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AN ANALYSIS UPON THE GROWTH OF GENOTYPING TECHNIQUES FOR METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

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An Analysis upon the Growth of Genotyping Techniques for Methicillin-Resistant Staphylococcus Aureus

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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.

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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.

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 al., 2000b). Under optimal conditions, cell division occurs approximately every 20min with cell diameter ranging from 0.5- 1.5 uni. S. aureus is able to grow on a range of nutrient media and appears as smooth, convex colonies with diameters of ~l-3mm. The golden pigmentation, caused by carotenoids. is usually visible, and this is thought to protect cells against ultra-violet radiation.

Staphylococcus aureus is a major cause of healthcare associated infections. Although several epidemiological studies have provided information on the prevalence of methicillin-resistant Staphylococcus aureus (MRSA) colonization in different hospital settings and in emergency department personnel, limited data exist on MRSA colonization rates for patients seen in emergency departments. The cost benefit of screening for MRSA colonization is not overwhelming and the subject is controversial. A limitation of both culturebased and molecular methods of MRSA screening is that current tests determine only the presence or absence of

colonization with no information on the colonizing strain type. As only small subsets of MRSA clones have been responsible for the major epidemics in the United States screening might have greater value if future studies demonstrate that certain clones are more likely to cause serious infections than others.

We have developed a rapid molecular surveillance assay based on PCR coupled to electrospray ionization mass spectrometry (PCR/ESI-MS) that identifies and provides detailed information about S. aureus strains. A previous version of this assay has been shown to accurately genotype S. aureus strains and to identify the clonal complex of a series of isolates from the Centers for Disease Control (CDC). Excellent agreement with multilocus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE) results was reached using in the PCR/ESI-MS assay a set of eight primer pairs that target the same genes as those for MLST. This set of primer pairs was designed to optimize the information content of the amplicons and distinguished 99% of 710 distinct S. aureus sequence types. Using a second set of eight primer pairs, PCR/ESI-MS has also been used to characterize virulence factors, toxinencoding genes and antibiotic resistance determinants on isolated colonies and to detect MRSA in nasal swabs with sensitivity and specificity similar to culture methods.

S. AUREUS GENOTYPING

Bacterial typing is the act of subdividing bacteria into smaller groups that share observable traits. Typing using the traditional phenotypic methods such as bacteriophage typing, have largely been replaced by powerful molecular genotyping methods. Genotyping characterises bacteria by the production of unique genetic "fingerprints" that are recognisably different among strains of the same species. These characteristic fingerprints are generated by using common methods such as PGR amplification, enzyme digestion, electrophoresis, hybridisation and DNA sequencing techniques.

The level of typing discrimination attained among strains is dependent upon the method used, and the diversity within the collection of isolates tested. There are two general types of genotyping methods: comparative, and library. Comparative genotyping methods determine whether isolates are the same or different and are useful for either preventing or managing outbreaks by distinguishing between background and infecting isolates. Library methods demonstrate extended capabilities, as they report the genetic changes that define the genotype. This feature is essential when reconstructing population structures, from micro-environments (such as hospitals), to large-scale global surveys. To date, a standard MRSA genotyping protocol has yet to be implemented globally. Within the last decade, numerous genotyping methods have been devised to accommodate this need.

Restriction Digestion Methods-

Pulsed-Field Gel Electrophoresis (PFGE) : Pulse-Field gel electrophoresis (PFGE) was a method developed in 1984 to separate and display yeast chromosomes for the purpose of karyotyping. Unlike conventional electrophoresis that is limited to separating fragments of less than 50Kb using a constant and unidirectional electric field, the PFGE apparatus alters the electric field orientation after each pulse. The programmed reorientation of the field forces the DNA fragments to frequently realign their migratory path. The time taken to realign determines the overall migration distance; the larger fragments reorientate at a slower rate and therefore do not progress along the field as far as smaller fragments during each pulse. PFGE has become a particularly important tool for bacterial genotyping.

Amplified Fragment Length Polymorphism (AFLP) : Amplified fragment polymorphism (AFLP) is another broadly applicable genotyping method that creates highly discriminatory DNA fingerprints. AFLP generates DNA profiles by selective PGR amplification of restriction fragments followed by electrophoresis. The technique involves three steps. Firstly, total genomic DNA is digested using one frequent and one rare cutting enzyme, followed by ligation of oligonucleotide linkers. This dual enzyme approach allows control over the total number of fragments to be amplified whilst the linker seives as the PGR primer attachment site. The use of multiple enzymes is not crucial as an AFLP technique using only a single enzyme has been reported.

