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A Review of the Occurrence and Developmental Stages in Laboratory Diagnosis of Methicillin Resistant *Staphylococcus aureus* (MRSA) Infections

			
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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) was discovered in 1961 and has since remained one of the most important multidrug-resistant bacterium globally. The resistance is acquired either through the production of an enzyme *Penicillinase* or a mutational change in the proteins attacked by methicillin which is produced by a chromosomally acquired gene called *mecA*. Early and accurate diagnosis is germane to treatment and control of infections caused by MRSA, hence nations/regions prepared and approved guidelines for the diagnosis of MRSA. Diagnosis of MRSA starts with first identifying the organism to be *S. aureus* through cultural, microscopical and serological methods. MRSA can then be diagnosed through, susceptibility tests, automated methods, quenching fluorescence method, microcalorimetry method and molecular methods e.g PCR. Some other diagnostic methods are specific for strain typing in epidemiological investigations; these include Pulsed-Field gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST) and whole genome sequencing (WGS). It is, however, important to know that whatever method one intend to choose, nationally approved infections guidelines, availability of reagents and equipment, time frame, cost, technicality, sensitivity and specificity must be carefully considered.

INTRODUCTION

Historical Background of MRSA

Plaque supposedly caused by *Staphylococcus aureus* was dated back to early Bible times when after the release of the 6th Plaque on Egypt, men and livestock developed festering eruptions called 'shhin', which when translated means boils and was believed to have been caused by an organism (Richard, 2005) However, the modern day recognition of *S. aureus* stemmed from the work of a Scottish Surgeon called Sir Alexander Ogston in the late 19th Century. In 1881, Alexander named the organism *Staphylococcus pyogenes aureus*, giving consideration to its microscopic morphology, formation of golden colonies on culture medium and involvement in acute suppuration (Reynolds and Tansey, 2008) *Staphylococcus aureus* is a germ many of us carry in our noses, armpits or other humid areas of the body where they cause no disease except when given the opportunity to do so. Infection with *S. aureus* can lead to boils, carbuncles and abscesses, scalded skin syndrome, toxic shock and food poisoning, septicaemia, wound infection and osteomyelitis (Reynolds and Tansey, 2008) Alexander Fleming, in a frustrated study of the properties of staphylococcal variants, accidentally discovered Penicillin from a 'contaminant' (*Penicillium notatum*) which inhibited the growth of *S. aureus*, for which he won a Nobel price in 1945. He noted that no two strains of Staphylococci behaved the same way when exposed to antibiotics and warned that inappropriate use of penicillin might lead to the development of resistance by either mutation or adaptive changes (Richard, 2005) By the 1950s, Flemings prediction came to fulfilment as resistance to penicillin among strains of *S. aureus* became major problem in the hospitals. The mechanism of resistance was through the production of an enzyme – *penicillinase* (β -*lactamase*). The emergence of these resistant groups led to the production of semi-synthetic penicillins (Cephalosporins) that are able to withstand the enzymes (Abraham and Chain, 1940). Methicillin was the first product in this category, which was produced by Beecham Research Laboratories in 1960, closely followed by Cloxacillin, which was better tolerated orally. Just as for Penicillin, within a year of use (1961), resistance to methicillin was detected. The mechanism involved this time was a mutational change in the proteins attacked by methicillin, which allow the bacteria now to avoid the consequences of exposure to the drug. Methicillin is stable to *Staphylococcal* β -*lactamase* producing staphylococci, but MRSA possesses a modified penicillin-binding protein (PBP2a or PBP2) with reduced affinity to methicillin. The modified PBP is synthesized by a chromosomally acquired gene (*mec A*), located on a transposon, which enables spread between Staphylococcal species.

