*European Journal of Epidemiology* **18**: 139–145, 2003.


# Distribution of *mecA* among methicillin-resistant clinical staphylococcal strains isolated at hospitals in Naples, Italy

E. Galdiero[1], G. Liguori[2], M. D'Isanto[3], N. Damiano[3] & L. Sommese[3]

[1]*Dipartimento di Fisiologia Generale ed Ambientale, Sezione Igiene e Microbiologia, Facoltà di Scienze Biologiche, Università Federico II;* [2]*Dipartimento di Medicina Pubblica, Clinica E Preventiva, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli;* [3]*Dipartimento di Medicina Sperimentale – Sezione Microbiologia, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli, Italy*

Accepted in revised form 18 April 2002

**Abstract.** Two hundred and twenty strains of *Staphylococcus* isolated in Naples, Italy, were surveyed for the distribution of the *mecA*, the structural gene for penicillin-binding protein 2a, which is the genetic determinant for methicillin-resistance in staphylococci. Screening by a cloned *mecA*, revealed that of 220 strains, 43 were methicillin-resistant (19.5%) and 177 were methicillin-susceptible (80.5%). Among the 43 resistant strains 23 (53.5%) carried mecA in their genome and 20 (46.5%) did not carry mecA, in spite of their resistance to methicillin. Every group was submitted to the AP-PCR profiling. A quantitative analysis of the patterns divided strains into four different clusters for methicillin-resistant mecA-negative and two different clusters for methicillin-resistant mecA-positive with primer 1, while no clusters were noted with primer 7. We conclude that these clinical isolates from our area, were not found to belong to a single clone, although the predominance of four methicillin-resistant mecA-negative genotypes were noted.

**Key words:** *mecA*, Methicillin-resistant staphylococci, Molecular epidemiolology

## Introduction

*Staphylococcus aureus* (*S. aureus)* is a major cause of nosocomial infection [1], and produces numerous and serious infections in humans. Bacteriophage typing is the method of choice for typing *S. aureus* [2]. Prompt and accurate identification of the staphylococci species is crucial because control methods vary depending on the identity of the epidemic strains. Some isolates are now non-typable, or type only with 100× routine test dilution (RTD) of phage. Alternatives have therefore been proposed based on molecular methods [3, 4]. Therefore, a rapid and sensitive method such as PCR, appears to be a specific assay to detect staphylococci.

In particular, increased frequencies of methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) and methicillin-resistant coagulase-negative (CNS) (MR-CNS) infections are associated with a high mortality and morbidity [1, 5, 6]. The increasing resistance of staphylococci to β-lactam antibiotics has become a major clinical problem. In staphylococcal species, resistance to methicillin and other β-lactam antibiotics is primarily mediated by the overproduction of a penicillin-binding protein 2a (PBP2a), an additional altered penicillin-binding protein with extremely low affinities for β-lactam antibiotics [7]. The *mecA*, encoding PBP2a, is highly conserved in the methicillin-resistant species, but is absent from susceptible strains [8, 9]. Thus, it is a useful molecular marker for β-lactam resistance in all staphylococci. Other chromosomally determined factors such as the *femA–femB* operon that act as regulator genes, are essential for the expression of methicillin-resistance in *S. aureus* [3, 10, 11]. However, the mechanism of *mecA* and *femA* cooperation is not well understood.

Because, many environmental factors such as inoculum size, incubation time, temperature, pH, salt concentration of the medium and exposure to β-lactam antibiotics influence the phenotypic expression of resistance [12–16], it is difficult to classify clinical isolates by standard methods. Moreover, there is often a category of isolates that many investigators define within a category of borderline or low level resistance. This implies a mechanism other than PBP2a [11, 14, 17–19].

In this study, a molecular epidemiological survey was undertaken with the purpose of estimating the percentages of methicillin-resistant staphylococci isolated in our area and to determine the possible prevalence and the clonal relatedness. We compared phenotypic and genotypic resistance by PCR assay using primer sets specific for the *mecA*, the structural determinant encoding PBP2a. Four categories were identified, two of methicillin-resistant isolates both *S. aureus* (MRSA) and CNS (MR-CNS) and two categories of methicillin-susceptible isolates both *S. aureus* (MSSA) and CNS (MS-CNS). The four

categories were studied to determine how *mecA* is distributed among clinical staphylococcal isolates. The methicillin-resistant strains (MR) were tested by arbitrary amplifying variable regions in the bacterial genome (AP-PCR) to see whether some isolate-specific DNA fingerprints could be obtained in a rapid and reproducible manner and to investigate the genetic relationship among resistant clinical isolates.

