Fingerprinting methicillin-resistant *Staphylococcus aureus* by the immunoblot technique

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Summary. A series of 133 isolates of methicillin-resistant *Staphylococcus aureus* was fingerprinted by the immunoblot technique. Extracts were prepared by lysostaphin degradation of overnight cultures and peptides were separated by SDS polyacrylamide gel electrophoresis. The peptides were transblotted on to nitrocellulose membranes and probed with (1) a hyperimmune rabbit serum raised against a methicillin-resistant *S. aureus* isolate, (2) a hyperimmune rabbit serum raised against an isolate of *S. epidermidis*, and (3) serum from a patient who had recovered from an infection with a methicillin-resistant *S. aureus*. This typing method confirmed the existence of an epidemic strain that accounted for 102 of the isolates. The remaining 31 isolates were grouped into a further seven types which correlated with the results of phage typing and antibiograms.

Introduction

Recently there has been an increase in interest in methicillin-resistant *Staphylococcus aureus* (MRSA) as a cause of serious nosocomial infection in hospitals. Some affected hospitals report that MRSA may account for 20–40% of all clinical isolates of *S. aureus* (Combined working party, 1986). Resistance to methicillin means that treatment with flucloxacillin and the other isoxazolyl penicillins is contraindicated (Sanderson, 1986). These isolates are usually resistant also to penicillin, gentamicin, cephalosporins, erythromycin and tetracyclines. Therefore, the mainstay of treatment is vancomycin (McDonald *et al.*, 1981; Marples *et al.*, 1986). This antibiotic is toxic and needs regular monitoring; to avoid its unnecessary use, the correct identification of methicillin-resistant isolates is important.

There has been much debate about the pathogenicity of MRSA strains and it has been suggested that the genetic material required for multiple antibiotic resistance might limit their ability to spread from patient to patient (Lacey and Chopra, 1975). More recent epidemiological data have suggested that there are strains of MRSA that are as virulent as methicillin-sensitive strains (Crossley *et al.*, 1979; Boyce *et al.*, 1981; Peacock *et al.*, 1981) whereas others are less virulent (Lacey *et al.*, 1986).

The results of phage typing and antibiograms have confirmed that there is more than one type of MRSA and have demonstrated a predominant outbreak strain. This strain is recognised by its typing weakly with phage 85, occasionally strongly with phage 84, and strongly with experimental phages 88A and 932 (Marples *et al.*, 1986). Isolates of this type have caused major outbreaks (Hone *et al.*, 1981; Bradley *et al.*, 1985; Shanson *et al.*, 1985) the control of which is expensive and time consuming. Clearly it would be advantageous to be able to differentiate between isolates of the epidemic MRSA (EMRSA) type and other methicillin-resistant staphylococci without recourse to the reference laboratory. This would confirm the results of antibiotic testing and allow the rapid instigation of control measures.

The examination of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of different staphylococcal species has shown that each species produces a pattern distinct for that species. However, the patterns could not be used for typing isolates within species (Clink and Pennington, 1987). SDS-PAGE typing with [³⁵S]-methionine has been valuable in fingerprinting isolates but this is an expensive technique that requires the use of radiolabels and it has been applied to only a few isolates (Stephenson *et al.*, 1986). Isolates of methicillin-sensitive *S. aureus* have been typed by immunoblotting culture supernate against human antiserum (Krikler *et al.*, 1986). The results agreed with those found by phage

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typing but the technique was not extended to methicillin-resistant strains. Isolates were grouped after visual inspection of immunoblots on the basis of the possession of bands unique to a group, band intensity and the presence or absence of bands common to several groups. A single antibody probe was used.

We developed a similar immunoblot fingerprinting system for *Candida albicans* (Lee *et al.*, 1986) and demonstrated that the enzymic breakdown of whole cells produced a better antigenic preparation in terms of the amount of protein available for typing and reproducibility. Isolates identical on fingerprinting with one antibody probe remained indistinguishable with a second probe although the overall pattern might have been quite different (Burnie and Matthews, 1987). It was shown that a difference in three antigenic bands, as defined by the presence or absence of a particular band or a very great variation in its density, was necessary to prove that two isolates were definitely different. Discrimination could be improved by examining the results obtained with multiple antibody probes (Lee *et al.*, 1986).

