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An Assessment upon Various Improvements of Rapid Genotyping Techniques for MRSA

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Abstract – Methicillin-resistant Staphylococcus aureus (MRSA) is an important human pathogen that is endemic in hospitals all over the world. It has more recently emerged as a serious threat to the general public in the form of community-acquired MRSA. MRSA has been implicated in a wide variety of diseases, ranging from skin infections and food poisoning to more severe and potentially fatal conditions, including; endocarditis, septicaemia and necrotising pneumonia. Treatment of MRSA disease is complicated and can be unsuccessful due to the bacterium's remarkable ability to develop antibiotic resistance.

The considerable economic and public health burden imposed by MRSA has fuelled attempts by researchers to understand the evolution of virulent and antibiotic resistant strains and thereby improve epidemiological management strategies. Central to MRSA transmission management strategies is the implementation of active surveillance programs, via which unique genetic fingerprints, or genotypes, of each strain can be identified. Despite numerous advances in MRSA genotyping methodology, there remains a need for a rapid, reproducible, cost-effective method that is capable of producing a high level of genotype discrimination, whilst being suitable for high throughput use. Consequently, the fundamental aim of this paper was to develop a novel MRSA genotyping strategy incorporating these benefits.

A comprehensive MRSA genotyping strategy requires characterization of the clonal background as well as interrogation of the hypervariable Staphylococcal Cassette Chromosome mec (SCCmec) that carries the p-lactam resistance gene, mecA.

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INTRODUCTION

Staphylococcus aureus is a major cause of health-care facility and community- acquired disease. It is now well established that the transmission of Staphylococcal disease can be reduced by stringent public health and infection control measures. Consequently, there is ample justification for monitoring the dissemination of these organisms at all scales, from within individual health care facilities to between continents. Tracking bacterial dissemination requires assigning isolates to subgroups within the species. This is known as typing, and if the typing is based on genetic analysis, it is usually known as genotyping. An ideal S. aureus genotyping method would be rapid, cost effective, and be easily portable among laboratories. It should also provide high informative power in relation to the population diversity and structure that is easily understood. This is necessary for reliable testing of hypotheses regarding epidemiological linkage. It also potentially facilitates drawing inferences from genotypes regarding virulence and resistance phenotypes. Developing such a

Staphylococcus aureus is a prominent human pathogen that was first reported in 1881 by Alexander Ogston. After microscopic analysis of purulent infections, Ogston discovered grape-like clusters of round, golden cells. Following this observation he replicated the original infections by injecting experimental subjects with the putative infectious organism. It is now established that S. aureus is a Gram-positive, facultative aerobe that produces catalase and coagulase and can tolerate reduced water activity (Madigan et ah, 2000b). Under optimal conditions, cell division occurs approximately every 20min with cell diameter ranging from 0.5- 1.5um. S. aureus is able to grow on a range of nutrient media and appears as smooth, convex colonies with diameters of ~4-3mm. The golden pigmentation, caused by carotenoids, is usually visible, and this is thought to protect cells against ultra-violet radiation.

S. AUREUS ANTIBIOTIC RESISTANCE

Staphylococcus aureus is the leading cause of bacterial infections involving the bloodstream, lower respiratory tract, and skin and soft tissue in many developed countries, including the United States. Penicillin was initially highly effective against staphylococcal infections, but penicillinase-producing S. aureus emerged in the mid-1940s, and its

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prevalence increased dramatically within a few years. This increase was entirely attributable to widespread use of penicillin, which selected for bacteria containing resistance genes. Several “epidemic waves” of antibiotic resistant S. aureus have occurred since then. Penicillin resistant S. aureus was pandemic in the 1950s and early 1960s.

These infections, both in hospitals and in the community, were caused primarily by one S. aureus clone known as phage-type 80/81. Pandemic phage-type 80/81 S. aureus infections declined after the introduction of methicillin in 1959. Within 2 years, however, Jevons and colleagues reported the emergence of methicillin resistant S. aureus (MRSA), and the first cluster of cases was reported shortly thereafter.

