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Identifying MRSA with Multiplex Sequencing

Nosocomial Surveillance Programs Benefit from Sequence-Based Analysis of Genetic Markers

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These days, patients with suspected nosocomial infections are assessed by traditional culture methods, and a positive result leads to appropriate antibiotic therapy. Routine screening for methicillin-resistant *Staphylococcus aureus* (MRSA), however, only occurs at admission. With the increase in need for patient isolation and long-term in-hospital patient management, hospitals are seeking a benchmark assay that could generate relevant evidence for implementing an appropriate intervention program as early as possible.

There are key genetic factors such as antibiotic-resistance genes and toxin genes that make a resident microbe a nosocomial pathogen. Corresponding variants of these genes may increase the pathogenicity, as reflected in the minimum inhibitory concentration (MIC) value. Most of these genetic markers are carried by mobile genetic materials (plasmids and transposons) and hence are transferable across species barriers.

Furthermore, based on selection pressure (e.g., antibiotic usage), the incidence and genetic makeup of these markers will vary but they can also be purged from the carrier organism in the absence of selection pressure.

The genes carried by the *Staphyloccus* chromosome cassette (SSCmec) vary, and mere identification of the cassette itself may not provide information about the genes it carries nor their variations. Therefore, the key deciding factors for monitoring nosocomial infections are the nosocomial-associated genes themselves and their respective variants, rather than the carrier vehicles such as organisms, plasmids, transposons, or genetic cassettes.

Although there are methods to type the carrier organisms, they seldom

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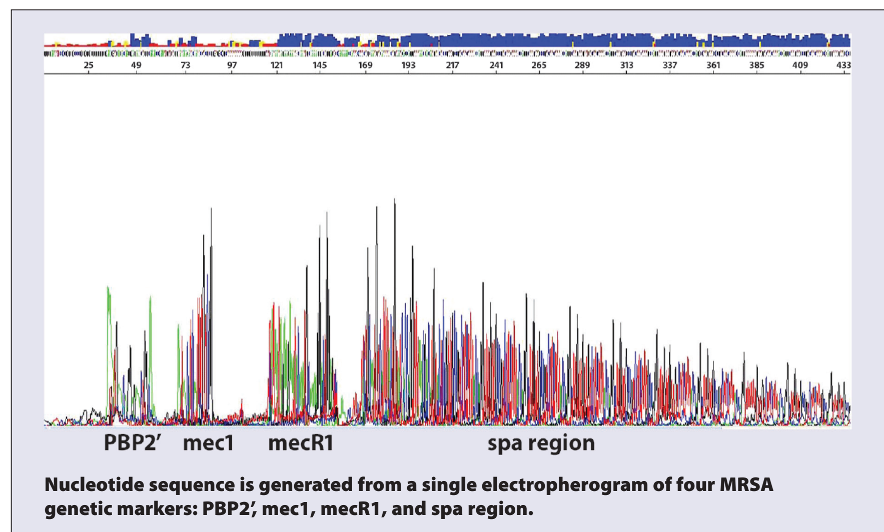

Nucleotide sequence is generated from a single electropherogram of four MRSA genetic markers: PBP2′, mec1, mecR1, and spa region.

include identifying the genes that are associated with nosocomial infections. Thus, there is a need to periodically characterize selected nosocomial-associated genes to monitor their changes and spread in the local microbial gene pool.

Although the need for intervention programs is well recognized, the lack of appropriate technology has prevented implementation at most healthcare facilities. In this article, we describe the application of a multiplex-sequencing technology that can be tailored to generate data on relevant microbial genetic factors and thereby facilitate implementation of an evidence-based surveillance program.

The data presented here was simultaneously generated to identify the methicillin-resistance marker (mecA), markers that distinguish community-acquired from hospital-acquired infections (mecR1 and mec1), and a marker (spa) for source identification. This assay not only accurately identifies methicillin resistance, it also subtypes the MRSA as community acquired (CA-MRSA) or hospital acquired (HA-MRSA). In addition, the hyper-variable region of the spa gene can be used for tracking and source identification.

