Two approaches to *Staphylococcus aureus* typing

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Typing of *Staphylococcus aureus* is carried out to detect outbreaks, monitor transmission and infer virulence and resistance properties. There are now many different extant typing methods. This article summarises the broad classes of typing approaches and describes two methods. One is based on micro-array technology while the other is based upon the interrogation of polymorphic sites using real-time PCR.

S. aureus is a ubiquitous microbe that has adapted to thrive in disparate ecological niches. The organism can be found as a normal commensal of the human microbiota, an irritating agent of superficial skin infections or as a dangerous invasive nosocomial or community-acquired pathogen. Its success lies in an extraordinary genetic flexibility. The mobile 'meta-genome' within the species includes genes that specify virulence factors and antibiotic resistance, and this provides a potent genetic resource that allows the species to continually evade attempts to eliminate or control its dissemination and associated morbidity.

Therefore, there are three related reasons for carrying out *S. aureus* typing. Firstly, typing allows the detection of transmission events and patterns at all scales, from within a single healthcare facility, to between continents, and so is central to the practices of infection control and public health microbiology. Secondly, typing allows the inference of virulence and resistance properties of specific isolates and so can directly influence clinical decisions. However, it must be stated that the relationship between gene content and virulence is not fully understood, and is an area of very active and sometimes controversial research. Finally, typing reveals the population structure and provides insight into the general principles of the species' natural history.

Currently extant genotypic methods for typing *S. aureus* may be divided into two broad categories – those that are based upon the conversion of the genome into an electrophoretic banding pattern, and those that are based on the interrogation of known polymorphic sites or genes.

The most prominent example of a banding pattern-based typing method is pulse field gel electrophoresis (PFGE). PFGE has been used for nearly 2 decades and has become a 'gold standard' *S. aureus* typing technique for most laboratories [1]. PFGE can measure evolutionary/genetic distance over a significant dynamic range and in particular can discriminate even closely related strains. This makes it very useful for detecting short time-scale epidemiological linkage, e.g. in the context of infection control in a healthcare facility. However, these methods are becoming less popular; this may be attributed in large part to their technically challenging and time-consuming nature. Also, specific genetic changes cannot be directly inferred from changes in banding patterns, and it is an exacting task to standardise the protocols such that the data may be reliably compared between laboratories.

The focus in the last decade has been upon typing methods that interrogate known polymorphisms or polymorphic genes. Multi-locus sequence typing (MLST) has become very prominent since its development around the turn of the century [2]. The *S. aureus* population structure revealed by MLST is fascinating. The current model is that over time alleles are re-assorted throughout the species, but successful lineages expand clonally and accumulate diversity primarily through mutation rather than recombination. Such lineages, which are known as clonal complexes, are therefore somewhat isolated and well defined genetically, and it has been suggested that they warrant species status [3]. Therefore, it would be advantageous for a typing method to be able to assign isolates to clonal complexes.

Despite the attractions of MLST, there are reasons that it is not

universally adopted. Firstly, determining seven sequences is still a significant task, particularly for large numbers of isolates. Secondly, MLST has insufficient resolution for revealing short time scale epidemiological linkage. Finally, a major part of the *S. aureus* genome is composed of mobile elements, not detected by MLST, some of which encode clinically relevant functions. These drawbacks have led to a recent focus upon methods that are based on the presence or absence of mobile genes (binary typing), and/or include the interrogation of rapidly evolving loci.

Scientists in the Gram-positive Bacteria Typing and Research Unit (GPBTRU) have pursued the adaptation of binary typing to array technology[4]. Recent advances have been made that allow the technique to be rapid, cost effective and user friendly in routine microbiology and typing laboratories. An oligonucleotide array that recognises staphylococcal species markers, accessory

