EPIDEMIOLOGICAL TYPING

Genotyping of European isolates of methicillin-resistant *Staphylococcus aureus* by fluorescent amplified-fragment length polymorphism analysis (FAFLP) and pulsed-field gel electrophoresis (PFGE) typing

RUTH GRADY*‡, DOMINIQUE BLANC†, PHILIPPE HAUSER† and JOHN STANLEY*

*Molecular Biology Unit, Central Public Health Laboratory, 61 Colindale Avenue, London and †Division Autonome de Médecine Préventive Hospitalière, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

A representative panel of 50 European MRSA isolates was subjected to genotype analysis by fluorescent amplified-fragment length polymorphism (FAFLP) and by macrorestriction pulsed-field gel electrophoresis (PFGE). Each isolate had a unique profile with FAFLP. To model genetic relationships within the continuing MRSA epidemic, cluster analysis of FAFLP data was made, revealing nine clone complexes of MRSA. Most of these were also found by PFGE. A number of isolates had FAFLP profiles significantly different from others, and might represent emerging epidemic strains. FAFLP analysis proved particularly suitable for surveillance of the MRSA epidemic at national and international levels.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a world-wide nosocomial pathogen, usually resistant to several antibiotics [1]. Currently, the leading effective therapy is intravenous vancomycin, which can be predicted to be effective before susceptibility testing of an MRSA isolate. In the UK in 1998, MRSA bacteraemia represented 32% of total *S. aureus* bacteraemia [2], the majority being acquired in hospital.

Epidemiological typing of isolates forms part of the effective infection control management of MRSA. Phage typing has been used to type clinical isolates of *S. aureus* for nearly 50 years [3]. Isolates are classified into broad 'phage groups' depending on their pattern of lysis by one or more of a set of internationally agreed phages. These groups can be subdivided into phage types, depending on particular

phage reactions. Although this simple phenotypic technique is easy to perform and convenient for large numbers of isolates, it has only limited discriminatory power. Moreover, in the USA, up to 20% of isolates are phage non-typable [4]. In England and Wales, 16 phage types of MRSA have affected more than one hospital, and are termed epidemic (EMRSA) [5]. In the 1990s, three of them (EMRSA-15, EMRSA-16 and EMRSA-3) predominated in England and Wales, while other phage types receded in clinical significance [2].

A widely used molecular method for typing MRSA is pulsed-field gel electrophoresis (PFGE). In Continental Europe, >20 strains of EMRSA have been defined by PFGE [6]. PFGE is currently employed in the USA for integrated national surveillance of the enteric pathogen *Escherichia coli* O157 [7]. Some authors report that PFGE may produce unstable banding patterns with MRSA, generating problematic variation in inter-laboratory studies [8]. The observed instability may be due to lysogenic integration of one or more phages in the *S. aureus* genome [9].

Fluorescent amplified-fragment length polymorphism (FAFLP) is based on selective amplification by PCR of a subset of restriction fragments from a digest of a whole bacterial genome. Typically, the DNA of an organism is digested with a restriction endonuclease

Received 20 Nov. 2000; accepted 8 Dec. 2000.

Corresponding author: Dr J. Stanley (e-mail: sevenwoods@hotmail.com).

‡Present address: Biomolecular Sciences, University of Manchester Institute of Science and Technology (UMIST), PO Box 88, Manchester M60 1QD.

with a six-base recognition sequence followed by digestion with a four-base recognition sequence endonuclease. Site-specific double-stranded adaptors are ligated to the ends of the fragments to generate primer binding sites. A subset of these restriction fragments amplified by PCR is separated on an automated sequencer. One of the site-specific primers is fluorescently labelled: only fragments cut with the corresponding enzyme are seen by the laser detector of the sequencer. Because a differentially labelled internal size standard is run in each gel track, all fragments are sized with precision (± 1 bp). FAFLP thus offers a valuable new approach to high-resolution genotyping of MRSA. When applied to isolates of the UK epidemic phage type EMRSA-15, it generated reproducible DNA fingerprints, resolving isolates as members of a clone complex [10]. Similarly, isolates of all 16 UK epidemic phage types were resolved by FAFLP into five clone complexes [11]. In the present study, FAFLP was used to examine the genetic diversity of a range of European MRSA isolates, with a view to modelling the genetics of the continuing MRSA epidemic; the majority of isolates had been analysed previously by multiprimer RAPD-PCR typing [12] and they were also subjected to PFGE analysis.

