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Comparative Evaluation of Traditional Susceptibility Testing for MRSA with the PCR Approach

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multi-drug resistant pathogen, which is responsible for increasing cases of serious diseases, including life-threatening diseases and nosocomial and community-acquired infections. Laboratory identification of MRSA is crucial and essential both for initiation of appropriate antimicrobial therapies and for effective infection control strategies that are designed to limit the spread of MRSA. In spite of the availability of commercial kits for MRSA detection in the market, the Clinical and Laboratory Standards Institute (CLSI) recommends the use of phenotypic methods, such as the disk diffusion method with oxacillin or with cefoxitin, as well as a serial dilution method with oxacillin. Nevertheless, some studies have shown that results obtained with such phenotypic methods are controversial. The aim of the study described in this paper was to comparatively evaluate the traditional susceptibility testing for MRSA with PCR as the gold standard assay. Analysis of collection (n = 68) isolates of *Staphylococcus aureus* revealed that the serial dilution method with oxacillin possessed the highest sensitivity (at 100%). In contrast, the disk diffusion methods with oxacillin and cefoxitin showed lower sensitivity (95.83%, 95% CI (78.81% - 99.30%)). Furthermore, the borderline value of zone inhibition diameters for cefoxitin might be considered as a risk, and they may give false-susceptible result.

Keywords

MRSA, mecA, Phenotypic Methods, PCR

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1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) (sometimes referred to as oxacillin-resistant *Staphylococcus aureus*) is one of the most important healthcare-associated pathogens, and it has gained global attention in recent years [1]. The major threat of MRSA to human health is associated with infectivity, possession of virulence factors and antimicrobial resistance [2] [3]. This is particularly with nosocomial and community-acquired infections, where the young, elderly and the immunocompromised are most at risk. In relation, it has been shown by Honda H *et al*. that patients who are colonized with MRSA have a greater risk for developing infection than with methicillin-susceptible *Staphylococcus aureus* (MSSA) in the intensive care unit [4]. The bacterium primarily commonly colonizes the anterior nares. Other potential sites for infection include the remainder of the respiratory tract, open wounds, intravenous catheters, and the urinary tract.

Methicillin resistance in *Staphylococcus aureus* is primary provided by the over production of the penicillin-binding protein 2a (PBP2a), which differs from other penicillin-binding proteins as its active site does not bind methicillin or other *β*-lactam antibiotics. 2a (PBP2a) is encoded in the mecA gene. This mechanism enables *Staphylococcus aureus* to survive in presence of penicillin-like antibiotics [5].

Patient screening upon hospital admission with nasal cultures is of great importance for controlling the risk presented by the bacterium. To define *Staphylococcus aureus* as MRSA in laboratory, either the mecA gene should be detected or a minimum inhibitory concentration (MIC) of oxacillin must be equal or higher than 4 mg/ml. However, some recent studies showed that some strains of *Staphylococcus aureus*, carrying the mecA gene have also demonstrated susceptibility to oxacillin [6] [7].

The molecular approach for defining of *Staphylococcus aureus* as MRSA by detection mecA gene with PCR is not yet available in every bacteriological laboratory; hence the phenotypic tests remain the methods of choice (and are the recommended diagnostic tests by the Clinical and Laboratory Standards Institute (CLSI)). However, the results obtained with phenotypic methods remain controversial (for example, incubation temperature is known to affect the test results). The traditional phenotypic screening method for methicillin resistance is oxacillin disk diffusion test (DDT); to add to the array of phenotypic methods Velasco *et al*. recently demonstrated that DDT with cefoxitin was more accurate for the detection methicillin resistance [8]. This is because Cefoxitin results are easier to interpret and are thus more sensitive for the detection of mecA-mediated resistance than oxacillin results.

The aim of our study was to evaluate the accuracy of the disk diffusion tests with oxacillin and with cefoxitin (with the PCR method serving as the gold standard for detection MRSA).

2. Material and Methods

2.1. Sample Collection and Bacterial Isolation

The collection of 68 stains of *Staphylococcus aureus* with methicillin resistance were obtained from patients with skin and soft tissue infections in Karaganda, Astana, Zhezkazgan and Semey cities from 2012 to 2014. Each of these strains of *Staphylococcus aureus* were obtained from different hospital departments, including intensive care unit, surgery, trauma unit, obstetrics and gynecology, and others.

