Epidemiol. Infect. (1994), **112**, 93–101

Ribotyping of *Staphylococcus aureus*: an assessment using well-defined strains

J. F. RICHARDSON, P. APARICIO*, R. R. MARPLES AND B. D. COOKSON

Laboratory of Hospital Infection, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK

(*Accepted 30 July 1993*)

SUMMARY

Ribotyping, with homologous or heterologous (*Escherichia coli*) r-RNA, of the propagating strains for phages of the international set for strains of *Staphylococcus aureus* of human origin was undertaken to determine the discrimination of this typing method. Ribotyping could distinguish between strains of different phage groups, but could not distinguish between seven phage group III strains of different phage type. Ribotyping may be a useful adjunct to phage typing in *S. aureus* but is unlikely to replace it as the primary method of epidemiological typing.

INTRODUCTION

Epidemiological typing of *Staphylococcus aureus* has been of interest to the hospital microbiologist for many years [1]. For general use a typing method must be inexpensive, easy to perform and, above all, rapid. Phage typing, organized internationally since the 1950s, fulfilled these criteria [2], but the international phage set, because of its derivation, is most useful for strains of Northern European origin. Many antibiotic resistant strains of *S. aureus* found in Britain and Europe are indeed phage typable [3–5]. However, elsewhere in the world this may not be the case. This observation, in conjunction with the occurrence in England and Australia of a methicillin-resistant strain which was poorly typable by the international phages [6], has led to the belief that multi-resistant strains of *S. aureus*, whether methicillin-resistant of susceptible, are non-phage typable. In consequence, molecular methods, particularly so-called genotyping methods, though slower and more costly than conventional methods have been used to type hospital strains of *S. aureus* [4, 7–10].

Human strains of *S. aureus* may be allocated to phage groups on the basis of their phage typing patterns [11]. In general, community strains belong to phage group I, skin isolates to phage group II and hospital isolates to phage group III, although strains of type 94/96 (phage group V), 95 or 81 may be found in both environments as a cause of local or general infection. There is some evidence to support the hypothesis that phage groups II and V are each of single strain origin [12, 13]. Phage groups I and III are less well defined but these may also be distinct restriction groups [14].

* Present address: Laboratorio de Infectíon Hospitalaria, Servicio de Bacteriología, Centro National de Microbiología, 2822D Majadahonda, Madrid, Spain.

The three components of any typing system are typability, reproducibility and discrimination. The typability by molecular methods is high, essentially 100%, and with the predominance of a single strain of *S. aureus* in any given hospital outbreak the second criterion has been adequately investigated [e.g. 4, 9]. There remains, however the question of discrimination, especially within sets of unrelated strains of the same phage group.

The discriminatory ability of a genotyping method, ribotyping [15] was assessed using the propagating strains of the international phages of *S. aureus* as the test series. It was hoped that testing this collection would confirm the genetic distance of strains of phage group II or V, the well-defined restriction groups, from strains of the less well defined groups I, III or I + III. Additionally, because many strains of MRSA belong to phage group III [16] it was thought that the range of ribotypes among the diverse, unrelated propagating strains of this phage group might give a coarse measure of the discriminatory power of this technique and thus a measure of its usefulness in the investigation of outbreaks of MRSA infection. The propagating strains are well-defined, lyophilized and readily available.

MATERIALS AND METHODS

Isolation details, characteristics of and pertinent references to the propagating strains, as available in the records of the National Collection of Type Cultures, are given in Table 1. There are currently 22 propagating strains supplied to national phage typing centres. Two, PS80 and PS81, are both representatives of the 80/81 pandemic strain prevalent in the late 1950s and early 1960s and both deposited in 1953 though no isolation date is available; otherwise these strains are independent. These strains were collected over a 30-year period and by definition cover the range of phage groups. None are resistant to methicillin or to gentamicin. These strains are lyophilized for long term storage. New ampoules were opened for this study and the phage type of the strain at routine test dilution (RTD) confirmed [2]. Additional phage typing at 100 × RTD with the international set and at RTD with a supplementary phage set was also carried out [5].

