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Relative Sensitivity of Conventional and PCR-based Approaches for the Detection of Clinical Methicillin-resistant *Staphylococcus aureus*

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Abstract

The aim of the present study was to investigate the use of PCR and disk diffusion methods in determination of methicillin-resistant *Staphylococcus aureus* (MRSA) strains and compare their accuracy and specificity. For this purpose a total of 150 clinical isolates obtained from burn patients were investigated; of these 55 (36.6 %) were identified as *S. aureus* by conventional and Vitek® 2 system. All isolates were subjected to 30 μg cefoxitin to identify MRSA isolates. Besides, PCR was utilized for molecular analysis. Among the results of disk diffusion methods in our study, the prevalence of MRSA was 92.7% and MSSA was 7.3% with the sensitivity and specificity of 92.15 % and 75 % respectively. Whereas, 87.3 % of *S. aureus* isolates were mecA positive and 12.7% were mecA negative in PCR assay. Our finding showed that the PCR is more accurate and allowed early and rapid identification of methicillin-resistant *S.aureus*.

Introduction

Staphylococcus aureus is known throughout the world as the predominant bacterial infection in humans. Despite being an asymptomatic colonizer of skin and anterior nares of healthy individuals, it can act as the cause of a large range of diseases with varying severity, ranging from soft tissue infections to pneumonia, septicemia, and septic shock syndrome [1] and [2]. It is known to be capable of causing death, and the fact that it can invade and live inside the cells of mammals –particularly within immune cells like neutrophils and macrophages- make it difficult to completely eradicate from the body, even when high doses of potent antibiotics are used against it [3]

Methicillin was introduced to clinical use in 1960 to fight against infections caused by penicillinase producing *S. aureus*, and methicillin resistant *Staphylococcus aureus* (MRSA) emerged shortly after and quickly became a problem all around the world [4]. The resistance occurs due to an altered protein called low-affinity penicillin binding protein (PBP2a) [5] which is encoded by mecA gene and this gene is located in chromosomal mobile genetic element called Staphylococcal cassette chromosome mec (SCCmec) [6].
MRSA has since become a widespread cause of nosocomial infections. Unfortunately, these bacteria can spread through hands, airborne particles and other environmental means, making them capable of spreading quickly [7].

Since MRSA are often resistant to multiple antibiotics, their treatment is challenging and often quite economically costly. Due to these facts, early diagnosis and detection of MRSA in patients are crucial in clinical practice, which is also important to prevent them from spreading [8].

Several methods have evolved for rapid detection of methicillin□resistant staphylococci, but the optimal method for the detection remains controversial. The most commonly used method in the laboratories is conventional phenotypic methods of culture and antibiotic sensitivity test. Molecular method is the

polymerase chain reaction (PCR) based method for detecting *mecA* gene, which remains the "gold standard" for diagnosing MRSA [23].

In the present study, we evaluated the performance of cefoxitin disc diffusion method and PCR assay for identification of methicillin resistant strain of *S. aureus* in clinical specimens obtained from patients with burn wound at the Emergency and Plastic Surgery Hospital in Sulaymaniyah.

Materials and Methods

a) *Study Site and Population*

A total of 150 non□duplicated, samples was collected from burn patients admitted to the Emergency and Plastic Surgery Hospital in Slemani during a 6-month period from July to December 2018. Wound swab samples from different anatomic locations received from the patients for bacteriological cultures were included in the study.

b) Isolation and Identification of S. aureus by conventional methods

Each sample was cultured on mannitol salt agar (LAB, UK) and incubated in 37° C for 24 h. Then all suspected *S. aureus* colonies were transferred onto blood agar medium. Gram staining and standard biochemical reactions, including catalase, slide and tube coagulase tests were used to identify the suspected colonies. Reference strain of the of *Staphylococcus aureus* ATCC® 6538™ was used in this study.

c) *Identification of S. aureus by Vitek® 2 System*

The vitek® 2 system was used according to the manufacturer's instructions; ID-Gram Positive Cocci cards_(BioMérieux, France-) for all samples that were detected as *S. aureus* by conventional methods as shown at Table 1.

Table 1. The Vitek 2 system result for identification of *S. aureus*.

Identification Information							**Analysis Time:**			**4.50 hours**					**Status:**		**Final**
Selected Organism							**99% Probability**			**Staphylococcus aureus**							
							Bionumber:			**010402062763231**							
Biochemical Details																	
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	-	39	ILATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

d) Antimicrobial susceptibility testing

1) Cefoxitin disk diffusion method

To determine the antibacterial resistance of the colonies, the Kirby-Bauer disk diffusion method was used as suggested by the Clinical and Laboratory Standards Institute (CLSI) [9]. The selected agent for the practice was 30 µg Cefoxitin per disk (Bioanalyses, Turkey).The standard bacterial suspension of *S. aureus* with turbidity equal to 0.5 McFarland was inoculated on Mueller–Hinton agar (MHA) (LAB, UK) with the subsequent application of a cefoxitin disk. The plates were incubated at 35°C for 24 h and the inhibition zone around the disk was then measured. The criteria provided by CLSI, which indicate that ≤21 mm meant resistant and ≥22 meant susceptible, were then used to evaluate the findings.

