4.0 MATERIALS AND METHODS

The present study, phenotypic and genotypic characterization of MRSA isolates in Sri Ramachandra Medical Center was carried out during January 2010 to December 2012. Out of the 750 MRSA isolated from various specimens during different points of the study period, 300 strains were evaluated.

4.1 Isolation of Staphylococcus aureus and phenotypic characterization of MRSA

MRSA strains were isolated from various specimens such as blood (48), exudates (226) [pus (70), wound swabs (80), ear swabs (50) and body fluids (26)] respiratory (10) [BAL (5), non-BAL (4), plural fluid (1)] and urine (16). A total of 300 isolates of MRSA were selected as per sample size from the 750 MRSA strains.

The strains were identified as S. aureus by their morphology on Gram staining, hemolysis on blood agar, growth on chocolate agar, oxidation-fermentation test and salt tolerance.

Biochemical characterization was determined by catalase production, coagulase production (slide and tube), mannitol fermentation and urease activity.

To obtain a pure culture the isolates were cultured on Mannitol Salt Agar. Lipase activity was determined with addition of 5% v/v Egg yolk emulsion.

Antibiogram was carried out for all the strains for the following drugs Erythromycin (30µg), Ciprofloxacin (5µg), Gentamycin (10µg), Clindamycin (2µg) and Linezolid (30µg) as per CLSI guidelines.

All the strains were resistant to the beta-lactum antibiotics such as Ampicillin (10µg), Cephalaxin (30µg), Cefotaxime (30µg), Cloxacillin (30µg).
ATCC 25923 (MSSA) and ATCC 43300 (MRSA) control strains were obtained from Hi-media Pvt Ltd.

4.1.1 Kirby-Bauer Test for Antibiotic Susceptibility

A true antibiotic is an antimicrobial chemical produced by microorganisms against other microorganisms. Mankind has made very good use of these antimicrobials in its fight against infectious disease. Many drugs are now completely synthetic or the natural drug is manipulated to change its structure somewhat, the latter called semisynthetics. Bacteria respond in different ways to antibiotics and chemosynthetic drugs, even within the same species. For example, *Staphylococcus aureus* is a common normal flora bacterium found in the body. If one isolated this bacterium from five different people, the 5 isolates would likely be different strains, that is, slight genetically different. It is also likely that if antibiotic sensitivity tests were run on these isolates, the results would vary against the different antibiotics used. The Kirby-test for antibiosisusceptibility, called the disc diffusion test, is a standard at has been used for years. First developed in the 1950s, it was refined and by W. Kirby and A. Bauer, then standardized by the World Health Organization in 1961. It has been superseded in clinical labs by automated tests. But the K-B is still used in some labs, or used with certain bacteria that automation does not work well with. This test is used to determine the resistance or sensitivity of aerobes or facultative anaerobes to specific chemicals, which can then be used by the clinician for treatment of patients with bacterial infections. The presence or absence of an inhibitory area around the disc identifies the bacterial sensitivity to the drug. The basics are easy: The bacterium is swabbed on the agar and the antibiotic discs are placed on top. The antibiotic diffuses from the disc into the agar in decreasing amounts the further it is away from the disc. If the organism is killed or inhibited by the concentration of the antibiotic, there will be **NO** growth in the immediate area around the disc: This is called the zone of inhibition. The zone sizes are looked up on a standardized chart to give a
result of sensitive, resistant, or intermediate. Many charts have a corresponding column that also gives the MIC (minimal inhibitory concentration) for that drug. The MIC is currently the standard test run for antibiotic sensitivity testing because it produces more pertinent information on minimal dosages. The Mueller-Hinton medium being used for the Kirby-Bauer test is very high in protein.

Objectives:
Determine the susceptibility of various bacterial species to various antibiotics and synthetic agents.

Materials Needed Per Table
1. 2 Mueller-Hinton agar plates
2. 24 hr old cultures sterile swabs 2
3. Antibiotics
4. Ethanol
5. Forceps

The Procedures:
Swab a Mueller-Hinton plate with ONLY 2 of the bacteria (tables will run different combinations of the 4 bacteria). Dip a sterile swab into the broth and express any excess moisture by pressing the swab against the side of the tube.

Swab the surface of the agar completely (you do not want to leave any unswabbed agar areas at all). In the pictures below, you can see what happens when the plate is not swabbed correctly with even coverage of the bacterium over the entire agar.

After completely swabbing the plate, turn it 90 degrees and repeat the swabbing process. (It is not necessary to re-moisten the swab). Run the swab around the circumference of
the plate before discarding it in the discard bag.

Allow the surface to dry for about 5 minutes before placing antibiotic disks on the agar.

