of SDS-PAGE also distinguished discrete subspecies groups. These groups possessed the characteristic profile of their species but were distinguished by a group of variable polypeptides. Species-specific banding patterns were also obtained by immunoblotting of whole-cell polypeptides. Differences between immunoblot and SDS-PAGE profiles could be attributed to variations of antigenicity of particular polypeptides. However, both SDS-PAGE and immunoblotting provided reproducible and sensitive methods for characterization of coagulase-negative staphylococci. Standardization of these techniques could provide the basis for a primary typing scheme.

Coagulase-negative staphylococci are commonly found on the body surfaces of both humans and animals (18). At least 11 species are indigenous to various sites on human skin (15). The potential of coagulase-negative staphylococci to cause opportunistic infections has been recognized, and they are now regarded as the principal cause of nosocomial bacteremia in the United States (2). *Staphylococcus epidermidis* is the species most commonly isolated in such infections (8). However, characterization of strains by more extensive typing methods have demonstrated that 26% of staphylococci causing nosocomial infections are not *S. epidermidis* (11). *S. saprophyticus* and *S. haemolyticus* cause urinary tract infections (17, 29). A significant number of postoperative infections are caused by *S. warneri* and *S. hominis* (16, 26). Recently, two new species, *S. lugdunensis* and *S. schleiferi*, have been isolated from clinical specimens (14).

Previously, taxonomic schemes for coagulase-negative staphylococci have been based primarily on panels of biochemical tests (4, 19, 20). These methods have also been applied in tandem with other systems: plasmid profile analysis (3), phage typing (12), DNA base composition (21), and antibiotic susceptibility (10). However, these methods do not always discriminate usefully between different species. Recently, cleavage of coagulase-negative staphylococcal DNA by restriction endonucleases to produce a "fingerprint" has been investigated as a typing technique (6). Analyses of the whole-cell polypeptides of coagulase-negative staphylococci have included polyacrylamide gel electrophoresis (PAGE) (9), ^{35}S-sodium dodecyl sulfate (SDS)-PAGE (27), and immunoblotting (7). By using SDS-PAGE, characteristic and reproducible banding patterns were obtained for four staphylococcal species (9). Immunoblotting has been used to type clinical isolates of coagulase-negative staphylococci (7). The requirement for [^{35}S]methionine in ^{35}S-SDS-PAGE is expensive, limiting the utility of this technique for routine studies (27). In the present study, SDS-PAGE and immunoblotting of whole-cell polypeptides were used to characterize a total of nine coagulase-negative species.

MATERIALS AND METHODS

Bacterial strains. A total of 13 type strains and 87 nontyped isolates representing nine species of coagulase-negative staphylococci were obtained from several different sources. *Staphylococcus* type strains obtained from the American Type Culture Collection, Rockville, Md., were *S. warneri* 27836 and 27837 and *S. hominis* 27844 and 27845. *S. cohnii* 20260 and 20261, *S. xylosus* 20266 and 20267, and *S. haemolyticus* 20263 and 20264 were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany. *S. simulans* 11046, *S. saprophyticus* 11092, and *S. haemolyticus* 11042 were obtained from the National Collection of Type Cultures, London, United Kingdom. The nontyped isolates included 13 *S. epidermidis*, 13 *S. capitis*, 10 *S. warneri*, 4 *S. cohnii*, 7 *S. xylosus*, 11 *S. hominis*, 14 *S. simulans*, 5 *S. saprophyticus*, and 10 *S. haemolyticus* isolates.

Nontyped isolates were obtained from our routine diagnostic laboratories; the Public Health Laboratory Service, London, United Kingdom; W. E. Kloos, Raleigh, N.C.; and the British Antarctic Survey, Cambridge, United Kingdom. The Public Health Laboratory Service group comprised isolates from diverse clinical specimens collected from hospitals worldwide (24). Biochemical characterizations were performed by using the Kloos and Schleifer scheme (20); a number of isolates were biotyped by using the Baird-Parker scheme (4). However, many of these isolates had equivocal

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species attributions. Isolates collected from our diagnostic laboratories and from two separate research bases in the Antarctic were characterized by using API Staph galleries (API Laboratory Products, Basingstoke, United Kingdom).

