Technical methods

Immunoblot fingerprinting of coagulase negative staphylococci

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Coagulase negative staphylococci have become prevalent nosocomial pathogens following the implantation of foreign bodies such as prosthetic valves, cerebrospinal fluid shunts, joint prostheses and central venous catheters. They are also the most common contaminants of blood cultures so that a means of differentiating contamination from a true bacteraemia is important.¹ Assessment of the clinical importance of an individual blood culture isolate is currently based on the clinical condition of the patient and the presence of similar isolates with identical antibiograms from subsequent blood cultures.² Unrelated isolates from different patients, however, may have the same antibiogram,³ and retesting the same isolate does not always give reproducible results.⁴

Baird-Parker⁵ produced a scheme for differentiating six biotypes of coagulase negative *Staphylococcus* (SI-SVI) and eight biotypes of *Micrococcus*. Kloss *et al* used these for species specific groups,⁶ but the problem remained that isolates of biotype SII (*S epidermidis*) comprised 75% of all clinically important isolates so that further typing methods were required to subdivide these isolates.⁷ Results with phage typing have been inadequate both in terms of the number of isolates typable and in the degree of reproducibility.⁸ Examination of protein profiles obtained by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that these were species specific but could not subdivide isolates within the species.⁹ SDS-PAGE typing with sulphur-35 methionine has been of some value but this is an expensive technique requiring the use of radiolabels and has only been applied to a few isolates.¹⁰ Isolates of *Staphylococcus aureus* have been typed by immunoblotting culture supernatants against human antiserum.¹¹

In this paper we report the results of immunoblot fingerprinting of isolates of coagulase negative staphylococci. Isolates were examined from 17 unrelated patients with systemic infections to assess the ability of the technique to discriminate between different strains. Multiple isolates were also available from seven

patients in whom there was a clinical problem thought to be related to coagulase negative staphylococcus infection. In all cases multiple isolates were obtained from blood cultures, and it was important to decide whether these were several different contaminants or a single predominant pathogenic strain. All isolates were probed by a rabbit hyperimmune antiserum raised against the predominant strain of coagulase negative staphylococcus, *Staphylococcus epidermidis*, from one of these cases (case 3). Individual isolates from the 17 patients with systemic infections and all isolates from three of the multiply infected systemic cases (cases 5, 6, and 7) were also probed by the human antiserum from case 5 and a rabbit hyperimmune antisera raised against *Staphylococcus aureus*. The results were then compared with those obtained from phage typing, biochemical studies, and antibiograms.

Material and methods

Seventeen isolates were collected from 17 different patients with systemic coagulase negative staphylococcal infections. Seven additional patients had multiple isolates from blood cultures (table 1).

The coagulase negative staphylococcus from case 3 and an isolate of the epidemic methicillin resistant *S aureus* were grown overnight on blood agar plates. Each was harvested, washed in distilled water, and fragmented by a French press at 12 mPa. Fifty milligrams of the supernatant obtained by spinning at 12000 g for 15 minutes was mixed with 1 ml of complete Freund's adjuvant. New Zealand white rabbits were immunised on day 0 and day 14 by subcutaneous injection. The rabbits were bled at 28 days.

Table 1 Details of seven patients with multiple blood culture isolates

Case No	No of blood samples taken	No positive for coagulase negative staphylococci	Clinical comment
1	18	18	Aortic and mitral valve endocarditis
2	6	6	Aortic valve endocarditis
3	12	12	Hickman line infection
4	9	3	Candida septicaemia treated by amphotericin B
5*	14	12	Aortic valve endocarditis
6*	2	2	Infected central line
7*	3	3	Infected central line

*These three cases were all on the intensive care unit at St Bartholomew's Hospital at the same time.

Pooled human antiserum was obtained from case 5 and used to fingerprint the 17 individual isolates from different patients and all the isolates from cases 5, 6, and 7.

