Determination of vancomycin and methicillin resistance in clinical isolates of Staphylococcus aureus in hospitals of Ilam city

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Received; 2015/11/9 revised; 2015/12/21 accepted; 2016/1/21

Abstract

Introduction: In this study, using the phenotypic and genotypic methods, oxacillin susceptibility in *Staphylococcus aureus* (*S. aureus*) strains isolated from patients at two government hospitals in Ilam, Iran was tested.
Materials and methods: Out of 200 *S. aureus* isolates from different human clinical specimens consisting of blood (31%), wound (20%), urine (21%), catheters (7%), sputum (12%), others (9%) were collected. The methicillin resistant *S. aureus* isolates were investigated using disk diffusion methods and oxacillin (1µg) and cefoxitin (30µg), on Mueller-Hinton agar were used, and *MecA* and *vanA* genes were detected by PCR. In addition, the isolates were tested for their antibiogram profiles.
Results: Among 200 *S. aureus* strains included in this study, 35.96% were MRSA. The percentage of resistance by disk diffusion method was as below: penicillin 85.96%, vancomycin 0%, ampicillin 87.71%, gentamicin 48.25% erythromycin 54.25%, clindamycin 32.45%, amikacin 21.05%, ciprofloxacin 42.10%, tetracycline 51.75% and co-trimoxazole 42.10%. Phenotyping method by disk diffusion method using oxacillin and cefoxitin for detecting of MRSA showed sensitivity and specificity of about 33.33% and 35.96%, respectively. Presence of *MecA* and *vanA* genes in MRSA isolates by PCR were 35.96% and 0%, respectively. The oxacillin and cefoxitin disk diffusion methods showed 92.68% and 100% sensitivity, respectively, and 98.8% specificity.
Conclusion: Our finding showed that, the cefoxitin disk diffusion method is better in compared to the oxacillin disk diffusion similar to results from detecting of *MecA* gene in PCR as a golden test.

Keywords*:* *Staphylococcus aureus*, *MecA*, Methicillin, Vancomycin

Introduction

Staphylococcus aureus frequently is a member of the normal skin flora and nasal cavity often causes abscesses, infections of wound, skin, soft tissue, osteomyelitis, endocarditis, pneumonia etc. It may also cause staphylococcal scalded skin syndrome, a severe disease in infants or the toxic shock syndrome (1). The previous data provided that the high virulence potential of MRSA is associated with genes like capsule, clumping factor, toxins and some genes as vancomycin and methicillin resistance (2). An important result rises from previous researches suggest these that characterization of MRSA isolates requires diagnosis of mec element, which carries methicillin resistance determinant MecA (3). *Staphylococcus aureus* is a inherit pathogen causes a variety range of disease, such as wound infections, pneumonia,

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[DOI: 10.18869/acadpub.jbrms.3.3.1]

septicemia, etc., with beta-lactam antibiotics being the drugs of choice for therapy (4).Detection of MRSA strains is important for the treatment of infections caused by these strains(5).The aim of this study was to determination of Vancomycin and Methicillin Resistance in Clinical Isolates of *Staphylococcus aureus* in Ilam Hospitals using disc diffusion method and detecting of *MecA* gene by PCR.

Materials and methods

Samples collection: A total of 200 non-duplicated *S.aureus* isolates used in this study were randomly collected from in-patient and outpatient of tow government hospitals from Ilam city in Iran, during September 2012 to October 2013.The specimens of clinical consisted blood (31%), wound (20%), urine (21%), catheters (7%), sputum (12%), others (9%). Then all *S.aureus* isolates were stored frozen at -80C in Skim Milk broth, containing 10% glycerol.

Laboratory methods: Specimens were screened by Gram's stain and were cultured on 10% sheep blood agar and MacConkey's agar. All isolates were identified by catalase production, haemolysis on blood agar, oxidative-fermentative test, and production of bound and free coagulase, manitol fermentation and 7.5 percent NaCl tolerance and heat labile DNase. Tube coagulase production is considered "gold standard" for identification of *S.aureus* (6).