Materials and methods

Strains

A representative panel of 50 methicillin-resistant isolates and reference strains of *S. aureus* from diverse European countries was examined (Table 1).

Methods

Bacteria were grown overnight on blood agar at 37°C, in an aerobic atmosphere. Genomic DNA was isolated from plate cultures with lysostaphin-NaCl-cetyltrimethylammonium bromide [10]. The endonucleases *EcoRI* and *MseI* were used to digest *c.* 500 ng of genomic DNA from each isolate, and restriction fragments were ligated to double-stranded adaptors as described previously [10]. PCR was performed in a final volume of 25 μ l with 2 pmol of *EcoRI* adaptor-specific primer (*EcoRI*+0) labelled with 5-carboxy-fluorescein (FAM) and 5 pmol of *MseI* adaptor-specific primer (*MseI*+C). Amplified fragments were separated in a denaturing polyacrylamide 5% gel, on an ABI Prism 377 DNA automated sequencer as described previously [10]. The fragments were sized with the ABI software GeneScan 2.1 and peak height thresholds were set at 100. Electropherograms of fragment profiles were inspected visually for polymorphisms. The presence and absence of fragments were scored in a binary matrix and recorded as a text (tab delimited) file in Excel v.5.0 (Microsoft). Dice coefficients of similarity were computed and cluster analysis was performed by the unweighted paired group method with arithmetic averages (UPGMA; NEIGHBOR pro-

gram of PHYLIP). The resulting tree was displayed by using the TREEVIEW program [13] with a percentage similarity cut-off of 93% as described previously [11].

PFGE was performed as described previously [6]. The relatedness between the macrorestriction patterns was analysed with the comparative quantification module (with the band matching tolerance and optimisation set at 3 and 5 points respectively) in GelCompar software (Applied Maths, Kortrijk, Belgium).

Results

FAFLP and cluster analysis

Each isolate had a unique FAFLP profile. Among the 50 FAFLP profiles, there were 125 distinct amplified fragments (*EcoRI*+0, *MseI*+C), ranging in size from 55 to 320 bp. Thirty-four amplified fragments were common to all the MRSA isolates; the remaining 91 fragments were polymorphic. The average number of fragments constituting the FAFLP profile of an MRSA genome was *c.* 70. A percentage similarity cut-off of 93% was applied to the FAFLP UPGMA tree analysis of the binary matrix, revealing nine clusters of MRSA (Fig. 1). There were 10–31 fragment differences between any two FAFLP clusters; the number of fragment differences between isolates within a cluster varied from 1 to 10.

The 15 MRSA isolates from England and Wales were distributed among five FAFLP clusters. Clusters 1–3 corresponded to epidemic phage types EMRSA-16, EMRSA-15 and EMRSA-3. Other EMRSA phage types fell into two separate clusters (4 and 5 of Fig. 1), as has been described recently [11]. Six Continental European isolates also fell into clusters 3, 4 and 5. Cluster 1 contained a reference strain (E16) and four clinical isolates (X57, Y35, Y36, Y39) belonging to the epidemic phage type EMRSA-16. Cluster 2 (26–31 amplified fragment differences from cluster 1) contained a reference strain (E15) and three clinical isolates (X59, Y30, Y32) belonging to epidemic phage type EMRSA-15. A reference strain (E3) and a clinical isolate (Y8) belonging to phage type EMRSA-3 formed a third cluster; this cluster also included the Polish MRSA isolate X1, and had 22–24 amplified fragment differences from cluster 2. Cluster 4 contained the reference strain of EMRSA-1 (E1), an English clinical isolate (Y58) previously unassigned to a phage type, an epidemic isolate from Hungary (X6) and two sporadic MRSA isolates from Switzerland (X83, X123). Cluster 5, with 14–16 amplified fragment differences from cluster 4, included the reference strains of UK epidemic phage types EMRSA-2 (E2) and EMRSA-10 (E10), grouped with epidemic MRSA isolates from Germany (X17) and Switzerland (X110).