Ribotyping

DNA extraction

Two methods of DNA extraction were used:

(i) the modification of the method of Jordens and Hall [22] by Richardson and co-workers [23].

(ii) the guanidium thiocyanate method of Pitcher and co-workers [24] as modified for *S. aureus* [25].

Enzymic digestion

Total cellular DNA, *c* 8 μg per reaction was digested with the restriction endonucleases *Eco*RI, *Hin*dIII, *Pvu*II, *Hae*III, *Cla*I or *Cfo*I according to the manufacturer's instructions. Electrophoresis of the digests was carried out in 0·9% agarose gels in 1 × tris/borate buffer pH 8·2 [26] at 35v constant voltage for 18–20 h at 4 °C. A biotinylated *Hin*dIII digest of λ DNA (Boehringer Mannheim) was used as a molecular weight standard. Gels were stained with ethidium bromide

https://doi.org/10.1017/S0950268800057459 Published online by Cambridge University Press

Staphylococcus aureus *of human origin*

NCTC	PS	Lytic group of phage	Isolation data	Phage type (RTD) international phages	Supplementary phages	Reference
8331	29	I	Oxford (UK); 1943	29	NT	
8507	52	I	-*	52	NT	
8363	52A/79	I;I	Breast abscess; (UK) 1944	52A/79	NT	Wilson and Atkinson (17)
9789	80	I	Australia; 1953	80/81	NT	80/81 pandemic strain
8319	3A	II	Pemphigus; Cardiff (UK) 1943	3A	NT	Wilson and Atkinson (17)
8327	3C	II	Birmingham (UK); 1945	3C/55/71	NT	
8358	55	II	Birmingham (UK)	3C/55/71	NT	
9315	71	II	Birmingham (UK); 1950	3C/55/71	NT	Williams and Rippon (18)
8509	6	III		6/47/53/54/75/83A/84/85	616/617/620/622/623/626/629/630	Wilson and Atkinson (17)
8357	42E	III	breast Billericay (UK); 1945	42E	NT	
8325	47	III	corneal ulcer Oxford(UK); 1943	47/53/75/77/84/85	616/617/620/622/626	
8511	53	III	wound; 1948	53/54/75/77/84/85	617/622	Korman and Bearman (19)
8329	54	III	wound; 1948	47/53/54/75/77/84/85	616/617/618/620/622/626/630	
8354	75	III	Birmingham (UK); 1951	53/75/77/84/85	618/626	
8356	77	III	Birmingham (UK); 1951	77	617/622/626	
10039	83A	III	New York (USA); *c* 1963	6/47/53/83A/84/85	616/617/620/622/626/630	
10455	84	III	Colindale (UK); *c* 1963	84/85	616/617/620/622/626/630	Jevons and Parker (20)
10457	85	III	Newcastle upon Tyne (UK); *c* 1963	84/85	618/620/626	Jevons and Parker (20)
9719	81	miscellaneous	Canada; 1953	80/81	NT	80/81 pandemic strain
10971	95	miscellaneous	Dunedin (New Zealand); 1972	95	NT	
10970	94	V	Texas (USA); 1971	94/96	NT	Blouse and co-workers (21)
10972	96	V	Dunedin (New Zealand); 1972	94/96	NT	

Order of strains follows the convention used in the Staphylococcus Reference Laboratory, CPHL, Colindale, London, UK.
Phages 95 and 81 are distinct. *, no information.

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(7 mg/l) for 30 min and viewed under u.v. light. A photograph was taken as a permanent record.

Blotting and probing

DNA was transferred to nylon membrane (Hybond, Amersham International plc) by the method of Southern [27] in early experiments or by vacuum transfer (Vacugene System Pharmacia Ltd) in later experiments. Biotinylated cDNA to *E. coli* 16S+23S rRNA (Boehringer Mannheim) or *S. aureus* NCTC 10442 16S+23S r-RNA [10] labelled by the method of Pitcher and colleagues [28] was used as the probe. Prehybridization, hybridization and the development of genes were performed as given by Richardson and co-workers [25].

