Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 534 - 540

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

DETECTION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) BY AUTOMATED AND MANUAL METHODS

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* has become an enormous problem for health care providers because it is hard to treat and is sometimes called super bug. Early and accurate detection of methicillin resistance in *Staphylococcus aureus* is essential for the treatment of overt infections and the implementation of infection control practices. The phenotypic detection of mecA-mediated resistance has presented ongoing challenges due to low level of methicillin resistance and heterogeneous nature of methicillin resistance in *S.aureus.* This study compares the performance of three different methods, Vitek 2 compact system (bioMerieux, France), Cefoxitin disc diffusion method and chromogenic media for the detection of methicillin resistant *Staphylococcus aureus* recovered from patients in tertiary-care hospitals. A total of 107 clinical isolates of *Staphylococcus aureus* were tested for methicillin resistance by Vitek 2 compact, automated ID/AST system. As a result, 23 isolates of *Staphylococcus aureus* were detected as methicillin resistant. The cefoxitin disk diffusion tests detected 100% (23 isolates) of all the MRSA and Chrom ID MRSA also showed similar good results (100%) of sensitivity and specificity for the detection of MRSA after 24 h of incubation.

KEYWORDS:***Staphylococcus aureus*, methicillin resistant *S.aureus* (MRSA), Cefoxitin Disc Diffusion method**

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INTRODUCTION

Staphylococcus aureus is a common cause of serious infections in the hospital and the community.Methicillin resistant *Staphylococcus aureus* (MRSA) was first detected in the 1960s, and since that time it has spread worldwide, becoming a leading cause of nosocomial infections[6,7]. MRSA share a common feature of carrying the *mecA* gene on their chromosome. The *mecA* gene codes for the PBP2', a penicillin–binding protein with low binding affinities to practically all β- lactam antibiotics clinically easily available in the market[8]. The degree of MRSA clinical strains is quite diverse. Some strains exhibit a heterogeneous type of resistance which means that the grown bacterial population is composed of subpopulation of bacteria with different degrees of methicillin resistance[12]. Routine oxacillin disc diffusion tests often fail to detect very heterogeneous MRSA populations, which consequently are considered methicillin-susceptible *Staphylococcus aureus* (MSSA) because of their usual susceptibility to most non-β-lactam antistaphylococcal antibiotics[11]. Compared with infections due to MSSA, infections due to MRSA are associated with poor clinical outcomes and increased healthcare costs[4]. For these reasons, accurate detection of MRSA is of utmost importance to ensure effective treatment for the affected patient and to prevent further transmission. In recent years, the increase in the number of bacterial strains that show resistance to methicillin (MRSA) has become a serious clinical and epidemiological problem because this antibiotic is considered as the first option in the treatment of the staphylococci infection, and resistance to this antibiotic implies resistance to all β-lactam antibiotics[9]. Because of its high transmissibility, its multiple antibiotic resistance, and its consequences on patient management, isolation and identification of MRSA strains must be rapid. Methods to detect MRSA in clinical samples should have high sensitivity and specificity and achieve isolation of MRSA in one single step[14]. The burden of the rapid and accurate detection of antimicrobial resistance among *S.aureus* remains a continuous challenge for clinical microbiology laboratories. There are several methods for detecting methicillin resistance including conventional methods for determining MICs (E test, broth dilution), disc diffusion method (Kirby-bauer), screening techniques (chromogenic media),automated identification and susceptibility (ID/AST) testing systems and methods that detect the *mecA* gene or its protein product, PBP2'protein [16,10]. Detection of *mec*A gene is considered as the reference method for determining resistance to methicillin[3]. However, many laboratories throughout the world do not have the capacity or the experienced staff required to develop molecular techniques for detecting MRSA isolates. In addition, they cannot afford to have automated (ID/AST) systems for rapid and reliable reporting. Prompt and adequate empirical therapy is crucial in reducing morbidity and mortality and for the control of the spread of resistant strains. The main objective of this study was to evaluate the suitability of some of these methods as a reliable and routine method for detecting MRSA isolates.

MATERIALS AND METHODS

Altogether 107 *Staphylococcus aureus* were tested. They were derived from different patients with a variety of infection and were isolated from pus, blood, tracheal aspirates, urine etc. Isolates were identified as *S. aureus* by the standard microbiological procedures which include Gram staining, colonial morphology, catalase, slide and tube coagulase, growth on mannitol salt agar, β-haemolysis on blood agar.

