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A comparison of DNA and immunoblot fingerprinting of the SII biotype of coagulase negative staphylococci

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SUMMARY

Thirty-eight isolates of the SII biotype of coagulase negative staphylococci were examined by phage typing, antibiograms, DNA and immunoblot fingerprinting. Multiple isolates were available from 5 patients with clinically proven infections and from 12 further patients who were epidemiologically distinct. Only 3 of the isolates were phage typable and antibiograms produced only 9 types. All isolates were typable by both DNA and immunoblot fingerprinting and reproducibility was excellent provided the conditions were standardized. Discrimination was better with immunoblot fingerprinting (17 types) than DNA fingerprinting (11 types). Both techniques were successful in assessing the significance of multiple isolates from a single patient.

INTRODUCTION

Coagulase negative staphylococci have assumed an increasing clinical importance in the last few years. Their ubiquitous nature has posed problems in deciding the significance of an individual isolate. Assessment is based on the clinical condition of the patient and the presence of similar isolates from subsequent specimens with identical antibiograms (MacGregor & Beaty, 1972). This has been shown to be unreliable as there is a limit to the number of potential antibiograms, isolates do not always give consistent results and unrelated isolates may have the same antibiogram (Harsteine *et al.* 1987). Traditional typing methods such as phage typing are inadequate both in terms of the number of isolates typable and in the degree of reproducibility (De Saxe *et al.* 1981).

Biotyping has demonstrated that 75% of isolates from infected patients are biotype SII (Marples, 1986). These isolates are often untypable by phages so that new typing methods are required for their study. Coagulase negative staphylococci have recently been subdivided by immunoblotting (Burnie *et al.* 1988). Isolates were grown overnight in broth, fragmented by lysostaphin, run on a standard 10% SDS–PAGE gel and transferred onto nitrocellulose membrane. They were then stained by a modified ELISA with a rabbit hyperimmune antiserum raised against one of the isolates. The pattern of antigenic bands detected was the basis of the fingerprinting system. This technique had the advantages that all isolates were typable and within coagulase negative staphylococci the degree of

discrimination was excellent (Burnie *et al.* 1988). Its disadvantages were that it was based on phenotypic characteristics and it required standardized antibody probes which may be both difficult and expensive to produce (Burnie & Matthews, 1987). A similar technique has been developed for *Staphylococcus aureus* by Krikler *et al.* (1986).

Restriction endonuclease digestion has been developed for fingerprinting many pathogens including *Candida albicans* (Scherer & Stevens, 1987), *Bacteroides* species (Bradbury *et al.* 1985), *Vibrio chlora* (Feng-Ying *et al.* 1986) and *Campylobacter pylori* (Langenberg *et al.* 1986). The principal of the technique is that the DNA of the microorganism is extracted and cut by a restriction enzyme into fragments. The pattern of the fragments is governed by the DNA sequence of the host's genome and so is independent of phenotypic variation. The latter variation may be misleading as characters such as disaccharide utilization in *Saccharomyces cerevisiae* may be encoded on several sites in the genetic map (Carlson, Osmond & Botstein, 1980).

In this paper we report a new technique for DNA fingerprinting isolates of coagulase negative staphylococci. Strains were all biotype SII and were obtained from 12 unrelated patients with systemic infections. This was to assess the ability of the technique to discriminate between different isolates. Multiple isolates were also available from five patients in whom there was a clinical problem due to a biotype SII coagulase negative staphylococcus which required antibiotic treatment. Three isolates were available from each of three patients on chronic ambulatory dialysis with peritonitis (case 1–3). A fourth patient had aortic valve endocarditis (6 isolates) and a fifth an infected Hickman line (12 isolates). This suggested that in each individual patient the multiple isolates were related to each other. DNA and immunoblot fingerprinting were compared to phage typing and antibiograms.

