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Typing Multidrug-Resistant *Staphylococcus aureus*: Conflicting Epidemiological Data Produced by Genotypic and Phenotypic Methods Clarified by Phylogenetic Analysis

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An outbreak of an unusual tetracycline-sensitive, rifampicin- and ciprofloxacin-resistant, methicillin-resistant *Staphylococcus aureus* (MRSA) strain at a large teaching hospital was investigated. Two typing methods, phage typing and restriction fragment length polymorphism (RFLP) by pulsed-field gel electrophoresis (RFLP-PFGE), gave conflicting results which were clarified by phylogenetic analysis. Phage typing identified all the "epidemic-associated" strains as identical, while RFLP-PFGE further divided these strains into four pulsotypes. Phylogenetic analysis showed these four pulsotypes were related genetically and also recognized a second strain of MRSA causing a continuing cross-infection problem. Variation in the RFLP-PFGE pattern was shown to occur following lysogenization of phage-sensitive MRSA. These results indicate that in analyzing outbreaks caused by subgroups of clonal organisms like MRSA, it is necessary to use at least two typing methods and that conflicts between these could be resolved by phylogenetic analysis.

There is a growing consensus that restriction fragment length polymorphism (RFLP) using pulsed-field gel electrophoresis (PFGE) is the method of choice in the clinical laboratory for typing methicillin-resistant *Staphylococcus aureus* (MRSA) (1, 5). Phage typing using the Basic International Set of Typing Phages has shown less ability to type the current isolates of MRSA, although the use of experimental phage sets can improve the typeability rate (17). The largest study to date (12), having compared multiple techniques, concluded that each institution should routinely use two typing methods. The authors, however, did not comment on how to resolve discordant results which may be produced by such an approach.

We present a detailed study of an outbreak of MRSA in an eastern Australian university teaching hospital where a variety of typing methods were apparently discordant. Concord Repatriation General Hospital is a 700-bed tertiary institution with a large medically complicated, elderly patient population with multiple readmissions. Like other eastern Australian hospitals it has an endemic MRSA problem. In 1992, 50% of nosocomial *S. aureus* infections were MRSA. It is often difficult to assess whether isolates of MRSA reflect old infections or a current problem due to cross-infection and/or antibiotic prescribing.

The isolation from clinical cases, in 1992, of a number of MRSA isolates with an unusual tetracycline-sensitive, rifampicin- and ciprofloxacin-resistant antibiogram suggested that there may be a cross-infection problem at this hospital. In 1994 we compared these 1992 strains with the "background" population of historical and contemporary, clinical and environmental isolates to assess whether the unusual isolates represented an outbreak and whether the cross-infection measures then instituted had been necessary and effective.

The various typing methods we used produced conflicting groupings of MRSA strains. This problem was resolved by the application of sophisticated phylogenetic methods and analysis of the molecular mechanisms relating two of the typing methods, phage typing and RFLP-PFGE.

MATERIALS AND METHODS

Bacterial strains. A total of 50 isolates of MRSA from Concord Repatriation General Hospital were included in this study (Table 1). Eighteen clinical isolates (92-01 to 92-18) were from individual patients in various wards throughout the hospital in 1992. Twenty-three isolates (94-19 to 94-41) were from clinical cases occurring in three wards (110, 230, and ICU) of the hospital in the first 6 months of 1994. Nine environmental isolates (94-42 to 94-50) were collected from the three wards in February 1994. In addition, nine isolates (92-01, 92-08, 92-10, 92-11, 92-17, 94-20, 94-21, 94-24, and 94-43) were lysogenized with phage 1648 and analyzed for changes in phage typing and PFGE patterns.

Because one subsidiary purpose of this study was to test the specificity of strain typing methods, multiple isolates from some patients were included. Of the 1994 MRSA isolates, five (94-19, 94-20, 94-21, 94-22, and 94-23) were single isolates from five patients, two were from one patient from the same site on the same day (94-36 and 94-37), six were pairs taken from the same patients but from different sites on different days (94-34 and 94-35, 94-38 and 94-39, and 94-40 and 94-41), and six isolates represented multiple collections from one patient; with four (94-28, 94-29, 94-30, and 94-31) being from different sites on different days and two (94-32 and 94-33) being from the same site on the same day. The remaining four isolates were multiple isolates from one patient: two (94-24 and 94-25) from the same site on consecutive days and two (94-26 and 94-27) from different sites on different days.

