Characterisation of methicillin-resistant isolates of *Staphylococcus aureus* by analysis of whole-cell and exported proteins

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Summary. Thirty-four isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) from patients in Glasgow Royal Infirmary were studied. Whole-cell protein profiles obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were compared with banding patterns produced by immunoblots of exported proteins. Human plasma was used as a source of staphylococcal antibodies for the immunoblots. SDS-PAGE of whole-cell extracts did not usefully distinguish different isolates of MRSA. Reproducible banding patterns were obtained by immunoblots of exported proteins. Analyses of immunoblots by use of the Dice coefficient demonstrated that isolates of MRSA could be divided into two main groups. Immunoblots of exported proteins provided a rapid, reproducible and sensitive method for characterisation of MRSA.

Introduction

Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) were reported in Britain shortly after the introduction of methicillin (Barber, 1961; Jevons, 1961). Since the 1960s, MRSA have caused major nosocomial infections world-wide, most recently in Italy (Gelmi *et al.*, 1987), the German Democratic Republic (Witte *et al.*, 1986), the United Kingdom (Bradley *et al.*, 1985), the United States of America (Locksley *et al.*, 1982) and Australia (Pavillard *et al.*, 1982). Further significant outbreaks have been reported elsewhere (Casewell, 1986).

Despite the clinical importance of MRSA, a satisfactory scheme for the characterisation of strains has yet to be established. Various properties of MRSA have been investigated in attempts to facilitate typing—e.g., pigmentation (Lacey and Stokes, 1979); biochemical characteristics (Peacock *et al.*, 1981; Vickery *et al.*, 1983) lipolytic activity and metal ion resistance (Grieble *et al.*, 1981); esterase polymorphisms (Branger and Goullet, 1987); susceptibility to lysis by phage (Marples *et al.*, 1986; Vickery *et al.*, 1986; Witte and Bräulke, 1987; Richardson *et al.*, 1988); and possession of plasmids conferring resistance to antibiotics (Dowd *et al.*, 1983; Gillespie *et al.*, 1984; Lyon *et al.*, 1984; Gelmi *et al.*, 1987). However, results obtained by

these methods have proved difficult to interpret for the following reasons: biochemical tests did not assist strain differentiation (Vickery *et al.*, 1983); loss of susceptibility to certain routine phages (Lacey and Stokes, 1979; Marples *et al.*, 1986); significant numbers of MRSA isolates were non-typable with routine phage panels (de Saxe *et al.*, 1983); non-typability with routine phages has necessitated the introduction of additional test phages (Vickery *et al.*, 1983; Marples and Cooke, 1985) and rapid spread of plasmids among MRSA has been observed to alter both plasmid profiles and antibiotic resistance (Gillespie *et al.*, 1984). Other techniques such as electrophoresis of polymorphic enzymes (Branger and Goullet, 1987) involve lengthy experimental procedures. Analyses of MRSA whole-cell polypeptides by ³⁵S-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were unable to discriminate different strains (Stephenson *et al.*, 1986).

Immunoblotting of bacterial whole-cell polypeptides, by the Western blot technique, has been suggested as a method for “fingerprinting” pathogens (Burnie and Matthews, 1987). Poxton *et al.* (1984) used immunoblotting to characterise different strains of *Clostridium difficile*, and MRSA isolates have been divided into eight groups according to their whole-cell immunoblot profile (Lee and Burnie, 1988). In an alternative approach, immunoblotting of exported proteins has also been used successfully to type isolates of *S. aureus*

(Krikler *et al.*, 1986). The aim of the present study was to compare different MRSA isolates by SDS-PAGE of whole-cell proteins, and by immunoblotting of exported proteins.

Materials and methods

Bacterial strains

MRSA isolates from 34 patients were obtained from Dr J. Coia, Glasgow Royal Infirmary. All isolates were identified as *S. aureus* on the basis of colonial morphology, positive coagulase and Staphaurex[®] latex agglutination tests (Coia *et al.*, personal communication). Phage typing was performed at the Central Public Health Laboratory, Colindale, UK, with routine and experimental phages.

