# Comparison of antibiotic susceptibility tests, plasmid profiles and restriction enzyme analysis of plasmid DNA of methicillin susceptible and resistant–Staphylococcus aureus strains isolated from intensive care units

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## ABSTRACT

**Objective:** To differentiate methicillin-resistant *Staphylococcus aureus (S. aureus)* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains and detect the source of epidemic strains and prevent their access to patients.

**Methods:** All the procedures were carried out in the Department of Microbiology, Medical Faculty Hospital, Dokuz Eylul University, Izmir, Turkey from 1996-1998, and antibiotic susceptibility tests continued in the laboratory of King Fahad Hospital, Al-Baha, Kingdom of Saudi Arabia (KSA), from 2001-2004. A total of 81 *S. aureus* strains (71 MRSA, 10 MSSA) from Turkey were isolated from different sites of patients in Intensive Care Unit's (ICU's), evaluated by plasmid profile, Restriction Endonuclease Analysis of Plasmids (REAP), and antibiotic sensitivity tests. A total of 117 *S. aureus* strains (24 MRSA, 93 MSSA) from KSA were isolated from different sites of patients in ICU's, evaluated by antibiotic sensitivity tests as recommended by National Committee for Clinical Laboratory Standards (NCCLS).

**Results:** Seventy-one MRSA from Turkey were divided into 13 groups by antibiotic sensitivity tests and into 4 groups by plasmid profiles, in which 3rd and 4th groups subdivided into 2 subgroups, and into 5 groups by REAP. The 1st, 2nd, 3rd and 5th groups were subdivided into 2 subgroups. Ten MSSA were divided into 4 groups by antibiotic sensitivity tests, 3 in plasmid profiles and 2 in REAP tests. Twenty-four MRSA strains from KSA were divided into 9 groups by antibiotic sensitivity tests while 93 MSSA strains were divided into 7 groups.

**Conclusion:** In respect to epidemiological survey, plasmids profiles and REAP seems to discriminate more respect to antibiotic sensitivity tests but at the same time neither of them were 100% accurately differential. According to the plasmid profile of the 3rd MSSA (Turkey) group, a multi-drug resistance by antibiotic susceptibility tests were noticed and showed the same plasmid profile in MRSA first subgroup of the 3rd group, but the same groups were different in REAP tests. In order to distinguish the discriminatory power of the strains, where REAP is better than plasmid profile and antibiotic sensitivity tests, we may formulate the statement into the following; REAP > plasmid profile > antibiotic sensitivity tests. For typing and gathering of epidemiological data, it is suggested that all 3 methods should be employed in clinical laboratories as they are cheap, practical and easily interpreted.

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Methicillin-resistant *Staphylococcus aureus (S. aureus)* (MRSA) strains are continuously becoming a major cause of nosocomial and community acquired infections.[1] The important goal of microbiologists and other health care workers is to reduce the number of MRSA infections by detecting and eradicating the sources of the organisms or by interrupting their path of transmission to the patients. As the organisms are often resistant to multiple antibiotics, the infections caused by these organisms complicate the treatment of patients, prolong hospitalization, and increase the cost of medical care. As *S. aureus* and specially MRSA often colonize hospital staff,[2-4] attaining these goals depend, in turn, on the techniques in characterizing epidemic MRSA and distinguishing them from resident strains. A lot of epidemiologic markers, such as susceptibility profile, bacteriophage typing,[5,6] plasmid analysis,[1,7-12] and other new molecular techniques,[9,13-17] have been used to investigate dissemination of MRSA in hospitals. In this study, we compared 3 epidemiological tests used to differentiate MRSA strains and to distinguish between isolates from many apparently independent infections. The aim of Restriction Endonuclease Analysis of Plasmids (REAP), is to increase discrimination between molecules of similar size.

**Methods.** ***Microorganisms***. A total of 198 *S. aureus* strains analyzed from each source are listed in **Table 1**. All of the strains were isolated from patient specimens in ICU's. Strains from all sources were tested for coagulase reaction and oxacillin susceptibilities before their use in the study and *S. aureus* was used as a control strain.

***Antibiotic susceptibility disks.*** All disks were purchased from the appropriate pharmaceutical companies, and antibiotic susceptibilities were determined by the Kirby-Bauer disk diffusion method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS),[18] by using the following antimicrobial agent-containing disks: oxacillin (1 µg ), penicillin(10 U), erythromycin (15 µg), clindamycin (2µg), tetracycline (30µg), amoxicillin-clavulanic acid (20/10µg), cephalothin (30µg), trimethoprim-sulfamethoxazole (1.25/23.75µg), fucidic acid (10µg), gentamicin (10µg), amikacin (30µg), streptomycin (10µg), kanamycin (30µg), aztreonam (30µg), imipenem (10µg), ciprofloxacin (5µg), ofloxacin (5µg), and norfloxacin(10µg).

