Original Article

Evaluation of phenotypic with genotypic methods for species identification and detection of methicillin resistant in *Staphylococcus aureus*

Kunsang O Bhutia, T Shantikumar Singh, Shilpie Biswas[1], Luna Adhikari

Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, 5th Mile, Tadong, East Sikkim, [1]Molecular Biology Laboratory, Genetix Biotech Asia (P) Ltd, 71/1, First Floor, Shivaji Marg, New Delhi, India

Abstract

Background: Phenotypic methods for the detection of methicillin resistance are inadequate, due to presence of hetero-resistant population and dependence of environmental factors that may affect the phenotypic expression of resistance. **Aims:** Present study was conducted, to evaluate the efficacy of phenotypic methods for the identification of species and *mec-A* mediated resistance in *S. aureus* with polymerase chain reaction (PCR), and to assess the prevalence of the Panton-Valentine leukocidin (*pvl*) toxin in methicillin resistant *S. aureus* (MRSA) and overall *S.aureus* population. **Materials and Methods:** A total of 200 clinical isolates of *Staphylococci* were subjected to phenotypic and genotypic methods for the species identification and detection of MRSA. **Results**: The specificity and sensitivity of conventional methods in the detection of *S.aureus*, was found to be 100 and 97.59% respectively. However, the performance of phenotypic methods in the detection of MRSA were: Oxacillin disc diffusion (DD)-sensitivity 70.58%, specificity 75.75%; cefoxitin DD-sensitivity 86.27%, specificity 83.33%; and oxacillin agar dilution-sensitivity 92.15%, specificity 90.90%. PVL gene was detected in all *mec-A* positive isolates irrespective of their types. **Conclusion:** Phenotypic methods still preferred for the species identification, but for the reliable detection of MRSA an algorithm should include a combination of tests and apply a genotypic method for confirmation of resistance isolates showing discrepant results. Considering the high prevalence of PVL-MRSA, we recommend PCR as assay, as it has an advantage of simultaneous detection of *mec-A* and *pvl* genes by multiplex PCR.

Key words: *fem-A*, methicillin-resistant *Staphylococcus aureus*, phenotypic methods, polymerase chain reaction, *pvl*

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were first described in England in 1961[1] shortly after methicillin became available for clinical use. They have subsequently spread throughout the world and are an important cause of nosocomial and community associated infections. Therefore, rapid and accurate detection of methicillin resistant strains in staphylococci is very essential in order to choose appropriate therapy, to prevent unnecessary use of glycopeptides antibiotics and to take necessary measures for infection control. The mechanism of resistance is due to acquisition of the *mec-A* gene, which encode for low-affinity penicillin-binding protein 2a (PBP2a). Therefore, presence and absence of *mec-A* gene indicates methicillin resistance and methicillin susceptibility in staphylococci respectively. Polymerase chain reaction (PCR) for the amplification of the *mec-A* is presently considered the gold standard for the detecting methicillin resistance in *S. aureus*.[2] In spite of the growing consensus in the literatures for this method, it is not yet available in the all clinical laboratories, therefore phenotypic methods still remains a methods of choice in the resource-constraint settings. However, the performance of phenotypic methods in the detection of methicillin resistance is not consistent, time consuming and also encounter difficulties in detecting all the resistant

Address for correspondence: Miss. Kunsang. O. Bhutia, Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, 5th mile, Tadong, East Sikkim, India.
E-mail: ankusang@gmail.com.

Access this article online

Quick Response Code:

Website: www.ijabmr.org

DOI: 10.4103/2229-516X.106348

isolates, as many environmental factors,[3] and existence of various types of strains among *S. aureus* population limit its accuracy.[4,5]

The strains of *S. aureus*, which do not possess the usual genetic mechanism for oxacillin resistance, but appear resistance phenotypically are known as borderline oxacillin resistant *S.aureus* (BORSA) and modified *S. aureus* (MODSA). The BORSA is a strain of *S. aureus*, that hyper produce beta-lactamase and appear oxacillin resistant, whereas MODSA possess a modification of existing penicillin binding protein rather than a altered or new PBP2a which is encoded by *mec-A* gene, which is the mechanism of classical MRSA.[4] In yet another type of resistance, strains have the genetic information for methicillin resistance, but only a small number express the resistance *in vitro*, due to the presence of two sub-population (one susceptible and the other resistant), that may co-exist within a culture of staphylococci. [5] Such strain is termed as heteroresistance and occurs in *staphylococci* resistant to penicillinase-stable penicillins, such as oxacillin.

Cells expressing hetero-resistance grow more slowly than oxacillin-susceptible population and may be missed at temperature above 35°C and should incubate for full 24 hrs when isolates being tested against oxacillin.[6]

To overcome these difficulties, many studies have been carried out for the evaluation of phenotypic methods for the accurate detection of methicillin resistance and different recommendations have been made regarding the most reliable method for routine use.[2,4,7-10] In comparison to gold standard method of PCR, many authors have recommended that cefoxitin could be a surrogate marker for the detection of methicillin resistance in the settings where PCR is not feasible, as it is a better inducer of *mec-A* gene and disc diffusion test using cefoxitin give clearer endpoints and are easier to read than test with oxacillin.[4,8-9]

Besides its comparable accuracy to PCR, it was also found very efficient in the detection of BORSA strains, which can avoid the mis-categorization of Methicillin-sensitive *S. aureus* (MSSA) into MRSA.[4] PCR also encountered difficulty in discriminating MRSA from methicillin-resistant coagulase negative *staphylococci* (MR-CoNS), because epidemiological studies revealed that *mec-A* gene are also widely distributed among coagulase negative staphylococci (CoNS) and associated with methicillin resistance.[11-13] So with the detection that *fem-A* gene encodes a factor essential for methicillin resistance present universally in all *S. aureus* isolates, MR-CoNS can be excluded.