Preparation of samples

Isolates of MRSA were plated out on nutrient-agar plates and incubated at 37°C for 24 h. A sweep of colonies from these plates sufficient to give approximately 2 µg bacterial dry weight/ml was inoculated into 150-ml volumes of nutrient broth and incubated at 37°C overnight in an orbital incubator. The resulting broth cultures were centrifuged at 10 000 *g* for 15 min at 4°C and the upper 10 ml of each supernate was retained for gel analysis. The remainder of each supernate was discarded. The cell pellets were resuspended in 10-ml volumes of distilled water and washed by centrifugation at 2500 *g* for 15 min at 25°C. After resuspension in 1-ml volumes of distilled water, the pellets were washed again at 9000 *g* for 5 min at 25°C. The pellets were 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, England) emitting 45W at maximum power. Whole-cell sonicates were stored at -70°C, culture supernates were stored at -20°C. Protein concentrations were estimated by the method of Lowry *et al.* (1951).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Whole-cell sonicates and culture supernates were analysed by a modified version of the SDS-PAGE technique of Laemmli (1970). Samples were prepared for PAGE by mixing in proportions of two parts sample to one part 0.5 M Tris-HCl, pH 6.8, containing SDS 6% w/v, glycerol 30% w/v, 2-mercaptoethanol 15% w/v, and bromophenol blue 0.001% w/v and boiling for 5 min. Solubilised samples, containing an average of 120 µg and 40 µg protein for whole-cell extracts and culture supernates respectively, were applied to wells in a 3.6% acrylamide stacking gel over a 10% acrylamide separating gel. Electrophoresis was performed with a discontinuous buffer system in a BRL gel apparatus, model V16-2 (BRL, Gaithersburg, MD, USA). The gel was run at a constant

current of 30 mA until the bromophenol blue marker had reached the bottom, a period of 4–5 h. Whole-cell polypeptide gels were stained with Coomassie Brilliant blue R (Sigma) 0.25% w/v in methanol:acetic acid:water (45:10:45 v:v) and destained in the same solvent mixture before being swollen to original size in acetic acid 7% v/v.

Immunoblotting

Proteins from culture supernate gels were blotted on to nitrocellulose membranes in 25 mM Tris-HCl, 192 mM glycine buffer, pH 8.3, containing methanol 20% v/v (Burnette, 1981). Transfer was effected with a current of 100 mA overnight in a Bio-Rad Transblot cell. Free protein sites were saturated by incubation in blocking buffer containing newborn calf serum (Gibco, Paisley, Scotland) 10% v/v in phosphate-buffered saline, pH 7.4, Triton X-100 0.2% v/v for 30 min. The nitrocellulose membrane was then incubated in human plasma diluted 1 in 50 in blocking buffer for 1.5 h. Several samples of human plasma known to contain antibodies against a large number of staphylococcal polypeptides were pooled to provide test plasma (Krikler *et al.*, 1986). After washing three times for 15 min each in phosphate-buffered saline, pH 7.4, Triton X-100 0.2% v/v the nitrocellulose membrane was incubated with horseradish peroxidase-linked sheep anti-human IgG serum (Scottish Antibody Production Unit, Carlisle, Scotland), diluted 1 in 1000 in blocking buffer, for 45 min. After three further washes of 15 min each in phosphate-buffered saline, pH 7.4, Triton X-100 0.2% v/v the nitrocellulose membrane was rinsed in phosphate-buffered saline alone and stained with diaminobenzidine tetrahydrochloride (B.D.H., Poole, England) 0.05% w/v in 0.1 M Tris-HCl, pH 7.6, containing hydrogen peroxide 0.01% v/v.