Multiplex DNA Sequencing

MultiGen Diagnostics' (www.multigendiagnostics.com) MultiGEN technology allows simultaneous sequencing of multiple amplicons generated either from the same genome, different genomes, or a combination of both.

In this assay we used three relevant SCC-associated gene segments (Pb2´, mecR1, and mec1) and a region (spa) of the *S. aureus* genome.

The MultiGEN process includes the preparation of total nucleic acid, followed by amplification using four PCR primer pairs to produce four amplicons that are simultaneously sequenced at the 3´end. Expected read sequences of the genes are shown in the *Figure*. The assay was developed using *S. aureus* genomic DNA, which was later used as a positive control in the analysis of the isolates.

Twenty five clinical isolates on agar slants were obtained from the clinical laboratory of a local hospital. Bacterial colonies were picked and suspended in 200 µL of sterile water. Total DNA was extracted using the DNA Mini Kit.

All four genes (Pb2´, mec1, mecR1, and spa) were amplified using the MultiGEN-MRSA Amp kit. In a 50 µL reaction volume, 3 µL of the DNA extract was amplified with 5 units of Taq polymerase for 35 cycles. Amplicons were purified using Ampure. All four amplicons were simultaneously sequenced by cycle sequencing.

Read Sequences of Pb2´, MecR1, Mec1, and Hypervariable Spa Region

	Pb2´	MecR1	Mec1	Hypervariable Spa Region	
Accession number	AM292304.1	AM292304.1	AJ810122.1	AM407384.1	
BLAST search loci	14199-14221	16515-16533	2101-2134		
Read sequence	AATGAAACAAGGAGAAACTGGCA	AATAACATCAATTTGTCGG	TATAATAGAAGAAATACAAATGCAAAAGGACTGG		
Sample no. 2	AATGAAACAAGGAGAAACTGGCA	AATAACATCAATTTGTCGG	Negative	CTTCTT	TGTTGTTGT
Sample no. 3	AATGAAACAAGGAGAAACTGGCA	AATAACATCAATTTGTCGG	TATAATAGAAGAAATACAAATGCAAAAGGACTGG	CTTCTT	TGTTGTTGT
Sample no. 5	AATGAAACAAGGAGAAACTGGCA	AATAACATCAATTTGTCGG	TATAATAGAAGAAATACAAATGCAAAAGGACTGG	CTTCTT	TGTTGTTGT
Sample no. 6	AATGAAACAAGGAGAAACTGGCA	AATAACATCAATTTGTCGG	Negative	CTTCTT	TGTTGTTGT
Sample no. 7	AATGAAACAAGGAGAAACTGGCA	AATAACATCAATTTGTCGG	Negative	CTTCTT	TGTTGTTGT
ATCC staph genomic DNA	AATGAAACAAGGAGAAACTGGCA	AATAACATCAATTTGTCGG	TATAATAGAAGAAATACAAATGCAAAAGGACTGG	CTTCTT	TGTTG
17	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
18	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
19	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
20	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
21	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
22	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
23	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
24	Negative	Negative	Negative	CTTCTT	TGTTGT
26	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
27	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
28	Negative	Negative	Negative	CTTCTT	TGTTGT
29	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
30	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
31	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
32	Negative	Negative	Negative	CTTCTT	TGTTGT
33	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
34	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
35	Negative	Negative	Negative	CTTCTT	TGTTGTTGT
36	Negative	Negative	Negative	CTTCTT	TGTTGT
37	Negative	Negative	Negative	CTTCTT	TGTTGT

Unincorporated dye terminators were removed using Cleanseq. Then, 35 µL of eluate was freeze-dried in a speed-vac and resuspended in 10 µL of Hi-Di foramide. Samples were then analyzed by capillary electrophoresis. They were drawn into the capillary by electrokinetic injection at 2 Kv for 12 seconds, and electrophoresis was carried out at 15 Kv for 20 minutes.

