Original Article

Pathology

Sensitivity and Specificity of Phenotypic and Genotypic tests for Detection of Methicillin-Resistant Staphylococcus Aureus in Clinical Isolates

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ABSTRACT

Objective: Methicillin-resistant Staphylococcus aureus (S. aureus) (MRSA) is an important pathogen in hospitals and communities. There is advance in technologies but infections by MRSA are difficult to diagnose. The current study was conducted to compare three phenotypic methods for with a genotypic method.

Study Design: Cross-sectional study.

Place and Duration of Study: Methicillin resistance was tested in 450 isolates of S. aureus retrieved from various clinical samples received in Dr. Ziauddin Laboratory Department of Molecular Genetics (DMG), Karachi from January to October 2011.

Materials and Methods: Methods used were, oxacillin disc diffusion, oxacillin screen agar, cefoxitin disc diffusion and Polymerase Chain Reaction (PCR) for mecA gene, which was taken as the gold standard.

Results: The Kirby Bauer disc diffusion, using cefoxitin discs 30µg, identified a total of 174 (38.6%) S. aureus isolates as methicillin resistant. With oxacillin disk diffusion test, 152 (33.77%) strains were MRSA with sensitivity of 87.35% and specificity of 100%. Oxacillin-agar screen detected 168 (37.33%) strains with sensitivity of 96.5% and specificity of 100%. PCR method detected mec A gene in 169 (37.55%) isolates with sensitivity of 97.12%, specificity of 100%.

Conclusion: The cefoxitin disc diffusion, as suggested by the Clinical and Laboratory Standards Institute (CLSI), is a consistent method for MRSA diagnosis but must be augmented with other methods like oxacillin screen agar, so that no MRSA is overlooked. PCR although taken as the gold standard cannot be recommended as routine because of the high cost and time consumption.

Key Words: Cefoxitin Disk, Methicillin-resistant Staphylococcus aureus, Polymerase Chain Reaction

INTRODUCTION

Methicillin-resistant *S.* aureus (MRSA) is accountable for a growing number of serious nosocomial and community-acquired infections. The first case of MRSA was identified in 1961; since then there has been a global dissemination of infections produced by MRSA. In Pakistan, the frequency of infections caused by MRSA differs between 20-40%.[2-4] Methicillin resistance in S. aureus is related to expression of a changed penicillin-binding protein, a 78 kDa protein PBP2a, mediated by the mecA gene. A distinctive type of mobile genetic element is the staphylococci cassette chromosome (SCC), a diverse genetic component which can integrate at a precise chromosomal location (attBsc) of MRSA.[5] The SCC element has evolved to carry certain genes for virulence and antibiotic resistance; transfer of DNA from Coagulase Negative Staphylococci (CoNS) may be the origin and reservoir of SCC elements.[5,6]

Phenotypic heterogeneous drug resistance (heteroresistance) to antistaphylococcal beta-lactams make the detection of MRSA problematical[1]. Many MRSA isolates express heterogeneous resistance therefore one daughter cell out of 10^4 to 10^6 expresses resistance when tested by phenotypic methods.[2] These strains appear phenotypically sensitive to methicillin; other factors also guide the phenotypic appearance of resistance. Adding sodium chloride or sucrose to culture medium, incubation at 30 °C or culture with β-lactam antibiotics heightens the appearance of resistance[7].

Traditional methods for the detection of MRSA include oxacillin disc diffusion, and oxacillin screen agar methods. Recently, the Clinical and Laboratory Standards Institute (CLSI) endorsed the use of the cefoxitin disc diffusion method for MRSA detection[8]. Cefoxitin is a cephamycin type antibiotic and has been defined as an inducer of the PBP2a-encoding mecA gene.[3] Countless other methods for the detection of MRSA have been established. The latex agglutination assay, developed by Denka Seiken Co., Japan, employs specific monoclonal antibodies focused

towards the PBP2a antigen.[9] Also, CHROMagar MRSA is one more method to identity MRSA.[10]

In recent years, PCR detection of the mecA gene has become the gold standard for MRSA detection. However, this is a costly and laborious method, and use of this assessment is confined to reference centers and is not customarily carried out in all laboratories.(3)The present study was undertaken to compare three phenotypic methods for the detection of MRSA, namely, the cefoxitin disc diffusion method, oxacillin disc diffusion method, oxacillin screen agar. The sensitivity and specificity of each test were determined in an attempt to find out the most suitable method, for detection of MRSA in a routine diagnostic laboratory.