Comparison of Coomassie-stained and immunoblot protein profiles

The average similarity between any two staphylococcal isolates represented by either Coomassie-stained gels or immunoblots was assessed by use of the coefficient of Dice (1945), whereby

$$\text{average percentage similarity (\%S)} = \frac{\text{Number of matching bands} \times 2}{\text{total number of bands in both isolates}}$$

Both whole-cell sonicates and culture supernates of all isolates were analysed several times.

Results

Whole-cell proteins

Whole-cell proteins were analysed by SDS-PAGE. Reproducible gel profiles were obtained

after staining with Coomassie blue and typical results are shown in fig. 1. Each whole-cell protein profile comprised 49–53 bands, with an average of 52. Visual inspection of gels showed several differences between isolates. However, these were almost entirely due to variations in band intensity, rather than the presence or absence of bands. Calculation of Dice coefficients showed high degrees of similarity between different isolates of MRSA (table I). The results shown are typical of several observations. The least degree of similarity observed, 4.2%, occurred between isolate nos. 272 and 292, no. 292 possessing 4 more bands than no. 272. Dice coefficients for all other comparisons were higher with 61 pairs of tracks being indistinguishable from each other. All other pairs differed by only 1–3 bands. The average similarity value was 98.9%. No constantly variable polypeptides could be identified. Analyses of whole-cell polypeptides by SDS-PAGE did not discriminate clearly between different isolates.

Exported proteins

Culture supernates which contained exported proteins were analysed by immunoblotting. Each track contained between 35 and 52 bands, with an average of 43, slightly fewer than were apparent in whole-cell polypeptide profiles. Visually the isolates could be divided into two groups. The first group consisted of isolate nos. 272, 274, 278, 288, 290, 291, 294, 51 and 159 and the second group consisted of isolate nos. 273, 277, 283, 289, 292, 295, 298, 83 and 26. Typical results are shown in fig. 2. Within each group much more variation occurred than between any pairs of tracks in whole-cell polypeptide gels. The spread of percentage similarity values was thus wider than those recorded for whole-cell polypeptide profiles (table II). The least degree of similarity observed, 73.7%, occurred between strain nos. 270 and 83. Only 10 pairs of tracks were indistinguishable from each other (table II) compared with 61 pairs in Coomassie-blue stained gels (table I). Average percentage similarities shown in

Fig. 1. Whole-cell polypeptide profiles of 18 different isolates of MRSA. Isolate numbers refer to the order in which they were isolated.

Table I. Average percentage similarity (%S) of whole-cell protein profiles of MRSA isolates shown in fig. 1

Isolate no.	Percentage similarity with isolate no.																	
	272	274	278	288	290	291	294	51	159	273	277	283	289	292	295	298	83	26
272		97.8	98.9	100	98.9	97.8	98.9	97.8	98.9	97.8	97.8	98.9	98.9	95.8	98.9	98.9	98.9	100
274			98.9	97.9	98.9	100	100	98.9	100	97.9	100	100	98.9	96.9	98.9	98.9	100	100
278				100	100	100	100	98.9	100	100	100	100	98.9	96.9	100	98.9	98.9	98.9
288					97.0	99.0	99.0	98.0	100	98.0	100	100	99.0	97.0	99.0	99.0	100	100
290						100	100	100	100	100	98.9	98.9	98.9	98.9	98.0	98.9	98.9	100
291							98.9	100	100	98.9	98.9	98.9	98.9	96.9	100	100	98.9	100
294								98.1	100	98.1	99.1	100	98.1	96.6	98.1	99.1	98.1	99.1
51									100	98.2	97.3	97.3	98.2	96.5	98.2	99.0	100	99.0
159										100	99.1	99.1	98.1	96.4	98.1	97.2	98.1	100
273											100	100	100	97.0	100	100	100	100
277												100	100	97.0	98.9	100	98.9	100
283													100	97.0	100	100	98.9	100
289														98.0	100	100	98.9	100
292															97.0	98.0	98.0	98.0
295																100	100	100
298																	98.9	100
83																		98.9
26

Fig. 2. Immunoblot analysis of culture supernates from the isolates shown in fig. 1.

