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Epidemiological characterization of methicillin-resistant *Staphylococcus aureus* isolated in the North West of England by protein A (*spa*) and coagulase (*coa*) gene polymorphisms

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SUMMARY

In a comparative study, isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) with known pulsed-field gel electrophoresis (PFGE) and bacteriophage type were analysed by polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) for additional discriminatory subtyping information. PFGE was previously performed using standardized, commercially available kits and pre-programmed software. Isolates were examined for coagulase (*coa*) and protein A (*spa*) gene polymorphisms following PCR amplification of the *coa* hypervariable and *spa* repeat regions. *Coa* gene RFLPs produced a total of 38 distinct combined patterns after digestion with *Hae*III and *Alu*I and identified the predominant epidemic (EMRSA) types 15 and 16. A unique *Hae*III restriction site was identified by RFLP and sequence analysis in the *coa* gene for EMRSA 15 but not EMRSA 16. The *spa* gene PCR yielded a total of 14 different profiles ranging from 3–18 repeats with the 2 predominant EMRSA types falling into 2 distinct groups. PCR detection of *coa* and *spa* polymorphisms offer a rapid preliminary strain identification and discriminatory subtyping information for surveillance of MRSA.

INTRODUCTION

Outbreaks of methicillin-resistant *Staphylococcus aureus* (MRSA) have become a major cause of hospital-acquired infection [1] and are now a world-wide problem. Some MRSA strains termed epidemic MRSA (EMRSA) have the ability to spread rapidly among patients whereas other strains (SMRSA) cause sporadic infections. Although the basis for epidemic potential has not yet been established, rapid identification of such strains is crucial for hospital infection control and eradication [2]. Furthermore, the emergence and spread of dominant clonal epidemic strains such as EMRSA types 15 and 16 in England and Wales [3] does not allow differentiation between isolates for surveillance or outbreak investigation and infection control. The problem of discriminatory subtyping between isolates from these major clonal EMRSA types is made more acute by the growing numbers of hospitals with endemic strains of MRSA and the growing numbers of community-associated MRSA [3, 4].

The current typing method routinely employed in the UK is bacteriophage typing but this requires the establishment of specialized facilities able to maintain bacteriophage stocks and propagating strains and additionally, some MRSA strains are insensitive to bacteriophage. Molecular typing methods have been shown to provide improved discrimination versus phenotyping methods including bacteriophage typing and are now frequently used for subtyping nosocomial

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pathogens including MRSA [5, 6]. Pulsed-field gel electrophoresis (PFGE) [5, 7–9] is now widely used for bacterial typing and has been shown to be the most discriminatory molecular method for subtyping MRSA but is a relatively time-consuming procedure to perform [10]. The search for a rapid method that identifies a discriminatory marker(s), which would allow for a more selective implementation of infection control measures, is still a high priority. Restriction fragment length polymorphisms (RFLP) in hypervariable gene targets including the staphylococcal coagulase (*coa*) and protein A (*spa*) genes have been investigated using the polymerase chain reaction (PCR) with or without restriction enzyme digestion (PCR-RFLP) for discriminatory subtyping of MRSA [11–15].

The *coa* gene is specific to *S. aureus* and expression of this gene is currently used for its routine identification. It has been shown that numerous allelic forms exist due to variable sequences and repeats within the 3′ coding regions [11]. Differentiation of *S. aureus* strains is therefore possible based on this variation and has been previously investigated [11, 12].

The X region of the *spa* gene is involved in cell wall attachment and has been shown to be polymorphic, containing a variable number of 24 bp repeats [13]. By amplifying the region containing these repeats it is possible to size the resultant fragment and thus determine the number of repeats therein. Discrimination of MRSA strains using this *spa* gene target has also been utilized to investigate MRSA outbreaks [13–15].

Evolution at single gene loci may provide epidemiologically significant information which is not apparent from PFGE macrorestriction profiles. The aims of this study were to investigate molecular subtyping of MRSA isolates from the North West of England using *coa* and *spa* gene polymorphisms, in comparison with PFGE and bacteriophage typing.