The Antibiotic Disks:

a. You are using individual antibiotic dispensers.

b. You will probably have to use a pair of forceps to remove an antibiotic disc from the dispenser: the forceps have to be sterile. Place the forceps in alcohol, flame the forceps until
c. they catch on fire, let the flame go out—*sterile forceps*.

d. Lightly touch each disc with your sterile inoculating loop to make sure that it is in good contact with the agar surface. Incubate upside down and incubate at 37°C.

Interpretation:

Place the metric ruler across the zone of inhibition, at the widest diameter, and measure from one edge of the zone to the other edge. HOLDING THE PLATE UP TO THE LIGHT MIGHT HELP.

Use millimeter measurements. The disc diameter will actually be part of that number.

If there is NO zone at all, report it as 0—even though the disc itself is around 7 mm.

Zone diameter is reported in millimeters, looked up on the *chart*, and result reported as sensitive, resistant, or intermediate.

Record the results for your table.

4.2 Comparison of conventional and rapid phenotype methods for detection of MRSA (n=48)

4.2.1 Conventional method: Cefoxitin Disc Diffusion test
Cefoxitin disc diffusion test was carried out using a 30 µg disc of cefoxitin.

Muller Hinton Agar was boiled and autoclaved

Media poured in petriplates in a sterile manner and cooled

Lawn culture of the bacterial suspension (0.5 Mc Farland standards) was done on the agar plates.

The plates were incubated at 37°C for 18 to 24 hrs

Zone diameters were measured.

Zone diameters <19mm was reported as methicillin sensitive and zone diameters >22mm was considered as methicillin resistant. Colonies that grew within the zones were tested again and the zone of inhibition reported.

4.2.2 Rapid Phenotype methods:

4.2.2.1Slide latex Agglutination test:

Intended use:

MRSA-Screen is a qualitative slide latex agglutination test for the detection of PBP2a present in isolates of *Staphylococcus aureus* and is thus useful as an aid in identifying methicillin-resistant *Staphylococcus aureus* (MRSA).

Summary and explanation:
MRSA has become a worldwide concern owing to their increasing frequency in hospitals causing serious staphylococcal infections, including sepsis and endocarditis. Rapid and appropriate antimicrobial therapy, including the administration of vancomycin, is critical for effective treatment. However, conventional methods for identifying MRSA, such as disc susceptibility testing, are not always reliable since phenotypic expression of methicillin resistance is known to be heterogenous, depending on such factors as incubation time, temperature, NaCl concentration, etc. Difficulties in the differentiation of MRSA from borderline oxacillin-resistance *S. aureus* (BORSA), for example, may also occur. Recent research suggests that in the identification of MRSA, it is more accurate to either directly detect the gene encoding the methicillin resistance determinant (*mecA*) or its product, penicillin-binding protein 2'(2a) or PBP2' (PBP2a), which is found in the cell membrane of MRSA. However, as nucleic acid hybridization and DNA amplification techniques such as PCR for detecting the *mecA* gene are expensive and technically demanding, simple and more inexpensive techniques are required for routine use. MRSA-Screen was developed expressly for this purpose, providing results in 15 minutes with minimal labour and no specialized equipment.

Principle of test:

MRSA-screen consists of a latex reagent sensitized with monoclonal antibody against PBP2’ together with reagents to rapidly extract PBP2’ from the bacterial membranes of MRSA. Extracts are prepared by boiling a suspension of *S. aureus* cells under alkaline conditions, followed by neutralization and centrifugation step. The supernatant is then mixed with the latex reagent on a test card and visible clumping or agglutination within three minutes indicates the presumptive presence of PBP2’.

Reagents:
Sensitized latex in a dropper bottle (1x1.3mL) with a red cap. Latex particles are sensitized with a monoclonal antibody prepared against PBP2'. Contains 0.08 w/v% sodium azide as a preservative.

Control latex in a dropper vial (1x1.3mL) with a white cap. Latex particles are sensitized with a monoclonal antibody with no reactivity to PBP2'. Contains 0.08 w/v% sodium azide as preservative.

Extraction reagent 1 in a dropper vial (1x10mL) with a green cap. A solution of 0.1 mol/L NaOH.

Extraction reagent 2 in a dropper vial (1x2.5mL) with a yellow cap. A solution of 0.5 mol/L KH$_2$PO$_4$.

a. Test card, 30

b. Mixing stick, 110

c. Product insert

Shelf life and storage:

Store at 2-10°C. Reagents are stable before and after opening vials when stored under these conditions until the expiry date written on the box.

Warnings and Precautions:

This product is for in vitro diagnostic use only. As reagents in this kit (sensitized latex and control latex are prepared from biological materials, and due to the potential infectious nature of the isolates being tested, proper handling and disposal methods should be established and only trained personnel should be permitted to perform the procedures. Both latex reagents contain 0.08 w/v% sodium azide as a preservative. Disposal by flushing with copious
amounts of water is necessary since sodium azide may react with lead or copper piping to form highly explosive sodium azide.

