EPIDEMIOLOGICAL TYPING

Antibiogram-resistogram typing scheme for methicillin-resistant *Staphylococcus aureus*

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Summary. An antibiogram-resistogram (AR) typing scheme that can simply and rapidly differentiate methicillin-resistant *Staphylococcus aureus* (MRSA) isolates has been devised. Susceptibility to antibiotics and chemicals was determined by disk diffusion testing. Three disk diffusion methods and three control *S. aureus* strains were evaluated. A modified Stokes' technique in which *S. aureus* ATCC 25923 replaced *S. aureus* NCTC 6571 as the control organism was chosen. Susceptibility patterns against 18 antibiotics and four chemicals were used to determine AR types. AR subtypes were determined with reference to knowledge of the local MRSA population so that plasmid loss would not result in misclassification. AR typing was compared with phage typing and plasmid profiling and found to be more discriminatory than either of these typing methods. Representative isolates of the most frequently occurring AR patterns were further characterised by investigating enterotoxin production, MICs of gentamicin and amikacin, and restriction endonuclease analysis of plasmid DNA, to determine whether apparently different strains could have the same AR pattern and to devise confirmatory tests for any such similar patterns. One pattern was shared by two strains but isolates could be differentiated by susceptibility to minocycline. This typing scheme can be used in the diagnostic laboratory and results may be obtained within 24 h.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial pathogen and its incidence has increased worldwide in recent years.^{1–4} In Ireland, MRSA has been a major problem in Dublin hospitals since the late 1970s^{5,6} and recently the incidence has been rising in the group of hospitals served by this laboratory.⁷

Control of MRSA hospital infection requires the facility to distinguish different strains.^{2,8,9} Typing methods currently used to differentiate MRSA strains include antibiogram and resistogram typing, phage typing, plasmid DNA analysis, total cellular DNA analysis (often followed by Southern hybridisation), electrophoretic protein typing and immunoblotting.¹⁰ Other methods used are multilocus enzyme electrophoresis, capsular serotyping and biotyping.^{9,10} Recently MRSA strains have been analysed by a polymerase chain reaction (PCR) technique with random amplification of polymorphic DNA (RAPD-PCR).^{11,12} Many of these techniques are expensive

research tools that are labour-intensive and may require the services of a reference laboratory.

The three most common typing techniques used with *S. aureus* are antibiogram typing, phage typing and plasmid profiling.¹³ The value of phage typing is limited because most typable MRSA isolates belong to phage lytic groups I or III and an increasing proportion of strains are now non-typable.^{1,2} Plasmid typing is inherently variable because of loss or gain of plasmids and isolates lacking plasmids cannot be typed.⁹ Like plasmid profiling, antibiogram patterns vary if a resistant determinant is plasmid-borne and the plasmid is gained or lost.⁹ Inclusion of chemicals and antibiotics that are not in clinical use may help to overcome variation resulting from selective pressure exerted by antibiotics and, perhaps, disinfectants in the hospital environment.¹⁴

Gillespie *et al.* used a combination of antibiotics and chemicals to devise an MRSA phenotyping scheme that characterised MRSA isolates on the basis of whether resistance determinants were plasmid-encoded, chromosomally-borne or plasmid- and chromosomally-determined. This typing scheme differentiated MRSA strains that were indistinguishable

by either phage typing or plasmid profiling alone but loss of a plasmid dramatically altered the typing result.¹⁵

Antibiogram-resistogram (AR) typing might be more helpful if variation due to plasmid carriage could be taken into account during interpretation. Further benefit might accrue if the susceptibility testing methods in use in the diagnostic laboratory proved suitable so that typing could be performed locally. It would be an advantage to employ the susceptibility testing methods already in clinical use since they are standardised, rapid, economical and widely available.¹⁶⁻²⁰ However, such methods have been standardised for use with antibiotics in clinical use and may not apply to other antimicrobial agents.¹³ If methods in routine use in the diagnostic laboratory prove suitable, preliminary typing results could be taken from routine laboratory reports.

Although some form of antibiogram typing is frequently the first stage in the investigation of an MRSA population,^{9, 21-26} many different susceptibility test methods have been used in studies both within and between different countries.²²⁻²⁹ The use of different methods should not pose a problem if the techniques produce comparable results.

The first part of this study compared the Stokes' disk diffusion susceptibility test method as routinely used in this laboratory,³⁰ a modified disk diffusion method used to investigate local MRSA phenotypes²⁵ and a reference comparative disk diffusion technique.³¹ Three *S. aureus* control strains were evaluated to investigate whether *S. aureus* NCTC 6571 (the control specified for both the comparative and Stokes' techniques^{30, 31}) is the most suitable control organism for use with this resistant population. From the results of this preliminary study, a modified Stokes' disk diffusion technique was used to determine AR types of the current MRSA population and the results were compared with phage typing and plasmid profiling.

