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Simultaneous Detection of Methicillin- and Mupirocin-Resistant genes in Staphylococcus aureus Isolated with Multiplex PCR from Patients in the City of Darab, Fars Province, Iran

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ABSTRACT

Background and Objectives: Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most important pathogens in hospitals. Hospital staffs can be one of the main sources of this organism in nosocomial infections. Identification and control of colonized health care workers can reduce the incidence of methicillin-resistant Staphylococcus aureus (MRSA). Mupirocin is a topical antibiotic and inhibits the activity of the most of gram-positive Cocci. Mupirocin resistance emerges in a short time after taking this antibiotic. The main goal of this study is to evaluate the prevalence of asymptomatic carriers of methicillin- and mupirocin-resistant Staphylococcus aureus by Multiplex PCR method among Fasa Hospital Staffs, Fars Province, Iran. Materials and Methods: In this study, 150 samples of Staphylococcus aureus isolated from the hospital staffs were analyzed and confirmed by biochemical and microbial methods. Disk diffusion method was used to determine the level of strains susceptibilities toward methicillin and mupirocin. Then the presence of mec-A, mup, nuc genes was examined using Multiplex PCR. 150 people were included in this study, including 120 women and 30 men. Results: Of the 150 studied people, 53 cases including 42 women and 11 men were Staphylococcus aureus carriers. Four individuals among 11 studied men were resistant to methicillin and none of them was resistant to mupirocin. A number of 11 cases among 42 studied women were resistant to methicillin and 2 women were resistant to mupirocin. Conclusion: Prevalence of methicillin- and mupirocin-resistant Staphylococcus aureus among Fasa Hospital Staffs was moderate and relatively low. Permanent control of carriers and their treatment can prevent spread of the bacteria and those infections caused by them.

Keywords: Prevalence of Staphylococcus aureus, MRSA, Mupirocin, Methicillin, PCR

INTRODUCTION

Over the past 40 years, MRSA infections have become endemic in hospitals of developed countries (e.g., the United States and European countries), as well as developing countries. Among two million nosocomial infections in the United States, 260,000 cases were caused by *Staphylococcus aureus*. Unfortunately, the percentage of methicillin-resistant strains is increasing, so that MRSA strains increased from 14.8% in 1987 to 39.7% in 1998 [20]. This problem has also arisen in Iran, so that a research showed that MRSA strains were included in 38.6% of isolated *Staphylococcus* from patients hospitalized in Dr. Shariati Hospital and in Children's Medical Center Hospital at Tehran [12]. *Staphylococcus* structure is similar to other gram-positive bacteria and includes cytoplasmic membrane, cell wall and extracellular materials, which plays an important role in connection of the bacterium with the host or the environment. Thickness of bacterial cell wall is in the range of 18-25 nm and a capsule or an extracellular slime layer made of carbon nitrate can be seen around some bacteria. *Staphylococcus aureus* cell wall is composed of three parts, including peptidoglycan, teichoic acid and protein A. The combination of these materials helps to diagnose *Staphylococcus* from *Micrococcus* and *Staphylococcus aureus* from *Staphylococcus epidermidis* [15]. Presence of coagulase is the basis of *Staphylococcus* classification. High prevalence of antibiotic resistance in *Staphylococcus aureus* and in other *Staphylococcus* strains is due to horizontal transfer of genes (antibiotic and metal resistance genes and pathogenesis-related genes). Recent studies show that the horizontal transfer of genes in

