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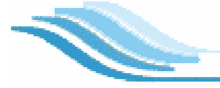

Methicillin Resistant Staphylococcus aureus (MRSA)

Staphylococcus aureus is responsible for a broad range of clinical infections, most notable of which are cases of bacteremia and endocarditis. Staphylococcus aureus is an important cause of serious infections in both hospitals and the community. Methicillin-resistant Staphylococcus aureus (MRSA) were first reported in 1961 and the first hospital outbreak of MRSA was reported in 1963. When MRSA strains first appeared, they occurred predominantly in the healthcare setting. Cases of community-associated MRSA (CA-MRSA) infections were first reported in the late 1980s and early 1990s. Health care-associated MRSA (HA-MRSA) is particularly efficient at developing resistance to antimicrobial agents. Methicillin resistance among staphylococci has steadily increased worldwide, especially among cases acquired in hospitals. It is associated with longer hospital stay and more infections in intensive care units and leads to more antibiotic administration. Asymptomatically colonized patients and health care workers are the major sources of methicillin-resistant Staphylococcus aureus (MRSA) in the hospital environment. MRSA-infected patients in burns units are particularly problematic because the big surface area of denuded skin can produce a large inoculum of organisms that can be easily transmitted to other patients via the hands of health care workers. Extensive skin lesions also result in heavy shedders of MRSA. The commonest site of MRSA carriage is the anterior nares. A significant risk factor for acquisition of MRSA is the duration of hospital stay. Prolonged stay in the hospital is likely with patients in orthopedics and dermatology wards, which may result in high rates of carriage observed in these patients. HACO (Health care-associated MRSA with a community onset) refers to community onset for a person associated with a hospital environment, e.g., a person living in a residential home, a health care worker, a dialysis patient, or an individual with a history of hospitalization within the previous year. Risk factors associated with MRSA bacteremia include the following: residence in an extended-care facility, prior antibiotic exposure, insulin dependent diabetes, prolonged hospitalization, urinary catheterization, nasogastric tube placement, prior surgery, and having an underlying disease. The elderly population (≥65 years old) is at a significantly higher risk of death due to MRSA bacteremia than are younger populations. MRSA bacteremia has been associated with an increased risk of acute renal failure, longer hospital and intensive-care-unit stays, development of ventilator dependency, and increased hospital costs. Fatality rates for patients that develop MRSA bacteremia are estimated to be between 23% and 54%. Nosocomial MRSA is remarkable for its clonal pattern of spread. Currently, 5 major MRSA clones account for approximately 70% of MRSA isolates in hospitals in the United States, South America, and Europe.

Brief timeline:

1940 Penicillin introduced
1942 Penicillin-resistant Staphylococcus aureus appears
1959 Methicillin introduced; most S aureus strains in both hospital and community settings are penicillin resistant
1961 Methicillin-resistant S aureus appears
1963 First hospital outbreak of methicillin-resistant S aureus
1996 Vancomycin-resistant S aureus (VRSA) reported in Japan

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Methicillin:

Meticillin or methicillin is a narrow spectrum beta-lactam antibiotic of the penicillin class developed in 1959 that was previously used to treat infections caused by beta-lactamase-producing Staphylococcus aureus. Methicillin is no longer manufactured because the more stable and similar penicillins such as oxacillin, flucloxacillin and dicloxacillin are used medically. The presence of the ortho-dimethoxyphenyl group directly attached to the side chain carbonyl group of the penicillin nucleus facilitates the ß-lactamase resistance. Methicillin has recently been renamed meticillin to comply with European law, which requires the use of the recommended international non-proprietary name (rINN). International convention has now renamed this agent as meticillin.