## Methods

### *Strains and culture conditions*

From 1998 to 1999 a total of 220 strains of staphylococci were isolated from hospitalized patients on the Medical Wards at the Faculty of Medicine, Seconda Università degli Studi di Napoli, Italy. All of the clinical isolates were obtained from several sites (blood, abscess, urine and tracheal culture). There was no duplication of patients from whom the strains were isolated.

Single colonies of isolated strains were cultured on to 5% sheep blood agar plates, incubated at 37 °C for 18–24 hour and were identified as *Staphylococcus aureus* or coagulase-negative staphylococci by colony morphology, Gram stain characteristics, catalase-test, coagulase-test, and latex slide agglutination test (Staphytect plus, Oxoid SpA, Italy). Bacteria were maintained in Trypticase Soy Broth (Oxoid SpA, Italy) to which 15% glycerol was added, at −80 °C.

### *Antibiotics and susceptibility tests*

Screening for methicillin resistance was performed by agar disk diffusion [20] testing with 5 μg of methicillin per disk (Oxoid SpA, Italy). The zones of inhibition were determined after 24 hour incubation at 35 °C.

Methicillin resistance was defined according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) [21]. MICs were determined by the plate dilution method with Mueller–Hinton agar containing 2% NaCl with an inoculation size of $10^4$ c.f.u. of bacteria. Growth of the cells was evaluated after incubation for 25 hour at 32 °C.

### *Genomic DNA isolation*

Single colonies of isolates were cultured in Brain Heart Infusion broth for 16 hour at 37 °C. An aliquot (0.1 ml) of overnight culture ($10^8$ c.f.u.) was pelleted by centrifugation, and resuspended in 300 μl of lysis buffer [50 mM Tris-HCl pH 8.0, 100 mM EDTA, 150 mM NaCl, 1% (vol/vol) sodium dodecyl sulfate] containing 100 μg of lysostaphin and 100 μg of RNase and incubated at 37 °C for 30 min. After adding 200 μg proteinase K at 37 °C for 30 min, samples were treated with 1 volume of phenol–chloroform–isoamyl alcohol (25:24:1) and then 1 volume of chloroform–isoamyl alcohol (24:1) prior to precipitation of the aqueous phase in 2 volume of 95% ethanol with 0.2 M NaCl for 1 hour at −20 °C. DNA was pelleted by centrifugation, washed with 80% ethanol, air dried, and resuspended in 200 μl of distilled water. The DNA concentration was determined by spectrophotometry at 260 nm; stock solutions of bacterial DNA were adjusted to a concentration of 5 ng/μl and stored at −20 °C [22].

### *PCR*

The DNA sequences of the primers used to amplify *mecA* (310 bp) of the MR staphylococcal DNA were M1 (885-5′TGGCTATCGTGTCACAATCG3′-904), and M2 (1194-5′CTGGAACTTGTTGAGCAGA-G3′-1175) [9]. Ten microliters of DNA samples were added to 90 μl of PCR mixture consisting of 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.25 mM (each) dNTPs, 100 pmol of each primer, and 1.25 U DNA Taq polymerase (Perkin-Elmer). DNA amplification was carried out for 34 cycles as follows: denaturation at 92 °C for 1 min, annealing at 56 °C for 1 min, DNA extension at 72 °C for 2 min. The reaction was achieved with a final extension at 72 °C for 3 min. For the identification of *mecA* positive strains, a discrete DNA fragment, the *mecA* specific product of 310 bp, was amplified. Each reaction series included a positive control (MR *S. aureus* strain ATCC 43,300), a negative control (*S. aureus* strain ATCC 29,213), a lysis solution blank and a water blank.

Ten microliters of PCR products were analyzed on a 2% agarose gel electrophoresis. Amplified, ethidium bromide-stained DNA fragments were then visualized on a UV transilluminator at 300 nm with standard XIV Marker (Boehringer Mannheim Meylan, France) as molecular weight markers.

### *Southern blot analysis of PCR products*

The probe for Southern hybridization (5′TGCTAA-AAGTTCAAAAGAGTAT) was a probe complementary to 21-mer oligonucleotides of the *mecA* used for the PCR. PCR products separated on a 2% agarose gel were transferred to a Hybond-N membrane (Amersham International plc, England) under alkaline conditions as recommended by the manufacturer [23].

