



Review Article

Mechanism of Resistance, Phenotyping and Genotyping of Methicillin Resistant *Staphylococcus aureus*: A Review

G.Jyothi Lakshmi*

Gandhi Medical College, Hyderabad-5000, India

*Corresponding author

ABSTRACT

Keywords

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Staphylococcus aureus is implicated in a variety of infections ranging from skin and soft tissue infections to infections of bone, pneumonia and endocarditis. Both community acquired and hospital acquired infections with *Staphylococcus aureus* have increased in the past two decade and this rise in incidence has been accompanied by a rise in antibiotic resistant strains, in particular Methicillin resistant *Staphylococcus aureus* (MRSA). Various mechanisms of resistance have been described. Typing of MRSA is essential to understand epidemiological trends and to initiate infection control strategies. Phenotypic methods are easy to perform and interpret, cost effective but less discriminatory. Genotypic methods have the advantage of being more discriminatory but are expensive and technically demanding. However there is no consensus on the single best typing method. Phage typing is preferred for epidemiological investigation. Pulsed Field Gel Electrophoresis (PFGE) remains the gold standard for characterization of strains. DNA sequencing methods require technical expertise and increased cost. Therefore the method used would depend on the purpose of study, facilities and technical expertise available.

Introduction

Staphylococcus aureus causes a variety of suppurative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as boils, styes and furuncles; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices.

Although methicillin-resistant *Staphylococcus aureus* (MRSA) have been entrenched in hospital settings for several decades, MRSA strains have recently emerged outside the hospital becoming known as community associated- MRSA (CA-MRSA) or superbug strains of the organism, which now account for the majority of staphylococcal infections. (Todar, 2012)

Methicillin Resistant strains of *Staphylococcus aureus* were first

recognized in 1961 one year after the antibiotic was used for treatment of *Staphylococcus aureus* infections. MRSA are resistant to all betalactum antibiotics. This includes all penicillins and cephalosporins- which become a challenge for treatment.

This review is done with an aim to discuss the different mechanisms of resistance, the techniques available presently for typing of MRSA isolates, the application of these methods in discriminating of strains and their use in epidemiological investigation as well as infection control practices.

Mechanism of resistance

Methicillin resistant strains typically carry the *mecA* gene that encodes for a low affinity penicillin binding protein (PBP) designated PBP2a. In most strains, *mecA* gene is a part of a chromosomally intergrated mobile genetic element called Staphylococcal Cassette Chromosome *mec* (SCC*mec*). This PBP2a has peptidoglycan transpeptidase activity, but low affinity for beta lactum antibiotics. PBP2a exhibits a reduced rate constant for acylation by beta lactams and elevated dissociation constants. These two factors acting together prevent acylation of PBP2a and this results in betalactamase resistance. (Fuda C, 2004)

Homogenous strains- of MRSA and penicillinase negative strains produce PBP2a constitutively. It has been suggested that, the MRSA PBP is a hybrid resulting from recombination between the inducible beta lactamase gene and a normal PBP gene. Hence MRSA PBP production is induced by the same mechanism that induces beta lactamase production in these strains (Boyce, 1987).

Autolytic activity – another factor mediating methicillin resistance is regulation

of autolytic activity. In Methicillin sensitive strains, autolysis is triggered by the inhibition of peptidoglycan cross linking as occurs with beta lactams.(Bruns W, 1987.)

Heteroresistance – in heteroresistant strains, all cells in the test population have the genetic elements (*mec A* gene) for oxacillin resistance, but not all cells express this resistance. The mechanism of heteroresistance in *S.aureus* is believed to involve the interaction of PBP2a and various gene products such as those encoded by *fem* (factor essential for Methicillin resistance) genes that are involved in cell wall peptidoglycan synthesis. (Maranan,1997.).

Acquired or Borderline Resistant *Staphylococcus aureus* (BORSA).- Mc Dougal and Thornsberry in 1984 described a mechanism of reduced susceptibility to Methicillin due to hyperproduction of normal Staphylococcal penicillinases (McDougal L.K.1986). Support for this hypothesis comes from the absence of PBP2a in their cell walls and from the observation that clavulanic acid and sulbactam lowers the MIC of oxacillin several fold (Montanari MP, 1990, Sierra-Madero JG, 1988, Chambers HF 1989.). The difference between the two mechanisms is resistance due to PBP2a is chromosomally mediated, while hyperproduction of beta lactamases in BORSA is plasmid mediated.