Panton-Valentine leukocidin (*pvl*) is a cytotoxin that causes leucocyte destruction and tissue necrosis, produced by less than 5% of *S. aureus* strains.[14] It has been preferentially linked to furuncles, cutaneous abscess and severe necrotizing skin infections.[15] Reports suggested the strong association of *pvl* toxin in Community-associated (CA) *S. aureus* and CA-MRSA infections.[14,16]

The purpose of the present study was to compare the efficacy of phenotypic methods for the identification of *S. aureus* and detection of MRSA with genotypic method of PCR by direct detection of the *S. aureus fem-A* gene (serve as internal control) and *mec-A* gene and secondly to assess the prevalence of the *pvl* toxin in the overall *S. aureus* population and MRSA by multiplex PCR with the simultaneous detection of *mec-A* and *pvl* genes.

Materials and Methods

A total of 200 isolates of *Staphylococci,* isolated from different clinical samples during the period from September 2009 to March 2011 in the tertiary-care teaching hospitals were studied. All the isolates were identified to the species level by using conventional techniques like colony morphology on 5% sheep blood agar, catalase test, slide and tube coagulase test, DNase test (Hi-Media, Mumbai, India), Phosphatase test (Hi-Media, Mumbai, India) and Modified Hugh-Leifson's test.

Case definition and source of data

Hospital-associated MRSA (HA-MRSA) isolate defined as one cultured from a clinical specimens obtained 72 hrs after patient's hospital admission or whose sources of isolation were associated with risk factors for HA-MRSA infection (e.g., recent hospitalization, recent surgery, residence in a long-term care facility, drug use, etc.,)[17,18] within one year of MRSA isolation date. In the CDC definition an infection is considered hospital-associated, if it occurs >48 hrs after admission; yet, we chose >72 hours as a cut-off to more conservatively capture hospital-associated infections, i.e., to minimize the mis-categorization of community-associated infections as hospital-associated infections.[19]

Community-associated MRSA (CA-MRSA) isolate were defined as one cultured during the first 72 hrs of a patient's hospital admission, or from patient's whose sources of isolation were not associated with risk factors for HA-MRSA infection as mentioned above.

The secondary data of the patients were obtained from the laboratory investigation register and from medical record file of patients. Data of the study subjects included basic demographic profiles, status of patients (out-patients/ in-patients), ward admitted, specimen type, length of hospital stay, clinical notes, and detailed of risk factors associated with HA-MRSA infections as mentioned above.

Detection of MRSA

Phenotypic methods

Disc diffusion methods

In all confirmed *S. aureus* isolates, oxacillin and cefoxitin disc diffusion (DD) methods were performed for the identification of methicillin-resistant. Four to five colonies from overnight growth was inoculated into 4 to 5 ml cation-adjusted Mueller-Hinton broth (Hi-Media, Mumbai, India) and incubated at 35°C until turbid to 0.5 McFarland standard, and inoculated on two separate Mueller-Hinton agar (MHA) plates, then 1 μg/ml oxacillin disc (Hi-Media, Mumbai, India) and 30 μg/ml cefoxitin disc (Hi-Media, Mumbai, India) placed aseptically and incubated at ambient air, 33-35°C for 16-18 hrs in cefoxitin and full 24 hrs for oxacillin disc diffusion method. Oxacillin DD test was interpreted as follows: Resistance (≤10 mm), moderately sensitive (11-12 mm) and sensitive (≥13 mm), whereas cefoxitin DD ≥ 22 mm as sensitive and ≤ 21 mm as resistant. Reference strains ATCC 29213 (MSSA) and ATCC 43300 (MRSA) included as a control strains in every batch of testing.[6]

Oxacillin agar dilution method

The preparation of oxacillin (Sigma-Aldrich, St Louis, USA) stock solution and testing conditions for oxacillin agar dilution was done as per Clinical and Laboratory Standards Institute (CLSI) guidelines.[6] The range of dilution used was 0.125 to 512 μg/ml. The preparation of test inoculum was similar to DD. Once it matched with standards, 1 μl of bacterial suspension was spot-inoculated on 2% Nacl MHA with a calibrated loop,[6,20] with various concentrations of oxacillin, starting with the lowest dilution and incubated at ambient air 33-35°C for 24 hrs. Reference strains MSSA (ATCC 29213) and MRSA (ATCC 43300) were included in each batch of testing along with drug free control plates to check the validity and purity of testing. The test result was interpreted as resistant (MIC of ≥ 4 μg/ml) and sensitive (MIC ≤ 2 μg/ml).