This paper presents data on 133 methicillin-resistant isolates of *S. aureus* immunoblotted against three antibody probes. Lysostaphin-degraded whole-cell extracts of all the isolates were tested against a rabbit hyperimmune antiserum raised against a French-press extract of an EMRSA strain. Examples of the different types generated were then fingerprinted with a serum from a patient who had recovered from a systemic infection with the EMRSA and against a rabbit hyperimmune antiserum raised against a French-press extract of an isolate of *S. epidermidis*.

Materials and methods

Isolates

The test series of 133 isolates was obtained from the London Hospital (87), the London Chest Hospital (3), St Bartholomew's Hospital (10), Reading (1), Oldchurch (2), Hull Royal Infirmary (8), St James' Hospital, Leeds (2), Pinderfields Hospital, Wakefield (6), St Luke's Hospital (9), Australia (4) and Egypt (1). They were all slide- and tube-coagulase positive.

Antibiograms

These were determined by a disk susceptibility method on Isosensitest Agar (Oxoid CM 471) with 10- μ g disks of methicillin, tetracycline, gentamicin, erythromycin, neomycin, fusidic acid and chloramphenicol, 2- μ g disks of rifampicin and clindamycin and 1- μ g disk of penicillin.

All tests were performed at 35°C with the exception of that with methicillin which was performed at 30°C.

Phage typing

This was performed by the Division of Hospital Infection, Colindale by the standard method (Parker, 1972).

Immunoblotting

Preparation of antigen. Isolates were grown overnight in 2 ml of Tryptone Soya Broth at 37°C. Cells were harvested by centrifugation at 3000 *g* for 10 min. The pellet was resuspended in 100 μ l of sterile distilled water with 100 μ l of lysostaphin (Sigma) solution, initial concentration 200 μ g/ml, and incubated for 30 min on a shaking tray at 37°C; 60 μ l of the resulting extract was loaded into each well.

Preparation of antiserum (i) Rabbit. An isolate of the EMRSA, as defined by antibiograms and phage typing (Marples *et al.*, 1986), was grown overnight at 37°C on Blood Agar (Oxoid). Growth was harvested, centrifuged at 3000 *g* for 10 min, resuspended and washed three times, all in distilled water. The washed cells were then treated in a French press at 12 m Pascals twice, and centrifuged at 12 000 *g* for 10 min. New Zealand White rabbits were inoculated with the resulting supernate; 25 mg was mixed with 1 ml of sterile water and 1 ml of Freund's complete adjuvant. Animals were inoculated twice, with 14 days between, and bled after 28 days. The hyperimmune rabbit antiserum against the *S. epidermidis* isolate was prepared similarly, as described by Burnie and Matthews (1987).

(ii) Human. Several samples of human serum were obtained from a patient with an abdominal aortic graft infected with the epidemic strain of MRSA and pooled. This serum was shown by immunoblotting to be hyperimmune to the EMRSA.

Gel electrophoresis. The enzyme extracts were solubilised in sodium dodecyl sulphate (SDS) 2.6% and 2-mercapto-ethanol 1.3% at 100°C for 5 min. Electrophoresis of these preparations was performed with 10% polyacrylamide gels in a discontinuous buffer system (Laemmli, 1970). The following mol. wt (10^3) standards were applied: myosin 200; β -galactosidase 116.25; phosphorylase B, 92.5; bovine serum albumin 66.2; ovalbumin 45; carbonic anhydrase 31; soybean trypsin inhibitor 21.5; and lysozyme 14.4.

