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Review on Recent Major Diagnostic Methods: The Diagnostic of Methicillin Resistant Staphylococcus Aurus

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Abstract
According to the World Health Organization (WHO) 20% of all documented *S. aureus* infections are attributable to methicillin resistant *staphylococcus aureus* (MRSA), although for some developing countries this value can exceed 80%. Thus the rapid and accurate detection of MRSA in low resource settings is becoming essential. Yet conventional microbial detection methods take from 1-5 days to identify MRSA. Recently, new types of automated laboratory methods as well as advances in nucleic acid testing, microfluidic technology, immunosensors, biosensors and point of care testing have reduced the time to detection to <1 hr. This review examines the current limitations and advances in methodologies employed in the rapid detection of MRSA.
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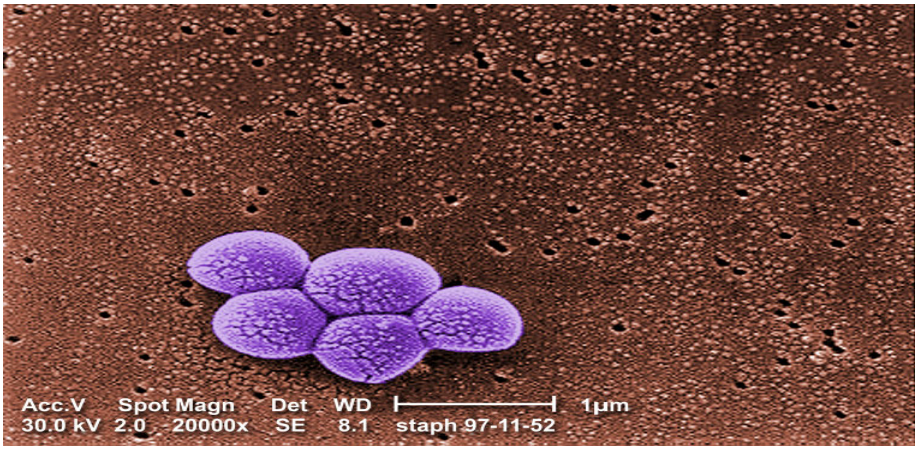
1. INTRODUCTION

Infectious diseases are the second leading cause of death worldwide and the third leading cause of death in developed countries. Antibiotics are powerful drugs, but they are not the cure for all thus diseases. Antibiotics, also known as antimicrobial drugs, are drugs that fight infections caused by bacteria. The rapid emergence and spread of drug-resistant organisms, such as *Staphylococcus aureus (S. aureus),* both in the healthcare setting and the community prompts great urgency in the development of and advocacy for prevention and treatment efforts (Fauci, 2001)

Staphylococcus aureus is a Gram positive, coagulase positive coccus in the family *Staphylococcaceae.* Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important antibiotic-resistant pathogens of humans and different animal species. The history of MRSA is mainly situated in human medicine and started in 1961, when MRSA was first isolated in a UK hospital. From then onwards, MRSA began to spread in hospitals all around the world, but at the end of the 1980's and during the 1990's its prevalence truly exploded in many countries (Panlilio AL, et al, 1992). In recent years, several studies have described livestock and companion animals as carriers of some specific lineages of MRSA, and they are therefore considered to be potential zoonotic bacteria. Moreover, MRSA have also been found in wild animals. The precipitous spread of methicillin-resistant strains of *S. aureus* (MRSA) has created new challenges for governments, healthcare systems, and drug development (Wardyn SE, 2012)

There are many antibiotic resistant strains of Staphylococcus aureus (SA) known to cause skin, soft-tissue and other infections in humans and animals (Malhotra K, et al, 2008). To date methicillin resistance (MR)-SA strains such as, hospital acquired (HA)-MRSA clones CC5, CC8, CC22, CC30 and CC45, and community acquired (CA)-MRSA represent the most serious challenge to public health (Witt, R, et al 2010). CA-MRSA can be distinguished from HA-MRSA by the presence of staphylococcal cassette micro chromosome (SCCmec) types IV and V and the Panton-Valentine Leukocidin (PVL) exotoxin, the latter often associated with necrotizing pneumonia and severe skin infections. HA-MRSA strains mainly harbor SCCmec types I, II and III and in contrast to CA-MRSA strains are multidrug resistant particularly to fluoroquinolones (Vandenesch, F. et al, 2003).

