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Full Length Research Paper

Evaluation of methicillin resistance *Staphylococcus aureus* isolated from patients in Golestan province-north of Iran

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Methicillin resistant *Staphylococcus aureus* (MRSA) is a main cause of nosocomial infections with consequence of increasing hospitalization, costs of treatment and rate of mortality. This study was aimed to demonstrate distribution of MRSA strains and their antibiotic resistance pattern. In this descriptive study, 185 clinical isolates of *S. aureus* that were collected from different infections during September 2008 to 2009 were tested by polymerase chain reaction (PCR) and micro dilution broth. All the MRSA and methicillin sensitive *Staphylococcus aureus* (MSSA) isolates were tested for antibiotic resistance pattern by disk diffusion method with 14 different antibiotics. Data were entered in SPSS software version 16 and analyzed by chi-square test. P value of <0.05 was considered significant. Of 185 tested *S. aureus*, 67(36.2%) strains were MRSA, which demonstrated 100% resistance to Penicillin, Ampicillin and CO-Amoxyclav and -80, 96.2 and 75% resistance to Cephotaxime, Nalidixic Acid and erythromycin, respectively. All *S. aureus* isolates was sensitive to vancomycin. All isolates with minimum inhibitory concentration (MIC) >8 µg/ml were *mecA* positive. MRSA is spreading worldwide with increasing levels of resistance, and accurate and early detection of these strains is encouraged.

Key words: *Staphylococcus aureus,* methicillin resistant *Staphylococcus aureus*, methicillin sensitive *Staphylococcus aureus*, minimum inhibitory concentration, mecA

INTRODUCTION

Infections caused by *Staphylococcus aureus* were the major cause of mortality in different communities, before the discovery of Penicillin by Alexander Fleming (Srifuengfung, 1994). *S. aureus's* infections, especially caused by methicillin-resistant *Staphylococcus aureus* (MRSA), are emerging as a major public health problem. MRSA has become a leading cause of nosocomial infections worldwide, since the first isolate was detected in 1961 in England (Srifuengfung, 1994; Zhang, 2001). *S.aureus* has a protein in its cell wall called penicillin binding protein (PBP), with trans-peptidase activity, play a key role in cell wall synthesis and are the target for β-lactam antibiotics. In addition MRSA strains produce a modified PBP called PBP2a or PBP2$^{/}$ with low affinity for β-lactam antibiotics (Hiramatsu, 2001; Zhang, 2005). Resistance to methicillin mediated by mecA gene, responsible for production of PBP2a, which have been observed in MRSA. mecA located on a region of chromosome called SCCmec. Five different types of SCCmec have been characterized as I, II, III, IV, V (Hiramatsu, 2001; Zhang, 2005). mecA expression in *invitro* condition was variable and depends on different factors such as temperature, cation concentration, incubation period and pH (Forbes, 2007). MRSA showed resistance to other β-lactam antibiotics such as Oxacillin and Nafcillin. MRSA is the main causes of nosocomial infections in the worldwide. Power of morbidity and simultaneously resistance to other antibiotics in MRSA strains is higher than methicillin sensitive *S. aureus* (MSSA). Regarding to increased prevalence of these strains in recent years and pathogenesis of *S. aureus*,

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accurate and early identification of these strains is very important (Chambers, 199; Gradie, 2001). MRSA strains can grow in the presence of 16 µg/ml or more of methicillin while sensitive strains are inhibited (Lewis, 2008; Gradie, 2001). Our recent investigation on healthcare staff showed 12.5% MRSA among *S. aureus* carriers (Unpublished). The aim of the present study was to demonstrate prevalence of MRSA by polymerase chain reaction (PCR) and micro dilution broth methods as well as their antibiotic resistance pattern.

MATERIALS AND METHODS

185 clinical samples were taken from various sites of infection including blood, wound, sputum, urine and others collected from five major hospitals in Golestan province named as: Taleghani, 5th Azar, Dezyani, Shohada and Shahid Motahari during June, 2008 and May, 2009. *S. aureus* identification performed based on standard tests such as Gram stain, catalase, DNase, growth on manitol salt agar, slide and tube coagulase (Forbes, 2007). Demographic data for all patients such as sex, age and site of infections were recorded. Methicilin resistance and molecular detection of mecA gene and susceptibility of strains to other antibiotics were performed as below.

Minimum inhibitory concentration (MIC)

MIC of methicillin was determined by micro dilution broth method, using Muller Hinton broth supplemented with 2% Nacl. Bacterium inoculation of 5 x 10^5 and incubation at 35℃ for 24 h was done according to Clinical and Laboratory Standards (CLSI) guide lines (Lewis, 2008; Gradie, 2001).