After being baked at 80 °C for 15 min, the membrane was prehybridized at 42 °C for 4 hour in 10 ml of hybridization solution consisting of 3× SSPE (0.54 M NaCl, 0.03 M sodium phosphate, 0.003 M EDTA pH 7.7), 5× Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.2 mM Tris-HCl (pH 8), 0.5% SDS, and 30% formamide and hybridized at 42 °C for 18 hour in 10 ml of hybridization solution with 10 pmol of the probe, which was labelled by using T4 polynucleotide kinase with [$\gamma$ $^{32}$P] ATP. The membrane was

washed three times in 200 ml of 2× SSPE and 0.1% SDS at 42 °C for 20 min and exposed to New RX film to detect hybridization.

*DNA amplification fingerprinting (AP-PCR)*

In order to assess the genetic relationship among MR found with the first PCR, all the 43 MR were subjected to fingerprinting with two oligonucleotide: primer 1 (5′GGTTGGGTGAGAA3′) and primer 7 (5′GTGGATGCGA3′) [24, 25]. The PCR reagents mixture consisted of: 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM of each dNTP, 100 pmol of each primer, 1.5 U Taq polymerase. Cycling was performed in Perkin-Elmer 9600 machine and consisted of the following steps: predenaturation at 94 °C for 4 min followed by 35 cycles of 1 min at 94 °C, 1 min at 25 °C, and 2 min at 74 °C. The reaction was achvied with a final extension at 72 °C for 4 min. Amplification products were separated by electrophoresis in 5 mm-thick 1.5% agarose gel.

Gels were run in 0.5× Tris-Borate-EDTA (TBE) at a constant current of 100 mA for about 3 hours. Gels were stained after electrophoresis by adding of 10 μl of ethidium bromide (10 mg/ml) to a total volume of 300 ml of 0.5× TBE.

*Computer-assisted analysis of the DNA banding patterns*

The AP-PCR types were analyzed using the Windows version of the Sigma Gel software version 5.0. The DNA fragments in the molecular size range of 400–550 bp were explored. Comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages and the Jaccard similarity coefficient applied to peaks (presence of band = 1; absence of a band = 0), was constructed. A tolerance of 1.5% in band position was applied during comparison of the DNA patterns. Identical DNA types were arbitrarily defined as those with AP-PCR homologies higher than 95%.

**Table 1.** Disk susceptibilities of 220 staphylococcal strains to methicillin

| Category | Number of strains | Methicillin zone diam. (mm) | MIC (μg/ml) |
|---|---|---|---|
| 43 | Resistant | ⩽9 | ⩾16 |
| 177 | Susceptible | ⩾14 | ⩽8 |

## Results

*Conventional staphylococci strain typing*

The results of standard susceptibility methods are summarized in Table 1. Of 220 clinical staphylococci isolated, 43 strains (19.5%) recognized as methicillin-resistant, had inhibition zone diameters ⩽9 mm and MIC ⩾16 μg/ml, as reported in Table 1.

Of the 220 staphylococcal isolates tested, 74 (33.6%) were identified as *S. aureus* and 146 (66.4%) identified as CNS as described in the Methods. Of 74 strains of *S. aureus*, 16 (21.6%) were methicillin-resistant (MRSA) and 58 (78.4%) were methicillin-susceptible (MSSA), while 27 of 146 CNS (18.5%) were methicillin-resistant (MR-CNS) and 119 (81.5%) were methicillin-susceptible (MS-CNS) as reported in Figure 1.

***PCR detection of mecA***

To know how extensively *mecA* is distributed among clinical staphylococcal isolates and to determinate