In humans, *S. aureus* is an opportunistic pathogen. Both methicillin-sensitive and methicillin-resistant strains can be found as normal commensals on the skin (especially the axillae and perineum), the nasopharynx, and anterior nares and/or in the gastrointestinal tract. From skin abscess and cellulitis to invasive bacteremia, endocarditis, and septic arthritis, capable of causing significant human disease. In animals, MRSA has been recovered from various sites in asymptomatic animals, including the nares, pharynx, mouth, skin, and rectum or cloaca and causes pneumonia, joint infections, osteomyelitis and septicaemia in poultry, subcutaneous abscesses, mastitis and pododermatitis in rabbits, dermatitis and cellulitis in horses, septicaemia in pigs, intramammary infections in cattle and small ruminants, leading to considerable economic losses in cattle farming (Bar D, et al, 2008).

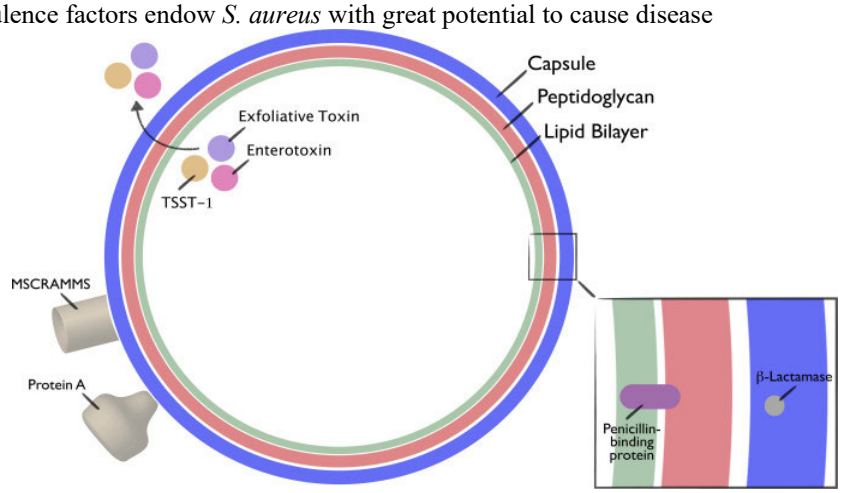


Source: (http://phil.cdc.gov/Phil/default.asp).

Figure: Image obtained from the Public Health Image Library of the Centers for Disease Control and Prevention showing MRSA .

2. PATHOGENESIS AND VIRULENCE FACTORS

A critical first step in the pathogenesis of S. aureus infection is colonization. Asymptomatic colonized host provide a reservoir for the animal to human and human-to-human spread of disease. The primary modes of transmission include direct skin-to-skin contact with a colonized source and, to a lesser extent, contact with colonized fomites. Disruption of the normal skin barrier (e.g., abrasion, burn) as well as immunosuppressive conditions (e.g., HIV, steroid use, genetic diseases) predispose colonized hosts to infection (Chambers and Deleo, 2009). When attacked (commensal and immunological) MRSA releases a barrage of toxins such as hemolysins, leukotoxins, exfoliative toxins (ETs), toxic-shock syndrome toxin, as well as a raft of virulence factors that include, coagulase, proteases, staphylokinases and fibronectin proteins, protein A and collagen adhesins(Wang, J. et al, 2015).A surface proteins and secreted virulence factors endow *S. aureus* with great potential to cause disease