Immunoblots. Proteins were blotted on to nitrocellulose paper (Bio-Rad Laboratories, Richmond, CA, USA) in 25 mM Tris, 192 mM glycine buffer, pH 8.3, containing methanol 20%. Transfer was performed at 25°C with a current of 0.5 A for 45 min in an LKB Trans-Blot cell. Free protein sites were saturated by incubation in bovine serum albumin (Sigma) 3% in buffered saline (NaCl 0.9% and 10 mM Tris, pH 7.4) at 4°C overnight. The nitrocellulose membrane was then incubated at 25°C for 2 h with either the rabbit antiserum (diluted 1 in 50) or the human serum (diluted 1 in 10) in bovine serum albumin 3% and

Tween 20 0.05%. After washing five times in saline 0.9% and Tween 20 0.05%, the nitrocellulose was incubated for 1 h at 25°C with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) or, if a human antibody was the probe, a mixture of anti-human-IgG and anti-human-IgM immunoglobulin (Sigma), used at a 1 in 1000 dilution in bovine serum albumin 3%. After washing again, the membrane was incubated for 15 min with freshly prepared mixtures of equal volumes of naphthol ASMX phosphate (Sigma; 0.4 mg/ml in distilled water) and fast red TR salt (Sigma; 6 µg/ml in 0.2 M Tris, pH 8.2).

Results

Immunoblotting detected multiple antigenic bands with mol. wts (10^3) 38–175; 15 of these were selected for the development of the fingerprinting system on the basis of their reproducibility between immunoblots. Probing with the rabbit hyperimmune anti-MRSA antibody confirmed the homogeneity of the EMRSA isolates. Immunoblots of extracts of six EMRSA isolates are illustrated in fig. 1, all from patients at the London Hospital. Tracks 2 and 3 were sequential isolates from the same patient. When the whole series of 133 isolates was examined eight different types were delineated

Fig. 1. Six isolates of the EMRSA (type 2) fingerprinted by the rabbit anti-EMRSA hyperimmune serum.

Table I. Results of immunoblot fingerprinting MRSA's

Type	Number of isolates	Comments
1	3	Rifampicin resistant variant of the EMRSA
2	102*	EMRSA
3	1	Indistinguishable from EMRSA except typed with phage 83A
4	6	All from Pinderfields Hospital. Not EMRSA
5	1	Penicillin sensitive. Not EMRSA
6	16	All from Hull Royal Infirmary, St. James' Hospital or St. Luke's Hospital. Not EMRSA
7	3	Restricted to Hull Royal Infirmary. Not EMRSA
8	1	Resistant to all antibiotics tested except vancomycin; non-typable

* 102 EMRSA isolates from the London Hospital (85), St Bartholomew's Hospital (10), Reading (1), Australia (4) and Oldchurch (2).

(table I). These produced identical patterns when stained by Coomassie blue (fig. 2) but different patterns when transferred on to nitrocellulose membrane and probed by the rabbit anti-MRSA serum (fig. 3), rabbit anti-*S. epidermidis* serum (fig. 4) and the human serum (fig. 5). Antibody binding to a particular antigenic band was categorised as absent, trace or strong (tables II–IV).

Fingerprinting systems can be assessed by three standards: typability, reproducibility and discrimination. All isolates of methicillin-resistant *S. aureus* were typable by this method. Reproducibility between immunoblots was assessed by repeating tests with each isolate at least three times on different gels. Previous immunoblot fingerprinting methods have advocated the sequential analysis of isolates, one representative from each type being run on each subsequent gel to avoid the necessity of comparison between gels. This system was necessary for the fingerprinting of *C. albicans* (Lee *et al.*, 1986) but the results for staphylococci were much more reproducible so that direct comparison between gels was possible. Track 2 in both figs 1 and 3 represents isolates of the EMRSA fingerprinted by the rabbit hyperimmune antiserum. The same pattern of antigenic bands was detected in both extracts although there was some difference in the intensity of the bands at mol. wts (10^3) 160, 132, 116, 75 and 57 because of the use of a different batch of rabbit antiserum.

The third criterion is discrimination. When isolates were typed by the rabbit hyperimmune

Fig. 2. Coomassie blue-stained extracts representing the eight types. The numbers refer to the type number given in table I. Mol. wts (10^3) are represented by the markers in the left hand lane.

Fig. 3. Immunoblot of the eight types stained by the rabbit anti-EMRSA hyperimmune serum. The numbers refer to the type numbers given in table I. Mol. wt (10^3) are represented by the markers in the right hand lane.

Fig. 4. Immunoblot of the eight types stained by the rabbit anti-*S. epidermidis* hyperimmune serum. The numbers refer to the type numbers given in table I.

Fig. 5. Immunoblot of the eight types stained by human serum. The numbers refer to the type numbers given in table I.