MATERIALS AND METHODS

Bacterial strains

A collection of 356 MRSA isolates obtained from 331 patients from 18 hospitals throughout the North West region of England were examined. The MRSA isolates were from both epidemiologically related and sporadic cases and EMRSA types 15 and 16 were well represented. A selection of isolates representing less frequently isolated EMRSA types were also included in the study (supplied by Central Public Health Laboratory, 61 Colindale Ave, London, UK).

Culture conditions

Isolates were recovered from Protect vials (Prolabs, Neston, Wirral, UK) stored at −80 °C by overnight culture on blood agar plates.

Methicillin resistance

Methicillin resistance was determined using the E-strip test (AB Biodisk, Solna, Sweden) [16]. The medium used was Mueller–Hinton containing 5% $NaCl_2$.

Bacteriophage typing

Bacteriophage typing was performed by modification of the method described by Blair and Williams [17] with the group III international set of bacteriophages as maintained at Manchester Public Health Laboratory. Isolates not susceptible or lysed by a single bacteriophage at the routine test dilution (RTD) were retested at 100 × RTD. The bacteriophage types were defined by the numbers of the bacteriophage to which they were susceptible with weak reactions taken into account to define the final bacteriophage reaction.

Pulsed-field gel electrophoresis

Plug preparation

DNA from the samples was prepared according to the manufacturers protocol (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) with all reagents or concentrates of them being supplied in the GenePath kit. Briefly, overnight cultures grown in 3 ml of nutrient broth were harvested by centrifugation at 12000 ***g*** for 2 min, resuspended in 150 ml of cell suspension buffer and kept at 55 °C. To this suspension 6 μl (0·15 mg) of lysozyme and 150 μl of 1% molten agarose was added and the resultant suspension poured into a plug mould. After setting for 15 min the agarose plugs were placed into 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 buffer containing 20 μl of Proteinase K (12 U). Following these lysis steps, six wash steps were performed using 1 ml of 1 × wash buffer, one wash in 1 ml of 0·1 × wash buffer and a final wash in 500 μl of *Sma*I buffer. The plugs were then placed in 300 μl of *Sma*I buffer, with the addition

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of 5 μl (25 U) of *Sma*I enzyme and incubated at 25 °C overnight. After restriction digestion the plugs were either stored in 1 × wash buffer or electrophoresed.

Gel electrophoresis

Agarose gels (1%) from the GenePath gel kit (Bio-Rad) were prepared according to the manufacturer's instructions with run parameters preprogrammed on the GenePath system for staphylococcal species. The resultant profiles were visualized by staining with ethidium bromide and documented using the GelDoc 1000 system (Bio-Rad).

Macrorestriction profile analysis

The macrorestriction profiles were analysed using Molecular Analyst Image Analysis followed by the Fingerprinting and Fingerprinting Plus software (Bio-Rad). PFGE macrorestriction profiles were defined by guidelines advocated by Struelens and colleagues [18] and Tenover and colleagues [19].

DNA extraction for amplification

DNA was extracted from staphylococci using DNAzol (Gibco–BRL, Paisley, UK). Approximately ten colonies were resuspended in 100 μl of sterile deionized H_2O (ddH_2O) followed by the addition of 1 ml of DNAzol and incubated at room temperature for 15 min. DNA was precipitated by the addition of absolute alcohol (0·5 ml) and incubation at room temperature for 15 min. The samples were then centrifuged for 10 min at 12000 ***g***. The supernatant was discarded and the resultant pellet washed in 1 ml of absolute alcohol, vortexed, then centrifuged for 5 min at 12000 ***g***. The supernatant was again removed and the DNA pellet allowed to dry before re-suspension in 50 μl of ddH_2O.

Coa gene amplification

Primers were designed upon the hypervariable region of the *coa* gene using the Primer Select software (DNASTAR Inc, Madison, USA).

Forward primer:
5′-^{1301}GAACAAAGCGGCCCATCATTA^{1322}-3′.
Reverse primer:
5′-^{2153}TAAGAAATATGCTCCGATTGTCG^{2131}-3′.

Base pair numbers refer to the positions on the *coa* gene (accession number X17679).

Extracted DNA (5 μl) was added to a 50 μl PCR reaction containing 1·5 μl (0·6 μM) of each primer, 5 μl of 10 × PCR buffer, 0·25 μl of each deoxynucleoside triphosphate (dNTP) (200 μM) (Amersham Pharmacia Biotech, St Albans, Hertfordshire, UK), 0·4 μl of *Taq* polymerase (1 U) (Life Technologies, Paisley, UK), 1 μl of W1 detergent, 4 μl of 4 mM $MgCl_2$ and 31·2 μl of ddH_2O.