Methicillin Intermediate *Staphylococcus aureus* (MODSA)- Tomasz and colleagues suggested a third mechanism of reduced susceptibility to Methicillin, wherein strains with Methicillin MIC 's of 4µg/ml and oxacillin MIC's of 1µg/ml to 2µg/ml possess "normal" PBP's of modified affinity, but not the unique PBP2a. They were designated MODSA for modified PBP's. These strains produce PBP's 1 and 2 of normal molecular size, but with low affinity for beta lactum antibiotics,

MODSA, in contrast to intrinsically resistant MRSA, which contains PBP2a of different molecular size, produce Staphylococcal PBP's of typical molecular size but with modified drug reactivity.(Tomasz A, 1989).

Thus presently there are two mechanisms to describe *S.aureus* strains with borderline resistance or diminished susceptibility to semisynthetic penicillins BORSA and MODSA. Both these groups are genetically distinct from MRSA.

Detection and typing of MRSA

Methicillin Resistant *Staphylococcus aureus* (MRSA) is a cause of nosocomial infections all over the world. The use of efficient and accurate epidemiological typing methods is a prerequisite for monitoring and for limiting the occurrence and spread of epidemic clones within and between hospitals. Typing of *S.aureus* mostly relied on phenotyping strain characteristics (susceptibility to phage or antibiotics) but over the past 2 decades a variety of molecular technologies have been developed (Stommenger B, 2008).

Phenotypic methods

ANTIBIOGRAM- AntibioGram typing involves comparison of susceptibilities of isolates to a range of antibiotics. Isolates which differ in their susceptibilities to antibiotics are considered as different strains. This technique is easy to perform, inexpensive, gives rapid results and is available in all microbiology laboratories.

Cefoxitin Disc Diffusion – A 0.5 Mc Farland standard suspension of the isolate is made and lawn culture done on MHA plate. A 30µg cefoxitin disc is placed and plates incubated at 37°C for 18 hours and zone diameter measured. A zone of >20mm is

interpreted as sensitive and <19mm is considered as resistant.(Mimica MJ, 2007)

Oxacillin Agar Screen -6 g/ml oxacillin is incorporated into muller hinton agar plates containing 4% NaCl. A 0.5 Mc Farland suspension of the test organism is streaked in one quadrant and incubated at 35 C for 24 hours. Any growth after 24 hours is considered oxacillin resistant. (Mimica MJ 2007)

Broth Microdilution – Two fold dilutions of Methicillin or oxacillin are incorporated into Muller Hinton broth with 2% NaCl. An inoculum density of 5×10^5 cfu/ml and incubation at 33-35°C for 24 hours is required.

E Test For MIC of Oxacillin – Muller Hinton plates supplemented with 2% NaCl are used. The inoculum is standardized to 0.5 McFarland turbidity. E test strips are placed and incubated at 35°C for 24 hours.

MRSA Screen Latex agglutination test – Nakatomi and Sugiyama developed a simple and rapid slide agglutination assay to detect penicillin binding protein (PBP2a) from isolates of Staphylococci. The tool contains latex particles sensitized with a monoclonal antibody against PBP2a (Nakatomi Y,1998)

Biotyping – Coia et al proposed a biotyping scheme based on biochemical and metabolic properties using Tween-80 hydrolysis, production of urease, production of pigment on Tween 80 agar and Gentamicin resistance (Krishna Prakash S, 1993. Coia JE, 1990).

Phage Typing - Staphylococcus strains contain temperate phages, which lyse other bacteria of the same species. Phages that have a narrow host range are used in groups to produce a pattern of lysis characteristic of the individual strain. Phage typing technique

was standardized by the international subcommittee on phage typing of Staphylococci. Strains are classified according to their susceptibility to a set of phages. An internationally accepted set of 23 phages are used for typing *Staphylococcus aureus* (Blair JE, 1961).

Serotyping - Serotyping of *Staphylococcus aureus* detects differences in the antigenic properties of the the capsular polysaccharide, membrane proteins and lipopolysaccharides. (Schlichting C, 1993.). Serotyping is performed using different serological tests like bacterial agglutination, latex agglutination, co-agglutination and enzyme labeling assays.

Protein Electrophoretic Typing- Various electrophoretic methods such as whole cell protein typing, immunoblotting, multilocusenzyme electrophoresis (MLEE) and zymotyping have been used for typing of MRSA strains.

Whole cell protein –Proteins are extracted from the culture of a strain, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained to compare with those of other strains. However this test has poor discriminatory power.

Imunoblotting – in immunoblotting the electrophoresed products of SDS-PAGE are transferred to nitrocellulose membrane and then exposed to antisera raised against specific strains. The bound antibodies are then detected by enzyme labeled anti-immunoglobulins.

Multilocus enzyme electrophoresis(MLEE)- MLEE involves extraction of enzymes from the strain, their separation by electrophoresis and examination by selective staining. Enzyme migration depends on its amino

acid composition. Typability and reproducibility are good for this method.