Environmental swabs in wards 110, 230, and ICU were taken from bed frames, cabinets, telephones, monitors, window ledges, and sink areas. Swabs were plated on blood agar and mannitol salt agar plates and incubated for 18 and 48 h, respectively, at 37°C. Suspected staphylococcal colonies were picked off, and purified, and MRSA isolates were confirmed.

All isolates were DNase positive, slide coagulase positive, and tube coagulase positive (overnight colony suspended in 0.5 ml of 1:10 diluted rabbit plasma incubated at 37°C for 2 h). Strains were stored in Protect Bacterial Preserver Beads (Technical Service Consultants Ltd, Heywood, Lancashire, United Kingdom) at −70°C.

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TABLE 1. Typing of MRSA isolates

Isolate	Source[a]	Patient	Ward	Antibiogram	Phage pattern	PFGE pulsotype
92-01	C	1	LDH	1	42E/54/83A/85/81/88//1648/87M	I
92-02	C	2	510	1	42E/54/83A/85/81/88//1648/87M	I
92-03	C	3	SMC	1	42E/54/83A/85/81/88//1648/87M	I
92-04	C	4	2	1	42E/54/83A/85/81/88//1648/87M	I
92-05	C	5	110	1	42E/54/83A/85/81/88//1648/87M	I
92-06	C	6	330	1	42E/54/83A/85/81/88//1648/87M	I
92-07	C	7	110	1	42E/54/83A/85/81/88//1648/87M	V
92-08	C	8	3	1	42E/54/83A/85/81/88//1648/87M	V
92-09	C	9	230	1	42E/54/83A/81/88//87M	IV
92-10	C	10	110	1	42E/54/83A/85/81/88//1648/87M	I
92-11	C	11	610	1	42E/54/83A/85/81/88//1648/87M	I
92-12	C	12	230	2	(83A/85/95)wk/88//87M	II
92-13	C	13	ICU	2	(83A/85/95)wk/88//87M	II
92-14	C	14	EAC	2	83A/85/88//56C/87M	II
92-15	C	15	LDH	3	29/79/95/88//56A/87M/13M	VI
92-16	C	16	530	4	42E/54/83A/85/81/88//1648/87M	I
92-17	C	17	120	4	(83A/85/95)wk/88//87M	II
92-18	C	18	CLIN	5	29/95/88//87M	IV
94-19	C	19	110	1	42E/54/83A/85/81/88//1648/87M	I
94-20	C	20	110	1	42E/54/83A/85/81/88//1648/87M	I
94-21	C	21	ICU	1	42E/54/83A/85/81/88//1648/87M	I
94-22	C	22	110	1	42E/54/83A/85/81/88//1648/87M	I
94-23	C	23	110	4	42E/54/83A/85/81/88//1648/87M	VII
94-24	C	24	ICU	2	85/88//87M/13M	III
94-25	C	24	ICU	2	85/88//87M/13M	III
94-26	C	24	ICU	2	85/88//87M/13M	III
94-27	C	24	ICU	8	85/88//87M/13M	III
94-28	C	25	230	2	83A/85/88//56C/87M	II
94-29	C	25	230	2	83A/85/88//56C/87M	II
94-30	C	25	230	2	83A/85/88//56C/87M	II
94-31	C	25	230	2	83A/85/88//56C/87M	II
94-32	C	25	230	2	83A/85/88//56C/87M	II
94-33	C	25	230	2	83A/85/88//56C/87M	II
94-34	C	26	230	6	84/90/88//90A/87M	III
94-35	C	26	ICU	6	84/90/88//90A/87M	III
94-36	C	27	ICU	6	84/90/88//90A/87M	III
94-37	C	27	ICU	6	84/90/88//90A/87M	III
94-38	C	28	110	6	84/85/88//87M	IX
94-39	C	28	110	6	84/85/88//87M	IX
94-40	C	29	ICU	4	85/95/90/88//90A/1648/87M/13M	X
94-41	C	29	230	7	(83A/85/95)wk88//87M	II
94-42	E		110	1	42E/54/83A/85/81/88//1648/87M	I
94-43	E		110	1	42E/54/83A/85/81/88//1648/87M	I
94-44	E		110	2	(83A/85/95)wk/88//87M	II
94-45	E		110	2	(83A/85/95)wk/88//87M	II
94-46	E		110	2	(83A/85/95)wk/88//87M	II
94-47	E		110	2	(83A/85/95)wk/88//87M	II
94-48	E		110	2	(83A/85/95)wk/88//87M	II
94-49	E		ICU	9	84/85/90/88//90A/1648/87M/13M	VIII
94-50	E		ICU	9	84/85/90/88//90A/1648/87M/13M	VIII