Vitek 2 Compact

All the isolates were tested with the Vitek 2 compact identification and susceptibility testing (ID/AST) system (bioMerieux, Marcy l'Etoile, France) using gram positive test panel (AST-GP-67 card) for susceptibility and minimum inhibitory concentration (MIC), according to the manufacturer's instructions. Gram-positive susceptibility card included a cefoxitin well for cefoxitin screening and the detection of methicillin-resistant staphylococci. Colonies of an 18-24 h old culture of *S. aureus* were

inoculated in a 0.45% NaCl solution and adjusted to concentration equivalent to a 0.5 Mc Farland standard. The card was then loaded with the inoculum and placed in the Vitek 2 compact system for incubation and reading. The current clinical and laboratory standard institute (CLSI) breakpoints were applied for oxacillin susceptibility as MICs of ≤2 μg/ml and resistant as MICs of ≥4 μg/ml.

Cefoxitin disc diffusion test

We evaluated the validity of disc diffusion test by using cefoxitin (30μg/ml). The Cefoxitin disc diffusion method was carried out on Muller-Hinton agar by using a 30 μg cefoxitin disc (HiMedia, Mumbai). The bacterial suspensions were prepared by inoculating three to five colonies of *S. aureus* into 5 ml saline water and the turbidity was adjusted equivalent to 0.5 Mc Farland standards (107 cfu/ml). One hundred microliter of culture was taken and spread on the surface of the Muller-Hinton agar and the plate was air dried for 15 min. The Disc was placed on the surface of the inoculated plates. The plates were inverted and incubated at 35°C for 24 h and the results were recorded. . *Staphylococcus aureus* (ATCC 25923) was tested with each batch. The isolates were considered as resistant when the diameter of zone of inhibition was ≤21mm, and as sensitive when the diameter was ≥22 mm.

Detection of MRSA by chromogenic media

Chrom ID MRSA agar is a chromogenic medium for the screening of methicillin-resistant *S. aureus* (Chrom ID MRSA). MRSA agar was consisted of a rich nutritive base combining different peptones, chromogenic α-glucosidase substrates and a combination of several antibiotics which favoured the growth of MRSA including hetero-resistant strains. The samples were directly inoculated onto the chrom ID MRSA agar and the plates were incubated at 37°C in aerobic conditions and were examined after 24 h of incubation. Any bacterial growth after 24 h or 18 h, resulting in intense blue colonies was indicative of resistance to methicillin. A well-defined collection consisting of 23 MRSA strains and 84 MSSA isolates was used (confirmed by Vitek system). After incubation, the plates were observed for bacterial colonies.

RESULT

A total of 107 clinical isolates of *Staphylococcus aureus* were tested for methicillin resistance by Vitek 2 compact, automated ID/AST system. The Vitek 2 system (by using AST-GP-67 card with cefoxitin screening) detected 23 isolates of MRSA with positive cefoxitin screening and remaining 84 as MSSA (Table 1). The oxacillin MICs for MSSA were ranged from 0.25 to 2μg/ml and showed negative cefoxitin screening. These Oxacillin MICs were <=0.25μg/ml for 56 isolates and equal to 0.5 μg/ml for 25 and 1μg/ml for 2 isolates. The cefoxitin disk diffusion tests detected 100% (23 isolates) of all the MRSA (Figure 1). Chrom ID MRSA also showed similar good results (100%) of sensitivity and specificity for the detection of MRSA after 24 h of incubation (Figure 2). MICs of vancomycin were measured for all 107 isolates and MIC for vancomycin was in the susceptible range for all isolates ranging from <=0.5μg/ml to 2 μg/ml. The susceptibility level to glycopeptides was observed and isolates with vancomycin MIC of ≥ 2 mg/L is 4.6%. Similarly, MIC for Quinupristin- dalfopristin ranged from 0.25 to 0.5 μg/ml which is also within the susceptible range (<=1 μg/ml) with most isolates with MIC of 0.25 μg/ml.