Antimicrobial Susceptibility Test: Disk diffusion test of Penicillin (10 U), vancomycin (30µg), Ampicillin (10µg), Gentamicin (10µg), Erythromycin (15µg), Clindamycin (2µg), Amikacin (30µg), ciprofloxacin (5µg), Tetracycline (30 µg), Co-trimoxazole (25µg) (Mast, Merseyside, United Kingdom), was carried out using Kirby-Bauer Method according to CLSI guidelines 2011. The inoculums (turbidity equivalent to that of a 0.5 McFarland Standard) of the *S.aureus* clinical isolates were cultured on Mueller-Hinton agar plates and after 24h incubation at 37℃, the Zone diameters were measured as CLSI guideline (7). The minimal inhibitory concentration (MIC) of oxacillin, cefoxitin, and vancomycin was determined only for MRSA isolates by agar dilution method. Briefly, gradient plates of Mueller-Hinton agar (Hi Media India) were prepared with vancomycin, cefoxitin and oxacillin (0.5-256 mg/l, Sigma- Aldrich). 0.5 McFarland equivalent inoculum prepared using 18-24 h old culture was spotted on to gradient plates and incubated at 37°C for 24 h before assessing the visible growth as recommended by the National Committee for Clinical Laboratory Standards (8). MSSA (ATCC 6538) and MRSA (ATCC 33591) were included as control strains for disk diffusion (2).

Detection of methicillin resistant isolates: Detection and confirmation of methicillin resistant *S.aureus* isolates was carried out using disk diffusion method and following disks were used: oxacillin (1µg), and cefoxitin (30 µg). The plates were incubated for 24 h at 37 °C and antimicrobial activity was evaluated by determining the diameter of the inhibition zone as recommended by the CLSI (9, 10). Then results were compared with *MecA* gene detecting using PCR.

DNA Extraction: Total genomic DNA was extracted using phenol-chloroform isoamyl alcohol (Merck, Darmstadt, Germany). The specimens were pelleted followed by adding 250µL buffer I and buffer II containing RNase (CinnaGen, Tehran, Iran). A 550µL volume of phenol was added to the aliquots before being centrifuged at 10000 g for 5 min, the supernatant clear phase was then collected into new eppendorf tube and the latter stages was repeated twice in order to wash cell derbies. A 0.1 volume of sodium acetate 0.1 M was then added to the tubes, washed twice using ethanol 100 and 80%, respectively. The tubes were then centrifuged at 12000 g for 15 min. The pellet was finally dried and re-suspended in 30µL Tris-EDTA buffer and used as template in PCR screening (11).

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PCR- based detection of Vancomycin and Methicillin resistant genes in isolates: In all coagulase positive isolates the resistance genes were detected by PCR. They included genes for methicillin resistance, and vancomycin resistance (12). The PCR primers used to detect resistance genes are listed in Table 1(13, 14). The PCR mixture was prepared in a final volume of 25 ml. The amplification mixture consisted of 2.5 ml template DNA, 2ml primers, 2 ml of a 10-fold concentrate PCR buffer, 2 ml dNTP, 0.5 mM MgCl2, 15 ml D.W. and 1 U of Taq DNA polymerase (CinnaGen, Tehran, Iran). A thermocycler (Master cycler gradient; Eppendorf, Hamburg, Germany) was programmed for genes with the following parameters:

Detection of *MecA*: Initial denaturation at 94ºC for 3 min was followed by 30 cycles of amplification with 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 30 s (except for the final cycle, which had an extension step of 4 min). The PCR products were submitted to electrophoresis on 1.5% agarose gel (MBI Fermentas) containing 0.4 ml/ml of ethidium bromide and visualized by using UV trans illumination, and photographed (BioDoc- Analyse; Biometra, Goettingen, Germany).