MRSA has since spread and is endemic in most hospitals worldwide. In the United States, S. aureus is the number one cause of hospital-associated infections, and a high percentage of these are caused by MRSA. The mortality rate associated with invasive MRSA infections is approximately 20%, and in the United States these infections are probably the leading cause of death by any single infectious agent; fatalities resulting from these infections are estimated to surpass those caused by HIV/AIDS. Thus, it is clear that S. aureus, especially MRSA, remains a major problem for health care facilities.

The rapid emergence of community-associated MRSA (CAMRSA) infections (i.e., those that occur outside of health care facilities in otherwise healthy people) is one of the most surprising events in infectious diseases in recent years. This is in part because community-associated S. aureus infections were traditionally caused by methicillin-susceptible S. aureus (MSSA), rather than the antibiotic-resistant strains so prevalent in hospitals. Thus, it remains a mystery why these antibiotic-resistant bacteria emerged in a niche (the community) not obviously under the relatively high selective pressure exerted by antibiotics in the hospital setting. CAMRSA was first reported in Western Australia in the 1990s and has since emerged worldwide (19) and become epidemic in the United States. In addition to the increased prevalence of infections, CA-MRSA strains can cause severe or fatal disease. In this Review, we discuss key components of the recent emergence of CA-MRSA, including transmission and virulence, and we highlight some of the genome-wide approaches used to understand the success of this pathogen. Due to space constraints, we focus mainly, but not exclusively, on the epidemic in the United States.

The most effective treatment presently available against multi-resistant MRSA infections is the glycopeptide vancomycin: however, like penicillin and its derivatives, vancomycin resistance has also evolved. Isolates demonstrating low to moderate levels of resistance are termed vancomycin-intemiediate S. aureus (VISA) and have been reported since 1996 from various locations, including Japan. United States (Smith et al.. 1999) and Europe. While the mechanism of resistance has not been fully elucidated, observations of abnormally thick cell walls indicate that increased cell wall synthesis maybe important. It has been hypothesized that the excess peptidoglycan ensnares the vancomycin molecule within the cell wall, therefore preventing access to the cytoplasmic target of the N- acetyl-muramic acid precursor (Hiramatsu. 2001). The first high level vancomvcin-resistant S. aureus (VRSA) isolate was reported from the United States in 2002 (Anonymous. 2002).

Unlike VISA. VRSA resistance is mediated by ran A. which is identical to the mechanism utilised by vancomycin-resistant Enterococci (VRE) (Weigel et al., 2003: Woodford. 2001).

S. AUREUS GENOME SEQUENCES AND STRUCTURE

Staphylococcus aureus is a human pathogen that causes both nosocomial and community-acquired infections. The emergence of strains resistant to many antibiotics (methicillin-resistant S. aureus [MRSA]) and of highly virulent community acquired MRSA that can cause fatal infections such as necrotizing pneumonia is of considerable concern even in countries with well-developed health surveillance systems. In order to study mechanisms of staphylococcal antibiotic resistance and virulence, whole genome sequences of several different S. aureus strains have been determined. MRSA strains N315 and Mu50 were the first staphylococcal genomes to be sequenced, which were followed by nine additional strains. All staphylococcal genomes are approximately 2.8 Mbp in size with a relatively low G_C content.

Comparative analysis revealed that most regions of the staphylococcal genome are well conserved, whereas several large sequence blocks display high variability. S. aureus strains likely acquired these genomic islands horizontally and, at least initially, their integration into the genome must have required dedicated DNA recombination (integrase) genes. Furthermore, variable blocks of genome sequence frequently carry virulence and antibiotic resistance determinants that aid in the development of staphylococcal diseases. Variable regions can be classified as prophages, pathogenicity islands, or staphylococcal cassette chromosomes. The overall combination of variable sequence elements and the encoded spectrum of virulence properties varies from strain to strain and appears to be reflective of the overall large spectrum of clinical disease manifestations in humans.