Also, the following general precautions should be followed:

1. The MRSA-Screen test is intended for in vitro diagnostic use.

2. Read instructions completely and carefully before performing the test.

3. Do not freeze the reagents nor use past the expiration date as this may result in poor reagent performance.

4. Bring the kit to room temperature before use each time.

5. Ensure that latex reagents form a homogenous suspension before use by gently inverting and shaking vials. Avoid extreme or excessive shaking, vortixing, etc. As this may impair reagent performance.

6. Do not interchange reagents between different lot numbers, or caps between different reagent vials, etc.

7. Use aseptic laboratory techniques and never mouth pipette.

8. If reagents come into contact with the skin, mucous membranes or eyes, wash immediately with plenty of water. Extraction reagents 1 and 2 are slightly basic and acidic, respectively. Seek medical treatment if serious reactions develop.

9. Sterilize all specimens, spills, inoculated products and equipment used in this test by one of the following methods:

   a. Soaking in 0.5w/v% hypochlorite for one hour or more

   b. Autoclaving at 121°C for 20 minutes or more
Specimen handling:

In general, standard microbiological procedures should be followed and only isolates with biochemical, growth and morphological characteristics of *S. aureus*, i.e., gram positive cocci that are coagulase positive, or, as identified by a rapid *S. aureus* identification test, should be used. Isolates should be fresh, 18-24 hour cultures grown at 35°C. Recommended culture media for use with this kit are blood agar plates, such as tryptic soy agar with 5% sheep blood, Columbia agar with 5% sheep blood and Mueller-Hinlton agar. Finally, after extraction of PBP2′ and separation of the test specimen for use in the latex test, perform the test that same day. The test specimen may be stored refrigerated at 2-10°C for batch testing later in the day or at -80°C for long term storage.

Important procedural note:

When dispensing latex reagents, hold the vial in a completely vertical position and pause slightly between dispensing of latex reagent drops, such as when performing more than one test at a time. Do not allow reagent vial nozzle tips to come in contact with the specimen on the side card. After use, ensure that the reagent vial caps are snugly but not too tightly capped.

Test procedure:

Materials and reagents necessary for the test

**MRSA-Screen**

I The following materials are required but not provided:

II Specimen: isolated colonies of *S. aureus* grown on blood agar plates for less than 24hrs.

III Equipment: Micropipette and tips (50μL)
- Microcentrifuge tubes (safe lock)
- Microbiological loop: Internal volume 1.0-1.5µL (or µL) (1mm internal diameter, 0.6mm thickness)
- Boiling water bath or heating block (95°C or greater)
- Centrifuge/Microcentrifuge
- Rotary platform (optical)

IV Reagent preparation

All reagents are used as supplied but bring kit to room temperature and ensure latex reagents form homogenous suspensions before use.

V PBP2′ Extraction Procedure

4 drops viz., 200µl of extraction Reagent 1 was taken in a microcentrifuge tube.

Using sterile bacterial loop (1mm internal diameter) sufficient bacterial colonies were taken and thoroughly suspended in the tube.

The centrifuge tube was placed in a heating block at 100°C and heated for 3mins.

The tube was removed and cooled to room temperature.

1 drop viz., 50µl of extraction Reagent 2 was added into the tube and mixed well

The tube was centrifuged at 4500rpm in microcentrifuge
After centrifugation the supernatant was removed and used as the test specimen.

VI Latex agglutination procedure

1. For each specimen, allot and label two circles on the test card, one as test and the other as control.

2. Place 50µL of the specimen onto each of the test and the control circles.

3. To the test circle, add 1 drop (25µL) of sensitized latex and to the control circle, 1 drop (25µL) of control latex.

4. With separate mixing sticks, thoroughly mix each reagent with specimen over the area of the circle.

5. Rotate the test card by hand or mechanical rotary platform for 3 minutes. Place the test card on the bench and read the agglutination patterns by eye. Record the results.

Interpretation:

Table: 4.2.2.1 Interpretation and Degree of Positivity PBP2’ of Latex Agglutination Test

<table>
<thead>
<tr>
<th>Interpretation</th>
<th>Test Latex</th>
<th>Control Latex</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong agglutination (3+)</td>
<td>+</td>
<td>-</td>
<td>+(PBP2’ positive)</td>
</tr>
<tr>
<td>Agglutination against slightly turbid background (2+)</td>
<td>+</td>
<td>-</td>
<td>+(PBP2’ positive)</td>
</tr>
<tr>
<td>Slight agglutination against turbid background (1+)</td>
<td>+</td>
<td>-</td>
<td>+(PBP2’ positive)</td>
</tr>
<tr>
<td>No agglutination</td>
<td>+</td>
<td>+</td>
<td>Indeterminate</td>
</tr>
</tbody>
</table>

