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ORIGINAL ARTICLE

Evaluation of different methods for detection of hospital-acquired methicillin resistant *Staphylococcus aureus*

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ABSTRACT

Background*: Methicillin-resistant Staphylococcus aureus (MRSA) has arisen as a hospital-acquired pathogen worldwide. Rapid and accurate detection of MRSA is important to prevent its hospital-acquired infections.* ***Objective****: make the comparison between various phenotypic approaches for the detection of the mecA gene with PCR based method as a gold standard.* ***Methodology****: Our study was carried out in the Clinical Pathology Department, Faculty of Medicine, Menoufia University between January 2019 to July 2019. A total of 100 clinical isolates of S. aureus, which were collected from various clinical specimens, was a subject of this study. Methicillin resistance was determined by cefoxitin disc diffusion and Chromogenic MeReSa media, the results of these approaches were compared with mecA gene-based PCR method.* ***Results****: Among 100 isolates of S. aureus, 78 (78%) isolates were positive for the mecA gene by PCR method.* ***Chromogenic media*** *had identified (76) out of (78) as methicillin-resistant Staphylococcus aureus (MRSA) and (21) out of (22) as methicillin-sensitive Staphylococcus aureus (MSSA) with sensitivity 97.4%, specificity 95.4%* ***Cefoxitin disc*** *had identified (77) out of (78) as MRSA and (21) out of (22) as MSSA with sensitivity 98.7%, specificity 95.4%.* ***Conclusion****: The chromogenic media method is less sensitive for the detection of MRSA than the cefoxitin disc diffusion method but they have the same specificity. The phenotypic identification of MRSA has high sensitivity and specificity in agreement with PCR. In addition, they are simple, rapid and not expensive so we can use them in the screening of patients and staff members in health care settings in order to decrease the risk of hospital-acquired infections.*

INTRODUCTION

Staphylococcus aureus is one of the major resistant pathogens in clinical practice; it is a main source of hospital-acquired infections, such as infections associated with indwelling medical devices and infections of surgical wounds. It also causes toxic shock syndrome by release of super-antigens into the blood stream.[1] Penicillin was widely considered as a "magic bullet" which can kill all Gram-positive bacteria without harming human hosts.[2] However, penicillin resistance was observed in a hospital setting, as early as 1942, just two years after the introduction of penicillin for clinical use. Within two decades, about 80% of both hospital- and community-acquired *S.aureus* solates were observed to have developed resistance to penicillin. Resistant strains expressed Penicillinase; a specific type of β-lactamase that shows specific activity against penicillin through hydrolysis of the β-lactam ring. Penicillin is inactivated and loses its ability to inhibit the synthesis of the cell wall.[3]

In response to this crisis, new classes of β-lactam antibiotics, which include penicillin, methicillin, Dicloxacillin, nafcillin, Oxacillin, and Cephalosporin, (which inhibit *S. aureus* growth by inhibiting penicillin-binding proteins, which normally catalyze cross-linking of bacterial cell walls) were introduced. Unfortunately, within a year of their introduction, methicillin-resistant staph aureus (MRSA) appeared in the clinical setting.[4]

The mechanism is associated to the alteration in the site of action of β-lactamase by producing a new penicillin-binding protein, PBP2a, which has a low affinity for antibiotics and is absent in susceptible *staphylococci*.[5]

The *mecA* gene encodes PBP2a; this gene is located on a mobile genetic element, which is called SCC *mec* (*staphylococcal* cassette chromosome *mec*) which is widely distributed among *staphylococci*.[6]

PBP2a acts as a trans-peptidase that resumes cell wall synthesis functions when other PBPs are inhibited, thus ensuring the integrity of the bacterial cell in the presence of β-lactams.[7] Although MRSA strains have been known for many years, they were rare in the 1960s, sporadic in the 1970s, epidemic in the 1980s, and have been widespread and endemic in the hospitals since the 1990s. Methicillin-resistant *staphylococcus aureus* (MRSA) is now well established in the health care settings. They are among the most common causes

of nosocomial infections as surgical wound infections, intravenous catheter-associated infections, and ventilator-associated pneumonia[8]. Appropriate and rapid identification of MRSA in clinical microbiology laboratories is an essential issue for treatment and epidemiological purposes. There are many different laboratory methods for the detection of MRSA, such as detection of *mec*A gene by PCR and other phenotypic methods (chromogenic media, cefoxitin disc diffusion).[9]

The main goal of this study was to evaluate different phenotypic approaches in relation to detecting the *mec*A gene. This evaluation helps us in choosing a reliable routine method for the rapid detection of MRSA in our microbiology laboratories in order to decrease the risk of hospital-acquired infections.