Genotypic methods

DNA isolation

The test inoculum was prepared by inoculating two to three isolated colonies of *S. aureus* into 3 to 4 ml of Brain-Heart infusion (BHI) broth (Hi-Media, Mumbai, India) and incubated overnight at ambient temperature of 35-37°C. The DNA was extracted by using the HiPurA™ Bacterial and Yeast Genomic DNA Miniprep Purification Spin kit (Hi-Media, Mumbai, India). The DNA concentration was determined as micrograms per millilitre according to A_{260} values by Nanodrop ND-1000 Spectrophotometer (Welmington, USA)

Monoplex polymerase chain reaction for the detection of fem-A gene

The monoplex PCR was performed for the detection of *fem-A* gene as an internal control to validate that all the isolates tested were *S. aureus*. The primers, GFEMAR-1 (5'-AAAAAAGCACATAACAAGCG-3') and GFEMAR-2 (5' -GATAAAGAAGAAACCAGCAG-3') with 132 bp amplicon size for the amplification of *fem-A* gene were taken from published sequence by Mehrotra *et al*.,[21] and checked for specificity against available *S. aureus* genomes with the BLAST utility available through the National centre for Biotechnology Information website (www.ncbi.nim.nih.gov) under GenBank (accession No. X17688.1) and commercially obtained from Sigma-Aldrich, Bangalore, India. PCR was performed by using Qiagen Taq PCR Master mix Kit (Hilden, Germany). A 25 μl final reaction volume consisted of 12.5 μl of master mix, 1 μl of each forward and reverse primers (0.4 μM), 7.5 μl of RNase free water and 3 μl of DNA template. PCR was performed in thermocycler (Biometra Goettingen, Germany) with the thermocycling conditions of initial denaturation (95°C, 5 mins), followed by 35 cycles of three step cycling conditions of denaturation (94°C, 30 sec), annealing (57°C, 1 min) and extension (72°C, 30 sec) followed by final extension (72°C, 7 min) and soaked at 4°C. Then 5 μl of amplified products were mixed with 2 μl of ethidium bromide (Fermentas, St. Leon-Rot, Germany) and were loaded on a 2% agarose gel (Amresco, Solon, USA) along with GeneRuler™ 100 bp Plus DNA Ladder (Fermentas, St. Leon-Rot, Germany); electrophoresis was performed at 100 volt for 50-60 min and visualized under UV transilluminator (Bio-Doc analyzer, Biometra, Goettingen, Germany).

Multiplex polymerase chain reaction for the detection of mec-A and pvl gene

The primers for the amplification of *mec-A* gene GenBank (accession No. Y00688) and *pvl* gene GenBank (accession No. X72700) were MECAP4 (5'-TCCAGATTACAACTTCACCAGG-3') and MECAP7 (5'-CCACTTCATATC TTGTAACG-3') as described by Oliveria *et al*.[22] and *luk-PV*-1 (5'-ATCATTAGGTAAAA TGTCTGGACATGATCCA-3') and *luk-PV*-2 (5'-GCATCAA GTGTATTGGATAGCAAAAGC-3') as described by Mclure *et al*.,[23] respectively. PCR was performed by using Qiagen Multiplex PCR kit (Hilden, Germany) with slight modification. A 25-μl final reaction volume consisted of 12.5 μl mastermix, 2.5 μl primer mix (0.2 μM of each primer), 3 μl of DNA template and 7 μl of RNase free water. DNA samples were subjected to thermocycling conditions with initial inactivation step (95°C, 15 min) with three steps cycling condition of denaturation (94°C, 30 sec), annealing (60°C, 90 sec) and extension (72°C, 90 sec) for 35 cycles with final extension (72°C, 10 min) and soaked at 4°C. Then 5 μl of amplified products were mixed with 2 μl of ethidium bromide (Fermentas, St. Leon-Rot, Germany) and loaded on a 2% agarose gel (Amresco, Solon, USA) along with GeneRuler™ 100 bp Plus DNA Ladder (Fermentas, St. Leon-Rot, Germany); electrophoresis was performed at 100 volt for 50-60 min and visualized under UV transilluminator (Bio-Doc analyzer, Biometra, Goettingen, Germany).

Results

A total of 200 isolates of *Staphylococci* were tested; 119 were detected as *S. aureus* and 81 were detected as CoNS. Of the 119 *S. aureus*, *fem-A* gene were detected in 117 (98.36%) isolates, whereas none reported from CoNS [Table 1, Figure 1]. The performance of conventional method in the accurate identification of *S. aureus* was evaluated by keeping the PCR (*fem-A*) as gold standard. All *fem-A* positive isolates were also identified as *S. aureus* by standard methods, however out of 83 *fem-A* negative isolates, 81 were detected as CoNS and 2 were detected as *S. aureus* by conventional techniques (sensitivity 100% and specificity 97.59%) [Table 2].

Of the 117 *fem-A* positive isolates, 51 (43.58%) were amplified by *mec-A* (162 bp) and 54 (46.15%) were amplified by *pvl* (433 bp) genes respectively; whereas the presence *mec-A* and *pvl* genes were not shown by 66 (56.41%) and 63 (53.84%) isolates respectively [Figure 2].

Among 51 *mec-A* positive isolates, 36 (70.58%), 41 (80.39%) and 47 (92.15%) isolates were identified as MRSA by oxacillin DD, cefoxitin DD and oxacillin agar dilution methods respectively [Table 3]. Whereas, of 66 *mec-A* negative isolates, 44 (66.66%), 48 (72.72%) and 60 (90.90%) isolates were correctly detected as MSSA by oxacillin DD, cefoxitin DD and oxacillin agar dilution methods respectively. The sensitivity, specificity, positive and negative predictive values for oxacillin DD were 70.58, 75.75, 69.23 and 76.92% respectively; for cefoxitin DD were 86.27%, 83.33%, 80% and 88.70% respectively; and for oxacillin agar dilution were 92.15%, 90.90%, 88.67% and 93.75% respectively [Table 4].