Note: the degree of positivity should not be interpreted to indicate levels of antibiotic resistance.
Precautions for Interpretation:

1. A positive reaction is indicated by the clear development of an agglutination pattern within 3 minutes. Although the size and appearance of the aggregates of latex particles may vary, agglutination is visible to the unaided eye. A negative reaction is indicated by a homogenous suspension or milky appearance of both latex reagents, although traces of granularity may be seen due to the particulate nature of the latex reagents. Care should be taken in not over reading such granularity as true positive reactions. (Also, see limitation of the procedure below)

2. Specimens showing indeterminant results should be retested, taking care to follow the protocol exactly as written, particularly with respect to the length of boiling, centrifugation and agglutination steps. Boiling for less than 3 minutes or insufficient centrifuging may result in non-specific reactions and boiling for over 5 minutes may decrease sensitivity.

3. Specimens repeatedly showing indeterminate results should be tested by another method.

Quality control:

1. Proper reactivity of the sensitized latex and control latex should be confirmed with previously identified MRSA and MSSA upon receipt of this kit and periodically in accordance with the laboratory’s standard quality control practice to serve as both a reagent and procedural control.

2. Recommended strains for this purpose are S.aureus ATCC 43300 (MRSA) and S. aureus ATCC 25923 (MSSA), respectively. The former should show clear agglutination with the sensitized latex reagent with strength of about 2+ within 3
minutes, while the latter should show a negative reaction. The control latex must not show agglutination with either organism.

Table 4.2.2.1.1 : Interpretation of Latex Agglutination Test for Control Strains

<table>
<thead>
<tr>
<th>Control Strain</th>
<th>Test Latex</th>
<th>Control Latex</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus ATCC 43300</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>S. aureus ATCC 25923</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Limitations of the procedure:

1. Negative results obtained with this kit should be considered with other clinically relevant data when diagnosing an MRSA infection. In particular, retesting should be performed if during the course of a *S. aureus* infection, prognosis indicates treatment failure, etc.

2. In very rare cases, false negatives will occur if the strain produces low amounts of PBP2'. Accordingly, other antibiotic susceptibility testing methods as recommended by the current clinical and laboratory standards institute (CLSI) should be performed.

3. It is strongly recommended that laboratory personnel using this kit thoroughly familiarize themselves with the procedure and reading of agglutination patterns before employing the test routinely in a clinical setting.

4. Other mechanisms of methicillin resistance exist which are not detected by this kit, including the hyperproduction of beta-lactamase (BORSA) and other altered PBPs (MODSA).

5. The test should not be used to detect mecA in coagulase-negative staphylococci.

6. The test should not be performed on a direct specimen such as blood culture, etc.
Performance characteristics:

MRSA-Screen has been evaluated at a number of geographically diverse laboratories using clonally distinct isolates of *S. aureus* (1, 5, and 6, 7, 8, 9 and other on file). The test was also evaluated and compared to the CLSI reference methods for microbroth dilution and oxacillin agar screen tests with 6µg/mL oxacillin on 726 clinical isolates of coagulase-positive *S. aureus* collected at 3 North American sites to challenge the test for a wide range of phenotypically distinct strains of *S. aureus* as well as on 201 fresh isolates of *S. aureus* at 4 North American sites. Below summarized agreement between MRSA-Screen and oxacillin agar screen.

4.2.2.2 ORSAB with Selective Supplement (ORSAB: Oxoid Ltd. Basingstoke, England):

Directions:

Suspend 51.75g of oxacillin resistance screening agar base in 500ml of distilled water and bring gently to the boil to dissolve. Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of one vial of ORSAB selective supplement (SR0195), reconstituted as directed below. Mix well and pour into sterile petri dishes.

Description

ORSAB in intended as a medium for the screening for methicillin-resistant *Staphylococcus aureus* (MRSA) directly from routine swab samples. The screening of patients and staff for the early detection of MRSA colonisation is essential if epidemics are to be prevented.
ORSAB is based on traditional Mannitol Salt Agar. CM0085 with a reduction in salt concentration from 75g/l (7.5%) to 55g/l (5.5%). This lower level of salt is still sufficient to inhibit most bacteria other than staphylococci whilst optimising growth of low numbers of MRSA.  oxacillin resistance screening agar base is a nutritious and selective medium containing peptones for growth, a high salt concentration and lithium chloride to suppress non-staphylococcal growth with mannitol and aniline blue for the detection of mannitol fermentation. The antibiotics contained in ORSAB selective supplement are oxacillin at 2mg/litre to inhibit methicillin sensitive staphylococcus aureus (MSSA) and polymyxin B for the suppression of other bacteria able to grow at such a high salt concentration, e.g. *Proteus* spp. typical colonies of MRSA are intense blue in colour on a colourless background enabling the organism to be more easily identified in mixed culture than the pale yellow colonies seen on Mannitol Salt Agar.

