IJHSR International Journal of Health Sciences and Research
www.ijhsr.org ISSN: 2249-9571

Original Research Article

Comparison of Phenotypic & Genotypic Methods for Identification of MRSA in Tertiary Care Hospital, Rajasthan

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Received: 06/08/2015 *Revised: 24/08/2015* *Accepted: 26/08/2015*

ABSTRACT

We evaluated the diagnostic ability of phenotypic tests in detecting methicillin resistance in 100 *Staphylococcus aureus* isolates collected between 2012-2013 in India, The four phenotypic methods studied were: Oxacillin screening agar, Cefoxitin disc diffusion, Chromogenic media, and Hicomb methicillin MIC test. Their performance was compared against the PCR assay result which detected 58% as MRSA. We found Oxacillin screen agar to be the most sensitive test in detecting methicillin resistant *S. aureus* (0.966). By analyzing the performance of these tests in combinations of two and three, we find that the best overall performance comparable to PCR, is achieved by combining cefoxitin disc diffusion and chromogenic media tests (sensitivity: 0.983, specificity: 0.833). Although 100% sensitivity can be achieved by combining oxacillin screening agar with either cefoxitin disc diffusion or chromogenic media test (for both combinations, sensitivity: 1, specificity: 0.762). Thus, diagnostic ability of these phenotypic tests can be sufficiently enhanced by combining them to facilitate detection of resistant *S. aureus* strains in resource-limited settings.

Key Words: *Staphylococcus aureus*, MRSA, cefoxitin, oxacillin, methicillin.

INTRODUCTION

Staphylococcus aureus remains a leading cause of morbidity and fatality in developing countries despite the availability of numerous effective anti-staphylococcal antibiotics. [1] This is largely due to widespread circulation of Methicillin-resistant *Staphylococcus aureus* (MRSA), a strain that is resistant to several antibiotics making successful treatment increasingly difficult.

Asymptomatic patients harbouring the organism and health care workers are the major sources of MRSA in hospital environments; however, community-associated MRSA infections have increased promptly in the past decade and affect populations that lack the hospital-associated MRSA risk-factors such as exposure to the health-care system. [2,3] Resistant phenotype is determined by testing for presence of *mecA*gene that encodes for protein PBP2A

that binds to antibiotics thus nullifying their anti-staphylococcal activity of inhibiting bacterial cell-wall synthesis. Infecting strains are hetero-resistant if the *S. aureus* population contains a mixture of *mecA*-positive and *mecA*-negative sub-strains. Such a heterogeneous bacterial population can exhibit variable resistance and can often be incorrectly diagnosed as susceptible, leading to improper treatment that results in bacteria evolving complete resistance. Thus, early and meticulous detection of resistance is of convincing importance in deciding the optimal treatment strategy as well as in containing the prevalence of resistance especially if hetero-resistant bacterial strains are circulating in a region.

In this study, we have studied the performance of phenotypic tests in detecting MRSA in clinical isolates obtained in the northwestern Indian state of Rajasthan. Since PCR cannot be performed to positively detect *mecA* gene for each isolate in resource-limited settings, we determine if combining simple phenotypic tests can enhance their diagnostic ability to detect resistance in *S. aureus* infections.

MATERIALS AND METHODS

STRAINS -

We evaluated 100 *Staphylococcus aureus* strains isolated from various clinical specimens submitted to the Bacteriology section of the Microbiology Department of S.M.S. Medical College, Jaipur (Rajasthan) from April 2012 to March 2013. The clinical samples included isolates from pus, blood, sputum, throat swab, ear swab, high vaginal swab, CSF, urine, pleural fluid, semen, bile, and corneal swab. Presence of *S. aureus* was detected by looking for distinctive growth on blood agar, MacConkey agar, gram stain and various other biochemical test which included catalase test, free and bound coagulase test and anaerobic mannitol fermentation.