Materials and methods

Identification of MRSA

Bacterial isolates were identified as *S. aureus* by testing for the production of clumping factor, coagulase and thermostable DNAase.^{32, 33} Since 5 April 1993, isolates were also identified with a *S. aureus* identification kit (Pastorex[®] Staph-Plus; Sanofi Diagnostics Pasteur).

Methicillin susceptibility was tested at 30°C on Columbia blood agar (CBA) with 25-µg methicillin strips (Mast) and a heavy inoculum.^{34, 35} The methicillin-resistant *S. aureus* NCTC 10442 was included as a positive control.³⁶

Group A isolates (1988-1989) were used to evaluate susceptibility testing methods. Group B isolates (collected between Dec. 1992 and April 1993) and group C isolates (May-Aug. 1993) were investigated in the AR typing study.

Evaluation of susceptibility test methods

Bacterial isolates (group A). The test methods were evaluated with a collection of 104 isolates from 24 patients—102 MRSA and two methicillin-sensitive *S. aureus* (MSSA)—collected during 1988-1989. Isolates were stored on nutrient agar (NA) slopes at room temperature. In addition to identifying isolates as MRSA, all were characterised by phage typing, plasmid profiling, restriction endonuclease analysis of plasmid DNA, plasmid curing, biotyping, protein A detection, enterotoxin production and gentamicin MIC determination.

Susceptibility testing. Susceptibility testing was performed on Diagnostic Sensitivity Test Agar (DST; Oxoid) by three disk diffusion methods (table I). For the comparative and modified comparative methods, inocula were prepared by making a 10³ saline dilution of 18-h trypticase soy broth (TSB) cultures grown at 35°C with shaking at 150 rpm; for sulphonamide and trimethoprim testing, isolates were also grown in Luria Bertani (LB) broth. For the Stokes' method, inocula were prepared by suspending sufficient growth from an overnight culture grown on CBA in 4 ml of nutrient broth (NB no. 2; Oxoid) to produce "dense but not confluent growth" when inoculated on to DST agar plates and incubated for 18 h at 35°C. In addition to DST, sulphonamide and trimethoprim were also tested on LB agar. Zone sizes were measured with calipers and recorded.

Antimicrobial agents were tested at the concentrations used in the diagnostic laboratory and in the modified comparative method used to define local MRSA phenotypes.²⁵ The agents used, their concentrations and sources are listed in table II. Where possible, commercially available disks were used. Non-commercial disks were prepared by dosing blank disks (Oxoid) with 20 µl of the required concentration of the antimicrobial agent.³⁷ During the methods evaluation, the ciprofloxacin concentration used was 5 µg. Recent recommendations suggest that 1 µg is a more suitable concentration; hence, subsequent work was performed with this concentration.²⁰

Antibiogram/resistogram (AR) typing

Bacterial isolates. Group B: 321 fresh isolates from 238 patients (collected between Dec. 1992 and April 1993) were AR typed and their phage susceptibility and plasmid profiles were determined. Restriction endonuclease analysis was performed on 63 isolates and 49 representative isolates of the most frequently occurring AR types were further investigated by biotyping, protein A detection, enterotoxin production and gentamicin and amikacin MIC determination.

Group C: 187 isolates from 163 patients collected between May and Aug. 1993 were AR typed only. These isolates were either tested fresh or were frozen at -70°C on cryoprotective beads (Protect; Technical Service Consultants Ltd) and batch tested. One isolate

Table I. Methods, controls and interpretative criteria used to evaluate susceptibility test methods

Method	Reference control strain	Controls evaluated	Zone diameter (mm)*	Interpretation
Comparative ³¹	NCTC 6571	RN4220 ATCC 29213 ATCC 25923	≤ 12	Resistant
			< 12	
			> 6 < control	Moderate
			= control	Sensitive
Modified comparative ²⁵	RN 4220	†	≤ 6 < control	
			Pre-determined zone sizes	†
			≤ 12	Resistant
			< 12	
Stokes ³⁰	NCTC 6571	ATCC 29213 ATCC 25923	> 6 < control	Moderate
			= control	
			≤ 6 < control	Sensitive

* These criteria apply to antimicrobial agents that produce zones sizes of 16–30 mm diameter.³⁰

† These results are unaffected by the control used.

Table II. Antimicrobial agents used to evaluate susceptibility test methods

Agent (abbreviation)	Disk content (μg)	
	Oxoid*	SJH (source)†
Gentamicin (Gn)	10	—
Amikacin (Ak)	30	—
Kanamycin (Kn)‡	30	20 (Sigma)
Neomycin (Nm)‡	30	20 (Sigma)
Streptomycin (Sm)‡	25	50 (Sigma)
Tobramycin (Tb)	10	—
Netilmicin (Nt)	30	—
Spectinomycin (Sc)‡	25	50 (Sigma)
Penicillin (Pn)	1.5 units	—
Ampicillin (Ap)	10	—
Erythromycin (Er)‡	5 15	—
Clindamycin (Cli)	2	—
Lincomycin (Ln)	2	—
Fusidic acid (Fd)‡	10	20 (Leo)
Tetracycline (Te)	10	—
Vancomycin (Vn)	30	—
Rifampicin (Rf)	2	—
Ciprofloxacin (Cp)	5	—
Chloramphenicol (Cl)	10	—
Mupirocin (Mp)	35	—
Sulphonamide (Su)	100	—
Trimethoprim (Tp)	1.25	—
Ethidium bromide (EB)	—	20 (Sigma)
Cadmium nitrate (CdN)	—	10 ⁻⁶ mols (Aldrich)
Phenyl mercuric acetate (Pma)	—	10 (Sigma)
Mercuric chloride (MC)	—	10 (BDH)

* Commercially-available disks (Oxoid).