Oxacillin Resistant Screen Agar Base: Preparation of the media

ORSAB is a selective media developed to detect MRSA in clinical specimens. The medium uses aniline blue to detect mannitol fermentation in *S. arueus*. The antibiotic supplements (oxacillin, 2.0 µl; polymixin B, 50, 000 IU/l) and of 5.5% NaCl reduce the growth of non-staphylococcal organisms and helps in the selection of MRSA. The test was carried out as per the manufacturer’s instructions.

51.75g of ORSAB was suspended in 500ml of distilled water and boiled to dissolve the contents.

The medium was sterilised by autoclaving at 121°C for 15minutes, cooled to 50°C
Asceptically the contents of one vial of the antibiotic supplement was added to the medium after reconstituting it in 2ml of sterile water.

20ml of the medium was poured in sterile petriplates and cooled to solidify.

Sufficient amount of each culture was taken in a loop and mixed in saline to bring it to 0.5 McFarland standards.

The plates were subsequently inoculated and incubated at 37°C for 48 hrs.

Technique

Take a routine swab sample from the patient or person to be screened. Rub the swab onto an ORSAB plate in one set of streaks near the plate perimeter. The sample material should then be streaked across the plate using the diminishing sweep technique. Incubate at 37°C for 24 hours. Examine after 24 hours for blue colonies. Confirm suspected MRSA with coagulase test staphytect plus DR0850 or Dryspot staphytect plus DR0100 and PBP2′ DR0900. Re-incubate negative plates for a further 24 hours if necessary. Do not re-incubate positive plates.

Storage conditions and shelf life:

Store the dehydrated medium at 10-30°C and use before the expiry date on the label. Salmonella selective supplement should be stored at 2-8°C. Store the prepared medium at 2-8°C.

Culture medium, colony colour:

- Positive negative
- ORSAB intense blue on straw/No growth
- CM1008 & SR 1095 colourless media
- Mannitol Salt Agar pale yellow on red pink-red/no growth
- CM0085 media

Vial contents (each vial is sufficient to supplement 500ml of medium) per vial

per litre

1. polymyxin B 25,000 IU 50,000 IU

2. Oxacillin 1mg 2mg

4.2.2.3 Epsilometer Test: Oxacillin Ezy MIC™ Strip (OXA) (0.016-256 mcg/ml), antimicrobial susceptibility testing for in vitro Diagnostic use

It is unique MIC determination paper strip which is coated with oxacillin in a concentration gradient manner, capable of showing MICs in the range of 0.016mcg/ml to 256 mcg/ml, on testing against the test organism.

Introduction:

Ezy MIC™ strip is used for quantitative determination of susceptibility of bacteria to antibacterial agents. The system comprises of a predefined quantitative agents. The system comprises of a predefined quantitative gradient which is used to determine the Minimum
inhibitory concentration (MIC) in mcg/ml of different antimicrobial agents against microorganisms as tested on appropriate agar media, following overnight incubation.

Ezy MIC™ strip features and advantages:

- Ezy MIC™ strip exhibits several advantages over existing plastic strip.
- Ezy MIC™ strip is made up of porous paper material unlike plastic non-porous material
- Ezy MIC™ strip has MIC values printed on both sides identically.
- The antimicrobial agent is evenly distributed on either side of the Ezy MIC™ strip and hence it can be placed by any side on the agar surface.
- For Ezy MIC™ strips, MIC values can be read without opening the lid of the plate as most commonly translucent medium such as Muller Hinton Agar is employed.
- Once placed, Ezy MIC™ strip is adsorbed within 60 seconds and family adheres to the agar surface.
- Unlike the plastic material, it does not form air bubbles underneath and hence there is no need to press the strip once placed.

CLSI recommendation for detection of oxacillin resistance:

- Of the pencillinase-stable penicillins, oxacillin is preferred for in vitro testing. Oxacillin is more resistant to degradation in storage and is more likely to detect heteroresistant staphylococcal strains. Oxacillin susceptibility results can be applied to the other penicillinase-stable penicillins like cloxacillin, didoxacillin, methicillin, flucloxacillin and naficillin.
Addition of 2% sodium chloride is required for dilution testing of oxacillin to improve the detection of heteroresistant MRSA.

The use of direct colony suspension method for preparation of inoculums is necessary.

Incubate tests to detect MRSA for full 24 hours at 35±2°C when using oxacillin (testing at temperature above 35°C may not detect MRSA).

Method and use of Ezy MIC™ strips guidelines for preparation of the medium:

Prepare the medium of choice from dehydrated powder according to the directions specified on the label. Cool the sterilized molten medium to 45-50°C and pour in sterile, dry petri plates on a levelled surface, to a depth of 4±0.2mm. Just before use, check for the presence of any excess surface moisture on the medium. The surface should be appropriately moist but no droplets should be present on the surface of the medium or on the petri plate cover.