[a] C, clinical isolate; E, environmental isolate.

Antimicrobial susceptibility. Susceptibility tests were performed using a replica plate technique (19) and according to National Committee for Clinical Laboratory Standards methods (document M7-A2). Five colonies, from an 18-h culture on blood agar, of each strain were inoculated into 5 ml of peptone water, to give a final concentration of 1×10^7 to 5×10^7 organisms per ml which was replicated in 1.0-μl aliquots onto the antibiotic agar. The concentrations of antibiotics in the test agar were as follows (in milligrams per liter): penicillin, 0.125; methicillin, 4; erythromycin, 1; tetracycline, 4; chloramphenicol, 8; fusidic acid, 1; rifampicin, 1; vancomycin, 4; trimethoprim, 1; gentamicin, 2; and ciprofloxacin, 1. Plates were incubated at 37°C, with duplicate methicillin plates also incubated at 30°C. Results were read after 18 h and strains were considered resistant if more than three colonies of visible growth were present.

Phage typing. Phage typing was carried out according to the method described by Blair and Williams (3). The 23 phages of the Basic International Set of Typing Phages were supplemented by three experimental phages, 187, 90, and 88, issued by the International Centre, Colindale, and nine experimental phages isolated at Royal Prince Alfred Hospital, Sydney, Australia (2). Phage 1648 quoted in this study is synonymous with phage C (2, 18). All phages were used at 100× routine test dilution (RTD). Isolates were considered to be of different phage type if they differed in sensitivity to two or more phages.

Lysogenization. Staphylococcal strains were lysogenized by spotting undiluted phage onto a lawn of cocci. After incubation overnight at 30°C, a small quantity of the secondary bacterial growth in the area of lysis was streaked onto blood agar plates. After incubation overnight at 37°C, single colonies were picked into broth and tested for the presence of the lysogenizing phage, for resistance to it, and for alteration in phage typing and RFLP-PFGE patterns. The lysogenized strains were numbered in the usual way; e.g., strain 92-08(1648) indicated strain 92-08 lysogenized with phage 1648.

PFGE. PFGE was done by a procedure modified from those described by Udo and Grubb (14). Eight colonies from an overnight culture on blood agar were inoculated into 8 ml of brain heart infusion broth, incubated with shaking in a 37°C water bath until the optical density (A_{600}) measured 0.75. The culture was

chilled on ice for 5 to 10 min before being pelleted by centrifugation. The pellet was suspended in 10 ml of ice-cold ST buffer (1 M NaCl, 10 mM Tris HCl [pH 7.6]) and then recentrifuged. The pellet was then suspended in 2 ml of 50 mM EDTA, pH 8.0. Then, 200 μl of this cell suspension was mixed with an equal volume of 1% low-melting-point agarose (Sigma Chemical Co., St. Louis, Mo.) and cast into blocks (10 by 20 by 1 mm) in a perspex mold (BioRad, Richmond, Calif.). Each plug was placed in an Eppendorf tube containing 500 μl of lysis solution (1 M NaCl, 100 mM EDTA [pH 8.0], 6 mM Tris HCl [pH 7.6], 0.5% Sarkosyl, 0.2% sodium deoxycholate, 0.5% Brij 58, 1 mg of lysozyme per ml, 50 μg of lysostaphin per ml) and left overnight at 37°C in a shaking water bath. After lysis the plug was transferred to a sterile Eppendorf tube containing 500 μl of proteolysis solution (1% sodium dodecyl sulfate, 0.05% Sarkosyl, 5 mM Tris HCl [pH 7.6], 50 mM EDTA [pH 8.0], 2.5 mg of proteinase K) and the tubes were left for 24 h at 50°C with shaking. The agarose inserts were then washed twice in 50 mM EDTA (pH 8.0) over 2 h at room temperature. The plugs were then transferred to fresh 50 mM EDTA (pH 8.0) where they were stored at 4°C.

