

Distribution Patterns of Methicillin Resistance Genes (*mecA*) in *Staphylococcus aureus* Isolated from Clinical Specimens

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ABSTRACT

There is a growing concern about the application of molecular methods in epidemiological studies of infectious diseases. The objective of this study was to determine the genetic stability of methicillin resistance genes in *Staphylococcus aureus* for the evaluation of resistance strain distribution. One hundred and fifteen *S. aureus* isolates from patients with staphylococcal infection were collected. The isolates were screened for methicillin resistance by minimum inhibitory concentration (MIC) examination. The stability of methicillin resistance genes was examined by physical curing and PCR screening methods. The results showed that methicillin resistance *Staphylococcus aureus* (MRSA) had risen up to 43% in Nemazi Hospital (Shiraz, Iran). Indeed, the incidence of MRSA in our hospital was 10% during the last four years. The ability to lose (curability) of methicillin resistance genes (*mecA*) was examined by physical curing method in 49 isolates with $\text{MIC} \geq 16 \mu\text{g ml}^{-1}$. No sign of curability of *mecA* gene was observed where 500 colonies from each strain have been studied and exhibited by the same MIC values before and after curing test. Positive PCR results for isolates with $\text{MIC} \geq 16 \mu\text{g ml}^{-1}$ before and after curing experiment have been achieved. These data confirm the results of curing method, indicating that stable genetic determinants confer methicillin resistance. These results support the hypothesis that resistance isolates may be selected due to clonal selection under antibiotic pressure used in clinics rather than transmission of mobile genetic determinant. The high prevalence of MRSA immerged in our Hospital could be originated due to antibiotic pressure and poor control measures.

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INTRODUCTION

Staphylococcus aureus is a major pathogen within hospital and in the community. Treatment of *S. aureus* before 1950s involved in the administration of penicillin G, however, by the late 1950s, *S. aureus* strains resistant to penicillin G were causing increasing concern [1]. Resistant strains typically produce an enzyme, called β -lactamase, which inactivates the β -lactam antibiotics. Efforts were made to synthesize penicillin derivatives that were resistant to β -lactamase hydrolysis. This was achieved with the synthesis of methicillin in 1959.

Unfortunately, as soon as methicillin was

used clinically, methicillin resistant *S. aureus* (MRSA) strains were isolated [2, 3]. The resistance was not due to β -lactamase production but due to the expression of additional penicillin-binding protein (PBP2a), acquired from another species [4]. The available data show that the structural gene of this PBP (*mecA*) is present in resistant strains but not in the susceptible ones [5]. In methicillin resistant cells, PBP2a with its low affinity for binding β -lactam antibiotics, can be substituted for essential functions of high-affinity PBP at concentrations of antibiotics that are otherwise lethal [6].

The origins of *mecA* are obscure. A *mecA* homolog with 88% amino acid similar to *mecA* of

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methicillin-resistant staphylococci has been identified in *Staphylococcus sciuri* [7]. These and other data support the fact that *mecA* originated in a coagulase negative staphylococcus species, possibly a close evolutionary relative of *S. sciuri* [8, 9]. All MRSA are clonal descendants from the few ancestral strains that acquired *mecA* [10]. How *mecA* was acquired by methicillin-resistant staphylococci is not known, but transposition is a plausible mechanism. The presence of one or more copies of the IS257 (Insertion sequences), a putative mobile element, is often associated with genes encoding a variety of resistance genetic determinants [11]. Within *mec*, inverted repeats of the end of *mec*, and identification of two open reading frames within *mec* that may encode recombinases, all suggest that *mecA* and its associated DNA are mobile elements [12]. Furthermore, recent studies of methicillin-sensitive *S. aureus* (MSSA) and MRSA isolates collected over many years and analyzed by multilocus sequence typing (MLST) and Staphylococcal cassette chromosome *mec* (SCC*mec*) typing revealed that the evolution and spread of MRSA occurred due to the introduction of SCC*mec* element into several different clonals of MSSA [13]. Therefore, if resistant isolates spread from small number of MRSA clone, it could be indistinguishable by MLST [13].

Although MRSA originated from mobile elements, it is not known if distribution of MRSA in the hospitals and communities occur due to the transfer of resistant determinants or as a consequence of clonal selection of resistant strains by antibiotics pressure. The possibility of antibiotic resistance genes being transferable can be assessed by physical or chemicals curing methods. This means treatment of bacteria with certain chemical agents such as ethidium bromide or acridine orange or incubation at high temperature can cause elimination of transferable elements [14-16].