Table II. Results of the MRSA rabbit antiserum fingerprinting of the eight type strains

Band Mol. wt (10 ³)	Presence of the band in type							
	1	2	3	4	5	6	7	8
38	+	+	+	+	+	+	+	+
40	-	-	-	-	-	-	+	+
50	+	+	+	+	+	+	+	+
54	-	-	-	+	-	-	+	-
57	tr	tr	tr	+	-	-	+	-
61	+	+	+	+	+	+	+	+
68	-	-	-	-	-	-	+	+
70	tr	tr	tr	-	tr	-	+	+
75	+	+	+	+	+	+	+	+
92	+	+	+	tr	tr	tr	+	+
116	+	+	+	tr	-	tr	+	-
132	+	+	+	+	+	+	-	+
145	-	-	+	-	-	-	+	-
160	+	+	+	+	+	+	+	+
175	-	-	-	+	-	+	-	-

- = Absent; tr = trace; + = strong.

Table III. Results of the *S. epidermidis* rabbit antiserum fingerprinting of the eight type strains

Band Mol. wt (10 ³)	Presence of the band in type							
	1	2	3	4	5	6	7	8
38	tr	+	tr	-	-	-	+	+
40	-	+	-	-	-	-	+	+
50	+	+	+	+	+	+	+	+
54	+	+	+	+	+	+	+	+
57	-	-	-	+	-	-	+	-
61	-	-	-	-	-	-	+	+
68	+	+	+	+	+	+	+	+
70	+	+	+	-	+	-	+	+
75	+	+	+	+	+	+	+	+
92	-	+	+	-	+	+	+	+
116	+	+	+	+	+	+	+	+
132	+	+	+	+	+	+	+	+
145	-	-	+	tr	tr	-	+	tr
160	+	+	+	+	+	+	+	+
175	-	-	-	+	-	+	-	-

- = Absent; tr = trace; + = strong.

MRSA antiserum, types 1 and 2 were indistinguishable and type 3 showed one difference at mol. wt 145×10^3 from these two types (table II; fig. 3). When the isolates were fingerprinted with the *S. epidermidis* antiserum, there were three differences between types 1 and 2, and 2 and 3, and two differences between types 1 and 3, and 7 and 8 (table III, fig. 4). When fingerprinted with the

human antiserum, there were two differences between types 1 and 2, 1 and 3 and only one difference between types 7 and 8. All other combinations with each antiserum showed at least three differences. When the results of the three antibody probes were combined only the combinations of type 1 versus type 2 (four differences) and type 1 versus type 3 (five differences) produced less than six differences between strains. The combination of type 2 and type 3 produced seven differences. The antibiograms (table V) and phage-typing results (table VI) confirm the similarity between these three types. Type 1 is a rifampicin-resistant variant of the epidemic MRSA (type 2). Type 3 was phage-typed with phage 83A but was otherwise a typical epidemic MRSA. The other types all showed considerable differences from the epidemic MRSA in both antibiograms (table V) and phage typing (table VI).

Discussion

This paper described a new technique for fingerprinting MRSA strains. It confirms the finding that the Coomassie blue-stained protein profiles are virtually identical for all isolates within the species (Clink and Pennington, 1987). It demonstrates that there are at least eight different types when the same antigenic extracts are immunoblotted against three different antibody probes.

The system differed from phage typing in that all isolates were typable. Type 8 did not type with any

Table IV. Results of the human antiserum fingerprinting of the eight type strains

Band Mol. wt (10 ³)	Presence of the band in type							
	1	2	3	4	5	6	7	8
38	+	+	tr	tr	tr	tr	+	+
40	+	+	tr	tr	tr	tr	+	+
50	tr	+	tr	tr	tr	tr	+	+
54	tr	tr	tr	+	tr	tr	+	+
57	-	-	-	+	-	-	+	+
61	+	+	+	+	+	+	+	+
68	+	+	+	+	+	+	+	+
70	+	+	+	+	+	+	+	+
75	+	+	+	-	tr	tr	+	+
92	+	+	+	+	+	+	+	+
116	+	+	+	+	+	+	+	+
132	+	+	+	-	tr	+	-	-
145	+	-	+	-	+	-	-	-
160	+	+	+	+	+	+	tr	-
175	-	-	-	+	-	+	tr	tr

- = Absent; tr = trace; + = strong.