Majority of *mec-A* positive isolates (70.58%) which were resistant to both cefoxitin and oxacillin DD had MIC of ≥16 μg/ml, whereas 15.68% isolates (8/51) which were sensitive to oxacillin but resistant to cefoxitin had MIC value of 8 μg/ml. However, variations in the MIC values were seen among the isolates, which was sensitive to both DD methods. Of the seven sensitive isolates by both DD methods, three and four isolates had MIC value of 4 μg/ml and 1-2 μg/ml respectively [Table 5].

Similarly, among the *mec-A* negative isolates, the majority of isolates [75.75% (50/66)] had MIC value in the range of 0.125-1 μg/ml, and 7.5% (5/66) isolates which were resistant to oxacillin but sensitive to cefoxitin had MIC of 2 μg/ml. Whereas among the 11 *mec-A* negative isolates, which were resistant to both DD methods, 5, 3 and 3 isolates had MIC value of 2 μg/ml, 4 μg/ml and 8 μg/ml respectively [Table 6].

Of the 51 *mec-A* positive (MRSA) isolates, 36 (70.58%) isolates were categorized as CA-MRSA and 15 (29.41%) isolates were categorized as HA-MRSA. Of the 54 pvl positive *S. aureus* isolates, 51 (94.44%) were reported from MRSA and 3 (5.55%) were reported from MSSA. All CA- and HA-MRSA were found to be harbouring *pvl* gene.

Table 1: Comparison of phenotypic and genotypic methods for the identification of *S. aureus*

PCR	No of isolates (*n*=200)	*Staphylococci* (*n*=200)	
		S. aureus (*n*=119)	CoNS (*n*=81)
fem-A positive	117	117	0
fem-A negative	83	2	81

PCR: Polymerase chain reaction; CoNS: Coagulase negative *Staphylococci*

Table 2: Performance of phenotypic method in the identification of *S. aureus* as defined by PCR

Methods	*Staphylococci* (*n*=200)		
Genotypic	*fem-A* positive (*n*=117)	*fem-A* negative (*n*=83)	
Phenotypic	TP=117	FP=2	PPV (98.31%)
	FN=0	TN=81	NPV (100 %)
	Sensitivity (100%)	Specificity (97.59%)	

Note: TP: True positive; FP: False positive; FN: False negative; TN: True negative; PPV: Positive predictive value; NPV: Negative predictive value

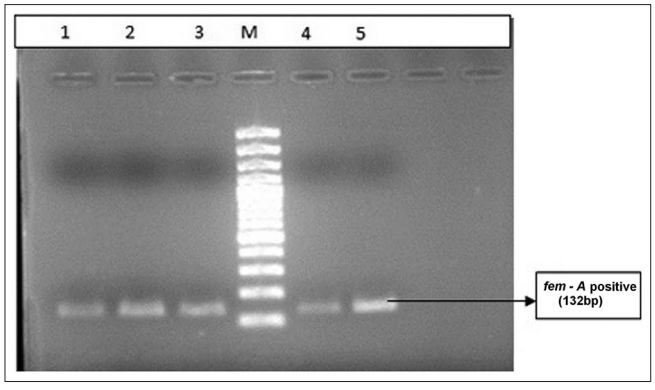

Figure 1: Monoplex PCR for the detection of *fem-A* (162 bp), Lane 1 to 5 = Positive for *fem-A* gene, M = (Marker 100 bp)

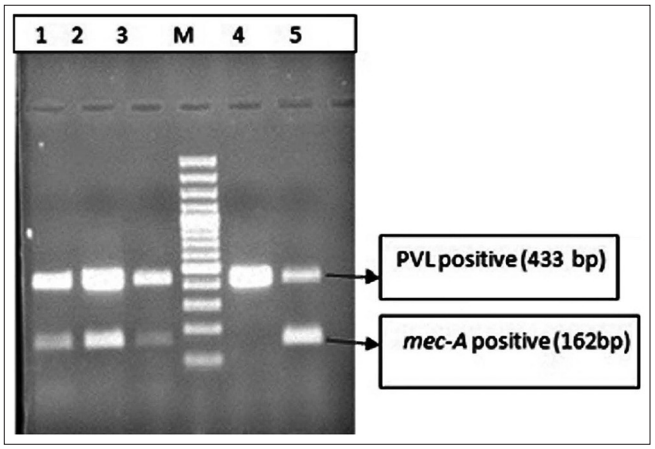

Figure 2: Multiplex PCR (*mec-A* and PVL gene). Lane 1,2,3,5 = Positive *mec-A* (162 bp) and PVL (433 bp), M = Marker (100 bp DNA ladder), Lane 4 = Negative *mec-A* (162 bp) and positive PVL (433 bp)

Table 3: Comparison of phenotypic and genotypic methods for the detection of MRSA

Presence of *mec-A* gene by PCR	*fem-A* positive isolates (*n*=117)	Number of MRSA by phenotypic methods		
		Oxacillin DD (1 μg/ml)	Cefoxitin DD (30 μg/ml)	Oxacillin agar dilution
mec-A positive	51	36	44	47
mec-A negative	66	16	11	6

MRSA: Methicillin resistant *S. aureus*; DD: Disc diffusion

Table 4: Performance of phenotypic methods for the detection of MRSA as defined by *mec-A* gene detection by PCR

Phenotypic methods	Results	Presence of *fem-A* gene as detected by PCR (*n*=117)			
		mec-A positive (*n*-=51)	*mec-A* negative (*n*=66)	Predictive values (%)	Sen/Spec (%)
Oxacillin disc diffusion (1 μg/ml)					
	MRSA	TP=36	FP=16	PPV (69.23)	Sen: 70.58
	MSSA	FN=15	TN=50	NPV (76.92)	Spec: 75.75
Cefoxiitn disc diffusion (30 μg/ml)					
	MRSA	TP=44	FP=11	PPV (80)	Sen: 86.27
	MSSA	FN=7	TN=55	NPV (88.70)	Spec: 83.33
Oxacillin agar dilution					
	MRSA	TP=47	FP=6	PPV (88.67)	Sen: 92.15
	MSSA	FN=4	TN=60	NPV (93.75)	Spec: 90.90