Preparation of Inoculum:

Direct colony suspension method has to be used while testing oxacillin Ezy MIC™ strips. Prepare a direct colony suspension, from 18-24 hour old non-selective media agar plate in broth or saline. Adjust the turbidity to that of standard 0.5 McFarland. Alternatively, the inoculums can be standardized by other appropriate optical method (0.08-0.13 OD) turbid suspension at 625 nm).

Test procedure:

The E-test (Oxacillin Ezy MIC™ Strip, Himedia Laboratories Pvt. Ltd.) for determining oxacillin MICs were performed on Muller-Hinton agar supplemented with 2% ,
NaCl. Addition of 2% NaCl enhances the detection of heteroresistant MRSA.

Direct colony suspension was prepared from 18-24hrs old non-selective media agar plate in broth

Turbidity adjusted to 0.5 McFarland standards

Muller-Hinton Agar plates prepared with 2%NaCl

Non-toxic cotton swab on a wooden applicator was dipped into the standardized inoculums

Soaked swab rotated firmly against the upper inside wall of the tube to express excess fluid

Agar plate streaked with the swab three times, turning the plate at 60° angle between each streaking

Ezy MIC™ strip container removed from cold and kept at room temperature for 15–30 minutes

One applicator removed from the self sealing bag stored at room temperature

Applicator held in the middle and the broader sticky side gently pressed on the centre of Ezy MIC™ strip

Applicator lifted along with attached Ezy MIC™ strip

Strip placed at a desired position on the pre-streaked agar plate
Applicator detached by gently turning it clockwise

Isolates incubated at 37°C for 24 hours

Precautions:

1. Incubate the plates at 35°C±2°C for 24 hours for ORSA and 48 hours for ORSE

2. Read the plates only when sufficient growth is seen.

3. Read the MIC where the ellipse intersects the MIC scale on the strip.

4. Always read at the point of complete inhibition of all growth including hazes and isolated colonies.

5. Since Ezy MIC™ strip has continuous gradient, MIC values “in-between” two fold dilutions can be obtained.

6. Always round up these values to the next two-fold dilution before categorization. For example: oxacillin showing reading of 0.75mcg/ml should be rounded up to next concentration i.e. 1.0mcg/ml.

7. If the ellipse intersects the strip in between 2 dilutions, read the MIC as the value which is nearest to the zone 3.

Interpretation:

<table>
<thead>
<tr>
<th>When testing</th>
<th>Interpretative criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;S</td>
</tr>
<tr>
<td><em>S. aureus</em> and <em>S. lugdunensis</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Coagulase-negative Staphylococci</em></td>
<td>0.25</td>
</tr>
<tr>
<td>except <em>S. lugdunensis</em></td>
<td></td>
</tr>
</tbody>
</table>
Quality control:

Quality control of Ezy MIC™ strips is carried out by testing the strips with standard ATCC cultures recommended by CLSI on suitable medium incubated appropriately.

Table: 4.2.2.3.1 The reference MIC values (mcg/ml) range for Oxacillin.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium used</th>
<th>Incubation</th>
<th>Std. Quality control limits (mcg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>Mueller</td>
<td>35-37°C for</td>
<td>0.125 0.25 0.5</td>
</tr>
<tr>
<td>ATCC 29213</td>
<td>Hinton Agar</td>
<td>18hrs.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>Mueller</td>
<td>35-37°C for</td>
<td>16-32-64</td>
</tr>
<tr>
<td>ATCC 43300</td>
<td>Hinton Agar</td>
<td>24hrs. + 2% NaCl</td>
<td></td>
</tr>
<tr>
<td>(MRSA)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References:

Performance standards of antimicrobial disc susceptibility tests, M100-520 CLSI vol.30 No. 1, Jan 2010.

Storage and shelf-life:

Once the consignment is received, store applicators at room temperature and Ezy MIC™ strips container at -20 to +8°C. Use before expiry date on the label.

Packing:

Each pack contains following material packed in air-tight plastic container with desiccator’s capsule.

- Oxacillin Ezy MIC™ strips (30/60/90/120 strips per pack)
- Applicator sticks
4.3 Genotypic studies: Rapid identification of methicillin-resistant *Staphylococcus aureus*

By Polymerase Chain Reaction (PCR)

Materials and Methods:

Genomic DNA extraction: Medox kit stored at room temperature.