† SJH, disks prepared in SJH, (source) source of antimicrobial agent for disks prepared in SJH.

‡ These antibiotics were used at both concentrations.

from each patient was included unless routine susceptibility test results indicated the presence of more than a single strain.

AR typing. On the basis of the results of the susceptibility test methods evaluation, susceptibility to the panel of antimicrobial agents listed in table III was

determined by the modified Stokes' disk diffusion technique in which *S. aureus* ATCC 25923 replaced *S. aureus* NCTC 6571 as the control organism. The antimicrobial disk concentrations in the panel were chosen because, with the exception of streptomycin and neomycin, there was good agreement between the results obtained with different content disks (table IV).

Table III. Most frequent AR patterns encountered in 1993

AR type	Gn 10	Ak 30	Kn 30	Nm 30	Sm 25	Tb 10	Ap 10	Er 5	Ln 2	Fd 10	Te 10	Vn 30	Rf 2	Cp 1	Cl 10	Mp 35	Su 100	Tp 1-25	EB 20	MC 10	CdN 10 ⁻⁶ **	Pma 10
1†	R	S	R	S	R	M	R	R	R	S	R	S	S	S	S	S	R	R	R	R	R	R
1.1††	S††	S	S††	S	R	S††	R	R	R	S	R	S	S	S	S	S	M††	R	S††	R	R	R
6-3	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S
6-5	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S
7	S	M	R	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	R**	S
7-2	S	M	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	R**	S
7-4	S	M	R	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	R**	S
7-6	S	M	R	R	S	R	R	R	R	S	S	S	R	R	S	S	S	S	S	S	R**	S
11	S	M	R	R	S	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	R	R
11 (Fd R)	S	M	R	R	S	R	R	R	R	R	S	S	S	S	S	S	S	S	R	R	R	R
11-1	S	M	R	R	S	R	R	R	R	S	S	S	S	R	S	S	S	S	R	R	R	R
13	R	M	R	R	R	R	R	R	R	S	S	S	S	R	S	S	S	S	R	R	R	R
13-1	R	M	R	R	R	R	R	R	R	S	S	S	S	R	S	MR	S	S	R	R	R	R
13-2	R	M	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R	R	R
14	R	M	R	R	R	R	R	R	S	S	S	S	S	R	S	S	S	S	R	R	R	R
14-1	R	M	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	R	R	R	R
14-2	R	M	R	R	R	R	R	R	S	S	S	S	S	R	S	R	S	S	R	R	R	R
15	R	M†	R	S	R	R	R	R	R	S	R	S	S	S	S	S	R	R	R	R	R	R
22	R	R	R	R	R	R	R	R	R	S	R	S	R	R	S	S	M	S	R	R	R	R

R, resistant; S, sensitive; M, moderate; other abbreviations, see table II.

* Cadmium nitrate concentrations 10⁻⁶ mols.

** May be sensitive to higher concentration (1 mM) of cadmium nitrate.

† AR 1 and AR 1.1 included because these patterns were prevalent in 1988 and 1989.

†† Pattern resulting from plasmid loss in AR 1.

‡ Correct recognition of moderate results is important to distinguish types 1 and 15.

Table IV. Effect of disk content on susceptibility test method results

Agent	No. of discrepancies	Analysis of discrepancies (%)	
		R → M	M → R
Fd	2	—	—
Sm	5	20	80
Kn	1	—	—
Nm	14	7.1	92.9
Sc	0	—	—
Ap	0	—	—
Er	1	—	—
Ap/Pn	0	—	—
Ln/Cl	3	—	—

R → M, isolates resistant to high content disks were moderately sensitive when tested with lower content disks; M → R, isolates moderately sensitive to high content disks were resistant with lower content disks. For other abbreviations, see table II.

With streptomycin and neomycin, 89% of discrepancies occurred because an isolate interpreted as moderately sensitive with the higher content disk appeared resistant with the lower content disk. Thus, with the exception of neomycin, concentrations in commercially available disks were suitable.

