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A Study on Antibiogram of *Staphylococcus Aureus* Isolates with Special Reference to MRSA Screening Methods in a Tertiary Care Hospital in Kerala

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Abstract
Introduction: *Methicillin resistant S. aureus (MRSA), which are often resistant to several classes of antibiotics, is the most common cause of nosocomial infections.*
Aim: *The objective of the study was 1)to find out antibiotic susceptibility pattern of clinically significant Staphylococcus aureus isolated from various samples and, 2)to compare four methods for MRSA detection.*
Materials and Methods: *This is a cross sectional study conducted in the Department of Microbiology, Govt. Medical College, Thiruvananthapuram. During the study period 208 strains of Staphylococcus aureus were collected and were characterized. Antibiotic susceptibility was performed by disc diffusion test using Clinical Laboratory Standards Institute guidelines. Polymerase chain reaction was done for the detection of mec A gene. D test was performed for those strains which were resistant to Erythromycin. The present study was undertaken to compare four phenotypic methods for the detection of MRSA, namely, the Cefoxitin disc diffusion method, Oxacillin screen agar, HiChrome agar and Mannitol salt agar with Cefoxitin.*
Results: *Among MSSA,(152) resistance to Penicillin, Erythromycin, Ciprofloxacin, Tetracycline, Gentamicin, Clindamycin and Cotrimoxazole were 94.73%, 9.8%, 59.86%, 3.94%, 36.84%, 7.89% and 15.78% respectively. Among MRSA,(56) resistance to Erythromycin, Ciprofloxacin, Tetracycline, Amikacin, Clindamycin and Cotrimoxazole were 57.14%, 73.21%, 67.85%, 14.28%, 28.57% and 57.14% respectively. Vancomycin E strip test was done for all MRSA isolates (56), and all of them had MIC below 2µg.(sensitive) Sensitivity of Oxacillin screen agar was 91.07% and specificity was 100%.For other methods sensitivity and specificity was 100%.*
Keywords: *MRSA, Erythromycin induced Clindamycin resistance, Vancomycin E test.*

Introduction

Staphylococcus aureus has acquired resistance to various antibiotics and is a leading cause of hospital and community acquired infections, manifesting from minor skin diseases to life threatening infections. Methicillin resistant *Staphylococcus aureus* (MRSA) has emerged as one of the most important nosocomial pathogens

especially in the last two decades .The presence of gene mecA codes for PBP2a and confers resistance in *Staphylococcus aureus*. Now CA MRSA has resulted an increase in skin and soft tissue infections primarily in young individuals. Even though Clindamycin is effective for MRSA, inducible Clindamycin resistance has resulted in treatment failure. In microbiology laboratories D tests should be done routinely for all Staphylococcal isolates which are resistant to Erythromycin.

Materials and Methods

Clinically significant isolates of Staphylococci from Microbiology Laboratory Medical College Hospital Thiruvananthapuram, were taken for study from July 2012 to June 2013. During the study period 208 strains of Staphylococci were collected and were characterized. In case of MRSA, only those which were repeatedly isolated from the same site with evidence of infection were taken for study. A detailed history was taken from all patients using a pretested proforma. It included name, age, sex, short history of case, nature of specimen, antibiotic dose and duration.

All strains were confirmed by gram stain, catalase test and coagulase test. All strains were subjected to antibiotic susceptibility testing by Kirby Bauer disc diffusion method in Mueller Hinton agar according to CLSI guidelines. *Staphylococcus aureus* ATCC 25923 was used as the standard strain. The number of discs per plate was limited to six.

The Antibiotics tested were Penicillin (10µg), Erythromycin (15µg), Gentamicin (10µg), cepha-losporin first generation (30µg), Cefoxitin (30µg), Amikacin (30µg), Rifampicin (5µg), Linezolid (30µg), Clindamycin (2µg), Ciprofloxacin (5µg), Tetracycline (30µg), Cotrimoxazole (1.25/23.75µg), Teicoplanin (30µg), Vancomycin (E test). The zone of inhibition was interpreted according to CLSI guidelines. E test was used for finding out the MIC of Vancomycin. All strains that were Erythromycin resistant were tested for the presence of iMLSB resistance by double disc diffusion assay (D test) according to CLSI guidelines. The test was done on Mueller Hinton agar with Clindamycin (2µg) and Erythromycin (15µg) placed 15 mm apart on the same plate. Flattening of zone of inhibition adjacent to the Erythromycin disc is referred to as a D-zone. D-zone indicates inducible Clindamycin resistance.