***Plasmid isolation and restriction enzyme digestion.*** All plasmid DNAs was isolated from the microorganisms by alkaline lysis method.[5,19] Half of each plasmid preparations were applied to adjacent wells on horizontal, 10-by 15-cm, 0.8% agarose gels in triborate ethylenediaminetetraacetic acid (TBE) buffer. The DNA markers (Hind III digest of λ phage DNA) were applied to control wells in each gel. Electrophoresis was at 120 V for 3-4 hours at room temperature. Other half of each plasmid preparations was digested with 10 U of EcoR1 (New England Biolabs) for 3 hours at 37°C. Digested samples were applied on wells in agarose gels with control and electrophoresis at 120 V for 3-4 hours at room temperature. Digested and undigested gels were stained for 30 minutes with 0.5 µg of ethidium bromide per ml in TBE buffer and photographed on a 302 - nm - wavelength transilluminator onto Polaroid type 665 films through an orange filter. We used the semi logarithmic papers to construct a standard curve relating the electrophoretic mobilities of the marker molecules to the logarithm of their size and to estimate the sizes of *S. aureus* plasmid and plasmid fragments from the standard curve.

**Results.** Ten methicillin-sensitive *Staphylococcus aureus (*MSSA) strains from Turkey showed 4 different patterns by antibiotic sensitivity tests **(Table 2)**, and 93 MSSA strains from the Kingdom of Saudi Arabia (KSA) showed 8 patterns in the same tests **(Table 3)**. On the other hand, 71 MRSA strains from Turkey showed 13, and 24 strains from KSA showed 9 different patterns in this tests **(Tables 4 & 5)**. Ten MSSA strains from Turkey were divided into groups of 3 in plasmid profiles and 2 in REAP tests. However, 71 MRSA strains were divided into 4 groups in plasmid profiles in which 3rd and 4th groups also subdivided into 2 subgroups **(Table 6)**, and 5 groups in REAP tests in which 1st, 2nd, 3rd and 5th groups were subdivided into 2 subgroups **(Table 7)**. One of 10 MSSA strains has no plasmid, and this was on the first group. The second group was divided into 2 subgroups, the first subgroup contain 2 light plasmid and the second subgroup contain 3 plasmid (one heavy and 2 light plasmids that were the same in the first subgroup), and the 3rd group contain 2 plasmids (one heavy and the same in the 2nd subgroup, and one light plasmid which was different from the others). As seen in the **Table 6**, 2 strains of MRSA contained no plasmid DNA for the 1st group ($P_0$), 6 strains, each containing one plasmid for the 2nd group ($P_1$), 13 strains, each containing 2 plasmids for the 3rd group ($P_2$), 37 strains where each contains at least 3 same plasmids for the 4th group ($P_3$), which was divided into 2 subgroups; 22 of them were containing 3 plasmids ($P_{3a}$) and 15 were containing 4 plasmids ($P_{3b}$).The last 13 strains each containing 4 plasmids for the 5th group ($P_4$), which was also divided to 2 subgroups; 3 of them were containing 2 heavy and 2 light plasmids ($P_{4a}$), and other 10 strains were containing 2 heavy and 2 light plasmids, but one of light plasmids was different ($P_{4b}$).

**Table 1 -** Sources and types of samples.

| Samples source of samples | Blood | Tracheal aspiration | Catheter | Others* | Total |
|---|---|---|---|---|---|
| ***Turkey*** | | | | | |
| AICU | 6 | 21 | 6 | 2 | **35** |
| IICU | 15 | 4 | 5 | 8 | **32** |
| CICU | 5 | - | - | - | **5** |
| THCSICU | 2 | 6 | - | 1 | **9** |
| Subtotal | 28 | 31 | 11 | 11 | **81** |
| ***Saudi*** | | | | | |
| ICU | 13 | 15 | 10 | 2 | **40** |
| NICU | 16 | 17 | 17 | 4 | **54** |
| PICU | 7 | 8 | 7 | 1 | **23** |
| Subtotal | 36 | 40 | 34 | 7 | **117** |
| **Total** | **64** | **71** | **45** | **18** | **198** |

ICU - intensive care unit, AICU - anesthesia intensive care unit, IICU - Intern ICU, CICU - coronary ICU, THCSICU - thorax heart coronary surgical ICU, NICU - neonate ICU, PICU - pediatrics ICU
*wound, sputum, nasal swabs

