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Antimicrobial Susceptibility Pattern of *Staphylococcus aureus* Isolated from Clinical Specimens from Wound Samples

Prashant Peshattiwar[1], Sachin Mishra[1*], Sonali Mishra[2] and Pragati Khanorkar[2]

[1]*Department of Microbiology,* [2]*Department of Biochemistry, Index Medical College Hospital and Research Centre, Index City, Nemawar Road, NH-59A, Indore, M.P. 452016, India*
*Corresponding author

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Staphylococcus aureus (*S. aureus*) is a major pathogen associated with serious community and hospital-acquired infections. Methicillin resistant *S. aureus* (MRSA) is now endemic in India. Methicillin-resistant *Staphylococcus aureus* (MRSA) has recently emerged as a major nosocomial pathogen worldwide with a significant morbidity and mortality. Early detection of emerging trends in antimicrobial resistance may facilitate implementation of effective control measures. The present study thus attempts to characterize the MRSA isolates and explore the antibiotic susceptibility pattern of MRSA isolated from wound samples at a tertiary care hospital, Central India. The present study was conducted to characterize 120 MRSA isolates, isolated from wound samples and their antibiotic susceptibility patterns. MRSA was identified by oxacillin disc diffusion test, cefoxitin disc diffusion test and resistance to oxacillin by the Minimum inhibitory concentration (MIC) method. Study also detected inducible clindamycin resistance in *Staphylococcus aureus*. About 120 MRSA isolates from various clinical samples such as such as pus swabs and aspirates, blood, urine, sputum and endotracheal tube aspirates were collected and processed in the laboratory for various tests. All the 120 MRSA strains were 59.17% resistant to mupirocin, 55.83% resistant to ciprofloxacin, 46.67% resistant to Erythromycin, 48.33% to co-trimoxazole, 30% to tetracycline, 21.67% to gentamycin. However, all (100%) MRSA strains were sensitive to vancomycin and linezolid. MRSA showed highest distribution in medical ward as it is a nosocomial pathogen and patients usually acquire it during hospital stay. The treatment of MRSA can become a challenge in the near future. Overuse and misuse of antibiotics along with self-medication should be avoided.

Introduction

S. aureus occurs as a component of normal microbiota of a Healthy individual in 25 – 50% cases. *S. aureus* is the most common species of staphylococcus to cause infections and is a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies. *Staphylococcus aureus* is a Gram-positive coccus belonging to the family Micrococcaceae. (Murray *et al.,* 2003)

Staphylococcus aureus is one of the most harmful species of staphylococci encountered. It is the leading cause of bacteremia, pneumonia, myocarditis, acute endocarditis, pericarditis, osteomyelitis, encephalitis, meningitis, chorioamnionitis, mastitis, and scalded skin syndrome. (Murray *et al.,* 2003)

Human morbidity and mortality in hospital settings are largely caused by staphylococcal bacteremia. The pathogenic capacity of *Staphylococcus aureus* is clearly dependent on its production of exoproteins and toxins (Klevens *et al.,* 2007).

Early detection of emerging trends in antimicrobial resistance may facilitate implementation of effective control measures. The antibiotic susceptibility contributes directly to patient care and the expertise of microbiology laboratory can have a powerful influence on antibiotic usage and hence on the pressure that facilitates the emergence of antimicrobial drug resistance (Karmakar *et al.,* 2016).

Prolonged hospitalization, extensive use of antimicrobial agents, surgical procedures, and close contact with a patient with MRSA colonization or infection are considered to be the risk factors for hospital-acquired (HA) MRSA. Recently, community acquired MRSA infections have been found in healthy individuals although underlying diseases such as diabetes mellitus, chronic skin diseases, intravenous drug use, and recent antibiotic use are the known risk factors (Karmakar *et al.,* 2016; Sachdev *et al.,* 2003; Gopal Rao *et al.,* 2007).

After discharge from the hospitals, patients with HA-MRSA colonization spread these organisms to their family members and other individuals who have contact with them (Cassandra *et al.,* 2003). Therefore, effective prevention of the dissemination of MRSA throughout the communities requires effective control of HA-MRSA transmission. The high endemicity of MRSA poses a problem for antibiotic therapy because of the possible development of resistance to glycopeptides, which could lead to untreatable infections. In addition, MRSA imposes economic burdens on the health care system. Multidrug resistant

(MDR) Staphylococcus isolates in hospital have been recognized as one of the major challenges in the hospital infection control.