Species identification of microorganisms was performed using Matrix-assisted laser desorption/ionization-Time-of-flight (MALDI-TOF) (Microflex, Bruker) with a score of >1.9 (Biotyper, Bruker). This method uses a type of mass spectrometry to measure a unique molecular (protein) fingerprint of a microorganism.

2.2. Phenotypic Identification of MRSA

All *Staphylococcus aureus* strains were tested for methicillin resistance using the oxacillin and cefoxitin disk diffusion methods (DDM) according to the Clinical and Laboratory Standards Institute (CLSI). Plates with MHA + 2% NaCl and commercial disks (Bio-Rad) with oxacillin were incubated at 35˚C for 24 h. and with cefoxitin (Bio-Rad) at 37˚C for 18 - 24 h to allow the microorganisms to grow. Following this, zone inhibition diameters were measured. The results with zone inhibition diameter for oxacillin (1 µg per disc) ≤ 10 mm and for cefoxitin (30 µg per disc) ≤ 21 mm indicated resistance to methicillin [9] [10].

The method of serial dilution used Mueller-Hinton broth (MHB) with oxacillin to detect MRSA. In the 96 well plates (Nunc) with broth, samples were inoculated with 10 µl of 0.5 McFarland suspensions of the bacterial strains and then incubated at 35˚C for 24 h. This procedure was performed using an automatic station, Evo 150

(Tecan). The criterion to define MRSA was ≥4 µg/ml, as described in CLSI [9].

2.3. Genotypic Identification of MRSA

Isolates were grown on Mueller-Hinton at 35˚C for 18 to 24 h. A single colony of *Staphylococcus aureus* was used for extraction of DNA using DNAzol Reagent (Invitrogen, USA), according to the manufacture's instruction. The genomic DNA was used as template for detection of mecA gene by PCR.

The primers used to amplify the mecA gene were: GMECAR-1 5'-ACTGCTATCCACCCTCAAAC-3' and GMECAR-2 5'-CTGGTGAAGTTGTAATCTGG-3' (163 bp) as described [11]. Each PCR mixture was 12.5 µl SYBR Select Master Mix (Applied Biosystems, USA) (2.0×), 0.5 µl of each primer (10 mM), 9.5 µl sterile distilled H_2O and 2 ml DNA template. The PCR program included an initial step at 94˚C for 5 min followed by 35 cycles of 92˚C for 2 min, 57˚C for 2 min, and 72˚C for 1 min, and ended with a final extension step at 72˚C for 7 min. The *Staphylococcus aureus* strains ATCC 43300 and ATCC 25923 (American Type Culture Collection, USA) were included as positive and negative controls for mecA gene, respectively. The products of PCR amplification were detected with 2% agarose gel.

2.4. Quality Control

Quality control of discs with antibiotics was performed with using *S. aureus* ATCC 25923 in accordance with recommendation CLSI. The presence of methicillin resistance was controlled with standard strains: *S. aureus* ATCC 25923 as negative control and *S. aureus* ATCC 43300 as positive control (**Figure 1**).

2.5. Diagnostic Test Evaluation

The PCR results with detection mecA gene (gold standard) were considered as true values. Calculations of sensitivity, specificity and positive/negative predictive values of phenotypic methods in comparison with genetic assay were calculated using MedCalc for Windows, version 10.2 (MedCalc Software, Ostend, Belgium) [12].

3. Results

The collection of clinical *Staphylococcus aureus* (n = 68) were obtained from different hospital departments of Karaganda, Astana, Zheskasgan and Semey (Kazakhstan), including intensive care unit, surgery, neurosurgery, trauma unit, obstetrics and gynecology, pediatry, general and others. The summarized data relating to the isolates is presented in **Table 1**.

S. aureus ATCC43300 MRSA | *S. aureus* ATCC25923 non-MRSA

Figure 1. Phenotypic identification of MRSA.

Table 1. List of nosocomial strains of *Staphylococcus aureus* isolated in hospitals of Astana, Karaganda, Semey and Zheskasgan (Republic of Kazakhstan, 2012-2014).