Four further FAFLP clusters were delimited; among them were distributed 18 Continental European MRSA

Table 1. Strains examined in study

Isolate no.*	Country of origin	FAFLP†	Cluster PFGE‡	Multiprimer RAPD§
Y56	England	—	ND	ND
X9	Germany	8	4	2b
XF1	France	8	—	2b
X30	Belgium	8	—	2b
X24	Belgium	8	4	2b
X40	Switzerland	8	4	2b
X7	Portugal	8	4	2b
X5	Portugal	8	4	2b
E10 (NCTC 13137)	England	5	ND	ND
X17	Germany	5	5	2b
E2 (NCTC 12232)	England	5	ND	ND
X110	Switzerland	5	5	2b
X83	Switzerland	4	—	2a
X123	Switzerland	4	—	2a
Y58	England	4	ND	ND
E1 (NCTC 11939)	England	4	ND	ND
X6	Hungary	4	—	2a
X18	Austria	7	—	2a
X3	Poland	7	—	2a
X78	Switzerland	—	—	—
XF11	France	6	—	1
X12	Germany	6	3	1
X65	Switzerland	6	—	1
X37	Switzerland	6	3	1
X1	Poland	3	—	1
Y8	England	3	ND	ND
E3 (NCTC 13130)	England	3	ND	ND
X80	Switzerland	—	—	—
X114	Switzerland	—	—	—
X77	Switzerland	—	1	3
X59	England	2	1	3
Y32	England	2	ND	ND
Y30	England	2	ND	ND
E15 (NCTC 13142)	England	2	ND	ND
X103	Switzerland	—	—	—
X84	Switzerland	—	—	—
X118	Switzerland	—	—	5
X108	Switzerland	9	2	5
X21	Germany	9	2	5
X46	Canada	9	2	5
X29	Belgium	9	2	5
X124	Switzerland	9	2	5
X33	Switzerland	9	2	5
Y39	England	1	ND	ND
Y35	England	1	ND	ND
Y36	Wales	1	ND	ND
E16 (NCTC 13143)	England	1	ND	ND
X57	England	1	6	5
X76	Switzerland	—	6	4
X131	Switzerland	—	—	4

—, not assigned to a cluster; ND, no data.

*X strains are described in more detail in reference [12]. 'Y' isolates are clinical isolates and 'E' strains are reference epidemic MRSA strains from England and Wales as described in reference [11].

†FAFLP cluster number derived from Fig. 1.

‡PFGE cluster number derived from Fig. 2.

§Clustering defined with multiprimer RAPD-PCR as described previously [12].

isolates and the sole North American MRSA isolate in this study. Cluster 6 had 15–19 amplified fragment differences from cluster 3, and contained non-epidemic isolates from Switzerland (X37, X65) and epidemic strains from Germany (X12) and France (XF11). Cluster 7 had 12–18 amplified fragment differences from cluster 4 and contained epidemic isolates from Poland (X3) and Austria (X18). Epidemic isolates from Portugal (X5, X7), Germany (X9), France (XF1), Belgium (X24, X30) and a non-epidemic isolate from Switzerland (X40) fell into an eighth cluster with 10–

15 amplified fragment differences from cluster 7. The ninth cluster (>25 fragment differences from either cluster 1 or cluster 8) contained epidemic isolates from Switzerland (X33, X108, X124), Belgium (X29) and Germany (X21), as well as a Canadian MRSA strain (X46).

A number of isolates did not fall into the well-defined FAFLP clusters described above. They included nine non-epidemic MRSA isolates from Switzerland (X118, X76, X131, X84, X103, X77, X80, X114, X78) and

Fig. 1. UPGMA radial cluster tree derived from FAFLP data showing genetic relationships of MRSA. Each branch represents the unique FAFLP profile of a single isolate. See Table 1 for explanation of the labels. Horizontal scale bar represents 10% divergence.

one from England (Y56). Profiles of all these isolates differed by at least 10 amplified fragments from those delimited in the nine major clusters.

