Molecular Evaluation of Methicillin Resistant Staphylococcus *Aureus* Isolates at a Tertiary Care Hospital in Lahore

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ABSTRACT

Background and Objective: *The versatility of Staphylococcus aureus has been transformed as "Methicillin resistant Staphylococcus aureus". Among phenotypic methods for detection of MRSA, feasible technique, i.e., cefoxitin disc diffusion test and/or oxacillin disc diffusion test can significantly contribute to-wards reliable detection. The objective of the study is to determine methicillin resistance in Staphylococcus aureus by disc diffusion methods; and to evaluate their accuracy with mecA gene PCR for MRSA detection.*

Methods: *A total of 750 staphylococcus aureus isolates were screened with oxacillin disk (1 μg) and cefoxitin disk (30 μg) by Kirby-Bauer method using CLSI guideline (2016); to get 105 continuous, non-repetitive clinical isolates of MRSA. All the methicillin resistant staphylococcus aureus isolates were further amplified by polymerase chain reaction for mecA gene detection.*

Results: *All the 105 MRSA isolates were resistant by both oxacillin and cefoxitin disk diffusion tests. On PCR amplification, out of 105 MRSA (oxacillin resistant) isolates, 83 (79.04%) isolates were positive for mecA gene. Among 105 MRSA (cefoxitin resistant) isolates, 89 (84.76%) were mecA gene positive, by PCR amplification. The sensitivity (96.73%) and diagnostic accuracy (94.28%) of cefoxitin disc diffusion method was higher than oxacillin disc diffusion technique, with reference to PCR as a gold standard.*

Conclusion: *Cefoxitin disc diffusion method can be employed reliably for detection of mecA gene in MR-SA isolates in settings with limited resources, where molecular methods are not available. This can efficiently reduce the misdiagnosis and dissemination of resistant strains.*

KEYWORDS: *Methicillin resistant Staphylococcus aureus, Oxacillin, Cefoxitin, PCR.*

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are recognized across the world equally in advanced and growing countries as a reason of recurrent hospitalizations and persistent infections associated with notable abnormal illness, high death rate and increased treatment costs[1]. In recent years, MRSA strains have shown resistance to antibiotics other than β-lactam groups, thus treatment of *Staphylococcus aureus* infections becoming extensively bothersome and distressing.[2]

Considering origin of MRSA, it is assumed that methicillin-susceptible *Staphylococcus aureus* (MSSA) attained the *mec*A gene through parallel transmission from coagulase-negative staphylococci. The genetic attainment of staphylococcal commensal types assisted the potential of *Staphylococcus aureus* to inhabit, persist infection and fight antibiotic treatment.[3]

It has been recognized that methicillin resistance in *Staphylococcus aureus* has been related with alterations in the penicillin binding proteins (PBPs) subsequently generating an added penicillin-binding protein, PBP2a or PBP2'; with diminished affinity for β-lactam antibiotics.[4] PBP2a is determined by the *mec*A gene which is passed on a large moveable genetic portion called Staphylococcal cassette chromosome *mec* (SCC*mec*).[5] Addition and acquirement of a staphylococcal cassette chromosome *mec* (SCC*mec*) portion into the chromosome alters drug-sensitive staphylococcal lineages into the notorious methicillin-resistant *Staphylococcus aureus* (MRSA).[6]

MRSA is believed to be a potential "Super Bug" because it is resistant to numerous antibiotics and is afore most hazard to hospital infection control. Laboratory analysis and susceptibility testing are vital steps in specific recognition, treatment, regulation and inhibition of MRSA infections.[7]

Currently the main phenotypic methods being used for the detection of MRSA include traditional disc diffusion method (Modified Kirby–Bauer and Stokes methods); broth microdilution method determining minimal inhibitory concentration (MIC); E Test method; breakpoint method; agar dilution method (oxacillin/methicillin screen agar, mannitol salt agar, iso sensitest agar, chromogenic agar)[8]; automated

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system methods: Vitek 2, Microscan Walkaway[9]; and latex agglutination method to detect *mec*A gene product i.e., PBP2a.[10] The genotypic methods confirm the existence or nonexistence of *mec*A gene in methicillin resistant *Staphylococcus aureus* isolates by polymerase chain reaction.[11].

In disc diffusion tests, hyper-producers of penicillinase may show small methicillin or oxacillin zones of inhibition, whereas most true methicillin/ oxacillin-resistant isolates give no zone.[12]

Detection of MRSA by phenotypic methods exhibits a lot of discrepancies due to external parameters that influence the degree of heterogeneity and resistance. These parameters include in oculum size, salt concentration, pH, composition of medium, osmolarity and temperature.[13] After screening by phenotypic methods in clinical laboratories, chances of uncertainty are still present due to conditional manifestation of PBP2a. The results have to be confirmed by a gold standard which is fast, precise, sensitive and also independent of growth conditions[14].