When the 30- and 20-μg neomycin disks were compared in the comparative method, 14 isolates appeared moderately sensitive with the 30-μg disk, but the 20-μg disk gave a resistant result. The comparison was repeated with the modified Stokes' technique on 133 isolates, and 7.5% of isolates gave this result. All isolates giving moderate results produced zone sizes ≤ 15 mm diameter. Hence, for convenience, and because commercially available disks have the advantage of standardised production, the commercially available 30-μg neomycin disks were used but zone sizes of

15 mm were interpreted as resistant. (Although 20-μg neomycin disks are not available routinely commercially at present, suppliers can provide this concentration disk if requested.)

There was no advantage in testing both penicillin and ampicillin but it was helpful to include a β-lactamase-sensitive penicillin to indicate isolates lacking β-lactamase activity.

Lincomycin was chosen in preference to clindamycin because the zones were easier to read. All isolates were resistant to spectinomycin, so this agent was not included in the AR typing panel. Netilmicin was also discontinued because a large proportion of isolates were classified as moderately sensitive.

Zones smaller than the control were measured with calipers and the zone sizes were recorded. Susceptibility categories were interpreted according to the Stokes' technique criteria.

Phage typing

Isolates were phage typed with the international basic set of typing phages at routine test dilution (RTD) and at RTD × 100.³⁸ The experimental phages 88A, 90 and 932 were also used (at RTD × 100).

Plasmid typing

Plasmid DNA screening was performed with a plasmid "mini-prep" kit (Magic Mini Prep[®]; Promega). This kit provides DNA suitable for restriction endonuclease digestion but required modification to make it suitable for use with *S. aureus*. Isolates, suspended in 10 units of lysostaphin (Sigma) in Tris EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5) in 50-μl volumes were incubated at 37°C for 1 h. After

addition of 150 μ l of the resuspension fluid from the kit, DNA extraction was completed according to the manufacturer's instructions. *S. aureus* NCTC 50850 (SK18) which carries plasmids of 28.4 kb, 4.5 kb and 1.5 kb was used as a plasmid size marker.²⁶

Restriction endonuclease analysis

Plasmid DNA from 63 isolates was digested with the restriction endonucleases *Eco*RI (Promega) and *Hind*III (Northumbria Biologicals Ltd) according to the manufacturers' instructions. Bacteriophage λ DNA cleaved with *Eco*RI and *Hind*II was included as a molecular size marker.

Further characterisation

From the Group B organisms, 49 isolates representative of the most frequently encountered AR types were further characterised by determining MICs of gentamicin and amikacin (by the E test[®]; AB Biodisk, Sweden), testing for production of enterotoxins A, B, C and D by a reverse passive latex agglutination method (SET RPLA; Oxoid) and performing extra antimicrobial susceptibility tests with minocycline (30 μ g), novobiocin (5 μ g) and bacitracin (10 units). In addition to these 49 isolates, minocycline, novobiocin and bacitracin susceptibility testing was also performed on another 51 group B isolates.

AR typing nomenclature

Isolates were assigned an AR type number on the basis of the susceptibility pattern produced. AR type numbers were assigned chronologically with AR types 1, 2, 3 etc. derived from group A isolates. AR subtypes were determined from information obtained from the full characterisation of these isolates. Thus AR type 1 isolates carry a plasmid that encodes resistance to gentamicin, kanamycin, tobramycin and ethidium bromide. AR type 1.1 is the sensitive variant that occurs following plasmid loss. AR type 1 was present during 1988–1989 but was not isolated during 1993 and the plasmid-free AR type 1.1 was isolated from one patient only.

Another factor taken into account when devising nomenclature was the emergence of resistance to ciprofloxacin in some strains.³⁹ For example, AR type 7 is ciprofloxacin sensitive but AR type 7.4 is its ciprofloxacin-resistant variant. Similarly the nomenclature took account of emerging mupirocin resistance (largely associated in this hospital group with one hospital and one AR type) by assigning the mupirocin-resistant isolate to a subtype; i.e., the mupirocin-sensitive type is 13 and its mupirocin-resistant variant is 13.1. Other factors taken into account included current antibiotic therapy: for example, the emergence of rifampicin or fusidic acid resistance in a patient being treated with either of these antimicrobials

resulted in the resistant variant being classified as a subtype. The most common AR types and subtypes are listed in table III.

Because it is difficult to compare AR patterns with an increasingly large number of AR types and subtypes, a commercially-available IBM[™]-compatible database (DataEase[™]; Sapphire Corporation) was used to simplify pattern recognition. We have described previously a system whereby phage typing results, antimicrobial susceptibility testing and patient demographics can be correlated by this database.⁷ This computer system allows the addition (and correlation) of AR types.

Plasmid profiles were named alphabetically.

Reproducibility and stability

Forty-eight isolates were re-tested after storage at -70°C for 8–12 weeks to determine whether the susceptibility testing method was technically reproducible. To investigate the reproducibility of the AR typing method, repeat isolates were collected from 56 patients. The stability of the typing patterns was investigated by comparing the typing results of repeat isolates from 42 patients collected after a minimum interval of 1 month.

