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A COMPARATIVE STUDY OF DIFFERENT PHENOTYPIC METHODS FOR DETECTION OF METHICILLIN RESISTANCE IN STAPHYLOCOCCUS AUREUS

Dr Niraj Ghanwate* Assistant Professor, Department of Microbiology, Sant Gadge Baba Amravati University, Amravati, India. *Corresponding Author

Dr Prashant Thakare Associate Professor, Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati, India

ABSTRACT Methicillin resistant Staphylococcus aureus (MRSA) is a well recognized problem pathogen all over the world both in the nosocomial as well as in the community. Accuracy and promptness in the detection of methicillin resistance is of key importance to ensure correct doses of antibiotic treatment in infected patients as well as control of MRSA isolates in hospital environments. The present study was aimed to compare the efficacy of the susceptibility testing methods as prescribed by CLSI guidelines. Agar dilution, Disk diffusion, E test, Ezy- MIC and Hi- comb test were compared for detecting high level methicillin resistance in S aureus. The results for these phenotypic methods were compared using PCR amplification of the mecA gene as gold standard.

A total of 106 strains of S aureus were isolated from clinical samples like blood, surgical specimens, wounds, burns and urine, from a tertiary care hospital of Central India. Out of 106 S aureus isolates 98 are mecA positive and 8 are mecA negative. When compared with PCR for mec A, Disk diffusion- 95.91% sensitivity and 87.5% specificity, Agar dilution method- 96.93% sensitivity and 87.5% specificity, Hi-Comb test- 97.95% sensitivity and 87.5% specificity and E-test and EZY- MIC shows excellent results with 100% sensitivity, specificity, PPV and NPV. the most appropriate and accurate test giving 100% concurrence with the Gold standard was E-test and EZY MIC, with EZY MIC being much advanced in performance and reading results.

KEYWORDS : MRSA, Oxacillin, mecA, EZY-MIC, PCR

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) strains emerged soon after the introduction of methicillin in clinical practice. Increase in the number of MRSA has become a serious clinical and epidemiological problem[1]. MRSA is found endemically in hospitals and has also appeared as a community pathogen. The severity of resulting diseases and high costs of health care justify an investment in prevention and control guidelines. It is therefore imperative for health services to carry out systematic MRSA surveillance and disseminate the findings to health professionals [2]. Accuracy and promptness in the detection of methicillin resistance is of key importance to ensure correct doses of antibiotic treatment in infected patients as well as control of MRSA isolates in hospital environments. Despite guidelines published by Clinical Laboratory Standards Institute (CLSI) [3,4] for the testing of susceptibility to oxacillin for *Staphylococcus aureus*, the optimum phenotypic method for detecting methicillin resistance remains controversial[5]. The present study was aimed to compare the efficacy of the susceptibility testing methods as prescribed by CLSI guidelines. Agar dilution, Disk diffusion, E test, Ezy- MIC and Hi-comb test were compared for detecting high level methicillin resistance in *S aureus*. The results for these phenotypic methods were compared using PCR amplification of the *mecA* gene as gold standard.

MATERIALAND METHODS

Strains

A total of 106 strains of *S aureus* were isolated from clinical samples like blood, surgical specimens, wounds, burns and urine, from a tertiary care hospital of Central India. All the strains were isolated from different patients and none of the two isolates were from the same patient. *S. aureus* was identified by standard methods[6]. The methicillin-susceptible *S. aureus* (MSSA) ATCC 29213 and MRSA ATCC 43300 were used as control strains in disk diffusion, MIC and PCR test. All isolates were maintained as glycerol stock in Brain Heart Infusion broth and were frozen at $^{-}70^{o}$ C.

Disc diffusion test: - The disk diffusion test was performed with oxacillin disk (1µ/ml) and an inoculum of isolate 0.5 McFarland standards. The oxacillin disk was placed on Muller Hinton agar plates with NaCl (2%wt/vol).The plates were incubated at 35°C for 24 h.

