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Susceptibility testing and resistance phenotypic detection in Staphylococcus aureus by conventional and molecular methods: Importance of automated (Vitek 2) system

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Abstract
Background: ***Once identified as Staphylococcus aureus, it is imperative to understand their methicillin resistance status for institution of appropriate therapy. Conventional disc diffusion tests may not accurately identify MRSA strains and may not be in concordance with PCR results.***
Objectives: ***Relative abilities of phenotypic and molecular methods to detect MRSA strains was evaluated to understand possible causes of disagreement between these tests, if any, by using automated microbial identification and antibiotic sensitivity test system.***
Methods: ***70 clinical isolates of S. aureus from North Karnataka were collected and phenotypically characterized as methicillin resistant or sensitive by disc diffusion test. PCR was done to detect mec A gene positivity. Isolates having disagreement between PCR results and disc-diffusion test were analyzed using Vitek 2 automated system (bioMerieux, France. Software version: 05.02).***
Results and Conclusion: ***There was 98.5 % (69/70) agreement between PCR and cefoxitin disc diffusion while it was 80% (56/70) between oxacillin and PCR. 14 isolates (20%) showed disagreement between either of the two disc diffusion tests and PCR. 10 of these were classified as MSSA by Vitek 2 with acquired penicillinase activity while 4 were categorized under MRSA by modification of mec A. Since automated systems provide additional information on likely penicillinase /modified mec A activity, use of these systems help clinicians treat patients with such MSSA infections like cases of MRSA.***

Keywords—MRSA, non mecA resistance, acquired penicillinase, PBP modification, Vitek 2 system.

I. INTRODUCTION

Staphylococcus aureus, is one of the greatly feared pathogen causing variety of life threatening infections and treatment against which is increasingly becoming difficult especially with the emergence of strains resistant to several antibiotics [1]. Therefore it is not enough to detect the *S. aureus* but also to know its resistance and sensitivity patterns for institution of appropriate treatment. Broadly, *S. aureus* are grouped into two major classes viz. Methicillin Sensitive *S. aureus* (MSSA) and Methiclin Resistant *S. aureus* (MRSA). MRSA strains have been associated with nosocomial or hospital acquired infections world over and have also emerged as an important cause of community acquired infections [2]. Resistance to methicillin is mostly determined by the presence of *mec A* gene encoding altered penicillin binding protein which shows low affinity to β-lactam antibiotics [3]. Although non *mecA* mediated resistance, i.e due to acquired penicillinase or due to effect of additional genes like *fem B,* is also reported [4,5]. Inaccurate antibiotic susceptibility report may result in treatment failure, rapid spread of resistance or unwarranted administration of higher antibiotics. Clinical laboratories had been using oxacillin disc (1 mcg) diffusion test for determination of MRSA status of *S.aureus* since long but now oxacillin discs have been replaced with cefoxitin (30mcg) as per CLSI 2006 guidelines [6]; cefoxitin being a potent inducer of the *mec A* regulatory system. With the advent of PCR in early 1980's and its consequent use till date for detection of *mec A* gene in MRSA isolates, new impetus has been brought to the study at molecular level and is increasingly becoming popular. However at times discrepancies have been observed between the results of these tests [7] that require detailed analysis and explanations. We undertook this study to understand the relative abilities of conventional disc diffusion using oxacillin and cefoxitin, and PCR in differentiation of MRSA strains. Vitek 2 Compact automated microbial identification and sensitivity test system (bioMerieux, France. Software version: 05.02) was used to analyze the disagreement in the results of phenotypic tests and PCR.

II. METHODOLOGY

Sample Collection and Identification:

A total of 70 isolates of *S.aureus* were collected after obtaining due ethical clearance from microbiology laboratory of a tertiary care hospital in Belgaum that receive samples from various diagnostic laboratories, primary and secondary care centers as well as outdoor and indoor patient departments of their attached hospitals. Samples identified as *S.aureus* by

standard microbiology methods at the tertiary care Centre were reconfirmed at the research laboratory.