These strains are resistant to multiple antibiotics and act as reservoir for drug resistant genes (Majumder *et al.,* 2001). Most of *S. aureus* infections are caused by methicillin sensitive *Staphylococcus aureus* strains (MSSA) that are susceptible to all other classes of anti-staphylococcal antibiotics. Methicillin resistant *Staphylococcus aureus* strains (MRSA) are implicated in serious infections and nosocomial outbreaks. These strains show resistance to a wide range of antibiotics, thus limiting the treatment options to very few agents such as vancomycin and teicoplanin. Rapid and accurate diagnosis of MRSA is important for proper management, prevention of transmission and to start correct treatment.

The present study thus attempts to characterize the MRSA isolates and explore the antibiotic susceptibility pattern of MRSA isolated from wound samples at a tertiary care hospital, Central India.

Materials and Methods

A cross-sectional observational study carried out in a 750 bed tertiary care hospital in Central India. The study commenced after obtaining approval from institutional ethics committee and continued for a span of 10 months. Data were analyzed at the end of study.

Inclusion criteria

Subjects with confirmed MRSA positive wound infections

Subjects 18 years of age and older
Subject's willing to participate

Exclusion criteria

Subjects less than 18 years of age

Subjects not agreeing to participate

Pregnant females

Any condition resulting in severe learning disability (e.g. brain injury) or

Those unable to comprehend for other reasons will be excluded from the study.

A proforma was prepared for recording patient related information and 120 MRSA isolates from clinical materials which were preserved from Oct 2013 to July 2014, from various clinical samples such as pus swabs and aspirates, blood, urine, sputum and endotracheal tube aspirates, were included in this study. Consecutive isolates of MRSA from same patients were excluded.

Study technique

All clinical samples were processed in the laboratory as per standard guidelines. *S. aureus* isolates were identified by standard laboratory procedures. Only strains obtained from a pure culture were included. Only the first strain from each patient was included.

All the strains were collected aseptically, transferred into mannitol salt agar (MSA) media, HiMedia (Mumbai), and incubated overnight at 37°C. (Biswas *et al.,* 2015)

Phenotypic characterization

Samples were plated on 5% sheep blood agar, MacConkey agar and Thioglycolate medium respectively. Plates were incubated at 37°C. After overnight incubation colony morphology and hemolysis was observed duly. Gram's stain was done on white colony showing lysis

on blood agar. Colonies showing Gram positive cocci arranged in clusters were subjected to coagulase test by slide and tube coagulase.

Coagulase positive strains were subjected to antibiotic susceptibility by the modified Kirby Bauer method and isolates showing methicillin resistance in the screening by Cefoxitin disc diffusion (MRSA) were included in study.

Further characterization of the MRSA was done by using Oxacillin screening agar, Oxacillin disc diffusion. MIC for Oxacillin was done for all the isolates by agar dilution and broth dilution and inducible Clindamycin resistance was detected by using the D test (Lancette and Tatini, 1992).

Cefoxitin disc diffusion test

All the isolates were again subjected to Cefoxitin disc diffusion test (for reconfirmation) using a 30 µg disc. A 0.5 Mc Farland standard suspension of the isolate was made and lawn culture done on MHA plate.

Plates were incubated at 37°C for 18 h and zone diameters were measured. An inhibition zone diameter of ≤ 19 mm was reported as oxacillin resistant and ≥20 mm was considered as Oxacillin sensitive. (Lancette and Tatini, 1992)

Oxacillin disc diffusion test

All the isolates were subjected to Oxacillin disc diffusion test using a 1 µg disc. A 0.5 Mc Farland standard suspension of the isolate was made and lawn culture done on MHA plate. Plates were incubated at 37°C for 18 h and zone diameters were measured. An inhibition zone diameter of ≤ 10 mm was reported as Oxacillin resistant, 11-12 intermediate resistance and ≥13 mm was considered as

Oxacillin sensitive. (Lancette and Tatini, 1992)

Inducible clindamycin resistance detection

Sensitivity to erythromycin (15 g) and clindamycin (2 g) was performed by disc diffusion method as per NCCLS guidelines. Isolates which displayed erythromycin resistant but Clindamycin susceptible were subjected.