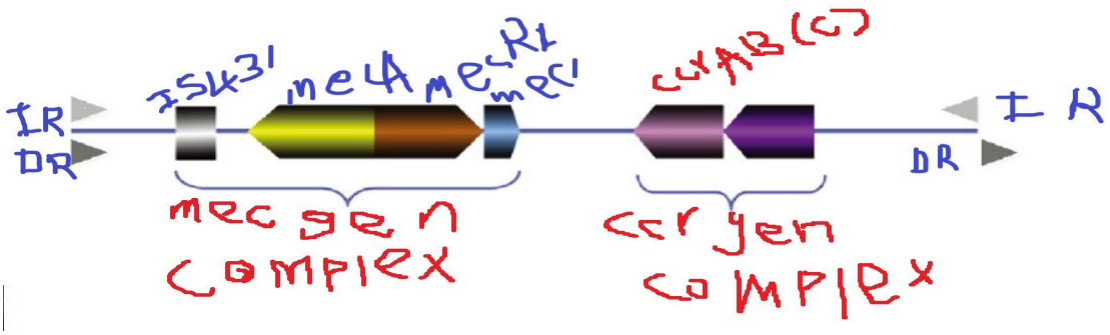
Source: Gordon et al., 2008.

Figure 8: Schematic diagram of *S. aureus*, depicting basic structure and a selection of virulence factors.

3. MECHANISMS OF *S. AUREUS* RESISTANCE TO METHICILLIN

In order to evade the bactericidal properties of methicillin, *S. aureus* has developed several modes of resistance. Such mechanisms include the expression of a methicillin-hydrolyzing β lactamase as well as the expression of an alternative penicillin binding protein2a (PBP2a) that binds to methicillin with lower affinity and with higher rates of methicillin release.

Most MRSA carry the mecA gene, nested within the mec element, which resides on a large mobile genetic element called the staphylococcal chromosomal cassette mec (SCCmec). This gene codes for a penicillin binding protein 2a, PBP2a, which interferes with the effects of beta lactam antibiotics on cell walls. It confers virtually complete resistance to nearly all beta-lactam antibiotics including semi-synthetic penicillins such as methicillin, oxacillin, or cloxacillin. Therefore, when the normal staphylococcal PBPs are inhibited by β-lactams, PBP2a can resume cell wall assembly, enabling viability in the presence of methicillin (Stapleton, and Taylor, 2002).



Source: Elsevier Science, 2014
Figure 9: The structure of SCCmec.

SCCmec is composed of two essential gene complexes. One is *mec*-gene complex, encoding methicillin resistance (*mecA* gene) and its regulators (*mecI* and *mecR1*), and the other is *ccr*-gene complex that encodes the movement, of the entire SCC element. Abbreviations: IR, inverted repeat; DR, direct repeat.

4. METHODS OF DETECTION

Identification of MRSA relies on the detection of SA specific genes, such as spa, nuc and fem in combination with the mecA gene. The mecA gene codes for the penicillin binding protein (PBP2a) and is carried by the staphylococcal cassette micro chromosome (SCCmec) a mobile genetic element. Due to its low affinity for B-lactam antibiotics PBP2a enables SA to survive in the presence of methicillin (Mantiona et al, 2015).

Activation of the mecA gene is regulated by the mecI repressor and anti-repressor mecR1. Eleven types (I-XI) of SCC elements have been reported all of which carry the mec and cassette chromosome recombinases (CCR) gene complexes. CCR's are the second essential component of the SCCmec element encoded by different allotypes of the ccrAB and ccrC genes. CCR's orchestrate site-specific integration and excision of the element at the integration site sequence (ISS) of SCCmec located at the 3′ end of the chromosomal orf (open reading frame) X gene (Dupieux, C. et al , 2015).