**Table 2 -** Methicillin sensitive *staphylococcus aureus* resistant patterns from Turkey.

| Pattern | Disks which are resistant | No of isolates in ICU's AICU | IICU | CICU | THCSICU | Total |
|---|---|---|---|---|---|---|
| $R_1$ | ATM | - | 1 | - | - | **1** |
| $R_2$ | E, ATM | 1 | 2 | - | - | **3** |
| $R_3$ | Te, ATM, S | 1 | 1 | 2 | - | **4** |
| $R_4$ | ATM, S, GM, AN, CIP, OFX, NOR | - | 1 | 1 | - | **2** |
| **Total** | | 2 | 5 | 3 | - | **10** |

ATM - aztreonam, E - erythromycin, Te - Tetracycline, S - streptomycin, GM - gentamicin, AN - amikacin CIP - ciprofloxacin, OFX - ofloxacin, NOR - norfloxacin, ICU - intensive care unit, AICU - anesthesia intensive care unit, IICU - Intern ICU, CICU - coronary ICU, THCSICU - thorax heart coronary surgical

**Table 3 -** Methicillin sensitive *staphylococcus aureus* from the Kingdom of Saudi Arabia.

| Pattern | Disks which are resistant | No of isolates in ICU'S ICU | NICU | PICU | Total |
|---|---|---|---|---|---|
| $R_0$ | - (sensitive to all disks) | 1 | - | 6 | **7** |
| $R_1$ | P | 19 | 16 | 9 | **44** |
| $R_2$ | P, E | 4 | 1 | - | **5** |
| $R_3$ | P, FA | 1 | 1 | - | **2** |
| $R_4$ | P, Te | 7 | 5 | 1 | **13** |
| $R_5$ | P, Te, FA | 4 | 3 | 2 | **9** |
| $R_6$ | P, E, Te, SXT | 5 | 2 | - | **7** |
| $R_7$ | P, E, Te, FA | 4 | 1 | 1 | **6** |
| **Total** | | **45** | **29** | **19** | **93** |

P - penicillin, E - erythromycin, FA - fusidic acid, Te - tetracycline SXT - Trimethoprim-sulfamethoxazole, c NICU - neonate ICU, PICU - pediatrics ICU

By restriction of enzyme profiles, 9 of MSSA, which contains plasmids; 3 of them showed no fragments, and other 6 strains was divided into 2 groups. These 2 groups were sharing only one fragment (8.800 kb). All of 69 MRSA strains, which contains plasmids were divided into 5 groups and again were subdivided into groups according to the size of the fragments as shown in **Table 7**. Three epidemiological markers for differentiation of microorganisms are compared in **Table 8**, where most microorganisms were included into more groups except 9 isolates from thorax heart coronary surgery ICU, which was one group by plasmid profiles and 3 groups by others. All 2 strains from coronary ICU was one group by the 3 methods. The important observation of these tests was that REAP is able to differentiate strains epidemiologically more than the plasmid profiles as seen in 9 strains of thorax heart coronary surgery ICU that was one group in plasmid profiles and divided into 3 groups by REAP and from which 6 strains were in the same group.

**Discussion.** We studied 198 strains of *S. aureus* by using sensitivity tests, where 81 strains were isolated using plasmid profiles and REAP. Antibiotic sensitivity tests worked reasonably well, but due to the instability of resistance pattern, which

**Table 4 -** Methicillin resistant *staphylococcus aureus* resistant patterns from Turkey.

| Pattern | Disks which are resistant | AICU | IICU | CICU | THCSICU | Total |
|---|---|---|---|---|---|---|
| $R_1$ | ATM, Te, E | - | 1 | - | 1 | **2** |
| $R_2$ | ATM, RA,GM, AN, CIP, NOR, K, S, IPM | 1 | - | - | - | **1** |
| $R_3$ | ATM, RA, GM, AN, CIP, NOR, K, S, OFX | - | 1 | - | - | **1** |
| $R_4$ | ATM, GM, AN, CIP, NOR, K, S, IPM, OFX | - | 4 | - | - | **4** |
| $R_5$ | ATM, RA, GM, AN, CIP, NOR, K, S, IPM, OFX | 5 | - | - | - | **5** |
| $R_6$ | ATM, RA, GM, AN, CIP, NOR, K, S OFX, AmC | 2 | - | - | - | **2** |
| $R_7$ | ATM, RA, GM, AN, CIP, NOR, K, S, OFZ, E, AmC | - | 1 | - | - | **1** |
| $R_8$ | ATM, RA, GM, AN CIP, NOR, K, S, E, AmC, IPM, Te | - | 1 | - | - | **1** |
| $R_9$ | ATM, RA, GM, AN, CIP, NOR, K, S, E, AmC, IPM, Te | 2 | - | - | - | **2** |
| $R_{10}$ | ATM, RA, GM, AN, CIP, NOR, K, S, OFX, E, AmC, IPM | - | 1 | - | - | **1** |
| $R_{11}$ | ATM, RA, GM, AN, CIP, NOR, K, S, OFX, AmC, IPM, Te | 5 | 1 | - | 1 | **7** |
| $R_{12}$ | ATM, RA, GM, AN, CIP, NOR, K, S, OFX, E, AmC, IPM, Te | 5 | 9 | - | 7 | **21** |
| $R_{13}$ | ATM, RA, GM, AN, CIP, NOR, K, S, OFX, E, AmC, Te | 13 | 8 | 2 | - | **23** |
| **Total** | | **33** | **27** | **2** | **9** | **71** |