Sen: Sensitivity; Spec: Specificity; TP: True positive; FP: False positive; FN: False negative; TN: True negative; PPV: Positive predictive value; NPV: Negative predictive value MRSA: Methicillin resistant *Staphylococcus aureus*; MSSA: Methicillin-sensitive *Staphylococcus aureus*

Table 5: Comparison of MIC value of *mec-A* positive isolates (MRSA) with the results of disc diffusion methods (*N*=51)

Results of disc diffusion methods	MIC value (μg/ml)	No of isolates (%)
Resistance to oxacillin and cefoxitin	≥16 μg/ml	36 (70.58)
Sensitive to oxacillin and resistance to cefoxitin	8 μg/ml	8 (15.68)
Sensiitve to oxacillin and cefoxitin	4 μg/ml	3 (5.8)
Sensiitve to oxacillin and cefoxitin	1-2 μg/ml	4 (7.8)

Table 6: Comparison of MIC value of *mec-A* negative isolates (MSSA) with the results of disc diffusion methods (*N*=66)

Results of disc diffusion methods	MIC value (μg/ml)	No of isolates (%)
Sensitive to oxacillin and cefoxitin	0.125-1 μg/ml	50 (75.75)
Resistance to oxacillin and sensitive to cefoxitin	2 μg/ml	5 (7.57)
Resistance to oxacilin and cefoxitin	2 μg/ml	5 (7.57)
Resistance to oxacilin and cefoxitin	4 μg/ml	3 (4.54)
Resistance to oxacilin and cefoxitin	8 μg/ml	3 (4.54)

Discussion

For the past 50 years *S. aureus* has been a dynamic human pathogen that has gained the deepest respect of clinicians, since the first report of MRSA infection in Boston city hospital in 1961.[1] Since then MRSA has become widespread all over the world. As methicillin resistant strains are widely distributed in *S. aureus* as well as in CoNS, therefore with the detection of a marker specific to *S. aureus* along with *mec-A* gene proved to be a more reliable indicator to identify MRSA by differentiating it from *mec-A* positive CoNS.[4,24,25] In our study, we have used *fem-A* gene as a specific marker to *S. aureus*, as besides being a unique feature to *S. aureus*,[26] it also act as a regulator gene in the expression of high-level methicillin resistant in *S. aureus*.[24] So, with the inclusion of *fem-A* gene in our study, it has added advantage of accurate identification of *S. aureus* along with the detection of any influence of *fem-A* gene on methicillin resistant in *S. aureus*.

In the present study, the detection rate of *fem-A* gene among *S. aureus* population was 98.31% (117/119) and none detected in CoNS by monoplex PCR, against detection rate of 89.4% and 97% of *fem-A* and *fem-B* genes by Kobayashi *et al*.[24] with the detection of three genes (*fem-A, fem-B* and *mec-A*) in a single run PCR (multiplex). This difference could be due to simultaneous detection of three genes in a single run by multiplex PCR,[24] against one gene (*fem-A*) in present study by monoplex PCR, that may increase the detection rate of *fem-A* gene in the present study.

Similarly, on evaluating the efficacy of conventional methods in the detection of *S. aureus* vs Monoplex PCR (*fem-A*); 100% *fem-A* positive and 2.4% *fem-A* negative isolates of *S. aureus* were identified by conventional methods (sensitivity 100% and specificity 97.59%) in the present study. This is in contrast to other studies having detected *fem-A* gene in few CoNS isolates, but similar in having good co-relation of standards methods in the detection of *S. aureus* in comparison to PCR.[24-26] Though, the detection rate of *fem-A* gene varies slightly in different geographical regions, but expression of *fem-A* gene is a unique feature of *S. aureus* and it can be reliably used as a marker in the differentiation of *S. aureus* from CoNS. The good performance of conventional techniques in the detection of *S. aureus* in comparison to PCR (*fem-A*) suggest that the conventional methods still remain a method of choice in the accurate detection of *S. aureus*.

On further evaluating the involvement of *fem-A* gene in the expression of methicillin resistance, it was found that 43.58% isolates were *fem-A, mec-A* positive (PCR confirmed MRSA) and 56.41% isolates were *fem-A* positive, *mec-A* negative (PCR confirmed MSSA) isolates. This is in concordance to other studies where prevalence of *fem-A* gene in *mec-A* negative isolates were found to be higher[24] or almost equivalent[25] to *mec-A* positive isolates. This finding indicate that the influence of *fem-A* gene on the methicillin resistance in the *S. aureus*

strains is slight as prevalence of *fem-A* gene was found higher in MSSA (*mec-A* negative) in comparison to MRSA (*mec-A* positive). From these results, it has become evident that methicillin resistance in *S. aureus* may be significantly regulated by other genes like *mecR1* and *mec I* genes or some other unidentiifed factors, rather than *fem-A* gene.