We interpreted the results according to three phenotypes:

1) **MS Phenotype** - Staphylococcal isolates resistant to Erythromycin but sensitive to Clindamycin giving a circular zone of inhibition around Clindamycin
2) **iMLSB Phenotype** - Staphylococcal isolates resistant to Erythromycin while being susceptible to Clindamycin and giving a D shaped zone of inhibition around Clindamycin
3) **cMLSB Phenotype** - Staphylococcal isolates which showed resistance to both Erythromycin and Clindamycin MRSA screening

1. Cefoxitin Disk Diffusion Method

This was done by Kirby baur method in Mueller Hinton agar plate by using cefoxitin 30µg disk .Test strains with zone diameter less than 22 mm is taken as MRSA.

2. Oxacillin Screen Agar Method

Suspend 51.75 grams in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure at 121°C for 15 minutes. Cool at 45-50°C and aseptically add 6µg Oxacillin. Mix well and pour into sterile petri dishes. Prepare suspension of strains to obtain 0.5 McFarland turbidity. Using a 1µl loopthat was dipped in suspention, spot an area 10-15mm in diameter into the agar. Incubate for 24 hours at 33-35^{0}C.Examine carefully with transmitted light for more than one colony. More than one colony indicates oxacillin resistance. No growth indicates that the organismis susceptible to oxacillin.

3. Screening with Mannitol Salt Agar Cefoxitin Medium

Suspend 111.02 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Add cefoxitin 4µg/ml to the medium, mix well and pour into petri dishes. Prepare suspension of strains having 0.5 McFarlands turbidity and spot inoculate into the agar. Incubate at 37^0C for 24 hours. Any growth after 24 hours of incubation indicates methicillin resistance.

4 HiCrome MRSA Agar Base

It is recommended for the isolation and selective identification of Methicillin Resistant Staphylococcus aureus (MRSA) from clinical isolates.

Suspend 41.65 grams in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Aseptically add sterile rehydrated contents of 1 vial of Selective Supplement and 1 vial of Cefoxitin supplement. Mixwell and pour into sterile petri dishes. Emulsify the colony to be tested into peptone water and adjust to 0.5 McFrlands turbidity and spot inoculate to the MRSA screen agar plate. Incubate at 30-35°C for 18-48 hours. Any growth indicates MRSA. It will appear as bluish green in colour.

Results

In the present study 152 (98.02%) MSSA and 56 (78.57%) MRSA were isolated from pus. Highest number of isolates of MSSA (46) were obtained from the age group 41-50 years, and MRSA (19) from 51-60 years. The isolation rate of Staphylococci were more from males (MSSA-113, MRSA-44). A total of 56 MRSA were sent for mecA gene detection to the National Institute of Epidemiology, Chennai and mecA gene was detected in all.

Among MSSA, resistance to Penicillin, Erythromycin, Ciprofloxacin, Tetracycline, Gentamicin, Clindamycin and Cotrimoxazole were 94.73%, 9.8%, 59.86%, 3.94%, 36.84%, 7.89% and 15.78% respectively. Among MRSA, resistance to Erythromycin, Ciprofloxacin, Tetracycline, Amikacin, Clindamycin and Cotrimoxazole were 57.14%, 73.21%, 67.85%, 14.28%, 28.57% and 57.14% respectively. Vancomycin E test was done for all MRSA isolates, all of them had MIC below 2µg.The antibiotic resistance pattern of isolates is given in Table.1.

Table 1. Antibiotic resistance pattern of Staphylococcal isolates

Isolates	P (10µg)	G (10µ)	Ak (30µ)	Ce I (30µg)	Cn (30µg)	Co (1.25-23.75µg)	E (15µ)	Cip (5µg)	T (30µ)	L (30µ)	R (5µg)	Cl (2µg)	Te (30µg)
MSSA	144 (94.73 %)	56 (36.84 %)	0 (0%)	0 (0%)	0 (0%)	24 (15.78%)	15 (9.8%)	91 (59.86 %)	6 (3.94%)	0 (0%)	0 (0%)	12 (7.89%)	0 (0%)
MRSA	NT	51 (91.07 %)	8 (14.2 8%)	NT	56 (100%)	32 (57.14%)	32 (57.14 %)	41 (73.21 %)	38 (67.85 %)	0 (0%)	0 (0%)	16 (28.57 %)	0 (0%)