Analysis of results

Digestion of every strain with each enzyme were performed at least in duplicate. Strains were compared using the restriction fragment pattern (fingerprint) as defined by the total number of bands developed after probing *Eco*RI, *Hin*dIII or *Cla*I digests. Each band was considered a separate locus. Migration distances were measured to the nearest 0·5 mm. More than 60 loci were therefore defined. Conclusions were based on inter-gel data. One worker (PA) performed this analysis. Similarity [29] between each pair of strains was calculated and strains were clustered by simple matching using an unweighted pair group method with averages (UPGMA) program [30].

RESULTS

Phage typing

The results of phage typing at RTD with both the international and supplementary phages are given in Table 1. Typing with the supplementary phages distinguished between PS 47 and PS 54, which are indistinguishable at RTD by the international phages and also increased the distinction between the other propagating strains of lytic group III phages. Typing with the international phage set at 100 × RTD also distinguished between PS 47 and PS 54: at 100 × RTD PS 47 was lysed, additionally, by phages 29, 52, 80 and 83A, thus indicating that the strain belongs to phage group I + III, while PS 54 did not show any additional reactions.

Ribotyping

The six enzymes used: *Eco*RI, *Hin*dIII, *Cla*I, *Hae*III, *Pvu*II and *Cfo*I, all digested *S. aureus* DNA. Blots were successfully hybridized with probes generated from either *E. coli* or *S. aureus* r-RNA. For any given enzyme digest the probe pattern generated for any given strain was independent of the bacterial source of the r-RNA probe, though when *S. aureus* r-RNA was used as the template the colour tended to develop more quickly and to become more intense. This difference in speed and intensity of development was only appreciated when *E. coli* and *S. aureus* probes labelled at the same time, using the same reagents and developed in parallel using solutions made at the same time were examined. Inter-run differences, irrespective of the r-RNA used as the template caused significant variation in colour development time ranging from 10 min to 12 h.

Qualitative discrimination between strains was poor with *Cfo*I, *Pvu*II and

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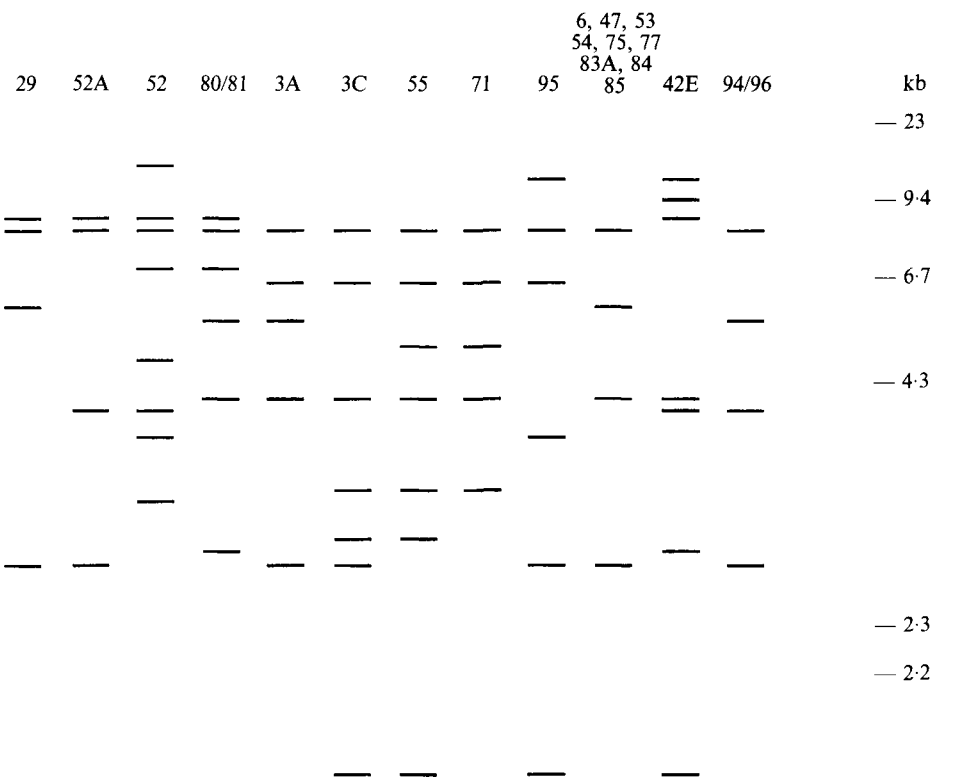

