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Construction of a database to identify *Staphylococcus* species

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SUMMARY A database was constructed for the routine identification of *Staphylococcus* species, isolated from man. The method comprised 15 conventional characterisation tests using substrates incorporated into agar plates and a multipoint inoculation system. The database was constructed from results of 125 reference strains and 1567 clinical isolates. In an evaluation trial, using a probability profile index generated from the database, 529 of 559 (94·6%) further clinical isolates were identified to species level. A further 20 (3·6%) gave low discrimination between two species. The proposed scheme was rapid, reliable, and inexpensive.

The scheme of Baird-Parker[1] defined the first generally accepted subgroups among the coagulase negative staphylococci. Schleifer and Kloos[2] and Kloos and Schleifer[3] amended the descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and proposed seven new species of coagulase negative staphylococci: *S cohnii; S xylosus; S warneri; S capitis; S hominis; S haemolyticus;* and *S simulans*. Subsequent studies have shown that coagulase negative staphylococci are clinically important pathogens.[4,5] In particular, *S epidermidis* may cause infection in association with various prosthetic implant devices, notably artificial heart valves, vascular grafts, peritoneal dialysis catheters, joint prostheses and cerebrospinal fluid shunts.[6]

The coagulase negative staphylococci have also been shown to be responsible for bacterial endocarditis and septicaemia,[7] wound infections,[8] and urinary tract infections.[9] Many methods are available for the identification of fermentative Gram negative bacilli; fewer exist for staphylococci.

Simplified schemes available for the identification of staphylococci include Kloos and Schleifer's method.[10] They selected 13 key characters and identified more than 80% of strains. Two to three days' incubation was necessary, however, for some of the tests used.[11] A dichotomous key scheme[12] could only identify 68% of strains.

A commercial micromethod, API Staph (API Bio Mérieux, Basingstoke, Hampshire, England) is available, and trials have shown broad agreement with conventional methods.[13] We report here a simple system for the identification of clinically important staphylococci. The system comprises conventional tests, using substrates incorporated into agar plates and a multipoint inoculation system similar to that used for Gram negative bacilli,[14] and antibiotic susceptibility testing using the breakpoint method.[15] The method was developed and evaluated using computer assisted technology.

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Material and methods

One hundred and twenty five strains obtained from culture collections and representing the 12 species included in the database were tested. Many were used in the taxonomic study of Feltham.[16]

The reference strains studied were as follows: *S aureus* (n = 13); *S sciuri* subspecies *sciuri* (n = 7); *S sciuri* subspecies *lentus* (n = 4); *S epidermidis* (n = 11); *S saprophyticus* (n = 10); *S hominis* (n = 16); *S haemolyticus* (n = 24); *S warneri* (n = 12); *S cohnii* (n = 6); *S simulans* (n = 6); *S capitis* (n = 10); *S xylosus* (n = 6). A further 23 reference strains representing species not normally associated with infections in man were also tested, although they are not included in the database: *S intermedius* (n = 6); *S auricularis* (n = 1); *S caprae* (n = 1); *S carnosus* (n = 1); *S caseolyticus* (n = 1); *S gallinarum* (n = 1); *S arlettae* (n = 1); *S equorum* (n = 1); *S kloosii* (n = 1); *S hyicus* subspecies *chromogenes* (n = 1); *Micrococcus maripuniceus* (n = 1); *M varians* (n = 3); *M roseus* (n = 2); *M luteus* (n = 1); and *M cyaneus* (n = 1).

A total of 1567 catalase positive, Gram positive cocci were collected from clinical specimens and were tested by the multipoint methods.

MEDIA AND QUALITY CONTROL

All tests were performed using a 21 pin multipoint inoculator on agar plates incorporating substrates. Three pin positions were used for internal quality control of all batches of media prepared. The organisms used were *S aureus* (NCTC 8178), *S haemolyticus* (CCM 1798), and *S sciuri* subspecies *sciuri* (ATCC 29059). These strains cover positive reactions on all the tests.