Detection of *VanA*: Initial denaturation step of 2 min at 94ºC; 30 cycles of 15 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final elongation at 72ºC for 7 min.

Statistical Analysis

All data analyses of data was carried out using SPSS software version 11.5 for Windows. By using the w2-test and Fisher's exact test, P-Values <0.05 were considered statistically significant.

Results

Diagnosis of isolates using microbiological methods: Out of 200 isolates of Staphylococci collected. The results showed 114 (57%) were coagulase positive and 86 (43%) were coagulase negative *staphylococcus aureus* (CNSA).

Antibiogram profile of isolates: Tables 1 and 2 show the results of antibiotic resistance testing of the MRSA and CNSA isolates to 10 antibiotics studied. The patterns of MICs of oxacillin, cefoxitin, and vancomycin on the MRSA isolates were determined with concentrations varying from 4µg/ml for vancomycin and 64 to 128µg/ml for oxacillin and 32µg/ml for cefoxitin.

Table 1. The primer sequencing for detection of *vanA* and *MecA* genes.

Primer	Primer sequence	Size of product (bp)
MecA forward	AAAATCGATGGTAAAGGTTGGC	532
MecA reverse	AGTTCTGCAGTACCGGATTTGC	
vanA forward	CATGAATAGAATAAAAGTTGCTGCAATA	1032
vanA reverse	CCC CTT TAA CGC TAA TAC GAT CA	

Table 2. Antibiotic resistant pattern of MRSA isolates.

Antibiotic	Pen	Van	Amp	Gn	E	Da	Ak	Cip	Te	Ts
Number of MRSA	98	0	100	55	62	37	24	48	59	48
Percentage (%)	85.96	0	87.71	48.25	54.38	32.45	21.05	42.10	51.75	42.10

Pen= Penicillin, Van= Vancomycin, Amp= Ampicillin, Gn= Gentamicin= Erythromycin, Da=Clindamycin, Ak= Amikacin, Cip= Ciprofloxacin, Te= Tetracycline, Ts= Co-trimoxazole.

Table 3. Antibiotic resistant pattern of CNSA isolates.

Antibiotic	Pen	Van	Amp	Gn	E	Da	Ak	Cip	Te	Ts
Number of CNSA	86	0	86	64	68	46	31	40	66	54
Percentage (%)	100	0	100	74.41	79.06	53.48	36.04	46.51	76.74	62.79

Pen= Penicillin, Van= Vancomycin, Amp= Ampicillin, Gn= Gentamicin, E= Erythromycin, Da=Clindamycin, Ak= Amikacin, Cip= Ciprofloxacin, Te= Tetracycline, Ts= Co-trimoxazole.

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Detection of methicillin resistant isolates: Out of 114(57%) coagulase positive staphylococci 41 isolates (35.96%) were resistant to cefoxitin and 38 isolates (33.33%) were resistant to oxacillin, using disc diffusion method.

Detection of *MecA* and *VanA* genes by PCR: PCR revealed the presence of the *MecA* gene in 41 isolates 35.96%, (Figure 1), and all isolates were negative for presence of *vanA* gene (data not shown).