A 0.5 Mc Farland standard suspension of the isolate was made and lawn culture done on MHA plate and erythromycin (15 μg) and Clindamycin (2 μg) disks were put 17 mm apart edge to edge on the same plate. Plates were incubated at 37 °C for 18 h. Inhibition of the circular zone (flattening of the zone towards Erythromycin disc) around the Clindamycin disc was considered positive for inducible resistance. (Lancette and Tatini, 1992)

Drug susceptibility test

Susceptibility to following antibiotics was studied using modified Kirby Bauer method.Zone sizes were measured and interpreted according to NCCLS guidelines (National Committee for Clinical Laboratory Standards, 2002; Ericsson and Sherris, 1971).

Minimum Inhibitory Concentration (MIC)

MIC of Oxacillin for all the 120 isolates was determined by standard agar dilution and broth dilution method, according to NCCLS guidelines (National Committee for Clinical Laboratory Standards, 2002; Ericsson and Sherris, 1971).

Agar dilution method (Jorgensen and Turnidge, 2007)

Procedure

Dilution of antimicrobial agent: Oxacillin pure

powder was used. Dilutions were made by sterile distilled water, which is the solvent and diluent for Oxaciilin. A stock of Oxacillin was prepared and further dilutions were made.

Preparation of the media

Mueller Hinton agar medium is prepared in tubes, autoclaved and allowed to cool in 50°C water bath. The diluted antibiotic solutions are added to the melted and cooled medium, in a ratio of 1:10 (i.e., 2 ml antibiotic and 18 ml MHA medium). Pure powder of Oxacillin was incorporated into liquefied Mueller Hinton agar medium at 45 – 50°C, mixed, poured in Petri dishes and is solidified. A series of Petri dishes were prepared with increasing concentration of drug (log 2 serial dilution). A control plate containing the test medium without antibiotic was prepared for each series of dilutions. After the plates have set they were dried at 37° C.

Standardization of the inoculum

3- 5 colonies of the strain was inoculated into 2 ml Mueller Hinton Broth and incubated at 37°C for 4 hours. Then turbidity was adjusted to 0.5 Mac Farland Standard (1.5 X 108 CFU/ml). Plates were inoculated within 15 minutes of preparation of the suspension so that the density does not change.

Inoculation of the test plate

The inoculum was applied as a spot that covers a circle about 5- 8 mm in diameter. A wire loop calibrated to deliver 10 μl of the inoculum was used to spot inoculate the 150 strains. Inoculated plates were left undisturbed until spots of the inoculum have dried. The plates are then inverted and incubated at 35°C for 16-18 hrs. ATCC (American type culture collection) *Staphylococcus aureus* was used as control strain.

Interpretation

The control plate should show the growth of the quality control test organism. The MIC of the control strain should be in the expected quality control range. The end point is the lowest concentration of antibiotic that completely inhibits growth. A barely visible haziness or single colony is disregarded. The results are reported as the MIC in microgram (µg) per ml. After overnight incubation, MIC end point was read as the lowest concentration of drug that inhibits the growth.

Broth dilution (Jorgensen and Turnidge, 2007)

Antibiotic sensitivity test (AST) by minimum inhibitory concentration (MIC) of Oxacillin for 120 isolates of, were determined by broth dilution method according to NCCLS guidelines.

Performance of test

MIC by microbroth dilution for above stated antibiotics were performed in a microtitre dilution plate having 96 wells. Controls wells were one for viability (growth) control which had in it 100 µl each of sterile Mueller Hinton Broth (MHB) and test organism. Other was sterility control well with 100 µl of Mueller Hinton Broth only.

All other wells were loaded with 100 µl of MHB, 90 µl appropriate antimicrobial dilutions and finally 10 µl of standardized inoculum (adjusted to 0.5 MacFarland standards). Reading was done by a parabolic magnifying mirror and tray stand that allows clear visual inspection of the under sides of the titre plates. The growth control well was examined for the organism viability and MIC for the control strain was confirmed. Then, the MIC of the test organism was recorded.