Inocula for the test plates were cultures of strains in brain heart infusion broth (BHI; Oxoid Ltd, Basingstoke, Hampshire), incubated for two to three hours at 37°C. Pre-dried, substrate incorporated agar plates were then inoculated with this culture and incubated for 18–24 hours at 37°C.

Deoxyribonuclease production was tested using DNase agar (Oxoid). After 18–24 hours' incubation the plates were flooded with 1M hydrochloric acid. A clear zone around the colonies indicated deoxyribonuclease activity.

Phosphatase production was tested using a medium containing 38 g/l Mueller Hinton agar (Oxoid), 3·5 g/l, anhydrous sodium carbonate 1·5 g/l, sodium bicarbonate and 0·3 g/l p-nitrophenyl phosphate disodium salt. Sigma 104 phosphatase tablets were used for this (Sigma Chemicals, Poole, Dorset). The pH was adjusted to 5·6. After 18–24 hours' incubation phosphatase activity was indicated by a yellow zone around the colonies. To prevent diffusion of positive colour reactions each inoculum patch was segregrated by cutting the agar with a sterile scalpel after inoculation.

Novobiocin resistance was tested using Mueller Hinton agar (Oxoid) containing novobiocin at a final concentration of 1·6 mg/l. Novobiocin resistant species produced colonies. Sensitive strains gave no growth or occasional microcolonies.

Acid production from maltose, mannitol, sucrose, lactose and xylose were tested using carbohydrate peptone agars (Mast Laboratories Ltd, Bootle, Merseyside, media codes IDM 5, 6, 10, 12 and 13). The indicator was bromo-thymol blue, and a positive fermentation reaction was indicated by a yellow halo around the colony on a blue plate.

Acid from trehalose, n-acetyl glucosamine, ribose, mannose and cellobiose were tested using carbohydrate base (Mast IDM 14) to which the substrate was added at a final concentration of 1% (w/v). All media were sterilised at 121°C for 15 minutes, with the exception of ribose, which was sterilised at 115°C for 10 minutes. The final pH was 7·2. Urease production was tested using urea agar base (Mast IDM 32) urea 1% (w/v). A positive reaction was indicated by a purple halo or colony on an orange-yellow plate.

Haemolysis was tested using a layered horse blood agar plate. The bottom layer was Columbia agar (Oxoid). The upper layer was Columbia agar (Oxoid) supplemented with 7% defibrinated horse blood.

To 0·5 ml of an overnight BHI culture was added 0·5 ml of a 1/10 dilution of fresh rabbit plasma in saline for the tube coagulase test. Tubes were incubated in a water bath at 37°C and read hourly for four hours and after standing at room temperature overnight. Any degree of clotting was regarded as a positive result.

A heavy suspension of cells in distilled water was prepared on a microscope slide for the slide coagulase test. One loopful of rabbit plasma (Wellcome Diagnostics, Dartford, England) was added and the suspension was mixed well. A positive test was indicated by clumping within 10 seconds. If the cells clumped in the distilled water alone the reaction was recorded as autoagglutinable.

Thermostable nuclease activity (TNase) was determined by the method of Lachica *et al.*[17] An overnight BHI culture was heated in a boiling water bath for 15 minutes. Wells of 3 mm were cut into toluidine blue-DNA agar and 30 μl of the heated culture was added to the well. The plates were incubated at 37°C and examined hourly for up to four hours. The presence of pink haloes indicated nuclease activity.

API Staph strips were used according to the manufacturer's instructions.

Lysostaphin susceptibility was tested using discs impregnated with lysostaphin (Roche Diagnostica, Welwyn Garden City, Hertfordshire, England).[18]

DATABASE CONSTRUCTION

A preliminary database using 17 tests was prepared based on the results obtained from 125 reference strains using the multipoint method. This was used to produce a skeleton profile list. Using this list 1567 strains taken at random from the routine clinical laboratory were identified. Of these, 782 strains were tested for coagulase (tube coagulase test and slide coagulase test), DNase and TNase. Isolates which did not identify were tested by API Staph. The multipoint methods were repeated on strains identified by API Staph, and if consistent with previous results the "new" profile was incorporated into the list.