S. aureus strain Newman was isolated in 1952 from a human infection and has been used extensively in animal models of staphylococcal disease due to its robust virulence phenotypes. Thirty genes that are required for staphylococcal pathogenesis were

identified in S. aureus Newman after a screen of 1,736 bursa aurealis mutants with transposon insertions in different genes. Both well-characterized virulence genes and genes with unknown function were shown to be involved in the pathogenesis of staphylococcal infections.

S. aureus genomes display an overall conservation of sequence and structure; however this uniformity is disrupted with small pockets of heterogeneity (Lindsay and Holden. 2006). The conserved portion of the genome is generally comprised of genes responsible for common species function and is referred to as the "core" genome. The non-essential sequence blocks not found uniformly in the species are deemed the "accessory" genome. Each genome component contains variation that accounts for the observed genotypic and phenotypic differences.

MULTIPLE-LOCUS, VARIABLE-NUMBER TANDEM REPEAT-BASED METHOD FOR RAPID GENOTYPING OF S. AUREUS

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community-acquired infections. The ability to rapidly and reliably identify relatedness between clinical isolates is crucial for the investigation of outbreaks and also for the epidemiological surveillance of strain dissemination. To date, several methods for the genotyping of S. aureus isolates have been reported. These methods are based on either phenotypic characterization or molecular techniques. Phenotyping usually relies on (i) antimicrobial susceptibility panels, (ii) arrays of specific antibodies directed against bacterial surface components, or (iii) phage susceptibility patterns. These techniques may suffer from poor discriminatory power, time consumption, or low throughput. Most molecular techniques employ PCR amplification but use different analytical methods for characterization of the amplified DNA fragments. This includes gel electrophoresis for amplicon size estimation, possibly after digestion with a specific restriction enzyme. Sequencing of the amplified fragment often serves as a definitive identification method for amplified DNA fragments. Direct digestion of the intact chromosome followed by size separation (pulsed-field gel electrophoresis [PFGE]) or genotyping by nucleic acid probes (ribotyping) are two techniques that do not require PCR amplification. However, PCR-based techniques appear to be advantageous for the study of slowly growing or difficult-to-grow organisms.

PFGE is considered the reference technique for the typing of S. aureus strains. However, despite large collaborative efforts, its use remains limited by suboptimal reproducibility between laboratories, demanding protocols, low throughput, and high costs. Arbitrarily primed PCR methods are less expensive in terms of reagent costs, but they also suffer from poor interlaboratory reproducibility. Ribotyping is limited to use with bacterial species that contain several copies of the ribosomal locus and has a lower interstrain discriminatory power than the other techniques. Multilocus sequence typing (MLST) shares the advantages of PCR-based methodologies, and the variability of the data obtained by MLST appears to be limited. MLST is currently the “gold standard” method for assessment of the evolutionary relatedness

between strains it must be kept in mind, however, that unrelated clinical isolates sometimes display common profiles. When S. aureus is considered, spa typing has been proven to be an inexpensive and rapid typing approach and is based on variable numbers of repeats in the protein A gene. Recent sequencing programs have enabled the development of genome-wide microarrays that allow detailed evaluation of the genome contents of the pathogen. However, despite its exquisite resolution power, this technology is still too expensive to be used in clinical laboratories.

A more recent approach consists of combination of the advantages of different methods with the same panel of strains. For example, two PCR amplifications probing for the presence of toxin genes and the hypervariable region of the mec element were applied to a large collection of clinical isolates. This technique proved successful for classification of methicillinresistant S. aureus (MRSA) isolates recovered during outbreaks or collected from clinical specimens, while it simultaneously permits evaluation of the potential virulence of these strains. Combination of MLST with SCCmec type determination also permits the analysis of collections of MRSA isolates and the detection of new MRSA clones.

We report here on the development of an assay that provides S. aureus genotyping together with the assessment of known virulence factors and which facilitates short turnaround times, high sample throughput, and low per-sample costs. We selected a total of eight primer pairs in regions flanking repeatcontaining genes that encode bacterial adhesins, a protease, and an immunoglobulin G (IgG)-binding protein. Additionally, a primer pair was selected from the mecA gene. Most of these target genes have been previously described as potentially important in the pathogenicity of S. aureus.