MATERIALS AND METHODS

Immunoblot fingerprinting

Each isolate was grown overnight at 37 °C in tryptone-soya broth. After centrifuging, pellets were resuspended in 100 μl of sterile distilled water and 100 μl of lysostaphin (Sigma; 200 μg/ml) and incubated at 37 °C for 30 min. Protein concentration was measured by the Lowry method and standardized to 10 mg/ml.

Rabbit hyperimmune antiserum. The isolate from case 1 was grown overnight at 37 °C on multiple blood agar plates (Oxoid), harvested in distilled water, pelleted at 12000 **g** for 10 min and disrupted by an Xpress at −20 °C. The supernatant, after centrifugation at 12000 **g** for 10 min was mixed with full Freund's adjuvant and distilled water in a 1:1 ratio. Twenty-five milligrams of protein was added to 1 ml of distilled water. Rabbits were inoculated at 0 and 14 days and the serum obtained at 28 days pooled for subsequent experiments.

Gel electrophoresis. The enzyme extracts were solubilized in 2·5% sodium dodecyl sulphate (SDS) and 1·3% 2-mercaptoethanol at 100 °C for 4 min. Electrophoresis was carried out on a 10% polyacrylamide gel in a discontinuous buffer system. Extract (60 μl) was added to each well. The rainbow standards (Amersham, UK) were the molecular-weight markers.

Immunoblotting. The preparations were transferred from gel to nitrocellulose membrane in a LKB Trans-Blot cell with a current of 0·6 A for 45 min at 25 °C. A 25 mM Tris 192 mM glycine buffer (pH 8·3) containing 20% methanol was used. Free protein sites were saturated by incubation in 3% bovine serum albumin in buffered saline (0·9% sodium chloride and 10 mM Tris pH 7·4) at 4 °C overnight. The membrane was then incubated at 25 °C for 2 h with the rabbit antiserum diluted 1 in 20 in 3% bovine serum albumin and 0·05% Tween 20. After washing five times in 0·9% sodium chloride and 0·05% Tween 20 the nitrocellulose was incubated for 1 h with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) at a 1 in 1000 dilution in 3% bovine serum albumin. After washing again, the membranes were incubated for 5–15 min at 25 °C with a 100 ml buffer (100 mM Tris-HCl, pH 9·5, 100 mM NaCl, 5 mM MgCl) containing a mixture of 660 μl nitroblue tetrazolium (NBT 50 mg/ml in 70% *B,N*-dimethyl formamide) and 330 μl 5-bromo-4-chloro-3 indolyl phosphate (BCIP 50 mg/ml in 70% *N,N*-dimethyl formamide). The reaction was stopped by washing in water and the results for each antigenic band interpreted as absent (−), present (+) or double (DB).

DNA fingerprinting

Preparation of genomic DNA. A modification of the procedure for isolating DNA, described by Scherer & Stevens (1987) was employed. A dense suspension of each isolate was made by inoculating a loopful of colonies from blood agar plates into a 5 ml tryptone soya broth (Oxoid) and incubated at 37 °C overnight. Cells were harvested in Eppendorf tubes after centrifugation for 5 min (MSE microcentaur, high speed). They were then washed once in 1 ml of 10 mM Tris-hydroxymethylaminomethane pH 8 and 1 mM ethylenediaminetetra acetic acid (EDTA), pH 8 (TE). Then 0·5 ml of fresh TE containing 100 μl of 1 mg/ml lysostaphin (Sigma) was added to each pellet and the mixture was incubated in a 37 °C water bath for 1 h. Subsequently 0·5 ml of a solution containing 0·05 M EDTA (pH 8·5) and 2 mg/ml sodium dodecyl sulphate was added followed by 30 μl of diethyl pyrocarbonate (Sigma). The solution was mixed and incubated in a 68 °C water bath for 30 min. It was centrifuged for 5 min and the supernatant pipetted into 1 ml cold ethanol. This was mixed and left at −20 °C for 30 min. The mixture was clarified by centrifugation for 5 min and the supernatant discarded. The precipitate was washed with 1 ml cold 70% ethanol and the ethanol removed. The precipitate was vacuum dried for 10 min in a speed vacuum concentrator (Savan). The dried precipitate was resuspended in 0·1 ml TE, pH 8 containing 12 μl RNase (Ribonuclease-A from Bovine Pancreas Type 1-AS, Sigma) which had been previously treated by boiling for 10 min in a TE solution (10 mg/ml). The suspension was incubated at 37 °C for 30 min. One-tenth volume (10 μl) of 3 M sodium acetate (pH 5·5) and two volumes (200 μl) of cold absolute ethanol were added and kept at −20 °C overnight. This was then centrifuged and the precipitate washed with 70% cold ethanol. It was then vacuum dried and resuspended in 90 μl TE, pH 8. To dissolve the prepared DNA in TE, it was incubated at 55 °C for 30–60 min. This was then stored at −20 °C.