	Hospital department											Laboratory detection of methicillin resistance			
	Gynecology	ENT	General	ICU	Neurosurgery	Surgery	Traumotology	Pulmonology	Pediatry	Ophthalmology	Uknown	DDM (OXA)	DDM (FOX)	MIC (OXA) µg/ml	MecA
Astana				20,051								R	R	256	+
		23,644										R	R	8	+
						24,362						R	R	64	+
						27,770						R	R	512	+
		27,774										R	R	16	+
									24,361			S	S	2	−
		22,719										S	S	0.5	−
		22,720										S	S	0.5	−
			22,721									S	S	2	−
			22,722									S	S	0.5	−
			22,724									S	S	0.5	−
											23,645	S	S	0.5	−
Karaganda							23,259					R	R	16	+
						24,418						R	R	512	+
						24,680						R	R	1024	+
			26,321									R	R	1024	+
			26,322									R	R	512	+
				27,040								R	R	256	+
					27,041							R	R	256	+
				27,042								R	R	1024	+
						27,043						R	R	1024	+
		27,076										S	S	2	−
		27,078										R	R	512	+
		27,799										R	R	512	+
		27,801										R	R	1024	+
		28,021										S	S	2	−
						28,044						S	S	0.25	−
								23,984				S	S	1	−
				26,520								S	S	0.1	−
		27,077										S	S	0.5	−
									23,140			S	S	0.5	−
									23,141			S	S	0.5	−
									23,142			S	S	0.25	−
									23,143			S	S	0.25	−

Continued

							23,256	S	S	0.25	−
			23,257					S	S	1	−
			23,258					S	S	2	−
					23,259			S	S	0.25	−
		23,758						S	S	0.25	−
		23,759						S	S	0.25	−
		24,634						R	S	128	+
Semiey						27,336		R	S	8	+
						27,337		R	S	16	+
						27,353		R	R	128	+
					21,871			S	S	1	−
				22,830				R	R	32	+
						24,008		S	S	4	−
			24,219					S	S	4	−
	24,598							S	S	256	+
		24,601						R	R	16	+
		23,982						S	S	0.5	−
		23,991						S	S	0.5	−
						24,000		S	S	1	−
				24,003				S	S	2	−
				24,214				S	S	1	−
				26,589				S	S	0.5	−
Zheskasgan					21,863			S	S	1	−
							21,867	S	S	1	−
		21,873						S	S	0.25	−
		21,874						S	S	1	−
		21,875						S	S	0.125	−
					22,828			S	S	0.25	−
			22,901					S	S	0.25	−
				22,903				S	S	1	−
		22,908						S	S	1	−
			22,940					S	S	0.25	−
			22,941					S	S	2	−
		22,948						S	S	0.25	−

The 68 clinical isolates of *Staphylococcus aureus* were tested for methicillin resistance with PCR by amplifying mecA gene. 24 of 68 were positive isolates based on predicted 167 bp PCR product (refer to **Figure 2**).

The comparison of phenotypic detection methods, for defining MRSA (DDM with oxacillin and with cefoxitin and method of serial dilution with oxacillin), with the PCR method (detection of mecA gene), acting as “the gold standard”, is summarized in **Table 2**.

