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Detection of Methicillin Resistant Strains of *Staphylococcus aureus* Using Phenotypic and Genotypic Methods in a Tertiary Care Hospital

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A B S T R A C T

Detection of Methicillin resistant strains of *Staphylococcus aureus* using phenotypic and genotypic methods in a tertiary care hospital Institution: Institute of Microbiology, Madras Medical College. Abstract: Purpose: Methicillin resistant *S. aureus* (MRSA) are significant pathogens that have emerged over the past 30 years to cause both nosocomial and community acquired infections. This study has been undertaken to compare the conventional and molecular methods in detecting MRSA. *S. aureus* was isolated from pus samples and identified by Gram stain and Catalase test. Further confirmation was done by slide and tube coagulase test, growth on MSA, DNA se test. Methicillin resistance was determined by disc diffusion method, oxacillin screen agar, micro broth dilution test and also by detection of *mecA* gene by multiplex PCR method. The antimicrobial susceptibility pattern of the isolates was determined by Kirby-Bauer disc diffusion method. Vancomycin resistance was determined by disc diffusion and E-test method as per CLSI guidelines. Results: A total of 150 *S. aureus* was isolated from pus samples. Phenotypic methods detected 54% of isolates as methicillin resistant and 46% as methicillin susceptible. Detection of *mecA* gene was done by multiplex PCR in 50 isolates which showed 29 isolates (58%) as methicillin resistant. All the isolates were sensitive to Vancomycin by disc diffusion method and E test. Antibiotic sensitivity results showed that MRSA strains were more resistant to antibiotics compared to methicillin sensitive *Staphylococcus aureus* (MSSA) isolates. Conclusion: The conventional phenotypic methods were comparable with that of *mecA* gene detection, the gold standard and hence it can be used in the detection of MRSA isolates.

Introduction

Skin and soft tissue infection are common type of infection that may contribute to longer hospital stays, significantly increase the cost of medical care and are likely to have an important role in the development of antimicrobial resistance[1]. Most of these infections are superficial and readily treated with a regimen of local care and antibiotics. However soft tissue infections involving deeper layers like fascia and muscle can rapidly progress to systemic sepsis and prove fatal[2]. *Staphylococcus aureus* is one of the common organisms associated with soft tissue infection[3]. Infections caused by *S. aureus* used to respond to beta-lactam group of antibiotics. Penicillin resistant *S. aureus* strains began emerging in 1940. Resistance to methicillin was noticed as early as in 1961[4].

The prolonged hospital stay, indiscriminate use of antibiotics and the lack of awareness are possible predisposing factors for MRSA emergences[5]. Many of the MRSA strains are multi-drug resistant and are susceptible only to glycopeptide antibiotics such as vancomycin[6]. Hence this study has been undertaken to compare the conventional and molecular methods in detecting MRSA and also in detecting the antimicrobial susceptibility pattern of the isolates.

Materials and Methods

Selection of sample

The study was carried out at Institute of Microbiology, Madras Medical College. Pus samples were collected from skin and soft tissue infections from patients admitted in General Surgery and allied specialties at Government General Hospital, Chennai. Ethical and research clearance was obtained from the Institute of Ethical Committee Government General Hospital and Madras Medical College.

Sample processing

The collected pus samples were subjected to direct Gram stain and inoculated onto nutrient agar, blood agar and Mac Conkey agar. The plates were incubated at 37ºC and inspected after overnight incubation. *Staphylococcus aureus* was identified by its colony morphology, Gram stain and catalase test. Further confirmation was done by slide and tube coagulase test, growth on Mannitol salt agar and DNase test by standard microbiological techniques as recommended by CLSI guidelines[7].

Detection of methicillin resistance

Disc diffusion method

Methicillin resistance was determined by using 1µg Oxacillin and 30µg Cefoxitin by Kirby Bauer disc diffusion method and incubating at 35ºC for 24 hours[7].

Oxacillin screen agar

Oxacillin working solutions 6µg/ml is prepared and added to Mueller Hinton agar with 4% NaCl. *Staphylococcus aureus* culture corresponding to 0.5 McFarland turbidity standard is prepared and spot inoculated on the agar. The plates were incubated for 24 hours at 35ºC. The appearance of even a single colony on Oxacillin screen agar indicates Oxacillin resistance[8].

Determination of MIC – Oxacillin by broth microdilution method

Inoculums were prepared by inoculating single purified colonies of bacterial and control strains into Mueller Hinton Broth and incubated overnight at 37ºC. Optical density was measured with a spectrophotometer at 546nm. The density of the inoculum was adjusted to 10^5 CFU/ml and used in MIC determination. MIC was determined in duplicate in Mueller Hinton Broth containing serial two-fold dilutions of Oxacillin with inoculated bacterial suspensions of 10^5 CFU/ml as outlined by CLSI. The results were recorded after overnight incubation at 37ºC. The MIC was defined as the lowest antibiotic concentration with no visible growth[9].