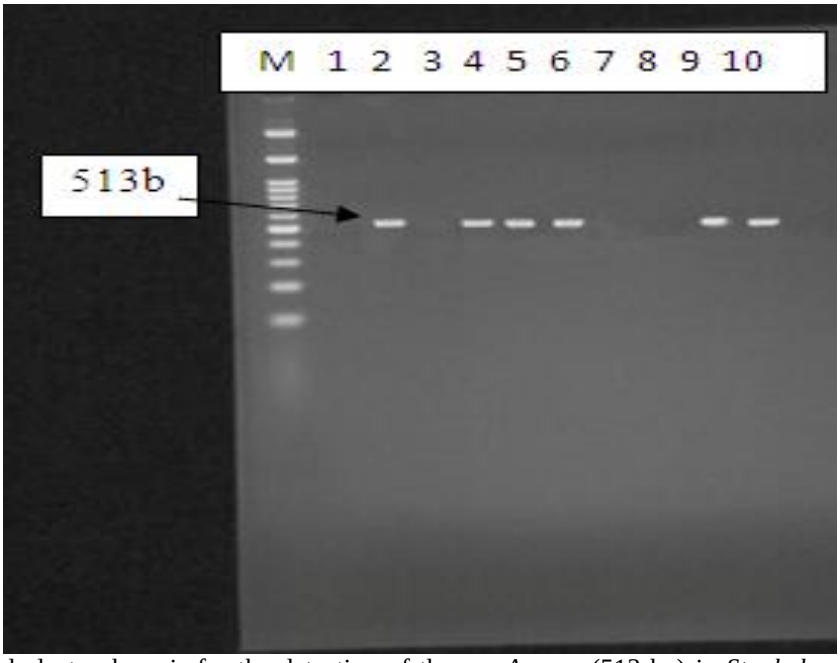


Figure 1. Agarose gel electrophoresis for the detection of the *mecA* gene (513 bp) in *Staphylococcus aureus* strains by PCR. Lanes 4, 5, 6, 9, 10: positive strains; lanes 3, 7 and 8: negative strains; lane 2: positive control; lane 1: negative control; lane M: molecular weight marker (100 bp).

Discussion

Methicillin-resistant *S.aureus* produces a low affinity penicillin-binding protein (PBP 2a) in addition to usual PBP (15). The structural genes of this PBP (*MecA*) are present in resistant strains but not in susceptible ones (15). Two mechanisms of resistance described: inactivation of oxacillin and the other is modified resistance, called MOD-SA, due to the production of modified intrinsic PBPs with affinity for altered oxacillin (10). In the present study, 200 *S.aureus* strains were collected from in-patient and outpatient of two government hospitals from Ilam city in Iran and tested for MRSA. Our finding showed that, the percentage of MRSA isolated from all patients was only 35.9%. Similar results have been reported by Mohajeri et al, in Kermanshah, western of Iran, with a percentage of MRSA of 36% (16). Our result also is in agreement to data conducted from Salimnia and Brown among staphylococcus isolates in outpatient and inpatient in Detroit Medical Center (DMC) and from Outreach specimens (17), and Malathi et al. in 2009 with percentage of 36.4% MRSA isolated from patient (6). Furthermore, the prevalence of MRSA observed here was lower than other reports in Iran and other countries. In a study, conducted by Japoni et al., 2004, in the Nemazi hospital Shiraz, Iran the rate of MRSA 56% was reported (18) and also the prevalence of MRSA infection of 75% was reported by Izadi et al., in Tehran, Iran (19). In the United

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States, a prevalence of MRSA infection of 47% was reported in a hospital in Texas in 2003 (20). In our study, phenotypic tests such as the oxacillin disk diffusion method and cefoxitin disc diffusion method were compared with a genotypic method (*MecA* gene detection by PCR). The cefoxitin disk diffusion method presented 100% sensitivity and was superior to the oxacillin disk diffusion method (93.18% sensitivity) in terms of the detection of oxacillin-resistant *S.aureus* using PCR method for detecting of *MecA* gene. Our data is in agreement to results reported by Velasco et al (21). They reported that, the cefoxitin disk diffusion method, showed 100% sensitivity and 98% specificity. Skov et al reported a result, with a sensitivity of about 100% and specificity of 99% for cefoxitin disk method (22). Cauwelier et al. reported 100% sensitivity and 99% specificity, for cefoxitin disk diffusion method whereas sensitivity fell to 91.7% in the oxacillin disk diffusion test (23). According to our data, compared to the gold standard (*MecA* gene detection by PCR), the disk diffusion method with 30µg cefoxitin is preferable to the1µg oxacillin disk diffusion method for the detection of MRSA.

Acknowledgment

This study was supported by University of Ilam. Iran. Authors are thankful to the Head of the Department of Biology Research Center, Ilam University, Ilam, Iran.

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