These resistant strains are known as “methicillin- resistant *Staphylococcus aureus*” or MRSA, even though methicillin is no longer marketed and was replaced with more active derivatives of penicillin (Tansey and Reynolds, 2000). Early MRSA isolates were solely associated with hospital environment (HA-MRSA), until 1990 when Community Associated MRSA (CA-MRSA) was reported. CA-MRSA generally differs in genetic background from HA-MRSA because it is associated with SSCmec 1V, V, VII and tends to be resistant to fewer antibiotic classes. However, genetic interchange between MRSA strains makes molecular classification as HA- or CA- complicated (Goss and Muhlebach, 2011; Lindsay, 2010; Lo, 2007)

EPIDEMIOLOGY

Methicillin-resistance *Staphylococcus aureus* (MRSA) is a Gram-positive cocci, shaped bacterium that is often found in the hospital environment where it causes various infections. *S. aureus* became a notable cause of severe morbidity and death especially during the World War 1, where *Staphylococcal pneumonia* was a major cause of morbidity among young healthy military personnel (Chambers, 2001). MRSA is generated when methicillin-susceptible *S. aureus* exogenously acquires a staphylococcal cassette chromosome mec (SCCmec), a methicillin-resistant gene *mecA*, carried by a mobile genetic element, which is transmissible across staphylococcal species (Tsubakishita *et al.*, 2010; Ito *et al.*, 1999) Skin and soft tissue infection which often progress to sepsis was common with MRSA. In pre-antibiotic era, an 82% mortality rate was reported among patients with Staphylococcal infection while in another study, a 2% survival rate was reported (Binh, 2006; Chambers, 2001). The prevalence of MRSA varies from country to country as reported for the year 2008 in USA (23.7%), France (8.5%), Ireland (11.0%), Australia (3.0%), Canada (3.2%), Belgium (8.2%) and United Kingdom (2.7). (Lyczak *et al.*, 2002; Rosenthal *et al.*, 2008; Ren *et al.*, 2007; Sawicki *et al.*, 2009; Muhlebach *et al.*, 2011; Dasenbrook *et al.*, 2008; Sanders *et al.*, 2010)

DIAGNOSIS

Early and accurate diagnosis is key to successful treatment and control of infections caused by MRSA. Many continents and countries have established protocols and procedures for the identification of MRSA, thereby leading to availability of various international guidelines that provide recommendations for best practices in MRSA diagnosis and treatment. A few of the guidelines sources are: Clinical and Laboratory Standards Institute (CLSI) that produced Surveillance of MRSA Principles, Practices and Challenges (CLSI, 2010) Joint Working

Party of the British Society for Antimicrobial Chemotherapy, the Hospital Infection Society and the Infection Control Nurses Association (BSAC/HIS/ICNA) that produced evidenced-based Guidelines for the laboratory diagnosis and susceptibility testing of methicillin-resistant *S. aureus*- MRSA (Andrews, 2008) and Sociedad Espanola de Infectologia Microbiologia clinica (SEIMC) that produced Protocols for Diagnostic Microbiology (Cano *et al.*, 2009) and European Antimicrobial Resistance Surveillance System –EARSS (EARSS, 2005). The choice of which protocol to adopt in a particular situation is affected by many factors ranging from cost, speed of result, facilities available, sensitivity, specificity and manpower. However, identification of MRSA starts with identification of *S. aureus* from clinical and non-clinical samples.

Tests for Identification of MRSA:

a. Cultural methods:

- i. ***Use of Mannitol Salt Agar (MSA):*** Mannitol Salt Agar consist of 7.5- 10% mannitol salt and phenol red as pH indicator. Pathogenic Staphylococci (MRSA and MSSA) grow well in high salt environment and turn MSA to yellow through the release of acid. When supplemented with Cefoxitin, it can be used for MRSA screening.
- ii. ***Blood Agar*** can also be used, where haemolytic ability of *S. aureus* is demonstrated through the production of exotoxin that causes the lysing of sheep red blood cells used in the Blood agar.
- iii. ***Plate Screening:*** this involves the use of Mueller-Hinton agar supplemented with 4% NaCl and 6mg/L of oxacillin. Inoculate with the test organism and incubate at 35⁰C for 2 hrs. Any growth recorded is indicated of resistance (MRSA). This method is cheap and reliable.
- iv. ***Chromogenic agar:*** Chromogenic agar media specific for MRSA which allow rapid detection of colonies through colored reactions are used. Examples are i. *S. aureus* ID (bioMerieux), ii. Chromagar MRSA (Chrom agar) and iii. Chromogenic MRSA agar (Oxoid). This method is appropriate for rapid Screening of MRSA and has a high sensitivity (>98%)
- v. ***Polymixin B and Novobiocin Susceptibility:*** *S. aureus* is resistant to 10U of polymyxin B when plated on agar medium incorporated with it, whereas *S. aureus* is sensitive to 5µg of novobiocin when added to agar plate. Both Polymyxin B and Novobiocin discs are relatively cheap and easy to use, but should only be used as part of a series of diagnostic tests.