Staphylococcus is more than previously thought [2]. *Staphylococcus aureus* has gained and/or are becoming resistant to all types of applied and known antibiotics such as beta-lactams, glycopeptides, aminoglycosides, quinolones, etc [6]. MRSA was restricted to hospitals in the past and called hospital-acquired MRSA (HA-MRSA). By emergence of methicillin-resistant strains in societies, resistant to methicillin obtains much more importance. The above strains which are commonly called as community-associated MRSA (CA-MRSA) are resistant to methicillin, but their pharmaceutical sensitivity pattern toward other antibiotics other than beta-lactams, is similar to methicillin-sensitive *Staphylococcus aureus* (MSSA) [7]. CA-MRSA strains often create skin abscesses, furunculosis, severe necrotizing pneumonia and shock [8]. The study on prevalence of MRSA colonization in developing countries with no higher levels of health has great importance, since this bacterium creates skin and soft tissue infections [9]. Resistance to penicillin in *S. aureus* strains was reported by Rimmel and Kemp a year after its discovery [4]. The resistance was toward beta-lactamase, which its gene is encoded in a related plasmid. Mucosa of anterior part of the nose and the throat is the original place for establishment of organisms. Resistance in MRSA is due to protein PBP2A (penicillin binding protein 2A) encoded by mecA gene located on staphylococcal chromosome cassette (SCCmec). Different types of SCCmec cassette have been studied extensively by PCR techniques. Resistance mechanism includes alterations or defects caused by mutation on mecA gene that creates antibiotic resistance in organisms. These genes are highly resistant among *Staphylococcus aureus* strains (5). Mupirocin is a topical antibiotic that prevents from isolation of tRNAsynthetase and stops formation of isoleusin-tRNA ligase. Universal application of mupirocin is widely for elimination of *S. aureus* colonization and it is applied as a mean to prevent staphylococcal infections associated with health. Surgical area and blood vessels infections in hemodialysis patients with increased MRSA associated with mupirocin population are now used as carriers in prevention from common infections and in stopping spread of MRSA-related skin infections [19]. Precise tests for diagnosis of bacterium and methicillin resistance are faced with a problem due to non-uniform expression of mecA [8]. Mupirocin (pseudomonic acid A) is similar to isoleucine and by its application the amino acid activation is prevented by isoleucyltRNAsynthetase encoded by ileS gene and as a result protein synthesis is prevented in bacterium. This research was performed based on evaluation of phenotypic and genotypic mupirocin resistance and determination of ileS-1, mupA and mupB genes using PCR method. Finally, resistance to mupirocin in the strains of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* and coagulase-negative *Staphylococcus* was studied [10].

MATERIALS AND METHODS

In this study within 6 months, 150 staffs of Vali-e-Asr Hospital in the city of Fasa, Fars Province, Iran, were examined in terms of antibiotic resistance in *S. aureus*. Sampling from the cases was performed from anterior portion of the nose using a sterile swap. Each swap was placed in Tryptic Soy Broth Medium. The obtained samples were transported to laboratory less than 2 hours and were incubated for 48-72 hours at 37 ˚C on mannitol-salt agar medium. After growth of bacteria, they were identified using Gram staining, catalase test, coagulase test and DNase test.

Identification of Resistance to Methicillin and Mupirocin

Sensitivity test to antibiotics was performed by Kirby-Bauer diffusion method. Disks were placed with forceps on mannitol-salt agar medium and by distinct distance and they were incubated for 24 hours in 37 ˚C. The bacteria growth inhibition zone diameter was measured then, and they were classified into three categories of sensitive, semi-sensitive and resistant after comparison with the standard Table (1).

DNA Extraction by Boiling Method

After identification of *S. aureus*, DNAs of bacteria were extracted using boiling method. The method was easy, fast and cheap and all contents of cell including, DNA and plasmid were removed from it by bacterial lysis. Bacterial remnants deposited by centrifugation and DNAs remained in solution. The solution containing DNAs could be used for PCR reaction.

Molecular Methods

After identification of mupirocin- methicillin-resistant *Staphylococcus aureus* and using PCR reaction, set up steps were primarily done and optimization was performed. Then, nuc, mecA, mup genes were simultaneously reproduced using Multiplex PCR.

PCR products were evaluated and observed in this step. Agarose gel electrophoresis technique was used to evaluate the products. Position and quality of bands became observable and were evaluated by putting gel in UV illuminator device and by help of ultraviolet radiation at wavelength 254 nm. For further investigation on bands, gel images could be directly printed and/or recorded on floppy disks and be examined with Photoshop software. Thus, according to different patterns of antibiotic resistance in each target area of this study, evaluation of frequency of

asymptomatic carriers of mupirocin- and methicillin-resistant *Staphylococcus aureus* was performed among Fasa Hospital staffs by Multiplex PCR method.

RESULTS AND DISCUSSION

Resistant *S. aureus* stains were identified using the following method, Gram Staining, DNase, fermentation of carbohydrate (sugar) and mannitol, catalase test, coagulase test as well as biochemical methods and disk diffusion method. The study population was 150 cases including, 120 women and 30 men. Among this population, 53 cases (42 women and 11 men) were carriers of *S. aureus*. Among 11 men with positive *S. aureus*, 4 cases were resistant to methicillin and no one was resistant to mupirocin. Among 42 women with positive *S. aureus*, 11 and 2 cases were resistant to methicillin and mupirocin, respectively (statistical analysis using SPSS statistics).