(P-Penicillin, G-Gentamicin, Ak-Amikacin, Ce 1-Cephalosporin first generation, Cn-Cefoxitin, Co-cotrimoxazole, E-Erythromycin, Cip-Ciprofloxacin, T-Tetracycline, L-Linezolid, R-Rifampicin, Cl-Clindamycin, Te-Teicoplanin, NT-not tested)

Among 208 isolates 47 were resistant to Erythromycin. These isolates, when they were subjected to the D test, showed 28 (59.57 %) constitutive MLSB (cMLSB) phenotype, 19(40.42%) isolates showed clindamycin sensitivity. Out of these, 15 (31.91%) strains were D-test positive i.e. of the inducible MLSB (iMLSB) phenotype, while 4(8.51%) were MS phenotype. Of the 28 cMLSB phenotypic strains, 16 isolates were MRSA and 12 were MSSA, while among iMLSB phenotype 3 strains were MRSA and 1 was MSSA.(Fig 1-3)The results of D test is given in Table 2.

Table 2. Analysis of isolates by D test

Isolate	Total	iMLSB phenotype	cMLSB phenotype	MS phenotype
MRSA	32	13	16	3
MSSA	15	2	12	1
Total	47	15	28	4

Fig-1, iMLSB phenotype

Fig-2,c MLSB phenotype

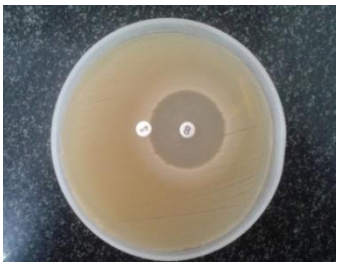

Fig-3,MS phenotype

Discussion

MRSA is now endemic in India. MRSA is of serious concern not due to its sole resistance to Methicillin, but also because of resistance to many other antimicrobials that are used on a regular basis in hospitals. Across the globe, it was found to be the most common cause of bacteremia, respiratory, and skin infections. Its risk factors remain constant.

There are three main mechanism of resistance to the penicillins: (i) Cleavage of the β-lactam ring by β-lactamases/penicillinases, (ii) alterations in the target PBPs and (iii) a permeability barrier preventing penetration of the antibiotic into the cell. The first two mechanisms are especially important to β-lactam resistance in *S. aureus*[1].

Hospital-associated MRSA characteristically colonizes or infects hospitalized individuals with predisposing risk factors like surgery, presence of indwelling medical devices, an immunocom-promised state or prior antibiotic exposure. It is usually isolated from wound infections, line-associated bacteremia and ventilator associated pneumonia.

CA-MRSA infections have been identified often in the context of dramatically rising prevalence of MRSA in hospitals with MRSA isolation rates approaching 50% of *S. aureus* infections. It is actually estimated that 80-95% of CA-MRSA infections involve the skin and soft tissues.

According to INSAR group the overall prevalence of Methicillin resistance during the study period 2008 to 2009 was 41 per cent[2]. In the present study MRSA isolation rate is 26.92%.In a study conducted by N Pal et al.[3] and Lahari Sakia et al[4], which showed incidence of MRSA 30% and 31% respectively and is almost similar to the present study. A study by Arti Tyagi et al reported 15.38% MRSA from 2080 pus samples[5] and similar study conducted by Krishna B V S et al., reported 18.1% of MRSA strains isolated from various samples[6], which are lesser compared to our study. In a study conducted by S Anuprabha et al[7] showed 54.85% of MRSA from 549 samples which was higher compared to our study.

In studies conducted in the Department of Microbiology, Coimbatore, showed a resistance of 63.2% to Erythromycin in the year 2006[8], 80% by S. Anupruba et al[7], 83% by Habeeb Khadri et al[9] and 32.4% by V. Deotale et al[10]. In the present study the resistance to Erythromycin for MSSA and MRSA was 22.17% and this is lesser compared with the above studies, may be due to less usage of this antibiotic for treating Staphylococcal infections.

The cross-resistance for 3 antibiotic families that share a common binding site-macrolides (eg, Erythromycin, Clarithromycin, Azithromcyin), lincosamides (eg, Clindamycin), and group B Streptogrammins (eg,Quinupristin), occurs due to enzymatic dimethylation of an adenine residue in these antibiotics' binding site in the 23S rRNA component of the 50S ribosomal subunit. The methylase enzyme is encoded by a gene *erm*,

which occurs predominantly as variants *erm* (C) or *erm* (A) in staphylococci. A negative D-test (round zone) indicates efflux-mediated macrolide resistance with retained Clindamycin susceptibility.