vi. **DNase and heat-stable nucleases:** this method is based on detection of specific DNase and heat-stable nucleases common to *S aureus* strains. DNase agar and metachromatic agar diffusion for heat-stable nucleases are commonly used. (CLSI, 2009; CLSI, 2010; Brown *et al.*, 2005; van Griethuysen *et al.*, 2001)

b. **Microscopy:** Staphylococci spp are stained purple (Gram +ve), that is, they take up the color of the primary stain (Crystal violet) without decolourisations with an alcohol when Gram's staining technique is adopted. Microscopically, they appear roundish (cocci) and usually in clusters. This is done with many other tests as Gram's staining reaction is not specific for *S aureus* but helps to classify it as Gram-positive organism.

c. Serological methods:

i. **Catalase test:** Catalase is produced abundantly by *S. aureus* which reacts with 3% hydrogen peroxide produces oxygen which bubbles almost immediately. This method is rapid and cheap, but cannot be performed on colonies grown on Blood agar because blood too, contains catalase which can lead to false positive result.

ii. **Coagulase test:** Coagulase, an enzyme which could be produced bound or free, converts fibrinogen to insoluble fibrin in the presence of plasma, resulting in clotting. The tube test is used to detect free coagulase while the slide test detects cell-bound coagulase. The test distinguishes pathogenic Staphylococci (MRSA, MSSA) from non-pathogenic strains. It is known that some Coagulase negative Staphylococci (CoNS) now contain a *mecA* gene or its gene product- PBP2a which may be easily spread to other CoNS and thereby classified as Methicillin Resistant Coagulase Negative Staphylococci (Mayhall, 2004; Bergy, 1984)

iii. **Latex agglutination test:** the principle is based on the presence of protein (protein A, clumping factor group specific antigen, capsular polysaccharides) recognized by specific latex spheres, resulting in aggregation. Commercially available latex agglutination kit specific for *S. aureus* include Staphaurex and Staphaurex Plus produced by Wellcome Diagnostics, UK. (Ojo, 1993; Bergy, 1984)

d. MRSA Susceptibility Tests:

i. **Disc diffusion (Kirby –Bauer) methods:** Cefoxitin or oxacillin discs are placed on the surface of Mueller-Hinton or Columbia agar (supplemented with 2% NaCl) already inoculated with the test organism. This is incubated at 30 – 35⁰C for 24hrs after which zones

of inhibition is seen around the discs. Cefoxitin gives a more reliable result than oxacillin because some hyper-producers of *penicillinase* give no zone of inhibition and will be incorrectly reported as MRSA (Bauer *et al.*, 1966; Cheesbrough, 2006)

ii. Minimum Inhibitory Concentration determination: This is done by assessing growth of MRSA in difference dilutions of cefoxitin or oxacillin in broth or agar to reveal the Minimum Inhibitory Concentration. The method is relatively cheap but more cumbersome than disc diffusion, E-test or agglutination methods (Bauer *et al.*, 1966; Cheesbrough, 2006)

iii. E-test: This method is based on the use plastic strips containing a predefined gradient of 15 antibiotic concentrations. Inoculum concentration of 0.5–1.0 McFarland standard is seeded on Mueller-Hinton agar plate, incubate at 35⁰C for 24 hrs after which growth is assessed along gradient to determine MIC. The method is easy to use but expensive for routine work (Tokue *et al.*, 1992)