1. Column and collector
2. TE buffer
3. 96%-100% ethanol
4. Proteinase K
5. Eppendorff tubes - 1.5ml (around 100)
6. Micropipette - 20-200 micro litre and tips
7. Digestion solution
8. Wash buffer

Amplification

1. PCR tubes - 0.2ml
2. Micropipette: 20-200 micro litre and tips
   2-20 micro litres along with the required tips
3. PCR Master mix (P.T.O)

Detection of Amplicon

1. Agarose
2. TAE buffer
3. DNA ladder - 100-1000 bps

4. Ethidium Bromide (2µl for each gel cast)

DNA isolation: Medox-Bio™ ultra pure genomic DNA spin minipreps kit from bacteria — Protocol

1. Spin appropriate number of bacteria (about 10^6-10^7) at 8,000 rpm for 5 min at room temperature. Remove supernatant completely and discard, then resuspend in 200µl cold TE, continue the procedure with Step 2.

2. Apply the mixture to column that is in a 0.2ml collection tube. Spin at 8,000 rpm for 1 minute.

3. Discard the flow-through in the collection tube. Add 500µl of wash solution, and spin at 8,000 rpm for 1 minute.

4. Repeat washing step 4.

5. Discard flow-through. Spin at 1,000 rpm for an additional minute to remove residual amount of wash solution.

6. Place the MX-10 column into a clean 1.5ml microfuge tube. Add 30-50µl elution buffer into the centre part of membrane in the column. Incubate the tube at 37 or 50°C for 2 minutes. Incubate at 37 or 50°C could increase recovery yield.

7. Spin at 10,000 rpm for 1 minute to elute DNA from the column.

8. Measure DNA quantity by UV absorption at A_{260} (1.0 OD unit is equivalent of 50µg). Assess genomic DNA quality by an analytical 0.7% agarose gel. Isolated genomic DNA should not contain RNA. Its length should be over 50kb.
PCR Protocol

Standardization of a Multiplex PCR targeting \textit{mecA}, \textit{nuc} and PVL genes (n=48).

The detection of \textit{mecA} gene in MRSA isolates was considered as the reference method for establishing the rapidity, sensitivity, specificity of each of the technique studied.

A multiplex PCR was standardized targeting \textit{mecA}, \textit{nuc} and PVL genes for the simultaneous detection of hospital and community strains of methicillin resistant \textit{Staphylococcus aureus}.

The primers were designed using bioinformatics tools (Primer BLAST) for the target sequences (FASTA) taken from the comparative microbial genome database (CMGB) and optimized to PCR conditions.

Table: 4.3 Sequences of primers designed in accordance with published nucleotide sequences

<table>
<thead>
<tr>
<th>Name of the gene</th>
<th>Primer Sequence</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>MecA_F</td>
<td>AGAGTAGCACTCGAATTAGGGCAGT</td>
<td>400bp</td>
</tr>
<tr>
<td>MecA_R</td>
<td>TCTGCAGTACCGGATTTGCCA</td>
<td></td>
</tr>
<tr>
<td>Nuc_F</td>
<td>ACGGGTCCTTTTCAAAAAAGGGGA</td>
<td>294bp</td>
</tr>
<tr>
<td>Nuc_R</td>
<td>ACGCCAATGTCTACCATAGCG</td>
<td></td>
</tr>
<tr>
<td>PVL_F</td>
<td>TCAGTTGTACATCAATTCGATTGCCAT</td>
<td>135bp</td>
</tr>
<tr>
<td>PVL_R</td>
<td>ATCGGATCTGATGTTGATGAGTGT</td>
<td></td>
</tr>
</tbody>
</table>
A 20µl PCR reaction consisted of 16µl of master mix [PCR buffer (2X), dNTP mix (400mM), Taq DNA polymerase (250 U) and MgCl₂ (3mM)], 2µl primer, 2µl of template DNA and 1µl of distilled water. DNA isolation done using Medox genomic isolation kit.

4.3.4 Amplification 20µl assay

- 5X Hot-start PCR master mix - 10µl
- Forward primer – 0.2µl
- Reverse primer – 0.2µl
- Template DNA - 4µl
- Double distilled water (milliQ water) – 5.6µl

PCR conditions:

Initial TAQ activation: 95°C for 10mins

PCR cycle:

- 95°C for 30 seconds
- 58°C for 60 seconds
- 72°C for 60 seconds 35 cycles

Used each 3 pmols of forward and reverse primers for 20µl assay. [primer sequence – see text].

Amplicon detection was carried in Agraose Gel Electrophoresis

Development and Validation of the Multiplex PCR

The assay development was carried out using ATCC control strains and a well characterized clinical isolate (positive for mecA, nuc and PVL genes).
Prior to optimization of the multiplex PCR, a single target PCR protocol was conducted for each of the primer pairs with the above control strains.

The assay validation for the multiplex PCR was performed by simultaneous comparison with the results of single-target PCR assay and the antibiotic susceptibility phenotypes of 34 clinical isolates.