Restriction endonuclease digestion of DNA. Ten microlitres *Eco* RI (10×) reaction buffer and 4 μl *Eco* RI enzyme (NBL Enzymes Division) containing 40 U

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were added to 90 μl of DNA in TE. Endonuclease digestion was carried out to completion at 37 °C for a period of 2·5 h. The digestion was stopped by heating the mixture to 70 °C for 10 min. Thirty microlitres *Hin* dIII digested DNA (150 μg/ml) was used as a DNA marker. Thirty microlitres of bromo-phenol blue buffer (40 mg dissolved agarose nutrient agar in 20 ml 10 mM Tris, pH 7·5; 20 mM EDTA; 10% glycerol and 0·01% bromophenol blue) was added to the digested DNA sample and mixed.

Gel electrophoresis and photography. The digested DNA samples prepared as above were electrophoresed overnight at 40 V (constant voltage) in a horizontal gel containing 0·8% agarose with the Tris Borate-EDTA buffer system previously described (Langenberg *et al.* 1986). After electrophoresis the gel was visualized under u.v. light and photographed with Polaroid type 57 film.

Antibiograms

This was performed by a disk susceptibility method on Isosensitest agar (Oxoid CM 471). Ten microgram disks of methicillin, tetracycline, gentamicin, erythromycin, fusidic acid and chloramphenicol were used as well as 2 μg disks of rifampicin and clindamycin. Penicillin was tested with a 1 μg disk. All tests were performed at 35 °C except methicillin which was performed at 30 °C.

Phage typing

Isolates were phage typed by the Division of Hospital Infection, Colindale.

RESULTS

Immunoblot fingerprinting

This detected multiple antigenic bands with molecular weights varying from 35 to 190 kDa. Each isolate was immunoblotted at least twice and the results summarized in Table 1. The multiple isolates from case no. 1 are illustrated by lanes 1–3 in Fig. 1. The immunoblot fingerprints of the multiple isolates from cases 4 and 5 have previously been reported (Burnie *et al.* 1988).

In all five cases the multiple isolates from each individual patient were identical to each other and different from the other isolates.

The results of the 12 isolates from the unrelated systemic infections are summarized by Table 1 and strains 6–10 are illustrated in Fig. 1. All isolates were typable and reproducibility was excellent as the same batch of antiserum was used throughout the study. The degree of discrimination was high as all 17 stains produced at least three antigenic band differences between each other.

DNA fingerprinting

This detected consistently 11 bands with apparent molecular weights from 9·0 to 1·7 kb. Each isolate was restricted at least twice and the results summarized in Table 2. The multiple isolates from case 3 are pictured by tracks 1–5 in Figure 2 and two of the isolates from case 4 are illustrated by tracks 6 and 7 (Fig. 2). Strains 3 (Fig. 2, track 8), 1 (Fig. 3, track 1), 6 (Fig. 3, track 3), 7 (Fig. 3, track 6), 8 (Fig. 3, track 7), 10 (Fig. 3, track 5), 11 (Fig. 3, track 4) and 15 (Fig. 3, tract 2) are also shown. All isolates were typable and reproducibility between gels was excellent