Mechanism of resistance:

Penicillin-resistant strains of S. aureus appeared as early as the 1940s, but for many years these remained susceptible to ß-lactamase-stable penicillins. Then, in the mid-1980s, S. aureus strains emerged that were resistant to the ß-lactamase-stable penicillins. These strains were termed "methicillin resistant S. aureus" (MRSA), because methicillin was initially used to detect their resistance to ß-lactamase-stable penicillins (oxacillin, methicillin, nafcillin). Even though the drug methicillin is no longer the agent of choice for treatment, the acronym MRSA continues to be used. Later use of oxacillin as an alternative to methicillin in susceptibility tests resulted in the term 'oxacillin-resistant S. aureus' (ORSA). These designations are used interchangeably in the literature and are synonymous. In S. aureus, resistance to penicillins occurs through 2 mechanisms: the production of the ß-lactamase enzyme and the presence of the *mec*A gene. Majority of S. aureus strains today produce ß-lactamase and are thus resistant to penicillin. Some of these strains produce excessive amounts of ß-lactamase, which makes them appear borderline resistant to oxacillin. These strains are termed borderline oxacillin-resistant S. aureus (BORSA), and they can be difficult to differentiate from classic MRSA. Methicillin-resistant isolates with alterations to existing PBPs have been described. These isolates have been termed 'moderately resistant S. aureus' (MODSA). They are not frequently reported, the resistance is low-level and their clinical significance is unclear.

Methicillin resistance in S. aureus is primarily mediated by the *mec*A gene, which codes for the modified penicillin-binding protein 2a (PBP 2a or PBP 2'). PBP2a is located in the bacterial cell wall and has a low binding affinity for ß-lactams. Although all cells in a population of S.aureus may carry the *mec*A gene, often only a few of the cells will express the gene. Thus, both resistant and nonresistant bacteria can exist in the same culture. *mec*A expression can be constitutive or inducible. *mec*A is carried on a mobile genetic element, the staphylococcal cassette chromosome *mec* (*SCCmec*), and at least five types of *SCCmec* elements have been reported. Characteristics shared by all *SCCmec* elements are carriage of the *mec* and *ccr* (cassette chromosome recombinases) gene complexes and integration in the S. aureus genome at the 3' end of an open reading frame (*orf*X) with an unknown function. Four and five types of *mec* and *ccr* genes have been identified, respectively, and *SCCmec* elements can be differentiated based on various combinations of these alleles.

MRSA infections in patients without any established risk factors too is on the rise in the community where skin and soft-tissue infection, necrotizing fasciitis and serious necrotizing pneumonia have been reported to cause epidemics. These infections have been attributed to dissemination of genetically distinct clonal strains, which contain Panton-Valentine leukocidin (*luk*S-PV and *luk*F-PV) and possesses different staphylococcal cassette chromosome *mec* (*SCCmec*) genetic elements (type IV a–d and type V). *SCCmec* types I to IV are annotated

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primarily by using the multiplex PCR. Isolates that are nontypeable (NT) by this method are further investigated by PCR to assess the *ccr*A, *ccr*B, and *ccr*C recombinase genes and the mec class or by multiplex PCR to distinguish *SCCmec* subtypes IVa to IVh. These strains tend to be more susceptible to antimicrobial agents compared with the *SCCmec* types I–III. On the other hand, HA-MRSA strains mainly harbor *SCCmec* types I, II, and III and in contrast to CA-MRSA strains tend to be multidrug resistant with hallmark resistance to fluoroquinolones.

Detection of resistance:

Several organizations have recommended that patients be screened upon admission to hospitals and persons identified as colonizers are placed on contact isolation. Nasal colonization has been shown to be predictive of the likelihood of patients to develop an infection. Active surveillance cultures from patients for carriage of MRSA facilitate an early contact isolation (and even treatment), thus preventing spread in the hospital and reducing costs. The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by S. aureus.