PCR assay is being used as a golden bench mark for detection of methicillin resistance for more than three decades, also in epidemiological studies for identification of *mec*A resistant genes. Detection of MRSA at molecular level has the capability to assist steward-ship efforts by sidestepping use of broad-spectrum antimicrobials as well as decreasing antibiotic consumption by up to 80%.[15,16]

Keeping in vision, the challenging risk of resistant infections thorough, competent and operative infect-ion control strategy has to be proposed and highlight-ted for appropriate extermination and eradication of MRSA by systematic examination and surveillance strategy.

METHODS

This descriptive cross-sectional study was conducted at Pathology Department of Postgraduate Medical Institute Lahore, Pakistan during the period from January, 2015 to December, 2015. Clinical specimens were obtained from patients admitted in various clinical wards of Lahore General Hospital (LGH). The study was approved from Institutional Ethical Board and all clinical samples were processed according to standard operating guidelines in microbiology laboratory of Pathology department, PGMI, Lahore.[17]

All the specimens were inoculated on blood agar and McConkey agar (prepared as instructions given by the manufacturer). The plates were incubated at 35-37°C aerobically. Following standard microbiological techniques; primary identification of *Staphylococcus aureus* isolates was done by spotting the colony morpho-logy on agar plates, finding gram positive cocci in clusters on Gram staining and positive Catalase test. Further biochemical tests like coagulase and DNAase were performed for the confirmation of *Staphylococcus aureus*. Control strains i.e., MRSA ATCC 33591 and MSSA ATCC 25923 were used as positive and negative control respectively, for each test mentioned above.[4]

Initial screening was performed by disk diffusion test following guidelines recommended by CLSI (2016). A bacterial suspension of each strain (0.5 McFarland standards) was inoculated on Mueller Hinton agar (MHA). Oxacillin disk (1μg) was applied and plates incubated at 35°C for 24 hrs. An inhibition zone of ≤ 10 mm was considered as oxacillin (methicillin) resis-tant.[25]

The phenotypic resistance to methicillin was ascertained by modified Kirby-Bauer using 30μg cefoxitin disc (Oxoid) on MHA according to CLSI (2016) guiding principles. For each strain, a bacterial suspension adjusted according to 0.5 McFarland turbidity standards was prepared and inoculated on Mueller Hinton agar. The plates were incubated at 35°C and zone of inhibition was determined after 24 hours. The results were interpreted according to CLSI criteria, i.e. zone of ≤ 21 mm was considered as resistant and ≥ 22 mm was considered to be sensitive.[25]

All the MRSA isolates were grown in nutrient broth by incubating in a shaking incubator at 37°C for 24 hrs. Boiling method was used for DNA extraction. The supernatant was collected and stored at -20°C for PCR re-action.

PCR was carried out to confirm the existence of *mec*A gene in methicillin resistant isolates of *Staphylococcus aureus*. The *mec*A gene was detected using primers for *mec*A gene (Table-1). DNA Amplification was perfor-med as follows: An initial denaturation step of 5 min at 94°C; followed by 35 cycles of denaturation step at 95°C for 45 s, annealing step at 58°C for 45 s, and ex-tension step at 72°C for 45 s; and a final extension at 72°C for 5 min. The PCR amplification products (310 bp) were analyzed by electrophoresis on 1.2% agarose gel stained with ethidium bromide (10 mg/ml), using DNA ladder (1kb) and visualized under UV light.[11]

STATISTICAL ANALYSIS:

All the data was entered and analyzed by using SPSS Version 20.0. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy (DA) of all the tests was calculated using *mec*A gene PCR as a gold standard.

RESULTS

Among 750 Staphylococcus *aureus* isolates, 105 consecutive, non-duplicate methicillin resistant strains *of Staphylococcus aureus* from different clinical specimens were included in this study. All MRSA isolates were resistant to both oxacillin (1 μg) and

Table-1: *Primers for mecA gene by PCR Amplification.*

Primers	*Oligonucleotide sequence (5' – 3')*	*Specificity*	*Product size (bp)*
MecA1 (F) MecA2 (R)	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	*mec*A	310

cefoxitin (30 µg) by disk diffusion method. On PCR amplification, out of 105 MRSA (oxacillin resistant) isolates, 83 (79.04%) isolates were positive for *mec*A gene. Among 105 MRSA (cefoxitin resistant) isolates, 89 (84.76%) were *mec*A gene positive, by PCR amplification.