For restriction endonuclease digestion, each agarose gel plug was cut in half (containing approximately 1 μg of DNA) and placed in a sterile Eppendorf tube with 500 μl of ice-cold water. This was left on ice for half an hour. The water was then replaced with 500 μl of *Sma*I restriction enzyme buffer (Promega, Sydney, Australia), and the tube was left on ice for half an hour. Then, 40 U of *Sma*I restriction enzyme (Promega) was added and the tube was again left on ice for half an hour and then at room temperature for 4 h.

Chromosomal RFLPs were analyzed by loading the agarose plugs into the wells of a 1% agarose running gel (BioRad) prepared in 0.5× Tris borate-EDTA and then sealed with 1% agarose. Bacteriophage Lambda Ladder DNA (BioRad) was used in each gel as a molecular weight marker. Electrophoresis was performed with the BioRad CHEF Mapper Pulsed-Field Electrophoresis Chamber with the following parameters: 200 V; initial pulse time of 0.47 s; final pulse time of 1 min, 3.8 s; for 24 h at 9°C.

The gels were stained with ethidium bromide and visualized using a transilluminator (Ultraviolet Products Inc., Sydney, Australia) and photographed with Polaroid film type 665 in a Polaroid Land camera.

A four-band difference indicated a different pulsotype (13). If isolates differed by up to three bands then they were classified as subtypes of the pulsotype.

Phylogenetic analysis. The degree of nucleotide sequence similarity of chromosomal DNA in the MRSA isolates was estimated using the modification of the methods of Nei and Li (9) and Upholt (15) as suggested by El-Adhami et al. (4). The proportion of shared fragments between any two isolates was calculated as follows: $F = 2n_{xy}/(n_x + n_y)$, where n_x is the total number of fragments from isolate X, n_y is the total number of fragments from isolate Y and n_{xy} is the number of fragments shared by the two isolates. The fraction of nucleotides different between two isolates was measured by the following relationship: $\rho = 1 - \{[(F^2 + 8F)^{1/2} - F]^{1/n}/2\}$, where F is the coefficient of similarity and n is the number of base pairs recognized by the restriction endonuclease, which has a value of 6 for *Sma*I. Using the estimated ρ values a dendrogram illustrating the degree of sequence similarity was constructed by the neighbor-joining algorithm of Saitou and Nei (10) and Drawtree, accessible in the PHYLIP group of programs (distributed by J. Felsenstein, Department of Genetics, University of Washington, Seattle) through the Australian National Genome Information Service.

Reproducibility. In order to ensure accuracy of each typing method, each isolate was typed at least twice and the pattern was interpreted by two independent observers.

RESULTS

In order to measure the relatedness of the strains according to the typing methods, all 50 isolates were tested "blind" and then correlated epidemiologically.

Antibiotic sensitivities. All 50 isolates were resistant to penicillin, methicillin, erythromycin, trimethoprim, and gentamicin and sensitive to vancomycin. Forty-one isolates (82%) were resistant to ciprofloxacin, 32 (64%) were resistant to tetracycline, 23 (46%) were resistant to rifampicin, 2 (4%) were resistant to fusidic acid, and 2 (4%) were resistant to chloramphenicol.

Therefore, according to their various sensitivities to these five latter antibiotics the 50 isolates could be grouped into nine antibiograms (numbered 1 to 9) (Table 2). Group 1 contained 11 1992 isolates (associated with the "apparent MRSA outbreak"), and four clinical and two environmental 1994 isolates. Group 2 contained three 1992 clinical isolates, nine 1994 clinical isolates, and five environmental isolates. The remaining 16 isolates from both 1992 and 1994 gave various antibiograms, groups 3 to 9.

