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Original Research Article

Rapid and Economical Bench Detection of Methicillin Resistance in Staphylococcus Aureus in a Resource Poor Setting

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ABSTRACT

Background: With the first reports of methicillin resistance in early 1960's shortly after its introduction, MRSA has been emerging as nosocomial and community pathogen worldwide. In developing world like ours there are many challenges for finding highly sensitive and economical test for detection of methicillin resistance in Staphylococcus aureus. The aim of the present study was to compare different phenotypic methods with PCR based method as a gold standard for detection of mecA gene.
Methodology: A total of 400 S. aureus isolates which were isolated from different clinical samples at a teritiary care hospital in Kashmir. Methicillin resistance was determined by oxacillin screening agar, cefoxitin disc diffusion, chromagar, latex agglutination test. The results were compared with mecA based PCR method as a gold standard.
Results: Among 400 isolates of S. aureus, 179 (43.5%) were positive for mecA gene by PCR method. Cefoxitin disc diffusion method and latex agglutination test were the most sensitive with 98. 8% and 100% sensitivity respectively.
Conclusion: Latex agglutination test and cefoxitin disc diffusion method with very high sensitivity and specificity can be used as an alternative for MRSA detection in resource poor settings.
Keywords: MRSA, mecA, Cefoxitin disc diffusion, Latex agglutination, PCR

INTRODUCTION

With the first reports of methicillin resistance in early 1960's shortly after its introduction. [1] It soon became evident that the entire groups of beta lactams were rendered ineffective by acquisition of mecA gene Staphylococcus aureus. The unfathomable consequences of such an occurrence were felt across the globe, as it not only posed serious challenges for the treatment strategies for MRSA but at the same time challenged the microbiologists for finding effective laboratory tests for rapid and reliable detection of MRSA.

Though, PCR for detection of mecA gene remains as a gold standard for testing of MRSA, it did not find widespread acceptability because of the need for costly equipment's and technical skills which are largely unavailable in most of the laboratories especially in the underdeveloped world. Hence alternatives like Cefoxitin disc diffusion, Oxacillin screening agar, E test, Chromagar and Latex agglutination test were used by different researchers to circumvent this problem. In the current study we tried to compare these alternate methods with PCR for detection of mecA gene so that an alternative choice can be provided to a large number of laboratories in our part of world that cannot afford to go for highend tests like PCR.

MATERIALS AND METHODS

This was an observational cross sectional study carried out at a tertiary care centre of north india (Department of microbiology, Government medical college Srinagar and associated hospitals) for a period of two years from January 2017 to December 2018.Due proper approval was obtained from institutional ethical committee. A total of 400 consecutive non duplicate Staphylococcus aureus isolates identified by standard microbiological procedures, were recovered from different clinical samples like pus, wound swabs, blood, urine, sputum and other body fluids from both inpatients and outpatients and were subjected to PCR for mecA detection and other phenotypic methods as discussed below. [2] All isolates were stored at 4C on nutrient agar slants.

MRSA detection by genotypic method

Frozen bacteria were subcultured twice on 5% sheep blood agar prior to DNA extraction. 1 to 5 colonies were suspended in 50 µl sterile distilled water, heated at 99^{O} C for 10 minutes followed by centrifugation at 30,000 rpm for 1 minute. Following this 2 µl of supernatant was used as a template in a 25 µl of PCR reaction. [3]

A trivalent multiplex PCR reaction was setup using three primer sets for genotypic confirmation of Staphylococcus aureus, detection of MRSA (mecA) gene and PVL toxin gene by amplification of 756 bp specific for 16s r Rna of Staphylococcus aureus, 189 bp specific for mecA gene and 433 bp specific for PVL gene. The primers used (Integrated DNA technologies, USA) are shown in Figure (1)