Strains that possess *mec*A gene are either heterogeneous or homogeneous in their expression of resistance. The heterogeneous expression occasionally results in minimal inhibitory concentrations that appear to be borderline and consequently the isolates may be interpreted as susceptible. MRSA strains expressing heterogeneous resistance (a predominantly low-level-resistance population coexisting with a small proportion of highly resistant cells) are often mistaken for methicillin-sensitive S. aureus (MSSA) by conventional culture and represent a hidden reservoir in hospitals. The presence of both resistant and nonresistant bacteria, along with the fact that the resistant bacteria often grow more slowly, can make it difficult to detect methicillin resistance. Their detection using methicillin and oxacillin is aided by following changes:

- Neutral pH,
- Incubation temperature of 33ºC-35ºC,
- Mueller-Hinton agar or broth infused with 2%-4% NaCl , and
- 24-hour incubation time.

Several selective media have been used for screening MRSA, such as mannitol salt agar with oxacillin, Oxacillin blood agar and CHROM agar. Occasionally, heteroresistant *mec*A-positive strain is not detected due to low expression of resistance. Oxacillin agar screen generally does not detect borderline resistant strains.

Phenotypic detection systems:

Phenotypic expression of resistance can vary depending on the growth conditions (e.g. temperature, osmolarity and culture medium supplements such as NaCl or sucrose), making susceptibility testing by standard microbiological methods potentially difficult.

Agar dilution test:

A minimum of four to five colonies isolated from an overnight growth are transferred to sterile saline. The suspension is adjusted to a 0.5 McFarland standard (10^8 cfu/ml) and spot inoculated on Mueller–Hinton agar plates supplemented with 2% NaCl and containing 256–0.125 µg oxacillin/ml in serial doubling dilutions. The oxacillin Mueller–Hinton plates are incubated at 35°C for 24 hours. MIC of ≥4 µg/ml is considered resistant and MIC of ≤2 is considered susceptible.

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Broth microdilution:
This involves the use of Mueller-Hinton broth with 2% NaCl, an inoculum density of 5 x 10^5 cfu/mL and incubation at 33–35°C for 24 hours.

Breakpoint methods:
Breakpoint methods include both agar and broth methods and are essentially similar to dilution MIC methods but test only the breakpoint concentration (2 mg/L oxacillin, 4 mg/L methicillin).

Disc diffusion test:
A direct colony suspension of each S. aureus isolate is prepared to a 0.5 McFarland standard and plated on Mueller-Hinton agar containing 2-4% NaCl. An oxacillin (1 µg) disk is placed on the surface and incubated at 35°C for 24 hours. Oxacillin disk is more resistant to degradation in storage and more likely to detect heteroresistant strains. The zone of inhibition must be read with transmitted light and not reflected light. Zone diameter of ≤10 mm is considered as resistant, ≥13 mm as susceptible whereas 11-12 mm is considered as intermediate. If intermediate results are obtained for S. aureus, testing for *mec*A, PBP2a, cefoxitin disk test, oxacillin MIC test or oxacillin-salt agar screen test may be performed. Any discernable growth within the zone of inhibition when seen using transmitted light is indicative of oxacillin resistance. It may be possible that some of the oxacillin disk test positive isolates are hyper-beta-lactamase producers, thereby accounting for non-*mec*A-mediated methicillin resistance. In disc diffusion tests, hyper-producers of penicillinase may show small methicillin or oxacillin zones of inhibition, whereas most true methicillin-/oxacillin-resistant isolates give no zone. A 5µg methicillin disk can also be used but is not a popular choice. Zone diameter of ≤9 mm is considered resistant, ≥14 mm is considered resistant whereas a diameter of 10-13 mm is considered intermediate.

Etest oxacillin MIC test:
The inoculum is standardized to 0.5 McFarland turbidity and plated on Mueller-Hinton agar supplemented with 2% NaCl. Etest strips are placed and incubation at 35°C for a full 24 hours. The Etest has an advantage over other MIC methods in that it is as easy to set up as a disc diffusion test.