Phage typing. All 50 isolates were phage typeable, at 100× RTD, with the Basic International Set of Typing Phages and with an experimental set of phages. Eleven reproducible phage patterns were discernible (Table 1).

TABLE 2. Antibiograms of MRSA isolates

Group	Antibiotic sensitivity[a]					No. of isolates	
	T	C	F	Ri	Cip	1992	1994
1	S	S	S	R	R	11	6
2	R	S	S	S	R	3	14
3	R	S	S	R	S	1	0
4	R	S	S	R	R	2	2
5	R	R	S	R	S	1	0
6	R	S	S	S	S	0	6
7	R	R	S	S	R	0	1
8	S	S	S	S	S	0	1
9	R	S	R	S	R	0	2

[a] T, tetracycline; C, chloramphenicol; F, fusidic acid; Ri, rifampicin; Cip, ciprofloxacin; S, sensitive; R, resistant.

Phage type 42E/54/83A/85/81/88//1648/87M was the most frequently isolated phage type: 11 (57.9%) of the 1992 isolates and 7 (21.9%) of the 1994 isolates. Ten of eleven 1992 isolates and six of seven 1994 isolates of this phage type were tetracycline sensitive and represented the "epidemic-associated" strain. The two tetracycline-resistant isolates with this phage type were isolates 92-16 and 94-23.

Isolate 92-09, showing phage type 42E/54/83A/81/88//87M, was tetracycline sensitive and differed from the epidemic-associated phage type showing no lysis with phage 1648 and phage 85.

One of the 1992 isolates and six of the 1994 isolates gave phage type 83A/85/88//56C/87M, and three of the 1992 and six of the 1994 isolates gave phage type (83A/85/95)wk/88//87M. Phage types 29/79/95/88//56A/87M/13M and 29/95/88//87M were shown by 1992 isolates, and phage types 85/95/90/88//90A/1648/87M/13M, 84/85/90/88//90A/1648/87M/13M, 84/85/88//87M, 84/90/88//90A/87M, and 85/88//87M/13M were shown by 1994 isolates.

PFGE analysis. The 50 isolates (analyzed by PFGE) could be divided into 10 reproducible pulsotype patterns, I to X (Table 1). Figure 1 shows a representative PFGE pattern of each pulsotype. Pulsotype I included nine isolates from 1992 and six from 1994. All these isolates were phage type 42E/54/83A/85/81/88//1648/87M and, except for isolate 92-16, were

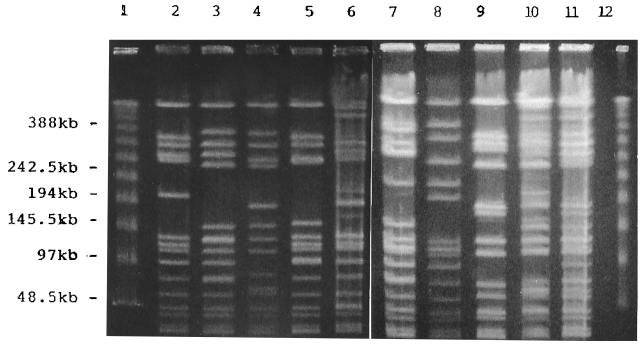

FIG. 1. PFGE patterns of *Sma*I digests of total DNA from representatives of each pulsotype. Lanes: 1 and 12, lambda DNA concatamers used as molecular size markers; 2, pulsotype I (94:22); 3, pulsotype II (92:13); 4, pulsotype III (92:25); 5, pulsotype IV (92:09); 6, pulsotype V (92:08); 7, pulsotype VI (92:15); 8, pulsotype VII (94:23); 9, pulsotype VIII (94:50); 10, pulsotype IX (94:38); 11, pulsotype X (94:40).