PCR

All *S. aureus* isolates were subjected to DNA extraction followed by amplification for the mecA gene by PCR with primers P1- (mecAF 5' GTAGAAATGACTGA ACGTCCGATGA 3') P2 -(mecA R 5' CCAATTCCACATTGTTTCGGTCTAA 3') 5μL of the extracted DNA present in the supernatant was added to the master mix solution and loaded in the thermal cycler with initial denaturation at 4 min at 94°C. This was followed by 30 cycles, with 45-s denaturation step at 94°C, a 45-s annealing step at 56°C and a 30-s extension step at 72°C and 2 min extension step at 72°C and a holding step at 4°C. [4]

The amplified product was observed by 1% agarose gel electrophoresis with 2.0μl ethidium bromide staining and visualized under UV light, which was found to be 310bp sequence. All strains confirmatory for the mecA gene were classified as MRSA. For PCR, positive and negative control strains [ATCC 43300 (mecA-positive) and ATCC 29213 (mecA-negative), respectively] were used.

PHENOTYPIC DETECTION METHODS

All isolates of *S. aureus* were tested by cefoxitin disc diffusion (CD), oxacillin screen agar (OS), growth on CHROMagar (CA) & Hi comb methicillin MIC test (MIC). A standard strain of MSSA (ATCC 29213) and a PCR-positive control strain [ATCC 43300 (*mecA*-positive)] has been used as controls for all the mentioned methods.

CEFOXITIN DISC DIFFUSION TEST

All strains were tested with 30 μg cefoxitin discs (Hi-Media) on Mueller–Hinton agar plates. [5] The bacterial suspension for each strain was adjusted to 0.5 McFarland. The zone of inhibition was calculated after 16–18 hr incubation at 35 °C. Zone size were defined according to CLSI criteria (23)(24): susceptible, ≥22 mm;

resistant, ≤21 mm. ATCC 25923 MSSA was used as negative control. (Fig 1)

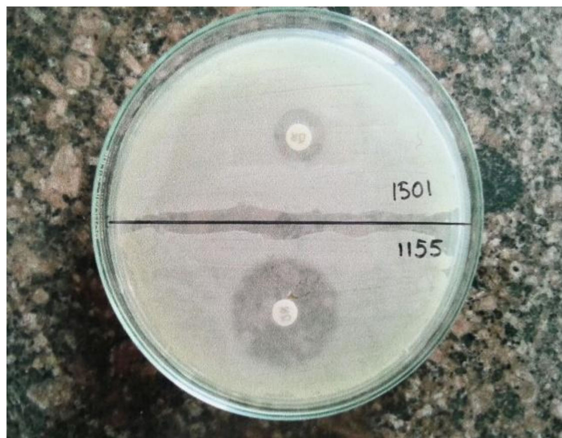

Fig 1: Methicillin resistant and Methicillin sensitive Staphylococcus *aureus* by cefoxitin disc diffusion test

Fig.2: Growth of MRSA on Oxacillin screen agar.

OXACILLIN SCREEN AGAR TEST

A bacterial suspension of each strain was made and turbidity was adjusted to 0.5 McFarland. Mueller Hinton agar plate containing 4% NaCl and 6μg/ml of oxacillin were prepared(Hi-Media) & inoculated with 10 μl of 0.5 Mc Farland suspension of the isolate by streaking on one quadrant of the plate & incubating at 35℃ for 24 h. Any strain showing characteristic growth on the plate containing oxacillin, were deemed resistant to methicillin. (Fig 2)

CHROMAGAR

CHROMagar (Hi-Media) is a novel and advanced chromogenic medium for the identification of MRSA. [6] For the identification of the MRSA among the isolates of *S. aureus*, HIMEDIA HiCromeMeReSa Agar Base (M1674) was used. *S. aureus* strains were inoculated onto the Hi Crome Me Re Sa agar and incubated at 35°C for 24 h. All cultures showing characteristic blue colored growth were taken as MRSA positive strains and the rest were reported to be MSSA strains. Fig. 3