METHODOLOGY

The study was approved by the Research and Ethical committee of Clinical Pathology Department, Faculty of Medicine, Menoufia University. From January 2019-July 2019, 100 clinical isolates of *S. aureus* were collected. The strains were gathered from diverse clinical specimens; pus, urine, sputum, and blood. These isolates were cultured on blood agar (BIO-RAD), nutrient agar (BIO-RAD), at 37°C for 18-24h.

Isolates were identified by Gram staining, catalase test, coagulase test and mannitol salt agar (BIO-RAD).

All of the isolates were kept frozen at -70 °C in trypticase soy broth containing 15% glycerol until performance of susceptibility testing and MRSA detection.[10]

Detection of the *mec*A Gene by PCR

DNA extraction and purification

This was accomplished by the use of the QIAamp® DNA Mini extraction kit (Germany) for the extraction and purification of genomic DNA and Lysozyme from chicken egg white (Sigma Aldrich, Germany) at concentration 20mg/dl. Extraction steps were consistent with manufacturer steps. In the beginning, bacteria were pelleted by centrifugation for 10 min at 5000 x g (7500 rpm) then the bacterial pellet was incubated in 180 µl of the lysozyme 20 mg/ml for 30 min at 37°C. 20µl proteinase K and 200µl Buffer AL were added. The mixture was mixed by vortexing and then was incubated at 56°C for 30 min followed anther 15 min at 95°C.

After that, the mix was Centrifuged for few seconds, 200 µl ethanol (96–100%) were added to the sample and mixed by pulse vortexing, the formed mixture was applied to QIAamp Mini spin column and was centrifuged at 6000 xg (8000 rpm) for 1 min. QIAamp Mini spin column was opened and 500 µl. Buffer AW1 was added then was centrifuged at 6000 xg (8000 rpm) for 1 min. QIAamp Mini spin column was opened and 500 µl Buffer AW2 was added. The cap was locked then centrifuged at (20,000 x g; 14,000 rpm) for 3 min. 200 µl Buffer AE or distilled water were added then incubated in room temperature for 1 min, then centrifuged at 6000 x g (8000 rpm) for1 min.

DNA amplification

PCR assay was done by Thermal Cycler (Biometra, Germany), primers of the *mec*A gene were used for amplification of the 162 bp fragment primers used for reaction.[11]

Forward primer:

5′-TCCAGATTACAACTTCACCAGG-3′

Reverse primer:

3′- CCACTTCATATCTTGTAACG-5′,

12.5µl Taq Green PCR Master Mix (2x) were gently vortexed after thawing and added for each 25µl reaction as follows: 1µl forward primer, 1µl reverse primer, 2µl templet DNA and 8.5µl nuclease-free water.

DNA sequencing and alignment

The *mec*A gene was amplified via the pre denaturation of the reaction mixture for 3 min at 94°C. then followed by 30 cycles at 94°C for 1 min, 1 min for annealing at 54°C, then the PCR reaction was completed at 72°C for 1 min, and a final elongation for 7 min at 72°C.[12]

Preparation of agarose gel:

The agarose gel (2%) preparation was done by adding 2 gm agarose to 100ml of 1 x TBE buffer. The agarose was dissolved then boiled for 4 min. The agarose solution was allowed to cool to 50°C, and then 5 µl of ethidium bromide (10 mg/ml) were added for later visualization of the bands.[13]

The prepared agarose was allowed to cool then poured into the cast to create wells for loading samples. The gel was poured in an electrophoresis tray and allowed to be solid then the tray was flooded with 1 x TBE buffer just enough to cover the gel to the depth of about 1mm.[14]Ten microliters of the amplification products were slowly loaded into the slits of the submerged gel. A DNA molecular weight marker (50 bp ladder) was run in parallel. The electrophoresis was carried out by the gel electrophoresis apparatus and was run at 100 Volts for 45 min. The bands were visualized and photographed by a digital camera[15].