Fig. 1. Diagrammatic representation of the 12 ribotypes obtained after probing a *Cla*I digest of the propagating strains.

*Hae*III. When the *Cfo*I digests were probed the fragments which hybridized with either probe were all under 6·7 kb. Therefore analysis was not attempted. Probing of *Pvu*II digests only yielded six profiles. Within strains of phage groups I and III there was little discrimination. PS 80 and PS 81 were separated from this main group, as was PS 42E, otherwise strains of these phage groups were indistinguishable. Probing of the *Hae*III digests generated 10 profiles. PS 80, 81 and 3A were indistinguishable as were PS 3C, 55 and 71 although PS 6, PS 47 and PS 83A could be separated from the main group III complex. PS 6 and PS 47 could not be distinguished by this enzyme. The probe profiles of PS 94 and PS 96, though indistinguishable from each other were most 'different' from those of the other propagating strains.

Hybridization of *Eco*RI, *Hin*dIII or *Cla*I digests yielded 13, 13 and 12 patterns respectively. The number of bands per pattern ranged from 4–12. The patterns generated after probing the *Cla*I digest are shown diagrammatically in Figure 1. When this enzyme was used PS 80 and PS 81 could not be distinguished, neither could PS 94 and PS 96. A set of nine group III propagating strains: 6, 47, 53, 54, 75, 77, 83A, 84 and 85 were indistinguishable. When the *Eco*RI digest was probed PS 47 and 84 were distinct from this complex, but PS 3C and 71 were then indistinguishable. The *Hin*dIII probe profile, however, again allowed distinction between PS 3C and PS 71 but PS 47 was included in the PS 6 complex. Examples of probe profiles are shown in Figures 2 and 3. When the probe patterns generated using all three enzymes were combined a total of 64 band positions was possible.

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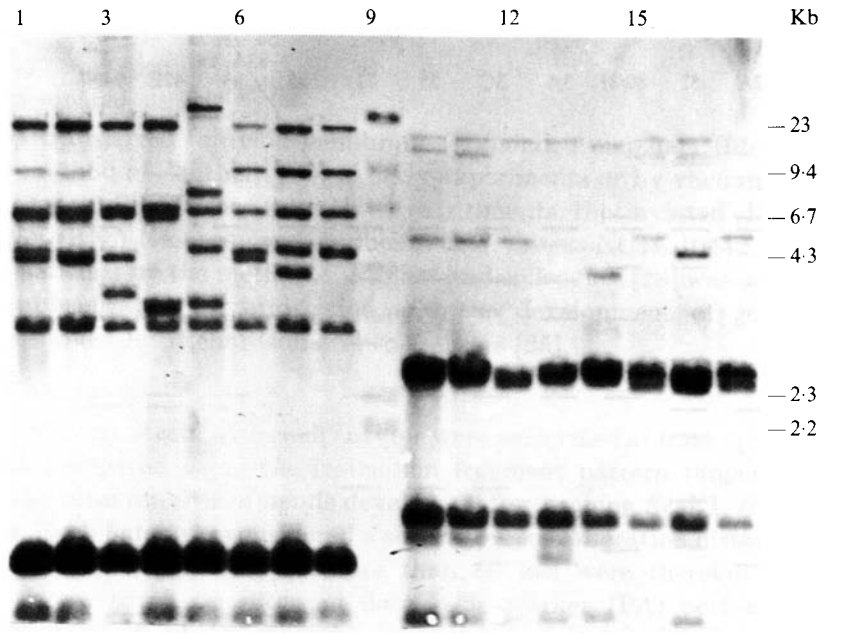