4.1. Traditional Culturing

The first step in traditional culturing is sample collection. Swabs are collected samples from organic (skin or nasal cavity) and inorganic surfaces (metal or plastics). The condition of sample specimens such as blood, urine, feces, pus, sputum and bodily fluids can vary significantly depending on the mode and the size of the container used to transport them. Until the late 1990's conventional culturing methods utilized methicillin and oxacillin chromogenic agars to distinguish MRSA from methicillin susceptible SA (MSSA) and SA. However cefoxitin and cephamycin agars are now preferred as they show enhance induction of penicillin-binding protein 2a (PBP2a) and thus better selectivity for MRSA (Shin, J.H. et al, 2013).

4.2. Enzyme-linked Immunoassay (ELISA)

In recent decades both labeled and non-labeled optical based immunoassays have been used to directly or indirectly detect MRSA. Direct, competitive and sandwich are the main assays formats used to detect whole cell MRSA and secreted enterotoxins. Produce some kind of observable color change to indicate the presence of MRSA.

4.3. Nucleic Acid Amplification Technologies (NAAT)

One of the advantages of nucleic-acid-based detection assays is the high level of specificity, as they detect specific nucleic acid sequences in the target organism by hybridizing them to a short synthetic oligonucleotide complementary to the specific nucleic acid sequence. Several different types of nucleic-acid-based assays, including amplification, hybridization, microarrays, and biochips, have been developed for use as rapid methods.

Simple PCR Method

Traditional PCR techniques are used in the amplification, identification and quantification of MRSA (RNA and DNA). In this method, double-stranded DNA is denatured into single strands, and specific primers or single-stranded oligonucleotides anneal to these DNA strands, followed by extension of the primers complementary to the single stranded DNA, with a thermostable DNA polymerase. DNA extraction of *S. aureus* isolates was conducted by DNA extraction kit to identify the presence or absence of mecA gene in the S. aureus isolates, the following pairs of primers were used for the amplification (Chaieb K, 2006):

MecA F: 5'-TAGAAATGACTGAACGTCCGATAA-3'
MecA R: 5'-CCAATTCCATGTTTCGGTCTAA-3

One of the major problems with traditional PCR is at low concentrations of bacteria an enrichment step is still

required. Depending on sample quality conventional PCR methods can take from 24 to 48 hr to detect MRSA. Traditional PCR is an excellent technique for detecting antibiotic resistant strains of SA however some studies have shown traditional PCR fails to detect genetically distant SCCmec (CoNS) subtypes. For this reason different alternative are used.

Real-time PCR (RT-PCR)

Real-time PCR (RT-PCR) or QPCR that monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR capable of continuously monitoring PCR products and is often employed in the detection of antibiotic resistant bacteria (Malhotra-Kumar, S. et al, 2008). Their methodology consisted of an overnight selective broth enrichment step, followed by mecA, mecC, and S. aureus-specific (SA442 gene) RT-PCR assays with subsequent confirmation using a staphylococcal cassette chromosome mec element (SCCmec)-orfX-based real time PCR assay (GeneOhm MRSA assay). Moreover, the real-time monitoring of the process means no need for post-amplification treatment of the samples, such as gel electrophoresis, reducing the time of analysis.

Multiplex PCR

A cheaper alternative to RT-PCR is multiplex PCR (M-PCR) refers to the use of polymerase chain reaction to amplify several different DNA sequences simultaneously which can target two or more sequences specific for MRSA. Recently a novel M-PCR assay was developed for the differentiation of MRSA and methicillin resistant coagulase negative staphylococcus (MR-CNS) isolates by PCR amplification of 16S RNA, mecA, and nuc gene fragments (Liu ete al, 2016). The selectivity, sensitivity and accuracy of M-PCR improve depending on the number of targets that are screened.