Multi-locus Sequence Typing (MLST) -

Multi-locus sequence typing (MLST) was first reported in 1998 as a method for unambiguously characterising bacterial isolates . The S. aureus MLST scheme is based on PC'R amplification and sequencing of seven internal fragments from widely dispersed core housekeeping genes. As housekeeping genes are essential for cell viability, they are ubiquitous within members of a species and exhibit a slow rate of SNP accumulation. For clonal bacteria such as S. aureus, the mutation rate allows MLST to excel in the step-wise reconstruction of evolutionary relationships within the species. The utilisation of an interactive website (www.mlst.net) for data storage and comparative analysis is fundamental to the success of MLST as a modem genotyping method.

Genotyping by Repetitive DNA Analysis -

Both eiikaiyotic and prokaiyotic genomes encode numerous and diverse regions of DNA hyper-variability. The repeat regions, particularly of bacteria, are often associated with MSC'RAMMs and other cell surface proteins that project binding domains into the extracellular environment. DNA sequence hyper-variability occurs due to slip strand mispairing during

cell division; however these regions do not require explicit conservation, as their role accommodates variation in amino acid sequence and length. This characteristic creates disparity between the evolutionary mutation rates of the repeat regions and the core genome, so much so that isolates from the identical genetic lineage can be differentiated. For this reason, hyper-variable regions are used commonly for bacterial genotyping. and those pertinent for S. aureus are discussed below.

SCCmec Genotyping -

The SCCmec element is a recently characterised chromosomal island that is responsible for transmission of p-lactam resistance. The SCCmec locus varies considerably in content and length and therefore is suited for genotypic analysis. In addition to defining MRSA clones, inherent variation and mobility is invaluable for augmenting the resolution of other methods, such as MLST and PFGE. Considering the benefits resulting from MRSA genotyping, a variety of methods have been developed for this task.

MOLECULAR METHODS FOR THE EPIDEMIOLOGICAL ANALYSIS OF MRSA

Staphylococcus aureus is able to cause a wide variety of different diseases, ranging from superficial skin inflammation to severe invasive infections such as bacteraemia. Methicillin-resistant Staphylococcus aureus (MRSA) has become a major problem worldwide. MRSA can cause both community-acquired and nosocomial infections. In Finland, an increase in the annual numbers of MRSA cases was observed from 121 in 1997 to 1772 in 2008. However, the MRSA situation improved in 2009, when 1267 cases were registered in the National Infectious Diseases Register. Accurate genotyping methods are required for national MRSA surveillance, outbreak investigations and international comparisons. Several different genotyping techniques for MRSA have been used in Finland.

Macrodigestion of the whole bacterial chromosome, pulsed-field gel electrophoresis (PFGE), served as a primary typing method to distinguish different MRSA strains for several years. However, spa typing, which analyses the polymorphic Xregion of protein A of S. aureus, replaced PFGE, and is now used as the first-line typing tool in Finland. Multilocus sequence typing (MLST) of seven housekeeping genes and analysis of a methicillin resistance genetic element, staphylococcal cassette chromosome mec (SCCmec), are used as additional typing methods. In addition, MLST and SCCmec typing are valuable for understanding the evolutionary relationship between MRSA clones.

Streptococcus pneumoniae causes severe invasive infections such as meningitis, bacteraemia and pneumonia, and non-invasive infections such as sinusitis and acute otitis media worldwide. Pneumococci are also normal inhabitants of nasopharyngeal mucous membranes in healthy children and adults. Although pneumococcal conjugate vaccine (PCV) is increasingly being used, pneumococcal infections caused by non-vaccine serotypes and the antimicrobial resistance of S. pneumoniae strains have become a major public health issue. In

Finland, over 800 new bacteraemic S. pneumoniae infections are registered annually. Careful monitoring of the serotype distribution, antimicrobial susceptibility pattern and clonality of the strains is needed for active national surveillance of the disease burden and the efficacy of the recently launched national immunisation of all children with PCV. Molecular typing methods, such as MLST, provide excellent

tools for genotyping of the strains and for better understanding of pneumococcal transmission in outbreaks. In addition, analysis of pneumococcal virulence protein genes is of interest for understanding the pathogenesis of pneumococcal diseases.