A collection of 20 strains extensively characterized by PFGE, MLST, and randomly amplified polymorphic DNA (RAPD) analysis was used for an initial validation of this new procedure. The resolution power of this novel automatically analyzed method was then evaluated with a collection of _200 strains from four documented nosocomial outbreaks and 59 pairs of strains collected from long-term carriers.

Genotyping results were consistent with PFGE and MLST data for both strain collections.

S. AUREUS GENOTYPING UTILIZING NOVEL REAL-TIME PCR FORMATS

Bacterial genotyping is a field in which there is continuous technological innovation. Many methods have been described, with pulsed-field gel electrophoresis, amplified fragment length polymorphism, multilocus sequence typing (MLST), and variable number tandem repeat analysis currently being used widely. In general, the whole-genome/electrophoresis-based methods such as pulsed-field gel electrophoresis and amplified fragment length polymorphism give very high resolution and are used for establishing epidemiological linkages in short time scales, MLST is used for research into large scale population structures, while variable number tandem repeat analysis is showing some promise at being amenable to both classes of application.

We have devised a systematic approach to the development of new bacterial genotyping methods. The central hypothesis is that, given a defined data set of genetic diversity within a bacterial species, an appropriate means of analyzing those data, and a numerical description of the required resolving power of a genotyping method, it is in principal always possible to identify a set of polymorphic sites that, if interrogated, will provide the required resolving power in an efficient manner.

Robertson et al. described the “Minimum SNPs” software that can derive highly informative sets of single-nucleotide polymorphisms (SNPs) from DNA sequence alignments and the application of this to the development of SNP-based genotyping methods for Neisseria meningitidis and Staphylococcus aureus. The SNPs used were derived from MLST databases.

The most generally applicable SNP sets were optimized through maximization of Simpson’s index of diversity (D). This work was extended by Stephens et al., who showed that, in the case of S. aureus, the genotypes defined by a seven-member high-D SNP set were concordant with the population structure of the species, and that it is possible to identify a small set of binary markers (genes present in some isolates but not others) that efficiently add genotyping resolution.

Real-time PCR is a single-step closed tube method that is amenable to automated setup and data analysis. Real-time PCR devices are increasingly cost-effective to purchase and run, standardized real-time PCR-based procedures can incorporate the interrogation of different classes of polymorphisms (e.g., SNPs plus genes that exhibit binary variability), and realtime PCR-based procedures have the potential to incorporate primary diagnosis and target quantification. An inherent disadvantage of real-time PCR is that it is not very amenable to multiplexing, so interrogating multiple genetic targets normally requires multiple reactions. In our previous reports of the application of real-time PCR to SNP-based bacterial genotyping, the technique used was allele-specific PCR in the real-time format (kinetic PCR). The advantage of kinetic PCR is that it is simple and robust and requires only generic mastermix and unlabeled primers. The disadvantage is that it requires at least two reactions per SNP, making the interrogation of multiple SNPs somewhat unwieldy and expensive.

Therefore, an objective of this study was to develop variants of kinetic PCR that preserve the advantages but reduce the cost and the number of reactions required. Here we report the development of two novel formats for allele-specific real-time PCR, the application of these methods to the genotyping of a large number of S. aureus isolates, and

automated methods for DNA preparation, reaction setup, and data analysis. The utilities of these methods have been compared using S. aureus as a model system. This study has yielded a robust, cost-effective, and automated procedure for S. aureus genotyping. The small number of genetic targets interrogated means that this approach is especially amenable to adaptation to emerging technologies such as “lab-on-a-chip” devices and dedicated, fully automated real-time PCR machines.

MRSA GENOTYPING USING A SMALL SET OF POLYMORPHISMS

Methicillin-resistant Staphylococcus aureus (MRSA) continues to be a significant human pathogen. For many years it has been a common cause of nosocomial infections, and variants capable of causing infections in the community [community-acquired MRSA (CA-MRSA)] are an emerging and serious public-health issue (Diekema et al., 2001). The CA-MRSA phenomenon is remarkable in that these organisms appear to have arisen independently in disparate lineages within the S. aureus species (Okuma et al., 2002).