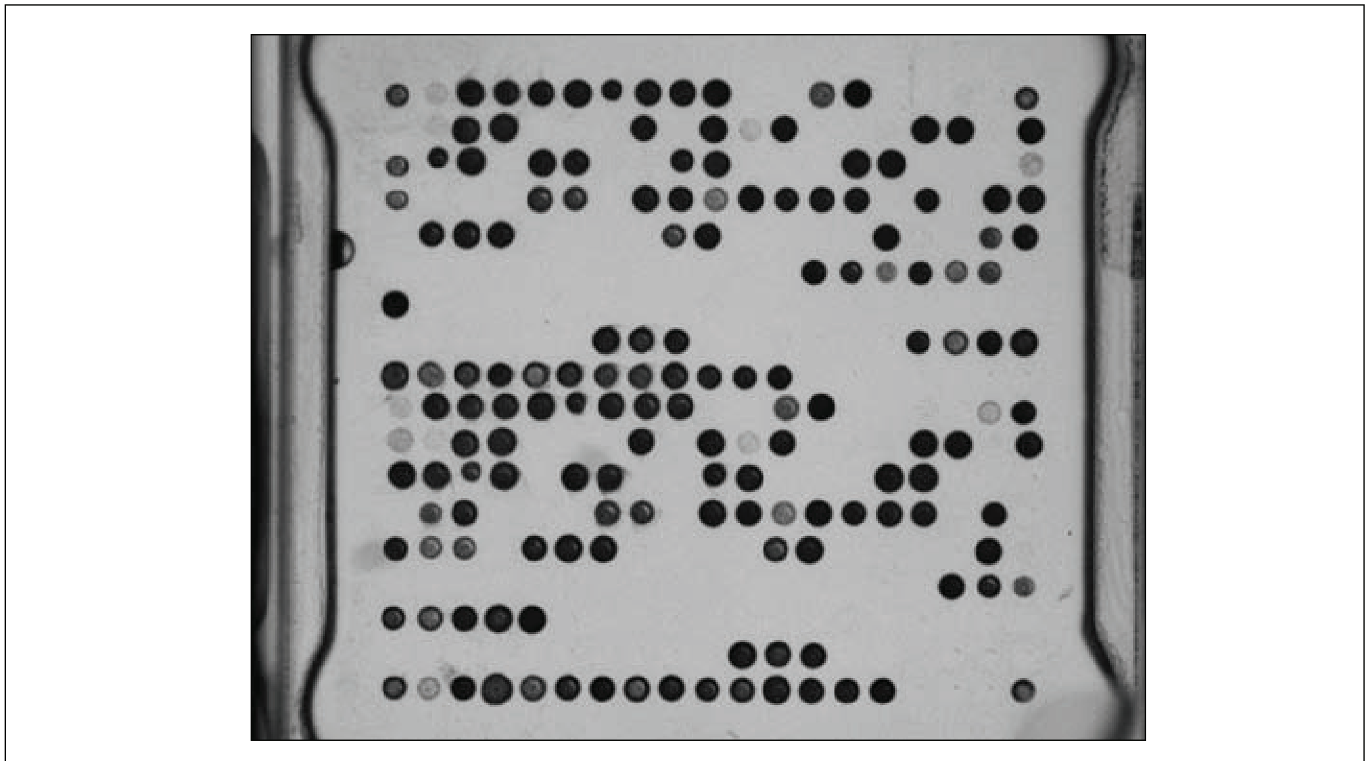

Figure 1a. Image of the hybridised and developed array. The actual size of the array is 2.4 x 2.4 mm.

<table>
<tr><td>X</td><td>set7</td><td>set8</td><td colspan="2">set9</td><td>set12</td><td>set21</td><td colspan="6">setB</td><td>setC</td><td colspan="3">set variants</td><td>Napp</td><td>X</td></tr>
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<tr><td>X</td><td colspan="4">rrn staph. species markers</td><td colspan="10">S. aureus markers</td><td colspan="3">agrI</td><td>X</td></tr>
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Figure 1b. Probe layout of genes detected by the array. Green background indicates staphylococcal species ribosomal RNA markers and specific *S. aureus* genes, orange indicates global regulators and *agr* types, blue indicates antibiotic resistance genes, grey indicates ribosomal RNA markers that should always be positive and yellow indicates virulence genes. Spots designated X indicate biotin markers whose asymmetrical layout allows correct alignment of the array to the grid.

gene regulator types, and genes for antibiotic resistance, superantigens, bi-component toxins, haemolysins, exotoxins, biofilm formation, adhesion molecules and immune evasion together with markers for staphylococcal cassette chromosome *mec* (SCC*mec)* types has been developed (Figure 1). The reaction involves the linear amplification of multiple genes in a single multiplex PCR reaction that incorporates biotin-labelled dUTP into amplicons, which are hybridised to an array chip and detected by an enzymatic reaction. A positive reaction is detected by a reading device and processed with manufacturer-designed software. As well as antimicrobial resistance and virulence potential, it has been shown that genetic lineages can also be inferred from the arrays [5].