Table 1. *Details of the immunoblot results for the multiple isolates, strains 1–5 and the single isolates, strains 6–17*

Band molecular weight (kDa)	Strain number																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
185–190	+	−	+	+	−	+	−	+	+	+	+	−	−	−	+	+	−
140	−	+	−	−	−	−	+	DB	−	DB	−	+	−	−	+	−	−
120	+	−	+	+	+	−	−	+	+	DB	+	−	−	−	−	DB	DB
114	+	+	−	−	−	−	−	+	+	DB	−	−	−	−	−	−	−
107	DB	−	−	−	−	−	−	+	DB	+	−	−	−	−	−	−	−
102	+	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−
98	+	−	−	+	−	+	+	+	+	+	−	+	−	−	−	+	−
94	+	−	−	+	−	−	+	+	+	+	−	−	−	−	+	−	−
91	+	−	−	+	−	−	+	+	+	+	+	−	−	−	−	−	−
88	+	−	−	+	+	−	+	+	+	+	+	−	+	+	+	−	−
68	+	+	+	+	−	−	−	+	+	+	−	+	+	+	−	+	+
64	+	+	+	−	−	DB	−	+	+	+	−	+	−	−	+	−	−
60	+	−	−	+	+	+	−	−	+	+	−	−	+	−	+	+	+
56	+	−	−	−	−	+	+	−	−	+	+	−	−	−	−	+	−
54	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	+	−
50	+	+	+	−	−	−	−	−	+	DB	−	+	+	−	−	+	−
46	+	−	−	+	+	+	−	−	−	+	+	−	−	−	−	−	−
40	+	−	−	+	−	+	−	−	+	+	−	−	−	−	−	DB	−
37	+	−	−	−	−	−	+	−	−	+	+	−	−	DB	−	+	−
35	−	−	−	−	−	+	−	−	+	+	−	+	−	−	−	+	−

+, present; −, absent; DB, double band.

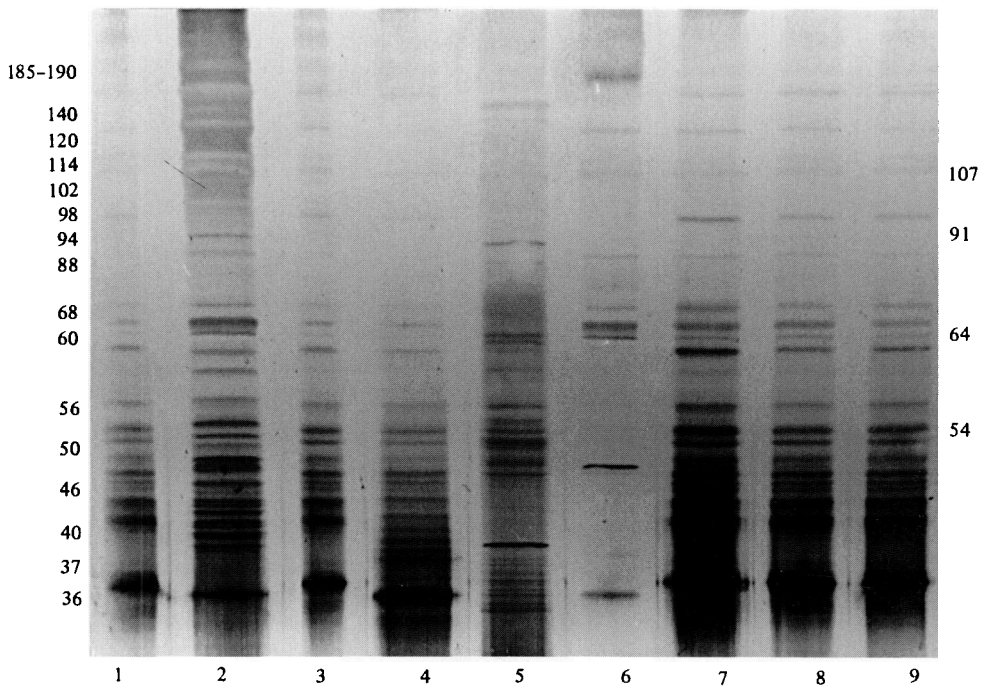

Fig. 1. Immunoblot fingerprints of the isolates from case no. 1 (tracks 1–3), and strains 6–10 (tracks 4–8).