Oxacillin screen agar:
Mueller-Hinton agar (MHA) plates containing 4% NaCl and 6 µg/ml of oxacillin are inoculated with 10 µL of 0.5 Mc Farland suspension of the isolate by streaking in one quadrant and incubated at 350C for 24 h. Plates are observed carefully in transmitted light for any growth. Any growth after 24 h is considered oxacillin resistant. Induction with oxacillin requires an extended period for full expression. Hence, oxacillin-containing media achieve sufficiently high sensitivities only after 48 hours of incubation.

Cefoxitin disc diffusion test:
Cefoxitin, which is a potent inducer of the *mec*A regulatory system is being widely used as a surrogate marker for detection of *mec*A gene-mediated methicillin resistance. MRSA strains exhibiting inducible resistance to methicillin grow much more readily in the presence of cefoxitin than oxacillin, due to an enhanced induction of PBP 2a by cefoxitin. CLSI has recommended cefoxitin disc diffusion method for the detection of MRSA. A 0.5 Mc Farland standard suspension of the isolate is made and lawn culture done on MHA plate. A 30 µg cefoxitin disc is placed and plates are incubated at 37°C for 18 h and zone diameters are

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measured. The zone diameter must be measured in reflected light. An inhibition zone diameter of ≤21 mm is reported as methicillin resistant and ≥22 mm is considered as methicillin susceptible. In one study a 10-μg cefoxitin disk has been shown to be superior to the 30-μg disk with IsoSensitest agar and semiconfluent growth. Recent studies indicate that disc diffusion testing using cefoxitin disc is far superior to most of the phenotypic methods like oxacillin disc diffusion and oxacillin screen agar testing and is recommended by CLSI. Cefoxitin will detect only MRSA with a *mec*A-mediated resistance mechanism.

Chromogenic media for MRSA:
Currently available chromogenic media for MRSA detection include ChromID, MRSA Select, CHROMagar MRSA, Chromogenic MRSA/Denim Blue agar, ORSAB (oxacillin resistance screening agar base), MRSA Ident agar and Chromogen oxacillin S. aureus medium. The chromogen in ChromID targets the α-glucosidase enzyme of S. aureus, and the inhibition of competing flora is brought about by the incorporation of cefoxitin (4 mg/liter), resulting in green-colored colonies of MRSA. ORSAB, a modified version of mannitol salt agar, is made selective by the addition of oxacillin (2 mg/liter) to inhibit MSSA and polymyxin to suppress gram-negative bacteria. This medium incorporates aniline blue as a pH indicator, giving MRSA colonies a characteristic blue color. Colonies of MRSA on MRSA Ident agar are dusky pink or ruby-colored due to a chromogenic phosphatase substrate and an antibiotic supplement including cefoxitin. Chromogen oxacillin S. aureus medium characterizes MRSA colonies by a pink-mauve color. MRSA Select incorporates a cephamycin derivative and characterizes MRSA colonies by a pink color. CHROMagar contains cefoxitin (6 mg/liter) and a chromogen that also results in rose to mauve MRSA colonies. The chromogen in Chromogenic MRSA or Denim Blue agar detects phosphatase activity in S. aureus strains and, coupled with a selection with cefoxitin, produces denim blue colonies of MRSA. The currently available chromogenic media for MRSA detection show almost uniformly high specificities after 24 h of incubation, although sensitivities tend to vary widely both between media and between studies. Prolonging incubation time to 48 h can improve sensitivities; however, specificities are adversely affected, necessitating confirmatory tests before reporting MRSA.

PBP2a latex agglutination kit:
The method involves extraction of PBP2a from suspensions of colonies and detection by latex agglutination. The kit contains latex particles sensitized with a monoclonal antibody against PBP2a. Visible agglutination indicates a positive result and the presence of PBP2a, the *mec*A gene product. The test is rapid (10 minutes for a single test) and very sensitive and specific with S. aureus, but may not be reliable for colonies grown on media containing NaCl. Isolates producing small amounts of PBP2a may give weak agglutination reactions or agglutinate slowly. Reactions tend to be stronger if PBPa production is induced by growth in the presence of a penicillin. Rare isolates may give negative reactions.