Zymotyping – zymotyping is based on the electrophoretic properties of esterase enzymes . *S. aureus* possess three esterases designated A,B and C. Results are analysed according to differences observed in the mobility of esterases in different strains.(Schlichting C, 1993.)

CHROMOGENIC AGAR - chromogenic selective media, which utilize a chromogenic enzymatic substrate and a selection of antibiotics, have also been introduced commercially for the detection of MRSA in clinical specimen. The commercially available media include-MRSA select, CHROMagar MRSA, MRSA ID, Spectra MRSA, HiCrome MeReSa agar, Chromogenic MRSA, ORSAB (Oxacillin resistance screening agar base), Chrom ID. (John D. Perry, 2003)

Genotypic methods

Plasmid analysis –The first molecular technique used for epidemiological investigation of MRSA was plasmid analysis. The isolates are differentiated according to the number and sizes of plasmids carried by an isolate. Reproducibility of this method is difficult due to plasmids existing in different forms such as supercoiled, nicked or linear, each of which migrates differently on electrophoresis.(Gaston MA, 1988.)

Restriction enzyme analysis (REA) of chromosomal DNA- Chromosomal DNA is too large for complete analysis and must be cut into smaller pieces using restriction enzymes which recognize and cleave specific sequences. The number and size of these restriction fragments generated depends on the recognition sequence of the

enzyme and composition of DNA. These fragments are separated according to their size on agarose gel electrophoresis. The patterns are stained by ethidium bromide and examined under UV light. All strains of MRSA can be typed with good reproducibility.(Jordens JZ, 1988.)

SOUTHERN BLOT ANALYSIS –in Southern blot analysis, the restriction fragments generated by the digestion of DNA by endonucleases, separated by gel electrophoresis and are transferred onto nitrocellulose membrane. The fragments containing specific sequences are detected by labeled DNA probes. Variations in number and sizes of the fragments detected are referred to as restriction fragments length polymorphism (RFLP) and make the basis of discrimination strains. (John V Hookey, 1998.)

RIBOTYPING –Ribotyping is used for MRSA typing with some variation in the restriction enzyme and type of probe employed. The probes generally used are either labeled with radioisotope or biotinylated.(Prevost G, 1992.)

BINARY TYPING –This is a modification of southern type hybridization. It is based on detecting the presence or absence of a combination of genetic loci by PCR. Amplified products can be detected by various methods including gel electrophoresis or real time PCR. Clinically diverse strains can be genotyped by means of binary typing using strain specific DNA probes.(van Leeuwen W, 1999.)

PULSED FIELD GEL ELECTROPHORESIS –This method uses enzymes which produce fewer large fragments of the chromosome. These segments do not separate easily by conventional electrophoresis. In PFGE the direction of the charge is kept constant for

short pulses of time which causes the DNA fragments to stop and reorient to a new course. Discriminatory ability is better when compared to antibiogram, phage typing, ribotyping and zymotyping. PFGE has been recommended as a ‘gold standard’ for typing of MRSA strains.

PCR based typing

PROTEIN A GENE TYPING – Based on heterogenicity within a specific fragment of the coagulase gene, PCR is used to amplify this region. Genes for protein A(*spa*) is used for typing, *spa* typing combined with BURP (based upon repeat pattern) grouping analysis is a frontline tool in epidemiological typing of *Staphylococcus aureus*. (Stommenger B, 2008).

AP-PCR/RAPD – Arbitrarily primer PCR or Random Amplified Polymorphic DNA is based on the assumption that there will always be some DNA sufficiently similar to the primers, for them to anneal in random fashion. It involves random amplification of segments of target DNA using small primers of arbitrarily sequences of nucleotides of unknown homology with a target sequence. The technique is simple and rapid and useful in studying outbreak strains but not suitable as reference method for typing MRSA.(Mehndiratta PL,2012.)

Rep- PCR – repetitive element sequence based, PCR is a method to sample the whole chromosome. Primers are used which hybridise to sequences known to be repeated throughout the chromosome but with variable number and position. Where the DNA between these binding sites is amplified the length polymorphism produces a fingerprint.

PCR-RFLP – relies on the amplification of a defined fragment of DNA and subsequent

digestion of the amplified product with a restriction enzyme to generate restriction fragment length polymorphism.(Mehndiratta PL,2009.)