Data analysis

All data were entered in a Microsoft Excel 2007 spreadsheet. Data were analyzed at the end of study, where possible, demographic data were analyzed with parametric tests, while the Chi squared test was employed for categorical data. All p values which were < 0.05 were considered to be statistically significant. Statistical analyses for outcome measures were performed by repeated measures analysis of variance using the Statistical Package for the Social Sciences (Windows version 17.0; SPSS Inc, Chicago [IL], US / Graphpad prism / Medcalc). A level of significance of p<0.05 was accepted for the study.

Results and Discussion

About 120 MRSA isolates from various clinical samples such as such as pus swabs and aspirates, blood, urine, sputum and endotracheal tube aspirates were collected and processed in the laboratory for various tests.

Among 120 isolates subjected to alkaline phosphatase test, 114 were found to be phosphatase positive while 6 being phosphatase negative. Among 120 clinical MRSA isolates were processed for urease test, where 116 were found urease positive and 4 were negative. About 120 strains under test were inoculated onto the Mannitol salt agar medium along with appropriate controls and incubated at 37°C for 24 hrs. 112 were found to be positive while 8 were found to be negative. The 120 isolates under the test were inoculated on DNase agar medium. Out of 120 samples, 115 were found to be DNase positive while 5 were DNase negative. Out of 120 isolates subjected to gelatin liquefaction test with appropriate controls, 104 were found to be positive while 16 were tested negative. About 120 isolates were subjected to various tests like Oxacillin disc diffusion test,

Cefotoxin disc diffusion test, MIC of oxacillin and Oxacillin Screening Agar test for detection of Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Sensitive *Staphylococcus aureus* (MSSA) [Table 2].

Inducible clindamycin resistances were detected in 53 isolates (44.2%) [Table 3].

Susceptibility to following antibiotics was studied using modified Kirby Bauer method; zone sizes were measured and interpreted according to NCCLS guidelines (National Committee for Clinical Laboratory Standards, 2002) (Fig. 1–3).

For the past 50 years, *S. aureus* has been a dynamic human pathogen that has gained the deepest respect of clinician since the report of MRSA infection in US at a Boston city hospital in 1961.Since, then MRSA has become wide spread all over the world. The incidence of Methicillin resistant *S. aureus* (MRSA) in India ranges from 30-70% (Raygada and Levine, 2009).

High prevalence of MRSA is an emerging problem in India. Several authors have reported a substantial increase in the prevalence of MRSA in India. It has increased from 12% in 1992 to 40% in 2009 (Verma *et al.,* 2000; Indian Network for Surveillance of Antimicrobial Resistance group, India, 2013). Increasing resistance of MRSA in recent years has had a significant impact on several aspects of patient care and infection control. Our findings showed high prevalence of MRSA in pus swabs (73.33%) followed by blood (9.17%) and endotracheal tube aspirates (6.67%) respectively. This is in accordance with previous studies where most MRSA were found in pus samples.

Staphylococcus aureus is a major human pathogen that is very common and highly virulent. Increased antimicrobial resistance for such an organism is, therefore a cause of concern. As new anti-staphylococcal agents have become available, there has been a subsequent increase in *Staphylococcus aureus* resistance to them. In recent years there has been an alarming increase in the *Staphylococcus aureus* strains showing resistance to methicillin and reduced susceptibility to Vancomycin. The potential reservoirs of MRSA include infectious patients, hospital personnel and hospital environment. This has driven the search for even more drugs and for ways to control the spread of the organisms (Hartstein and Mulligan, 1996).

There are two major types of MRSA infections: health care-associated MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA). HA-MRSA is usually associated with people who have compromised immune systems and who have had frequent or recent contact with hospitals or other long-term care facilities such as nursing homes and dialysis centers. It is commonly transmitted via the hands of health care workers and is associated with severe, invasive diseases in hospitalized patients (Hartstein and Mulligan, 1996). MRSA is now endemic in India. The incidence of MRSA varies according to the region, 25% in western part of India (Patel *et al.,* 2010) to 50% in South India (Gopalakrishnan and Sureshkumar, 2010). Community acquired MRSA (CA-MRSA) has also been increasingly reported from India. For the past 50 years, *S. aureus* has been a dynamic human pathogen that has gained the deepest respect of clinician since the report of MRSA infection in US at a Boston city hospital in 1961.