- Binomial test showed that there was a significant difference at 1% level in total number of the samples between studied women and men (P-Value=0.000).
- Binomial test showed that there was a significant difference at 1% level in total number of the samples between *S. aureus* samples and the others (non-*S. aureus* samples)(P-Value=0.000).
- Binomial test showed that there was a significant difference at 1% level in *S. aureus* samples between studied women and men (P-Value=0.000).
- Pearson Chi-Square test showed that there was no significant difference in total number of the samples between gender of the carriers and *S. aureus* and non-*S. aureus* samples (P-Value=0.864).

Table 1. Standard Antibiograms

Antibiotic names	Sensitive	Semi-sensitive	Resistance
Methicillin	≥ 15	-	≤ 14
Penicillin	≥ 29	-	≤ 28
Ciprofloxacin	≥ 21	16-20	≤ 15
Tetracycline	≥ 19	15-20	≤ 14
Amoxicillin	≥ 29	-	≤ 28
Rifampin	≥ 20	17-19	≤ 16
Erythromycin	≥ 23	14-23	≤ 13
Mupirocin 5	≥ 27	7-26	≤ 6

Table 2. Relative frequency of staffs with positive *Staphylococcus aureus* on the basis of gender

Samples / Gender	*Staphylococcus aureus*	Others	Total
Women	42(28%)	78(52%)	120(80%)
Men	11(7.3%)	19(12.7%)	30(20%)
Total	53(35.3%)	97(64.7%)	150(100%)

It was found from Fisher's Exact Test that there was no significant difference in *S. aureus* positive samples between gender of the carriers and resistant type (P-Value=0.818).

Table 3. Frequency of methicillin- and mupirocin-resistant genes in *Staphylococcus aureus* based on gender

Gender of carriers X, resistance				
Samples / Gender	Methicillin	Mupirocin	Others	Total
Women	11 (20.8%)	2(3.8%)	29(54.7%)	42(79.2%)
Men	4(7.5%)	0(0%)	7(13.2%)	11(20.8%)
Total	15(28.3%)	2(3.8%)	36(67.9%)	53(100%)