Discrimination and AR type confirmation

The possibility that two different strains could produce the same AR pattern was investigated by comparing the AR types of groups B and C isolates with the patterns produced by group A isolates. Apparently similar isolates were further studied to determine what additional tests would be necessary to distinguish such strains. The patterns produced by isolates from groups B and C were analysed to see if any patterns were sufficiently similar (or if results varied sufficiently) to cause two patterns to be confused.

The validity of the AR classification was confirmed by detailed characterisation of representative isolates with frequently occurring AR patterns. Isolates were characterised by biotyping, enterotoxin production and gentamicin and amikacin MIC determination.

Results

Evaluation of susceptibility test methods

Sulphonamide susceptibility test results showed great variation between the methods used, and zones were difficult to interpret, so these results were excluded from the evaluation. With phenyl mercuric acetate, resistant isolates produced zones of 17–20 mm which the modified comparative method criteria considered sensitive, hence phenyl mercuric acetate results were also excluded (zone sizes produced by sensitive isolates were in the range 38–40 mm).

Table V. Discrepancies between results obtained from comparison of three disk diffusion susceptibility test methods and different control organisms

Methods compared	Discrepancies (%) with control <i>S. aureus</i> strains			
	NCTC 6571 RN 4220 ATCC 29213 ATCC 25923	RN 4220 ATCC 29213 ATCC 25923	NCTC 6571	ATCC 25923
Comparative	6.9	0.35	—	—
Comparative, modified comparative	8.7	2.9	—	—
Comparative, modified comparative, Stokes'	—	—	13.3	4.9
Comparative, Stokes'	—	—	8.2	3.1 (2.6)*
Comparative, modified comparative	—	—	8.4	2.7†
Modified comparative, Stokes'	—	—	10.2	4.2 (2.6)*

* Results with antibiotics where resistance was shown to be encoded by a labile plasmid have been excluded.

† Results with the comparative and modified comparative methods were unaffected because they were read from the same plates.

Table VI. Characteristics of isolates belonging to the most commonly encountered AR types during 1993

AR type	Number of patients	Phage type	Percent of isolates (n = 321)	Plasmid profile*	Enterotoxins produced (n = 49)	Gentamicin MIC (mg/L)		Amikacin MIC (mg/L)	
						Mean	Range	Mean	Range
1†	0	84 NT	8 92	B	A	30.8	8–96	S	
1-1†	1	84 NT	3 97	N	A	S		S	
6	22	NT 42E	69 31	B, C or N	None or C	S		S	
7	42	83A 83A/‡ NT	63 14 23	B	None	S		11.6	8–12
11	40	75/83A 83A NT	70 11 19	A	None	S		8	8
13	89	NT	100	A	A	48	48	12	12
14	75	NT	100	A	A	50.7	48–64	12	12
15	8	NT	100	B	A	64	64	6	6
22	16	6/47/54/75/ 77/84/85§	100	B	None	> 256	> 256	60.8	48–64

* Plasmid profile of majority of isolates (but see *authors' note*); N, no plasmid detected; S, sensitive.

† Characteristics determined from 1988–1989 isolates.

‡ 47/54/75/83A or 75/83A.

§ Some isolates were lysed by bacteriophage 29 also.

There were 178 discrepancies when results obtained with the comparative method were interpreted with the four controls evaluated. Table V shows that when *S. aureus* NCTC 6571 was excluded, the discrepancy rate was reduced from 6.9 to 0.35%.

When results obtained with all three methods were compared, discrepancies were reduced by an average of 6.3% if *S. aureus* ATCC 25923 was used instead of *S. aureus* NCTC 6571. Although the results obtained with *S. aureus* ATCC 29213 and RN 4220 correlated well with the results with *S. aureus* ATCC 25923, the latter was chosen for further work because it is a recognised disk diffusion susceptibility testing control and does not produce β -lactamase.

Because the control organisms were inoculated on to separate plates in the comparative and modified comparative methods, test isolate susceptibility zones

from one plate could be interpreted according to the comparative or modified comparative methods' criteria and also with reference to each control *S. aureus* evaluated. In contrast, the Stokes' technique required that separate susceptibility tests had to be performed for each control used. Hence, in this evaluation, there was greater potential for variation with the Stokes' technique. A further variable appeared during the study because a number of the MRSA test isolates were found to harbour a labile plasmid that encoded resistance to aminoglycosides and ethidium bromide. To minimise the effect of variation due the loss of this plasmid, results were re-calculated excluding the antibiotics whose resistance determinants were encoded by this plasmid. When *S. aureus* ATCC 25923 was the control, the modified comparative and the modified Stokes' techniques produced a similar number of

Fig. 1. Plasmid profiles of MRSA isolated in the Federated Dublin Voluntary Hospitals and St James's Hospital between Dec. 1992 and Aug. 1993. Lane 19 contains plasmid DNA from *S. aureus* NCTC 50580 which carries plasmids of c. 28.4, 4.5 and 1.5 kb; lanes 9–13 show examples of plasmid profile A; lanes 1, 3–8, 14 and 15 show examples of plasmid profile B; 16 and 17 contain plasmid DNA from an isolate imported from Croatia which exhibits an unusual plasmid pattern; C denotes the position of residual chromosomal DNA.

discrepancies compared with the reference comparative method.