e. Automated methods: There are various cartridges produced which are used for the diagnosis of *S. aureus*. These products include Vitek/ Vitek 2 (bio Merieux) Phonix (Becton Dickinson) and Microscan (Dade Behring). The method is simple and fast, however, few false-positive results have been reported (Louie *et al.*, 2000; Jureen *et al.*, 2001)

f. Quenching fluorescence method: This method is reliable but requires several hours of incubation. It involves the use of Crystal MRSA (Becton Dickinson). Inhibition of growth of an isolate by oxacillin is indicated by the quenching of fluorescence of an oxygen-sensitive fluorescent indicator by oxygen remaining in the broth (Knapp *et al.*, 1994; Zambardi *et al.*, 1996; Smyth *et al.*, 2001)

g. Microcalorimetry method: This is a highly sensitive, rapid and useful technique that allows the measurement of heat generated by biological processes in the living cell. The principle is based on the replication of microorganisms in an appropriate culture medium, resulting in an exponential increase of heat that can be recorded in real-time (i.e. heat – flow curve). Bacterial inocula for the calorimetry samples were prepared from discrete colonies of *S. aureus* freshly grown on Columbia 5% sheep blood agar and resuspended in 0.85% sterile saline to a McFarland turbidity of 5. This high turbidity is to allow early heat measurement and to increase the chances of including methicillin-resistant colonies when testing strains heterogeneous in the expression of resistance. A 48-channel batch calorimeter (thermal activity monitor) was used to measure the heat flow at 37⁰C. Heat was measured

continuously in 4-ml glass ampoules and expressed as heat flow over time in watts (W). Total heat was determined by the integration of the area under the heat flow-time curve. Data analysis was accomplished using the manufacturer's software and prism 4.0a (Baldoni *et al.*, 2009; Brown *et al.*, 2005; Niemeyer *et al.*, 1996; Spink and Wadso, 1976)

h. Molecular Methods: The principle is based on the fact that MRSA contains species-specific genes such as nuclease (nuc) coagulase (coa) protein A (spa) Fem A and Fem B, 16 RNA fibrinogen-binding protein genes and mecA gene, which encodes penicillin-binding protein 2a (PBP2a). Methods for assessing the presence of these specific genes vary from PCR (Simple, Multiplex and Real-time), DNA sequencing and Hybridization-based techniques. All molecular identification processes are preceded by DNA extraction. The method is now simplified with the availability of many extraction kits (QIA amp DNA Mini Kit) that give improved yield. This step (extraction) is very important because if the product is contaminated, the integrity of the whole process would have been compromised (Vautor *et al.*, 2005 Makgotlho *et al.*, 2009)

i. Southern Blot Hybridization of DNA: The bacterial genome DNA will be digested with Hind III, electrophoresed on a 1.0% agarose gel, and transferred to a nitrocellulose membrane by the method of Southern (Southern, 1975, Tokue *et al.*, 1992)

ii. Polymerase Chain Reaction: The DNA extract (1µg) will be amplified by PCR in 100µl of a reaction mixture containing 200µM (each) deoxynucleoside triphosphates, 1µM (each) primers, 2.5 U of Taq DNA polymerase, 50Mm KCL, 10Mm Tris-HCl (Ph 8.3), 1.5 Mm MgCl₂ and 0.01% gelatine. The procedural steps are as follows: denaturation, 30s at 94⁰C; annealing, 30s at 55⁰C; primer extension, 2 min at 72⁰C. Each step will be repeated 25 times. Ten microliters of the reaction mixture will be loaded to 1.0% agarose gel with ethidium bromide. The band of amplified DNA will be visualized under UV light (Tokue *et al.*, 1992; Tokue *et al.*, 1991)

iii. Restriction Fragment Length Polymorphism (RFLP): This is done on the coagulase (coa) and the clumping factor B (cl +B) genes. Amplification of the genes (cao and cl+B) will be done by PCR and the products will be digested by restriction enzymes, analyzed on 2.0% agarose gels with ethidium bromide and observed under UV light (Vautor *et al.*, 2005)

i. **Epidemiological typing:** These are methods commonly employed for establishment of relationship (indistinguishable, closely related, possibly related and different strains) in epidemiological study of outbreaks.