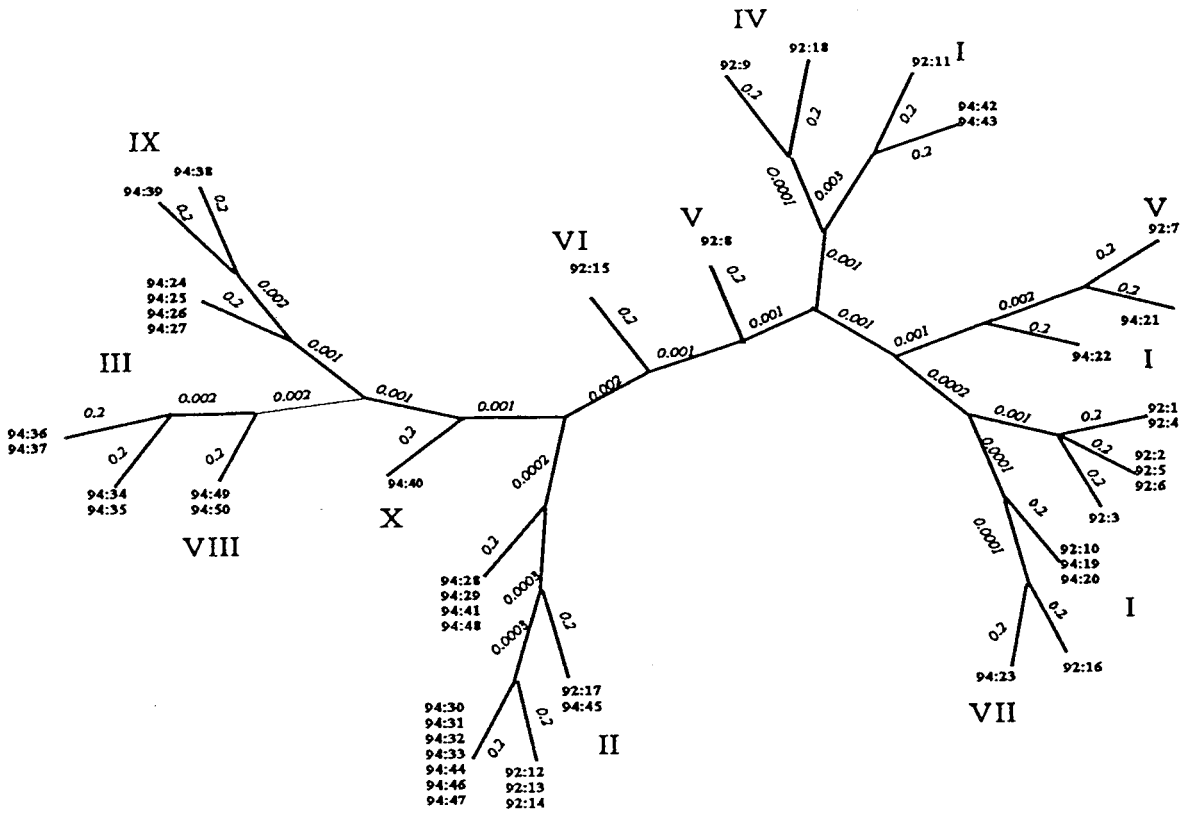

FIG. 2. Dendrogram calculated from the ρ values for genomic DNA from all isolates. Pulsotypes are represented by Roman numerals. The genetic distance (ρ) is indicated between each node of the tree. The methods for calculating and construction of the dendrogram are described in Materials and Methods.

tetracycline sensitive. Pulsotype II included 16 isolates, 4 from 1992 and 12 from 1994; 7 of these isolates were phage type 83A/85/88//56C/87M, and 9 were phage type (83A/85/95)wk/88//87M. Pulsotype III included eight isolates: four were phage type 84/90/88//90A/87M, and four were phage type 85/88//87M/13M. Pulsotype patterns IV, V, and VI were shown by 1992 isolates, and patterns VII, VIII, IX, and X were shown by 1994 isolates.

Phylogenetic analysis. A dendrogram (Fig. 2) was constructed as described in Materials and Methods. Zero genetic distance corresponds to identical PFGE patterns. Each terminal branch of the dendrogram represents a pulsotype subtype. The dendrogram showed that isolates of pulsotypes I, IV, V, and VII grouped together. Pulsotype II isolates grouped together, and isolates of pulsotypes III, VIII, IX, and X grouped together.