Disk diffusion test

Resistance to other antibiotics evaluated by Kerby bauer disk diffusion method based on CLSI guide lines (Brown, 2005; Kohner, 1999). Antibiotic disks (Himedia-India) that were used in this study is listed as: Nalidixic Acid (30 µg)-Ceftriaxone (30 µg)-Penicillin(10 unit)-Sulphamethoxazol-Trimethoprime (50 µg)-Ciprofloxacin(5µ g)-Chloramphenicol(30 µg)-Cefotaxime(30 µg)-Ampicillin(10 µg)-Vancomycin(30µ g)Gentamicin (10 µg)-Erythromycin(15 µg)-Tetracycline (30 µg)-Co-amoxyclave(10 µg) and Cefoxitin (30 µg).

Molecular detection of mecA gene

DNA extraction was performed by lysing *S. aureus* as following procedure: one milliliter of overnight growing bacteria in BHI broth transferred to 2 ml sterile microtube and centrifuged at 8000 rpm. 300 ul TE (tris EDTA pH=8, sigma- Germany) buffer was added to pellet and vortexed vigorously. 25 ul lysozyme (10 mg/ml) was added to suspension and incubated at 37℃ for 30 min. Re-incubation at 37 ℃ for 1 h was done by adding 300 ul sarcozyl 2% and 30 ul proteinase K (10 mg/ml). DNA were extracted and purified by phenol-chloroform-isoamylalcohol (25:24:1) and cold pure ethanol method.

PCR for detection of mecA gene was carried out by using primers as below: mecA-F: 5-'AAAATCGATGGTAAAGGTTGGC-3' and mecA-R: AGTTCTGCAGTACCGGATTTGC-3' (Cinnagen, Iran) for coding a 533 bp mecA. 50 ul PCR reaction mixture were made consist of 10 pmol of each primers, 200 uM Dntp (Roche-Germany), 2.5 ul (50 mM $mgcl_2$), 0.5 ul Taq pol (2.5 u) (Roche-Germany), 5 ul PCR buffer 10x (Roche-Germany) and 5 ul DNA-template. *S. aureus* COL strain was used as a positive control. Cycling condition performed in 40 cycles including, denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, extension at 72℃ for 1 min and final extension at 72℃ for 5 min (Nimmo, 2003; Louie, 2000). PCR products were visualized on 1.7% agarose gel with ethidium bromide dye under UV transilluminator. Amplicon of 533 bp were consistent with mecA gene amplification.

RESULTS

In this study 185 *S. aureus* were isolated from patients. Median age of patients were 29.2 ± 28 years (from less than one year to 84 years) that 96 (52%) of them were male. The clinical specimens, 57 (30.8%), 49 (26.5%), 42 (22.7%), 20 (10.8) and 17 (9.2%) of them were isolated from urine, wound, blood, sputum and other specimens such as abscess respectively.

Based on MIC value, 67(36.2%) of isolates were MRSA (MIC≥16 µg/ml). In 5 (2.7%) strains MIC≥256 µg/ml were seen with highly resistance to methicilin. In 82 (44.3%) MIC was between 4 and 8 µg/ml. Only 36 (19.5%) of isolates had MIC less than 2 µg/ml and showed full sensitivity to methicillin (Table 1).

We have found mecA gene in 65 (35.1%) isolate of *S. aureus* by PCR method (Figure 1). None of mecA containing isolates were shown MIC≤2 µg/ml and in 8 (6.7%) *S. aureus* isolates without mecA gene with MIC≥16 µg/ml were seen (Table 1).

Rate of MRSA strains isolation in wound and sputum were higher than other samples with 42.9% and 40% respectively (Figure 2).

According to the sex, rate of MRSA and MSSA strains among men and women were 37 (38.5%) and 30 (33.7%) respectively, showing significant relation (P<0.05). Frequency of antibiotic resistance in MRSA strains was significantly higher than MSSA strains (P<0.05). This high resistance was seen more in MRSA strains to Cephotaxim, Erythromycin, Cephtriaxon and Gentamycin in compare with MSSA isolates (Table 2). Our finding showed that multi drug resistant property among MRSA strains was high, so that highest level of resistance was observed with Penicillin, Ampicillin, Nalidixic Acid, Co-amoxyclave, Ceftriaxone and Erythromycin. In MSSA strains resistance to Penicillin, Ampicillin and Co-amoxyclave was high in comparison with other antibiotics (Table 2). All isolates were sensitive to vancomycin.

DISCUSSION

Our study demonstrated that 36.2% of *S. aureus* isolates were MRSA, this prevalence are similar to some other studies performed in various region in Iran such as Tehran as well as neighbor countries like Saudi Arabia and Kuwait, and also European country, France and American nation, Brazil (Fatholahzadeh, 2008; Mdani, 2001; Udo, 2008; Oteo, 2004). Instead of the abundance of MRSA isolates across the world are not the same; the most MRSA isolates have been reported from Taiwan, Canada and

Table 1. Comparison of MIC and PCR results in MRSA detection.