The reaction mixtures consisted of 2 µl of the extracted DNA template of the bacterial isolates, 5 µl 10× PCR buffer (75 mMTris-HCl, pH 9.0, 2 mM MgCl2, 50 mMKCl, 20 mM (NH4)2SO4), 1 µldNTPs (40 µM), 1 µl (1U AmpliTaq DNA polymerase), (GeNei india) 1 µl (50 pmol) from the forward and reverse primers (Integrated DNA technologies). Three sets of primer pairs were used in each reaction mixture and the volume of the reaction mixture was completed to 50 µl using distilled water. The PCR tubes were placed in thermal cycler and subjected to the following programme as follows: 94°C for 10 min, followed by 10 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, and 25 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min, followed by final extension at 72°C for 1.5 min. The PCR products were stored in the thermal cycler at 4°C until they were collected for further processing. [4]

The amplicons so obtained were separated on 2% agarose gels in 1X TAE buffer (GeNei india) containing0.5 µg/ml ethidium bromide using a 100-bp reference ladder (GeNei india). Gels were documented under a UV transilluminator for the presence and absence of specific bands of interest.

MRSA detection by phenotypic method

Oxacillin screening agar. Mueller Hinton Agar (MHA) with 4% NaCl was prepared and Oxacillin powder was added to achieve a final concentration of 6µg/ml. The test strain were spot inoculated along with known positive (ATCC 43300) and negative controls (ATCC 25923). Strains able to grow were designated as MRSA.

Cefoxitin Disc Diffusion method. A 30µg disc of cefoxitin was applied on Mueller Hinton agar and plates incubated at for at 37°C for 24 hours in ambient air. An inhibition zone of ≥ 20 mm was interpreted as sensitive and ≤ 19 mm was considered as MRSA. [5]

MRSA Chromagar- (Hichrome Me Re Sa agar, M1674, HiMedia, Mumbai, India) was prepared and dispensed in petri dishes according to manufacturer's instructions. S. aureus strains were inoculated and plates incubated at 37°C for 18-24 hours in ambient air. MRSA grew as bluish-green colored colonies of MRSA on this medium. Known positive control strain and negative control was included in each set. [6]

E test Strips (AB Biodisk, Solna)– MIC determination by E Test strips of Oxacillin strips (Range 0.016-256 µg/ml) were applied on lawn culture of Staphylococcus

aureus on MHA plates supplemented with 2% Nacl according to manufacturer's instructions. The plates were incubated at 37°C in ambient air for 24 hours and the MIC values were read as the intersection of the inhibition eclipse with the MIC scale on the test strip. Interpretations of the results were done in accordance with the CLSI breakpoints. [5]

Latex agglutination method: All Staphylococcus aureus strains were tested for presence of mecA gene product PBP2a by Latex agglutination method (Slidex MRSA from bioMerieux). The test was performed as per instructions by the manufacturer. [7]

The results were interpreted as follows:

- Agglutination observed with the Sensitized latex (R1) but not with the Negative control latex (R2) -------- > PBP2' positive (MRSA)
- No agglutination or very fine granulation with both the sensitized latex and negative control latex --------- > PBP2' negative (MSSA)

All the Staphylococcus aureus isolates were subjected to antimicrobial susceptibility testing by Kirby bauer disk diffusion method using: penicillin (10 units), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), cotrimoxazole (1.25/23.75 µg), linezolid (30 µg) vancomycin (30 µg). [8] The resulting inhibition zone sizes were interpreted as sensitive or resistant as per CLSI guidelines. [5]

Statistical analysis

The sensitivity, specificity, PPV, and negative predictive value (NPV) were calculated using the website http://vassarstats.net/clin1.html. A p-value of <0.05 was considered statistically significant. SPSS statistics version 17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

RESULTS

Out of total 400 Staphylococcus aureus isolates tested for mecA gene by PCR (Gold standard) and other methods compared with it, 174 isolates were confirmed as MRSA 174/400 (43.5%). PVL was detected in 228/400 (57.5%) isolates. Figure (2), Table (2)

Majority of the MRSA were recovered from pus (44.8%) and wound swabs (29.7%) Table (3). Among the phenotypic methods, tested, cefoxitin disk diffusion and latex agglutination test were the most sensitive (98.8%) and specific (99.6%) with very less number of discrepant results. The concordance with the gold standard for the two tests was highest. The oxacillin screening agar showed a sensitivity of 95% and specificity of 98% while E test showed sensitivity of 91% and specificity of 98%. Lowest sensitivity of 81% was reported with chromagar screening agar which also gave a specificity of 97%. Most number of discordant results was seen with chromagar. Table (5)