Several criteria should be considered when evaluating, validating and comparing typing methods. These can be divided into performance and convenience criteria. The selection of the optimal and most applicable typing method depends on the purpose of the analysis. After the typing method has been selected, it is very important to carefully match this technology with the speed at which the molecular changes occur. The use of typing tools in epidemiological studies requires understanding of both the strengths and limitations of the chosen typing method as well as the epidemiological study design to be able to address the research question. In addition, laboratory and epidemiological evidence validate each other, and typing results must therefore be interpreted in the context of the epidemiological evidence as well as the characteristics of the bacteria. To enable comprehensive epidemiological surveillance and international comparability, often one typing method alone is not sufficient, and a combination of various molecular typing techniques is used.

Typing methods can be classified into phenotyping and genotyping methods. Phenotyping techniques detect the characteristics expressed by bacteria, while genotyping methods assess the variation in the chromosomal or extrachromosomal nucleic acid composition of bacterial isolates. Phenotyping involves methods for detecting differences in

biochemical reactions, morphology, and the environment. Biotyping is a widely used phenotyping method to assess biochemical characteristics varying within a given species, and is usually used in diagnostics and species identification and to separate the members of a particular species.

Other commonly used phenotyping methods include serotyping, antimicrobial susceptibility testing, and bacteriophage and bacteriocin typing. Less frequently used phenotyping methods include multilocus enzyme electrophoresis (MLEE), mass spectrometry (MS) and SDS-PAGE of cellular and extracellular components.

Different genotyping methods are likely to reveal different degrees of genetic variability. Genetic diversity arises by various molecular processes, including the accumulation of spontaneous point mutations, diverse types of genetic rearrangements, and the loss and acquisition of chromosomal and extrachromosomal DNA sequences. Genotyping methods define variation in the genomes of bacterial isolates with respect to the composition, overall structure, or precise nucleotide sequence. The increasing availability of bacterial genome sequences has had a significant impact on the evolution and improvement of genotyping methods.

Bacterial genotyping methods can be classified into three main categories: hybridisation-mediated, fragment-based and sequence-based methods. Hybridisation-mediated methods include ribotyping, which has been used for a long time, and novel DNA array-based methods such as DNA macro- and microarrays, cDNA microarrays and oligonucleotide microarrays. Fragment-based methods can be separated into methods where fragments are generated by cleavage of DNA using restriction enzymes, including methods such as plasmid typing, restriction fragment length polymorphism (RFLP), restriction endonuclease analysis (REA), and pulsed-field gel electrophoresis (PFGE). PCR fingerprinting (e.g. BOX, arbitrarily primed PCR), repetitive sequencing-based PCR (REP-PCR), and multilocus variable number tandem repeat analysis (MLVA) are fragment-based methods where fragments are generated by amplification of DNA. A combination of both restriction enzyme digestion and DNA amplification includes methods such as PCR-RFLP and amplified fragment length polymorphism (AFLP) . Sequence-based methods include single- locus sequence typing (SLST), multilocus sequence typing (MLST), single nucleotide polymorphism (SNP) genotyping and genome sequencing, which is currently still too expensive and time demanding to be used in routine genotyping.

The interpretation of typing data is an important phase in the typing process. Depending on the typing method, the interpretation of the experimental data leading to correct identification can be complicated and demanding. This may be due to technical factors relating to the typing method used or the fact that an epidemic strain can evolve during an ongoing outbreak. Some typing methods are more stable than others. For example, PFGE patterns of MRSA strains have been demonstrated to be relatively stable over periods of weeks to months. However, the use of PFGE in long-term laboratory-based surveillance can be misleading. Interpretation rules should provide well-defined guidelines for unambiguous data interpretation, whether the strain is unique or a part of an outbreak.

TANDEM REPEAT-BASED METHOD FOR RAPID AND HIGH-THROUGHPUT GENOTYPING OF STAPHYLOCOCCUS 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.

Each method mentioned above allows the definition of re- lationships between strains, but direct comparison between respective data sets failed to yield comparable results. PFGE is a suitable method for the establishment of clonal relationships, but it has been described to be poorly informative for long-term epidemiological surveillance. To the opposite of this, MLST has been validated for long-term and global epidemiological studies.

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.