Agar dilution test:-MIC of oxacillin was determined by agar dilution test as per the guidelines of CLSI[2].Culture suspension prepared in sterile saline was adjusted to 0.5 McFarland standards, diluted 1:10,and inoculated on Muller Hinton agar plates with NaCl (2%wt/vol). Spot inoculation was performed with 10^4cfu in each spot. The plates were incubated at 35° C for 24 h.

E- test: - Oxacillin MIC were determined by E-test (AB, Biodisc, Sweden), performed according to manufacturer's instructions. Muller Hinton agar plates with 2% NaCl (wt/vol) were inoculated by swabbing with 0.5 McFarland standard suspensions of *S aureus*. E-strips were placed on the swabbed plates and incubated at 35°C for 24 h. Results were interpreteted as per CLSI guidelines.

HI Comb test: - Muller Hinton agar plates with 2% NaCl (2%wt/vol) were inoculated by swabbing with 0.5 McFarland standard suspensions of *S aureus.* HI Comb strips of oxacillin were placed on agar surface and incubated at 35°C for 24 h.

Ezy MIC test:- Muller Hinton agar plates with 2% NaCl (2%wt/vol) were inoculated by swabbing with 0.5 McFarland standard suspensions of *S aureus.* Ezy MIC™ strip container was kept at room temperature for 15-30 minutes before opening. Holding the applicator in the middle and gently pressing its broader sticky side on the centre of Ezy MIC™ strip. Applicator is lifted along with attached Ezy MIC™ strip. The strip is placed at a desired position on agar plate pre-spread with test culture. Gently the applicator is turned clockwise with fingers. With this action, the applicator is detached from the strip. Table 1 shows the standard methods used for susceptibility testing.

Table 1.Susceptibility test methods used in this study

Sr. No	Method*	Oxacillin conc.	Interpretive guideline
1	Disk diffusion	1 µg disk	Susceptible -13mm Intermidiate- 11 to 12 mm Resistant- ≤ 10 mm
2	Agar dilution	0.016-256 µg/ml	Susceptible <2 Intermidiate 2-4 Resistant >4
3	E-test (AB BIODISK, Sweden)	0.016-256 µg/ml	Susceptible <2 Intermidiate 2-4 Resistant >4
4	Hi-Comb test (Hi-Media, India)	0.016-256 µg/ml	Susceptible <2 Intermidiate 2-4 Resistant >4
5	EZY MIC (Hi-Media, India)	0.016-256 µg/ml	Susceptible <2 Intermidiate 2-4 Resistant >4

*All media prepared in house.
Culture was adjusted to 0.5 McFarland standards.
Incubation for all tests for 24 to 48 h
Medium for all test were MH (Hi-Media, India)

Amplification of mec A gene:- *S aureus* isolate suspension (0.5 McFarland) was lysed directly in amplification tube in Thermal cycler

T-personal (Biometra, Germany). PCR reagent mix containing 200 µg dNTPs, 10 mM Tris (pH8.3), 50 mM KCl, 1.5mM $MgCl_2$, primer (0.25µM each) and Taq polymerase (1.25 µl, Fermentas,US). PCR for detection of *mecA* gene was carried out by using following primers[7].

F5'AGTACCGGATTTGCCAATTAG'3
R5'TAAAAATCGATGGTAAAGGTTGGCAA'3

Amplification reaction was carried out with initial denaturation at 95° for 5 mins followed by 35 cycles of amplification (denaturation at 95 ° C for 1 min, annealing of primers at 58°C for 1 min, extension at 72 ° C for 1 min), followed by final extension at 72ºC for 5 mins. Amplified DNA was fractionated on 1.5% agarose gel at 100 V for 1 h. Positive results were indicated by presence of an amplicon of 540 bp.

RESULTS AND DISCUSSION

The sensitivity and specificity of the four phenotypic tests as compared to genotypic tests are given in Table-2. Out of 106 *S aureus* isolates 98 are mecA positive and 8 are *mec*A negative. By taking PCR as a gold standard method, all phenotypic methods demonstrated following results: Disk diffusion- 95.91% sensitivity and 87.5% specificity, Agar dilution method- 96.93% sensitivity and 87.5% specificity, Hi-Comb test- 97.95% sensitivity and 87.5% specificity and E-test and EZY-MIC shows excellent results with 100% sensitivity, specificity, PPV and NPV (Table 2). No growth failure occurs with any method using MH agar (HiMedia, India).