PFGE analysis

When macrorestriction and PFGE were applied, all isolates had unique profiles, with the exception of isolates X29 and X33 that displayed profiles identical to each other. A total of 63 distinct fragments was observed, which, in accordance with the methodology, we were not able to size precisely. The number of fragments constituting a PFGE profile varied from 14 to 19 (Fig. 2).

When a cut-off of *c.* 65% was used for analysis of the macrorestriction PFGE data, six clusters were found, broadly confirming the FAFLP results (Fig. 2). The lower percentage cut-off for PFGE data compared with FAFLP data reflects the considerably reduced number of data points in these profiles. Certain isolates not assigned to a cluster by FAFLP were clustered by PFGE. For instance, isolate X77 had a PFGE pattern related to isolate X59 (six band differences) and they were classed in the same PFGE cluster, but were not clustered by FAFLP (see above). Similar considerations also apply to isolates X76 and X57. Conversely, isolates XF1, XF11, X3, X30, X65, X83 and X123

clustered (although not identical) with other isolates by FAFLP, as also by RAPD-PCR [5], were distinct by macrorestriction PFGE.

Discussion

In this study, FAFLP defined nine clone complexes among European isolates. Two of these (clusters 1 and 2) correspond to EMRSA-16 and EMRSA-15, the most prevalent phage types currently found in England and Wales. FAFLP detected differences between all isolates and strains in these two tight clusters. There were a further seven FAFLP-defined clone complexes, as well as 10 'free-standing' isolates, which could have the potential to give rise to newly emerging epidemic strains. Three FAFLP clusters (clusters 4, 6, 8 in Fig. 1) included isolates not associated with epidemic spread in a community. High-resolution FAFLP genotyping should assist in mapping the short-term evolution and dissemination of MRSA clones. Its high discriminatory power suggests that it may be particularly useful for resolving issues in local infection control. As it can display putative genetic relationships between current and even potential epidemic strains, it may be used to generate a practical model of the MRSA epidemic, both in local and in global terms.

Fig. 2. UPGMA dendrogram derived from PFGE data and PFGE banding patterns. Each branch represents the unique macrorestriction profile of a single isolate.

A valid epidemiological typing system should exhibit high discriminatory power, be reproducible, easy to use, and capable of typing all isolates [14]. FAFLP analysis fulfils these criteria, exhibiting particular advantages with respect to reproducibility and discriminatory power [10, 15–18]. While PFGE exhibits most of these criteria for many organisms, and has been applied with notable success in the PulseNet system for national epidemiological typing of *E. coli* O157 in the USA [7], it had lower discriminatory power than FAFLP in the present study of MRSA, and poorer reproducibility in an earlier study [10]. The ease-of-use of FAFLP analysis is likely to be further improved by robotics, automated sample loading and adaptation to multi-channel capillary sequencers. This high-resolution genotyping technique has now been applied successfully to the definition of epidemiological clonality in a range of other important bacterial

pathogens including *E. coli* [15], *Mycobacterium tuberculosis* [18], *Streptococcus pyogenes* [16, 17], *Neisseria meningitidis* and *Salmonella enterica* serotype Enteritidis (unpublished observations). All these studies are characterised by unusually high reproducibility and discriminatory power. All polymorphic amplified fragments, which act as anonymous markers of genotype, were sized to within one base pair, an important and novel feature in the definition of a clone. Furthermore, experiments in this laboratory show that significant amplified fragments can readily be cloned for nucleotide sequencing. Although there is no published genome sequence for *S. aureus* at the time of writing, in the case of *E. coli* and *M. tuberculosis*, FAFLP analysis was modelled on, and relatable to, the published genome sequences. There are compelling theoretical reasons to believe that FAFLP can act as a bridge between the evolving database of whole

bacterial genome sequences and the requirements of contemporary epidemiology.

Special thanks for provision of MRSA to B. Willey, H. de Lencaste, I. Sanches, J. Etienne, M. Struelens, T. Berendt, I. Bowler, W. Hryniewicz, K. Trzinski, W. Witte, G. O'Neill and B. Cookson.

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