Table II. Average percentage similarity (%S) of immunoblot profiles of MRSA isolates shown in fig. 2

Isolate no.	Percentage similarity with																																
	Group 1 isolates nos.														Group 2 isolates nos.														Group 3 isolates nos.				
	270	272	274	278	279	288	290	291	294	297	51	159	87	305	180	271	273	275	277	283	286	287	289	292	293	295	296	298	83	26	156	86	276
270	87.4	86.4	88.9	97.1	94.7	83.6	85.2	93.0	79.2	76.9	76.6	89.5	73.5	41.0	51.2	34.3	40.8	47.1	45.2	75.7	52.9	40.9	33.3	44.4	35.1	40.9	43.2	31.8	40.9	42.6	40.0	70.9	65.2
272	96.6	96.6	90.9	90.9	95.8	82.6	97.8	65.5	86.8	93.6	100	75.4	57.9	56.3	65.6	50.0	33.3	35.9	54.5	51.4	33.3	43.9	64.9	50.0	48.6	32.4	58.8	60.0	47.1	58.8	78.9	81.1	
274	91.9	93.3	91.8	92.8	93.2	90.9	73.0	86.9	94.7	95.7	86.9	62.1	64.5	53.8	52.6	39.0	34.3	57.9	58.8	61.5	52.6	54.5	50.0	61.5	43.5	60.4	64.7	51.1	46.8	80.9	70.9		
278	87.9	95.1	90.9	88.5	89.7	70.2	89.5	86.7	93.8	73.7	53.8	56.3	52.0	55.6	42.1	44.4	51.9	57.1	47.8	45.2	61.9	46.2	56.0	40.0	50.8	58.8	54.5	35.0	71.8	65.0			
279	91.2	88.2	85.4	95.0	81.5	73.9	81.6	87.2	78.6	39.0	55.6	26.3	34.8	40.0	50.0	60.6	58.8	43.2	43.5	34.3	33.3	44.9	37.5	38.3	42.9	40.6	42.9	77.4	61.3				
288	94.5	84.9	95.5	71.9	85.2	94.7	97.6	76.9	68.3	55.0	50.0	57.9	48.8	47.4	53.5	55.6	39.1	55.0	58.5	46.2	56.4	51.4	51.3	53.3	56.0	52.8	75.0	69.8					
290	96.7	89.2	92.9	100	92.3	83.3	94.1	52.4	53.8	72.7	58.5	38.3	35.3	55.3	50.9	66.7	43.1	63.8	32.7	64.3	42.6	60.4	48.0	47.5	53.9	65.3	69.4						
291	84.4	94.4	91.7	86.3	84.4	94.1	66.7	52.2	73.8	60.4	45.8	43.5	55.0	48.9	63.2	53.3	57.8	44.9	75.7	45.5	58.3	60.5	52.8	46.2	62.7	72.0							
294	78.8	94.7	100	97.6	85.2	70.9	59.5	52.6	51.6	42.1	55.0	60.6	55.0	54.9	51.3	63.6	47.6	59.5	46.2	51.3	51.3	59.6	56.6	79.1	77.3								
297	94.7	88.0	80.0	88.6	55.8	59.3	63.8	70.4	52.6	55.6	51.9	56.1	56.5	66.7	58.3	66.7	70.4	61.9	51.8	66.7	60.4	80.0	78.3	67.5									
51	92.0	96.2	93.9	48.6	51.2	75.9	59.6	60.9	52.0	54.5	47.8	67.9	62.7	51.2	50.2	59.6	40.0	61.8	50.0	66.7	76.7	66.7	74.5										
159	100	92.1	43.2	52.6	64.2	59.5	55.8	54.1	47.1	48.8	57.8	63.8	42.1	60.0	52.6	44.4	51.3	48.5	73.3	76.9	72.7	77.3											
87	85.2	68.6	48.9	65.6	63.8	43.9	43.2	51.3	58.5	57.1	55.8	50.0	48.9	65.1	46.2	45.5	76.2	53.3	54.2	84.4	68.9												
305	50.0	50.0	68.8	57.1	47.8	40.0	47.8	46.5	67.8	46.8	64.7	47.8	60.5	36.4	70.8	57.9	50.9	50.9	66.7	81.8													
180	77.4	81.4	86.9	75.7	93.0	80.6	83.7	80.0	84.6	78.9	89.6	95.0	80.9	93.6	87.6	93.1	75.0	56.0	72.7														
271	90.8	72.2	83.9	88.0	95.0	97.3	88.2	85.0	89.7	74.4	81.1	87.5	70.3	81.3	88.4	68.3	53.3	55.6															
273	91.7	91.4	81.1	85.6	84.2	97.3	97.6	57.1	97.6	95.5	100	95.8	80.9	97.8	70.0	57.1	50.0																
275	75.5	72.7	89.8	89.8	93.9	76.4	70.5	76.4	98.2	70.6	94.3	71.7	92.8	73.3	56.0	72.7																	
277	96.7	74.1	80.0	93.2	91.4	82.4	92.3	78.3	92.3	100	88.9	85.0	80.0	45.2	55.6																		
283	81.3	70.6	86.9	93.0	73.3	95.0	81.8	100	87.8	88.9	80.9	61.1	37.5	51.6																			
286	93.8	82.4	85.6	90.9	74.4	83.2	77.4	66.7	83.3	89.5	65.0	58.8	52.5																				
287	80.0	81.8	97.3	79.2	86.9	80.0	75.6	85.0	89.5	77.1	50.0	52.6																					
289	97.9	64.9	98.0	96.8	93.3	95.8	71.4	96.4	68.1	62.2	61.2																						
292	93.3	100	95.5	95.8	98.1	76.4	96.2	74.4	52.6	69.6																							
293	91.3	60.5	97.9	92.3	62.5	80.9	80.9	60.0	75.0																								
295	95.5	100	100	70.0	98.1	75.0	56.4	69.4																									
296	83.0	95.5	81.6	95.4	84.8	60.0	69.8																										
298	100	97.9	88.9	66.7	61.5	55.6																											
83	97.4	94.9	81.6	60.0	56.3																												
26	78.0	70.6	66.7	56.4																													
156	82.1	65.2	58.3																														
86	63.4	62.9																															
276	82.3																																
285																																	