PCR result \ MIC	≤2 (%)	4-8 (%)	≥16 (%)	Total (%)
mecA+	0 (0)	6 (9.2)	59 (31.9)	65 (35.1)
mecA-	36 (30)	76 (63.3)	8 (6.7)	120 (64.9)
Total	36 (19.5)	82 (44.3)	67 (36.2)	185 (100)

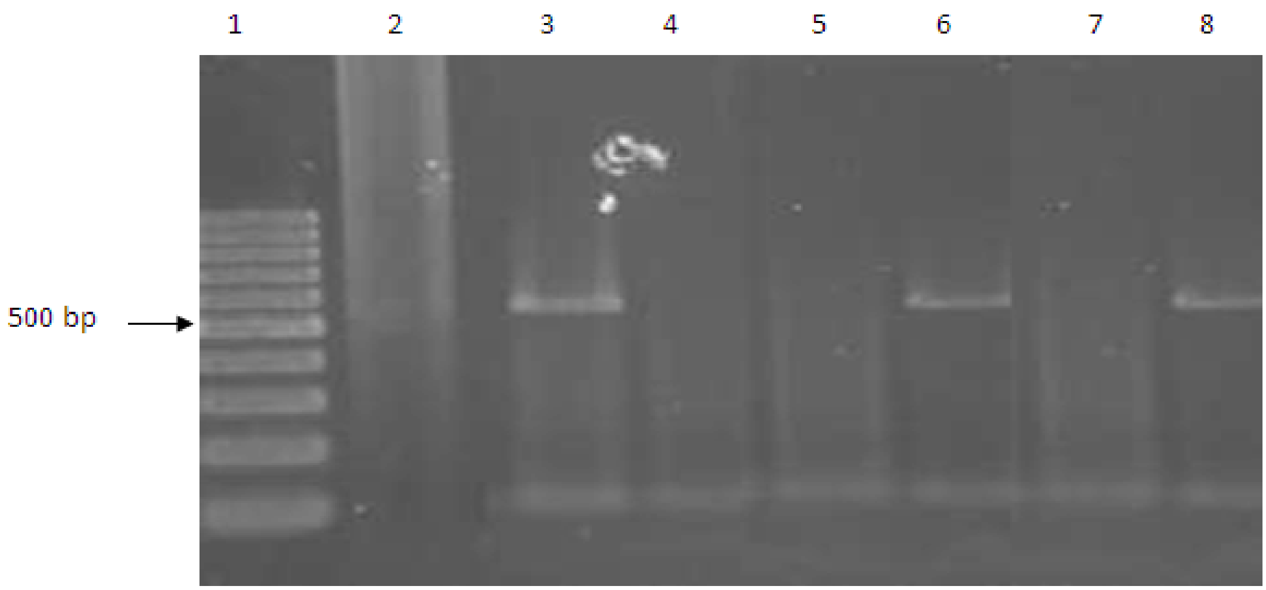

Figure 1. Gel image of representative PCR *mec*A gene products –line1-marker (100 bp), line 2 negative control, line 3 positive control (533 bp band) and line4 to 8 patient specimens.