Fig. 2. Ribotypes of individual group I and group II propagating strains. PS 29: lanes 1 and 10. PS 52; lanes 2 and 11, PS 52A; lanes 3 and 12. PS 80; lanes 4 and 13. PS 3A; lanes 5 and 14. PS 3C; lanes 6 and 15. PS 55; lanes 7 and 16. PS 71; lanes 8 and 17. Lane 9; biotinylated *Hin*dIII digest of λ DNA. Lanes 1–8; *Hin*dIII digests. Lanes 10–17; *Hae*III digest. Digested DNA transferred by capillary transfer.

Addition of the *Pvu*II profile did not increase discrimination therefore the combined probe profiles of *Hin*dIII, *Eco*RI and *Cla*I digests were used to generate a dendrogram based on simple matching (Fig. 4). The relationships between propagating strains of different phage groups may be seen schematically in this dendrogram. As expected, within the major phage groups there was a 75% or greater degree of similarity, with strains of phage group III, excluding PS 42E, forming the tightest group. PS 80 and 81 were the furthest away from the remainder of the group I strains. Also, as expected, PS 95 and PS 42E were the most distinct, with strains of phage groups I and III showing a greater degree of similarity to each other than either did to strains of group II or group V. PS 94 and PS 96, the propagating strains of the group V phages were related to PS 3A and the 85% level and to the remainder of the group II phages at 75% level.

DISCUSSION

Ribotyping is a method of genotyping which uses a probe derived either directly from cloned r-RNA genes or labelled cDNA to 16S+23S r-RNA [15]. The r-RNA used may be homologous or heterologous. In staphylococci, ribotyping has been applied most frequently, but not exclusively, to methicillin-resistant strains of *S. aureus*, with little regard to its discriminatory power. The propagating strains for the international set of typing phages for *S. aureus* of human origin were used as the test set because not only are they well characterized and easily available but they also represent the majority of 'types' of strains found in human clinical material.

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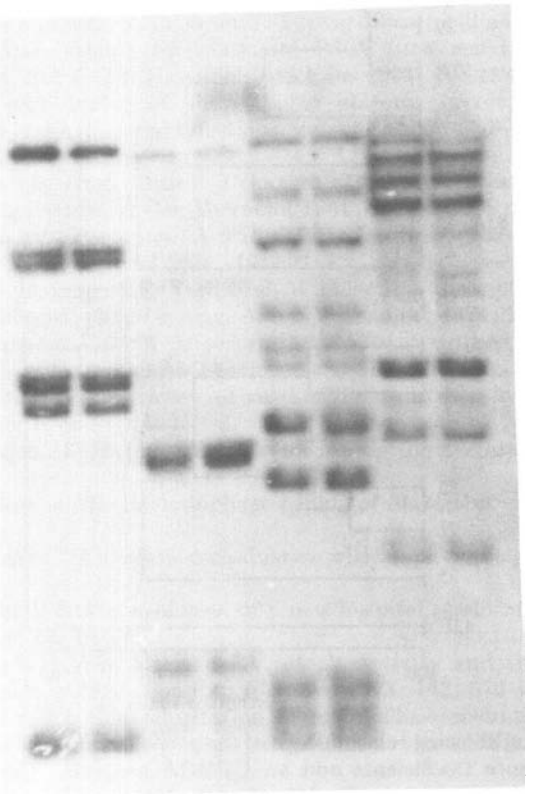

Fig. 3. Ribotypes of PS 80 and PS 81. PS 80; lanes 1, 3, 5, 7. PS 81; lanes 2, 4, 6, 8. Enzymes used: lanes 1 and 2: *Hin*dIII, lanes 3 and 4; *Hae*III, lanes 5 and 6; *Eco*RI, lanes 7 and 8; *Pvu*II. Digested DNA transferred by capillary transfer.

