Rapid determination of hospital-acquired meticillin-resistant *Staphylococcus aureus* lineages

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Multilocus sequence typing (MLST) and multi-strain microarray analysis have shown that most human *Staphylococcus aureus* strains belong to ten dominant clonal complexes (CCs) or lineages, each with unique surface architecture. Meticillin-resistant *S. aureus* (MRSA) strains currently belong to six of these lineages (CC1, CC5, CC8, CC22, CC30 and CC45), each of which has independently acquired mobile genetic elements (MGEs) carrying antibiotic resistance genes. MLST and microarrays are expensive and time consuming methods for routine determination of *S. aureus* lineage. A restriction-modification (RM) test has now been developed that is rapid, simple, inexpensive and accurately determines lineage of hospital-acquired MRSA. The RM test is based on three PCRs for *hsdS* gene variants, as *hsdS* genes likely control the independent evolution of *S. aureus* lineages. The RM test correctly identified 102 MRSA isolates as belonging to one of the six lineages/CCs. Real-time MRSA typing can be used to identify and track changes in local MRSA outbreaks, and provide support for targeting infection control strategies. Simple and accurate typing methods will also support large scale epidemiological studies, and could lead to greater understanding of the carriage, spread and virulence of different MRSA lineages.

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INTRODUCTION

We have recently shown that most human *Staphylococcus aureus* belong to one of ten independent lineages, and that each lineage has variable surface proteins and structures that interact with the host (Lindsay *et al.*, 2006). We have also shown that *S. aureus* from different lineages exchange DNA at a lower frequency than *S. aureus* of the same lineage, via a restriction-modification (RM) system, and this is likely to contribute to the independent evolution of the lineages (Waldron & Lindsay, 2006). Currently, six of the dominant *S. aureus* lineages have acquired SCCmec on mobile genetic elements (MGEs) to become meticillin-resistant *S. aureus* (MRSA) that are widespread in hospitals and the community (Robinson & Enright, 2003). The geographical distribution of hospital-acquired MRSA (HA-MRSA) continuously evolves, although typically each geographical location will

only have a limited number of MRSA clones in their hospitals (Table 1). These clones are often of different lineages, making them potentially easy to discriminate using targeted biochemical or genetic tests. It is important that hospital infection control teams can detect the introduction of new strains, particularly when such introductions herald the beginning of major population shifts. These can include the introduction of more pathogenic strains (Edgeworth *et al.*, 2007), or community-acquired MRSA (CA-MRSA) strains in hospitals (McDougal *et al.*, 2003).

There have been major advances in our understanding of *S. aureus* populations and typing techniques in the last few years, in particular due to multilocus sequence typing (MLST) (Enright *et al.*, 2000), *spa* typing (Harmsen *et al.*, 2003) and comparative genomics by multi-strain whole genome microarray (Lindsay *et al.*, 2006) based on the *S. aureus* sequencing projects (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Holden *et al.*, 2004; Gill *et al.*, 2005). However, these typing technologies and the widely used PFGE (Tenover *et al.*, 1995; McDougal *et al.*, 2003), are expensive, time consuming and technically demanding, and, therefore, are not performed by diagnostic laboratories or in large scale epidemiological studies.

Abbreviations: CA-MRSA, community-acquired MRSA; CC, clonal complex; HA-MRSA, hospital-acquired meticillin-resistant *Staphylococcus aureus*; MGE, mobile genetic element; MLST, multilocus sequence typing; MRSA, meticillin-resistant *Staphylococcus aureus*; RM, restriction modification; ST, sequence type.

Details of the isolates and assays are available as supplementary material with the online version of this paper.