The heterogeneous nature of methicillin resistance in *S. aureus* limits the accuracy and reliability of phenotypic methods such as DD and dilution methods.[10] In the present study, among the phenotypic methods, the performance of oxacillin agar dilution (sensitivity 92.15%, specificity 90.90%) was found to be better in comparison to cefoxitin DD (sensitivity 86.27%, specificity 83.33%) and oxacillin DD (sensitivity 70.58%, specificity 75.75%) in the detection of *mec-A* mediated resistance. In contrast, recently, many studies have reported 100% accuracy of cefoxitin DD test (100% ssensitivity and specificity),[4,9] in comparison to oxacillin DD (sensitivity 100%, specificity 56%),[4] (sensitivity 87.5%, specificity 100%),[9] and oxacillin agar dilution (sensitivity 100%, specificity 90%).[4] Similarly, Bosselemez-Tmaz *et al*., reported better performance of cefoxitin DD (sensitivity 99.19%, specificity 100%) over oxacillin DD (sensitivity 95.96%, specificity 100%) in comparison to PCR.[8] A few studies reported that the oxacillin DD (100% sensitivity and specificity) approaches the accuracy of PCR in the detection of MRSA.[7] However, in concordance to the present study, one study reported that none of the phenotypic methods was reliable for the detection of the methicillin resistance in *S. aureus*.[10]

In the present study, the higher false negativity of DD methods (oxacillin 29.41%, and cefoxitin 13.7%) was reported in comparison to 7.84% of oxacillin agar dilution. All four isolates were oxacillin sensitive *S. aureus* (OSSA) by all three phenotypic methods but *mec-A* positive, suggesting these isolates probably non-PBP 2' producing strains have been detected earlier and referred as cryptically methicillin resistant strain.[24,27] This was supported by the fact that MIC value of all four isolates was in the range of 1-2 μg/ml. Therefore it is recommended that the isolates with MIC value in this range should be further confirmed with PCR, as clinical problem with such strains like beta-lactam antibiotics induced production of PBP 2' may be seen during chemotherapy, leading the conversion of the strain into oxacillin resistant *S. aureus* (ORSA) as demonstrated *in vitro*.[28]

The percentage of cryptic isolates reported in our study, is comparatively less than the study conducted by Kobayashi *et al*., (16.7%),[24] but higher than the study conducted by Nikbakht *et al*., (3.75%).[27]

The rest 11 (21.56%) isolates were OSSA by oxacillin DD and 3 (5.8%) by cefoxitin DD methods, but all were ORSA by oxacillin agar dilution; suggesting it as heteroresistant strains of MRSA instead of cryptic strains. Heteroresistant strains have usual mechanism for methicillin resistance, but few expresses *in vitro*, suggesting that the expression of resistance sub-population to be suppressed by over expression of susceptible sub-populations.[9] This too corroborated by the fact, that all isolates had MIC value in the range of 4-8 μg/ml. The specificity of the DD methods is often affected by high false positive isolates. The high false positivity of DD methods (oxacillin 24.24% and cefoxitin 16.66%) in the present study could be due to hyper-production of beta-lactamase, leading to phenotypic expression of oxacillin resistance. Such strains are called BORSA strains, because despite the absence of genetic mechanism for methicillin resistance (*mec-A* gene), these starins express resistance phenotypically.[4] However, wide variation was seen in the MIC value of BORSA strains. The MIC value all false positive isolates by all three phenotypic methods (9.09%) was in the range of 4-8 μg/ml. Similar oxacillin MIC range was reported earlier for very high percentage (45%) of BORSA strains as detected by oxacillin DD,[4] but no such isolates had been reported by cefoxitin DD.

The rest 5 (7.57%) isolates which were resistant to both DD methods but sensitive to oxacillin agar dilution (MIC-2 μg/ml) and another 5 (7.57%) isolates resistant to oxacillin DD but sensitive to cefoxitin DD and oxacillin agar dilution (MIC of 2 μg/ml). The results suggest that such isolates may have another mechanism of oxacillin resistant than PBP 2a for methicillin resistance. Such strains are labelled as modified *S. aureus* (MODSA) strains, which possess a modification of existing penicillin binding proteins rather than acquisition of new PBP as in the mechanism for classical MRSA. Probably, these MODSA strains under antibiotic pressure may evolve into fully resistant isolates in future, as MIC value of such isolates (2 μg/ml) were higher than the isolates (0.125-1 μg/ml) which were sensitive by all three phenotypic methods in concordance with PCR; and labelled as true MSSA. Occurrence of various types of strains among *S. aureus* population complicated the accurate detection of MRSA. Though the cefoxitin was proved to be better predictor of *mec-A* mediated resistance and the performance was equivalent to PCR,[4,8,9] it was also found to be unsatisfactory. However, not absolute but better co-relation was seen between oxacillin agar dilution and PCR in the detection of *mec*-mediated resistance. The determination of the MIC of those isolates showing discrepancy results with DD methods give additional information about the different strains of *S. aureus* that may complicate the treatment and patient management. Comparing the MIC value of *mec-A* positive and negative isolates it was found that majority of isolates showing discrepant results with DD methods was in the MIC range of

2-8 μg/ml, suggesting this MIC range to be critical (doubtful) breakpoint and the isolates with MIC within this range should always be confirmed further with PCR.