Disc Diffusion Assays with oxacillin and cefoxitin:

All isolates were subjected to phenotypic antimicrobial susceptibility tests by Kirby Bauer disc method using oxacillin,1 mcg and cefoxitin,30 mcg(Hi-Media, India) following CLSI guidelines 2010[8]. The zone of inhibition was measured after 24 hrs incubation at 35°C with the criteria for oxacillin being considered susceptible when zone diameter is ≥13 mm; intermediate when 11-12 mm; and resistant when zone diameter is ≤10 mm and susceptible to cefoxitin when zone diameter is ≥22 mm; and resistant when ≤21 mm at incubation at 37°C. Standard MRSA strain (ATCC 43300) and MSSA strain (ATCC 25923) were included in each batch.

PCR for detection of *mecA* gene:

Bacterial DNA was extracted from overnight cultures of *S. aureus* by CTAB- NaCl method (Hexadecyltrimethylammonium bromide- Sodium Chloride) [9]. The quality and quantity of isolated DNA was determined using Nanodrop 1000 spectrophotometer (JH Biosciences, USA .Model: ND1000) at 260/280 nm as well as visually by horizontal gel electrophoresis in 1% agarose. PCR for the detection of *mecA* was carried out following the method of Unal *et al.*,1995[10]. Primer sequence used for *mecA* detection are *mec A* (F): GTA GAA ATG ACT GAA CGT CCG ATA A , *mec A* (R): GTA GAA ATG ACT GAA CGT CCG ATA A (Bangalore Genei, India). Briefly, 1 µl of 60 ng of the extracted DNA was added to 24 µl of PCR amplification mix consisting of 16 µl of doubled distilled autoclaved water, 2.5 µl of 10X Taq buffer (Tris with 15 mM $MgCl_2$), 1µl of 2.5 mM dNTP mix (Merck, India), 0.5 µl of 3U/µl Taq polymerase (Merck, India), and 0.5 mM of each primer (synthesized by Sigma, India). The *mec A* gene was amplified using the primers as described by Jonas *et al*., 1999. Amplifications were carried out in a thermal cycler (iCycler, BioRad Inc., USA) with conditions that consisted of 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1 min with a final extension at 72°C for 2 min. The PCR products were subjected to agarose gel electrophoresis using gel red dye and images were acquired using Alpha Imager gel documentation system (JH biosciences, USA. Model: D E 400).

Automated microbial identification and sensitivity (Vitek 2; bioMerieux, France) system:

For this study, 14 isolates of *S. aureus* that showed disagreement in their MRSA/MSSA status in any of the disc diffusion or PCR based assays, were subjected to analysis by Vitek 2 Compact automated antimicrobial identification and sensitivity system. Standard MRSA strain (ATCC 43300) and MSSA strain (ATCC 25923) were included as internal controls. Cultures (18-24 h old) of specified density (0.5- 0.63 McFarland standard, measured with colorimeter supplied with the system) were inoculated into sterile normal saline (supplied by bioMerieux, France) and loaded on to Vitek 2 Compact system with software version 5.02 (bioMerieux, France) following manufacturer's instructions for sensitivity using AST GP-67 (sensitivity detection) cards. Each of the inoculums put into Vitek 2 was cultured again and standard microbiological tests followed by PCR were carried out to reconfirm their identity. Routine sterility checks were done by plating the saline used and checked for possible contaminations.

III. RESULTS

Among 70 *S. aureus* strains, 40 were clearly identified as MRSA by all methods used while 16 were identified as MSSA giving an overall agreement of 80% between oxacillin and cefoxitin disc diffusion and PCR (Table 1).

Oxacillin disc diffusion method detected 44 clinical isolates as MRSA and 17 as MSSA out of total 70 strains, whereas 9 isolates showed intermediate resistance and was therefore inconclusive (3 PCR positive and 6 PCR negative). 43 isolates were MRSA and 27 were MSSA by cefoxitin disc diffusion method (Table 1). Total 44 isolates out of 70 were found to be *mec A* gene positive by PCR. There was 98.5% agreement between cefoxitin disc diffusion and PCR while it was 80% between oxacillin disc diffusion and PCR (Table 1).