PHAGE OPEN-READING FRAME TYPING METHOD

Methicillin-resistant Staphylococcus aureus (MRSA) remains an important pathogen as a cause of nosocomial infections. Among these infections, MRSA bacteremia is one of the most serious, with a high mortality rate. Pulscd-field gel electrophoresis (PFGE) of chromosomal DNA digested with restriction enzymes has become one of the most reliable genotyping techniques for MRSA typing, since it is a highly discriminative, stable, and reproducible method. A new strain differentiation method for MRSA, the phage open- reading frames (ORFs) typing (POT) method, was reported by Suzuki ct al. in 2006. This is a rapid, PCR-based method, amplifying the ORFs of phage genomes lysogenized in MRSA together with Tn554 transposase B subunit and kdpC on SCCm^c type II. In this report, we dcscribc the usefulness of the POT method in the epidemiological study of MRSA isolated from bloodstream infections.

Nagoya University Hospital is a 1,035-bed, tertiary-care, university-affiliated hospital with an intensive carc unit (ICU) and a neonatal intensive carc unit (NICU). A total of 92 MRSA strains were investigated, 14 isolated in 1991 -1992 and 78 isolated in 1996-2003. The strains were recovered from blood culture samples of individual patients suffering from MRSA bacteremia. Antibiotic susceptibility tests for oxacillin, gen- tamicin, arbekacin, minocycline, clindamycin, levofloxacin, and vancomycin were performed using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Staphylococcal toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins A to D (SEA, SEB, SEC, and SED), and coagulase type were performed as reported previously. PFGE using Sma\ was performed on a CHEF-DR II apparatus at 6 V/cm, for 22 h with pulse times running from 1-50 s in 0.5X TBE buffer at 14°C. PFGE patterns in the dendrogram were analyzed with Molecular Analyst Software Fingerprinting II. PFGE genotypes were distinguished by the DICE coefficient of 80% and designated as A to U. PFGE subtypes were divided within the same PFGE genotypes, analyzed by the DICE coefficient of 100%, and designated as Arabic numbers added after each alphabetical genotype. The POT method was performed according to the report by Suzuki et al.. Two representative MRSA strains were subjected to analysis to test the reproducibility and stability of the POT method. For reproducibility analysis, the DNA preparations from the two MRSA strains were subjected to a set of PCR reactions five times. To confirm stability, a set of PCR reactions was performed using the two MRSA strains with repeated subcultures up to 10 times.

The POT method consistently gave the same results for the two representative MRSA strains in the serial PCR reactions and after 10 times passage, which indicated sufficient reproducibility and stability of the method. The results of POT were given as 16 digits of binary code divided into two parts which were then transformed into decimal numbers. Data given as decimal numbers are easier for inter-laboratory com- parison.

Usually, in the dendrogram analysis of the PFGE profiles, the cut-olf of the epidemiological relatedness was the DICE coefficient of 80%. Using the DICE coefficient of 100%, 92 isolates were divided into 48 PFGE subtypes. Among genotype A, major subtypes A1 and A2 comprised 16 and 13 isolates, respectively.

However, these six isolates were recovered from 1991 through 1992, and appeared to be epidemiologically related. Three out of six patients were hospitalized in the same ward during the same period, and five out of six were temporally admitted to the ICU after a surgical operation in which they were

exposed to MRSA. Furthermore, the phenotypes of these strains were identical, i.e., coagulase type II, TSST-1 positive, SEC and had the same antibiotic susceptibility profile. The PFGE profile of these six strains was identical with type 1 reported by Ichiyama et al. (data not shown), suggesting that these strains were involved in an outbreak. In contrast, among the three major POT types 159-87, 159-118, and 191-246, all but three isolates belonged to PFGE genotype A, indicating that the discriminatory power of these methods was quite similar in this limited number of isolates. As a whole, the discrimination index (DI) of the PFGE genotype, PFGE subtype, and POT was 0.719, 0.953, and 0.988, respectively. These results show that POT typing had greater discriminatory power than PFGE typing in this analysis of serial blood culture isolates.

CONCLUSION

In conclusion, the prevalence of S. aureus and MRSA colonization in a convenience sample of patients seeking care in Emergency Department was 29.5% and 13.5%, respectively. A substantial fraction of the S. aureus-colonized patients were co-colonized with CoNS and highlevel mupirocin-resistant CoNS. These findings highlight the importance of active surveillance studies to detect MRSA colonization and/or infection among emergency department patients. The molecular genotypes were directly identified from nasal swabs by PCR/ESI-MS. The ability to identify unique molecular signatures from MRSA may allow infection control resources to be effectively focused and will enable tracking origins of outbreaks in hospital settings. Determining the molecular genotype of S. aureus during intake screening may prove valuable if certain molecular genotypes become associated with increased infection risk.

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 carnage 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.

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