The nucleotide sequences generated were verified by BLAST search to confirm the authenticity of sequences generated from the isolates. The assays were carried out with negative controls and genomic DNA from *S. aureus* was used as positive control.

The process from extraction to generating nucleotide sequences took less than eight hours. Our initial panel consists of the following genetic markers: SCCmec that expresses the PBP2´ gene (mec A), accessory genes (membrane-bound transducer gene mecR1 that derepresses the repressor gene mec1) that control the expression of PBP2´, and the hypervariable spa region.

Electropherograms of the positive control *S. aureus* showed all four gene-specific read sequences of PBP2´, mecR1, mec1, and spa (*Figure*). BLAST search of the generated sequences showed 100% alignment to that of the GenBank sequences (*Table*). Similarly, corresponding sequences generated from the isolates were BLAST searched confirming 100% analytical specificity.

Analytical sensitivity was determined by using serially diluted *S. aureus* genomic DNA and generating satisfactory read signals for PBP2´ at amounts of less than 1.5 pg per sample. In order to demonstrate absence of nonspecific amplification, a sample containing 15 ng of *S. aureus* genomic DNA was spiked with DNA extract (5 µg) from *Treponema pallidum* and positive signals were recorded for PBP2´.

All of the 25 isolates carried the spa region confirming that they were *S. aureus* (*Table*). Out of the 25 isolates, only five carried the methicillin-resistance gene PBP2´, and the other 20 were methicillin sensitive. Methicillin resistance has been noted previously to be able to transpose to independent replicons, and hence could have been purged in the absence of selection pressure.

Out of the five isolates positive for mecA resistance, two carried both mecR1 and mec1 genes confirming them as HA-MRSA. Three of the isolates positive for mecA resistance were negative for repressor gene mec1 but did carry the transducer-gene mecR1.

Absence of the mec1 repressor would have allowed maximum expression of PBP2´ and therefore would have increased the MIC against methicillin. Although the presence or absence of the ancillary genes helps to group them as HA-MRSA or CA-MRSA, respectively, it is important to recognize that there are variants among these two respective genes that may also harbor insertion sequences IS431 and IS1272. Because of such variations, the relevant nucleotide sequence of selected markers should be determined in order to predict their pathogenicity and accurately categorize them as CA-MRSA or HA-MRSA.

Conclusion

How can this type of genetic analysis be utilized by healthcare facilities? As each healthcare facility has its own policy for antibiotic usage, the selection pressure on the local microbial flora will vary among hospitals. By using this multiplex sequencing assay, however, the baseline incidence and the spread of selected genetic markers could be determined by conducting a point-prevalence study, where all potential sites including patients, healthcare workers, and the hospital environment are sampled over a period of time.

Based on this study and the hospital-specific selection pressure (antibiotic usage), an assay with a specific set of genetic markers could be tailored for each healthcare facility.

In addition, based on the point-prevalence results, an ongoing surveillance program could be developed that includes sampling critical points in the facility on a regular basis.

The frequency of sampling would vary according to the baseline data and ongoing surveillance data. Data from these nosocomial-associated genetic markers would then enable identification of the carrier organism they "infect", an assessment of the overall incidence of MRSA, the clinical significance of the genetic changes, and the underlying cause (including selection pressure and influx from community). With this evidence in hand, effective surveillance and intervention programs could be implemented.

Effective nosocomial surveillance requires accurate identification of the incidence of the variable genetic markers in the microbial gene pool in the hospital environment and the sources and routes of transmission both inside and outside hospitals. In order to accurately identify these genetic variables, specimens need to be analyzed directly, rather than from subcultured isolates.

Compared to present identification methods by culture, labeled probes, or molecular typing technologies, sequence-based nucleotide analysis of the relevant genetic markers provides more accurate and valuable information by which effective surveillance and intervention programs could be implemented and audited. GEN