The profiles obtained from the 1567 clinical isolates and 125 reference strains were analysed by a computer program PROFMAT (our unpublished observations) to generate a new database. The data for the program consisted of a list of bacterial taxon names, their corresponding profiles, and the number of occasions that each profile for that taxon had occurred. The program carried out the analysis in three stages: it sorted all bacterial names together with corresponding profile data; it calculated sequential summated results for each taxon; it constructed and printed probabilities of positive results for each taxon. This database was

Table 1 *Sample of entries from full profile list*

Profile	*Taxon name*	*Wilcoxon probability*
03260	*S haemolyticus*	0·636377
	S hominis	0·363168
03262	*S haemolyticus*	0·995144
03270	*S hominis*	0·990967

further analysed using the programs CHARSEP[19] and DIACHAR[20] to define the 15 most useful tests. Ease of interpretation and reproducibility were also considered.

Based on these results the number of tests was reduced from 17 to 15 and a change in the test order was made. The quality of the 15 test database was analysed using the programs OVERMAT,[21] MOSTYP,[22] and OUTLIER.[23] This database was used to generate a set of profiles (the profile index), using the computer programs PROFGEN, PROFSORT, and PROFPRINT (our unpublished observations). PROFGEN generated a list of the most likely five digit profiles with the corresponding name for each taxon in the matrix. PROFSORT used the data produced by PROFGEN to print a numerical list of profiles with the taxon name beside each profile. PROFPRINT used the output from PROFGEN to give the profile, the single highest taxon name, and the probability of identification, using the Wilcoxon probability coefficient,[24] provided that the highest probability was at least 0·90. If the value was less than 0·60 this was reported as "unidentified". For values between 0·61 and 0·89, the profile, the top two taxon names, and their corresponding probability scores were printed (table 1). The profile booklet is available on request.

The final 15 test database was evaluated by testing 559 strains isolated in the clinical laboratory. The profiles obtained from these strains were compared with the final profile index.

Forty five strains representing all 12 species in the database, including reference and clinical strains, were selected, randomly coded, and tested in triplicate. Interpretation of results was carried out by both an experienced and an inexperienced operator.

Results

Table 2 is the final database showing the percentage of positives for each species for the tests: 100% and 0% are shown as 1% and 99% which is usual for probabilistic systems of this type. Table 1 shows a sample of the profile list.

Table 3 shows the results of 559 clinical strains tested using the database. Several strains seemed to be intermediate between *S epidermidis* and *S hominis*, between *S simulans* and *S hominis*, and between *S haemolyticus* and *S hominis*. Whether this reflects a spectrum of variation between these species or is due to the small number of tests in the present system is not clear. These strains accounted for 20 (3·6%) of the 559 clinical isolates. Their uncertain identity is unlikely to be of clinical importance and one would hope that any cross infections by such strains could be recognised by their distinctive profile numbers.

Table 4 gives the results of 782 clinical strains tested

Table 2 *Final database*

	Strains tested	*DNase* 1	*Phosphatase* 2	*Novobiocin* 3	*Mannitol* 4	*Trehalose* 5	*Sucrose* 6	*Xylose* 7	*Maltose* 8	*Lactose* 9	*n-acetyl glucosamine* 10	*Haemolysis* 11	*Urease* 12	*Mannose* 13	*Ribose* 14	*Cellobiose* 15
S aureus	538	99	99	1	97	97	98	1	97	79	92	35	54	94	73	1
S epidermidis	497	1	82	1	1	3	97	1	99	97	1	20	32	6	3	1
S saprophyticus	132	1	1	99	94	87	99	1	99	95	7	1	99	1	3	1
S cohnii	20	1	50	99	85	99	1	1	95	50	35	20	30	35	10	1
S xylosus	21	10	81	99	99	99	99	99	86	67	57	5	71	71	38	1
S haemolyticus	219	3	1	1	76	93	99	1	99	84	99	99	1	5	59	1
S hominis	50	3	1	1	2	90	98	1	94	50	52	12	68	6	1	1
S warneri	68	3	1	1	99	96	99	1	99	12	6	16	59	22	66	1
S simulans	97	12	1	1	87	97	99	1	1	99	76	15	29	35	1	1
S capitis	18	39	1	1	67	1	72	1	6	1	1	1	1	72	1	1
S sciuri	17	82	88	99	99	99	99	12	99	53	6	1	1	99	99	99
S lentus	15	7	67	99	87	99	99	99	87	99	87	1	1	99	99	99