4.4 To characterize MRSA strains by molecular typing of Staphylococcal Cassette Chromosome (SCCmec typing) [Boyle et al. 2007] (n=48)

A multiplex SCCmec PCR was performed on the previously characterized 34 clinical isolates. Four primer sets were designed to ensure amplification of two DNA targets from SCCmec type IV and two targets from SCCmec type V. The targets were chosen so that one target would be amplified from each of SCCmec types I–III (Table 2).

Materials: Same as 4.3

Table 4.4: Buffers and their Concentrations

<table>
<thead>
<tr>
<th>S. No</th>
<th>Buffer</th>
<th>Working Solution</th>
<th>Stock Solution Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TAE</td>
<td>1X 40mM Tris-acetate 1mM EDTA</td>
<td>50X 242g of tris base 57.1 ml of glacial aceticacid 100ml of 0.5M EDTA (pH 8.0)</td>
</tr>
<tr>
<td>2</td>
<td>TBE</td>
<td>0.5X 45mM Tris-borate 1mM EDTA</td>
<td>5X 54g of Trio base 27.5g of boric acid 20ml of 0.5M EDTA (pH 8.0)</td>
</tr>
<tr>
<td>3</td>
<td>TPE</td>
<td>1X 90mM Tris-phosphate 2mM EDTA</td>
<td>10x 108g of tris base 15.5 ml of phosphonic acid (85%, 1.679g/ml)</td>
</tr>
<tr>
<td>4</td>
<td>Tris-glycine</td>
<td>1X 25mM Tris-Cl 250mM glycine 0.1% SDS</td>
<td>5X 15.1g of Tris base 94g of glycine (electrophoresis grade) 50ml of 10% SDS (electrophoresis grade)</td>
</tr>
</tbody>
</table>

DNA isolation: same as 4.3.2
SCC\textit{mec} typing Primers: table 4.4

PCR Protocol

The PCR reaction was standardized at 4\mum of each forward and reverse primers for 25\mum assay.

Cycling condition:

- 94°C for 5mins
- 94°C for 45seconds
- 54°C (annealing) for 30seconds
- 72°C (extension) for 45seconds
- 72°C (final extension) for 7minutes

DNA extraction performed using bacterial genome DNA isolation kit (Shrimpex Biotech Services)

PCR was performed in a total volume of 50 \mL containing 1x AmpliTaq PCR buffer, 1.5 mM MgCl$_2$, 200 \mM each dNTP and 1 U of AmpliTaq DNA polymerase. Based on optimisation experiments, primer concentrations were as follows: primers β and α3, 0.2 \mM each; ccrCF and ccrCR, 0.25\mM each; 1272F1 and 1272R1, 0.08 \mM each; and 5RmecA and 5R431, 0.1 \mM each.

Amplification: Cyclic conditions—4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C, with a final extension for 4 min at 72°C.

PCR products (5 \mL) analysed by electrophoresis on agarose 1.5% w/v gels, staining with ethidium bromide.
The SCC\textit{mec} type was determined on the basis of the band pattern obtained (Table 4.4). Isolates with no visible bands, or with a band pattern that was not in agreement with one of the five predicted band patterns, were classified as non-typeable (NT).

PCR Trouble Shooting

Long non-specific products in PCR: When running an agarose gel, spot larger non-specific PCR bands that are not the right size.

Solutions to long non-specific products in PCR

1. Decrease annealing time

2. Decrease extension time

3. Decrease extension temperature to 62°C - 68°C

4. Increased KCl (buffer) concentration to 1.2X – 2X, but keep MgCl2 concentration at 1.5-2mM

5. Increased MgCl2 concentration up to 3-4.5 mM but keep dNTP concentration constant.

6. Use less primers

7. Take less dna template

8. Take less taq polymerase

9. If none of the above works, check the primer for repetitive sequences (blast align the sequence with the database) and change the primer (s)
10. Combination of some/all the above

PCR primer dimmers

When running an agarose gel, we may spot some very small PCR bands. These are the size of your primer or about the size of both your primers (A and B) together. These are termed “primer dimers” and are formed by the annealing of the primer with itself or with the other primer.

Solutions to primer dimers in PCR

1. Use less primer

2. Re-design primer and order a new batch. Make use of primer design software and check for self-annealing and that the primers do not share a large percentage of complementary sequence. Check primers carefully for homo-dimer and hetero-dimer formation with oligo analyzer.

3. Conduct PCR with or without formamide.

4. Titrate Mg2+ (MgCl₂ – 1.5, 2.0, 2.5 and 3.0mM) concentration, increase DNA template amount (concentration).

5. Increase annealing temperature [try optimizing using a gradient PCR machine to find optimal temperature for annealing.]

6. Try adding DMSO upto 5% try using hotstart PCR instead of regular taq polymerase PCR.

7. Combination of some/all the above.