Recent years have seen a rapid increase in the understanding of the comparative genomics and population biology of S. aureus, which is leading to an equally rapid increase in the understanding of the relationship between genotype and phenotype.

This in turn is facilitating the development of genotyping procedures based upon the interrogation of known genetic polymorphisms (Francois et al., 2004). It is now known that the S. aureus genome consists of a ‘backbone’ that is composed of genes that are essentially common to all variants within the species, and which evolves primarily by mutation, punctuated by rare recombination events of unknown mechanism. Superimposed upon this is a complement of mobile genes that give rise to binary gene variation between and within lineages. The staphylococcal cassette chromosome mec (SCCmec)

is an example of an accessory element whose diversity is able to demonstrate the multiple insertion events that have occurred in the evolution of MRSA.

Our overarching aim is to develop microbial genotyping methods based upon small sets of polymorphisms selected from known genomic diversity on the basis of their optimal combinatorial informative power. Such methods would be suitable for low-density array, or real-time PCR technology platforms, and would facilitate routine surveillance, infection control, and rapid and cost-effective identification of clones of interest. We have previously reported a computerized method for identifying sets of single nucleotide polymorphisms (SNPs) in mutilocus sequence typing (MLST) databases that provide high Simpson's index of diversity (D) values with respect to the databases, and the application of this to S. aureus (Robertson et al., 2004). A set of seven SNPs was identified that provides a D of 0?95 with respect to the S. aureus MLST database, and a real-time PCR method for interrogating these SNPs was reduced to practice. Robertson and co-workers did not determine the concordance between SNP profiles and the S. aureus population structure, i.e. whether the SNP-based genotyping clusters closely related isolates and discriminates more distantly related ones. Accordingly, one of the principal aims of this study was to address this question as it relates to MRSA, and also to apply the SNP-based genotyping method to a larger collection of MRSA isolates.

An ideal molecular typing method would indicate the position of the isolate within the species population structure, and also economically and conveniently serve as a high-resolution comparative method. The 'progressive hierarchical resolving assays using nucleic acids' (PHRANA) concept articulated by Keim et al. (2004) is very helpful in devising such typing methods. The underlying principle of PHRANA is that slowly evolving polymorphic sites do not provide high resolution, while rapidly evolving markers are unreliable at indicating the position of the isolate within the population structure and are subject to homoplasy if used by themselves. However, rapidly and slowly evolving markers in combination provide a usefully complete picture. Therefore, the other principal aim of this study was to identify a small set of binary markers that adds resolving power to the SNP set. The markers identified are four virulenceassociated genes and three SCCmec-associated integrated plasmids. Polymorphisms in SCCmec and the agr locus have previously been used in combination with MLST to define MRSA clones (Oliveira et al., 2001), so we have also assessed the informative powers of SCCmec and agr typing in this context. The genotypes defined by the combination of SNPs and binary markers are very easy to obtain and digitize, consistent with previous studies, and provide easily understood genetic fingerprints for the well-known MRSA clones.

CONCLUSION

Suitable bacterial genotyping methods are essential for the management of infectious diseases in both healthcare and community environments. Subspecies identification is conducted within hospitals and research institutions for many applications, including for identifying an outbreak source during epidemiological investigations, for routine surveillance of hyper-invasive, multi-resistance or simply carriage strains, and for small or large scale population structure analyses. Infectious disease genotyping is also utilised for biodefence in order to identify the source of biological agents.

The bacterial genotyping methods currently available vary in then resolving power, cost effectiveness, and the technological platform required to employ them. Based on these factors, the most appropriate genotyping method for any given application can be determined. Studies involved in detailed investigations of virulence or other complex traits require whole genome sequencing or microarray analysis. Both approaches are time consuming and expensive. In contrast, routine analysis of clinical isolates requires genotyping methods that are rapid, cost effective, and generate portable results of appropriate resolution that can be interpreted easily.

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