The percentage of positives for each species for all tests is shown. 100% and 0% are shown as 1% and 99% which is usual for probablistic systems.

Table 3 *Results of 559 strains tested using final database*

Source	*Urines*	*Bloods*	*PDEs**	*NSGs†*	*IV tips‡*	*Total*
No of isolates	249	142	84	43	41	559
S aureus	34	17	10	4	0	65
S epidermidis	89	79	54	17	24	263
S saprophyticus	64	0	0	1	0	65
S cohnii	0	0	0	0	1	1
S xylosus	0	0	1	1	0	2
S haemolyticus	20	9	3	4	9	45
S hominis	10	11	1	3	2	27
S warneri	7	5	7	2	2	23
S simulans	9	3	0	2	0	14
S capitis	4	5	3	6	2	20
S sciuri	0	0	0	1	0	1
S lentus	0	0	0	0	0	0
S epidermidis/S hominis	4	7	2	1	1	15
S simulans/S hominis	3	0	0	0	0	3
S haemolyticus/S hominis	1	0	0	1	0	2
Micrococcus sp	1	1	1	0	0	3
Unidentified	3	5	2	0	0	10

*Peritoneal dialysis effluents.
†Isolates regarded as not clinically important.
‡Intravascular cannula tips.

for their ability to produce deoxyribonucleases, heat stable deoxyribonucleases, "bound" and "free" coagulases.

Table 5 shows the results of 10 staphylococcal species not usually associated with human disease. The eight reference strains of micrococci gave negative reactions with all tests. These strains were all resistant to lysostaphin. The six strains of *S intermedius* were all identified by the system as *S aureus*.

The number of discrepant results out of the 2025 (45 × 15) triplicates was 35 when read by the experienced worker, and 55 when read by the inexperienced one. When corrected for degrees of freedom by the method of Sneath and Johnson,[25] the error rate was 1·78% and 2·81%, respectively. This is very good for work of this nature. The least reproducible tests were n-acetyl glucosamine, urease, and mannose with average errors of 5·9%, 4·0%, and 3·7%, respectively.

Discussion

The multipoint indentification scheme described here was designed to identify the species of staphylococci most commonly occurring in man. The rapid identification of *S aureus* is of paramount importance and this scheme is not intended to replace the rapid slide tests currently available. It is, however, designed to identify *S aureus* as well as the coagulase negative staphylococci, and will give an accurate identification with atypical strains of *S aureus*.

This study shows that of 392 strains identified as *S aureus*, only 359 (91·6%) gave positive reactions with

Table 4 *Characteristics of 782 strains of catalase positive, Gram positive cocci*

		Strains identified as S aureus			
	No of strains	*Tube coagulase test*	*Slide coagulase test*	*Thermonuclease test*	*DNase test*
	359	+	+	+	+
	21	+	−	+	+
	9	−	+	+	+
	3	−	autoagglutinable	+	+
Total	392				
		Strains other than *S aureus*			
	320	−	−	−	−
	29	−	autoagglutinable	−	−
	19	−	−	+	+
	16	−	−	−	+
	5	−	autoagglutinable	−	−
	1	−	+	−	−
Total	390				