Analysis of discrepancies obtained with the modified comparative method showed that approximately half (46.2%) were due to zone sizes in the moderately sensitive category by the comparative or Stokes' methods being regarded as sensitive in the modified comparative method. For example, 26.2% of such discrepancies occurred with neomycin (30- μ g disk) where the average zone size was 15.5 mm. With the lower content (20 μ g) disk these isolates were resistant by all methods.

The modified Stokes' technique was chosen for all further work because in addition to comparing well with other methods and being a well established, easily performed technique, every antimicrobial agent tested is controlled.

AR typing

AR typing divided MRSA isolates from Dec. 1992 to Aug. 1993 into 31 AR types; seven types were further divided into subtypes. Of all isolates, 90% belonged to seven types (table III). Table III includes two patterns, AR type 1 and AR type 1.1. AR type 1 was not isolated during the 1993 study and its plasmid-free subtype AR 1.1 was isolated from one patient only, but these patterns were encountered among c. 70% of MRSA isolated in 1988 and 1989. AR type 1 isolates harbour a labile plasmid that encodes aminoglycoside resistance. AR type 1.1 is the pattern that results from plasmid loss. The numbers of patients

from whom the most frequently occurring AR types were isolated during 1993 are shown in table VI.

Phage typing and plasmid profiling

Analysis of phage typing results showed that 66% of isolates were non-typable; 10% were of phage type 83A and 12% were lysed by bacteriophages 83A and 75.

Amongst plasmid-bearing isolates, 90% showed plasmid profiles A or B (fig. 1).

Table VI correlates the most frequently isolated AR types with phage type, plasmid profile, MICs of gentamicin and amikacin, and enterotoxin production. To simplify data presentation, AR subtypes are not shown separately. All isolates were sensitive to novobiocin and bacitracin; results of minocycline susceptibility testing mirrored those obtained with tetracycline. With group A (1988–1989) isolates, tetracycline-resistant isolates of AR types 2, 3 and 5 were minocycline-sensitive.

Comparison with phage typing and plasmid profiling

Phage typing failed to distinguish AR types 13, 14 and 15 while 69, 23 and 19% of AR types 6, 7 and 11, respectively, were non-typable by phage typing (table VI). Although plasmid profiling may be helpful if an isolate has an unusual profile, for example, AR type 23 had a distinctive plasmid profile (fig. 1), 90% of isolates belonged to plasmid type A or B. RE analysis

failed to differentiate plasmid type A DNA from AR types 11, 13 and 14 and, although RE analysis did distinguish plasmid DNA from AR types 7 and 15, other AR types with plasmid type B profile exhibited similar RE patterns.

Reproducibility and stability

There was 98.9% agreement among results when susceptibility testing was repeated on stored isolates. The majority of discrepancies (67%) occurred with amikacin susceptibility in the moderately sensitive and sensitive categories.

Analysis of AR typing patterns from repeat isolates showed that 73% of isolates yielded the same AR types and 45% yielded different patterns. Three or more isolates were tested from 18% of patients and at least one isolate showed the same AR type as one of the pair of dissimilar isolates. Dissimilar AR types were frequently cultured from the same specimen (36% of such isolates).

When typing results of repeat isolates collected after a minimum interval of 1 month were analysed, the same AR type was isolated from 74% of patients and 31% yielded a different type (more than two isolates were tested from 5% of patients). These results indicate that the method is reproducible and the patterns are stable but patients may harbour more than one strain of MRSA and the MRSA population may be changing. The declining incidence of AR type 1 and AR type 1.1 confirms this observation. Further confirmation was obtained by an epidemiological study undertaken to assess the value of this typing scheme. This study is described in an accompanying paper.⁴⁰

Discrimination and AR type confirmation

Comparison of AR patterns among groups A, B and C isolates showed that one pattern, AR type 1.1, could be obtained from two different strains. AR type 1.1 is the pattern of AR type 1 isolates after plasmid loss and these isolates were tetracycline- and minocycline-resistant. One other group A isolate produced the same AR pattern, but this isolate carried a 28.4-kb plasmid and was tetracycline-resistant but minocycline-sensitive. Thus it may be useful to confirm the minocycline susceptibility of isolates showing the AR type 1.1 pattern.

AR types 1 and 15 may be confused if the result with amikacin is not recognised as moderately sensitive in AR type 15. These two types cannot be distinguished by either phage typing or plasmid profiling but RE analysis of plasmid DNA differentiated the isolates used in this study. In a subsequent study, the RE pattern of AR type 1 isolates varied when isolates stored on nutrient agar at room temperature were compared with isolates stored frozen at -70°C . Two RE patterns were produced by the latter and one pattern was similar to the pattern produced by AR

type 15 isolates. Until these results are further investigated, the relationship between AR types 1 and 15 remains unclear.