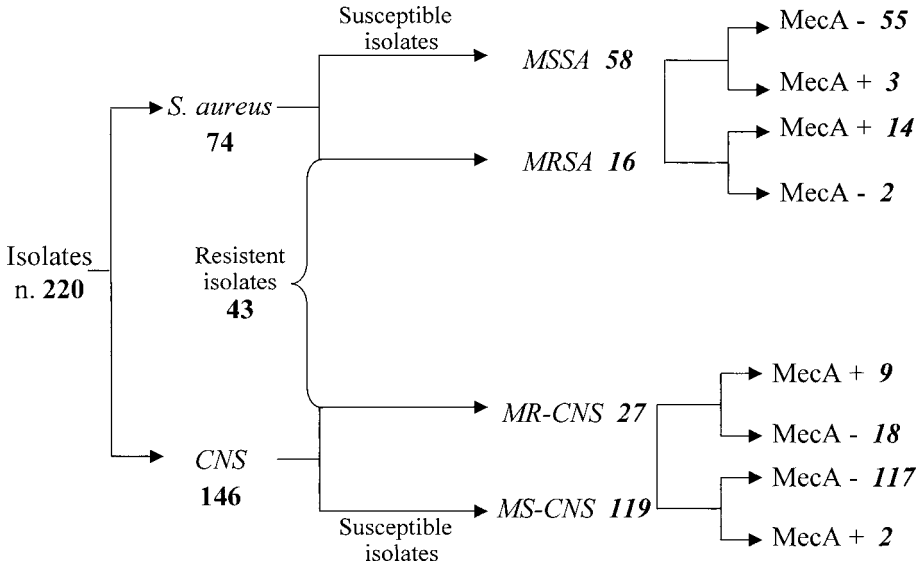


**Figure 1.** Bacteriological and molecular results.

whether other mechanisms of methicillin resistance are present, we analyzed the 43 methicillin-resistant clinical staphylococci on the basis of the hybridization analysis.

The PCR results indicate that only 23 (53.5%) of 43 methicillin-resistant staphylococcal strains isolated, were found to carry the mecA. In fact, as reported in Figure 1, after the DNA amplification, the mecA was detected only for 14 MRSA and nine MR-CNS strains, while the other 20 (46.5%) methicillin-resistant isolates, and in particular two MRSA and 18 MR-CNS, were found to lack the mecA in spite of their resistance to methicillin. Of 177 susceptible strains isolated (inhibition zone diameters $\geqslant$ 14 mm and MIC $\leqslant$ 8 μg/ml respectively) we found that five strains, and in particular three MSSA and two MS-CNS, carry the mecA gene in spite of their susceptibility (Figure 1). The phenotypic resistance of both 20 resistant strains and the five methicillin-susceptible strains were re-tested to confirm the obtained data and showed the same results (data not shown).

Furthermore, Southern hybridization of PCR products of 23 methicillin-resistant mecA positive and five methicillin-susceptible mecA positive staphylococcal strains isolated, yielded positive reactions (Figure 2). These results excluded any possibility of contamination.

*PCR fingerprinting*

In order to study the possible genetic relationships among methicillin-resistant isolates, we used primers 1 and 7 with different resolving powers.

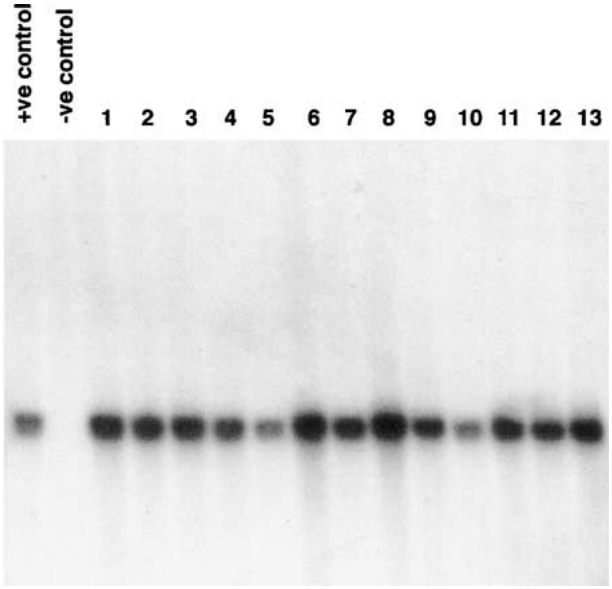


**Figure 2.** Southern blot analysis of PCR products of methicillin-resistance mecA positive (lines 1–10) and methicillin-susceptible mecA positive (lines 11–13); +ve control: MR *S. aureus* ATCC 43300; −ve control: *S. aureus* ATCC 29213.

AP-PCR products have been analyzed on agarose gel and the length of bands for each pattern was evaluated by Sigma Gel Software. We found that primer 1 gives from 21 to 3 kbp fragments, while primer 7 gives from 20 to 3 kbp fragments. When the length of the DNA fragments generated by the AP-PCR was investigated, it was found that primer 1 generated about 25 amplicons. For primer 7 about 21 amplicons were found and the mean values were about 1.1 and 0.4 kbp.

To find a relationship among the different fingerprints, obtained using primers 1 and 7, the dendrograms of methicillin-resistant staphylococci were elaborated. The results show that with primer 1 it is possible to have four different clusters for methicillin-resistant mecA-negative and two different clusters for methicillin-resistant mecA-positive isolates, while with primer 7 there were no clusters for methicillin-resistant mecA-positive and for methicillin-resistant mecA-negative isolates (data not shown).