The 3M™ BacLite™ Rapid MRSA test:
It is a rapid culture-based test that detects ciprofloxacin-resistant MRSA. The test measures adenylate kinase (AK) activity, an enzyme in cells that regulates adenosine triphosphate (ATP) AK catalyzes the conversion of adenosine diphosphate (ADP) to ATP. In the assay, AK detection is combined with selective broth enrichment. Magnetic microparticles coupled with a mouse anti-S. aureus monoclonal antibody are used to capture MRSA. Lysostaphin is added to lyse the S. aureus and release AK. ADP is provided as the substrate for the enzyme activity and production of ATP. The ATP is detected by the addition of firefly luciferin and luciferase, allowing a reaction that emits light when MRSA is present in the sample. This assay yields

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results in five hours. Few false positives due to methicillin resistant coagulase-negative staphylococci (CoNS) misidentified as MRSA have been encountered.

Quenching fluorescence method:
With the Crystal MRSA method (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. The method is reasonably reliable but requires several hours of incubation.

Molecular methods:

Detection of *mec*A gene by PCR is considered as the gold standard. DNA extraction is performed on the isolate and *mec*A gene is amplified using specific primers. The master mix containing PCR buffer, dNTP mix, primer, Taq DNA polymerase, and $MgCl_2$ and template DNA is subjected to hot start PCR. This is followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute and final extension step at 72°C for 3 minutes. PCR products are visualized on 2% agarose gel with ethidium bromide dye under UV transilluminator.

Another method that is designed to detect MRSA directly from clinical samples uses a front-end immunocapture of S. aureus followed by MRSA detection using a multiplex PCR that detects S. aureus-specific *fem*A and *mec*A. Staphylococcus aureus-specific targets used in various user-defined molecular assays for detection of MRSA:

- *nuc* Encodes heat-stable DNA nuclease gene
- *fem*A, *fem*B Encode enzymes important in cross-linking peptidoglycan
- *spa* Encodes S. aureus-specific protein A

The **hyplex StaphyloResist™** and **hyplex StaphyloResist™ plus** are qualitative multiplex PCR assays for the direct detection of clinically relevant staphylococci from swabs of the nose, skin, wounds, and endotracheal speciemns. The assay consists of PCR modules that contain labeled oligonucleotide primers, enabling simultaneous and specific amplification of different staphylococcal DNA regions in a single PCR reaction. The PCR is followed by recerse hybridization procedures using single-stranded specific probes immobilized on microtitre plates. Hybridization of PCR products with specific probes is detected using the ELISA principle.

In GenoType MRSA Direct, the IDI-MRSA, and the GeneXpert MRSA assay, the MRSA detection is based on the detection of a single amplicon, which includes the right junction of the *SCCmec* downstream of the mecA gene and a part of the adjacent S. aureus-specific *orf*X gene.
The **Genotype MRSA Direct** targets SCCmec types I to V in a multiplex PCR using biotinylated primers followed by a reverse hybridization step. 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. The **GenoQuick™ MRSA assay** is a direct test for MRSA from swabs of the nose, throat, skin, and wounds that uses a dipstick for qualitative detection of amplicons. Following DNA extraction and conventional PCR, the single stranded amplicon hybridizes with a fluorescein labeled probe included in the mastermix. The amplicon-probe complex is selectively labeled with gold and generated a band on the dipstick that is inserted in a tube that contains the amplicon. The assay aslo has an amplification control dipstick. It takes approximately 2 hours and 20 minutes to perform.

