MOLECULAR TYPING AND EPIDEMIOLOGY

Subtyping of methicillin-resistant *Staphylococcus aureus* isolates from the North-West of England: a comparison of standardised pulsed-field gel electrophoresis with bacteriophage typing including an inter-laboratory reproducibility study

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Bacteriophage typing is currently the recognised methodology for the typing of methicillin-resistant *Staphylococcus aureus* (MRSA) in the UK. Bacteriophage typing is less discriminatory and does not type all isolates compared with some molecular methods for typing MRSA. Chromosomal genotyping by pulsed-field gel electrophoresis (PFGE) is increasingly recognised as an improved method for typing MRSA, providing increased discrimination and typability. In this study the results of a comparison of bacteriophage typing and PFGE typing and subtyping are presented for a large collection of isolates from the North-West of England. Isolates belonging to the most frequently isolated epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA) bacteriophage types 15 and 16 were typed by PFGE with further discrimination of common PFGE types possible into a number of subtypes. These results for a large collection of isolates demonstrate the improved typing of MRSA with PFGE. The widespread acceptance of PFGE for typing MRSA isolates has been hampered by the lack of standardised methodologies. Recently, a standardised PFGE strain typing system, known as the GenePath system has become available. The results of an inter-laboratory comparison of PFGE typing for a collection of isolates demonstrated good reproducibility with this system.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infection [1]. Such infections are associated with high mortality and morbidity, creating a huge burden upon health care services. Many distinct strains of MRSA exist, some of which termed 'epidemic MRSA' (EMRSA) have increased epidemic potential [2], whilst others occur only sporadically (SMRSA). The ability to type MRSA isolates is critical in detecting outbreaks and for the early implementation of control measures.

In the UK, bacteriophage typing is currently used for typing MRSA, but is less discriminatory and fails to subtype a significant number of isolates compared with several molecular methods which have been examined [3–9], thus compromising outbreak identification and infection control. Furthermore, bacteriophage typing requires the maintenance of a large number of bacteriophage stocks and propagating strains, thereby restricting its use to only a small number of reference laboratories and limiting responsiveness to local outbreak situations.

Macro-restriction genomic profiles obtained by pulsed-field gel electrophoresis (PFGE) have been shown to yield the greatest discrimination between strains [3, 10]. Until now, a set of fully standardised protocols for PFGE, which can be applied between laboratories, has not been developed and this lack of standardisation has retarded the widespread acceptance of PFGE as the typing method for MRSA. Recently, a standardised PFGE system known as the GenePath strain typing system (BioRad) has been developed [11].

The GenePath strain typing system comprises pathogen-specific kits (including MRSA), a pre-programmed...
electrophoresis system and gel documentation system for macro-restriction analysis of bacterial chromosomal DNA. The combined benefits of the system enable standardised and inter-gel comparison, allowing the establishment of libraries of macro-restriction profiles and cataloguing of strain profiles. Furthermore, the electronic transfer of strain profiles for national or international surveillance of MRSA infection is possible with the gel documentation system.

The aim of this study was to compare bacteriophage typing with standardised PFGE for typing and subtyping MRSA in a large collection of isolates from the North-West of England. Secondly, the reproducibility of the system was examined by an inter-laboratory study of PFGE for MRSA typing by the GenePath system.

Materials and methods

Bacterial strains

A collection of 300 staphylococcal isolates from 267 patients from 18 hospitals in the North-West region of England, including coagulase-negative staphylococci (CNS) and representative isolates of known bacteriophage type (kindly provided by Dr J. Richardson, Central Public Health Laboratory, 61 Colindale Avenue, London) was examined. A smaller collection of 49 isolates was selected from this group for an inter-laboratory study.

Culture conditions

Isolates were recovered from Protect vials stored at −80°C by overnight culture on blood agar.

Methicillin resistance

Methicillin resistance was determined with E-test strips (AB Biodisk, Solna, Sweden).

Bacteriophage typing

Bacteriophage typing was performed by a modification of the method described by Blair and Williams [12] with the group III international set of bacteriophages maintained at Manchester Public Health Laboratory. Isolates not susceptible to any bacteriophage or lysed by a single bacteriophage at the routine test dilution (RTD) were re-tested at 100 × RTD. The bacteriophage types were defined by the number(s) of the bacteriophage(s) to which they were susceptible, with weak reactions taken into account to define the final bacteriophage reaction pattern.