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Table 1
Antimicrobial susceptibility results of Methicillin Resistant Staphylococcus aureus in terms of Minimum Inhibitory Concentration (MIC) to various antibiotics (n= 23)

S.N. O	CIP	CLI	ERY	GEN	LVX	LNZ	MFX	NIT	OXA	PEN	QDA	RIF	TCY	VAN
1	>4	<=.25	>4	4	>4	2	4	<=16	>2	>.25	0.5	>16	2	1
2	>4		>4	<=.5	>4	2	2	<=16	>2	>.25	<=.25	<=.5	<=1	1
3	>4	<=.25	1	>4			2	32	>2		<=.25	<=.5	<=1	
4	>4	<=.25	>4	4	4	2	2	<=16	>2	>.25	<=.25	<=.5	<=1	<=.5
5	4	<=.25	<=.25	4	4	2	1	<=16	>2	>.25	<=.25	<=.5	>8	<=.5
6	4	<=.25	>4	4	4	2	1	32	>2	>.25	<=.25	<=.125	>8	<=.5
7	4	<=.25	1	>8	4	2	1	<=16	>2	>.25	<=.25	<=.5	<=1	1
8	>4	<=.25	<=.25	1	4	2	1	<=16	>2	>.25	<=.25	<=.5	<=1	<=.5
9	>4	<=.25	>4	<=.5	4	2	1	<=16	>2	>.25	<=.25	<=.25	<=1	<=.5
10	>4	<=.25	1	>8	4	2	2	<=16	>2		<=.25	<=.5	<=1	<=.5
11	4	<=.25	1	>8	4	2	1	<=16	>2		<=.25	<=.5	<=1	<=.5
12	>4	<=.25	<=.25	4	4	2	1	<=16	>2	>.25	<=.25	<=.5	<=1	<=.5
13	>4	<=.25	>4	<=.5	4	2	2	32	>2	>.25	<=.25	<=.5	<=1	1
14	>4	<=.25	>4	>8	4	2	2	<=16	>2	>.25	<=.25	<=.5	>8	1
15	>4		>4	>8	4	2	1	32	>2	>.25	<=.25	<=.5	>8	
16	>4	<=.25	<=.25	>8	>4	2	>4	32	>2	>.25	<=.25	>16	>8	1
17	>4	<=.25	>4	2	>4	2		32	>2	>.25	<=.25	8	>8	1
18	4	<=.25	1	>8	4	2	1	32	>2	>.25	<=.25	<=.5	<=1	1
19	>4	<=.25	1	>8	4	2	1	<=16	>2	>.25	<=.25	<=.5	<=1	1
20	>4	<=.25	>4	<=.5	4			32	>2		<=.25		<=1	1
21	>4	<=.25	<=.25	4	4	2	1	<=16	>2	>.25	<=.25	<=.5	<=1	<=.5
22	>4	<=.25	1	>8	4	2	1	<=16	>2	>.25	<=.25	<=.5	<=1	<=.5
23	>4	>4	>4	>8	>4	2	4	<=16	>2	>.25	<=.25	<=.5	>8	<=.5

CIP – ciprofloxacin; CLI – clindamycin; ERY – erythromycin; GEN – gentamycin; LVX- levofloxacin; LNZ – linezolid; MFX– moxifloxacin; NIT – nitrofurantoin; OXA – oxacillin; PEN – penicillin; QDA- Quinpristin/daflopristin; RIF – rifampicin; TCY – tetracycline; VAN –vancomycin;

Figure 1
Detection of MRSA by Cefoxitin disc diffusion method

Disc diffusion method showing no zone of inhibition against cefoxitin and oxacillin but susceptible with vancomycin

Figure 2
Detection of MRSA by chromogenic media (Chrom ID MRSA)