From dendrogram-derived clusters of the 43 MR with primers 1 and 7, a representative pattern has been chosen and reported in Figures 3 and 4. Figure 3 shows the most representative AP-PCR patterns of each cluster obtained using primer 1. Each lane reports the pattern of the related cluster (panel A), both for the mecA-negative (lanes 1–4) and for the mecA-positive strains (lanes 5, 6) (panel B); all the studied patterns show a similar index of about 0.6–0.7. Figure 4 shows the most representative patterns obtained by DNA amplification of 43 MR using primer 7 (panel A), (lanes 1–6 mecA negative, lanes 7–12 mecA positive; panel B). Analysis of these fingerprints has not yielded any cluster. This could be due to the great differences existing between strains.

## Discussion

Of gram-positive bacteria and in particular staphylococci, resistance to methicillin is increasing and making these strains more resistant to other β-lactam agents. Methicillin-resistance is primarily mediated by the overproduction of PBP2a that has a low affinity for β-lactam antibiotics. The *mecA* is normally present and detained in MR, while it is absent in susceptible [8, 26].

The identification of methicillin-resistant staphylococci in the laboratory is sometimes complicated by the heterogeneous expression of resistance and the variables that influence this expression (pH, temperature, salt concentration). The unstable nature of methicillin-resistance has previously been reported [27, 28] and a further study [29] has described mecA-positive but phenotypically susceptible subclones, as well as mecA-negative ones, that arose from a methicillin-resistant strain after penicillinase plasmid elimination.

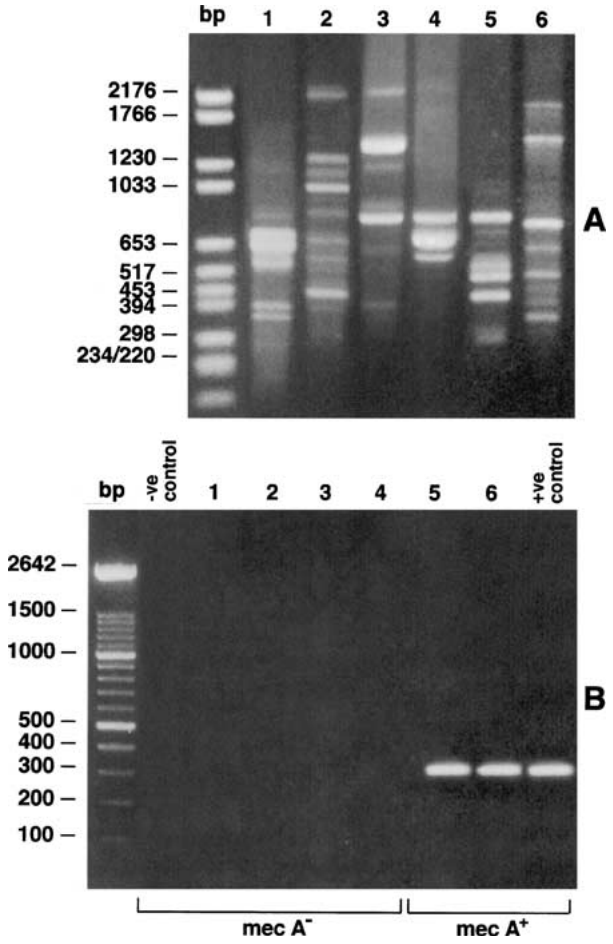


**Figure 3.** Agarose gel electrophoresis of PCR products. The major primer 1 AP-PCR patterns found among the 43 MRS clinical isolates are represented in panel A. MecA PCR analysis of the corresponding patterns reported in panel A, are represented in panel B (lanes 1–4 mecA-negative, lanes 5–6 mecA-positive, −ve control *S. aureus* ATCC 29,213, +ve control MR *S. aureus* ATCC 43,300). Band molecular sizes are indicated on the left of panel A and B.