Recently, there has been an increasing awareness that combinatorial bacterial typing methods that interrogate both the slowly evolving genome backbone and rapidly evolving and mobile genes are particularly efficient and informative [6]. The commonly used combination of MLST and SCC*mec* typing for defining MRSA clones is an example of this. Research carried out at the Queensland University of Technology node of the Cooperative Research Centre for Diagnostics has revealed that it is possible to derive, from the *S. aureus* MLST database, an eight member set of single nucleotide polymorphisms (SNPs) that resolves the major clonal complexes. Real-time PCR methods for interrogating these SNPs have been developed for use in the routine laboratory [7] (Figure 2). These SNPs are designed to be combined with rapidly evolving and binary loci.

Genome regions containing tandem repeats generally evolve rapidly because of slipped-strand mis-pairing during replication and intra-molecular recombination. The *S. aureus spa* locus possesses a very complex and rapidly evolving repeat structure; this is the basis of a single locus sequence typing method that is

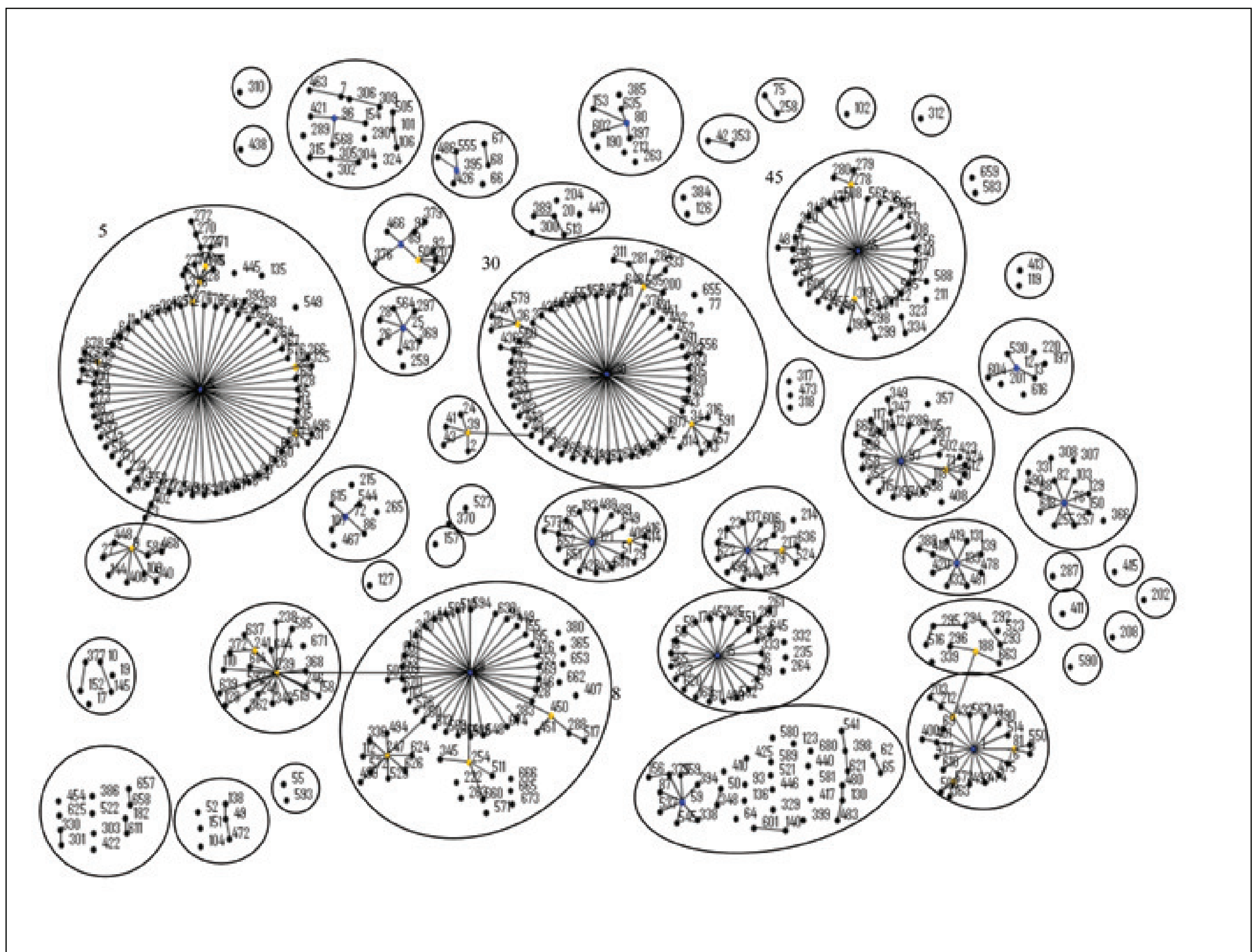

Figure 2. Relationship between the eBURST defined population structure of *S. aureus* and the genotypes defined by eight SNPs. These SNPs were derived from the *S. aureus* MLST database on the basis of maximisation of Simpsons Index of Diversity as calculated against all STs in the database. They are *arcC*210, *tpi*241, *tpi*243, *arcC*162, *gmk*318, *pta*294, *tpi*36 and *pta*383, with numbering on the basis of the gene fragments used for MLST. Each dot represents an ST, lines connect STs that differ by a single locus, and STs at the centre of the 'stars' are deduced founders of clonal complexes. SNP-based genotypes are defined by the ellipses. This diagram is an approximation – constraints on what it is possible to draw mean that a very small number of STs are mis-assigned in this diagram. Complete correct information has been published by Huygens and co-workers [7].

commonly known as *spa* typing[8]. A recent technical development is high resolution melt (HRM) analysis. This is now available on many real-time PCR devices. Very recently, HRM was shown to be very effective for discriminating between *spa* alleles[9]. This method is ideal for combining with, for example, real-time PCR interrogation of the clonal complex defining SNPs.