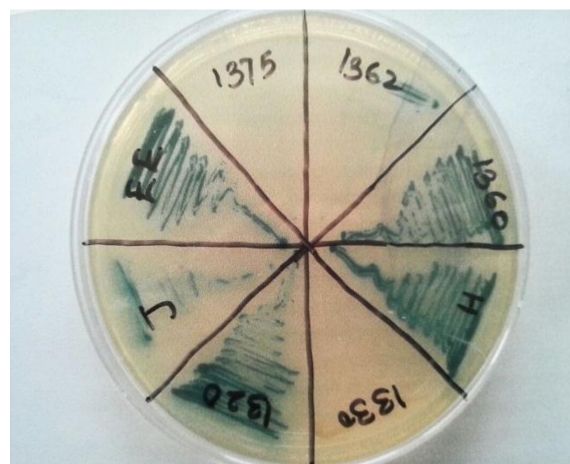

Fig 3: Blue colonies of MRSA on CHROMagar

HI COMB METHICILLIN MIC TEST

The test consists of a strip made of an inert material with 8 extensions that carry 4mm discs, resembling the 'tooth' of a comb. A pre-defined concentration of the antibiotic (methicillin) was loaded on each disc to form a gradient when placed on the agar plate. Hi comb (based on diffusion and dilution) consists of a gradient that covers a continuous range of 16 two-fold dilutions on 2 different strips (Part A & B) as per conventional MIC method. The gradient remains stable after diffusion, and the zone of inhibition is elliptical in shape. MIC value is defined as the value at which the zone of inhibition converges on the comb-like projections of the strips, and not the handle.

If there is no zone of inhibition, MIC will be reported as greater than the highest concentration on the strip. If the zone of inhibition is below the minimum concentration, MIC will be reported as less than the minimum concentration.(Fig. 4)

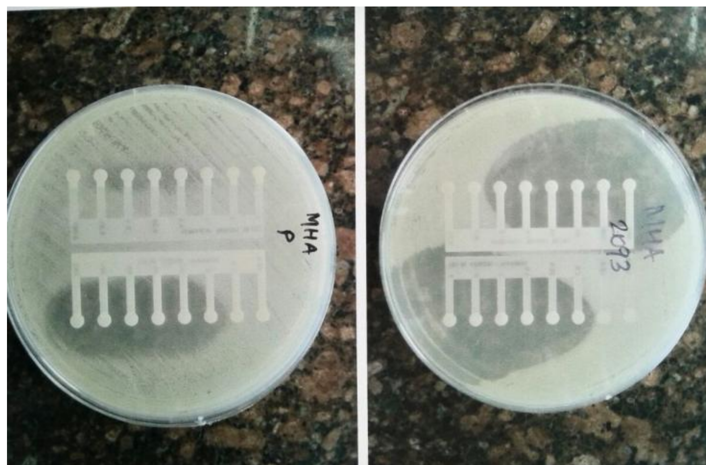

Fig.4 : Methicillin MIC test for MRSA and MSSA.

Statistical Analysis

To determine the performance of phenotypic tests or combinations thereof in detecting MRSA, we compared the results of these tests against that of the PCR (Table 1). For combination of two or more tests, phenotypic test result was considered 'Resistant' if *any one* test makes that conclusion, and was considered 'Susceptible' if *all* tests in the combination find the isolate as susceptible. Sensitivity (also called *Recall*) was calculated as TP/(TP+FN), Specificity as TN/(TN+FP), Positive Predictive Value (PPV or *Precision*) as TP/(TP+FP), and Negative Predictive Value (NPV) as TN/(FN+TN). To determine overall performance of each test, we calculate F-1 measure as the harmonic mean of precision and recall: $F_1 = 2 \cdot \frac{precision \times recall}{precision + recall}$ [7]

Table 1.Criteria for calculating performance of phenotypic tests in identifying MRSA

	PCR result	Phenotypic test result
True Positive (TP)	mecA positive	Resistant
False Negative (FN)	mecA positive	Susceptible
False Positive (FP)	mecA negative	Resistant
True Negative (TN)	mecA negative	Susceptible

RESULTS

A comparative table showing statistical analysis of all the methods is shown (Table 2).

Table 2. Performance of phenotypic tests in isolation

Test	Detected as MRSA by PCR	Sensitivity	Specificity	PPV	NPV	F_1-measure
PCR	58	1	1	1	1	100
Cefoxitin Disc Diffusion n = 59	55	0.948	0.905	0.932	0.927	0.940
Oxacillin Screening Agar n = 62	56	0.966	0.857	0.903	0.947	0.933
Chromogenic Agar n = 56	52	0.897	0.905	0.929	0.864	0.913
Hi comb methicillin MIC test n = 56	50	0.862	0.857	0.893	0.818	0.877

Table 3. Performance of phenotypic tests in combination

Tests*	Sensitivity	Specificity	PPV	NPV	F_1-measure
A. Double-tests					
CD+OS	1	0.762	0.853	1	0.921
CD+CA	0.983	0.833	0.891	0.972	0.935
CD+MIC	0.983	0.786	0.864	0.971	0.920
OS+CA	1	0.762	0.853	1	0.921
OS+MIC	0.966	0.810	0.875	0.944	0.918
CA+MIC	0.897	0.786	0.852	0.846	0.874
B. Triple-tests					
CD+OS+CA	1	0.69	0.817	1	0.899
CD+OS+MIC	1	0.738	0.841	1	0.914
CD+CA+MIC	0.983	0.738	0.838	0.969	0.905
OS+CA+MIC	1	0.738	0.841	1	0.914

*CD: cefoxitin disc diffusion; OS: oxacillin screen agar; CA: growth on CHROMagar; MIC: Hi comb methicillin MIC test.