MATERIALS AND METHODS

All clinical samples used were processed during a 10 month period ending October 2011. Clinical samples (n=450) (pus, sputum, urine, blood, high-vaginal swab, throat swab, ear swab and cerebrospinal fluid) were obtained from routinely referred patients. The molecular study was conducted at DMG, Dr. Ziauddin Hospital.

All strains were identified by (Gram's staining, Catalase, and Tube Coagulase test).The isolates were sub-cultured on slopes of Mueller-Hinton agar (Oxoid) stored at -70°C MSSA ATCC 25923 negative and MRSA ATCC 43300 positive controls were used.

Phenotypic Detection Methods

Cefoxitin Disc Diffusion Test: All the positive cultures were tested on Mueller-Hinton agar (MHA) media by the cefoxitin 30 µg disc. A 0.5 McFarland equivalent was used to make a lawn and incubated at 35˚C for 24 hours and zone of inhibition was calculated. A zone of inhibition of ≤ 20 mm was cefoxitin insusceptible and ≥21 mm was cefoxitin susceptible.[11]

Oxacillin Screen Agar: 10 µl of the isolate matched to 0.5 McFarland were inoculated in (MHA) media incorporated with 4% NaCl and 6 µg/ml of oxacillin , incubated at 35˚C for 24 hours, any growth was oxacillin resistant.[11]

Oxacillin Disk Diffusion Test: Oxacillin disc (1 µg) was placed on (MHA) inoculated with culture matched to 0.5% McFarland standard and incubated at 35°C for 24 hours. Zone size >13mm sensitive, equal to 13mm intermediate; <13mm was resistant.[11]

PCR

DNA Extraction: S. aureus isolates were grown over night in Brain Heart Infusion Broth (BHI) centrifugation was done at 3000 rpm for 25 minutes; the pellets were cleaned in 8 ml TE buffer (house-made) and suspended in 2 ml of TE buffer. DNA was extracted by DNA extraction kit (Promega, USA) with 1 ml lysozyme stock sol. (10 mg/ml in 0.25 M EDTA, pH 8) and lysostaphin (Roche) 2 mg/ml (from a stock solution of 600 µg/ml in 0.25 M EDTA to give a final concentration of 30 µg/ml) to obtain bacterial lysis.

Detection of *mecA* gene was done in accordance with the method of Geha et al. (Table 2)[12] 50 µl PCR solution comprised of 25 µl of master mix (Finnzymes usb) including PCR buffer (10X), dNTP mix (2.5mM of each), primer (10 µM), Taq DNA polymerase (0.5 U), and $MgCl_2$ (50 mM). Amplification of DNA was done in an Applied Biosystem 2720 Thermal Cycler. Cycling settings were initially hot temperature at 94°C for 3 minutes. Next 35 cycles at temperature 94°C for denaturation for 40 seconds, annealing at 54°C for 40 seconds, followed by extension at 72°C for 45 seconds and finally at 72°C for 3 minutes. PCR products 10 µl were loaded on 2% agarose gel stained with 0.5 µg ethidium bromide dye. 100 bp DNA ladder (Pennicon Usb) was also run. 310 bp amplicon corresponding with *mecA* gene were visulalized under transilluminator (Benchtop UV, Cambdrige, UK).

RESULTS

Out of 450 S. aureus samples, 174 (38.66%) strains were detected as MRSA by the Cefoxitin disk diffusion test (Table 1). (Figure 1).