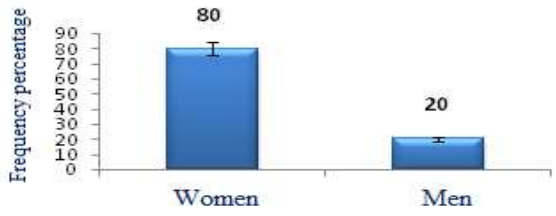

Chart 1. Frequency percentage of gender in the total number of the samples

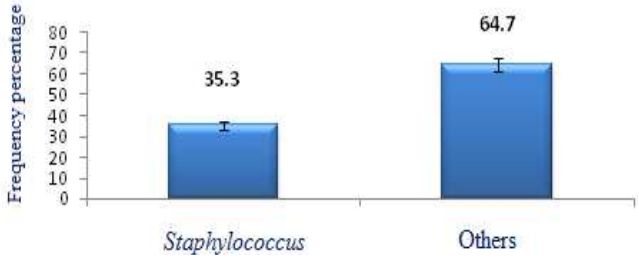

Chart 2. Frequency percentage of a sample in the total number of the samples

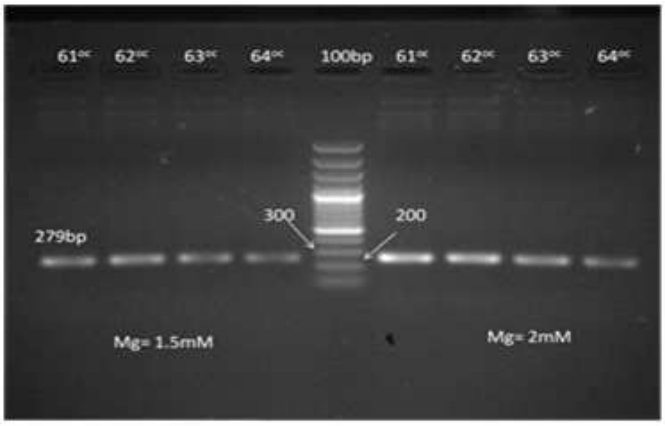

Figure 1. Electrophoresis results of set up PCR products for NUC

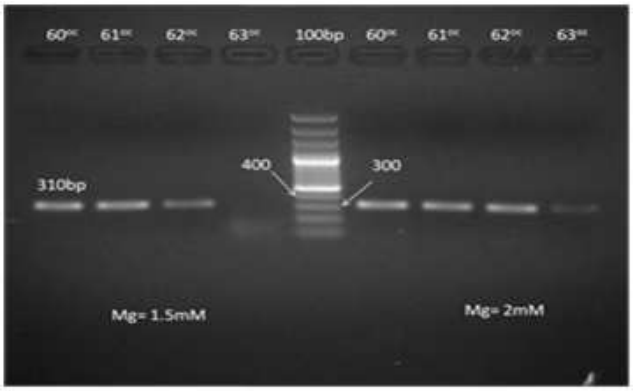

Figure 2. Electrophoresis results of set up PCR products for mecA

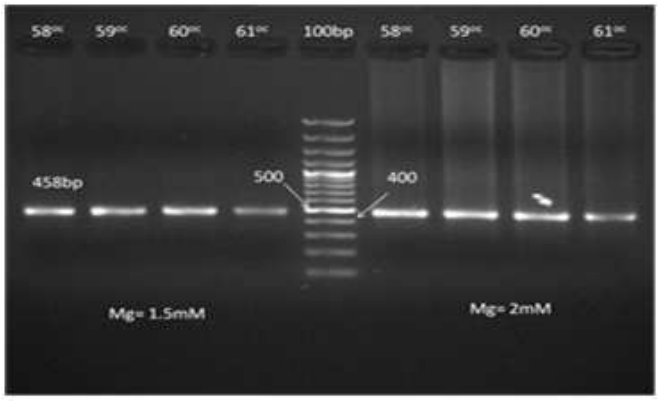

Figure 3. Electrophoresis results of set up PCR products for mup

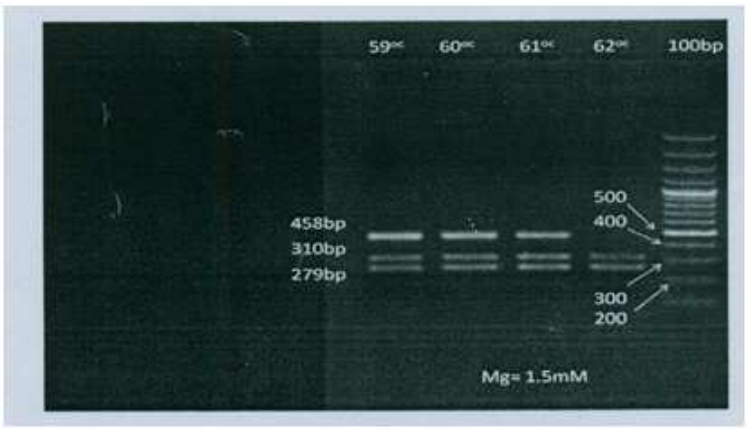

Figure 4. Electrophoresis results of set up Multiplex PCR products for all three genes

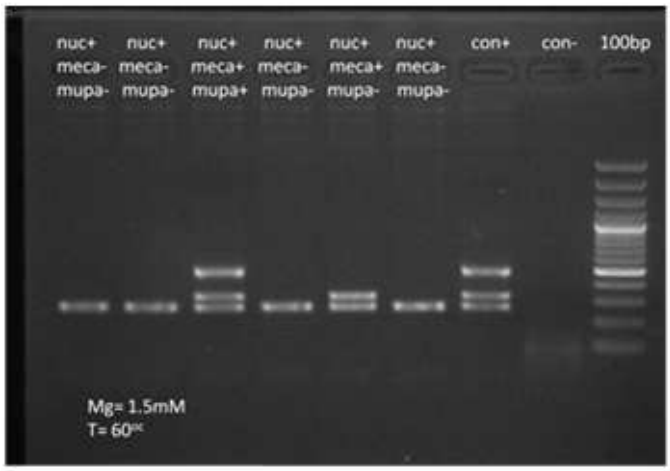

Figure 5. Electrophoresis results of Multiplex PCR products for all three genes and for all of the samples