Table V. Antibigrams of the eight types

Type	Sensitivity to										
	Clin	Ery	Fuc	Gent	Pen	Meth	Chlo	Neo	Rif	Tet	Vanc
1	R	R	S	R	R	R	S	S	R	R	S
2	R	R	S	R	R	R	S	S	S	R	S
3	R	R	S	R	R	R	S	S	S	R	S
4	R	R	S	S	R	R	S	R	S	R	S
5	R	R	R	S	S	R	S	R	S	R	S
6	R	R	S	S	R	R	R	R	S	R	S
7	R	R	S	R	R	R	S	R	R	R	S
8	R	R	R	R	R	R	R	R	R	R	S

R = resistant; S = sensitive. Clin, clindamycin; Ery, erythromycin; Fuc, fucidic acid; Gent, gentamicin; Pen, penicillin; Meth, methicillin; Chlo, chloramphenicol; Neo, neomycin; Rif, rifampicin; Tet, tetracycline; Vanc, vancomycin.

Table VI. Results of phage typing

Type	Phage type at	
	RTD	RTD × 100
1 ^a	NT	NT
2 ^a	NT	85
3	NT	83A/85
4	47/54/75	29/47/54/75/77/85
5	75	75
6	47/54	42E/47/53/54/75/85
7	NT	6/42E/53/54/75/85/81
8	NT	NT

^a typed with phages 88A and 932 in addition.
NT = non-typable.

of the phages tested and type 1 only typed with the experimental phages 88A and 932. Phage typing has the additional problem that there is considerable variation in the results obtained with the epidemic strain. Most type weakly with phage 85, some strongly with phage 84 and others with experimental phages 88A and 932 (Marples *et al.*, 1986). Reproducibility was much better than with a previous immunoblot fingerprinting system developed for *C. albicans* (Lee *et al.*, 1986), in which it was important to compare all isolates on the same gel. This approach also had to be used with the [³⁵S]-methionine labelled typing system for *S. aureus* (Stephenson *et al.*, 1986). Variation in the results obtained with the method of staphylococcal fingerprinting described here was sufficiently small for the direct comparison of immunoblots between gels to be possible, provided that the same batch of antiserum was used for probing.

The discrimination produced by each individual

antibody probe can be assessed by its ability to distinguish between two types. It was difficult to separate types 1, 2, and 3 with any of the three antibody probes, but these three types were also very similar on phage typing and antibiograms. The human serum and rabbit *S. epidermidis* antiserum identified only slight differences between types 7 and 8 despite considerable variation in their phage typing (table V), antibiograms, (table VI), and immunoblots probed by the rabbit anti-MRSA antiserum. Apart from these exceptions all of the antibody probes were capable of distinguishing between each of the types.

In terms of the epidemiology and pathogenicity of the MRSA, these results have several consequences. Type 2 represents the epidemic strain which is virulent and capable of spreading to cause major outbreaks. This type included four isolates from Australia confirming the similarity of the isolates from that country to the UK epidemic strain. Types 1 and 3 were very similar. The ability to identify these isolates by their immunoblot profile from other methicillin resistant staphylococci should facilitate documentation of the spread of the epidemic strain.

Type 4 consists of six isolates from Pinderfields Hospital. These fingerprinted as a unique group regardless of which antibody probe was used. They constitute a group of isolates with reduced virulence compared to the outbreak strain (Lacey *et al.*, 1986) and can now be seen to have a totally different fingerprint confirming that they constitute a different micro-organism. Types 5 and 8 are unusual organisms with unique antibiograms (tables I and VI). Types 6 and 7 represent strains from small clusters of cases. They did not cause large outbreaks but could be isolated from several patients in the

hospitals involved. These clusters were largely self limiting in contrast to the large outbreak caused by the EMRSA.

In summary, this paper demonstrates the use of immunoblotting to identify isolates of the EMRSA and differentiate them from other methicillin resistant strains. These strains, despite being

methicillin resistant, are often less virulent and less likely to cause major epidemics so that their distinction from the EMRSA is of considerable importance.

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