In addition to classic PCR, numerous variations in combination with a variety of detection chemistries and automated systems are being developed. Currently available PCR-based molecular tests for MRSA include the LightCycler Staphylococcus and MRSA detection kit (LC assay; Roche Diagnostics, Mannheim, Germany), the Hyplex StaphyloResist PCR (BAG, Lich, Germany), the GenoType MRSA direct assay (Hain Lifescience, Nehren, Germany), the IDI-MRSA assay (GenOhm, San Diego, CA; BD Diagnostics), and GeneXpert MRSA assay (Cepheid, Sunnyvale, CA).

The LC Staphylococcus assay from Roche amplifies and detects a species-specific internal transcribed spacer region of the rRNA operon, and differentiation of *S. aureus* from CoNS is based on a melting curve analysis. *S. aureus*-positive samples are further tested for mecA with the LC MRSA detection kit. Using a lysostaphin-lysozyme-selective extraction method, the LC assay showed 95.7% sensitivity, 90.8% specificity.

Hyplex StaphyloResist is a multiplex PCR–enzyme-linked immunosorbent assay that detects a species-specific genetic element of *S. aureus* and the mecA gene. The running time for the Hyplex StaphyloResist PCR is longer than real-time PCR (3 h 25 min versus 2 h 30 min), although hands-on times for both assays are similar (1 h 30 min and 1 h 15 min, respectively) (M. Michiels, 2007).

The Genotype MRSA Direct assay targets SCCmec types I to V in a multiplex PCR using biotinylated primers followed by a reverse hybridization step. Direct detection of MRSA from nose, throat, groin, axilla, wound, and other sites shows a high sensitivity, specificity, PPV, and NPV (94.59%, 98.73%, 85.37%, and 99.57%, respectively) in comparison to culture. An updated version of the assay, the Genoquick MRSA dipstick assay, does away with the reverse line hybridization step to reduce the total assay time from 4 h to 2 h 20 min (Holfelder, M., 2006)

The IDI-MRSA (also called GeneOhm MRSA) is a multiplex qualitative real-time PCR assay and is an FDA-approved assay for the direct detection of nasal colonization by MRSA. The assay's most recent version (V3) contains primers targeting the right-junction sequences of SCCmec types I, II, III, IVa, IVb, IVc, and V, combined with one consensus primer and three molecular beacons specific for the orfX gene. This assay showed a sensitivity, specificity, PPV, and NPV of 98%, 96%, 77%, and 99.7%, respectively, for direct detection from nasal swabs (Paule, S. M., 2007).

The GeneXpert MRSA is another FDA-approved real-time PCR assay and works on a fully automated GeneXpert platform. The assay proves expensive (US$35 to 55). However, total assay time is short (75 min), and since the assay is fully automated, both the hands-on time (2 min) and the level of expertise required for operation are minimal. A comparison of the GeneXpert and the IDI-MRSA showed similar sensitivities (98.5% and 97.1%, respectively) and specificities (90.4% and 89.2%, respectively) for MRSA detection from nasal samples (Mehta, M, 2007).

4.4. Biosensor-Based Methods

Biosensors have recently been defined as analytical devices incorporating a biological material (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products, etc.), a biologically derived material (e.g., recombinant antibodies, engineered proteins, aptamers, etc.), or a biomimic

(e.g., synthetic catalysts, combinatorial ligands, and imprinted polymers) intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic, or micromechanical. Biosensors are devices for pathogen detection and generally consist of at least three elements, including a biological capture molecule (e.g., probes or antibodies), a method of converting captures molecule-target interactions into a signal, and a data output system. The greatest advantageous aspects of biosensors are those that enable fast or real-time detection, portability, and multi-pathogen detection for both field and laboratory analyses (Lee and Deininger, 2004)

4.4.1. Optical Sensing

A variety of optical techniques such as Raman, infrared, fluorescence, absorption, reflection, biochemical luminescence, refractive index (RI) and surface enhanced Raman spectroscopy (SERS) have been employed in the detection of MRSA (Shen, F. et al, 2011). More recently Liu et al 2013 employed an efficient and versatile nanoparticle based luminescence resonance energy transfer (LRET) system to detect MRSA DNA sequences. The technique was based on the up conversion of nanoparticles (UCNs) and LRET between NaF4: Yb, Er UCNs and carboxytetramethylrhodamine (TAMRA), the energy acceptor. MRSA capture nucleotides were immobilized on the surface of UCNs and released in the vicinity of TAMRA labeled DNA reporter oligonucleotides. There are many types of label free refractive index sensors used to detect MRSA. Bandara et al. recently showed that an optical fiber long period grating conjugated with PBP2a monoclonal antibodies can detect 36 strains of MRSA.