Compared with HA-MRSA strains that are generally resistant to multiple drugs, CA-MRSA strains are sensitive to most antibiotics. For example, clindamycin, trimethoprim and sulfamethoxazole are medications used for treatment of CA-MRSA [11]. Methicillin-resistant factor in MRSA is mecA gene on bacterial chromosome and encodes a protein called PBP2a. This protein has no or little affinity to bind to methicillin. In methicillin-sensitive *Staphylococcus aureus* that lacking this gene, methicillin has more affinity to bind to PBP protein in bacterial cell wall, and this causes lysis of the wall [19]. Various studies have examined use of PCR technique to determine the prevalence of MRSA strains. In a study performed in UK, 39 (8.2%) isolated samples of MRSA were identified from a total number of 439 nasal swabs. The study pointed out that molecular identification, due to accuracy, speed and high sensitivity, had high value to identify MRSA strains [14]. In another study from Thailand, 100 isolated *S. aureus* obtained from nasal swaps were examined using phenotypic and molecular methods (PCR, mecA gene). This study noted that using phenotypic method, the possibility of separation of methicillin-sensitive strains which produce large amounts of penicillinase enzyme was less than MRSA strains and this group was considered as MRSA stains at first. So, it is necessary to examine the presence of mecA, as the indicator of being resistance, using molecular method [17]. A study conducted in Iran showed that 88% of clinical isolates of *S. aureus* are MRSAs. This study emphasized that the prevalence of MRSA in Iran is rising [16]. It is noted that heterogeneous expression of methicillin resistance among *S. aureus* strains can produce serious challenges for evaluation of resistance levels using phenotypic method [20, 21]. So, detection of the presence of mecA gene is considered as the gold standard [19]. Identifying the presence of mecA gene was also used in the present study as a confirmatory method and confirmed the results obtained from the phenotypic method. Despite a number of reports on prevalence of MRSA, few studies have been published in Iran about the prevalence of MRSA in kindergartens. In the only study performed in this field, the prevalence of MRSA in the noses of children in Hamedan Child Care Centers was reported equal to 4.1% by Sedighi et al, in 2011. The studies performed in Iran have indicated that the level of resistance in different parts of Iran including, Tehran, Hamedan, Shiraz, Babol and other places have had differences that are mentioned in the following: the prevalence of MRSA was reported 33.4% in a study conducted in Tehran in 2001. This amount was reported 39% in another study performed in Tehran in 2003. The prevalence of MRSA was reported 50% in a study conducted in Hamedan. The prevalence of MRSAs in another study carried out in Babol in

2006 was reported 42% and this amount was reported 47.6% in a study performed in 2007 in Tehran. All these results imply that the prevalence of MRSA stains is also increasing in Iran over time [1]. Several studies on mupirocin resistance in methicillin-resistant *Staphylococcus aureus*, methicillin-sensitive *Staphylococcus aureus* as well as coagulase-negative *staphylococci* were done in all around the world and Iran. Due to availability of mupirocin since 1986, steady increase in the level of resistance to it has been observed since 1990 [24]. Among 11 resistance strains identified by phenotypic method in a study performed by Hesami, ileS-1 was detected only in one strain with low resistance using E-test. MupA gene was recognized in 4 isolated strains with high resistance and this indicated the presence of plasmid containing mupA in these resistant strains. In the study performed by Yun, agar micro-dilution, PCR, and strains sequencing methods were used respectively to determine minimum inhibitory concentration, mupA gene and resistance to mupirocin and succeeded [23]. Unlike the study of Seah et al. no strain was observed in our study with very high resistance that can encode mupB plasmid gene [22]. Three groups of strains were also evaluated in 2013 by Jayakumar et al. Among 150 studied strains in this study, 3.3% of mupirocin-resistant strains were detected. In a study performed by Mohajeri in 2012, *Staphylococcus* strains were evaluated for resistance to mupirocin, and no resistance was detected. So, the antibiotic, due to lack of resistance in Kermanshah similar to Arak, had the ability to be used [13]. Several factors can influence on the prevalence of mupirocin-resistant *Staphylococcus* in different countries, including Iran. The factors include various policies on infection control programs, quality and level of antibiotic prescription, population, dominant strain type, and type of laboratory methodologies for detection of mupirocin-resistant *Staphylococcus*. According to increased resistance to mupirocin in central area of Iran, particularly with regard to a study performed by Saderi et al. in 2009 in Tehran, mupirocin resistance increased from 5% to 7.3% [18].

In summary, given effective role of mupirocin on most gram-positive *Cocci*, the antibiotic is of great importance in treatment of patients and carriers. So, evaluation of resistance on them could be an effective way to select an appropriate treatment for these types of infections [10].

CONCLUSION

Investigation of antibiotic resistance helps us to prevent spread of resistant strains and/or transfer of resistance to sensitive strains in MRSA samples. Awareness about frequency of clinical strains in patients, samples of medical personnel in hospitals and samples of healthy subjects provide the possibility to perform treatment regimen containing suitable MRSA strains to treat the patients and to control nosocomial infections. So, the results of the present study can be used by physicians to select appropriate measures for treatment and control of nosocomial infections with MRSA strains. The results can enhance the basic knowledge in the fields of microbiology and epidemiology of infectious diseases in the region and in the city of Fasa.

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