Preparation of samples. All isolates were plated out on nutrient agar plates and incubated at 37°C for 24 h. A sweep of colonies from these plates was inoculated into 150 ml of nutrient broth (final cell density, approximately 2 μg of bacterial dry weight ml^{-1}) and incubated at 37°C overnight in an orbital incubator. The resulting broth culture was centrifuged at 10,000 × *g* for 15 min at 4°C. The cell pellet was suspended in 10 ml of distilled water and washed at 2,500 × *g* for 15 min at 25°C. After resuspension in 1 ml of distilled water, the pellet was microcentrifuged at 9,000 × *g* for 5 min at 25°C. The pellet was finally resuspended in an equivalent volume of sterile distilled water, placed in an ice bath, and sonicated for 5 min with an ultrasonic probe (Rapidis 150; Ultrasonics Ltd., Shipley, United Kingdom) emitting 45 W at maximum power. Whole-cell sonic extracts were stored at −70°C. Protein concentrations were estimated by the method of Lowry et al. (23).

SDS-PAGE. Whole-cell sonic extracts were analyzed by using a modified version of the SDS-PAGE technique of Laemmli (22). Samples were prepared for SDS-PAGE by mixing in proportions of 2 parts of sample to 1 part of 0.5 M Tris hydrochloride, pH 6.8, containing 6% (wt/vol) SDS, 30% (wt/vol) glycerol, 15% (wt/vol) 2-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue and boiling them for 5 min. Solubilized samples, containing an average of 120 μg of protein for Coomassie gels or 50 μg of protein for immunoblots, were applied to wells in a 3.6% acrylamide stacking gel over a 10% acrylamide separating gel, 160 mm by 168 mm by 1.5 mm. Electrophoresis was performed by using a discontinuous buffer system in a gel apparatus (model V16-2; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The gel was run at a constant current of 30 mA until the bromophenol blue marker had reached the bottom, an interval of 4 to 5 h. Gels were stained with 0.25% (wt/vol) Coomassie brilliant blue R (Sigma Chemical Co. Ltd., Poole, United Kingdom) in methanol–acetic acid–water (45:10:45 by volume) and destained in the same solvent mixture before being swollen to original size in 7% (vol/vol) acetic acid. Polypeptides from whole-cell sonic extracts were also transferred to nitrocellulose membranes.

Immunoblotting. Polypeptides were blotted onto nitrocellulose membranes in 25 mM Tris hydrochloride–192 mM glycine buffer, pH 8.3, containing 20% (vol/vol) methanol (5). Transfer was effected with a current of 100 mA overnight in a Transblot cell (Bio-Rad Laboratories, Richmond, Calif.). Free protein sites were saturated by incubation in blocking buffer containing 10% (vol/vol) newborn calf serum (GIBCO, Paisley, United Kingdom) in phosphate-buffered saline (pH 7.4)–0.2% (vol/vol) Triton X-100 for 30 min. The nitrocellulose membrane was then incubated in human plasma diluted 1 in 50 in blocking buffer for 1.5 h. Several portions of human plasma known to contain antibodies against a large number of staphylococcal polypeptides were pooled to provide test plasma (28). After being washed three times for 15 min each time in phosphate-buffered saline (pH 7.4)–0.2% (vol/vol) Triton X-100, the nitrocellulose membrane was incubated with horseradish peroxidase-linked sheep anti-human immunoglobulin G serum (Scottish Antibody Production Unit, Carluke, United Kingdom) and diluted 1 in 1,000 in blocking buffer for 45 min. After three further washes of 15 min each in phosphate-buffered saline (pH 7.4)–0.2% (vol/vol) Triton X-100, the nitrocellulose membrane was rinsed in phosphate-buffered saline alone and stained with 0.05% (wt/vol) diaminobenzidine tetrahydrochloride (BDH, Poole, United Kingdom) in 0.1 M Tris hydrochloride, pH 7.6, containing 0.01% (vol/vol) hydrogen peroxide.