e) Detection of mecA gene by PCR technique

1) DNA Extraction

Commercial DNA extraction kits (Geneaid, USA) were used to extract the cellular DNA from the incubated *S.aureus* colonies, following the procedures provided by the manufacturer's instruction. For this

purpose, a 1 ml, 0.5 McFarland turbidity suspension of the incubated colony was taken into a tube for centrifuge at 14000 x g for 1 minute. The resulting supernatant was removed from the tube, and the remnants were added Gram (+) buffer (200 µl/sample) and lysosome (0.8 mg/200 µl). The samples were then vortexed and left for incubation at 37°C for ½ hours to completely dissolve. Proteinase K (20 µl) was then introduced to the samples, which were left for further incubation at 60°C for 10 minutes. Next, GB buffer was introduced to the samples (200 µl), and this time, the samples were incubated at 70°C for 10 minutes. Later, absolute ethanol (200 µl) was introduced to the sample tubes, which were vortexed for additional 10 seconds. The lysate was then transferred to the GD column in a 2 ml collection tube, where further centrifugation was performed at 14000 x g for 2 minutes. Four hundred µl of W1 buffer and 600 µl of wash buffer were used to wash the sample under additional 14000 x g centrifugation for 30 seconds. Next, the GD column in the 2ml collection tube was centrifuged for 3 minutes to dry the samples, after which they were taken into new 1.5 ml microcentrifuge tubes. Finally, preheated Elusion buffer (50 µl) was added to the samples, which were then centrifuged 16000 x g. The resulting supernatant was used as the base template for DNA in the PCR assay.

2) PCR Assay

T100 Thermal cycler (BIO-RAD, USA) device was used to amplify the DNA for the standard PCR assay method, which was utilized to determine the MRSA strains amongst the inoculated colony samples. A mecA-specific primer pairs were employed to amplify the 162 base pairs, which were: Forward, 5-TCCAGATTACAACTTCACCAGG- 3`, Reverse 5`-CCACTTCATATCTTGTAACG-3` as suggested by [10].

A volume of 5 µl of extracted DNA was added to a final volume of 25 µl PCR mixture containing 12.5 µl of 2x EasyTaq PCR supermix (Transgene,China), 1 µl forward primer, 1 µl reverse primer, and 5.5 µl ddH_2O.The thermal cycling protocol for PCR was comprised as initial denaturation at 94°C for 3 minutes, and follow-up denaturation of 30 cycles at 94°C for 30 seconds. The samples were annealed at 54°C for 30 seconds and extended at 72°C for 1 minute. Finally, the final extension process was applied with 72°C for 7 minutes. The amplified products were electrophoresed using 85 volts for 1 hour in a 1.5% agarose gel stained with prime safe dye (GeNet Bio, Korea) with a 100 bp DNA ladder (Transgene, China). Finally, for DNA visualization, the gel was examined and documented under Ultraviolet light [11].

Results

Bacterial isolates

Among 150 wound swab samples processed in the study with the above-explained methods, 55 (36 %) showed culture positivity for *S. aureus*. In terms of the gender distribution of *S. aureus* presence, 25 out of 55 samples were from male patients, while the remaining 30 were from females. The results also show that patients of age between 11 and 20 were more susceptible to *S. aureus* infections (Table 2).

Table 2. Age and gender distribution of patients with *S.aureus*

Age	Male (%) n=25	Female (%) n=30
≤ 10	2 (8)	5 (16.6)
11-20	7 (28)	10 (33.3)
21-30	4 (16)	3 (10)
31-40	3 (12)	0 (0)
41-50	4 (16)	4 (13.3)
51-60	2 (8)	5 (16.6)
61-70	3 (12)	3 (10)
71-80	0 (0)	0 (0)
≥ 81	0 (0)	0 (0)

Cefoxitin disk diffusion

From 55 *S. aureus* isolates, by using cefoxitin disk diffusion methods, 51 (92.7%) of *S. aureus* were identified as being methicillin resistant (MRSA), while the remaining 4(7.3%) were identified as methicillin-susceptible (MSSA) *Staphylococcus aureus*. Table 3 displays how sensitive and specific the cefoxitin disk diffusion method employed in the present study.

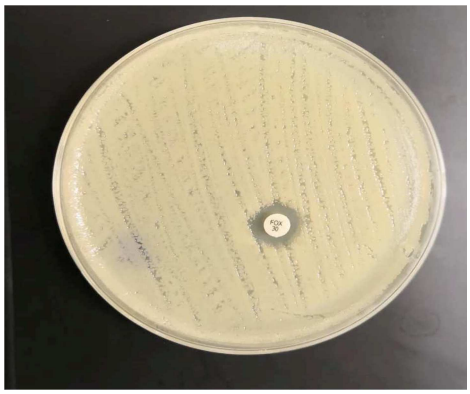

Figure 1. cefoxitin disk diffusion plate showing methicillin-resistant *Staphylococcus aureus*

Figure 2. Cefoxitin disk diffusion plate showing methicillin-sensitive *Staphylococcus aureus*

Table.3. Sensibility and specificity of the cefoxitin disk diffusion tests as compared with *mec*A detection by PCR technique.