Detection of the amplified product:

DNA bands were visualized on UV trans-illuminator and photographed. Gene was determined by the site of amplified products in comparison with known ladder bands *mec*A was at 162 bp. [16]

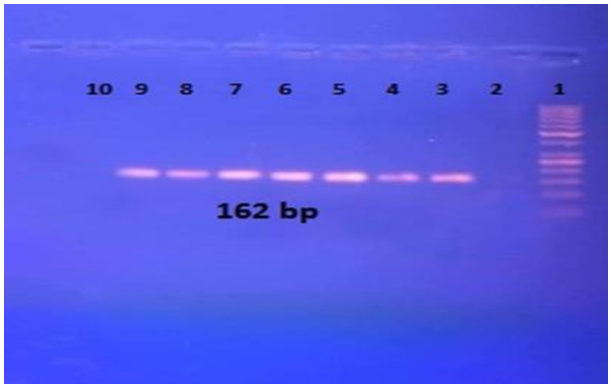

Fig. 1: Detection of *mec*A gene by PCR method at 162 bp. Lane (1) is a 50-bp DNA ladder, Lane (2) is a negative control (MSSA), Lane (3) is a positive control (MRSA), Lanes (4-5-6-7-8-9) are positive for *mec*A gene and lane (10) is negative for *mec*A gene.

Detection of methicillin resistant *S. aureus*

Disc diffusion methods

The disc diffusion method using cefoxitin disc was performed for all isolates on Mueller–Hinton agar for detection of MRSA as recommended by CLSI guideline 2017. Briefly, isolated colonies from overnight growth of *S. aureus* on Blood agar were suspended in 4-5 ml of sterile saline. The turbidity of suspension was adjusted to 0.5 McFarland standard turbidity. Then inoculated in Mueller–Hinton agar plate. A cefoxitin (30µg) disk (BD) (USA) was aseptically placed on the Mueller–Hinton agar and incubated at 37 for 18- 24 hours. The inhibition zone of antibiotic discs was measured as recommended by CLSI 2017; cefoxitin is used for Oxacillin resistance. Reporting Oxacillin susceptible or resistant was based on Cefoxitin disc diffusion zone diameter as shown in table 1. [17]

Table 1: Cefoxitin disc diffusion zone diameter

Antibiotic	Disc content	Resistant (mm)	Sensitive (mm)
Cefoxitin	30 µg	≤ 21mm	≥ 22 mm

Chromogenic media

Chromogenic MeReSa Selective Supplement (TS 206) & Cefoxitin supplement (TS 219) in combination, which is provided by Tm media A- 902A, RIICO Industrial Area, Phase III, and Bhiwadi-301019 were used.

41.65 grams were dissolved in 500 ml distilled water then was heated until boiling to dissolve the medium. The media was sterilized by autoclaving at 15-psi pressure (121°C) for 15 minutes. Cooled to 45-50° C, Aseptically sterile rehydrated contents of 1 vial of Chromogenic MeReSa Selective Supplement (TS 206) & Cefoxitin supplement (TS 219) for selectivity were added, after that, they were mixed well then poured into sterile Petri plates.

Green colonies after incubation at 30-35 °C for 18-48h were considered MRSA

RESULTS

The present study was conducted during the period from January 2019-July 2019. It comprised 100 isolates from patients who were admitted to different departments and ICUs of Menoufia University Hospitals, Their ages range from 1 year to 80 years old (mean ±SD: 39.87 ±22.62) years old. Different samples were collected from various patients, one specimen from each admitted patient. Validity tests for cefoxitin disc diffusion and chromogenic media (PCR for *mec*A is the gold standard method) are shown in table2. Antibiotic-resistant patterns of *S.areus* MRSA and MSSA are shown in table 3. ROC curve, which compared diagnostic performance of cefoxitin disc diffusion with chromogenic media, is shown in figure 2.

Table 2: Validity tests for the chromogenic media and cefoxitin disc diffusion (PCR for *mec*A is gold standard)

Method	Chromogenic media	Cefoxitin disc
True positive	76	77
False negative	2	1
True negative	21	21
False positive	1	1
Sensitivity%	97.4%	98.7%
Specificity%	95.4%	95.4%
PPV%	98. 7%	98.7%
NPV%	87.5%	95.4%
Accuracy% (Diagnostic efficacy)	96.0%	98%

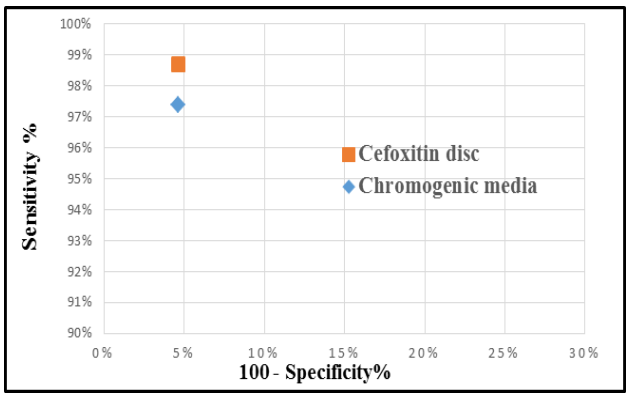

Fig 2: ROC curve for comparison between cefoxitin disc diffusion and chromogenic media