Lysogenization. Isolates 92-01, 92-08, 92-10, 92-11, 92-16, 94-20, 94-23, 94-42, and 94-43 were selected for lysogenization with phage 1648, because they were all lysed at RTD by this phage. Following lysogenization, these isolates showed no lysis with phages 1648 and 85 giving the phage type 42E/54/83A/81/88//87M. The PFGE patterns of all the lysogenized strains showed a two-band difference.

Relationship of antibiogram, phage typing, and PFGE analysis data to epidemiological data. Correlation of the typing methods with epidemiological data showed that of the 1992 isolates which had been identified as epidemic-associated isolates, by virtue of their sensitivity to tetracycline and resistance to rifampicin and ciprofloxacin, all belonged to the same phage type except 92-09, and all had the same pulsotype except isolates 92-07 and 92-08 (pulsotype V) and 92-09 (pulsotype IV). This "core" epidemic-associated strain, phage type 42E/54/83A/85/81/88//1648/87M and PFGE pattern I, was again isolated on six occasions in 1994. Four of these isolates, 94-19, 94-20, 94-21, and 94-22, were from separate clinical cases isolated over a period of 4 months. Three were from ward 110, and two isolates, 94:42 and 94:43, were from the environment of ward 110. Isolate 94-23 from a patient in ward 110 had the epidemic phage type and although resistant to tetracycline and PFGE pattern VII, grouped with the epidemic-associated isolates in the dendrogram.

In 1992, there were three clinical isolates 92-12, 92-13, and 92-14, with pulsotype II and antibiogram profile 2. However, they were divided into two groups by phage typing: phage type (83A/85/95)wk/88//87M and phage type 83A/85/88//56C/87M. In 1994 there were 12 isolates with pulsotype II; 11 gave antibiogram profile 2, and 1 gave antibiogram profile 7. These were again divided into two groups by phage typing. Five were environmental isolates, phage type (83A/85/95)wk/88//87M and antibiogram 2, from ward 110, and one was a clinical isolate from patient 29 (ward 230) with this phage type but antibiogram 7. Six clinical isolates, phage type 83A/85/88//56C/87M, were from patient 25 in ward 230. All these isolates of pulsotype II clustered together on the dendrogram.

Three other isolates in antibiogram group 2 were pulsotype III and phage type 85/88///87M/13M. They were isolated from the same patient, patient 24, but on different days. A fourth isolate, 94:27, from this patient gave the same PFGE and phage patterns but had a different antibiogram profile. All these isolated grouped together in the dendrogram.

Pulsotype III also contained four other isolates but with phage type 84/90/88//90A/87M and antibiogram profile 6.

These were isolated from two patients, 26 and 27, from the same ward, ICU, but 2 months apart and grouped together phylogenetically. No environmental isolates with these patterns were detected in this ward.

One patient, patient 28, had two isolates, 94-38 and 94-39, from different sites on different days which gave identical phage type (84/85/88//87M) and PFGE pattern IX.

Environmental isolates 94-49 and 94-50 were identified as the same by antibiotic profile, phage type, and PFGE patterns but were not associated with any clinical cases in this study.

DISCUSSION

The chief aims of this study were to determine if cross-infection had resulted in the dissemination of an epidemic MRSA with an unusual antibiotic sensitivity profile and whether improved infection control procedures had contained the outbreak.

By including among the background strains isolates which should be identical (same patient, site, and day) or very closely related but perhaps distinguishable (same patient, different site, and/or different day), we demonstrated the specificity and sensitivity of our typing methods.

Phage typing and RFLPs successfully identified the majority of the 1992 tetracycline-sensitive isolates as phage type 42E/54/83A/85/81/88//1648/87M and pulsotype I. Ten of the tetracycline-sensitive isolates and one tetracycline-resistant isolate were identical by phage typing while PFGE/RFLP separated these isolates into pulsotypes I and V. All these isolates clustered together on phylogenetic analysis, consistent with the epidemiological information.