Table 1: Isolation rate of MRSA and MSSA from inpatients

ISOLATES	NUMBER	PERCENTAGE
MRSA	174/400	43.5%
MSSA	226/400	56.5%
TOTAL	400	100%

Table 2: Frequency of mecA and PVL gene in Staphylococcus aureus isolates

GENE	POSITIVE	PERCENTAGE
mecA	174/400	43.5%
PVL	228/400	57.5%

Table 3: SAMPLE DISTRIBUTION OF MRSA ISOLATES

SAMPLE	PERCENTAGE	NUMBER
PUS	44.8%	78
SWAB	29.7%	50
BLOOD	8.6%	15
URINE	9.19%	16
SPUTUM	4.02%	7
MISSCELLENOUS	2.2%	4
BODY FLUIDS	2.2%	4
TOTAL	100%	174

Table 4: ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF MRSA AND MSSA ISOLATES

	MRSA	MSSA	P value
Penicillin	100%	90%	0.003
Erythromicin	43.2%	14%	0.001
Chloramphenicol	23.9%	6.6%	0.001
Gentamicin	78.8%	9.6%	0.001
Amikacin	32.1%	15%	0.001
Clindamicin	40.3%	5%	0.001
Ciprofloxacin	70.7%	8%	0.001
Vancomycin	0%	0%	-
Linezolid	0%	0%	-
Teicoplanin	0%	0%	-

NS: $p > 0.05$; Not Significant; $p < 0.05$; significant at 5%; $p< 0.01$; Significant at 1%; $p < 0.001$; Highly significant

Antibiotic susceptibility results of MRSA isolates showed 100% resistance to the penicillins followed by 78% to gentamicin, 70% ciprofloxacin and greater than 40% to erythromycin and clindamycin respectively. Vancomycin, linezolid were uniformly 100% active against all MRSA isolates. MSSA isolates showed highest resistance for penicillins (90%) while a moderate resistance was seen in erythromycin (14%) and amikacin (15%). There was statistically significant difference between resistance pattern of MRSA and MSSA. Table (4)

Table 5: PERFORMANCE OF VARIOUS PHENOTYPIC METHODS FOR MRSA DETECTION

METHOD	FALSE -	FALSE +	SENSITIVITY	SPECIFICITY	PPV	NPV
CEFOXITIN DISC DIFFUSION	2	2	98.8%	99.1%	98.8%	99.1%
OXACILLIN SCREENING AGAR	8	3	95.4%	98.6%	98.2%	96.5%
CHROMAGAR	32	6	81.6%	97.3%	95.9%	87.3%
E TEST	14	4	91.9%	98.2%	97.5%	94%
LATEX AGGLUTINATION TEST	0	1	100%	99.5%	99.4%	100%

TARGET GENE	PRIMER SEQUENCE (5′- 3′)	AMPLIFIED SIZE
***mec*A**	**GTG GAA TTG GCC AATACA GG**	**1339 bp**
	TGA GTT CTG CAG TAC CGG AT	
16s rRNA	**AACTCTGTTATTAGGGAAGAAC**	**756 bp**
	CCACCTTCCTCCGGTTTGTCACC	
lukS/F-PV	**ATCATTAGGTAAAATGTCTGGACATGATCCA**	**433 bp**
	GCATCAAGTGTATTGGATAGCAAAAGC	

Figure 1. Forward and reverse primers for mecA, I6s r RNA and PVL gene

Table 6:Comparison of different phenotypic methods with genotypic method (mecA)

		CDD	OSA	CA	E T	LAT
mecA +	174	172	166	142	160	174
mecA -	226	224	223	220	222	225

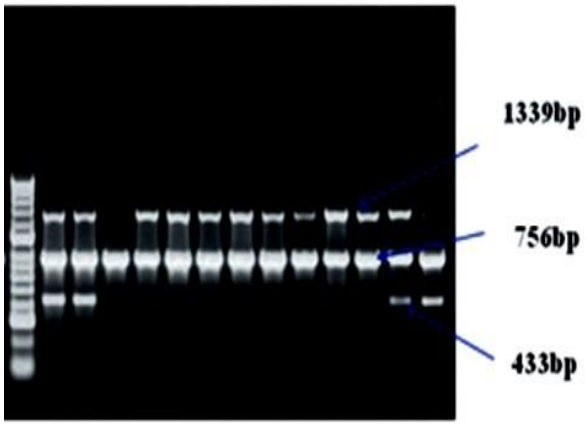

Figure 2: Gel electrophoresis picture showing positive amplification of 756 bp fragments specific for 16S rRNA of S. aureus, 1339 bp fragments specific for the mecA gene and 433 bp fragments specific for PVL gene. Left extreme has the 100 bp ladder