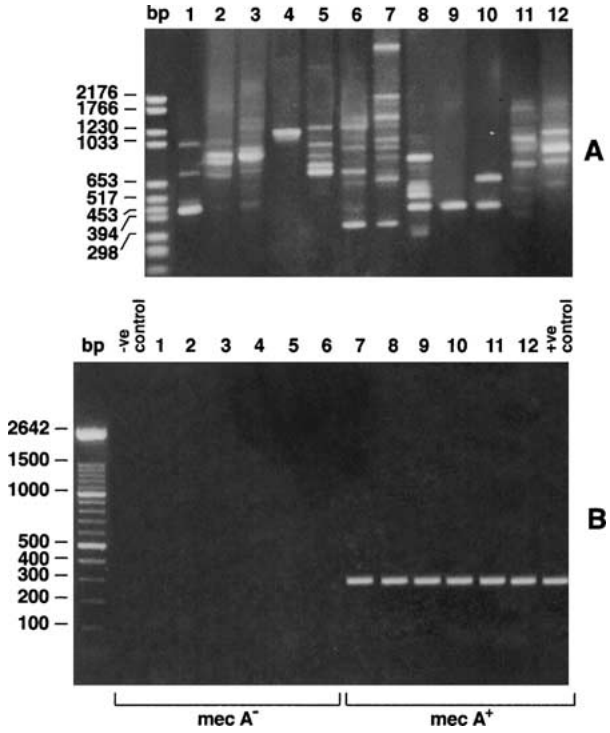


**Figure 4.** Agarose gel electrophoresis of PCR products. The major primer 7 AP-PCR patterns found among the 43 MRS clinical isolates are represented in panel A. MecA PCR analysis of the corresponding patterns reported in panel A, are represented in panel B (lanes 1–6 mecA-negative; lanes 7–12 mecA-positive, −ve control *S. aureus* ATCC 29,213, +ve control MR *S. aureus* ATCC 43,300). Band molecular sizes are indicated on the left panel A and B.

The occurrence of the MR variant described above implied that, during chemotherapy with β-lactam antibiotics, a typical resistant subpopulation developed in a cryptically methicillin-resistant staphylococci (a mecA-positive but not PBP2a producing strain), even when strains could not be identified as resistant by conventional susceptibility tests on the wards. It is possible that staphylococci, first selected as MR by β-lactam antibiotics, but later stopped the production of PBP2a with loss of their resistance. The overproduction of β-lactamase may be another contributor to the antibiotic resistance of our isolates [19].

Methicillin-resistant staphylococci could be successfully detected by PCR even for those defined as cryptically methicillin-resistant. These created a typically methicillin-resistant subpopulation, that should not be classified as methicillin-susceptible to β-lactam antibiotics, because of the possibility that the typically methicillin-resistant variants appeared during chemotherapy with β-lactam antibiotics. PCR allows accurate classification not only of highly resistant strains but also borderline-resistant ones [30]. Many investigators advocate recognition of different resistance mechanisms that are clinically important in determining appropriate therapy. These can involve non-PBP2a-dependent mechanisms such as hyperproduction of β-lactamase [17, 19], the presence of other low-affinity PBPs [11, 19], or production of a newly described methicillinase [31].

The wide distribution of the mecA gene in staphylococcal strains, demonstrated by several investigations, has now been confirmed in this study. The 220 clinical isolates of staphylococci, isolated in our geographical area, were studied to verify the distribution of the mecA gene and to analyze the DNA profile with the AP-PCR. Our results show that only 23 (53.5%) of 43 strains of staphylococci, determined as methicillin-resistant, carry the *mecA*, while the other 20 strains (46.5%), also phenotypically classified as methicillin-resistant, do not carry the *mecA*. This is probably due to a different MS and MR phenotypic correlation with the mecA genotype [32]. These discrepancies might be due to differences between the strains analyzed in our geographical area or to low level MR or to the so called borderline isolates.

To determine whether there was a genetic correlation between the antibiotic resistance phenotype and the presence or absence of mecA gene, we analyzed the fingerprints of methicillin-resistant staphylococcal strains. Among 220 isolates typed in our study by AP-PCR, about 20 different types have been identified, and each type had at least six-band differences from the other types. The primer 1 has led to the realization of six clusters, with a medium similarity index, while primer 7 has led to a lower similarity index, the fingerprints of clinically isolated strains are highly specific and the patterns are different from each other. The difference between the two primers studied, could be due to the different length, and therefore to the primer specificity. Because primer 7 is smaller than primer 1, it could recognize a larger number of staphylococcal DNA sequences and all this could be one of the causes for the greater difference between the fingerprints. Because primer 1 is longer than primer 7, it recognizes a lower number of staphylococcal genome sequences and this could lead to a lower probability of amplification and therefore the fingerprints have less differences from each other. The different sources, just as the frequent transfer of patients from different health care settings, may contribute to the genetic diversity observed. Moreover, the distribution of different patterns varies because of ancestral strain-to-strain mutational differences and because of the variations in the gene content of staphylococcal chromosome.