MATERIALS AND METHODS

Collection of specimens and isolated MRSA. One hundred and fifteen *S. aureus* isolates were obtained from different Wards in Nemazi Hospital in Shiraz (Iran). The isolates were identified as *S. aureus* by microscopic morphology observation, gram stain, catalase test and coagulase activity.

Susceptibility to methicillin. An agar screen plate was supplemented with 4% NaCl and 6 μ g methicillin ml^{-1} was used for gross screening of resistant isolates from sensitive ones. Methicillin (Sigma, St. Louis, USA) MIC were determined by agar dilution plates as recommended by NCCLS [17]. ATCC 25923 (American Typing Culture Collection) was used as an antibiotic sensitive control for MIC determination and curing study and ATCC 51153 was used as a control positive for PCR performance. After curing experiment, methicillin MIC tests for resistant isolates have been retested.

Curing experiment. Based on MIC determination, all the isolates with $\text{MIC} \geq 16 \mu\text{g ml}^{-1}$ were selected for the curing method assessment. Briefly, Muller-Hinton Broth (MHB) supplemented with 4% NaCl was used for growing bacteria and the inocula were incubated at 43°C over night [16]. This mid log growth suspension was then serially diluted in sterile PBS and plated out on Muller-Hinton Agar (MHA) with 4% NaCl. The agar plates containing single colonies were then stabbed into MHA containing 6 $\mu\text{g ml}^{-1}$ methicillin and MHA not supplemented with methicillin. For each isolate, 500 colonies have been screened using a sterile toothpick for each colony.

Detection of *mecA* genes by PCR. All methicillin resistant isolates were examined for *mecA* genes existence by total DNA extraction and PCR performance. Briefly, the isolates were swabbed on Trypticase Soy Agar (TSA), while the surface of agar medium was covered with standard vancomycin discs and incubated over night. The bacterial colonies from the edges of inhibition zone were then resuspended in sterile distilled water and matched to 0.5 MacFarland standard (approximately 10^8 cfu/ml). The bacterial suspension was heated at 95°C for 15 minutes and cooled in room temperature. The cured lysis (2.5 μl) was used as a DNA template for all isolates when PCR tests were carried out. To detect methicillin resistance genes, the 533-bp band (Fig. 1) from *mecA* has been amplified using two specific primers as described previously [18]. The *mecA* gene stability was then retested by PCR after curing experiment.

Table 1. Frequency of methicillin MIC for 115 *S. aureus* isolates.

MIC $\mu\text{g ml}^{-1}$	0.25	0.5	1	2.0	4.0	8.0	16.0	32.0	64.0	128.0	256.0	>256
Number	4.00	8.0	15	14.0	11.0	14.0	6.0	5.0	3.0	6.0	7.0	22
Percent	3.50	7.0	13	12.2	9.6	12.2	5.3	4.3	2.6	5.3	6.1	19
Total	MSSA*			BL†						MRSA‡		
Number/percent	27/23.5			39/34.1						49/42.4		
MIC range	MIC = 0.25-1 $\mu\text{g ml}^{-1}$			MIC = 2-8 $\mu\text{g ml}^{-1}$						MIC = 16->256 $\mu\text{g ml}^{-1}$		

*MSSA, methicillin sensitive *S. aureus*; †BL, borderline resistant of *S. aureus* (intermediate ranges); ‡MRSA, methicillin resistant *S. aureus*.

RESULTS

MIC values of one hundred and fifteen staphylococcal isolates to methicillin are shown in Table 1. Altogether, 27 isolates (23.5%) were methicillin sensitive (MSSS) but 39 (34.1%) which had MIC of between 2 to 8 $\mu\text{g ml}^{-1}$, were designated as borderline (BL), whereas 49 (42.4%) having MIC \geq 16 were classified as MRSA (Table 1). Data analysis based on the sites of infection showed that of 33 (28.7%) samples were taken from blood. The following samples included urine, nose discharge, sore bed, sputum, abscess, skin lesion, eye discharge, pleural fluid, bone, throat and intratracheal tube (ITT) had sequential ranks but joint fluid and cerebrospinal fluid (CSF) specimens showed lowest percent (Table 2). Distribution of staphylococcal aureus infection in different hospital wards (Table 3) showed that internal medicine ward with 41 isolates had the highest prevalence, while surgery ward with 23 isolates (20%), pediatrics wards with 19 isolates (16.5%), pediatrics ICU ward

Table 2. Distribution of *S. aureus* isolated from patients based on sites of sampling.