Table 1. Dominant HA-MRSA worldwide

	Lineage/CC*					Reference
	5	8	22	30	45	
Common ST	5	8, 239, 247, 250	22	36, 30	45	Robinson & Enright (2003)
Named clone	USA100 USA800 Paediatric NY Japan	USA300 USA500 Brazilian Iberian Hanover	EMRSA-15 Barnum	EMRSA-16 USA200	USA600 Berlin	Robinson & Enright (2003); McDougal <i>et al.</i> (2003)
Sequenced <i>S. aureus</i> strain	N315 Mu50	COL FPR3757 8325†		MRSA 252		Lindsay & Holden (2004)
Country/region						
UK			+	+		Moore & Lindsay (2002)
USA	+	+		+	+	McDougal <i>et al.</i> (2003)
Canada	+	+		+		Simor <i>et al.</i> (2005)
Germany		+	+		+	Ghebremedhin <i>et al.</i> (2005)
Spain		+		+		Perez-Roth <i>et al.</i> (2004)
Portugal		+	+			Aires de Sousa & de Lencastre (2003)
Italy	+	+				Aires de Sousa & de Lencastre (2003)
Greece		+		+		Aires de Sousa & de Lencastre (2003)
Czech		+				Aires de Sousa & de Lencastre (2003)
Turkey		+				Aires de Sousa & de Lencastre (2003)
Chile	+	+				Aires de Sousa & de Lencastre (2003)
Columbia	+					Aires de Sousa & de Lencastre (2003)
Argentina	+	+				Aires de Sousa & de Lencastre (2003)
Brazil		+				Aires de Sousa & de Lencastre (2003)
Japan/Korea	+					Ko <i>et al.</i> (2005)
Rest of Asia		+				Ko <i>et al.</i> (2005)
Australia		+	+			Coombs <i>et al.</i> (2004)

*Determined by MLST typing for *S. aureus*. Two isolates with identical sequences in seven out of seven housekeeping genes belonged to the same ST. Two isolates with five or more identical sequences belonged to the same CC.

†Meticillin-sensitive *S. aureus*.

The aim of this project was to develop rapid, simple and inexpensive methods that could classify HA-MRSA isolates into their appropriate lineage or clonal group. We exploited our recent understanding of how RM contributes to lineage evolution to design such a test. The test would be suitable for clinical diagnostic laboratories, infection control units and research laboratories undertaking large scale epidemiological analysis of strain collections.

METHODS

Strains. A total of 102 different reference and clinical MRSA isolates from hospitals all over the world were utilized in this study (Day *et al.*,

2001; Kuroda *et al.*, 2001; Moore & Lindsay, 2002; Baba *et al.*, 2002; Robinson & Enright, 2003; Gill *et al.*, 2005; Edgeworth *et al.*, 2007). These isolates were previously typed by either sequencing, MLST, Southern blotting or microarray, and represent the five dominant HA-MRSA lineages and the common CA-MRSA clonal complex (CC)1. The number of isolates from each lineage were: CC22 ($n=22$), CC30/sequence type (ST)36 ($n=49$), CC5 ($n=7$), CC8/239 ($n=21$), CC45 ($n=2$), CC1 ($n=1$). They included the dominant UK isolates MRSA-15 (CC22), MRSA-16 (CC30/ST36), examples of each of the earlier 'epidemic' UK MRSA-1 to -14 and isolates representing dominant lineages in Germany, USA and Japan. By MLST typing, two isolates with identical sequences in seven out of seven housekeeping genes belonged to the same ST. Two isolates with five or more identical sequences belonged to the same CC (Enright *et al.*, 2000) or lineage. ST36 isolates belonged to the CC30 lineage (Holden *et al.*,

Table 2. Primer sequences

Name	Sequence
AF	AGGGTTTGAAGGCGAATGGG
AR30	CAACAGAATAATTTTGTAGTTC
AR22	TCAGAGCTCAACAATGATGC
AR45	GGAGCATTATCTGGTGTTC
AR1	GGGTTGCTCCTTGCATCATA
BF	CCCAAAGGTGGAAGTGAAAA
BR8	CCAGTTGCACCATAGTAAGGGTA
BR5	TCGTCGACTTTTGAAGATTG

2004), while ST239 isolates belonged to the CC8 lineage but have a large insert from a CC30 donor (Robinson & Enright, 2004). Two further methicillin-sensitive *S. aureus* of CC1 were also studied (Day *et al.*, 2001). Further information about individual isolates is given in Supplementary Table S1, available with the online journal. All isolates were stored in 20% glycerol brain heart infusion (BHI) medium and freshly streaked onto BHI plates for any assay or preparation.

DNA extraction. DNA to be used as a template for the PCR assays was extracted using a bacterial genomic DNA purification kit from Edge Biosystems. Manufacturer's directions were followed with the following modifications: (i) 0.5 ml bacterial culture was used instead of 5 ml, (ii) the volumes of all reagents were scaled to one quarter of those described in the protocol, (iii) 3 µl lysostaphin (Sigma L738; 5 mg ml⁻¹ in 20 mM sodium acetate, pH 5.2) was added to each sample at the same time as the addition of spheroplast buffer.