Table.1 Frequency of MRSA in clinical specimens

Clinical Specimen	MRSA (n=120)	Percentage
Pus swabs and aspirates	88	73.33
Blood	11	9.17
Urine	6	5
Sputum	7	5.83
Endotracheal tube aspirates	8	6.67

Table.2 Pattern of MRSA or MSSA isolates among study samples

Tests	Detected as MRSA [n (%)]	Detected as MSSA [n (%)]	Total
Oxacillin disc	97 (80.83%)	23 (19.67%)	120
Cefoxitin disc	120 (100%)	0	120
MIC of Oxacillin	109 (90.83%)	11 (9.17%)	120
Oxacillin Screening Agar	92 (76.67%)	28 (23.33%)	120

Table.3 Detection of inducible clindamycin resistance in study wounds samples

Test		Total
Inducible Clindamycin Resistance Detected	53	53
No Inducible Clindamycin Resistance Detected	0	53

Table.4 Drug susceptibility test among wounds samples

Antibiotic	Resistant	Sensitive
Amikacin (Ak) - 30 µg	13 (10.83%)	107 (89.17%)
Co-Trimoxazole (Co) - 1.25+23.75 µg	58 (48.33%)	52 (51.67%)
Chloramphenicol (C) - 30 µg	4 (3.33%)	116 (96.67%)
Clindamycin (Cd) - 5 µg	22 (18.33%)	98 (81.67%)
Ciprofloxacin (Cf) - 5 µg	67 (55.83%)	53 (44.17%)
Erythromycin (E) - 15 µg	56 (46.67%)	64 (53.33%)
Gentamycin (G) - 10 µg	26 (21.67%)	94 (78.33%)
Linezolid (L) - 30 µg	0	120 (100%)
Mupirocin (M) - 5 µg	71 (59.17%)	49 (40.83%)
Netilmicin (Nt) - 30 µg	4 (3.33%)	116 (96.67%)
Rifampicin (R) - 5 µg	13 (10.83%)	107 (89.17%)
Tetracycline (T) - 30 µg	36 (30%)	84 (70%)
Vancomycin (V) - 30 µg	0	120 (100%)

Table.5 MIC of oxacillin among wounds samples

Tests	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml	<1 µg/ml	Total
MIC by Broth Dilution	52	32	19	8	3	5	1	120
MIC by Agar Dilution	52	32	19	8	3	5	1	120

MIC was done by both Broth dilution method and Agar dilution method.

The antibiotics used were as follows

Antibiotic	Concentration
Amikacin (Ak)	30 µg
Co-Trimoxazole (Co)	1.25+23.75 µg
Chloramphenicol (C)	30 µg
Clindamycin (Cd)	5 µg
Ciprofloxacin (Cf)	5 µg
Erythromycin (E)	15 µg
Gentamicin (G)	10 µg
Linezolid (L)	30 µg
Mupirocin (M)	5 µg
Netilmicin (Nt)	30 µg
Rifampicin (R)	5 µg
Tetracycline (T)	30 µg
Vancomycin (V)	30 µg

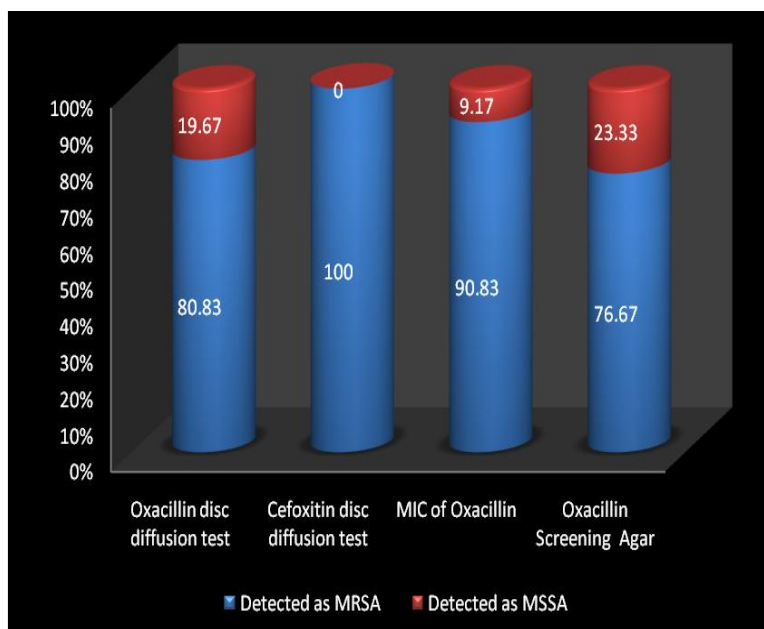**Fig.1** Pattern of MRSA or MSSA isolates among study samples