Determination of *mecA* gene by Multiplex PCR method

The mec A gene codes for Penicillin Binding Protein 2A (PBP2A) that is responsible for methicillin resistance. The *mecA* gene is highly conserved among Staphylococcal species, therefore presently detection of this gene by PCR is considered as ‘gold standard’ for detection of methicillin resistance in Staphylococci[10].

Molecular diagnostic assays based on the detection of the *mecA* gene encountered difficulty in discriminating MRSA from methicillin resistant coagulase negative Staphylococcus species (MR-CoNS) because the *mecA* gene is widely distributed in *S. aureus* as well as in MR-CoNS[11].

In this study multiplex PCR was used which allows the detection of MRSA by using primers specific for methicillin resistance and coagulase genes. The *coag* gene was used to differentiate between *S. aureus* and *CoNS*, a gene which allows species specific identification of *S. aureus*. In addition MRSA harbours the *mecA* gene encoding methicillin resistance, which is absent in methicillin susceptible Staphylococci[11].

Cell lysates of the isolates were used as DNA template for colony lysates PCR. Two sets of primers were used for multiplex PCR. The first pair of primers was derived from the region of the *mecA* gene.

The second pair of primers was derived from the region of the *coag* gene. Forty amplification cycles were performed with an automated thermocycler.

Amplified products were run using horizontal 1.5% agarose gel electrophoresis. The gel was visualized using a UV transilluminator.

The amplified PCR products and 100 base pair DNA molecular markers were seen as bright florescent bands[6]. A 533bp corresponds to *mecA* and 810bp corresponds to coag gene specific oligo nucleotides.

Detection of antimicrobial susceptibility pattern

The sensitivity to common antibiotics was done by Kirby-Bauer disc diffusion method as recommended by CLSI[7].

Determination of vancomycin resistance by disc diffusion

Testing for vancomycin resistance was done by using 30µg vancomycin disc by Kirby Bauer disc diffusion method[7].

MIC for vancomycin by E-test

Epsilometer (E-test) is based on a combination of both diffusion and dilution tests. It consists of a strip made of inert material with 8 extensions that carry the discs of 4mm, resembling the 'tooth of comb'.

A defined concentration of antibiotic is loaded on each of the disc so as to form a gradient when placed on agar plate. A 0.5 McFarland turbidity standard of *S. aureus* was inoculated as a lawn culture on Mueller Hinton Agar with 2% NaCl.

E-strips were placed on the agar surface and plates were incubated at 35ºC for 24 hours. MIC was read where the ellipse intersects the scale[11].

Statistical analysis used

Statistical package for social sciences (SPSS) and Epi- info

Results and Discussion

Methicillin resistance was determined for a total of 150 *S. aureus* isolates from pus samples by oxacillin and cefoxitin disc diffusion method, oxacillin screen agar, MIC-broth microdilution method and PCR for *mecA* gene. Most of the isolates were from orthopedics department 51 (34%) followed by general surgery 42 (28%) (Table 1).

53.3% of the strains were found to be resistant, 3.3% were intermediate and 43.3% were susceptible strains by oxacillin disc

diffusion method (Table 2) 46% of strains were sensitive and 54% were resistant by cefoxitin disc method and microbroth dilution method and oxacillin screen agar method (Table 3).

Among MRSA strains, high level oxacillin resistance ≥32µg/ml was found in 58 strains. Moderate level resistance ≤16≥8µg/ml was found in 19 strains (13%). Low level resistance 4µg/ml was found in 5 strains (3%) (Table 4).

Multiplex PCR for the detection of *mecA* gene and *coag* gene detected 29 strains (58%) as methicillin resistant and 21 strains (42%) as methicillin susceptible (Table 5). Among MRSA strains all strains (100%) were resistant to penicillin. More than 80% strains were resistant to cefotaxime, cephalexin, cefaclor, ampicillin, gentamicin and erythromycin. Moderate level of resistance was detected to ciprofloxacin, co-trimoxazole, amikacin and ofloxacin. However the strains were highly sensitive to chloramphenicol and rifampicin. 100% sensitivity was observed to vancomycin, teicoplanin and linezolid (Table 6). Among the MSSA strains, 95.6% strains were resistant to penicillin, moderate level of resistance were seen to ampicillin, co-trimoxazole and cephalexin. However majority of strains were sensitive to cefotaxime, erythromycin, chloramphenicol, ciprofloxacin, ofloxacin and rifampicin. All strains were sensitive to vancomycin, teicoplanin and linezolid (Table 7). All strains were sensitive to vancomycin by E-test method also (Table 8).