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Table 2. *Details of the DNA results for the multiple isolates, strains 1–5 and the single isolates, strains 6–17*

Band size (kb)	Strain number															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
9·0	+	+	+	−	+	−	DB	−	−	−	−	−	+	+	−	+	+
8·4	−	−	−	−	+	DB	−	−	+	−	DB	−	−	−	DB	−	+
5·5	−	−	+	−	DB	+	−	−	+	−	+	−	−	−	+	+	−
4·4	+	+	+	+	+	−	−	+	−	−	−	−	+	+	−	−	+
4·0	−	−	+	−	+	+	+	+	−	+	+	+	−	−	+	+	−
3·6	+	+	+	+	+	−	−	+	−	−	−	+	+	+	−	−	+
3·2	+	+	−	+	+	+	−	−	+	+	+	+	+	DB	+	+	−
2·9	+	−	+	+	+	+	−	−	−	−	DB	−	−	+	+	−	DB
2·3	−	−	+	+	+	+	−	−	−	+	+	−	−	+	+	−	+
1·9	DB	−	DB	DB	DB	DB	−	−	−	DB	DB	−	DB	DB	DB	−	−
1·7	−	−	−	+	−	+	DB	−	+	+	+	−	+	+	+	−	−

+, present; −, absent; DB, double band.