Table No.1: Frequency Distribution of Staphylococcus aureus and MRSA

Clinical Samples	MRSA frequency (%)	S. aureus frequency (%)
Pus and Wounds swab	93(57.4)	162 (36)
Urine	42(33.3)	126 (28)
High vaginal swab	31(46.96)	66 (14.7)
Blood	08(22.22)	36 (8)
Urethral Swabs	0 (0.00)	26 (5.78)
Ear Swab	0 (0.00)	19 (4.22)
Eye swabs	0 (0.00)	15 (3.33)
Total	174	450

Table No.2: Primers for Amplification of mecA Gene

Primer	Sequence	Product size(bp)
Forward	5'-GTA GAA ATG ACT GAA CGT CCG ATA A-3'	310
Reverse	5'CCA ATT CCA CAT TGT TTC GGT CTA A-3'	310

Table No.3: Zone of Inhibitions against Cefoxitin in MRSA Clinical Isolates.

Cefoxitin	n	Results of zone diameter (mm) of 30µg Cefoxitin discs
Resistant	174	<14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 74 40 24 36
Sensitive	276	106 92 78

Table No.4: The Sensitivity and Specificity of Phenotypic and Genotypic Tests.

Test method	Detected as MRSA	Sensitivity (%)	Specificity (%)
Cefoxitin disc diffusion (30 µg)	174	100	100
Oxacillin agar screen (6 µg)	168	96.9%	100
Oxacillin disc diffusion (1 µg)	152	87.6%	100
PCR for mecA gene	169	97%	100

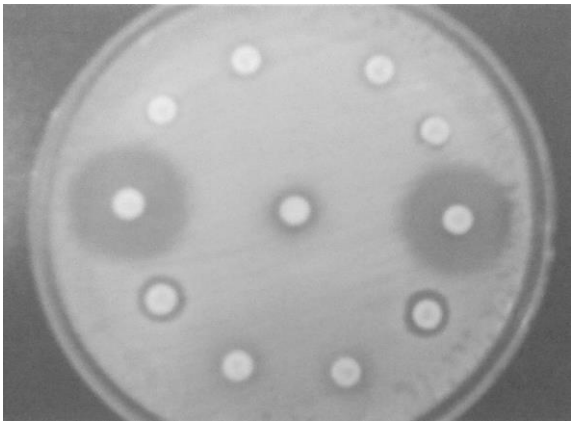
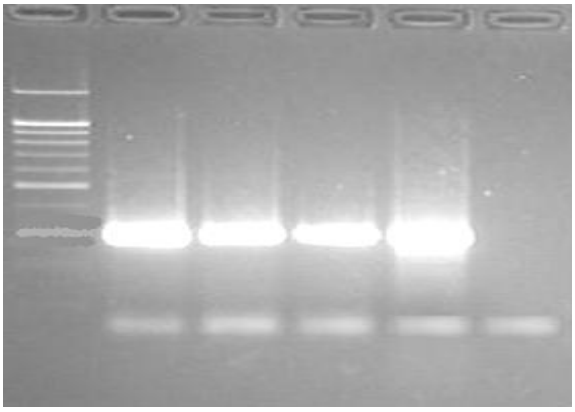


Figure No. 1: Cefoxitin disc-diffusion test showing Staphylococcus aureus (MRSA) isolated from clinical samples.



Lane 1: DNA markers, Lane 2-5: mec A positive
Lane 6: mec A negative control
Figure No. 2: Agarose gel showing 310 bp PCR products of mecA gene

Cefoxitin disc had an overall sensitivity and specificity of 100%, the Positive Predictive Value (PPV) and the Negative Predictive Value (NPV) were again 100% with highest accuracy, considering the proposed zone diameter breakpoints of R < 20 mm and S > 22 mm. (Table 3).Oxacillin disk diffusion test, 152 (33.77%) strains were detected as MRSA with sensitivity of 87.35% and specificity of 100%. Oxacillin-agar screen detected 168 (37.33%) strains with sensitivity of 96.5% and specificity of 100%. PCR method detected 169 (37.55%) isolates positive for the presence of *mecA* gene with sensitivity of 97.12% and specificity of 100% (Table 4) (Figure 2).