Table 2 Sensitivities, specificities, and positive and negative predictive values for phenotypic methods in comparison with the results of PCR for detection of oxacillin susceptibility among 106 *S.aureus* isolates.

Methods	MecA +ve(98)		MecA-ve(8)		Sensitivity	Specificity	PPV	NPV
	True +ve	False -ve	True -ve	False +ve				
Disk Diffusion	94	4	7	1	95.91	87.5	98.94	63.63
Agar Dilution	95	3	7	1	96.93	87.5	98.95	70
E-test	98	0	8	0	100	100	100	100
Hi-Comb test	96	2	7	1	97.95	87.5	98.96	97.95
Ezy MIC test	98	0	8	0	100	100	100	100

PPV-Positive predictive value
NPV-Negative predictive value

Table 3 shows the comparison of susceptibility testing methods to *mec*A gene analysis and MIC of resistant isolates in high, medium and low range. Number of isolates exhibiting resistance by disk diffusion method was 79% at 24h and 82% at 48h, by Agar dilution method it was 77% at 24h and 79% at 48h, by E-test and EZY- MIC it was 80% at 24h and 87% at 48h and by Hi-Comb test it was 80% at 24h and 87% at 48h. Isolates showing resistance were considered for further studies. Depending upon level of resistance, resistant strains were categorized as low-range MIC (4-16 µg/ml), medium-range MIC (>16-32 µg/ml) and high-range MIC (≥64-256 µg/ml). For all the methods except disk diffusion, 23 strains showed medium-range MIC (>16-32 µg/ml) and 41 stains shows high- range MIC (≥64-256 µg/ml). Rest of the isolates demonstrated low-range MIC (4-16 µg/ml) by all methods. Disk diffusion method has no ranges in MIC.

Table 3. Comparison of susceptibility testing methods to *mecA* gene analysis and resistant isolates MICs in high, medium and low range.

No. of strains (n=106)								No. of resistance strains MICs (µg/ml)		
Method	Hours	*mecA*[a] +ve (n=98)			*mecA*[a] -ve' (n=8)			Low Range (4-16)	Medium Range (16-32)	High Range (32-256)
		S[c]	I[c]	R[c]	S[c]	I[c]	R[c]			
Disc diffusion test	24	14	2	82	6		2	-	-	-
	48	12	1	85	6		2	-	-	-
Agar dilution test	24	15	3	80	5	1	2	18	23	41
	48	14	2	82	6		2	20	23	41
E- test	24	13	2	83	6		2	21	23	41
	48	12	1	85	6		2	23	23	41
Hi-comb test	24	13	2	83	6		2	21	23	41
	48	12	1	85	6		2	23	23	41
Ezy MIC	24	13	2	83	6		2	21	23	41
	48	12	1	85	6		2	23	23	41

[a]The *mecA* status of the strains was determined by PCR.
[b]disk diffusion has no low, intermediate and high MIC values.
[c]S, susceptible. I, intermediate. R, resistant.