Among 70 isolates taken for the study 14 isolates (20%), which showed disagreement between either of the two antibiotics used in disc diffusion tests or with PCR results, were subjected to analysis by Vitek 2 system. 10 of the 14 isolates were pronounced as MSSA by Vitek 2 with acquired penicillinase activity and 4 were pronounced MRSA by modification of *mec A.* (Table 1).

IV. DISCUSSION

Rapid and precise identification of MRSA is of utmost importance in the clinical microbiology laboratories for institution of appropriate treatment. However, there is no optimal phenotypic method for detection of methicillin resistance in *S. aureus* as conventional methods require special conditions e.g. 2% NaCl enriched media, up to 48 hours incubation time, ≤ 35°C temperature, etc. [11,4,6]. Despite necessary precautions and expertise in handling MRSA, discrepancies occur as far as phenotypic methods are concerned [12]. Anand *et al.,* 2009 [13] found cefoxitin disc method to be 100% in concordance with PCR while in some cases discrepancies have been found even while using cefoxitin disc for MRSA detection[14], PCR based genotypic methods, although often referred to as a gold standard are not able to detect the non-*mecA* mediated resistances. Even if an isolate possess *mecA* gene and positive in PCR, it does not necessarily imply that the strain is phenotypically MRSA, as there are occasions where the gene product is not expressed or the gene expression is suppressed [15].

In our study cefoxitin disc test results were in 98.5% concordance with PCR results. However, oxacillin disc test yielded imprecise results, often with intermediate values and misclassifying few strains. Results of oxacillin disc diffusion therefore may lead to incorrect therapy. Sometimes heterogeneous expression of methicillin resistance often affects the reliability of disc diffusion tests [16]. Availability of

automated microbial identification and sensitivity testing systems has made it possible to detect the phenotypic traits of *S.aureus* irrespective of their mechanism of acquiring resistance to methicillin. Despite being costly, these systems are therefore increasingly being used in diagnosing MRSA infections, particularly in resourceful laboratories [17]. Roisin *et al*., reported 97.5% accuracy in detection of oxacillin resistant *S.aureus* by Vitek 2 system [18]. Ligozzi *et al.,* showed 96% correct agreement of Vitek 2 compact system for gram positive bacillus and indicated it as accurate and acceptable means for performing antibiotic susceptibility tests with medically relevant gram positive cocci [19].

In our study status of 14 strains that were disputed by any of the tests employed, (oxacillin or cefoxitin in disc diffusion or PCR) were subjected to Vitek 2 automated system to get further information on their sensitivity status.