IMMUNOBLOT FINGERPRINTING

Preparation of isolates

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

Gel electrophoresis

The enzyme extracts were solubilised in 2.5% sodium dodecyl sulphate (SDS) and 1.3% 2-mercaptoethanol at 100°C for four minutes. Electrophoresis was carried out on a 10% polyacrylamide gel in a discontinuous buffer system. Extract (60 µl) was applied to each well. The following molecular weight standards were used: myosin, 200 000; betagalactosidase, 11 650; phosphorylase B, 92 500; bovine serum albumin, 66 200; ovalbumin 45 000; carbonic anhydrase 31 000; soybean trypsin inhibitor, 21 500, and lysozyme, 14 400.

Immunoblotting

The preparations were transferred from gel to nitrocellulose paper in a LKB Trans-Blot cell with a current of 0.6A for 45 minutes 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 nitrocellulose was then incubated at 25°C for two hours with the rabbit or human antiserum, diluted 1 in 50 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 one hour with alkaline phosphatase conjugated goat antirabbit IgG (Sigma), or alkaline phosphatase conjugated goat antihuman IgG and IgM (Sigma), used at a 1 in 1000 dilution in 3% bovine serum albumin. After washing again this was incubated for 15 minutes with equal volumes of naphthol AS-MX phosphate (Sigma; 0.4 mg/ml in distilled water) and fast red TR salt (Sigma; 6 mg/ml in 0.2 M, Tris, pH 8.2).¹²

OTHER TYPING SYSTEMS

Antibiograms

This was performed by a disc susceptibility method on Isosensitest agar (Oxoid CM 471). Ten µg discs of methicillin, tetracycline, gentamicin, erythromycin, fusidic acid and chloramphenicol were used as well as

2 µg discs of rifampicin and clindamycin. Penicillin was tested with a 1 µg disc. All tests were performed at 35°C except methicillin which was performed at 30°C.

Biochemistry and phage typing

Isolates were biotyped by the commercially available API Staph system (API SA System, La Balme, les Grottes, France) and phage typed by the Division of Hospital Infection, Colindale.

Results

Immunoblot fingerprinting detected multiple antigenic bands with molecular weights varying from 27 to 250 kD. The technique was evaluated by three criteria: typability, reproducibility, and ability to discriminate between two isolates that were considered to be distinct on clinical and bacteriological grounds. All isolates were typable by this method. Reproducibility has presented problems when analogous techniques have been applied to other micro-organisms.¹³ To compensate for this in the case of *C. albicans*, all the isolates were run on the same gel and prepared with the same batch of enzyme. When all the isolates of coagulase negative staphylococci were examined twice, however, the degree of reproducibility was sufficient for a direct comparison between two immunoblots.

A difference in the position and intensity of three antigenic bands has been the criterion used to decide whether two isolates are definitely different.¹⁴ The results of nine of the 17 systemic isolates from 17 different patients are shown in figs 1 to 3. The anti-*S. epidermidis* rabbit hyperimmune antiserum detected 19 antigenic bands and showed at least three antigenic band differences between all isolates with the exception of isolates 5 and 7 which were identical (fig 1). The

Fig 1 Nine systemic isolates stained by anti-*S. epidermidis* serum.

Fig 2 Nine systemic isolates stained by rabbit anti-*S aureus* serum.

anti-MRSA rabbit antiserum detected 18 antigenic bands and found at least three differences between all isolates with the exception of isolates 5 and 7 which were identical (fig 2). The human hyperimmune serum from case 5 identified 13 antigenic bands only and produced less than three antigenic band differences between isolates 5, 7, and 8 (fig 3). Isolates 5, 7, and 8 (table 2) were all non-typable by phages and were biotype SVI(I). Isolates 5 and 7 had the same antibiogram (table 2). The SII biotype isolates, shown by tracts 2, 4, 6 and 9, are clearly differentiated from each other by all three antisera. In the remaining eight isolates (data not shown) there were more than three differences between each set of isolates. Only two of the 17 isolates were typable by phages and eight were biotype SII. Antibiograms produced 11 different types compared with the 16 seen by fingerprinting with the rabbit anti-*S epidermidis* hyperimmune antiserum.

Fig 3 Nine systemic isolates stained by human antiserum.