MecA result	PCR No. of isolates tested	CDD	
		Positive	Negative.
Positive	48	47	1
Negative	7	4	3
Sensitivity		92.15%	
Specificity		75%	

CDD= cefoxitin disk diffusion tests

PCR Amplification mecA gene

Identification of MRSA strains was performed by detection of mecA gene in all *S.aureus* strains using PCR assay. The result revealed that 48 (87.3 %) were identified as methicillin-resistant and 7 (12.7%) of *S. aureus* isolates were identified as methicillin-sensitive (MSSA). The PCR amplification of mecA positive and negative genes are shown in figure 3.

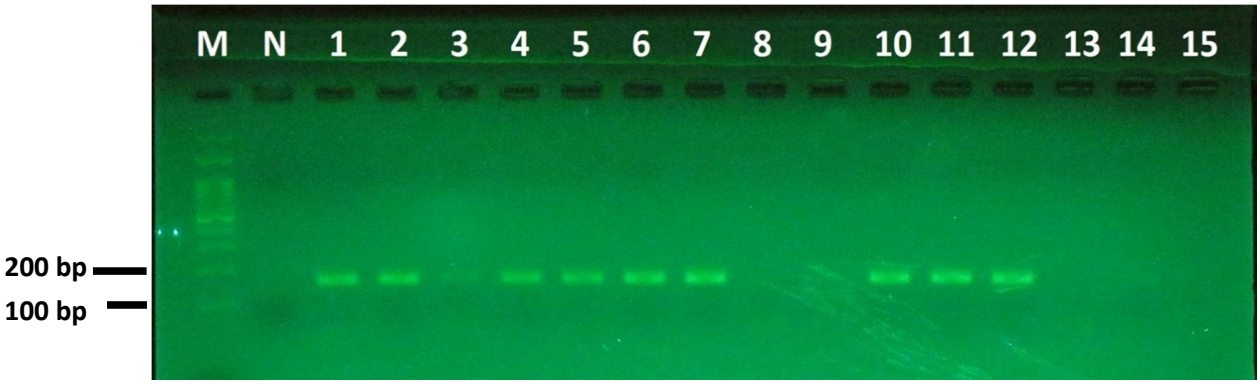

Figure 3. PCR amplification of mecA gene 162 bp. Lane M: ladder with a size of 100bp, lane N: negative control, lane 1-7,10-12 show positive mecA gene. Lane 8,9,13,14,15 show negative mecA gene.

Discussion

In a last few decades, the spread of MRSA strains all over the world increased, and made them a significant issue in terms of public health. These nosocomial pathogens are gaining resistance against even more types of antibacterial agents [12, 13]. Due to this reason, early diagnosis of the presence of such bacteria and detection of methicillin resistance is important to be able to prevent spreading of infections and reduction of mortality rate [14, 15].

Our results showed that the prevalence of MRSA infections was 87.3 % with an amplified band of 162 bp among culture-positive cases of *S. aureus* which is compatible with the studies done by [17] and [16] in which the same results were obtained. Our result agree with the results previously reported in a similar study conducted by Jalal in Sulaimai, Kurdistan-Iraq, on burn wound infection rate also has the same prevalence of MRSA infection with 87.7% [24].

This high prevalence rate of MRSA could be attributed to various factors like indiscriminate use of antibiotics, deficiencies in hygiene practices and lack of hospital infection control programs.

While the disk-diffusion method is easy to implement and can be performed even in laboratories with limited conditions, its sensitivity and specificity are low in case of MRSA due to the heterogeneous nature of the resistance developed in these types of bacteria [18] and it was also was affected by inoculation' size, incubation duration, pH and salinity of the media [21].

The findings of the present study indicate the sensitivity and specificity of cefoxitin disk-diffusion method as 92.15 % and 75 % respectively, when compared to the PCR method. The specificity of the disk diffusion methods is often affected by false positive isolates, which usually occur due to over□produced β-lactamase such strains are resistance (*mec□A* gene), phenotypically [19] and [20].

A similar study by [19] reported the low sensitivity and specificity of cefoxitin disk diffusion test as compared to PCR for detection of MRSA with a sensitivity of 86.27% and specificity of 83.33%.

This is why the rapid, reliable, sensitive PCR tests stand out in terms of accuracy, albeit their implementation involves costs and turnaround time, which the clinical laboratories have to optimize to be able to use them in infection control programs effectively [22].

The PCR method has numerous advantages as compared to conventional methods. The false□negative results of the conventional methods can be picked up by the PCR even during the earliest periods of the infection. Within 5 hours of isolation, PCR can determine whether mecA gene is present within the specimen or not, making it a rapid and useful tool for clinicians. While the conventional methods require 48-72 hours [23]

In conclusion, the findings of the present study show that PCR assay is faster and more reliable in the determination of MRSA presence compared to the cefoxitin disk-diffusion method. This is of great significance, as the ever-increasing number of drug-resistant MRSA strains require quick diagnosis to help reduce patient's mortality and to prevent them from spreading to other individuals.

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