Site of sampling	Number	Percent
Blood	33	28.7
Urine	13	11.3
Nose discharge	12	10.4
Sore bed	11	9.7
Sputum	9	7.8
Abscess	7	6.1
Skin lesion	6	5.3
Eye discharge	5	4.3
Pleural fluid	5	4.3
Bone	4	3.5
Throat and mouth	3	2.6
ITT*	3	2.6
Joint fluid	2	1.7
CSF	2	1.7
Total	115	100.0

*ITT, Intratracheal tube.

with 14 isolates (12.2%), newborn ward with 12 isolates (10.4%) and adult ICU ward with 6 isolates (5.2%) had sequential ranks, respectively.

Table 3. Frequency of *S. aureus* infection based on hospital ward distribution.

Wards	Number	Percent
Internal medicine	41	35.7
Surgery	23	20.0
Pediatrics	19	16.5
Pediatrics ICU	14	12.2
New born	12	10.4
Adult ICU	6	5.2
Total	115	100.0

Genetic stability of *mecA* genes in 49 isolates with MIC \geq 16 has been examined. Five hundred colonies from each isolate have been screened by physical curing method. All of these isolates showed *mecA* genes stability when stabbed into MHA containing 6 $\mu\text{g ml}^{-1}$ methicillin. The existence of *mecA* genes in all 49 methicillin resistant isolates were observed by PCR performance and MIC determination. Repetition of both of these examinations generates the same results after curing test. There was a correlation between the sharpness of PCR bands and MIC values (Fig. 1).

DISCUSSION

MRSA infection has recently become a serious problem in anti-microbial chemotherapy. This is why during the past four decades, MRSA has spread throughout the world and has become highly endemic in many geographical areas. It has been suggested that due to the changing pattern of antibiotic resistance in *S. aureus*, it would be wise to have a periodical surveillance of these changes once every 3 to 4 years [19].

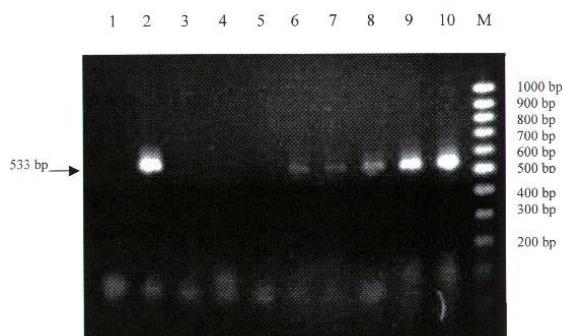


Fig. 1. Correlation between sharpness of PCR bands and MIC values. Line 1, control negative PCR (no DNA template); line 2, PCR from control positive (ATCC 51153); Lines 3, 4, 5, 6, 7, 8, 9 and 10, PCR from isolates with MIC 2, 4, 8, 16, 32, 64, 128 and 256 $\mu\text{g ml}^{-1}$, respectively; and M, 100 bp marker.

The stability of *meca* genes has been confirmed in all clinically isolated strains with $\text{MIC} \geq 16 \mu\text{g ml}^{-1}$. These results indicate that resistant determinants are located in non-transferable DNA. If resistant genes are located in non-conjugative plasmids or chromosome, one would expect their genetic stability [20], however there are more supporting evidence that *meca* genes are located in chromosome [21, 22]. Nevertheless, if resistant genes are recently integrated into the chromosome as a result of transposition or plasmid insertion, their genetic instability could be possible but at low frequency [23].

Despite the fact that existence of *meca* genes was observed in all resistant strains before and after curing test by positive PCR results and MIC determination, the majority of isolates showed $\text{MIC} < 8 \mu\text{g ml}^{-1}$ (57%). One possibility for this finding could be that *meca* genes are present in these low MIC isolates but unable to express themselves. It is known that a variety of genes regulatory elements such as *fem* factors (factor essential for methicillin resistance), *mecI* and *mecR1* regulatory genes are involved in *meca* expression and depression [24, 25]. Nevertheless, none of the isolates with $\text{MIC} < 8 \mu\text{g ml}^{-1}$ showed PCR positive results. Therefore, isolates with MIC between 4 to 8 $\mu\text{g ml}^{-1}$ (BL resistant) probably did not actually harbor *meca* genes. Indeed, these isolates may contain β -lactamase genes which gradually hydrolyze methicillin [19].