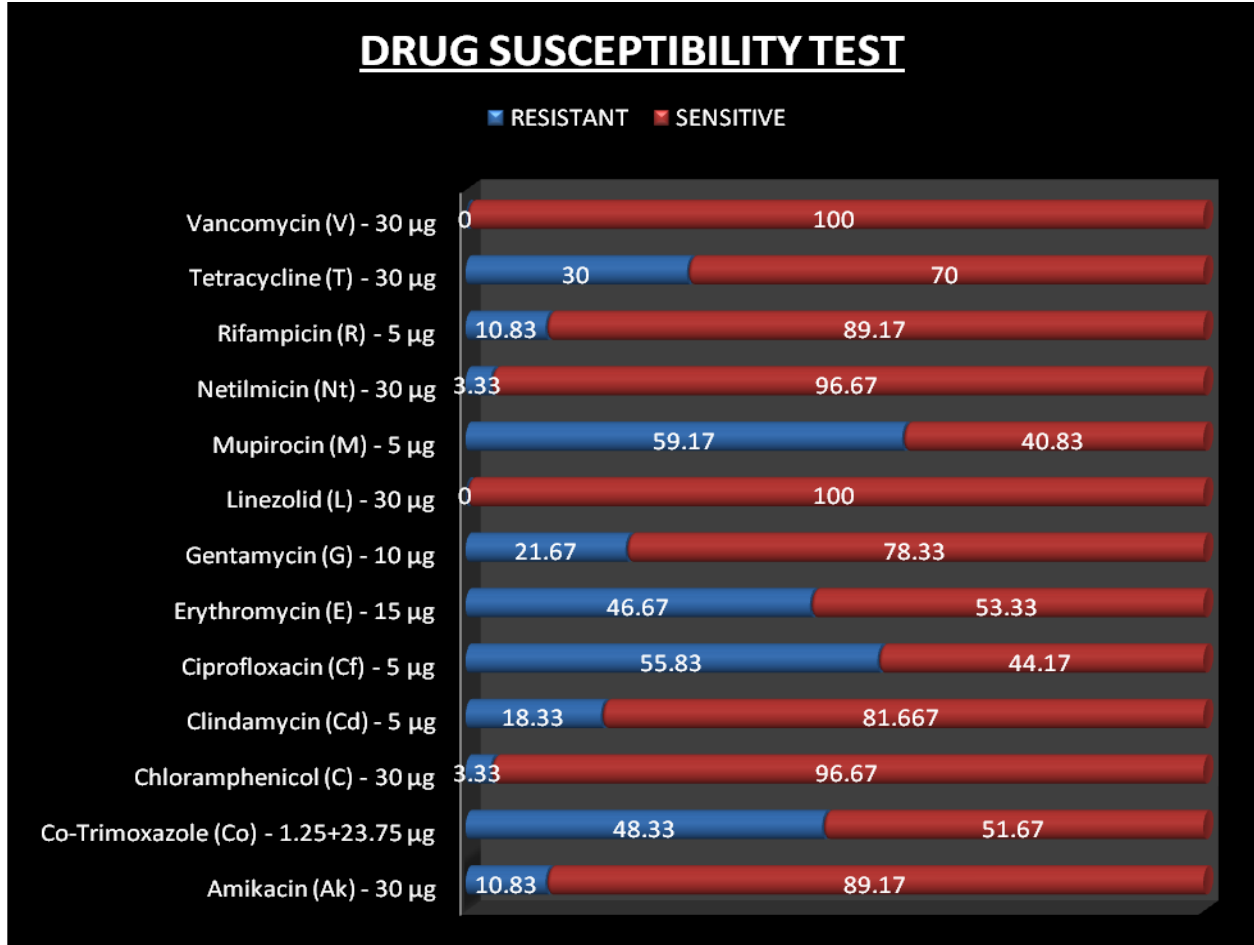**Fig.2** Pattern of drug susceptibility test among wounds samples

Fig.3 Colonies of *S. aureus* (MRSA) on nutrient agar (golden yellow pigment)

Since, then MRSA has become wide spread all over the world. The incidence of Methicillin resistant *S. aureus* (MRSA) in India ranges from 30-70%.