Table 4. Phenotypic tests ranked by their overall performance

Rank	Test(s)	F_1-measure	Sensitivity	Specificity
1	CD	0.940	0.948	0.905
2	CD+CA	0.935	0.983	0.833
3	OS	0.933	0.966	0.857
4	CD+OS	0.921	1	0.762
5	OS+CA	0.921	1	0.762

DISCUSSION

Precise, systematic and prompt detection of methicillin resistance is of prime importance in the prognosis of *S. aureus* infections. Although many phenotypic methods have been developed for this purpose but the detection of methicillin resistance is perplexed by the fact that the phenotypic expression in many strains of *S. aureus* is heterogeneous. This has led to the development of various laboratory techniques to reinforce the expression of this resistance *in vitro*.

In Jaipur (Rajasthan), we found the prevalence of methicillin resistant *S. aureus* to be 58% by PCR. Cefoxitin disc diffusion test appeared to be the most specific, with specificity of 0.905 and oxacillin screen agar yielded the best sensitivity of 0.966, followed by the cefoxitin disc diffusion (0.948).

OSA test, with its high sensitivity, stands out to be a reliable option but its low specificity restricts its use in detecting MRSA when used alone. Cefoxitin disc diffusion test is the most specific (0.905) besides being very sensitive, suggesting that this test is especially useful in detecting hetero-resistant strains. CHROMagar is a recently developed test that had specificity comparable to the cefoxitin disc diffusion test (0.905), but had relatively poor sensitivity of 0.897. The worst performer yet was the Hi comb methicillin MIC test (Hi Media), which is a simple and rapid test for determining antimicrobial sensitivity at an extremely cost effective range. We used drug methicillin in the comb test which is less stable than oxacillin and could explain the low sensitivity and specificity for this test in our study.

None of the techniques produced 100% sensitivity and specificity in isolation, and thus we compared the performance of these tests in all possible combinations of two and three (Table 3). We compute F_1-measure that computes the harmonic mean of sensitivity and specificity, giving a single measure of overall performance. However, this approach weighs sensitivity and specificity equally, while in clinical settings it may be more desirable to have high sensitivity at the cost of an acceptable false positive rate. Table 4 lists the tests ranked by their F_1-measure, and the decrease in specificity is immediately evident when a high sensitivity is achieved by combining tests. It is also interesting to note that combining more than two tests does not yield any increase in diagnostic power.

When combining tests, it is usually recommended to combine the tests with highest sensitivity and highest specificity,

which in our case will be oxacillin screening agar (OS) and cefoxitin disc diffusion (CD). However, we see that while this combination yields an increase of 4% in sensitivity compared to OS, specificity decreased by 15% compared to CD. We find that the best combination is CD+CA, with a 3% increase in sensitivity over CD test and a 7% decrease in specificity (Table 4). Still, OS can be combined with CD or CA if 100% sensitivity is desirable.

CONCLUSION

The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by S. aureus. Although multiple methods of detection of this resistance have been developed, they are often too slow or not sufficiently sensitive or specific to ensure appropriate treatment of the MRSA-infected patients. Nevertheless, none of the techniques compared showed 100% sensitivity and specificity, although if the mecA gene detection method (PCR) is not accessible or available, it is advisable to combine two methods, one with high sensitivity and the other with high specificity.

Thus, this study identifies the optimal combination of phenotypic tests for detecting MRSA in resource-limited settings, and finds that no more than two tests are needed to enhance their diagnostic ability.

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How to cite this article: Gupta PS, Sharma R, Gupta A et al. Comparison of phenotypic & genotypic methods for identification of MRSA in tertiary care hospital, Rajasthan. Int J Health Sci Res. 2015; 5(9):196-201.

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