DNA amplifications were performed using the following cycling parameters: denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 25 s, annealing at 52 °C for 40 s and extension at 72 °C for 1 min. A 5 min extension at 72 °C was included at the end of the final cycle.

Restriction enzyme digestion of the *coa* gene amplicon

PCR product (15 μl) was digested overnight with 1 μl (10 U) of the restriction endonucleases *Alu*I or *Hae*III (New England Biolabs Inc., Hertfordshire, UK), 11 μl of enzyme buffer and 15 μl of ddH_2O. The resulting fragments were electrophoresed in 12% polyacrylamide gels in the presence of ethidium bromide, visualized using u.v. illumination and recorded using the GelDoc 1000 gel documentation apparatus (Bio-Rad).

The RFLP patterns were then analysed using Molecular Analyst Image Analysis followed by the Fingerprinting and Fingerprinting Plus software (Bio-Rad). The normalization 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 upon the gel and the construction of libraries of profiles for classification into distinct RFLP patterns.

Spa gene amplification

Primers were designed upon the X-region of the *spa* gene using the Primer Select software (DNASTAR Inc).

Forward primer:
5′-^{1696}TCAAGCACCAAAAGAGGAAGA^{1712}-3′.
Reverse primer:
5′-^{2026}ACGACATGTACTCCGTTGCCG^{2005}-3′.

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Base pair numbers refer to the positions on the *spa* gene (accession number X61307).

Extracted DNA (5 μl) was added to a 50 μl PCR reaction containing 1·5 μl each primer (0·6 μM), 5 μl of 10 × PCR buffer, 0·25 μl each deoxynucleoside triphosphate (dNTP) (200 μM), 0·4 μl of *Taq* polymerase (1 U), 1 μl of W1 detergent, 4 μl of 1·5 mM $MgCl_2$ and 33·6 μl of ddH_2O. DNA amplifications were performed using the following cycling parameters: denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation of 94 °C for 25 s, annealing at 48 °C for 40 s, extension at 72 °C for 1 min. A 5 min extension at 72 °C was included at the end of the final cycle.

Electrophoresis and analysis of the *spa* gene repeats region

The amplicon was sized by first electrophoresing the producing on a 1·2% agarose gel in the presence of ethidium bromide and visualized using u.v. illumination. Using the GelDoc 1000 system, accurate sizing of the PCR product was possible and from this size the number of 24 bp repeats present was calculated by subtraction of the portion of the primers which were not contained within the repeat region (32 bp) and dividing the remainder by the repeat length (24 bp).

Stability of *spa* gene repeats region

Three MRSA isolates with diverse *spa* gene repeats (6, 10 and 15 repeats) were consecutively subcultured nine times.

DNA sequencing of *coa* amplicons

DNA sequences of the *coa* PCR products, amplified as above, from each of two EMRSA 15 and 16 isolates were obtained using a commercial DNA sequencing facility (MWG-Biotech, Waterside House, Peartree Bridge, Milton Keynes, UK).

RESULTS

Comparison of bacteriophage versus PFGE typing

The study group contained 151 EMRSA type 15 strains and of these 89·4% (135/151) were PFGE type 5 or a subtype of 5. The second predominant group were EMRSA type 16 strains of which there were 40; of these 92·5% (37/40) were PFGE type 1 or subtype of 1. PFGE profiles for representative isolates of the remaining bacteriophage types examined were distinct from each other as well as from EMRSA types 15 and 16.

Table 1. *Analysis of methicillin-resistant* Staphylococcus aureus (*MRSA*) *isolates belonging to PFGE types 1 and 5 by coagulase* (coa) *gene restriction fragment length polymorphisms* (*RFLP*) *and protein A* (spa) *gene repeats*

PFGE type	No. of isolates	No. of *coa* RFLP profiles	No. of *spa* repeats
1	53	6	8
1a	1	1	1
1b	1	1	1
1c	6	4	5
1d	1	1	1
1e	1	1	1
5	99	7	11
5a	1	1	1
5b	1	1	1
5c	9	2	2
5d	1	1	1
5e	4	2	2
5f	29	3	3
5g	1	1	1
5h	1	1	1
5i	13	1	3

Coa gene PCR amplicon RFLP

Digestion of the *coa* gene PCR products yielded 13 different RFLP patterns after *Hae*III digestion and 18 different patterns after *Alu*I digestion giving rise to 45 different combined RFLP profiles.