The multiple isolates from individual patients were thus fingerprinted by this antiserum and in cases 5–7 by the human antiserum from case 5.

The results of immunoblot fingerprinting of the multiple isolates from the patients correlated well with antibiograms, biotyping, phage typing and clinical history (table 3). In cases 1, 2, and 3 a pattern of a predominant isolate, which was untypable by phage typing, indistinguishable by biotyping, and identical by antibiogram and immunoblot typing, was observed (table 3). Fifteen of the 18 isolates from case one were identical on all the test systems examined and two of the remaining three showed distinct immunoblot patterns, phage types, biotypes and antibiograms. The final isolate had a distinct immunoblot pattern. Four isolates from case 3 are shown in fig 4. In case 4 all three isolates were untypable by phage typing and indistinguishable by biotyping but were distinguish-

Table 2 Details of phage typing, biotype, and antibiograms from 17 systemic isolates

Isolate No	Phage typable	Biotype	Antibiogram							
			P	M	F	R	C	E	G	V
1	—	M3	R	R	R	S	S	S	S	S
2	—	SII	R	S	S	S	S	S	S	S
3	—	SV	R	R	R	R	S	S	R	S
4	—	SII	R	S	R	S	R	R	R	S
5	—	SVI(I)	R	R	S	S	R	R	R	S
6	+ *	SII	R	S	S	S	S	S	S	S
7	—	SVI(I)	R	R	S	S	R	R	R	S
8	—	SVI(I)	R	S	S	S	S	S	S	S
9	+ †	SII	R	S	S	S	S	S	R	S
10	—	SVI(3)	S	S	S	S	S	S	S	S
11	—	SV	R	R	S	R	S	S	S	S
12	—	SII	R	R	R	S	R	R	R	S
13	—	SVI(3)	S	S	S	S	S	S	S	S
14	—	SV	R	R	S	R	S	S	R	S
15	—	SII	R	R	S	S	S	S	S	S
16	—	SII	R	S	S	S	S	S	S	S
17	—	SII	S	S	S	S	S	S	S	S

*phage type 27/155/A6C/A9C/71/275/48/456/471/A/459/275A/B1; †phage type 155/A6C/A9C/456/459.
P: penicillin; M: methicillin; F: fusidic acid; R: rifampicin; C: chloramphenicol; E: erythromycin; G: gentamicin; and V: vancomycin

Fig 4 Tracts 1-3, isolates from case 4; tracts 4-7, isolates from case three; tract 8, biotype SII isolates from case five; tract 9, biotype SII isolate from case six; tract 10, biotype SII isolate from case seven; All isolates stained by anti-*S. epidermidis* serum.

able by antibiograms and immunoblotting (fig 4, tracts 1-3). There was greater than three antigenic band differences between each of the isolates (fig 4, tracts 1-3). This was compatible with the clinical history, which suggested that the blood culture isolates were contaminants.

Cases 5, 6, and 7 formed a cluster on the intensive care unit at St Bartholomew's Hospital. All 17 isolates produced the same antibiogram and 13 were biotype SII (table 3). Phage typing split the isolates from case 5 into two main groups, but all produced the same pattern on immunoblot fingerprinting. The non-biotype SII isolates from all three cases produced greater than six differences from each other and the SII isolates on immunoblotting. An example of an SII isolate from each of the cases is shown in fig 4 (tracts 8,

9, and 10). These were different from each other, confirming the existence of a cluster of isolates on the unit rather than an outbreak. The pattern of results with the human antiserum was identical, although only eight antigenic bands were detected.

Discussion

Coagulase negative staphylococci are the organisms most commonly isolated from blood cultures.¹⁵ The identification of a true infection rather than multiple contaminants is based on the repeated isolation of organisms with an identical phenotype from several sets of blood culture bottles. This phenotype has been defined by markers which have included antibiograms, plasmid profiles, biotypes and phage types. Antibiograms can be difficult to reproduce and isolates from different patients may have the same pattern.^{4,16} In this study the 17 systemic isolates produced only 11 different antibiograms (table 2); the isolates from cases 5, 6, and 7 had identical antibiograms (table 3).