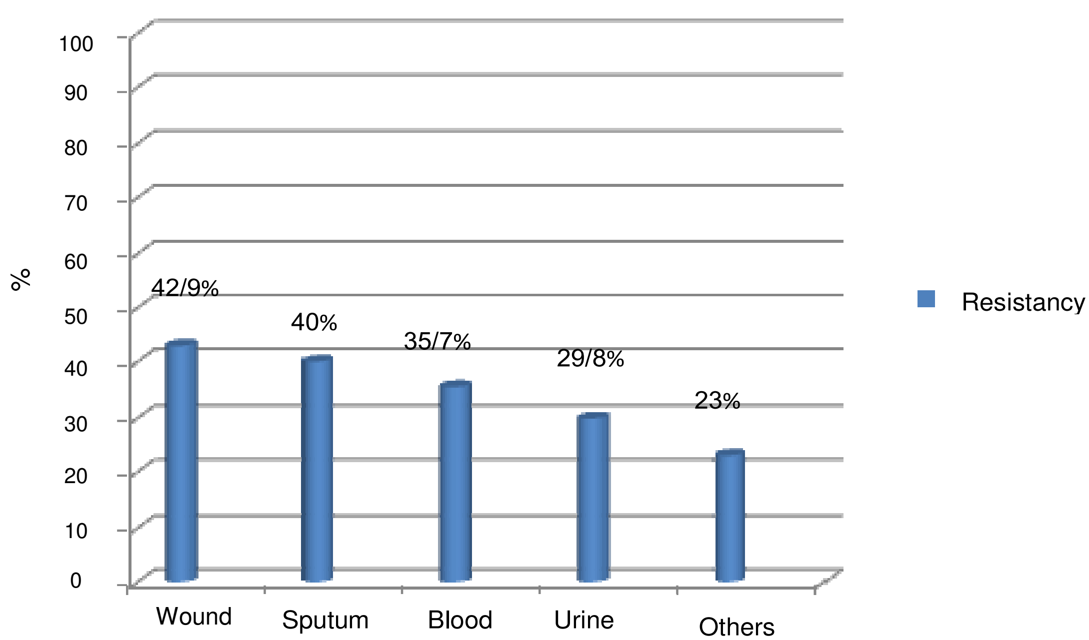

Figure 2. Distribution of mecA positive MRSA in clinical samples.

Table 2. Antibiotics resistance pattern in MSSA and MRSA.

Isolate / Antibiotic	MSSA (120)(65%)	MRSA (65)(35%)	Total (185)(100%)
Penicillin	115(95.6)	65(100)	180(97.3)
Ampicillin	115(95.6)	65(100)	180(97.3)
Co-amoxyclave	93(95.3)	63(97)	156(84.3)
Nalidixic Acid	40(33.3)	58(89.2)	98(53)
Cefotaxime	3(2.5)	48(73.8)	51(27.6)
Erythromycin	8(6.7)	46(70.8)	54(29.2)
Ceftriaxone	3(2.5)	44(67.7)	47(25.4)
Trimethoprime	16(13.3)	41(63.1)	57(30.8)
Ciprofloxacin	4(3.3)	38(58.5)	42(22.7)
Gentamicin	1(0.8)	37(56.9)	38(20.5)
Tetracycline	26(21.7)	32(49.2)	58(31.4)
Imipenem	1(0.8)	15(23.1)	16(8.6)
Chloramphenicol	8(6.7)	12(18.5)	20(16.7)
Vancomycin	0(0)	0(0)	0(0)

Australia as much as 66 to 77% (Fatholahzadeh, 2008; Hsueh, 2004; Merlino, 2002; Arbique, 2001). As our recent study showed that 24% of healthcare staff were *S. aureus* carriers. Of these 12.5% were MRSA showing 3% total prevalence of MRSA (unpublished). Also recent report about rate of MRSA in nosocomial infections in Isfahan, Iran showed that 67.2% of isolates were MRSA (Khorvash, 2008). Different epidemiological factors such as geographical, health system capability in running infection control program has role in variability of prevalence of MRSA. Most isolates of MRSA were observed in wound specimens (43%). Instead of many similar and independent studies that is not showing any relation between sex, age, site of infection and rate of MRSA. Only a few report has been shown increasing the rate of MRSA in elder people aged 64 and more significantly (Fatholahzadeh, 2008; Hsueh, 2004; Merlino, 2002; Arbique, 2001; Waness, 2010). It has been clear that age is a risk factor because of its role in long term hospitalization; lose of immunity and longer antibiotic therapy (Waness, 2010).

Multi drug resistance MRSA strains are made difficult treatment of MRSA infections. In our study 100% resistance to Penicillin, Co-amoxyclave and Ampicillin, and more than 70% resistance to Cefotaxime, Erythromycin, Ceftriaxone and Nalidixic Acid were showed. In compare, rate of MSSA sensitivity to other antibiotics was considerable. It is comparable with other study in our country showing such results (Fatholahzadeh, 2008; Khorvash, 2008).

We have found mecA gene in 65 (35.1%) isolates of *S. aureus* by PCR method. None of mecA containing isolates was shown MIC≤2 µg/ml whilst in 8 (6.7%) *S. aureus* isolates without mecA gene MIC≥16 µg/ml were seen (Table 1). We considered MIC as the reference or "gold standard" method for establishing the sensitivity and specificity of each of PCR technique studied. We have obtained specificity and sensitivity of 94.89 and 88% for PCR method respectively.

It is determined that 82 (44.3%) of isolates showed MIC between 4 and 8 µg/ml and 76 (63.3%) of them were mecA negative and categorized as border line in susceptibility to methicilin named as BORSA. High rate of BORSA strains is important in drug resistancy and it has been suggested that under pressure of antibiotics this isolates can probably shift to fully resistant MRSA (Mathews, 2010; Khorvash, 2008; Nelson, 2006).

In conclusion MRSA is spreading worldwide, growing epidemic and increasingly claiming victims. Accurate and early detection of these strains in hospitals and community is encouraged.

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