The remaining tetracycline-sensitive isolate (92-09) pulsotype IV, which grouped with I and V phylogenetically, was not recognized by phage typing as an epidemic strain but its phage pattern varied only by the lack of sensitivity to phages 1648 and 85. We have shown previously that lysogenization in vitro with phage 1648 also results in loss of sensitivity to phage 85 (2). Therefore this isolate may belong to the epidemic group but has additional prophage resulting in the altered phage sensitivity. As phage 1648 has been reported to be carried by many MRSA in Australia (2, 7), alteration in the carriage of this prophage may be a factor in typing variability seen in MRSA epidemiological studies (16). Smeltzer et al. (11) reported that *Sma*I defined RFLP patterns observed in the *S. aureus* strains they studied could be accounted for by the presence of prophage. In this study, the effect of lysogenization in vitro with phage 1648 in the epidemic strains produced observable changes in RFLP-PFGE patterns. The lysogenized strains remained in the pulsotype grouping of their respective original strains (data not shown), but by showing loss of two phage sensitivities they would be considered different by phage typing. This reflects the problems encountered when closely related groups of MRSA are typed.

In 1994, it appeared that the tetracycline-sensitive epidemic MRSA was now endemic in the hospital. RFLP-PFGE separated these isolates into two pulsotypes, I and VII. Phylogenetic analysis again clustered these 1994 isolates on the dendrogram with the 1992 group I, IV, and V pulsotypes.

Our phylogenetic analysis also suggested that there was a failure of infection control in 1992 which involved more than the easily recognizable tetracycline-sensitive strains. It identified a second group of MRSA, all pulsotype II, among the "background" 1992 isolates. Phage typing divided this second group of strains into two phage types. When MRSA with these characteristics were isolated from the environment and from two patients in 1994 it indicated that this second epidemic MRSA strain had also persisted despite the improved infection control measures.

A recent report suggesting that RFLP-PFGE is better than other methods for typing *S. aureus* was based on a study of isolates from various staphylococcal syndromes (1). It is known that some, at least, of these syndromes are caused by clonal groups within the general population of *S. aureus*, and one would expect RFLP-PFGE to readily distinguish between such clonal groups. However, it is more difficult to distinguish epidemic strains within a clonal group. Furthermore, it is likely that within a hospital with a long history of endemic MRSA infection, isolates of this organism will be more closely related than MRSA isolated from different hospitals. Assuming a molecular clock, our dendrogram, with its long terminal branches of roughly equal genetic distance (approximately 0.2) and its short internodal distances (0.001 to 0.002) could support the hypothesis that the MRSA isolates we studied are descendants of one intrahospital MRSA strain. Comparison with the MRSA analyzed by the same method by El Adhami et al. (4) similarly suggests that we are studying intrahospital subclones separated by intranodal distances with an order of magnitude of 10^{-3}.

It has been suggested that outbreaks involving MRSA should be investigated by using two typing methods as we have done, particularly when the isolates come from within one institution (8, 12). However, there is no current guidance on how to resolve the conflicting results which two methods may produce. The epidemic strain which initiated this study had a single phage type but was divided by RFLP-PFGE into three pulsotypes. On the other hand, the second epidemic strain identified by RFLP-PFGE was divided into two phage types. In both situations, then, one method was too discriminatory. Therefore, either RFLP-PFGE or phage typing may provide useful, relatively stable typing data as variants of a subclone gain-or-lose phage or restriction sites. Our phylogenetic analysis readily provided an epidemiological background on which the significance of these variants could be judged. This technique estimates the fraction of nucleotides different between isolates from changes in RFLP patterns and assumes that restriction sites are gained or lost by the random mutation of single nucleotides. It has been suggested in a study of *Enterococcus faecalis* that mobile genetic elements make a major contribution to changes in RFLP patterns (6). By contrast, our experiments on the effect of lysogenization on RFLP patterns in MRSA indicate that mobile genetic elements do alter RFLP patterns but not significantly.

The more sophisticated phylogenetic analysis of RFLPs produced by PFGE can be used to clarify the problem of two typing methods producing conflicting results and may be especially useful in demonstrating the long-term relationships of epidemic and endemic strains of MRSA.

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