a. **Pulsed-Field Gel Electrophoresis (PFGE):** This is a macro construction analysis of the chromosomal DNA from bacterial culture was performed with the restriction enzyme *SmaI* before testing, using the CHEF-DR III Pulsed Field Electrophoresis System as described by Vautor *et al.*, 2003. On each gel, 2 control plugs with the reference strain (CIP 57.10^b) were included (Vautor *et al.*, 2003; Tenover *et al.*, 1995)

b. **Multilocus Sequence Typing (MLST).** The Staphylococcus DNA array identifies sequence variation in 7 MLST targets (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *ygiL*) using the 4L tiling strategy. Six hundred microlitre (600µl) of a 0.5 MacFarland suspension, obtained from an overnight agar plate culture, will be added to a mixture of different diameter beads and vortexing was performed at maximum speed for 2 minutes. The supernatant will be directly used as target for PCR reactions. Probe arrays will be hybridized using a Gene Chip fluidics station. Biotin-labeled and fragmented DNA will be denatured, hybridized with the probe array, washed and the hybrids will be stained with streptavidin-R-phycoerythrin. The probe array will be washed again. The fluorescent signals emitted by hybrids will be detected at 570nm (phycoerythrin) using a GeneArray Scanner. Probe array fluorescence intensities, base call scores, sequence determination and reports will be generated by the Gene Chip Software. The percentage base-right score will be determined by the percentage homology between the experimentally derived sequence and the distinct reference sequence tiled on the array (Vautor *et al.*, 2005; van Leeuwen *et al.*, 2003; Brown, 2015)

c. **Bacterial Whole Genome Sequencing (BWGS):** This is the most reliable strain typing approach for investigation of suspected clinical outbreaks. The method involves DNA purification using an UltraClean microbial DNA isolation kit. Genomic DNA (30 to 100ng) will be digested for 60 to 90 min at 37⁰C in a 10µl volume using 0.3µl NEBNext double-stranded DNA (dsDNA) Fragmentase. DNA will be end repaired and A-tailed in a 40µl reaction mixture containing 1x Rapid ligation buffer, 0.1675 Mm (each) deoxynucleoside triphosphate (dNTP), 0.1ul *Escherichia coli* DNA polymerase I, 0.5µl T4 polynucleotide Kinase and 0.02µl Taq DNA polymerase and was incubated at 37⁰C for 30 min and 72⁰C for 20 min. Annealed Y-adaptors (0.2um) will be added and ligated at 25⁰C for 20min using T4 DNA ligase in Rapid ligation buffer. Following purification with Agencourt AMPure XP

beads, PCR amplification will be performed with KAPA HiFi HotStart ReadyMix using primer PRECAP.FWD.AMP.COMMON and sample-specific indexed primers. Cycling conditions are 95⁰C for 3 min, 10 cycles of 98⁰C for 20s, 65⁰C for 15s and 72⁰C for 1 min, and 1 cycle of 72⁰C for 5 min. PCR Products were purified using AMPure beads, pooled in equimolar amounts and sequenced on a MiSeq Sequencer using 150-bp paired-end reads with a custom index primer. The sequence reads will be aligned to completed reference genomes in GenBank using bwa and samtools (Salipante *et al.*, 2015; SenGutpta, *et al.*, 2014)

CONCLUSION

The laboratory diagnosis of MRSA both at Hospital and Community setups has been reviewed. Starting with simple and cheaper methods like culture and serology to more specific but expensive molecular methods. Methods for epidemiological studies have also been highlighted in this review. Some of the methods are labour-intensive, time-consuming and technically challenging while others are cheap and fast, ideal for routine screening. It should be noted that each method has its strengths and weaknesses.

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