PFGE

DNA from the MRSA isolates was prepared according to the manufacturer’s protocol (BioRad, Hemel Hempstead, Herts) with all reagents or concentrates of them supplied in the GenePath reagent kit. Briefly, overnight bacterial cultures grown in 3 ml of nutrient broth were harvested by centrifugation at 12 000 g for 2 min, resuspended in 150 μl of cell suspension buffer and kept at 55°C; 6 μl of lysozyme (0.15 mg) and 150 μl of molten agarose (1%) were added to this suspension and the resultant suspension was poured into a plug mould. The moulds were held at room temperature for 15 min to allow the agarose to set. The agarose plugs were placed in 500 μl of lysis buffer with 20 μl (0.5 mg) of lysozyme and incubated at 37°C for 1 h. The plugs were then washed in 1× wash buffer and incubated overnight in 500 μl of proteinase K buffer containing 20 μl (12 U) of proteinase K. Following these lysis steps six wash steps were performed with 1 ml of 1× wash buffer, one wash in 1 ml 0.1× wash buffer and a final wash in 500 μl of SmaI buffer. The plugs were then placed in 300 μl of SmaI buffer with 5 μl (25 U) of SmaI enzyme and incubated at 25°C overnight. After restriction endonuclease digestion the plugs were either stored in 1× wash buffer or electrophoresed immediately.

Agarose 1% gels were prepared with the GenePath Gel kit according to the manufacturer’s instructions with run parameters pre-programmed on the GenePath system for staphylococcal species. The resultant profiles were visualised by staining with ethidium bromide and documented by the GelDoc 1000 system (BioRad).

Macro-restriction profile analysis

The macro-restriction profiles were analysed by Molecular Analyst Image Analysis followed by the Fingerprinting and Fingerprinting Plus software (BioRad). The normalisation feature of the Molecular Analyst Fingerprinting and Fingerprinting Plus software allowed inter-gel alignment by the use of size standards electrophoresed on each gel. Analysis of the band patterns was possible by defining the band positions on the gel and archiving the banding patterns. An optional set of internal standards was available in the GenePath kit and was included alongside each set of samples. These controls acted as indicators of the success of both lysis and digestion steps.

Definition of PFGE macro-restriction profiles

PFGE macro-restriction profiles were defined by published guidelines [3, 13, 14]. Isolates were classified as genetically related if their PFGE patterns were consistent with a single genetic event resulting in a two or three band difference and a Dice coefficient (SD) of correlation of ≥80% calculated by the Molecular Analyst Fingerprinting and Fingerprinting Plus software. Isolates were classified as possibly genetically related if their PFGE band patterns were consistent with two genetic events, i.e., four to six band difference...
and an SD of 68–79%. Isolates were considered to be unrelated if their PFGE band pattern differed by six bands or more and an SD of ≤60%. All profiles were examined in consideration of each isolate’s detailed epidemiological information.

Reproducibility studies
To determine the reproducibility of the GenePath strain typing system for PFGE subtyping of MRSA four isolates were electrophoresed five times on the same and different gels to determine intra- and inter-gel reproducibility. A selection of 49 isolates which had been tested by the GenePath strain typing system in laboratory 1 (Manchester PHL) were shipped freeze-dried to laboratory 2 (Creighton University School of Medicine, Omaha, NE, USA) where they were analysed blind by the GenePath strain typing system.

Results
A comparison of bacteriophage typing and PFGE typing
The analysis of all staphylococcal isolates is summarised in Table 1. Isolates representing each of the EMRSA bacteriophage types were subdivided into additional subtypes by PFGE. PFGE electrophoretotypes were further subdivided into one or more subtypes. Isolates identified as one or other of the two most frequently isolated EMRSA strains (EMRSA 15 or 16) occurring within the UK were further subtyped (Figs. 1 and 2). Several isolates misidentified by bacteriophage type were identified as electrophoretotypes 5 and 1 and confirmed as EMRSA 15 or 16. Analysis by PFGE resulted in 90 distinct PFGE types. Further discrimination was also possible between isolates where subtypes were identified.

EMRSA strains
Amongst the EMRSA isolates, two macro-restriction profiles were associated with those MRSA strains bacteriophage typed as EMRSA 15 and EMRSA 16 (Figs. 1 and 2). The study group contained 98 isolates bacteriophage typed as EMRSA 15; of these 86% (85 of 98) were PFGE type 5 or a subtype of 5 (Fig. 1). The second predominant group were the 35 isolates bacteriophage typed as EMRSA 16; of these, 91% (32 of 35) were PFGE type 1 or a subtype of 1 (Fig. 2). PFGE profiles for representative isolates of the remaining EMRSA types 1–14 were distinct from each other as well as from EMRSA 15 and 16.

CNS strains
CNS isolates yielded profiles that were distinct from each other and also different from the MRSA isolates. This allows discrimination between MRSA and staphylococcal species other than S. aureus.