AR type 6.3 isolates may also require confirmation because, in this study, a patient harbouring AR type 6.5 was found to be carrying a mixed population of erythromycin-resistant (AR 6.5) and erythromycin-sensitive (AR 6.3) isolates. These isolates were non-typable by phage typing and produced enterotoxin C. When phage typing and enterotoxin production results of 14 AR type 6 isolates were correlated, all six isolates failing to produce enterotoxin belonged to AR type 6.3 and were lysed by bacteriophage 42E (one isolate gave a reaction in the “+” category). Seven enterotoxin C-producing isolates were non-typable by phage typing and were either AR 6.3 or AR 6.5. One such AR 6.3 isolate was cultured from a sibling of the patient mentioned earlier. This indicates that the AR 6.5 pattern may be unstable and information on phage type or enterotoxin production may be required to confirm the AR type of AR 6.3 isolates.

With the exception of AR type 6.3, these patterns did not cause a problem, because although AR type 1 was prevalent during 1988 and 1989, it was not isolated during the 1993 study and its plasmid-free subtype AR 1.1 was isolated from one patient only.

Discussion

The ideal epidemiological typing method should be simple, standardised, reproducible, stable, inexpensive, widely available, epidemiologically valuable and sufficiently sensitive to distinguish similar but not identical strains.^{10, 41, 42} If typing methods are to be of use in the diagnostic laboratory, they must be easy to perform, simple to interpret, utilise readily available reagents and follow a standard reporting system.^{9, 41} The modified Stokes' technique described here fulfils most of these criteria.

The Stokes' technique is widely used in Great Britain and Ireland. The only modification made to the standard method was to replace *S. aureus* NCTC 6571 with *S. aureus* ATCC 25923, but close attention must be paid to technical detail. It is important that the correct “dense but not confluent growth” is achieved for both test and control isolates. Equally important is the necessity to measure zone sizes in the moderate range (i.e., any zone > 12 mm diameter but smaller than the control). The effects of these two factors were most marked with amikacin, partly because the MICs of local isolates clustered in the 6–12 mg/L range and because amikacin susceptibility testing is particularly inoculum-dependent.⁴³ These difficulties were reflected in the results of the reproducibility study, where the majority of discrepancies occurred with amikacin. Incubation for a minimum period of 18 h is also important. The traditional “overnight” incubation that may often be ≤ 16 h will not produce reliable results, especially with clindamycin.

Brown and Kothari have shown that when four different disk diffusion sensitivity testing methods—the Comparative, Stokes', International Collaborative Study (ICS) and Kirby-Bauer methods—were compared in the same laboratory, 7.7% of results showed major differences. When the comparison was performed in different laboratories, the number of major differences was 8.7%.⁴⁴ In the study reported here, the use of *S. aureus* ATCC 25923 instead of *S. aureus* NCTC 6571 reduced the number of discrepancies by 6%. Although this work was performed as part of an investigation of MRSA susceptibility patterns, this finding may be relevant to routine sensitivity testing in the diagnostic laboratory. *S. aureus* ATCC 25923 is a widely used susceptibility testing control, being the designated control in the Kirby-Bauer method.¹⁸ Hence its use in the modified Stokes' technique would serve to increase standardisation among the commonly-used sensitivity testing methods.

Susceptibility test interpretative criteria have undergone a number of modifications in recent years. The previous criterion used to determine resistance was a zone diameter of 12 mm.⁴⁵ Most recent recommendations suggest 10 mm as the cut-off point.^{20,46} Adopting this criterion would have resulted in a proportion of resistant isolates being interpreted as moderately sensitive.

Many investigations of MRSA populations include some form of antibiogram typing.^{9,21–26} Potential disadvantages of antibiogram typing include variation due to loss or gain of resistance determinants carried on plasmid, transposon or phage DNA resulting from selective pressure exerted by antibiotics in the hospital environment.⁹ In theory, the effect of this variation can be minimised with resistogram typing where chemicals are used in the antimicrobial panel, because the genetic determinants encoding resistance to these compounds will not necessarily be subject to the same selective pressure.¹⁴ However, only a limited number of compounds produce sufficiently discriminatory results to warrant inclusion in a *S. aureus* resistogram typing panel and resistance to the heavy metal ions, mercury, lead, cadmium and arsenic may be plasmid-mediated. For example, loss of β -lactamase production may be accompanied by loss of resistance to some or all of these heavy metals.⁴⁷ If cognisance is taken of the resistance determinants that are known to be plasmid-borne in the study population, plasmid loss need not result in an incorrect typing result.⁴⁷ Inclusion of antibiotics that are not in clinical use may also help overcome variation resulting from antibiotic pressure.

Although the chemicals used to determine the resistogram pattern are readily available, they are toxic and require precautions for safe handling and disposal, and disks containing these compounds are not available commercially. For this typing scheme to be practical in the routine laboratory, disks should be available commercially.