Among the 208 *Staphylococcus aureus* strains which were studied, 47(22.17%) were Erythromycin resistant. These isolates, when they were subjected to the D test, showed 28 (59.57 %) constitutive MLSB (cMLSB) phenotype,15 (31.91%) strains were inducible MLSB (iMLSB) phenotype, while 4(8.51%)were of the MS phenotype. Of the 28 cMLSB phenotypic strains, 16 isolates were MRSA and 12 were MSSA, while among iMLSB phenotype 3 strains were MRSA and 1 was MSSA. In a study done by Patel M in 2006, among 402 *S. aureus* isolates, the overall prevalence of iMLSB was 52%, with 50% of MRSA and 60% of Methicillin-susceptible *S. aureus* isolates exhibiting iMLSB[11].In the present study out of 212 isolates, the overall prevalence of iMLSB is 7.07% with 6.13% of MSSA and 0.94% of MRSA and it is lesser compared to the above study.

In the present study resistance of MSSA and MRSA to Tetracycline was 3.94 % and 67.85% respectively. In a study done by Nasira et al. in 2011, resistance of MSSA and MRSA to Tetracycline was 68.8% and 100% respectively, and this is very much higher compared to the present study[12].In a study done by Mulla et al. in 2007, resistance of MSSA and MRSA to Tetracycline was 27.5% and 63.1% respectively[13]. According to this study, resistance of MSSA to Tetracycline is lesser compared to the present study, and to MRSA is comparable to the present study.

In the present study all strains of MRSA were sensitive to Linezolid and Rifampicin. Although Linezolid resistance has been reported for *S. aureus*, rates of resistance remain very low. Although linezolid is essentially bacteriostatic against *S. aureus* in vitro, a number of serious cases of MRSA, hVISA and VISA infections, including endocarditis, have been cured with Linezolid.

Susceptibility breakpoints established by CLSI for Vancomycin are currently as follows: susceptible, _2 mg/mL; intermediate (VISA), 4–8 mg/mL; and resistant (VRSA), _16 mg/mL[15].In the present study 56 MRSA isolates were tested for Vancomycin E test,all had MIC<2µg. While the increased prevalence of MRSA may seem alarming, the main concern now is the dissemination of Vancomycin-resistant *S. aureus* (VRSA).There are also reports of a strain termed hetero-VRSA. Although not VRSA, it seems to generate mm cells at a high frequency within its cell population. It is proposed to be the precursor stage of resistance to Vancomycin.

In the present study based on the antibiotic resistance pattern MRSA were catagorised into CA-MRSA and HA-MRSA. Among 56 MRSA, 24(42.85%) were sensitive to both Cotrimoxazole and Erythromycin and Amikacin, and grouped into CA-MRSA. The resistances of MRSA to non β-lactam antibiotics were the characteristics of HA-MRSA. With this regards, the present study result shows the mixing of HA-MRSA and CAMRSA in the community. Several researchers identified the possible mixing of HA-MRSA and CAMRSA. Susceptibility to more than two antimicrobials has been used as a proxy defining criterion to identify CA-MRSA.

In the present study four phenotypic methods for detection of Methicillin Resistant Staphylococcus aureus were compared with the gold standard method mec A gene detection. It was found that for Cefoxitin disc diffusion method, sensitivity was 100%, Sensitivity was lowest for Oxacillin screen agar. For HiChrome agar and Mannitol salt agar sensitivity was 100% and specificity was 100%. The oxacillin disk diffusion test which was used routinely earlier is showing low sensitivity and specificity compared to other methods. Our study strengthens the point that cefoxitin is superior to oxacillin as an indicator of MRSA for the detection of meticillin resistance.

Now genotyping is used to tracking outbreaks, also to distinguish between contaminating and infecting isolates and between separate episodes of infection and relapse of disease. Genotyping is also able to link certain *S. aureus* clonal types and disease syndromes, such as in cases of food poisoning and toxic-shock syndrome. This study highlights that an antibiotic policy and monitoring of susceptibility pattern of *Staphylococcus aureus* help in decreasing the prevalence of MRSA and Antibiotic resistance. Infection control measures like proper hand hygiene and surveillance.

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