In this study enzyme digests which gave the greatest numbers of probe patterns: *Eco*RI, *Cla*I and *Hin*dIII were examined initially. Combination of the data derived from probing these digests divided the strains into 13 groups (Fig. 4). In general the observations made from phage typing were confirmed; there was greater relatedness within a phage group than between phage groups, and groups II and V were most clearly 'different' from the others. PS 42E was clearly distinct from the remainder of the group III strains thus confirming previous observations [11]. PS 95, as expected, appeared to be quite different from the rest of the propagating strains. In contrast, PS 80 and 81, both representatives of the 80/81 pandemic strain [31] were indistinguishable from each other as were the two group V propagating strains [13]. However this method fails to distinguish between seven group III propagating strains (Fig. 4). Additional data obtained by examining the patterns obtained from the probed *Pvu*II or *Hae*III digests were unhelpful, although use of *Hae*III did allow distinction between PS 6, PS 83A and strains of the 53 complex but did not distinguish between PS 3C, 55 and 71 or between PS 29 and 52 or, more importantly, between PS 80/81 and 3A.

In this study ribotyping confirms empirical observations made about the relatedness of strains within or between phage groups [11–14] and underlines their genetic relatedness. It may therefore be useful in assigning phage-untypable

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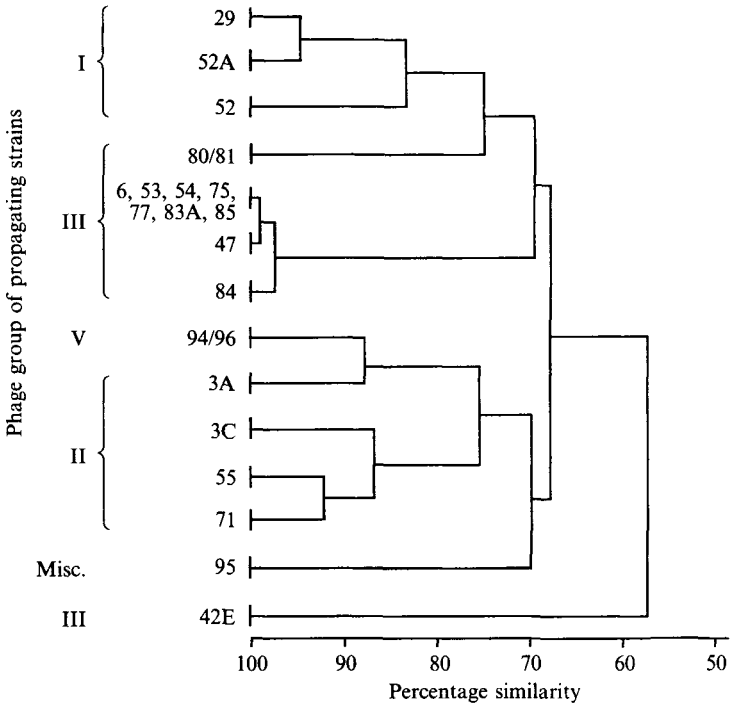

Fig. 4. Dendrogram, showing relationships of the propagating strains, generated using the Dice Co-incidence Coefficients and an UPMGA program. The phage group of individual propagating strains is given on the 'y' axis.

strains to phage group. However, because ribotyping fails to distinguish between PS 6 and PS 85, phenotypically very different group III propagating strains, and has proved disappointing in the definition of some epidemic MRSAs [32, 33] its value lies not as a method for the first line investigation of outbreaks of infection caused by strains of *S. aureus*, but as a useful adjunct to classical epidemiological methods.

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