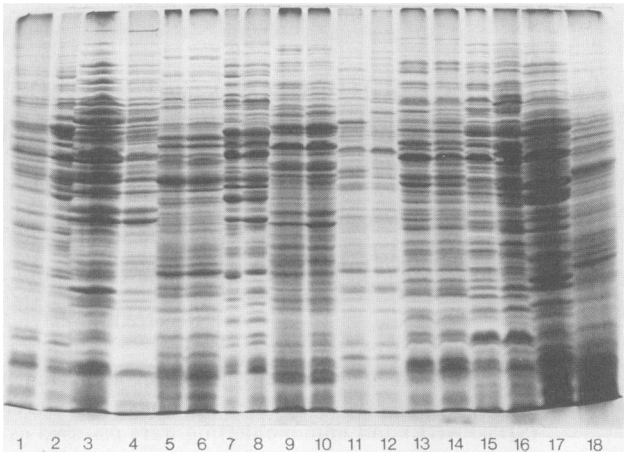

FIG. 1. Whole-cell polypeptide profiles of 18 different isolates of coagulase-negative staphylococci representing nine different species. Lanes: 1 and 2, *S. haemolyticus*; 3 and 4, *S. saprophyticus*; 5 and 6, *S. simulans*; 7 and 8, *S. hominis*; 9 and 10, *S. xylosus*; 11 and 12, *S. cohnii*; 13 and 14, *S. warneri*; 15 and 16, *S. capitis*; and 17 and 18, *S. epidermidis*.

Comparison of Coomassie blue-stained and immunoblot polypeptide profiles. The average similarity between any two staphylococcal isolates represented by either Coomassie blue-stained gels or immunoblots was assessed by using the coefficient of Dice (13), whereby average percentage similarity (% S) = number of matching bands × 2/total number of bands in both isolates. Both Coomassie blue-stained and immunoblot profiles of all isolates were analyzed several times.

RESULTS

SDS-PAGE of whole-cell polypeptides. Whole-cell polypeptides were analyzed by SDS-PAGE. Reproducible gel profiles were obtained for each of the nine species studied, after staining with Coomassie blue. Isolates belonging to each species had a characteristic banding pattern; they contained between 51 and 63 bands, with an average of 58 (Fig. 1).

Comparisons of the banding patterns of different isolates within a species showed various degrees of similarity (Table 1). Calculation of Dice coefficients showed average percentage similarity values ranging from 76.8 to 100%. Within each species, a range of similarity values was observed because of variation of individual isolates. However, most average percentage similarity values exceeded 95%, indicating high degrees of similarity among the different isolates analyzed. Significant numbers of isolates were indistinguishable from each other, i.e., they exhibited 100% similarity. Therefore, in general, high degrees of similarity were apparent between different isolates of any particular species.

A similarity matrix was constructed for each species from SDS-PAGE data. Each matrix included at least one type strain, except those of *S. epidermidis* and *S. capitis*. Three species, *S. saprophyticus*, *S. capitis*, and *S. cohnii*, were very homogeneous, having high average percentage similarity values, i.e., 99.7, 97.1, and 96.1%, respectively (Table 1). Of a total of 108 comparisons for these species, only one had

TABLE 1. Average percentage similarity of Coomassie-stained whole-cell polypeptide profiles for nine species of coagulase-negative staphylococci