DISCUSSION

The last ten years has seen a startling rise in MRSA cases all over the world. The data on the prevalence of MRSA in Pakistan is limited with some hospitals reporting less than 10% of *S. aureus* infections being MRSA while other studies reported it to be as high as 65%.[13] As early as 1999 a report by National Nosocomial Infection surveillance (NNIS) system in Atlanta Georgia in USA showed that MRSA accounted for >50% of *S.* aureus infections in intensive care units, however by 2004 the figure increased to 63 %.[14]

Precise and timely identification of methicillin resistance is critical for treatment of patients with invasive infections. There are phenotypic methods to differentiate between resistant and sensitive strains, the gaps in the sensitivity of these assessments may not warrant correct and suitable treatment of all MRSA-infected patients. The present gold standard for MRSA identification is the detection of the *mecA* gene. Nevertheless, use of molecular approaches for routine diagnosis may not be practicable in a resource poor country like ours Consequently, it is necessary to identify an accurate, quick and worthwhile phenotypic method for the detection of MRSA.[15] In our study, the Kirby Bauer disc diffusion method using cefoxitin discs, identified a total of 174 (38.6%) isolates as methicillin resistant. By PCR method, 169 isolates showed the presence of *mecA* gene, the isolates which were negative for *mecA* gene were phenotypically recognized as MRSA by cefoxitin disc. In previous studies similar strains have been identified, demonstrating that resistance in these strains is not inherently mediated by *mecA* gene.[16] MRSA strains negative for *mecA* gene are assumed to be due to some modifications in the penicillin-binding protein (PBP), result of which is over production of beta-lactamase . Nevertheless the non-*mecA* mediated resistance is clinically significant since the strain is still resistant to methicillin.[17,18] By oxacillin disk diffusion test, 152 strains were detected as MRSA. It has been shown that staphylococci that grow on Mueller Hinton media incorporated with 4% NaCl and 6µg/ml oxacillin typically are *mecA* positive.[16]

Different workers have demonstrated that the cefoxitin disc method has improved sensitivity than the oxacillin disc method for MRSA detection[3,19,20], This higher sensitivity to cefoxitin can be explained by the increased expression of the *mecA*-encoded protein PBP2a, cefoxitin being an inducer of the *mecA* gene.[3]. The drawback with the cefoxitin disc is that the inhibition zones are big and restrict the zones of

adjoining discs[20] Swenson et al.[18] concluded that sensitivity decreased when heterogeneous resistant strains were tried and specificity reduced with strains having borderline MIC. Among the recently developed technologies, CHROMagar MRSA shows good sensitivity and specificity. This sensitivity might be improved to 100% by increasing the incubation period of CHROMagar from 24 to 48 hour[10]. This medium is very expensive and cannot be recommended for routine detection of MRSA and we did not employ it in our study. Five of the strains that were negative for *mecA* by PCR, tested positive in the Cefoxitin Disk diffusion test. Sakoulas et al[21] encountered comparable results, they recommended the usage of several colonies for PCR in place of a single colony. Gene instability and primers design might also be suggested as one of the explanations for this inconsistency in results. Laboratory mistakes in the identification of MRSA have serious implications. False positivity leads to unnecessary usage of backup drugs like vancomycin and linezolid resulting in drug resistance and rise in health-care expenses. False-negative reports results in therapy failure and dissemination

CONCLUSION

We conclude cefoxitin disc, as endorsed by the CLSI, as a worthy method for MRSA detection but it should be complemented with some other technique so that no MRSA is lost. It is at all times desirable to pool two methods, one with high sensitivity and the other with high specificity. Accordingly cefoxitin disc diffusion method and oxacillin screen agar test can be recommended. Isolates that give a zone diameter of less than 20 mm can be straightforwardly described as MRSA and only those with zone diameters of 20–22 mm require to be further tested. PCR, although taken as a gold-standard is expensive and time consuming for routine testing.

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