On the basis of these results, a higher percentage (46.5%) of staphylococcal strains phenotypically MR have been observed but they do not carry the *mecA*. These results suggest that some MR might present a different resistance mechanism from that associated with the production of the PBP2a [8]. On the contrary, amplification product of 310 bp *mecA* fragment was detected in five (3 MSSA and 2 MS-CNS) isolates out of 177 (2.9%) methicillin-susceptible isolates. In spite of this, there is a discrepancy between MIC values and PCR analysis. The fact that five strains classified as methicillin-susceptible resulted *mecA* positive with PCR and Southern blotting, should be regarded as strains potentially methicillin-resistant bearing the mecA gene and simultaneously devoid of expression of PBP2a, which results in negative susceptibility tests [33].

Resistance to antibiotics is not a stable marker for typing staphylococci, and the character is often plasmid borne and may be gained or lost over time. A mutation or deletion in the mecA gene or in its regulatory sequences may explain such unreliability, but confirmation requires further investigation.

In our study, no direct correlation between genotypic and phenotypic analyses was found. The application of the *mecA* DNA probe for the detection of methicillin-resistance staphylococci gave results that sometimes contradicted with the results of a KB test [20] frequently used in clinical microbiology laboratories. Despite guidelines published by the NCCLS for the testing of susceptibility to methicillin for staphylococci, the optimal phenotypic method for detecting methicillin-resistance remains less vigorous for species other than the most communing encountered staphylococci such as *S. aureus*, *S. epidermidis* and *S. haemolyticus* [32].

Lack of identical antibiotic resistance patterns of the isolates might indicate that they do not share a common mechanism of developing antibiotic resistance, the AP-PCR profiles revealed a wide variability and no genetic correlation within isolates could suggest a completely different origin.

In conclusion, methicillin-resistant staphylococci could be successfully detected by the PCR technique employed here. The AP-PCR procedure seems particularly appropriate for rapid typing of nosocomial isolates and it is cost-effective as well. Strain-specific amplicons can be generated quite easily, even among clonally related isolates.

It can therefore be concluded that in our area, a high degree of genetic polymorphism was found among these staphylococcal clinical isolates. The observation of different patterns could be due to ancestral strain to strain mutational differences and to the variations in the gene content of staphylococcal genome.

## References


1. Al Ujayli B, Nafziger DA, Saravolatz L. Pneumonia due to *Staphylococcus aureus* infection. Clin Chest Med 1995; 16: 111–120.
2. Parker MT. Phage typing of *Staphylococcus aureus*. In: Norris JR, Ribbons DW (eds), Methods in Microbiology. 1972; 7B: 1–28.
3. Maslow JN, Mulligan ME, Arbeit RD. Molecular epidemiology: The application of contemporary techniques to typing of bacteria. Clin Infect Dis 1993; 17: 153–162.
4. Tenover FC, Arbeit R, Archer G, et al. Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. J Clin Microbiol 1994; 32: 407–415.
5. Fagon JY, Chastre J, Vuagnat A, et al. Nosocomial pneumonia and mortality among patients in intensive care units. JAMA 1996; 275: 866–869.
6. Rello J, Torres A, Riccart M, et al. Ventilator-associated pneumonia by *Staphylococcus aureus*. Comparison of methicillin-resistant and methicillin-sensitive episodes. Am J Respir Crit Care Med 1994; 150: 1545–1549.
7. Hackbarth CJ, Chambers HF. Methicillin-resistant staphylococci: Genetics and mechanisms of resistance. Antimicrob Agents Chemother 1989; 33: 991–994.
8. Suzuki E, Hiramatsu K, Yokota T. Survey of methicillin-resistant clinical strains of coagulase-negative staphylococci for mecA gene distribution. Antimicrob Agents Chemother 1992; 36: 429–434.
9. Ryffel C, Tesch W, Birch-Machin I, et al. Sequence comparison of mecA genes isolated from methicillin-

resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Gene 1990; 94: 137–138.
10. Berger-Bachi B, Barberis-Maino L, Strassle A, et al. FemA, a host-mediated factor essential for methicillin resistance in *Staphylococcus aureus*: Molecular cloning and characterization. Mol Gen Genet 1989; 219: 263–269.
11. Gerberding JL, Miick C, Liu HH, et al. Comparison of conventional susceptibility tests with direct detection of penicillin-binding protein2a in borderline oxacillin-resistant strains of *Staphylococcus aureus*. Antimicrob Agents Chemother 1991; 35: 2574–2579.
12. Hartman BJ, Tomasz A. Low-affinity penicillin-binding protein associated with β-lactamase resistance in *Staphylococcus aureus*. J Bacteriol 1984; 158: 513–516.
13. Madiraju MVVS, Brunner DP, Wilkinson BJ. Effects of temperature, NaCl, and methicillin on penicillin-binding proteins, growth, peptidoglycan synthesis, and autolysis in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 1987; 31: 1727–1733.
14. Sierra-Madero JG, Knapp C, Karaffa C, et al. Role of β-lactamase and different testing conditions in oxacillin borderline-susceptible staphylococci. Antimicrob Agents Chemother 1988; 32: 1754–1757.
15. Thornsberry C, Caruthers JQ, Baker CN. Effect of temperature on the in vitro susceptibility of *Staphylococcus aureus* to penicillinase-resistant penicillins. Antimicrob Agents Chemother 1973; 4: 263–269.
16. Watanakunakorn C. Effect of inoculum size on in vitro susceptibility of methicillin-resistant *Staphylococcus aureus* to eighteen antimicrobial agents. Eur J Clin Microbiol 1985; 4: 68–70.
17. McDougal LK, Thornsberry C. The role of β-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalosporins. J Clin Microbiol 1986; 23: 832–839.
18. Montanari MP, Tonin E, Biavasco F, et al. Further characterization of borderline methicillin-resistant *Staphylococcus aureus* and analysis of penicillin-binding proteins. Antimicrob Agents Chemother 1990; 34: 911–913.
19. Tomasz A, Drugeon HB, de Lencastre HM, et al. New mechanism for methicillin-resistance in *Staphylococcus aureus*: Clinical isolates that lack the PBP2a gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob Agents Chemother 1989; 33: 1869–1874.
20. Bauer AM, Kirby WMM, Sherris JC, Turch M. Antibiotic susceptibility testing by standardized single disk method. Am Clin Pathol 1966; 45: 493–496.
21. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. Eighth informational supplement, National Committee for Clinical Laboratory Standards, Wayne, Pa. 1998; 18, n.1.
22. Vannuffel P, Gigi J, Ezzedine H, et al. Specific detection of methicillin-resistant *Staphylococcus* species by multiplex PCR. J Clin Microbiol 1995; 33: 2864–2867.
23. Reed KC, Mann DA. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 1985; 13: 7217–7221.
24. Van Belkum A, Bax R, Prevost G. Comparison of four genotyping assays for epidemiological study of methicillin-resistant *Stapylococcus aureus*. Eur J Clin Microbiol Infect Dis 1994; 13: 420–424.
25. Van Belkum A, Bax R, Peerbooms P, et al. Comparison of phage typing and DNA fingerprinting by PCR for discrimination of methicillin-resistant *Staphylococcus aureus* isolates. J Clin Microbiol 1993; 31: 798–803.
26. Predari SC, Ligozzi M, Fontana R. Genotypic identification of methicillin-resistant coagulase-negative staphylococci by polymerase chain reaction. Antimicrob Agents Chemother 1991; 35: 2568–2573.
27. Al Salihy SM, James AM. Loss of methicillin-resistance from resistant strains of *Staphylococcus aureus*. Lancet ii 1972; 12; 2(7772): 331–332.
28. Grubb WB, Annear DI. Spontaneous loss of methicillin-resistance in *Staphylococcus aureus* at room temperature. Lancet 1972; 9; 2(7789): 1257.
29. Hiramatsu K, Suzuki E, Takayama H, et al. Role of penicillinase plasmids in the stability of the mecA gene in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 1990; 34: 600–604.
30. Chambers HF, Archer G, Matsuhashi M. Low-level methicillin resistance in strains of *Staphylococcus aureus*. Antimicrob Agents Chemother 1989; 33: 424–428.
31. Massiddia O, Montanari MP, Varaldo PE. Evidence for a methicillin-hydrolyzing β-lactamase in *Staphylococcus aureus* strains with borderline susceptibility to this drug. FEMS Lett 1992; 92: 223–228.
32. Gradelski E, Valera L, Aleksunes L, et al. Correlation between genotype and phenotypic categorization of staphylococci based on methicillin susceptibility and resistance. J Clin Microbiol 2001; 39: 2961–2963.
33. Barski P, Piechowicz L, Galinski J, et al. Rapid assay for detection of methicillin-resistant *Staphylococcus aureus* using multiplex PCR. Mol Cell Probes 1996; 10: 471–475.

*Address for correspondence*: Prof Giorgio Liguori, Dipartimento di Medicina Pubblica, Clinica E Preventiva, Facoltà di Medicina e Chirurgia, Seconda Università di Napoli, Via Luciano Armanni N 5, 80138 Napoli, Italy
Phone: +39-81-5666031; Fax: +39-81-5666013
E-mail: giorgio.liguori@unina2.it