Individual restriction enzyme digests identified *coa* RFLPs which were strongly associated with the major PFGE types 5 and 1. Considering the restriction enzymes individually, 87·5% (56/64) of PFGE type 1 by *Alu*I digestion gave *coa* RFLP type 1 whilst 68% (109/161) of the other predominant PFGE type 5 gave *coa* RFLP type 5. Interestingly, 25% (40/161) PFGE type 5 gave an *Alu*I RFLP type of 1. *Hae*III digestion of PFGE types 1 and 5 gave 75% (48/64) *coa* RFLP type 1 and 90% (145/161) *coa* RFLP type 5. One PFGE type 1 isolate did not yield a *coa* gene amplicon.

When the *Alu*I and *Hae*III RFLP patterns were combined, 72% (46/64) of PFGE type 1 strains were found to be RFLP type 1,1. Of the PFGE type 5 isolates, 68% (110/161), were classified as *coa* RFLP type 5,5. This demonstrates a high level of association between the MRSA macrorestriction profiles and *coa*

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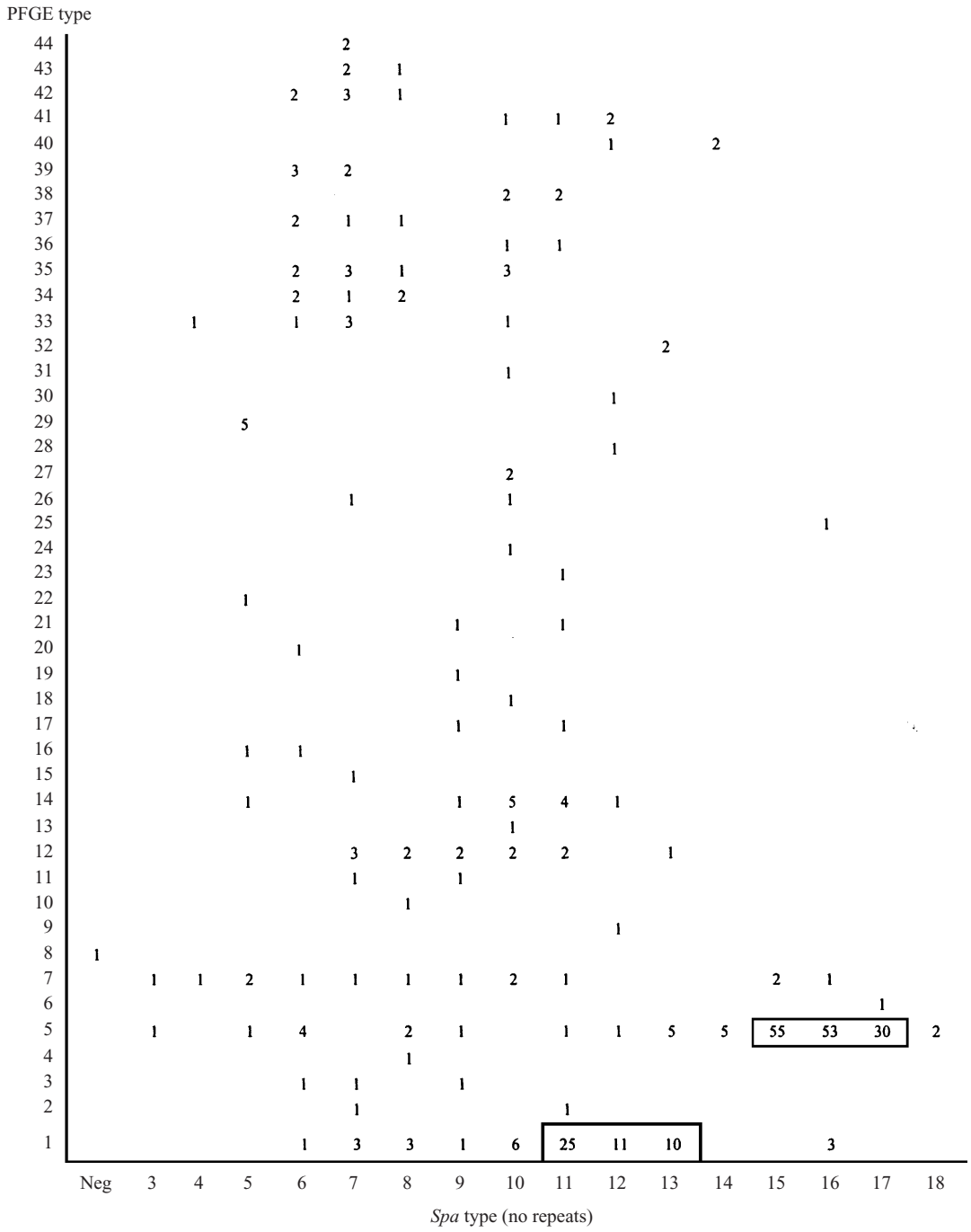