Table 1. Summary of data for staphylococcal isolates used in this study

<table>
<thead>
<tr>
<th>Staphylococcal isolates</th>
<th>Number of PFGE types (including subtypes)</th>
<th>Number (%) of isolates with methicillin MIC ≥ 8 mg/L.</th>
<th>Number of hospitals from which EMRSA isolates were referred*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMRSA 15</td>
<td>93</td>
<td>55 (91.4)%</td>
<td>12</td>
</tr>
<tr>
<td>EMRSA 16</td>
<td>35</td>
<td>32 (91.4)%</td>
<td>8</td>
</tr>
<tr>
<td>EMRSA 3</td>
<td>2</td>
<td>2 (100)</td>
<td>1</td>
</tr>
<tr>
<td>Other S. aureus</td>
<td>92</td>
<td>63 (68.5)%</td>
<td>16</td>
</tr>
<tr>
<td>Bacteriophage not typable</td>
<td>19</td>
<td>11 (57.9)%</td>
<td>6</td>
</tr>
<tr>
<td>CNS</td>
<td>59</td>
<td>22 (37.3)%</td>
<td>3</td>
</tr>
</tbody>
</table>

*From a total of 20 hospitals in the North-West of England.

1Not all isolates referred as MRSA were found to be methicillin resistant.

Fig. 1. PFGE types of those staphylococcal isolates designated EMRSA 15 by bacteriophage typing. *Other PFGE types (5), 1 (5), 7b (1), 8 (1), 11 (1), 12c (1), 14b (1), 14e (1), 18 (1).
Subtypes of PFGE type 1

Fig. 2. PFGE types of those staphylococcal isolates designated EMRSA 16 by bacteriophage typing.

Bacteriophage non-typable strains

Those MRSA isolates which were non-typable or bacteriophage type unknown (n = 24) in bacteriophage typing were all typed by PFGE; 63% (15 of 24) of these were PFGE type 5 (EMRSA 15) and one was PFGE type 1 (EMRSA 16) (Fig. 3).

Reproducibility

Four distinct MRSA isolates were repeatedly electrophoresed on the same and different gels. Intra- and inter-gel band size variation demonstrated good intra- and inter-gel reproducibility with band size coefficients of variation of ≤1.74% and ≤5.2%, respectively.

Inter-laboratory comparison of MRSA PFGE typing

There was complete correlation between the number of fragments produced in both laboratories over the size range 120–300 kb for 48 of the 49 isolates examined. A single isolate appeared to possess an extra band when analysed in the second laboratory (results not shown). All strains were classified as the same PFGE type or subtype by both laboratories.

Discussion

Until recently the traditional methods of typing MRSA have been bacteriophage typing and antibiograms. However, because of the problems of non-typability and ambiguous bacteriophage types, alternative molecular typing techniques have been examined, but have had limited application due to lack of standardisation. PFGE has been widely used to examine MRSA in outbreak situations [10, 15–17] and is now a recognised technique for subtyping MRSA [3]. Progress towards improved standardisation of techniques such as PFGE may be further advanced by the development of systems such as the GenePath strain typing scheme [11] for nosocomial outbreak investigation and surveillance. Furthermore, published guidelines for interpretation of PFGE profiles [13, 14], in combination with criteria for isolate submission for PFGE analysis, will allow the designation of strain types and subtypes.

EMRSA types 15 and 16 are the most common bacteriophage types found in the UK [18], which is reflected in isolates from the North-West of England. EMRSA 15 and 16 isolates were typed as PFGE types 5 and 1, respectively, and further subdivided into one or more subtypes by PFGE. The spread of EMRSA 15 and 16 throughout the North-West of England underlines the need for greater subtyping discrimination for improved outbreak identification and also emphasises the need for accurate epidemiological information.
accompanying isolates submitted for PFGE typing to determine the epidemiological significance of minor genetic events and the genetic relatedness of PFGE variants. This demonstrates the greater discrimination achieved with PFGE versus bacteriophage typing for typing MRSA. The remainder of the MRSA isolates were classified into different and distinct PFGE types. The data showing numbers of isolates with a single electrophoretotype such as type 14 demonstrate its usefulness for epidemiologically significant strains which previously remained unidentified as non-typable or unclassified.

An earlier inter-laboratory comparison of PFGE for the typing of MRSA [4] highlighted a number of problems involved in the standardisation of methodologies and analysis. Poor reproducibility was achieved in that study, which was performed in three different laboratories and involved two different techniques – field inversion gel electrophoresis (FIGE) and PFGE – and, where the same technique was compared between laboratories, different electrophoretic run conditions were used. The problems encountered by Cookson et al. [4] have been addressed in the present study in which good correlation between the participating laboratories was achieved.

Pattern analysis can be improved by the use of a gel documentation system and appropriate software for image capture, allowing the construction of large databases and eliminating human error and variation. The problems of inter-gel comparison and band pattern analysis can be overcome, as shown here, by the use of commercially available software incorporating a normalisation component which allows alignment to be made between any two gels provided that both gels have the universally selected size standard and appropriate software for image capture, allowing the construction of large databases and eliminating human error and variation. The problems of inter-gel comparison and band pattern analysis can be overcome, as shown here, by the use of commercially available software incorporating a normalisation component which allows alignment to be made between any two gels provided that both gels have the universally selected size standard.

Fully standardised protocols, reagents and electrophoresis conditions which can be pre-programmed are now available, allowing the establishment of PFGE for improved subtyping of MRSA.

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References