Species	No. of isolates	No. of comparisons	Avg	% Similarity range
S. hominis	13	78	95.7	90.2–100
S. saprophyticus	6	15	99.7	99.2–100
S. xylosus	8	36	91.8	76.8–100
S. epidermidis	13	78	95.7	88.7–100
S. capitis	13	78	97.1	85.5–100
S. warneri	12	66	98.4	90.9–100
S. haemolyticus	13	78	92.8	77.4–100
S. simulans	15	105	95.9	85.4–100
S. cohnii	6	15	96.1	93.7–99.1

a percentage similarity value of less than 90%, i.e., a value of 85.5%. All *S. saprophyticus* isolates showed similarity values greater than 95% and all *S. cohnii* isolates studied were more than 90% similar. Both *S. epidermidis* and *S. hominis* had an average percentage similarity value of 95.7%. Only one comparison from a total of 156 for these two species gave a value less than 90%, that value being 88.7%.

Other species were less homogeneous, and although high average percentage similarity values were obtained for *S. simulans* and *S. xylosus*, 95.9 and 91.8%, respectively, more intraspecies diversity was apparent (Table 1). Percentage similarity values for two *S. simulans* isolates ranged from 85.4 to 99.2%. All other *S. simulans* isolates had similarity values exceeding 90%. Comparable results were obtained for two *S. xylosus* isolates in that percentage similarity values ranged from 76.8 to 82.1%. The remainder of *S. xylosus* isolates were at least 95% similar in all other comparisons. Therefore, these four isolates differed significantly from the remainder of *S. simulans* or *S. xylosus* isolates studied.

The average percentage similarity value for comparisons of *S. warneri* isolates was 98.4%. The banding patterns obtained for four isolates were consistently incomplete; only the lower section of the profile, containing the smaller polypeptides, was apparent. However, the bands observed demonstrated high similarity to other *S. warneri* isolates, being at least 90.0% similar. Therefore, these four isolates also differed significantly from the other *S. warneri* isolates studied. Although these isolates of *S. simulans*, *S. xylosus*, and *S. warneri* had decreased intraspecies percentage similarity values, their taxonomic position appeared to be justified because these values were significantly greater than those obtained in interspecies comparisons (see Table 3).

S. haemolyticus was the most heterogeneous isolate studied. Although most isolates were at least 90.0% similar, 16 percentage similarity values of 80.0 to 89.9% were recorded, and one pair of isolates was only 77.4% similar. Despite the wide range of percentage similarity values recorded, the average was 92.8%, indicating significant similarity among *S. haemolyticus* isolates.

Comparison of the banding patterns of isolates from different species demonstrated low degrees of similarity (see Table 3). Calculation of Dice coefficients showed average percentage similarity values ranging from 49.5 to 68.9%. In contrast to intraspecies comparisons, average percentage similarity values did not exceed 59.9%, indicating little similarity among isolates of different species.

It was concluded that analyses of whole-cell polypeptides by SDS-PAGE could be used to distinguish different species of coagulase-negative staphylococci.

Immunoblots. Whole-cell polypeptides were analyzed by immunoblotting with pooled human plasma as a source of staphylococcal antibodies. Reproducible blot profiles were obtained for each of the nine species studied. Each species had a characteristic banding pattern containing between 35 and 44 bands, an average of 39 (Fig. 2). Fewer bands were apparent in the blot profile than in the Coomassie blue-stained gel profile for any isolate analyzed.