DNA sequence analysis based typing

MULTILOCUS SEQUENCE TYPING (MLST)- MLST has been reported to be useful for studying clonal evolution of MRSA. It is the genotypic descendant of MLEE and is based on sequence analysis of several housekeeping genes. Seven housekeeping genes have been studied *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*. The different sequences of each housekeeping gene are assigned as distinct alleles and each MRSA strain is defined by the alleles of the seven genes. MLST has been used in conjunction with PCR analysis of Staphylococcal Cassette Chromosome *mec* (*SCCmec*) element to define the clonal type of MRSA strains.(Enright MC, 2000.).

SINGLE LOCUS SEQUENCE TYPING (SLST) –SLST is used to compare sequence variation of a single target gene. The genes selected are usually of short sequence repeat (SSR) regions that are sufficiently polymorphic to provide useful resolution. Genes for protein A (*spa*) and coagulase (*coa*) in MRSA strains having 24bp and 81bp tandem repeats have been studied extensively and it has been reported that MRSA strains can be discriminated by determining the repeat sequence numbers within the X region of *spa* gene.(Frenay HM, 1996.).

STAPHYLOCOCCAL CASSETTE CHROMOSOME *mec* TYPING.-*Staphylococcus aureus* acquires methicillin resistance through mobile genetic elements Staphylococcal Cassette Chromosome *mec* that contains the *mec* A gene complex and *ccr* gene complex. Several *mec* and *ccr*

allotypes have been found among *SCCmec* element. Currently , eight main types of *SCCmec* (type I to type VIII) along with many subtypes have been distinguished among MRSA strains.(ref 42). Studies have found that healthcare associated MRSA (HA-MRSA) strains contain mainly type I, type II and typeIII *SCCmec* cassettes while community acquired (CA-MRSA) strains contain type IV and type V cassettes.(Arakere G, 2005.)

TOXIN GENE PROFILE TYPING –MRSA strains produce various toxins including toxic shock syndrome toxin (*tsstI*), enterotoxins and exfoliative toxins. Genes encoding for enterotoxins are carried on Staphylococcal pathogenicity islands. Other toxin genes like gene for Panton Valentine Leucocidin (PVL) are carried on bacteriophages and are easily transferred between lineages. Thus toxin gene profile of the strains can be used as an important epidemiological marker for typing of MRSA strains.Studies on toxin gene profile of MRSA have been reported that most of the CA-MRSA possess genes for PVL toxins and may have evolved from the established CA-MSSA (community acquired methicillin sensitive) strains (Cai Yongwe, 2007).

Recently reported methods include (Kumar VA, 2012.)

SmaI RESTRICTION SITE- BASED MULTIPLEX PCR (SmaI- multiplex PCR) typing(SMT) has been evaluated to investigate outbreaks and was found to be simple, reproducible and highly discriminatory without the need of expensive equipment or specialist expertise and has the potential to be used in routine clinical microbiology laboratories (Al-Zaharani IA,2011).

DHPLC -a novel application using denaturing HPLC (DHPLC) utilising the variability within the Staphylococcal protein A, or *spa* A, gene repeat region as a marker of short and long term , genetic variation for rapid, inexpensive characterization of *spa* gene amplification products, without the need for DNA sequence determination has been described.(Jury F, 2006.).

MICROARRAY – the isolates are grown overnight, harvested and enzymatically digested prior to DNA purification. Then a linear primer elongation reaction is used to simultaneously label and amplify 333 target sequences corresponding to about 170 genes and their alleles. These include resistance markers, SCC *mec* associated genes encoding virulence and adhesion factors. Resulting single stranded, biotin labeled DNA amplicons are hybridized to arrays with spotted specific oligonucleotides . Hybridisations are visualized by adding a streptavidin- horse radish peroxidase conjugate that triggers in a later step a local precipitation of a dye. Arrays are scanned and normalized intensities or spots determined based on their average intensities and on the local background. Results are regarded as negative if the normalized intensity for a given probe was below 25% of the median of predefined species markers and a staining control. If the normalized intensity for a given probe was higher than 50% of this median, it was interpreted as positive. Values between 25% and 50% were regarded as ‘ambiguous’ indicating technical issues, or for some genes a presence of low copy number plasmids (eg *aaaA –aphD*)as well as cross reactions with other alleles of the same gene or with closely related genes. (Monecke S, 2014. Monecke S, 2008.) Phenotypic methods are easy to perform and do not require the technical expertise that genotypic methods demand. They can be

performed in resource poor setting also. Macrorestriction analysis by pulsed field gel electrophoresis (PFGE), distinguishes highly related strains and would therefore be suitable for an outbreak investigation. However it is difficult to standardize and is more time consuming than PCR based methods, since it requires a culture of bacteria. Multi locus sequence typing (MLST), a PCR based method has been proven to be reliable and reproducible. The choice of typing method will depend on the requirement, available resources and expertise of the laboratory.

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