Blue coloured colonies of MRSA on chromogenic media

DISCUSSION

Detection of the *mec*A gene by PCR and/or DNA probe hybridization is a reliable and rapid method, but it is not yet routinely used in clinical laboratories[1]. CLSI recommends usage of cefoxitin instead of oxacillin when using the disc diffusion method to determine resistance against MRSA. In heterogeneous resistance all cells in an intrinsically resistant population carry the *mecA* gene, however, it is only expressed by a small percentage of cells. Due to this phenomenon, routine tests often fail to detect methicillin resistance in staphylococci[15]. Therefore, a number of parameters have been recommended to improve results: increasing the inoculum, growth at a low temperature, an oxacillin screen test with NaCl, or protracted incubation. In recent studies, cefoxitin disc diffusion method is considered a better predictor than oxacillin for the detection of heterogeneous methicillin resistance[1, 2,15]. Felten *et al*, (2002) studied total of 152 clinical isolates of *S. aureus.* Out of which 69 were MSSA (*mecA*-negative) and 83 were MRSA (*mecA*-positive) isolates, including 63 heterogeneous isolates and 26 class 1 isolates (low-level resistance). The cefoxitin disc diffusion tests detected 100% of all the MRSA classes which corroborated well with our findings. In the present study, total of 107 isolates of *S. aureus* were studied and 23 were confirmed as MRSA and the remaining 84 as MSSA by automated ID/AST system. This well defined collection was further confirmed by cefoxitin disc diffusion method. As a result, cefoxitin detected all the 23 MRSA isolates (100%).

None of the 107 tested isolates had reduced susceptibility to vancomycin with most MICs lying in the 0.5–1 μg/ml range. The term MRSA is based on the oxacillin breakpoint recommended by CLSI, where a strain for which the MIC is 8μg/ml is defined as resistant. According to our results, an *S. aureus* isolate that gives a cefoxitin diameter of <21 mm can be identified as MRSA.

Brown *et al*, (2005) The cefoxitin disc diffusion method detected 20 (36.4%) out of the 55 isolates as MRSA, which accounted for 100% sensitivity and specificity as compared to the *mec*A-based PCR which matched well with the present study[1]. Cefoxitin, a cephamycin, is a more potent inducer of the *mec*A regulatory system and an accurate surrogate marker for the detection of MRSA in the routine susceptibility testing. It has been suggested that no special medium or incubation temperature is required for cefoxitin as is required for oxacillin. Sakoulas *et al*, (2001) studies have indicated that disc diffusion testing by using the cefoxitin disc is superior to most of the currently recommended phenotypic methods like oxacillin disc diffusion and oxacillin screen agar testing and that it is now an accepted method for the detection of MRSA by many reference groups including CLSI[13]. Media utilizing chromogenic enzyme substrates have been developed to detect MRSA with high sensitivity and specificity combined with a short results reporting time[3].

The sensitivity of Chrom agar MRSA after 24 h of incubation was 95.4%, increasing to 100% after 48 h. The specificity was already 100% after 24 h whereas, in a number of studies, sensitivity and specificity of disc diffusion method have been reported between 61.3-100 and 50-99.1%, respectively[18]. Sakoulas *et al* (2001) have determined the sensitivities of Vitek 2 compact as 99% and 99.5% and the specificities as 100 and 97.2%, respectively.

The oxacillin disc method is most widely used for the detection of *mec*A-mediated MRSA; however, the oxacillin disc susceptibility results may not always be reliable. It has been shown that cephamycins are more potent inducers of the *mec*A gene. Therefore, CLSI recommends the use of the cefoxitin disc diffusion test for detecting oxacillin resistance. Felten *et al*, (2002) reported that the cefoxitin disk diffusion methods were the best-performing tests for routine detection of all classes of MRSA. For the class 1 MRSA isolates, the sensitivity of the Vitek 2 test was 92.3%, whereas those of disk diffusion method with cefoxitin were 100%. The resistance of *S. aureus* against different antibiotics tested was higher for inpatient as opposed to outpatient isolates for many antibiotics[17]. In this study, Cefoxitin proved to be superior for the selection of MRSA from other strains of *S. aureus*. Chrom ID MRSA show similar good results for the detection of MRSA after 24 h of incubation.

CONCLUSION

Resistance to commonly used antimicrobial drugs represents a critical public health problem because it complicates treatment and may result in longer hospital stay for patients. Accuracy and promptness in the detection of methicillin resistance is a key importance to ensure correct antibiotic treatment in the infected patients as well as control of MRSA isolates in hospital environments, to avoid them spreading. The enhanced recovery of MRSA, the identification of most MRSA isolates at 24 h and providing early information on methicillin resistance to practitioners will lessen the burden of infection and death from MRSA and will benefit the patients and decrease costs associated to infections caused by MRSA.

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