Vitek 2 automated system not only returned objective results regarding the methicillin resistance status of *S.aureus* isolates, it also interpreted the resistant phenotypes as being of wild type or different due to acquired penicillinase or modification of PBP. Interestingly, some strains like 5148, 5689, 5363, 6561, 5481, 5505, and 5639 that showed different susceptibility pattern with oxacillin and cefoxitin but were *mec A* gene negative, were confirmed as MSSA by Vitek 2 System (Table1). Cefoxitin screen (by Vitek 2) was negative (Organism is inhibited by cefoxitin) based on the value of oxacillin but Advanced Expert System (AES; software in Vitek 2 compact), detected the possible phenotypic resistance mechanism (Acquired Penicillinase). According to AES, these particular strains are producers of penicillinase enzyme through genetic exchange with other strains such as *Enterococcus*, *Streptococcus*, Extended Spectrum Beta Lactamase (ESBL) Gram negative bacilli and, even including *S.aureus* by any of the recombinant methods i.e, conjugation, transduction and transformation [5]. MIC values of these 7 strains favored classification of these organisms as MSSA, but were reported to be heterogenetically resistant to cefoxitin by Vitek 2 system with the indication that these isolates have the potential of becoming MRSA later on. These findings are clinically very significant as patients having infections with these MSSA strains, if put on conventional MSSA therapy may not show signs of recovery unless put on non penicillinase antibiotics and therefore need to be treated as cases of MRSA infections. Strains that were positive for *mec A* i.e. PC-2a and 5677 were not only correctly detected as MRSA by Vitek 2 but also clarified the cause of resistance as modification of PBP (*mecA*) (Table1). Strain 5810, though interpreted as cefoxitin screen negative by Vitek 2, was detected as MRSA by advance expert system of Vitek 2 system and further confirmed as *mec A* positive by PCR. Strain 6007 was mec *A* positive by PCR (Table1) though detected as MSSA having acquired penicillinase by Vitek 2 (AES), would not require any change in required antibiotic therapy as it has been already discussed that such cases with acquired penicillinase are to be treated as MRSA. The strains 6559 and 5635 were *mecA* negative and were correctly detected as MSSA by Vitek 2 system (Table 2). There was an interesting case of strain 5985 which had oxacillin MIC's ≥4 µg/ml, cefoxitin sensitive and was reported as MRSA by Vitek 2 because of its high MIC value, but when the isolate was put up for PCR it was *mec A* negative (Table1). This is actually a rare case of methicillin resistance due to factors other than *mec A*, so subsequently the isolate was saved and sent to the reference laboratory to get a more clear picture of its resistance. Figure 1 shows the specimen Printouts from Vitek -2 Compact (bioMerieux, France) indicating cause of resistance of *S.aureus*- acquired penicillinase/modified *mec A* mechanism.

V. CONCLUSION

Our study corroborates the use of cefoxitin disc diffusion as better method than oxacillin disc diffusion for identification of MRSA. It underscores the importance of using automated microbial identification and sensitivity systems that provides more information to the microbiologist. Currently it appears to be the only available technique that could detect cases of MSSA infections with acquired penicillinase activity likely due to non *mec A* mediated mechanisms that calls for the same treatment that are instituted for MRSA strains.