Table 3: Antibiotic-resistant patterns of MRSA & MSSA

Antibiotic	% MSSA	%MRSA
Ceftriaxone	55.5%	84.6%
Penicillin	59.1%	97.4%
Erytromycin	31.8%	73.1%
Cefoperazone	36.4%	83.3%
Tetracyclin	64.2%	88.5%
Rifampcin	77.3%	73.5%
Linezolid	10%	15.3%
Nitrofentoin	55.5%	76.1%
Ciprofloxacin	50.0%	87.2%
Gentamycin	54.5%	82.1%
Sulfamethoxazole Trimethoprim	68.2%	62.8%

DISCUSSION

S.aureus is one of the common causes of nosocomial and community-acquired infections with high mortality and morbidity. An increase in methicillin resistance among *Staphylococci* has posed great difficulty in managing its infections.[20] Hence, an accurate and rapid detection of methicillin resistance is essential not only to select the suitable antibiotic but also to control the spread of MRSA. Many phenotypic methods to detect MRSA have been established (cefoxitin disc diffusion, chromogenic media). Detection of *mec*A gene by PCR is the gold standard for MRSA identification. However, the use of molecular methods for routine practice is not affordable to many laboratories, because of its cost and greater technical and infrastructural requirements. Therefore, it is essential to develop a rapid, accurate and sensitive phenotypic method for detection of MRSA.[21]

In this study, we compared cefoxitin disc diffusion and chromogenic media with PCR for *mec*A gene, the results revealed that 78% of the *S.aureus* isolates were MRSA and 22% were MSSA by PCR.

Percentage was also high with Wijesooriya et al study, who found that among *S. aureus* isolates, 69.1% were MRSA & 31% were MSSA. [22] also the study of Dubey et al which found that 81.7% of S. aureus isolates were MRSA & 18.2% were MSSA. [23]

Chromogenic media correctly identified (76) out of (78) as MRSA and (21) out of (22) as MSSA with sensitivity 97.4% ,specificity 95.4%, positive predictive value 98.7%, negative predictive value 87.5%, and accuracy 96.0% in agreement with Kali et al. which found that chromogenic media sensitivity was 97.82 % and specificity was 91.11%.[24]

Cefoxitin disc correctly identified (77) out of (78) as MRSA and (21) out of (22)as MSSA with sensitivity 98.7%,specificity 95.4% ,positive predictive value 98.7%, negative predictive value 95.4%, and accuracy 98.0% in agreement with Farahani et al. which found that cefoxitin disk diffusion Sensitivity was 98.9% and specificity was 94.7%.[25]

ROC curve showed the highest diagnostic performance of cefoxitin disc diffusion in comparison to chromogenic media (the point of cefoxitin disc diffusion is nearer upper left corner than point of chromogenic media) as shown in figure 2. Regarding to antibiotic resistance of MRSA 84.6% of MRSA isolates were resistant to ceftriaxone, 97.4% were resistant to penicillin and 73.1% were resistant to erythromycin. It was also found that 83.3% were resistant to Cefoperazone, 88.5% were resistant to Tetracycline and 73.5% were resistant to Rifampicin. In addition, it was found that 76.1% were resistant to Nitrofentoin, 87.2% were resistant to Ciprofloxacin. 82.1% were resistant to gentamycin and 62.8% were resistant to Sulfamethoxazole Trimethoprim. On the other hand, there was 15.3% found to be resistant to linezolid, In agreement with our study Sharif et al. found that penicillin resistance was almost detected in all MRSA strains. [26] High level of sensitivity to linezolid also was reported by Chitnis et al. [27]

CONCLUSION

The chromogenic media method is less sensitive for detection of MRSA than the Cefoxitin disc diffusion method but they have the same specificity. Although PCR is the gold standard method for detection of MRSA, chromogenic media and cefoxitin disc diffusion tests have high sensitivity and specificity in agreement with PCR. They are also simple, rapid and not expensive. Therefore, we can use them for rapid diagnosis of MRSA and for screening of patients and staff members.

Conflict of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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