4.4.2. Electrochemical Sensing

Electrochemical sensors can be classified into amperometric, potentiometric, impedimetric and conductometric. Construction and modification of these sensors is inexpensive making them highly compatible with the latest materials. The system was based on the chromoamperomteric detection of MRSA via ferrocene labeled probes conjugated to gold nanoparticles (AuNPs). In addition detecte the mecA gene via a novel electrochemical DNA (e-DNA) based on isothermal strand displacement polymerization reaction (ISDPR). In short, methylene blue (MB) labeled hairpin probes were self-assembled on a gold electrode and hybridized with target DNA which resulted in a change in probe confirmation and movement of MB from the electrode surface. The primers were then annealed with the opened stems of the hairpin probes and extended via DNA polymerase. The process was repeated many times until a substantial reduction in signal current had been observed.

4.4.3. Integrated Sensing Platforms

Integrated microfluidic systems allow for several molecular processing steps to be performed on a single modular platform. By integration with modular microfluidic cartridge with a PCR/ LDR/universal array molecular assay for the rapid and efficient identification of MRSA with high specificity and multiplexing capabilities. SG16S, spa, femA, PVL, and mecA genes were targeted with 5 sets of primers followed by multiplex ligase detection reactions (LDR) which generated fluorescent ligation products appended with a zip code complement directing the ligation product to a particular address on a universal array. Results indicated that the modular system could differentiate CA-MRSA from HA-MRSA based on the presence/absence of the Panton-Valentine Leukocidin (PVL) exotoxin gene as well as from other Staphylococcal species in less than 40 min.

4.5. Mass Spectrometry

Mass spectrometry (MS) methods, including gas chromatography MS (GC-MS), high resolution tandem MS (LC-MS/MS) and liquid chromatography-MS (LC-MS) have recently been exploited for the identification of pathogenic bacteria in clinical samples. Specifically soft ionization techniques such as matrix assisted laser desorption ionization (MALDI-TOF) and electrospray ionization (ESI)-MS have facilitated the analysis of large molecules, whole cells, proteins and DNA. MALDI-TOF is readily adaptable to the direct analysis of bacterial isolates and is capable of returning an identification result in approximately 15-30 s. Potential advantages of ESI compared to MALDI-TOF include the ability to conduct identifications directly from specimens without subculture. A potential drawback of ESI is the requirement for pre analytic steps such as extraction and amplification of nucleic acids increasing turnaround times. One of the major disadvantages of MS techniques is their inability to correctly detect specific strains in polymicrobial cultures.

5. CONCLUSION

During the last decade the developed world has strived to reduce the number of MRSA and multi drug resist SA (MDR-SA) infections in hospital and community based environments by combining automated culturing processes with refined microfluidics devices and digitized PCR assays. Yet even with this level of automation an official or gold standard assay remains out of reach due in part to the hetergenous nature of bacterial cultures. Unfortunately MRSA still accounts for a significant portion of SA infections in the developing world where the need for even faster and cheaper diagnostics tests is felt the strongest. Moreover there is no single standalone rapid assay that can recover all MRSA strains. However, continued improvements in micro patterned paper devices combined with digitized fluidics have led to an increase in the availability of affordable, accurate point of care (POC) tests, which if employed will inevitability led to a reduction in the transmission and spread of multidrug resistant SA in those

regions.

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