RM test. Sequences of the two *S. aureus* *hsdS* genes, *sau1hsdS1* and *sau1hsdS2* (also known as *sau1IhsdS1* and *sau1IhsdS2*), vary

significantly between *S. aureus* lineages, but are highly conserved within lineages (Waldron & Lindsay, 2006). Each gene has a conserved 5' end, central region, and occasionally 3' end, with two highly variable regions in-between. We exploited this variation to design the primers (Table 2, Fig. 1). Three PCR assays were developed, each containing one forward and two different reverse primers. Thus each reaction could lead to one of three outcomes, either a large PCR product, a small PCR product or no product. The three PCR assays were named RM test 1 (UK), RM test 2 and RM test 3. The combination of primers used in each assay and the expected PCR product sizes for each lineage are shown in Figs 1 and 2.

All PCR was performed using Qiagen HotStar *Taq* and associated buffers according to manufacturer's directions. Primers were used at a concentration of 0.5 µM each. The PCR conditions were as follows: 5 min at 94 °C, then 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C. The products were separated on 1.5% agarose gels.

Biochemical assays. Twenty-seven different biochemical assays were trialled using representative isolates from CC22, CC30, CC8 and CC5. A tryptophan auxotroph assay and a urease assay were chosen for further trials using all the isolates, due to their reproducibility and ease of interpretation (for the complete list of assays trialled see Supplementary Table S2 available with the online journal).

For the tryptophan auxotroph assay, a chemically defined agar (CL) was prepared as described previously (Lindsay & Foster, 2001), although Chelex 100 was not added to chelate out the iron. A similar chemically defined media lacking tryptophan was also prepared (CLtrp-). For isolate screening, a 1 µl loop was inoculated with bacteria from an overnight BHI agar plate and resuspended in 1 ml sterile PBS. A 20 µl aliquot of the suspension was spotted onto both a CL and a CLtrp- plate. Up to nine samples were freshly spotted on each plate. These plates were then incubated at 37 °C for 24 h. Positive isolates grew on both plates, whereas negative isolates only grew on the CL plates (Fig. 3).

Fig. 1. Primer binding positions and predicted product sizes. On the left hand side, the expected PCR product size for each lineage is listed. On the right, the hybridization positions of the test primers on the variant forms of the *sau1hsdS* genes are shown. The expected PCR product sizes are illustrated in Fig. 2.

Fig. 2. PCR products from the 3 RM tests. The three agarose gels (1.5 %) contain typical PCR assay results for members of all 6 lineages. The representative isolates used as template in the reactions have their corresponding lineage name listed above the lanes in which the resultant products were run. A ladder (PCR marker; Sigma) was run in the left hand lane and selected band sizes from this ladder have been labelled by size (bp).

For the urease assay, 10 ml slopes were prepared with urea agar base (Oxoid) and 2 % urea. Loops (1 µl) of bacteria from an overnight BHI plate were resuspended in 200 µl sterile PBS, which was then used for inoculation. The yellow/orange coloured slopes were incubated at 37 °C for 24 h. Positive isolates turned the agar colour to pink (Fig. 3).

RESULTS AND DISCUSSION

The first attempt to develop a rapid discriminatory test involved screening representative MRSA isolates in 27 biochemical assays for association with lineage. Most of these assays (see Supplementary Table S2 available with the online journal) were ruled out, but two tests were useful. The urease test was uniformly negative for CC22 isolates, and positive for CC30, CC1, CC5 and CC45, while CC8 was variable. The tryptophan auxotroph test was negative for CC30, and positive for CC22, CC1 and CC45, and all but one isolate of CC5 and CC8. These tests may be valuable for screening populations dominated by lineages CC22 and CC30, such as those found in UK hospitals. However, the tests were not discriminatory enough to classify isolates into a wider range of lineages.

In contrast, the 3 RM tests correctly assigned all 102 isolates to their respective lineage (see Supplementary Table S1 available with the online journal). Whole genome microarray comparison of large populations of isolates have identified many large gene variations associated with lineage that could be targeted by rapid testing (Lindsay *et al.*, 2006). We focused on *hsdS* because as a single gene type it is particularly variable and discriminatory (Waldron & Lindsay, 2006). Furthermore, it is the key gene controlling genetic exchange between *S. aureus* lineages,

Fig. 3. Typical results of the biochemical tests: (a) tryptophan auxotroph assay, (b) urease test.

and therefore controls independent evolution of the different lineages. This means that *hsdS* is likely to be very stable, and any variation would be an early marker of a new emerging lineage.