Figure 1. EZY MIC test

Figure 2.Hi Comb test

Figure 3.E test

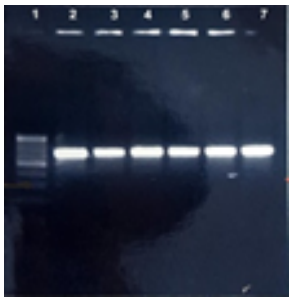

Figure 4.PCR for *mecA*

MRSA is a well recognized problem pathogen all over the world both in the nosocomial as well as in the community. In recent decades, MRSA rates have been increasing worldwide [8- 10]. Methicillin-susceptible Staphylococci are preferably treated with β-lactam antibiotics because these are more effective in treating such infections, and other agents, such as vancomycin, are reserved for treating infections caused by methicillin-resistant isolates [11]. Therefore, it is clinically crucial to rapidly determine whether *S. aureus* isolates are methicillin resistant or not because this determination is of paramount importance for both treatment and control measures. The strains in our study shows high resistance by all phenotypic methods and all strains were found to be *mecA* positive. By different phenotypic methods, out of all resistant strains, 41 strains showed high-range MIC, 23 strains showed medium range MIC and 18 strains shows low-range MICs. In India, the significance of MRSA had been recognized relatively late and it emerged as a problem during the 80s and 90s. Epidemic strains of these MRSA are also resistant to several other antibiotics. During the past 15 years, the appearance and world-wide spread of many such clones have caused major therapeutic problems in many hospitals [6].

Another significant observation our data indicated in this study was that some isolates were found to be susceptible after 24h incubation and after 48h of incubation they tuned into resistant ones. Chambers demonstrated that heterogeneous resistance to methicillin occurs among *S. aureus* isolates due to variations in the expression of the *mecA* gene, or alteration of constitutive PBPs [1]. Also some strains remains susceptible by all phenotypic methods despite carrying *mecA* gene. A study by Rao *et al* and Anand *et al* demonstrated that the results of cefoxitin disc diffusion test are in concurrence with the PCR for mecA gene[12,13]. Tim *et al* analysed 68 isolates of *Staphylococcus aureus* revealing that the serial dilution method with oxacillin possessed the highest sensitivity (at 100%). In contrast, the disk diffusion methods with oxacillin and cefoxitin showed lower sensitivity (95.83%, 95% CI (78.81% - 99.30%)). Furthermore, the borderline value of zone inhibition diameters for cefoxitin might be considered as a risk, and they may give false-susceptible result[14].

Comparing with other phenotypic tests, E-test and EZY MIC was found to be very effective in MIC determination with 100% sensitivity, specificity, and PPV and NPV values. The difference in these two tests is that the Ezy MIC™ strip has MIC values printed on both sides identically. Another advantage is that 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. Moreover for Ezy MIC™ strips, MIC values can be read without opening the lid of the plate as most commonly translucent medium such as Mueller Hinton Agar is employed. Ezy MIC™ strip is made up of porous paper material unlike plastic non-porous material. Unlike the plastic material, it does not

form air bubbles underneath and hence there is no need to press the strip once placed. E test strip is a non porous plastic material with antibiotic concentration on one side of the strip. The MIC reading scale is on the other side of the E test strip.

The epidemiology of MRSA has continued to evolve since its first appearance more than three decades ago. Vidhani et al, [15] indicates that the epidemiology of MRSA in our country is also changing (51.6%) over past few decades as compared to previous studies [16- 19] and indicates increasing emergence of highly resistant strains over the years.

CONCLUSION

Detection of MRSA from clinical specimens is of crucial importance for control and treatment of nosocomial pyogenic infections. Though PCR is the Gold standard, facility to perform it is not available in all laboratories. In order to find a suitable and appropriate method, the present study aimed at comparing various available methods for MRSA detection and compare with the Gold standard. Total 106 strains of *S aureus* were isolated from clinical samples and processed to determine Oxacillin susceptibility using Disk Diffussion test, Agar dilution test, E- test, HiComb test and EZY MIC test in comparison with PCR for *mecA.* When compared with PCR for mec A, Disk diffusion- 95.91% sensitivity and 87.5% specificity, Agar dilution method- 96.93% sensitivity and 87.5% specificity, Hi-Comb test- 97.95% sensitivity and 87.5% specificity and E-test and EZY- MIC shows excellent results with 100% sensitivity, specificity, PPV and NPV. By different phenotypic methods, out of all resistant strains, 41 strains showed high-range MIC, 23 strains showed medium range MIC and 18 strains shows low-range MICs. Comparing with other phenotypic tests, E-test and EZY MIC was found to be very effective in MIC determination with 100% sensitivity, specificity, and PPV and NPV values. It was concluded that the most appropriate and accurate test giving 100% concurrence with the Gold standard was E-test and EZY MIC, with EZY MIC being much advanced in performance and reading results.

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