Table.1 Distribution of the sample source

N = 150

Speciality	**No. of cases**	**Percentage**
1. Orthopedics	51	34
2. General Surgery	42	28
3. Neuro Surgery	17	11.3
4. Dermatology	30	20
5.Cardiothoracic Vascular Surgery	4	2.7
6. Otorhino layrngology	4	2.7
7. Gastroenterology	2	1.4

Table.2 Results of methicillin resistance in *S. aureus* as determined by oxacillin (1ug) disc diffusion method

Pattern of Resistance	**No. of cases**	**Percentage**
1) Susceptible	65	43.3
2) Intermediate	5	3.3
3) Resistant	80	53.3

Table.3 Results of methicillin resistance in *S. aureus* as determined by cefoxitin (30µg) disc diffusion method, microbroth dilution method and oxacillin screen agar method

N=150

Pattern of Resistance	**No. of cases**	**Percentage**
1) Susceptible	69	46
2) Resistant	81	54

Table.4 Pattern of Oxacillin Resistance among MRSA isolates

N = 82

Oxacillin Resistance Pattern	**MIC µG/ML**	**No. of cases**	**Percentage**
High-level	≥ 32 µg/ml	58	71
Moderate level	≤ 16 ≥ 8 µg/ml	19	23
Low level	4 µg/ml	5	6

Table.5 Results of *MEC A* gene detection by PCR

N =50

mec A	**Total no. of cases**	**Percentage**
Positive	29	58
Negative	21	42

Table.6 Anti-microbial susceptibility pattern of MRSA

N = 81

Antibiotics	**Susceptible (%)**	**Intermediate (%)**	**Resistant (%)**
Penicillin			100
Cefotaxime		4	96
Cephalexin	6		94
Ampicillin	8		92
Gentamicin	16.4	3.6	80
Erythromycin	10.5	9.5	80
Cefaclor	22		78
Ciprofloxacin	11	12	77
Co – trimoxazole	36		64
Amikacin	30.5	25.6	44
Ofloxacin	49	11	40
Chloramphenicol	78.1	7.3	14.6
Rifampicin	85.4	7.3	7.3
Vancomycin	100		
Teicoplanin	100		
Linezolid	100		

Table.7 Anti-microbial susceptibility pattern of MSSA

N = 69

Antibiotics	Sensitive (%)	Intermediate (%)	Resistant (%)
Penicillin	4.4		95.6
Ampicillin	22		78
Cephalexin	38		62
Cefotaxime	75		25
Cefaclor	71		29
Erythromycin	52.4	17.6	31
Chloramphenicol	92.6		8.8
Co – trimoxazole	46		54
Gentamicin	57.3	4.4	39.7
Amikacin	60.2	22	17.6
Ciprofloxacin	53	17.6	30.8
Ofloxacin	59.7	13.2	28.5
Vancomycin	100		
Teicoplanin	100		
Linezolid	100		
Rifampicin	98		2

Table.8 Results of vancomycin resistance determined by disc diffusion and E test method

N = 150

Pattern of resistance	No. Of cases	Percentage
Susceptible	150	100’
Intermediate	-	-
Resistant	-	-

Oxacillin disc diffusion method was not reliable for the identification of methicillin intermediate strains. Cefoxitin is a better inducer of *mec A* gene and disc diffusion tests using cefoxitin give clearer end points and are easier to read than tests with oxacillin. The present study reveals that using cefoxitin is a good alternative method for oxacillin disc diffusion method for the detection of MRSA especially in the identification of intermediate resistant strains of *S. aureus*.

The conventional MRSA detection assays are simple and relatively cheap in detecting methicillin resistance. Accurate determination of MRSA by conventional methods is subjected to variation in inoculum size, incubation time, temperature, pH and salt concentration. It is in such instance that detection of *mec A* gene is useful by molecular methods[12]. In this study, multiplex PCR was used to detect the presence of *mec A* gene and *coag* gene in 50 isolates. In this study, on comparing the phenotypic and genotypic results of the 50 isolates, the phenotypic methods such as oxacillin screen agar, cefoxitin disc diffusion, MIC-microbroth dilution methods, had sensitivity of 90%, specificity of 100% and accuracy of 94%. The phenotypic results are therefore comparable with that of mec A- the gold standard. In the present study, the antibiotic sensitivity results show that all MRSA isolates were more resistant to antibiotics as

compared to MSSA isolates. In this study disc diffusion method and E-test detected all isolates of *S. aureus* as vancomycin sensitive.

High methicillin resistance observed in this study warrants the need for screening for MRSA as a routine procedure in clinical laboratories.

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