The screening of *pvl* gene among MRSA has gained importance in recent years due to high involvement of *pvl* toxin in CA-MRSA infections.[16,29] The three major genotypic markers that distinguishes CA-from HA-MRSA isolates are: Their genetic lineage (ST), the architecture of mobile genetic element (SCC*mec* type) and presence of PVL toxin.[15] Almost 100% of CA-MRSA strains possess the *pvl* gene compared to <5% in HA-MRSA and MSSA.[30] Thus many researchers have highlighted the *pvl* gene as a reliable marker for CA-MRSA infections.[16,29,31-32]

In the present study, *pvl* gene was included to validate the molecular definition of CA-MRSA and test the hypothesis that "PVL toxin is a reliable marker for CA-MRSA infections". We found the prevalence of *pvl* gene as 46.15% (54/117), 94.44% (51/54), and 5.55% (3/54) among *S. aureus*, MRSA and MSSA isolates respectively. All MRSA were positive for *pvl* gene irrespective of their types (CA-36 and HA-15), This is in contrast to several studies, which reported presence of *pvl* toxin only in CA-MRSA and none from HA-MRSA isolates.[29,31-33] But our study is in agreement with few studies reporting that the presence of PVL toxin cannot be used as a sole marker for CA-MRSA.[34,35] Considering the high detection of *pvl* gene in MRSA (irrespective of their types) than MSSA, it can be rather hypothesize that MRSA is an important reservoir of *pvl* gene and is now being slowly acquired by MSSA strains.

It is concluded that phenotypic methods still remain the preferred choice for the species identification, but for the detection of MRSA none of the phenotypic methods showed 100% accuracy with PCR. However, considering the better performance of oxacillin agar dilution and also the additional information provided by this method for the isolates showing discrepancy results with DD methods, it is suggested that a combination of tests should be used and PCR method should be applied for confirmation of resistance, as assay has an advantage of rapid and simultaneous detection of *mec-A* mediated resistance along with the detection of any other gene (*fem-A* and *pvl*)) with multiplex PCR techniques.

Future work suggested

Further molecular typing of the MRSA isolates will be helpful to validate the molecular definition of CA- and HA-MRSA isolates, as *pvl* gene one of the important genotypic marker for CA-MRSA, was found to be an unreliable marker to differentiate it from HA-MRSA. It will be helpful in taking appropriate measures in control and prevention of further spread of MRSA.

Acknowledgment

We would like to thanks Dean, Sikkim Maniple Institute of Medical Sciences and Mr Arum Parkas (CEO), Genetix-Biotech Asia (P) Ltd, Dr Samar Husain Navqvi (Genetix-Biotech) for valuable guidance and allowing me to carry out molecular part of our work in Genetix-Biotech Molecular Laboratory, New Delhi.

References

1. Jevons MP. Celbenin-resistant *Staphylococcus*. Br Med J 1961;1:124-5.
2. Mohanasoudaram KM, Lalitha MK. Comparison of phenotypic versus genotypic methods in the detection of methicillin resistance in *Staphylococcus aureus*. Indian J Med Res 2008;127:78-84.
3. Brown DF. Detection of methicilin/oxacillin resistance in staphylococci. J Antimicrob Chemother 2001;48:65-70.
4. Mathew AA, Thomas M, Appalaraju B, Jayalakshmi J. Evaluation and comparison of tests to detect methicillin resistant *Staphylococcus aureus*. Indian J Pathol Microbiol 2010;53:79-82.
5. Bannermann TL. Staphylococcus, Micrococcus and other catalase-posiitve cocci that grow aerobically. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH, editors. Manual of Clinical Microbiology. 8th ed. Washington, DC: ASM Press; 2003. p. 384.
6. Clinical and Laboratory Standards Institute/NCCLS. Performance standards for Antimicrobial susceptibility testing; Eighteenth Informational supplement. Approveds tandard M100-S18; Vol, 28 No 1. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
7. Ercis S, Saneak B, Hascelik G. A comparison of PCR detection of *mec-A* with oxacillin disk susceptibility testing in different media and sceptor automated system for both *Staphylococcus aureus* and *Coagulase-negative Staphylococci* isolates. Indian J Med Microbiol 2008;26:21-4.
8. Bosgelmez-Tmaz G, Ulusoy S, Aridogan B, Coskun-Ari F. Evaluation of different methods to detect oxacillin resistance in *Staphylococcus aureus* and their clinical laboratory utility. Eur J Clin Microbiol Infect Dis 2006;25:410-12.
9. Anand KB, Agrawal P, Kumar S, Kapila K. Comparison of cefoxitin disc diffusion test, oxacillin screen agar and PCR for *mec-A* gene for detection of MRSA. Indian J Med Microbiol 2009;27:27-9.
10. Kaya EG, Karakoc E, Yagci S, Yucel M. Evaluation of phenotypic and genotypic methods for detection of methicillin resistance in *Staphylococcus aureus*. Afr J Microbiol Res 2009;3:925-9.
11. Ryffel C, Tesch W, Birch-Machin I, Reynolds PE, Barberis-Maino L, Kayser FH. Sequence comparison of *mec-A* genes isolated from methicillin resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Gene 1990;94:137-8.
12. Suzuki E, Hiramatsu K, Yokota T. Survey of methicillin-resistant clinical strains of coagulase-nagative staphylococci for *mec-A* gene distribution. Antimicrob Agents Chemother 1992;36:429-34.
13. Ubukata K, Nonoguchi R, Song MD, Matsuhashi M, Konno M. Homology of *mec-A* gene in methicillin resistant *Staphylococcus haemolyticus* and *Staphylococcus simulans* to that of *Staphylococcus aureus*. Antimicrob Agents Chemother 1990;34:170-2.
14. Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V, *et al*. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis 1999;29:1128-32.
15. Couppie P, Criebier B, Prévost G Grosshans E, Piemont Y. Leukocidin from *Staphylococcus aureus* and cutaneous infections: An epidemiologic study. Arch Dermatol 1994;130:1208-09.
16. Boyle-Vavra S, Daum RS. Community-acquired methicillin resistant *Staphylococcus aureus*: The role of Paton-Valentine leukocidin. Lab Investig 2007;87:3-9.