Table 5 *Reactions of 10 species not in database*

	DNase	Phosphatase	Novobiocin	Mannitol	Trehalose	Sucrose	Xylose	Maltose	Lactose	n-acetyl glucosamine	Haemolysis	Urease	Mannose	Ribose	Cellobiose
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
S intermedius	+	+	−	v	+	+	−	v	+	+	v	v	+	−	−
S auricularis	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−
S caprae	−	+	−	+	+	−	−	−	+	−	−	−	+	−	−
S carnosus	−	+	−	+	+	−	−	−	+	+	−	−	+	−	−
S caseolyticus	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−
S gallinarum	−	+	+	+	+	+	+	+	+	+	−	+	+	−	+
S arlettae	−	−	+	+	+	+	+	+	+	−	−	−	−	+	−
S equorum	−	−	+	+	−	+	−	+	+	−	−	−	+	−	−
S kloosii	−	−	+	+	+	−	−	+	+	−	−	+	−	+	−
S hyicus subsp *chromogenes*	+	+	−	−	+	+	−	−	−	−	−	−	−	+	−

v = variable reaction.
Six strains of *S intermedius* and one strain of each of the other species were tested.

tube coagulase test, slide coagulase test, DNase and thermonuclease test (table 4). If slide coagulase test and tube coagulase test were used alone only 368 (93·9%) and 380 (96·9%) of the strains, respectively, would have been correctly identified as *S aureus*.

Methicillin resistant strains (MRSA), which are of increasing importance in hospital-acquired infections, have been shown to be deficient in clumping factor.[26] Six MRSA strains were identified correctly as *S aureus* by this scheme, including one strain which was defective in both clumping factor and protein A production. Three mucoid variants of *S aureus* were also correctly identified.[27] In the past coagulase negative staphylococci were often dismissed as clinically unimportant or as contaminants and were often reported as *S epidermidis*. They have recently assumed far greater importance as true pathogens.

The ability of clinical laboratories to identify rapidly and reliably coagulase negative staphylococci may have important implications. For example, in deciding whether two or more blood culture isolates from the same patient are of the same species (more likely to be important) or different (less likely to be so). The system described here was designed with the aid of several computer programs which have aided in the test selection, identification profile generation, and database evaluation. The results from the database evaluation programs give added confidence in the use of the system. Of 559 strains tested, 93·2% were identified to species level and 3·6% gave low discrimination results between two species. *S hominis* occurred in all such cases, usually in association with *S epidermidis*. The close similarity between phosphatase negative strains of *S epidermidis* and *S hominis* is well documented.[28] The OVERMAT computer program predicted this occurrence with an overlap of 12% between these species.

The order of tests in the system is important and was chosen carefully. It allows more flexible schemes to be devised. For example, an abbreviated scheme can be simply devised, based on the first three tests (DNase, phosphatase, and novobiocin). This would be an identification screen for *S aureus, S epidermidis,* and *S saprophyticus*. Other tests can be added in any order for particular applications, making the scheme flexible.

Profiles produced by the 10 staphylococcal species not associated with human clinical infection were excluded from the profile index. The profile numbers of seven of these species were distinct from the clinically important species included in the database (table 5). All six strains of *S intermedius,* however, were identified as *S aureus*. *S intermedius* is primarily of veterinary interest because it produces a variety of canine infections and is coagulase positive. Though little is known of its importance in human infections, it will rarely need to be distinguished from *S aureus* in routine clinical work. If necessary it can be differentiated from *S aureus* because *S intermedius* is Voges Proskauer negative.

The strain of *S hyicus* subspecies *chromogenes* gave low probabilities for *S aureus* and *S capitis*.

The strain of *S kloosii* gave low probabilities for *S cohnii* and *S saprophyticus*. *S hyicus* subspecies *chromogenes* and *S kloosii* are of veterinary interest and are not thought to cause human infection. The system could be expanded to include these and other species if necessary.

Material costs were £0·12 per identification. Labour costs were similar to those described for an identification scheme for Gram negative organisms.[14]

The system may be used in conjunction with a multipoint antibiotic breakpoint method, thus becoming an integrated identification and susceptibility system.

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