ACKNOWLEDGEMENTS

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CONFLICT OF INTREST

None declared

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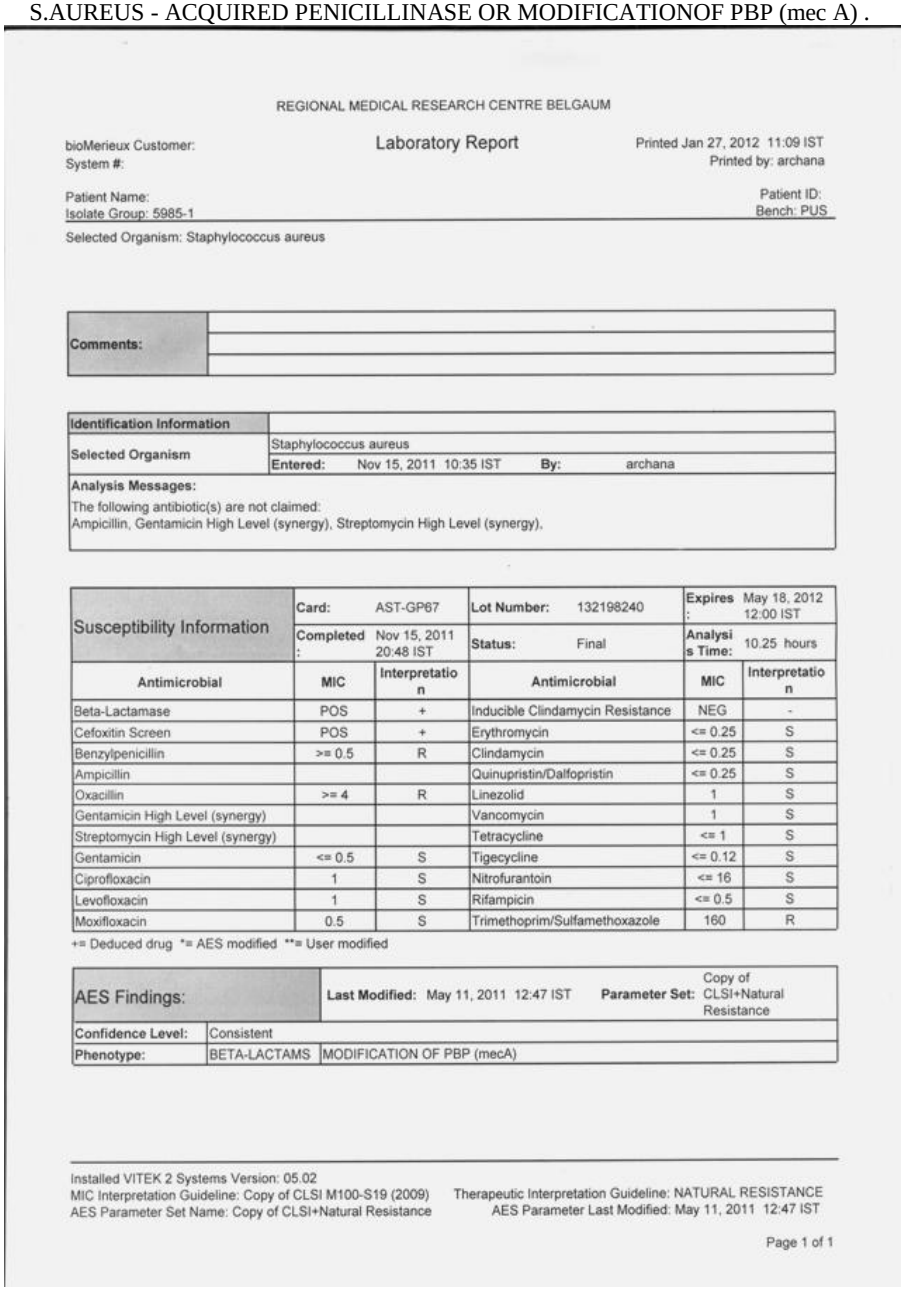
Table 1: **Comparative results of disc diffusion with oxacillin & cefoxitin discs, PCR and Vitek 2 System (bioMerieux,France).**

Phenotypic Tests (Disc Diffusion Test)		**PCR**	**Analysis by Vitek 2 Compact(bioMerieux, France)**						**No. of strains**	**Clinical strain No.**
Oxacillin R=Resistant S=Sensitive I=Intermediate	**Cefoxitin** R=Resistant S=Sensitive	***mecA***	**Benzyl penicillin**	**Cefoxitin**	**Oxacillin**	**Beta Lactamase**	**Advance Expert System(AES) Vitek 2 Compact**	**Interpretation by Vitek 2**		
R	R	+	Not done						40	
S	S	-	Not done						16	
I	R	+	≥0.5; R	POS	≥4 ; R	POS	Modification of PBP (mec A)	MRSA	2	PC-2a,5677
I	S	+	0.25 ;R	NEG	≥4; R	POS	Modification of PBP (mecA)	MRSA	1	5810
S	R	+	≥0.5 ; R	NEG	≤0.25; S	POS	Acquired penicillinase	MSSA	1	6007
I	S	-	0.25 - ≥0.5 ; R	NEG	≤0.25-0.5; S	POS	Acquired penicillinase	MSSA	6	5689,5363,6561,5481,5505,5639
R	S	-	≥0.5; R	NEG	0.5; S	POS	Acquired penicillinase	MSSA	1	5148
			≤0.03 ; S	NEG	≤0.25; S	NEG	Acquired penicillinase	MSSA	2	6559,5635
			≥0.5 ;R	POS	≥0.5 ;R	POS	Modification of PBP (mecA)	MRSA	1	5985
TOTAL (n)									**70**	

Figure 1: SPECIMEN PRINTOUTS FROM VITEK-2 (bioMerieux, France) INDICATING CAUSE OF RESISTANCE OF S.AUREUS - ACQUIRED PENICILLINASE OR MODIFICATIONOF PBP (mec A) .