17. Herold BC, Immergluck LC, Maranan MC, Lauderdale DS, Gaskin RE, Boyle-Vavra S, *et al*. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. JAMA 1998;279:593-8.
18. Garner JS, Jarvis WR, Emori TG, Horan TC, Hughes JM. CDC definitions for nosocomial infections. Am J Infect Control 1988;16:128-40.
19. Maree CL, Daum RS, Boyle-Vavra S, Matayoshi K, Miller LG. Community-associated Methicillin-resistant *Staphylococcus aureus* isolates causing healthcare-associated Infections. Emerg Infect Dis 2007;13:236-42.
20. Huang MB, Gay TE, Baker CN, Banerjee SN, Tenovar FC. Two percent sodium chloride is required for susceptibility testing staphylococci with oxacillin, when using agar-based dilution methods. J Clin Microbiol 1993;31:2683-8.
21. Mehrotra M, Wang G, Johnson WM. Multiplex PCR for Detection of Genes for *Staphylococcus aureus* Enterotoxins, Exfoliative Toxins, Toxic Shock Syndrome Toxin 1, and Methicillin Resistance. J Clin Microbiol 2000;38:1032-5.
22. Oliveira DC, de Lencastre H. Multiplex PCR Strategy for Rapid Identification of Structural Types and Variants of the mec Element in Methicillin-Resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2002;46:2155-61.
23. McClure J, Conly JM, Lau V, Elsayed S, Louie T, Hutchins W, *et al*. Novel Multiplex PCR Assay for Detection of the Staphylococcal Virulence Marker Panton-Valentine Leukocidin Genes and Simultaneous Discrimination of Methicillin-Susceptible from Resistant Staphylococci. J Clin Microbiol 2006;44:1141-4.
24. Kobayashi N, Wu H, Kojima K, Taniguchi K, Urasawa S, Uehara N, *et al*. Detection of *mec-A*, *fem-A* and *fem-B* genes in clinical strains of Staphylococci using polymerase chain reaction. Epidemiol Infect 1994;113:259-66.
25. Vannuffel P, Gigi J, Ezzedine H, Van Der Cam B, Delmee M, Wauters G, *et al*. Specific detection of methicillin-resistant *Staphylococcus* species by multiplex PCR. J Clin Microbiol 1995;33:2864-7.
26. Unal S, Hoskins J, Flokowitsch E, Wu CY, Preston DA, Skatrud PL. Detection of methicillin-resistant staphylococci by using the polymerase chain reaction. J Clin Microbiol 1992;30:1685-91.
27. Nikbakht M, Nahaei MR, Akhi MT, Asgharzadeh M, Nikvash S. Comparison of different methods (Disk agar diffusion, Oxacillin agar and PCR) in detection of methicillin resistant *Staphylococcus aureus* in strains in Tabriz, Iran. Int Med J 2008;69:46-55.
28. Ubukata K, Yamashita N, Konno M. Occurence of a β-lactam-inducible penicillin-binding protein in methicillin-resistant Staphylococci. Antimicrob Agents Chemother 1985;27:851-7.
29. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, *et al*. Community-acquired methicillin resistant *Staphylococcus aureus* carrying Panton-Valentine leukocodin genes: Worldwide emergence. Emerg Infect Dis 2003;9:978-84.
30. Borlaug G, Davis JP, Fox BC. Community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). Guidelines for clinical management and control of transmission. Available from: http://www.unc.edu/depts/spice/WisconsinCAMRSAGuide.pdf. [Last accessed on 2011 Jun 12].
31. Soo Ko K, Kim YS, Song JH, Yeom JS, Lee H, Jung SI, *et al*. Genotypic diversity of methicillin-resistant *Staphylococcus aureus* Isolates in Korean Hopitals. Antimicrob Agents Chemother 2005;49:3583-5.
32. McDonald RR, Antonishyn NA, Hansen T, Snook LA, Nagle E, Mulvey MR, *et al*. Development of a triplex real-time PCR assay for detection of Panton-Valentine leukocidin toxin genes in clinical isolates of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 2005;43:6147-9.
33. D'Souza N, Rodriques C, Mehta A. Molecular Characterization of methicillin-resistant *Staphylococcus aureus* with emergence of epidemic clones of sequence type (ST) 22 and ST 772 in Mumbai, India. J Clin Microbiol 2010;48:1806-11.
34. Roosney AS, Shore AC, Morgan PM, Fitzgibbon MM, O'Connell B, Coleman DC. The Emergence and importation of diverse genotypes of methicillin-resistant *Staphylococcus aureus* (MRSA) harboring the Panton-Valentine Leukociidn Gene (pvl) reveal that pvl is a poor marker for communtiy-acquired MRSA strains in Ireland. J Clin Microbiol 2007;45:2554-63.
35. Said-Salim B, Mathema B, Braughton K, Davis S, Sinsimer D, Eisner W, *et al*. Differential distribution and expression of Panton-Valentine leucocidin among community-acquired methicillin-resistant *Staphylococcus aureus* strains. J Clin Microbiol 2005;43:3373-9.

How to cite this article: Bhutia KO, Singh TS, Biswas S, Adhikari L. Evaluation of phenotypic with genotypic methods for species identification and detection of methicillin resistant in *Staphylococcus aureus*. Int J App Basic Med Res 2012;2:84-91.

Source of Support: Nil. **Conflict of Interest:** None declared.