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The BD GeneOhm™ MRSA assay (also called IDI-MRSA) is a multiplex assay comprised of six primers that amplify target sequences near the insertion site of SCCmec. Amplified targets within *SCCmec* and *orf*X genes are detected with four molecular beacons. This assay has an internal control not found in MRSA. The fluorescence emitted by each beacon is measured and interpreted. Results are displayed as postive, negative or unresolved. It can differentiate MRSA from MSSA and *mec*A positive CONS in clinical sample.

Interpretation of results:

Detection of mecA gene or its product, penicillin binding proteins (PBP2a), is considered the gold standard for MRSA confirmation. Isolates of S. aureus that carry *mec*A gene or that produce PBP2a should be reported as MRSA and isolates lacking mecA gene or not producing PBP2a should be reported as methicillin susceptible. For MRSA strains, other beta-lactam agents such as penicillins, ß-lactam/ ß-lactamase inhibitor combinations, cephems and carbapenem must be reported as resistant or not reported at all even though they may appear susceptible in vitro. This is because most MRSA infections have responded poorly or convincing data to prove their clinical efficacy are unavailable. For oxacillin susceptible S. aureus isolates, results for parenteral and oral cephems, ß-lactam/ ß-lactamase inhibitor combinations and carbapenems the results should be reported as routine interpretative criteria. Certain rare isolates of S. aureus that are mecA & PBP2a negative but have oxacillin MIC ≥ 4 µg/ml should be reported as oxacillin resistant. Penicillin resistant but oxacillin susceptible strains produce ß-lactamase and should be tested using 10 unit penicillin disk and not ampicillin disk. A positive ß-lactamase test predicts resistance to all ß-lactamase labile penicillins such as ampicillin, amoxicillin, carbenicillin, ticarcillin and piperacillin. For oxacillin resistant S. aureus penicillin susceptibility should not be reported or reported as resistant. The results of oxacillin resistance can be applied to other penicillinase-stable penicillins such as cloxacillin, dicloxacillin and flucloxacillin. Cloxacillin disks should not be used to detect MRSA because they may not detect oxacillin resistance.

Quality control strains:

Methicillin sensitive S. aureus (MSSA) ATCC 25923 and methicillin resistant S. aureus (MRSA) ATCC 43300 are used as quality controls.

Treatment options:

Daptomycin is an acidic lipopeptide with a mode of action requiring calcium. Daptomycin has recently demonstrated significantly better bactericidal activity than vancomycin against S. aureus and enterococci and has activity against a small number of glycopeptide-intermediate S. aureus strains and vancomycin resistant enterococcus. Mupirocin is a bacteriostatic antibiotic used exclusively as a topical agent. It exerts its antimicrobial effect by specifically and irreversibly binding to bacterial isoleucyl tRNA synthetase, thus preventing protein synthesis. It has been used widely for the clearance of nasal methicillin-resistant Staphylococcus aureus (MRSA) carriage during outbreaks and has been recommended for the decolonisation of methicillin-sensitive S. aureus (MSSA) in healthcare personnel. Intranasal application of mupirocin ointment is effective in reducing surgical site infections and the likelihood of bronchopulmonary infection.

Recently, there have been reports of vancomycin failure due to either relative vancomycin resistance or MRSA infections in sites that have poor vancomycin penetration. Few other drugs including linezolid (a synthetic oxazolidinone), tigecycline (a derivative of minocycline), and daptomycin (a cyclic lipopeptide) appear promising in treatment. Daptomycin should be

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avoided in the treatment of MRSA-associated pneumonia because it is inactivated by pulmonary surfactant. For initial empiric therapy, oral trimethoprim-sulfamethoxazole is a good choice. Other alternatives include minocycline, clindamycin, or a macrolide antibiotic, depending on local susceptibility patterns. Staphylococci may develop resistance during prolonged treatment with quinolones. Therefore isolates initially susceptible may become resistant within three to four days after initiation of therapy. Rifampicin should not be used alone for treatment. Macrolide resistance in S. aureus may be inducible or constitutive and the former can be detected by D-test.

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