Alternatively, these strains may lack *meca* genes but contain normal penicillin-binding proteins with modified penicillin capacity [26]. Another

possibility could be the mutation that occurs in sequence of *meca* genes, which is amplified by PCR. Our PCR results showed direct correlation between the sharpness of PCR bands and MIC values (Fig. 1). Although we have not carried out this test, but it can be conducted to detect the number of copies of *meca* genes in the chromosome quantitatively. This means isolates with $\text{MIC} \geq 256 \mu\text{g ml}^{-1}$ harbor a high copy number of *meca* genes and regulatory elements. Nevertheless, for all isolates we used same amount of templates DNA with similar quantitative concentrations. Therefore, the sharpness of PCR bands in samples with the higher MIC values could be due to the availability of sites for DNA amplification rather than fluctuation of template DNA concentration.

Data analysis shows a 10% increase in MRSA incidence (from 33% to 43%) from the last survey compared to the present study. It has been proposed that extensive methicillin usage may select pure homogeneous MRSA rather than heterogeneous resistant isolates. This phenomenon has also been observed experimentally when homogeneous isolates at methicillin concentration of 50 to 100 $\mu\text{g ml}^{-1}$ have been isolated [27]. MIC comparison of our clinical isolates confirms this hypothesis because 22 (19%) out of all the isolates exhibit $\text{MIC} > 256 \mu\text{g ml}^{-1}$. Furthermore, a steady increase in the incidence of MRSA that has periodically been observed, implies that resistant isolates were selected due to antibiotic pressure. The clinical application for this observation could be suggested to the clinician to use an alternative drug of choice to reduce selective pressure upon methicillin resistant strains. It has been documented that prescription of alternative antibiotics for patients infected with gentamicin-resistant MRSA leads to the reemergence of gentamicin-susceptible MRSA strains [28].

Data analysis also showed that 40% of *S. aureus* was isolated from blood and urine specimens (Table 2). The administration of β -lactams antibiotics in hospitalized patients may more penetrate into body fluids such as blood or urine. The high concentration of antibiotic in body fluids can be facilitate selection of methicillin resistant isolates. A recent study from France revealed that substantial portion of MRSA patients who had antibiotic-associated diarrhea and whose gastrointestinal tract was heavily colonized with MRSA had an accompanying bactemia by the same strain [29]. Our data also showed that 56% of specimens containing *S. aureus*

has been isolated from internal medicine and surgery wards. This could be happened since these two wards are patients crowded. Furthermore, administration of different β -lactam antibiotics, which were used for prophylactic and treatment purposes, could be responsible for the emergence of high percent resistant isolates.

If resistance gene transfer occurs by mobile genetic elements, one would expect that antibiotic resistant isolates emerge at upper ranges of MIC in a limited period with the high frequency while, the intermediate ranges of MIC occurs at low frequency [30]. However, resistance genes may have been acquired some time in the past and these genes gradually change into untransferable elements [8-10]. As a consequence, these genes are considered as stable genetic determinants. This possibility can be examined by sequence of resistant genes to find out any clues for transferable sequences such as direct or indirect repeated sequences flanking of the *mecA* genes [12]. The existence of these sequences can prove that resistant genes originate from transferable elements (transposon or integration of plasmid into chromosome) [23]. Nevertheless, MLST typing revealed that spread of MRSA in European hospitals has originated from small number of clones [16]. Therefore, in addition to antibiotic pressure which select resistant strains, other factors are taken into account. These factors are: routine screening of patients and personnel for carrying MRSA, wearing a mask and gown and gloves whenever entering the room of patients with MRSA and washing or disinfecting hands after removing the gloves [31]. Surprisingly, in our hospital, such aggressive control measures and routine screening of patients and personnel to detect MRSA have not been carried out so far.

These findings support the hypothesis that antibiotic pressure and lack of standard control measures could be responsible for the high prevalence of MRSA observed in our hospital as compared to very low prevalence of MRSA in northern and western European countries [31, 32]. But, the prevalence of MRSA is similar to or even lower than southern European countries [31]. Nevertheless, the factors most frequency mentioned for this phenomenon vary between countries regarding antibiotic use and control measures.

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