table III were lower than the 98.9% average recorded for whole-cell profiles. Despite the wide range of percentage similarity values recorded, strains of MRSA could be grouped according to immunoblot profiles. Two main groups were clearly apparent: group 1, comprising 14 isolates and group 2, comprising 18 isolates. A third minor group made up of only two isolates, nos. 276 and 285, was also identified (table II).

Within group 1 the spread of percentage similarity values was 65.5–100%, with an average similarity of 88.6%. The spread of percentage similarity values within group 2 was 57.1–100%, with an average

similarity of 84.9%. Isolate nos. 276 and 285 (group 3) were 82.3% similar. Comparisons between the two main groups demonstrated decreased percentage similarity of values, from 26.3% to 76.9%, with an average similarity 53.7%. Thus the visual designation of MRSA strains to either group 1 or 2 was confirmed because of the degree of dissimilarity between the groups.

Grouping of strains according to immunoblot profile shows concordance with their phage-typing pattern (table IV). Generally, group-1 strains were not susceptible to phages which lysed group-2 strains.

Group 3 was not as clearly defined by immunoblot profiles as groups 1 and 2. A significant degree of similarity, 72.8%, was observed with group 1, although similarity with group 2 was only 58.6%. Furthermore, isolate nos. 276 and 285 had different phage-typing patterns (table IV). It is possible that analyses of a larger number of strains could establish group 3 as a discrete set or as a subgroup of group 1.