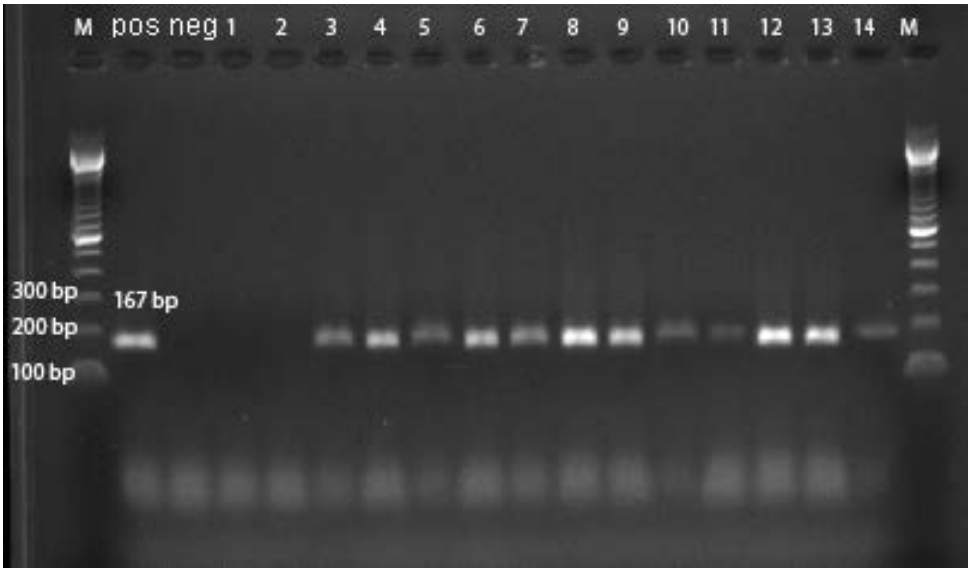

Figure 2. Detection the amplified PCR product of mecA gene (2% agarose gel). M-molecular markers, pos-positive control (*S. aureus* ATCC43300 strain), neg-negative control (*S. aureus* ATCC 25923 strain), 1 - 14—clinical isolates of *Staphylococcus aureus.*

Table 2. The comparison evaluation of the different phenotypic methods compared with PCR.

Phenotypic method	Positive*	Negative	Sinsitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
DDT with oxacillin						
Positive	23	0	95.83%	100%	100%	97.78%
Negative	1	6	(78.81% - 99.30%)	(91.88% - 100%)	(85.05% - 100%)	(88.19% - 99.63%)
DDT with cefoxitine						
Positive	20	0	83.33%	100%	100%	91.67%
Negative	4	6	(62.6% - 95.16%)	(91.88% - 100%)	(83.01-100%)	(80% - 97.63%)
Method of serial dilution with oxacillin						
Positive	24	2	100%	95.45%	92.31%	100%
Negative	0	4	(85.62% - 100%)	(84.5% - 99.31%)	(74.83% - 98.83%)	(91.51% - 100%)

*The presence of mecA gene in isolates of *Staphylococcu aureus* was considered to be a true positive result. PPV—positive predictive value, NPV—negative predictive value.

4. Discussion

Methicillin-resistant *Staphylococcus aureus* is considered to be a nosocomial pathogen with a wide range of clinical manifestations, including life-threatening diseases such as necrotizing pneumonia and endocarditis [13]. It has been shown that patients colonized with MRSA have an increased risk for developing infection compared with non-colonized patients [2] [14]. Therefore, rapid and accurate identification of multi-drug resistant *Staphylococcus aureus* is crucial and essential both for initiation of appropriate antimicrobial therapies and for effective infection control strategies to limit the spread of MRSA [15]. In spite of the fact that there are many new rapid and less costly methodologies, the traditional susceptibility testing, such as disk diffusion method with oxacillin or cefoxitin, is available in every bacteriological laboratory and these methods can be performed easily to detect MRSA. It also remains that such methods are the recommended ones of the CLSI.

5. Conclusions

Our study showed sufficiently high enough diagnostic characteristics from the traditional techniques for detec-

tion methicillin resistance of *Staphylococcus aureus*: such as the serial dilution method (100% sensitivity) and disk diffusion tests with oxacillin (95.83% sensitivity). These results are in accordance with previously reported studies [16] [17]. Although some publications revealed the higher sensitivity for disk diffusion test with cefoxitin (95% - 100%), in our research the sensitivity for DDT with cefoxitin was considerably lower (83.33% (95% CI 62.6% - 95.16%)). Interestingly, all discrepant PCR and disk diffusion test results with cefoxitin were associated with border zone inhibition diameters to breakpoint parameters for being susceptible (≥22 mm). Repeated tests on these discrepant results showed the "resistant" results were instead "susceptible" and this increased sensitivity of disk diffusion test with cefoxitin up to 95.83%. Thus, results with borderline zone inhibition can be considered as false-negative results. Having chance to compare phenotypic screening results with PCR allow double-checking the discrepant results, otherwise false-susceptible results can be considered as a true results. These results exemplify the concerns with biased and imprecise breakpoints in relation to the classical methods.

At the same time, examples of *Staphylococcus aureus* with borderline oxacillin resistance being mecA-negative have been reported to be resistant due to other mechanisms, such as the hyperproduction of *β*-lactamase [18]. In addition, isolates of *Staphylococcus aureus* having mecA gene have been reported to be phenotypically susceptible to oxacillin [7] [19] Whilst the PCR approach stands as an ultimate method for detecting resistance to methicillin where such technology cannot be incorporated, our results demonstrated that the combination of two or more pheonotypic tests will provide more confidence in the result obtained [20].

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