Cadmium susceptibility testing presented problems. Published reports show variations with the cadmium

compound (nitrate, acetate or sulphate) and concentrations used.^{25,26,28,47–49} In this study, cadmium nitrate (10^{-6} mols) was used,²⁵ but cadmium-sensitive MRSA produced small zones that were difficult to read. Increasing the concentration of cadmium produced larger easily read zones. It has been suggested that isolates with an MIC $\geq 10^{-4}$ mols be considered resistant, and that for disk diffusion testing, 1 mM cadmium nitrate (20 μ l/disk) is a suitable concentration.^{50,51} Isolates of AR type 7 were sensitive when higher content (1 mM) cadmium disks were used.

Despite these problems, this typing scheme is suitable for use in the routine laboratory and results can be obtained within 24 h. All isolates are typable, an important consideration in any typing scheme. The criteria for interpreting the susceptibility pattern follow standard recommendations and, with standard methods and an agreed panel of antimicrobial agents, susceptibility patterns obtained in different laboratories will allow comparison between MRSA from geographically different areas.

The AR typing nomenclature is arbitrary but subtypes were defined with reference to a well-characterised culture collection and took account of variation due to plasmid loss in the local population. In the epidemiological study described in an accompanying paper, it has been shown that the MRSA population in this hospital is changing.⁴⁰ Therefore, the AR typing scheme nomenclature requires flexibility to allow the addition of new types. Hence the nomenclature was devised to be open-ended. Criteria for the addition of new types would ideally include the isolation of the isolate on more than one occasion (preferably from more than one patient) and full characterisation of the organism before acceptance as a new type to prevent the inadvertent inclusion of plasmid-free isolates (or isolates that had lost other resistance determinants) as new types. This raises the question of whether there is a point at which plasmid-free (or other aberrant) isolates constitute new strains.

Although the AR patterns were more discriminatory than either phage typing or plasmid profiling, the possibility that two different strains could have the same AR type demonstrates the need to be familiar with other characteristics of the local MRSA population. Such strains could then be distinguished by confirmatory tests or AR types interpreted with due caution. Because extensive characterisation is impractical for the routine laboratory, a simple approach might be to determine the AR typing pattern in the routine laboratory and have detailed characterisation of representative isolates performed in a reference laboratory. When the local MRSA population has been sufficiently characterised, new patterns only might be referred to the reference laboratory. Thus the local laboratory has access to rapid results and the reference laboratory remains familiar with changes occurring in the MRSA population. In practice, the less frequently isolated AR types were often isolated from one patient only and further characterisation

Fig. 2. Plasmid DNA from phenotype II MRSA isolates that carry plasmids of c. 30.4 and c. 34.8 kb. DNA was extracted by a modified alkaline lysis method with 0.4 N NaOH and SDS 2% (lanes 2–5, 7); lanes 8–13 contain DNA extracted with 0.2 N NaOH and SDS 1%; lanes 1 and 14 contain DNA from *S. aureus* NCTC 50580 (SK18) which carries plasmids of 28.4, 4.5 and 1.5 kb. DNA was electrophoresed through agarose 0.6%.

may not be warranted unless the new type affects more than one patient.

This MRSA AR typing system is more useful than either phage typing or plasmid profiling, can provide results within 24 h and is suitable for use in the routine laboratory. The typing scheme has demonstrated the heterogeneity of the MRSA population in this hospital group, recognised the introduction of new strains and shown the extent to which this MRSA population has changed since 1989.

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Authors' note: Since this work was completed, further work on plasmid DNA extraction methods has shown that the Magic MiniPrep[®] kit may not yield intact DNA from plasmids > 20 kb. This is confirmed by recent instructions from the manufacturers who now market the kit as Wizard Minipreps[®]. After pre-treatment with lysostaphin, *S. aureus* plasmid DNA can be extracted by the alkaline lysis method of Birnboim and Doly^{52–54} but large plasmids may not be adequately resolved (fig. 2, lanes 8–13). If the NaOH concentration is increased from 0.2 N to 0.4 N and the SDS concentration from 1% to 2%, this alkaline lysis method can be used to extract plasmid DNA from which large plasmids can be separated (fig. 2, lanes 2, 3, 5 and 7). Fig. 2 shows plasmid DNA extracted from isolates of MRSA previously reported as phenotype II strains.²⁵ These MRSA may carry two large plasmids of 30.4 and 34.8 kb (fig. 2, lanes 2, 3, 5 and 7). When plasmid DNA was extracted from these isolates with the Magic MiniPrep[®] kit, a single large plasmid was observed but when the modified alkaline lysis method was used (fig. 2, lanes 2, 3, 5 and 7), the two plasmids are clearly visible. When this modified alkaline lysis method was used, a small number of isolates of AR types 1 and 2 showed additional plasmids. A number of AR type 6 isolates also showed extra plasmids. AR types 1 and 2 were not isolated during the present study. AR type 6 isolates presented classification problems and although the demonstration of additional plasmids did not resolve these difficulties, it indicates the need to be aware of the limitations of the plasmid DNA extraction methods used.

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