Fig. 1. The association between methicillin-resistant *Staphylococcus aureus* (MRSA) pulsed-field gel electrophoresis (PFGE) types and the number of protein A (*spa*) gene repeats. Boxed areas illustrate the correlation between PFGE type 1 and the numbers of *spa* gene repeats 11, 12 and 13 as well as PFGE type 5 and the numbers of *spa* gene repeats 15, 16 and 17.

polymorphisms following multiple restriction digests for these major EMRSA types.

As shown in Table 1, it was possible to achieve additional discrimination with *coa* gene RFLP over that yielded by PFGE. Several isolates identified as PFGE type 5 and 1 were associated with other RFLP types and vice versa. *Coa* gene RFLPs for the other PFGE types yielded patterns which were distinct from each other as well as those of PFGE types 1 and 5.

Spa gene repeats

The number of repeats found in the *spa* gene of each strain was used as its *spa* type designation. The

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number of repeats ranged from 3–18 though one isolate (PFGE type 8) tested negative by the *spa* gene PCR. Figure 1 illustrates the distribution of *spa* repeats associated with the different PFGE types. Of the PFGE type 1 strains 78% (50/64) were found to contain 10, 11, 12 or 13 repeats whereas 90% (145/161) of the PFGE type 5 strains contained 15, 16 or 17 repeats. Only 3 (4·8%) of PFGE type 1 strains contained 15, 16 or 17 repeats whilst 7 (5·9%) of the PFGE type 5 strains contained 10, 11, 12 or 13 repeats.

Of the 42 other PFGE types only 1 isolate of each of the PFGE types 6 and 25 and 3 isolates of PFGE type 7 were found to contain 15, 16 or 17 repeats whilst 21 of the other PFGE types contained 10, 11, 12 or 13 repeats. The only isolates which contained 14 repeats were 5 PFGE type 5 strains and 2 PFGE type 40 strains. Two PFGE type 5 isolates were found to contain 18 repeats. The *spa* gene types 6, 10 and 15, from the 3 MRSA isolates remained constant after 9 consecutive subcultures.

DNA sequencing of the *coa* amplicon

The DNA sequence data for each of 4 isolates (2 EMRSA type 15 and 2 EMRSA type 16) were analysed using the SEQNET MAP program. The *coa* amplicons from the 2 PFGE type 5 (EMRSA 15) isolates were found to contain 5 fragments following restriction digest by *Alu*I giving sizes of 80, 110, 273, 243 and 224 bp whilst digestion by *Hae*III gave 3 fragments of 689, 97 and 144 bp. Gene sequencing of the 2 PFGE type 1 (EMRSA 16) *coa* gene amplicons yielded 1 *Alu*I restriction site giving 2 fragments of 471 and 224 bp whilst digestion with *Hae*III also yielded a single restriction site with 2 fragments of 454 and 240 bp. The *coa* sequence data for the two pairs of isolates was consistent with the RFLP profiles 1,1 and 5,5 associated with the two major clonal PFGE types 1 and 5.