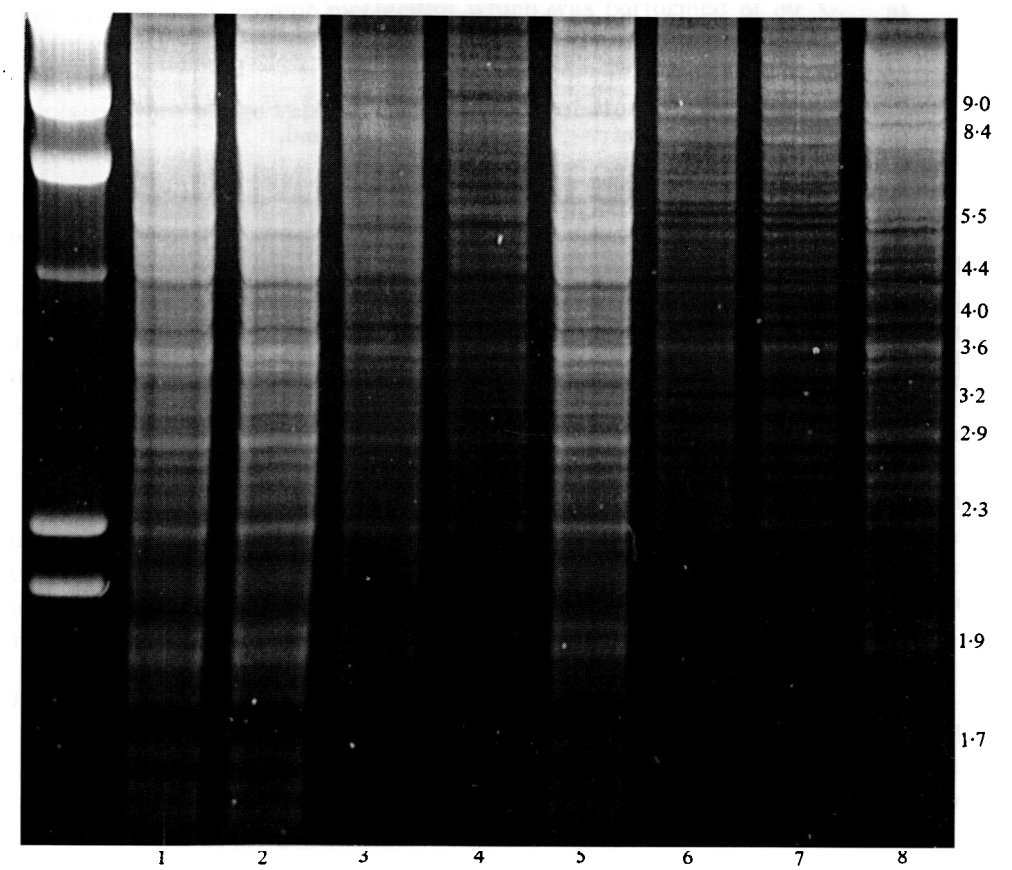

Fig. 2. DNA fingerprints. Isolates from case 3 (tracks 1–5). Isolates from case 4 (tracks 6 and 7). Isolate from case 3 (track 8).

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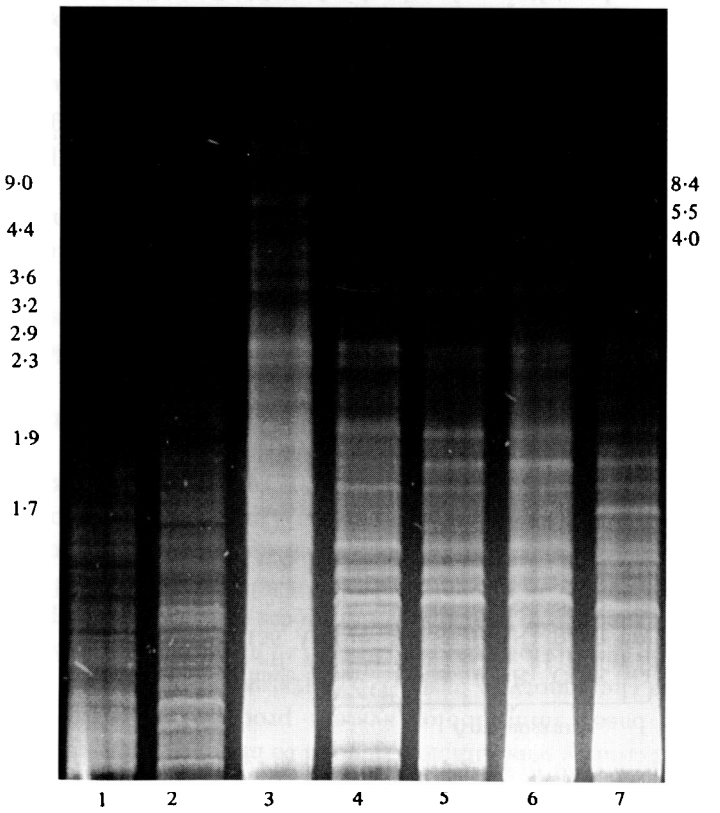

Fig. 3. DNA fingerprints. Strain 1 (track 1), strain 15 (track 2), strain 6 (track 3), strain 11 (track 4), strain 10 (track 5), strain 7 (track 6), strain 8 (track 7).

provided that the running conditions were standardized. When multiple isolates were examined from a single case these were identical to each other. The degree of discrimination between isolates was related to the number of antigenic band differences used as a differentiating criterion. Strains 6 and 15 were identical whilst strain 11 showed only one difference at 2·9 kDa. In the case of immunoblotting three antigenic band differences were required to confidentally differentiate isolates (Burnie & Matthews, 1987). If this is applied to DNA fingerprinting then isolates 1, 2 and 13 merge, isolate 4 is identical to isolate 14 and isolate 8 cannot be distinguished from isolate 12. The number of potential types amongst the 17 strains is thus reduced to 11.

Antibiograms

These are summarized by Table 3. The 8 antibiotics tested generated 9 separate patterns. Three of these had three isolates in them. In the five patients with multiple isolates these were identical to each other and distinct from the other isolates. Only 17 pairs of isolates had at least three antibiotic differences between each member of the pair.