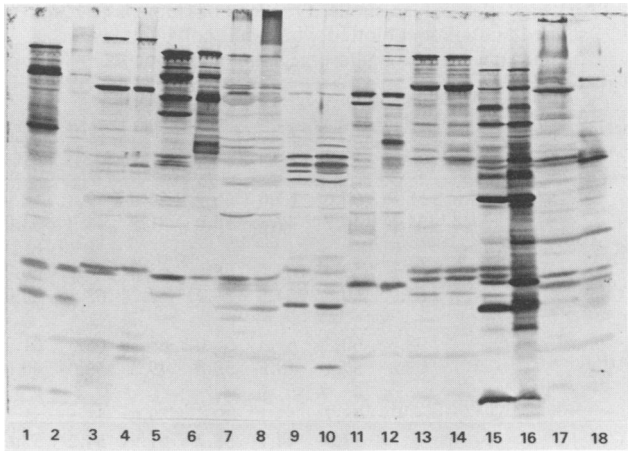

FIG. 2. Immunoblot analysis of whole-cell polypeptides from the species shown in Fig. 1. Lanes: 1 and 2, *S. haemolyticus*; 3 and 4, *S. saprophyticus*; 5 and 6, *S. simulans*; 7 and 8, *S. hominis*; 9 and 10, *S. xylosus*; 11 and 12, *S. cohnii*; 13 and 14, *S. warneri*; 15 and 16, *S. capitis*; and 17 and 18, *S. epidermidis*.

Comparisons of the blot profiles of different isolates within a species demonstrated various degrees of similarity (Table 2). Calculation of Dice coefficients showed average percentage similarity values ranging from 82.7 to 99.2%. In common with Coomassie blue-stained gels, most average percentage similarity values exceeded 95%, indicating high degrees of similarity among the different isolates analyzed. Within each species, a range of similarity values was observed because of differences between individual isolates. Individual variation was due to differences in antigenicity of particular polypeptides. The range of percentage similarity values for immunoblots was greater than that of those recorded for whole-cell polypeptide profiles (Table 1). However, significant numbers of isolates were indistinguishable from each other. Therefore, high degrees of similarity were again apparent between different isolates of any particular species.

Comparison of the blot profiles of isolates from different species demonstrated low degrees of similarity (Table 3).

TABLE 2. Average percentage similarity of immunoblot whole-cell polypeptide profiles for nine species of coagulase-negative staphylococci

Species	No. of isolates	No. of comparisons	Avg	% Similarity range
S. hominis	13	78	94.4	85.5–100
S. saprophyticus	6	15	99.2	98.3–100
S. xylosus	9	36	89.0	61.9–100
S. epidermidis	13	78	98.1	94.2–100
S. capitis	13	78	96.5	91.9–100
S. warneri	12	66	82.7	48.6–100
S. haemolyticus	13	78	91.0	75.7–100
S. simulans	15	105	95.7	86.4–100
S. cohnii	6	15	96.3	89.3–100

TABLE 3. Percentage similarity values of Coomassie blue-stained and immunoblotted whole-cell polypeptides for nine species of coagulase-negative staphylococci[a]

Species	% Similarity of species tested by Coomassie blue staining and immunoblotting								
	E	C	W	CO	X	H	SIM	S	HL
	94.8	68.6	58.7	60.6	63.0	66.7	54.8	59.8	56.0
E	**91.2**	**54.8**	**51.6**	**46.7**	**47.5**	**50.0**	**41.5**	**44.0**	**53.0**
		98.8	60.2	59.4	63.2	68.1	63.3	56.2	66.7
C		**97.9**	**53.7**	**60.5**	**54.5**	**46.2**	**63.7**	**53.3**	**50.0**
			98.3	60.2	63.3	58.0	65.3	65.3	61.2
W			**97.6**	**57.1**	**48.5**	**58.8**	**49.3**	**50.0**	**40.0**
				98.7	53.1	49.5	59.6	58.5	51.5
CO				**97.9**	**44.7**	**58.7**	**61.9**	**54.2**	**57.6**
					100	55.1	51.7	62.0	64.0
X					**97.7**	**55.1**	**55.0**	**53.1**	**56.1**
						94.3	53.5	54.0	64.2
H						**95.3**	**65.0**	**52.2**	**47.6**
							100	68.9	61.3
SIM							**91.7**	**54.0**	**52.9**
								99.3	54.2
S								**92.1**	**52.9**
									96.6
HL									**95.1**

[a] Abbreviations: E, *S. epidermidis*; C, *S. capitis*; W, *S. warneri*; CO, *S. cohnii*; X, *S. xylosus*; H, *S. hominis*; SIM, *S. simulans*; S, *S. saprophyticus*; and HL, *S. haemolyticus*. Boldface numbers indicate immunoblotted whole-cell polypeptides.