DISCUSSION

The identification of strains involved in outbreaks is crucial when designing the subsequent control measures to be implemented. Bacteriophage typing relies on a phenotypic event which is itself often caused by more than one genetic event. It therefore lacks a systematic biological basis and is beset by non-typable isolates and by unpredictable and uninterpretable variability amongst the typable ones. PFGE has become the new 'gold standard' for epidemiological subtyping of MRSA, providing good discrimination and complete typability, but is still relatively time consuming [10]. The PCR followed by RFLP analysis is a rapid, reproducible method for the discrimination of MRSA and a number of targets such as *coa*, *spa*, *mecA* (penicillin binding protein 2a), *femA* (cytoplasmic protein necessary for expression of methicillin resistance), *nuc* (thermostable nuclease) and 16S rRNA genes have been investigated for this purpose [6, 11, 12, 20–25]. In this study the *coa* and *spa* genes were selected to investigated their usefulness as discriminatory markers for the subtyping of MRSA strains isolated from the North West of England.

Good correlation was noted between PFGE type 1 and *coa Alu*I RFLP type 1 (93·2%) as well as PFGE type 5 and *coa Hae*III RFLP type 5 (93·3%). However, combination of both data sets provided the best discrimination of strains and still correlated with PFGE profiles (76·3% of PFGE type 1s and 78·2% of PFGE type 5s typing as *coa* gene RFLP types 1,1 and 5,5 respectively). Restriction maps of the DNA sequences of the *coa* gene amplicons confirmed the location of the restriction sites in both PFGE type 5 and 1 isolates.

Since the isolation of the Staphylococcus *coa* gene in 1987 [25], *Alu*I [6, 11, 12, 20–22], and to a lesser extent *Hae*III [25] RFLP digestion of the *coa* gene has been extensively used for differentiation of MRSA strains. Goh and colleagues [11] found excellent correlation between RFLP patterns and multi-locus enzyme electrophoresis (MLEE) in an analysis of 30 *S. aureus* isolates whilst Schwarzkopf and Karch [20] confirmed the data of Tenover and colleagues [6] that unrelated strains may be linked to outbreak clusters, thus limiting its effectiveness as a typing scheme. Kobayashi and colleagues [21] found that MRSA and methicillin-sensitive *S. aureus* (MSSA) were classified into 6 and 12 RFLP patterns respectively with 5 patterns detected frequently in both MRSA and MSSA. Nada and colleagues [22] analysed 42 MRSA isolates in an epidemiological investigation of hospital inpatients by *coa* RFLP typing and PFGE. PFGE detected 11 types compared with 4 by RFLP.

Frénay and colleagues [13] showed that the X-region of the *spa* gene of *S. aureus* was polymorphic due to a variable number of repeats. They suggested that the number of repeats contained within this region may allow strain discrimination. In concordance with this study, Frénay and colleagues [14] reported the number of repeats in the X region of the

spa gene was sufficiently stable for epidemiological typing of MRSA strains and that, in general, good correlation was achieved between bacteriophage typing and *spa* typing.

Recently Hoefnagels-Schuermans and colleagues [12] found RFLP patterns perfectly matched clonal delineation defined by PFGE. Similarly in this study the associations between PFGE types 5 and 1 and EMRSA types 15 and 16 demonstrate the clonality of these strains which is further evident by the associations between the PFGE types and *coa* and *spa* gene polymorphisms.

It has also been proposed [13] that the number of repeats contained within the *spa* X-region is related to the epidemic potential of the strain and that an extended X-region improves the strain's binding affinity for the Fc region of IgG thus facilitating infection of the skin. Evidence for this may be seen by the role of immunoglobulin G-binding proteins of group A streptococci in skin infections [27]. EMRSA 15 is isolated in higher frequency than EMRSA 16 and one possibility may be that the increased number of *spa* repeats contributes to the increased epidemic potential of this strain. Isolates with less common genotypes had consistently fewer numbers of repeats within the *spa* X region which may be reflected in a reduced epidemic potential.

In conclusion MRSA *coa* and *spa* gene polymorphisms showed a high degree of correlation with PFGE types for the major EMRSA types 15 and 16. The strength of these associations indicate that the rapid PCR-based identification of *coa* and *spa* RFLPs may provide useful preliminary strain identification and in combination with PFGE provide additional discrimination for subtyping MRSA for infection control. The differences in *spa* gene repeats amongst different MRSA isolates may be useful to monitor MRSA isolates for changes in epidemic potential.

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