DISCUSSION

Methicillin resistance in Staphylococcus aureus varies across the world ranging from less than 1% in Norway to as high as 50% in some parts of USA. [9] The Indian scenario is equally grim with reported resistance of 43.5% by Oberia et al. and 55.5% by Goyal et al. [10, 11] There are approximately 19000 deaths per year in USA attributable to MRSA infections surpassing those due to HIV/AIDS. [12] Cost estimates for treatment of MRSA infections in the USA is estimated to be in billions of dollars with a staggering economic impact. The detection of methicillin resistance in Staphyloccus aureus cannot be under emphasized as the same has strong implications for the treatment strategies. While a methicillin sensitive Staphylococcus aureus can be reasonably treated well with cheap, orally available, safe antibiotics like cloxacillin on the contrary, MRSA infection invariably rules out the possibility of treatment with the whole gamut of beta lactams leaving only toxic and more costly glycopeptides and oxazolidinones as the last option.

In the current study 43.5% isolates were MRSA similar results have been reported by Hanumanthappa et al who reported 43% of isolates as MRSA. [13] PVL

was detected in 57.5% of isolates, these results are comparable to a Study by Govindan et al. who reported 58.8% of MRSA strains were positive for PVL gene. [14] While comparing different phenotypic tests with the PCR we found cefoxitin disc diffusion and latex agglutination tests to be most sensitive (98.8%) and specificity of 99%. There were very few discordant results with these tests. Similar results have been reported by Velasco et al and Mohansundaraman et al. [15, 16] Etest gave sensitivity of 91% and specificity of 98% with overall 18 discordant results, 4 false positive and 14 false negatives which are in line with results reported in a study by Tiwari et al. who observed sensitivity of 89.4% for E test. [17] Oxacillin screening agar gave sensitivity of 95% and specificity of 98%, there were 11 discordant results with 3 false positive and 8 false negative which is similar to other studies. [10, 17] Using chromagar we got sensitivity of 81% and specificity of 97%, this test gave highest number of discordant results.

The cefoxitin disc diffusion gave overall superior results because cefoxitin is an efficient inducer of mecA gene and the test itself is simple to perform and doesn't require stringent conditions whereby enhancing its utility. [18] Latex agglutination test also provided excellent results as it detects mecA gene product, which is the altered penicillin binding protein PBP2a. Oxacillin screening agar gave relatively lower sensitivity compared to cefoxitin disc diffusion test as oxacillin is not an inducer of mecA gene and the test is tedious involving several steps which may interfere with the results. Moreover oxacillin screening agar has been reported to provide lower sensitivity with heteroresistant strains and low specificity with strains having borderline MIC's which are hyper producers of beta lactamases. [19]

CONCLUSION

Though the PCR for detection of mecA stands as gold standard for MRSA detection yet because of its requirements for costly equipments, consumables and technical expertise it is unlikely to find wide acceptance in the majority of laboratories especially in resource poor countries like ours. As such other alternatives like cefoxitin disc diffusion test and latex agglutination test can serve as efficient replacements because of their high sensitivity and specificity, user friendliness, low cost.

Application of research. Present study will help in establishing that cefoxitin disc diffusion method and latex agglutination test are excellent alternatives to highend methods like PCR in resource poor settings

Research category. Clinical microbiology

Abbreviations

MRSA- methicillin resistant staphylococcus aureus

PCR – Polymerase chain reaction

MSSA - methicillin sensitive staphylococcus aureus

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Author contribution. All authors contributed equally

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Ethical approval. This article doesn't contain any studies with animals or humans.

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