Calculation of Dice coefficients showed average percentage similarity values ranging from 41.5 to 65.0%. As with interspecies comparisons of Coomassie blue-stained gels, the percentage similarity values between isolates were much lower than the values obtained from intraspecies comparisons.

DISCUSSION

Analyses of 100 different isolates of nine staphylococcal species by SDS-PAGE and by immunoblotting gave reproducible and characteristic whole-cell polypeptide and banding profiles. These banding patterns were used to group staphylococcal isolates according to species, with type strains serving as reference points for specific identity. Profiles characteristic of a particular species were obtained from its constituent isolates, irrespective of their geographical origins or isolation sites. Both SDS-PAGE and immunoblotting provide reproducible and sensitive methods for characterization of coagulase-negative staphylococci.

Application of SDS-PAGE and immunoblotting techniques enabled attribution of species for ambiguous isolates. Conventional analyses with the Kloos and Schleifer scheme (20) or other biochemical methods had not permitted assignment of these isolates to any particular species. However, comparison of their SDS-PAGE or immunoblot whole-cell polypeptide profiles with those of type strains allowed unequivocal grouping. Species assignation according to the whole-cell banding patterns of more-defined isolates agreed with results obtained by other methods.

Our results indicate that SDS-PAGE or immunoblot analysis can be used to identify coagulase-negative staphylococci to species level. Although immunoblotting is a more sensitive technique, SDS-PAGE is a simpler procedure. In addition, all major bacterial proteins are examined in the latter technique, rather than just those which are active antigenically.

Previously, SDS-PAGE has been used to distinguish different staphylococcal species (9). The results reported here demonstrate that not only species but subspecies groups can be identified. These groups were apparent in *S. warneri*, *S. simulans*, and *S. xylosus*. The significant decrease in percentage similarity values of certain isolates indicated that discrete groups existed within these species. However, despite the distinct values observed for these isolates, intraspecies similarity values were always greater than those for interspecies comparisons, with divergent isolates maintaining a close resemblance to the characteristic whole-cell polypeptide profile of their assigned species. Grouping of methicillin-resistant *S. aureus* according to an immunoblot profile followed the same pattern of intragroup similarity and intergroup dissimilarity (28). Subspecies groups identified in some staphylococcal species (18) were not detected in the other coagulase-negative staphylococci studied here or in *S. aureus* (9). The heterogeneity of certain staphylococcal species has a parallel in *Haemophilus influenzae*, whose constituent strains exhibit significant variation (25). Analyses of larger numbers of staphylococcal isolates will be needed to define subspecies groups, and further work will be required to assess the taxonomic significance of these results.

Genetic recombination could account for intraspecies variation. However, whole-cell polypeptide profiles represent phenotypic expression only. Although the total complement of induced bacterial proteins can be studied, SDS-PAGE analyses do not index the genotype directly. Staphylococcal chromosomal DNAs have been analyzed previously by rRNA gene restriction patterns (F. Thomson-Carter, P. Carter, and T. Pennington, J. Gen. Microbiol., in press), and application of this method would establish genetic similarities both within and between species. Phenotypic comparisons with SDS-PAGE profiles did demonstrate that certain species are more closely related than others, e.g., *S. epidermidis* and *S. capitis* share more common polypeptides than *S. epidermidis* and *S. cohnii* do.

Adoption of SDS-PAGE and immunoblotting techniques as routine methods of characterization would require standardization of reference banding patterns. Methods for analysis of band position and intensity are available (1). Thus a data bank of reference banding patterns could be constructed with which any unknown isolate could be compared. The present study has demonstrated that SDS-PAGE and immunoblotting can be used in staphylococcal species and strain differentiation and could have application as typing tools.

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