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Table 3. *Details of the phage types and antibiograms of the 17 SII biotype isolates*

Strain no.	Phage type	Antibiogram P	M	F	R	C	E	G
1	155, A9C	R	S	R	S	R	R	R
2	—	R	R	S	S	S	S	R
3	—	R	S	S	S	S	S	S
4	—	R	R	S	S	S	S	R
5	—	S	S	S	S	S	S	S
6	—	R	R	R	R	R	R	R
7	—	R	R	R	S	S	R	R
8	—	R	S	S	S	R	R	R
9	155, A9C	R	R	S	S	S	R	R
10	—	R	S	S	S	S	S	R
11	—	R	R	S	S	S	R	R
12	—	R	R	S	S	S	S	R
13	—	R	R	R	R	R	R	R
14	71, 456	R	R	S	S	S	R	R
15	—	R	R	R	R	R	R	R
16	—	R	S	S	S	S	S	S
17	—	R	S	R	S	R	R	R

P, penicillin; M, methicillin; F, fusidic acid; R, rifampicin; C, chloramphenicol; E, erythromycin; G, gentamicin. All isolates were sensitive to vancomycin.

Phage typing

Only 3 of the 17 strains were typable by phages. Two of these were identical to each other despite coming from epidemiologically distinct patients.

DISCUSSION

This paper compares phage typing, antibiograms, DNA fingerprinting and immunoblot fingerprinting in their ability to type isolates of the SII biotype of coagulase negative staphylococci. Typing methods are judged by three criteria: typability, reproducibility and discrimination. All of the 17 strains produced antibiograms and could be DNA and immunoblot fingerprinted. Only three isolates were phage typable with this set of phages, a deficiency which has been compensated for in the past by the use of further experimental phages (Pulverer *et al.* 1975) and reverse phage typing (de Saxe & Notley, 1978).

Reproducibility has been a problem with phage typing (de Saxe *et al.* 1981), antibiograms (Christensen *et al.* 1983), and immunoblot fingerprinting (Burnie & Matthews, 1987). In the latter technique this was shown to be due to variations in the antibody probe. In the current work, the standardized antibody raised against the coagulase negative staphylococcus from case 1 produced reproducible results. The results for the DNA fingerprinting showed good reproducibility for the 11 bands which formed the basis of the typing system.

Discrimination for phage typing was poor (3 types) and for antibiograms, moderate (9 types), which invalidates their use as typing methods on their own. Immunoblot fingerprinting produced 17 distinct types even when differences were required at three separate antigenic band sites. DNA fingerprinting produced 11

types with this criterion but this rose to 16 types if a single difference was the marker. Reproducibility was sufficiently good to make this possible. Interestingly, the phenotypic based immunoblot system produced a better degree of discrimination than the genotype based DNA system. This may, in part, be due to the use of a single restriction enzyme, *Eco* RI. This cuts the DNA at a particular site throughout its length so that dissimilar pieces of DNA with approximately the same length will overlap on the gel. Only DNA with a total size of less than 20 kb will run on the gel which greatly reduces the range of DNA fragments which can be incorporated into the fingerprinting system. One possible way of compensating for this would be to combine the results of several restriction enzymes acting on different sites within the DNA.

Both DNA and immunoblot fingerprinting were capable of distinguishing true infection from multiple contaminants. In case 4, this was from a patient with endocarditis and in case 5 the multiple blood cultures were taken through an infected Hickman line. In the three patients with CAPD peritonitis there had been a clinical failure to respond to antibiotic therapy which suggested a recurrence of infection due to the same strain. In all five instances the isolates from each individual had identical phenotypes by immunoblot fingerprinting and identical genotypes by DNA fingerprinting.