The RM test is suitable for HA-MRSA, but will also correctly assign CA-MRSA strains to five of the seven lineages described (CC1, CC5, CC8, CC22 and CC30, but not ST80 and ST59). ST80 and ST59 are relatively rare *S. aureus* lineages that have acquired *SCCmec* and are spreading rapidly in the community worldwide. As more *hsdS* sequence becomes available additional RM tests to identify these and other lineages can be designed. Isolates that test negative in the three RM tests can be further typed with MLST, *spa* typing or multi-strain whole genome microarray to identify unusual lineages. Nevertheless, within a geographical area, because CA-MRSA and HA-MRSA usually belong to different lineages, the RM test will be a useful rapid method to identify movement of CA-MRSA into hospitals or vice versa, or the emergence of new lineages in particular settings. This may be particularly helpful in determining the source of paediatric outbreaks where CA-MRSA is emerging and HA-MRSA is less common. It may also be helpful to determine which strains are responsible for 'endemic' MRSA, so that new outbreaks are more easily identified.

The RM test is the simplest, cheapest and fastest reliable method for typing HA-MRSA, and provides the single most important piece of information: the lineage/CC that correlates with variation in hundreds of genes. However, in common with other typing systems, such as MLST and *spa* typing, the RM test does not discriminate between isolates of the same lineage that differ in their carriage of MGEs. Movement of MGEs (which often encode virulence and resistance genes) into and out of MRSA is common, and can be a useful marker for short-term epidemiology, though depending on the stability of an element, it can be less useful for tracing spread over longer distances and periods of time. MGE variation can be detected by band changes in PFGE. However, interpretation of PFGE is complex and dependent on technical skill and knowledge of local strains. PFGE band changes may be due to MGE movement or to differences in lineage, but neither of these can be determined by PFGE alone. Therefore, PFGE may benefit from combination with the RM test. Similarly, the multiple-locus variable-number tandem repeat analysis test (Sabat *et al.*, 2003), which compares variations in size of repeat regions in five surface proteins (including *spa*), does not provide lineage data, and could benefit from combining with the RM test.

Multi-strain whole genome microarrays are the most comprehensive method for detecting MGEs, as well as lineage (Lindsay *et al.*, 2006). However, this method is exceedingly complex and is not applicable to large scale or routine studies. However, there are several other options available for detection of MGE movement, which combined with the RM test could be of additional value. Testing for SCC*mec* type can be useful for discriminating clones from the same lineage (Oliveira & de Lencastre, 2002), and this is performed using a series of PCR tests. Testing for toxins and virulence factors that are found on MGEs, such as Panton–Valentine, toxic-shock syndrome toxin, enterotoxins A, B, C and H, and exfoliative toxins, could also be beneficial when subtyping isolates, and this can be done using PCR or biological assays. Another method that deserves consideration is the use of antibiotic resistance profiles. This method has previously been rejected for typing, as strains from different lineages can have the same profile. However, in combination with RM testing it could again be valuable. Diagnostic laboratories routinely perform antibiotic resistance profiling of MRSA isolates; therefore, additional work may not be required. It seems likely that the combination of the rapid tests for lineage we have described and antibiotic resistance data may be a useful method to rapidly discriminate between variants of the same lineage (Lindsay & Holden, 2004). Further studies are required to prove this in the clinical setting.

For any MRSA typing, the method chosen should be dependent on the question being asked, as well as logistical factors. The major advantages of the RM assay are that it is a rapid, inexpensive, simple and accurate test that can be performed in any routine laboratory, and can be scaled up.

It is, therefore, applicable in circumstances where typing is currently not done, due to technical, time or financial issues, but could aid the understanding of epidemiology or virulence. The RM test is not intended to replace the more comprehensive typing methods, which give additional data useful for determining evolutionary relationships. We expect the test will help clinicians, infection control teams and researchers to rapidly recognize unusual clonal outbreaks within particular hospitals (Edgeworth *et al.*, 2007), or differences in virulence, spread or carriage associated with lineage. In broader terms, we hope that this method will result in an increase in the amount of available typing data that is comparable worldwide, enhancing epidemiological, public health and pathogenicity studies.

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