Discussion

Analyses of different strains of MRSA by SDS-PAGE gave reproducible whole-cell polypeptide profiles. However, due to the high degrees of

Table III. Average percentage similarity (%S) of exported proteins of MRSA isolates shown in table II

Group*	%S
1	88.6
1v.2	53.7
1v.3	72.8
2	84.9
2v.3	58.6
3	82.3

* As indicated in table II.

Table IV. Results of phage typing of MRSA isolates

Group no.	Isolate no.	Phage type (RTD)	Phage type (RTD × 100)
1	274	53/85	29/52/52A/79/80/53/54/85/96
	278	53/85	29/52/79/80/95/53/85/96
	279	53/85	29/52/52A/79/80/95/53/85
	087	53/85	29/52/52A/79/80/95/53/54/85/96
	288	53/85	29/53/84/85
	270	53/85	29/79/80/53/85
	290	53/83A/85	29/52/79/80/95/53/54/83A/85/96
	159	53/83A/85	29/52/52A/79/80/53/54/83A/85/96
	272	53/83A/85	53/54/83A/85
	280	53/75/83A/85	6/47/53/54/75/83A/85
	294	6/53/75/83A/85	6/47/53/54/75/83A/85
	291	6/53/54/75/83A/85	6/42E/47/53/54/75/83A/85/81
	051	6/47/53/54/75/83A	29/52/52A/79/80/6/42E/47/53/54/75/83A/85/81
	305	29/52/52A/79/80/95/53/75/85	29/52/52A/79/80/95/53/75/77/83A/85
	273, 277, 298, 287, 156, 292, 295	NT	75
	275, 289, 283, 293, 271	NT	75/84
2	296	NT	53/75/84
	026	75/84	52/75/84
	180, 083, 086	Data not available	
3	285	NT	NT
	276	53/75/83A/85	29/52/52A/79/80/95/6/53/75/83A/85

NT = non-typable.

similarity observed among these banding patterns, SDS-PAGE analyses of whole-cell extracts could not be used to distinguish between MRSA isolates. In contrast, immunoblotting of extracellular proteins gave characteristic profiles which were used to discriminate different strains of MRSA. The 34 isolates tested could be divided into two main groups and a third minor group. The results show that immunoblotting can provide a rapid, reproducible and sensitive method for characterisation of MRSA.

The development of new methods for typing MRSA is desirable since existing methods often give ambiguous or misleading results. Similar methods to those used in this study have been applied previously. Analysis of whole-cell extracts by ³⁵S-SDS-PAGE was unable to discriminate between different isolates of MRSA, and it was therefore concluded that all isolates were closely related (Stephenson *et al.*, 1986). Similarly, immunoblotting of whole-cell extracts has been used to divide a collection of MRSA isolates from the United Kingdom, Australia and Egypt into eight groups (Lee and Burnie, 1988). As reported above, whole-cell polypeptide profiles cannot be used to distinguish MRSA. In comparisons where similarity values approach 100%, different isolates will be indistinguishable from each other. In our hands

immunoblots of whole-cell extracts of MRSA did not discriminate clearly between different strains (results not shown), and therefore culture supernates were chosen for analyses.

Use of a single hypoimmune antibody probe resulted in distinct and reproducible banding patterns. Therefore, MRSA isolates can be grouped satisfactorily without the requirement for specific hyperimmune antisera (Lee and Burnie, 1988).

In this study 34 isolates were allotted to three groups on the basis of immunoblot profile: group 1 consisting of 14 strains, group 2 of 18 strains, group 3 of 2 strains. Group 3 may prove to be a subgroup of either of the major groups. To define group 3 more clearly, further analyses of new isolates are required. An increase in sample size would also identify any other overlapping strains and confirm the major groupings. Comparisons with other MRSA isolates, e.g., European and Australian strains, would establish whether the groupings described here are global and could thus provide evidence for or against the clonal origin of MRSA.

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