In conclusion, the authors of this article have been involved in the development of typing methods using two different platforms – oligonucleotide array and real-time PCR/HRM analysis. However, there are significant commonalities between the two approaches as regards the use of multiple loci, and the emphasis on achieving consistency with other typing methods and the actual *S. aureus* population structure. The advantage of array-based methods is that they can interrogate a large number of targets. The advantage of real-time PCR based methods is that they are single step-closed tube, and can interrogate different classes of polymorphisms such as SNPs, binary markers and variable number tandem repeats. It is easy to envisage a convergence of these approaches with, for example, an on-chip amplification format that includes HRM analysis of the products.

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Detecting hVISA

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Rates of reduced vancomycin susceptibility (RVS) among isolates of methicillin-susceptible *Staphylococcus aureus* (MSSA), especially methicillin-resistant *S. aureus* (MRSA), have increased. Although vancomycin remains the therapeutic mainstay for MRSA, clinical response to vancomycin has been compromised by RVS among our hospital clones. Laboratories need simple procedures to detect these organisms. Whilst routine disc or MIC susceptibility testing is not reliable, some useful screening procedures are available.

The first vancomycin-intermediate *S. aureus* (VISA) isolate of MRSA was recognised in 1996[1]. The first Australian isolate with RVS was reported as hetero-VISA (hVISA) in 2001[2]. Isolates of hVISA contain subpopulations (10^{-5} to 10^{-6}) that grow at increased vancomycin concentrations. Patients with hVISA infections suffer persistent infection despite apparently adequate vancomycin therapy[3, 4]. Mortality is reported to be higher (63%) in patients with RVS strains versus 12% mortality in patients with susceptible strains[5].

Reduced susceptibility is also evidenced *in vitro*. Vancomycin MICs are increasing with higher proportions of MRSA, with MICs >1mg/L increasing from 26-70% over 4 years[6]; similar data exist