Plasmid profile analysis has been regarded as a more precise method, but is only marginally better in sensitivity, and difficult to interpret.¹⁷ Antibiotic resistant plasmids transfer between coagulase negative and coagulase positive staphylococci and between strains of coagulase negative staphylococci, which is an important factor to consider if they are the basis of a typing scheme.¹⁸

Compared with phage typing, the immunoblot technique has the advantage that all isolates are typable. It was clearly more sensitive than biotyping as biotype SII has been shown to cause up to 75% of infections.⁷ In the present series 42 isolates had this biotype so that it was essential to be able to subdivide

Table 3 Details of phage typing, biotype, and antibiograms of multiple isolates from cases 1-7

Case No	Isolate No	Phage typable	Biotype	Antibiogram							
				P	M	F	R	C	E	G	V
1	1-16	—	SV	R	R	S	S	S	S	R	S
	17	—	SVI(2)	S	S	S	S	S	S	S	S
	18	+*	SVI(3)	S	S	S	S	S	S	S	S
2	1-6	—	SII	S	S	S	S	S	S	S	S
3	1-12	—	SII	R	R	S	S	S	S	R	S
4	1	—	SII	S	S	S	S	S	S	S	S
	2	—	SII	R	R	S	S	S	S	S	S
	3	—	SII	R	R	S	S	S	S	R	S
5	1-4	—	SII	R	R	R	R	R	R	R	S
	5-10	+†	SII	R	R	R	R	R	R	R	S
	11	—	M6	R	R	R	R	R	R	R	S
6	12	—	SVI	R	R	R	R	R	R	R	S
	1	—	SII	R	R	R	R	R	R	R	S
	2	+‡	SII	R	R	R	R	R	R	R	S
7	1	—	SV	R	R	R	R	R	R	R	S
	2	—	SVI	R	R	R	R	R	R	R	S
	3	—	SII	R	R	R	R	R	R	R	S

*phage A6c; inhibition reaction 155/B1; †phage A6c; ‡phage 155/A9c.

P: penicillin; M: methicillin; F: fusidic acid; R: rifampicin; C: chloramphenicol; E: erythromycin; G: gentamicin; and V: vancomycin

it. On clinical grounds, excluding multiple identical isolates from the same patients, this represented 17 different isolates. Antibigrams identified nine separate patterns and four isolates produced distinct phage types. Immunoblot fingerprinting with the rabbit anti-*S. epidermidis* serum as probe produced at least three antigenic band differences between each of these isolates. Reproducibility has been a problem with other fingerprinting systems,¹³ but with staphylococci the lysostaphin based breakdown produced sufficiently similar extracts for gel to gel comparison.¹⁴ We confirmed this, provided that the same batch of antiserum was the antibody probe. The rabbit hyperimmune antisera were better than the human sera at differentiating between isolates. Isolates 5 and 7 of the 17 systemic cases were identical with all three antisera, isolate 8 could only be differentiated by the rabbit hyperimmune antisera.

Immunoblot fingerprinting also showed the similarity of multiple isolates from the same patient when these were thought to be a single pathogenic strain rather than multiple different contaminants. In case 1 of the 16 isolates which were biotype SV, 15 were identical on immunoblot fingerprinting. This confirmed the clinical importance of the strain and allowed one isolate to be selected for further antibiotic sensitivity testing. In case 5, another case of endocarditis, immunoblot fingerprinting showed the existence of one predominant strain despite the presence of two distinct phage types.

The clinical and epidemiological applications of the technique are well illustrated by the results from cases 5, 6, and 7. All three cases occurred on the same unit within seven days of each other. The antibigrams were identical, suggesting a cross infection problem. The isolates were fingerprinted and this rapidly confirmed that: 10 of the 12 isolates from case 5 were identical despite the presence of two phage types; the biotype SII, non-phage typable isolates of case 6 were different from those of case 5 despite identical antibigrams; the isolates from case 7 were all different from each other and from the isolates from cases 5 and 6. This indicated that the cluster was not due to cross infection from a single source. The problem resolved spontaneously and there had been no further cases on the unit at the time of writing (four months).

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