In our present study, drug resistance patterns of 120 MRSA isolated from clinical specimens was found to be highly variable. All the 120 MRSA strains were 59.17% resistant to mupirocin, 55.83% resistant to ciprofloxacin, 46.67% resistant to Erythromycin, 48.33% to co-trimoxazole, 30% to tetracycline, 21.67% to gentamycin. However, all (100%) MRSA strains were sensitive to vancomycin and linezolid.

Gentamycin resistance in MRSA is worldwide. Mechanism of resistance is drug inactivation by cellular transferase enzyme. Even when the organisms are sensitive either alone or with beta-lactams has proved to be less satisfactory for treatment of Staphylococcal infections. Maple *et al.,* (1989) found resistance to gentamycin, tobramycin, netilmicin and amikacin to be more than 90%, Pulimood *et al.,* (1996) reported 85.5% resistance to gentamycin. (Majumder *et al.,* 2001) reported 94.1% resistance to gentamycin and 20.5% by Rajaduraipandi *et al.,* (2006).

MRSA strains are also resistant to macrolides. Strains resistant to erythromycin are generally cross resistant to clarithromycin and azithromycin. Mechanism of resistance is target site alteration. Maple *et al.,* in 1989 recorded 90% resistance to erythromycin. Maple *et al.,* (1989) reported 17% resistance to ciprofloxacin, Pulimood *et al.,* (1996) reported 90% resistance and Majumder *et al.,* (2001) reported 22.8% resistance and 12.8% by Rajaduraipandi *et al.,* (2006). All the above studies show that the MRSA isolates are often resistant to multiple antibiotics. Therefore treatment of infections due to this organism and its eradication is difficult, and

also use of beta lactam antibiotics in MRSA infections will increase antibiotic selection pressure. In the present study linezolid and Vancomycin were found to be useful drugs in treating MRSA infections and similar findings were observed by Rajaduraipandi *et al.,* (2006) with 100% sensitivity for both the drugs. For life threatening Staphylococcal infections and especially for MRSA strains, Vancomycin thus is the drug of choice (Naimi *et al.,* 2004).

The resistance of MRSA to a wide range of antibacterials is well documented. This makes the empirical use of antibacterials effective against MRSA imperative. The regular surveillance of hospital associated infections including monitoring antibiotic sensitivity pattern of MRSA and formulation of definite antibiotic policy may be helpful for reducing the incidence of MRSA infections. The degree of resistance or sensitivity of MRSA towards commonly used antibiotics is recognized to be diverse from region to region and vancomycin was the only antibiotic found to give uniform sensitivity (100%). When antimicrobials including vancomycin are considered for treatment, choice inevitably requires the need for in vitro susceptibility testing of every isolate of MRSA in the clinical laboratories.

In conclusion, the degree of resistance or sensitivity of MRSA towards commonly used antibiotics is recognized to be diverse from region to region and vancomycin was the only antibiotic found to give uniform sensitivity (100%). When antimicrobials including vancomycin are considered for treatment, choice inevitably requires the need for in vitro susceptibility testing of every isolate of MRSA in the clinical laboratories. The study of prevalence of MRSA will not only provide the current antimicrobial situation but also help to devise the appropriate treatment of these infections. Hospitals are also

contributing to a great extent in spreading antibiotic resistance elevating MRSA. Our study is a preamble to enable epidemiologists to understand the nature of MRSA isolates in this part of India. MRSA showed highest distribution in medical ward as it is a nosocomial pathogen and patients usually acquire it during hospital stay. The treatment of MRSA can become a challenge in the near future. Overuse and misuse of antibiotics along with self-medication should be avoided. Improved diagnostic techniques can produce better results by promoting targeted therapy. Laws should be devised that will keep a check on the prescriptions made by doctors.

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