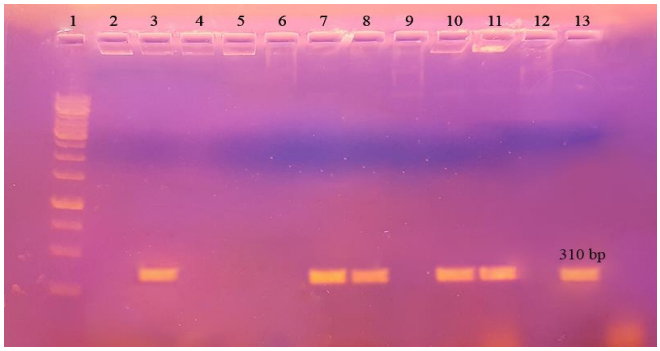

Fig. 1: *Agarose Gel Electrophoresis photograph of Isolates showing mecA gene after PCR Amplification (Lane1: 1 kb DNA ladder; Lane 2, 4, 5, 6, 9: Isolates negative for mecA gene; Lane 3, 7, 8, 10, 11: Isolates positive for mecA gene; Lane 12: MSSA ATCC 25923 (negative control); Lane 13: MRSA ATCC 33591 (positive control).*

Table- 2: *Comparison of Oxacillin and cefoxitin disc diffusion test with PCR for detection of mecA gene in MRSA.*

Parameters	*Oxacillin (1µg)*	*Cefoxitin (30µg)*
True positive	83	89
False positive	4	3
False negative	5	3
True negative	11	10
Sensitivity (%)*	94.31	96.73
Specificity (%)*	73.33	76.92
Diagnostic accuracy (%)*	91.26	94.28

*95% confidence interval

DISCUSSION

Careful detection of methicillin resistance in *Staphylococcus aureus* is exceptionally important to confirm efficient treatment for the affected patient and to prevent transmission of infection by implementing a com-prehensive infection control policy for this organism.[18] The rapid expansion of MRSA has to be restricted by early recognition, investigation and typing which are important for suitable treatment.[19]

A wide range of phenotypic methods have been developed to spot methicillin resistance in *Staphylococcus aureus* but they vary in sensitivity and specificity. Moreover, these tests may not confirm proper and timely treatment of all the patients suffering from MRSA infections. Detection of the *mec*A gene by PCR is the "gold standard", but not always offered in routine laboratories and is not affordable. Cefoxitin disc diffusion test for MRSA identification was found to be the most sensitive method for routine use in resource limited laboratories.[4]

In this study it was evaluated that the diagnostic capability of two antibiotics, i.e., oxacillin and cefoxitin by disc diffusion methods in detecting methicillin resistance in *Staphylococcus aureus*; in comparison with polymerase chain reaction as a gold standard. These methods were oxacillin disc diffusion test and cefoxitin disc diffusion test.

The data in this study showed, that the Oxacillin disc diffusion test has shown less performance values in comparison to cefoxitin disc diffusion method. MRSA detection by Oxacillin disc diffusion method showed sensitivity of 94.31% and specificity of 73.33%. Diagnostic accuracy of cefoxitin disc diffusion method (94.28%) was higher than Oxacillin disc diffusion test (91.26%). Similar results have shown by other researchers.[20-23] The study conducted by Panda et al. 2016 showed high sensitivity and specificity of cefoxitin disc diffusion method.

In the present study, it was found that out of 105 oxacillin and cefoxitin resistant MRSA strains, 83 (79.04%) oxacillin resistant isolates while, 89 (84.76%) cefoxitin resistant isolates were *mec*A gene positive on PCR amplification. Study by Bhattacharya et al. 2016 has revealed that by PCR, *mec*A gene was present in 96.25% among cefoxitin resistant *Staphylococcus aureus* strains.

CONCLUSION

In conclusion, it was found that oxacillin disc diffusion test was less sensitive and specific than cefoxitin disc diffusion test, using PCR as a gold standard. For meticulous identification of MRSA, Cefoxitin disc diffusion method can be used reliably in resource limited circumstances as an alternative to PCR.

LIMITATIONS OF STUDY

It is a single centre study on isolates and clinical profile of patients was not recorded. Clinical correlation could have added a significant impact to the study findings.

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AUTHOR'S CONTRIBUTION

AK: Conceptualization of study design and drafting of manuscript.
FA, NY: Acquisition and analysis of data.
MA, SA, AH: Drafting of manuscript.

CONFLICT OF INTEREST

None to declare.

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