The term fingerprinting has been applied to both techniques rather than typing due to problems with reproducibility (Burnie & Matthews, 1987; Scherer & Stevens, 1987). If the conditions are strictly standardized these can be overcome. In the clinical setting, when trying to assess the significance of a particular isolate, immunoblot fingerprinting is preferable to DNA fingerprinting as it is technically easier to perform and results can be obtained within 24 h. DNA fingerprinting has the advantage that it does not require antibody probes which must be made in advance and it measures genotype rather than phenotype.

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REFERENCES

Bradbury, W. C., Murray, R. G. E., Mancini, C. & Morris, V. I. (1985). Bacterial chromosomal restriction endonuclease analysis of the homology of *Bacteroides* species. *Journal of Clinical Microbiology* **21**, 24–28.

Burnie, J. P., Lee, W., Matthews, R. C. & Bayston, R. (1988). Immunoblot fingerprinting of coagulase negative staphylococci. *Journal of Clinical Pathology* **41**, 103–107.

Burnie, J. P. & Matthews, R. C. (1987). Immunoblot analysis: a new method for fingerprinting hospital pathogens. *Journal of Immunological Methods* **100**, 41–46.

Carlson, M., Osmond, B. C. & Botstein, D. (1980). SUS genes of yeast: a dispersed multigene family. Cold Spring Harbor Symposium. *Quantitative Biology* **45**, 799–804.

Christensen, G. D., Parisi, J. T., Bisno, A. L., Simpson, W. A. & Beachey, E. H. (1983). Characterization of clinically significant strains of coagulase negative staphylococci. *Journal of Clinical Microbiology* **18**, 258–259.

De Saxe, M. J., Crees-Morris, J. A., Marples, R. & Richardson, J. F. (1981). Evaluation of current phage typing systems for coagulase negative staphylococci. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infectionskrakheiten und Hygiene.* I Abteilung Originale 10 suppl., 198–204.

DE SAXE, M. J. & NOTLEY, C. M. (1978). Experiences with the typing of coagulase-negative staphylococci and micrococci. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infections-krankheiten und Hygiene*. I Abteilung Originale A 241, 46–59.

FENG-YING, C., LIN, C., GLENN MORRIS, J. JR, KAPER, J. B., GROSS, T., MICHALSKI, J., MORRISON, C., LIBONATI, J. P. & ISRAEL, E. (1986). Persistence of cholera in the United States: Isolation of *Vibrio cholera* 01 from a patient with diarrhoea in Maryland. *Journal of Clinical Microbiology* **23**, 624–626.

HARSTEINE, A. J., VALVANO, M. A., MARTHLAND, V. H. FUCHS, P. C., POTTER, S. A. & CROSA, J. H. (1987). Antimicrobial susceptibility and plasmid profile analysis as identity tests for multiple blood isolates of coagulase negative staphylococci. *Journal of Clinical Microbiology* **25**, 589–593.

KRIKLER, S. J., PENNINGTON, T. H. & PETRIE, D. (1986). Typing of strains of *Staphylococcus aureus* by Western blot analysis of culture supernates. *Journal of Medical Microbiology* **21**, 169–171.

LANGENBERG, W., RAUWS, E. A., WIDJOJOKUSUMO, A., TYTGAT, J. & ZANEN, H. C. (1986). Identification of *Campylobacter pylori* isolates by restriction endonuclease DNA analysis. *Journal of Clinical Microbiology* **24**, 414–417.

MACGREGOR, R. R. & BEATY, H. N. (1972). Evaluation of positive blood cultures. *Archives of International Medicine* **130**, 84–87.

MARPLES, R. R. (1986). Laboratory assessment in the epidemiology of infections caused by coagulase negative staphylococci. *Journal of Medical Microbiology* **22**, 285–286.

PULVERER, G., PILLICH, J. & KLEIN, A. (1975). New bacteriophages of *Staphylococcus epidermidis*. *Journal of Infectious Diseases* **132**, 524–531.

SCHERER, S. & STEVENS, D. A. (1987). Application of DNA typing methods to epidemiology and taxonomy of *Candida* species. *Journal of Clinical Microbiology* **25**, 675–679.