REGIONAL MEDICAL RESEARCH CENTRE BELGAUM

bioMerieux Customer:
System #:

Laboratory Report

Printed Jan 27, 2012 11:09 IST
Printed by: archana

Patient Name:
Isolate Group: 5985-1

Patient ID:
Bench: PUS

Selected Organism: Staphylococcus aureus

Comments:	

Identification Information	
Selected Organism	Staphylococcus aureus
	Entered: Nov 15, 2011 10:35 IST By: archana

Analysis Messages:
The following antibiotic(s) are not claimed:
Ampicillin, Gentamicin High Level (synergy), Streptomycin High Level (synergy),

Susceptibility Information	Card: AST-GP67		Lot Number: 132198240		Expires: May 18, 2012 12:00 IST
	Completed: Nov 15, 2011 20:48 IST		Status: Final		Analysis Time: 10.25 hours
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Beta-Lactamase	POS	+	Inducible Clindamycin Resistance	NEG	-
Cefoxitin Screen	POS	+	Erythromycin	<= 0.25	S
Benzylpenicillin	>= 0.5	R	Clindamycin	<= 0.25	S
Ampicillin			Quinupristin/Dalfopristin	<= 0.25	S
Oxacillin	>= 4	R	Linezolid	1	S
Gentamicin High Level (synergy)			Vancomycin	1	S
Streptomycin High Level (synergy)			Tetracycline	<= 1	S
Gentamicin	<= 0.5	S	Tigecycline	<= 0.12	S
Ciprofloxacin	1	S	Nitrofurantoin	<= 16	S
Levofloxacin	1	S	Rifampicin	<= 0.5	S
Moxifloxacin	0.5	S	Trimethoprim/Sulfamethoxazole	160	R

+= Deduced drug *= AES modified **= User modified

AES Findings:	Last Modified: May 11, 2011 12:47 IST	Parameter Set: Copy of CLSI+Natural Resistance
Confidence Level:	Consistent	
Phenotype:	BETA-LACTAMS	MODIFICATION OF PBP (mecA)

Installed VITEK 2 Systems Version: 05.02
MIC Interpretation Guideline: Copy of CLSI M100-S19 (2009)
AES Parameter Set Name: Copy of CLSI+Natural Resistance

Therapeutic Interpretation Guideline: NATURAL RESISTANCE
AES Parameter Last Modified: May 11, 2011 12:47 IST

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REGIONAL MEDICAL RESEARCH CENTRE BELGAUM

AES Detail Report

bioMerieux Customer:
System #:

Printed by: archana
Printed: Jan 30, 2012 17:15 IST

Accession Number: 6007-1

Date Tested: Oct 26, 2011 05:57 IST

Selected Organism: Staphylococcus aureus
AES Confidence: Consistent

ID Confidence:

Phenotypes

Antibiotic Family	Detected Phenotypes
BETA-LACTAMS	ACQUIRED PENICILLINASE
AMINOGLYCOSIDES	RESISTANT KAN (APH(3')-III),RESISTANT KAN TOB (ANT(4')(4")),WILD
QUINOLONES	PARTIALLY RESISTANT
MACROLIDES/LINCOSAMIDES/STREPTOGRAMINS	WILD
OXAZOLIDINONE	WILD
GLYCOPEPTIDES	WILD
TETRACYCLINES	WILD
FURANES	WILD
RIFAMYCINES	WILD
TRIMETHOPRIM/SULFONAMIDES	TRIMETHOPRIM RESISTANT,WILD

Therapeutic Interpretations
None

MIC/Test Differences
None

Antibiotic Deductions
None

Installed VITEK 2 Systems version: 05.02

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AES Parameter Set: Copy of CLSI+Natural Resistance
Last Modified: May 11, 2011 12:47 IST
Basis: CLSI+Natural Resistance

MIC Interpretation Guideline: Copy of CLSI M100-S19 (2009)
Basis: CLSI M100-S19 (2009)
Therapeutic Interpretation Guideline: NATURAL RESISTANCE
Basis: NATURAL RESISTANCE