* ATM - aztreonam, Te - tetracycline, E - erythromycin, RA - rifampin, GM - gentamicin, AN - amikacin
CIP - ciprofloxacin, NOR - norfloxacin, K - kanamycin, S - streptomycin, IPM - imipenem, OFX - ofloxacin
AmC - amoxicillin-Clavulanic acid, ICU - intensive care unit, AICU - anesthesia intensive care unit,
IICU - Intern ICU, CICU - coronary ICU, THCSICU - thorax heart coronary surgical

**Table 5 -** Methicillin resistant *staphylococcus aureus* resistant patterns from the Kingdom of Saudi Arabia.

| Pattern | Disks which are resistant | ICU | NICU | NICU | Total |
|---|---|---|---|---|---|
| $R_1$ | P, OX | 2 | - | 3 | **5** |
| $R_2$ | P, OX, FA | - | 1 | 1 | **2** |
| $R_3$ | P, OX, Te | - | 1 | 1 | **2** |
| $R_4$ | P, OX, CF, Te | - | 1 | - | **1** |
| $R_5$ | P, OX, CF, Te, GM, SXT | - | 1 | - | **1** |
| $R_6$ | P, OX, CF, Te, GM, SXT, FA | - | - | 1 | **1** |
| $R_7$ | P, OX, CF, Te, GM, SXT, FA, E | 2 | 1 | - | **3** |
| $R_8$ | P, OX, CF, Te, GM, SXT, FA, E, CC | 2 | - | - | **2** |
| $R_9$ | P, OX, CF, Te, GM, SXT, FA, E, CC, RA | 5 | 1 | 1 | **7** |
| **Total** | | **11** | **6** | **7** | **24** |

P - penicillin, OX - oxacillin, FA - fusidic acid, Te - tetracycline, CF - cephalothin,
GM - gentamicin, SXT - ttrimethoprim-sulfamethoxazole, E - erythromycin
CC - clindamycin, RA - rifampin, ICU - intensive care unit,
NICU - neonate ICU, PICU - pediatrics ICU

**Table 6 -** Plasmid profiles of 71 methicillin resistant *staphylococcus aureus* strains.

| Pattern | Subgroup | Plasmid (kb) | No of strains | Sources |
|---|---|---|---|---|
| $P_0$ | $P_0$ | | 2 | IICU (2) |
| $P_1$ | $P_1$ | 17.500 | 6 | IICU (4), AICU (2) |
| $P_2$ | $P_2$ | 23.130, 1.900 | 13 | IICU (6), AICU (7) |
| $P_3$ | $P_{3a}$ | 23.130, 1.600, 1.400 | 22 | IICU (13), AICU (4), CICU (2), THCSICU (3) |
| | $P_{3b}$ | 23.130, 1.900, 1.600, 1.400 | 15 | AICU (9), THCSICU (6) |
| $P_4$ | $P_{4a}$ | 29.000, 23.130, 3.800, 1.400 | 3 | AICU (3) |
| | $P_{4b}$ | 29.000, 23.130, 1.600, 1.400 | 10 | IICU (2), AICU (8) |

ICU - intensive care unit, IICU - intern ICU, AICU - anesthesia ICU, CICU - coronary ICU,
THCSICU - thorax heart coronary surgical ICU

**Table 7 -** Digestion profiles of methicillin resistant *staphylococcus aureus* plasmids.

| Pattern | Subgroup | Sizes of *EcoR1* fragments (kb) | No of strains | Sources |
|---|---|---|---|---|
| $P_1$ | $P_{1a}$ | 8.800, 5.000, 4.000, 2.950, 1.300 | 26 | AICU (14), IICU (6), THCSICU (6) |
| | $P_{1b}$ | 7.800, 5.000, 4.000, 2.950, 1.300 | 2 | AICU (2) |
| $P_2$ | $P_{2a}$ | 8.800, 5.400, 4.000, 2.950, 2.450, 1.200 | 12 | AICU (4), IICU (7), THCSICU (1) |
| | $P_{2b}$ | 8.800, 5.400, 4.000, 2.950, 2.000, 1.200 | 8 | IICU (6), CICU (2) |
| $P_3$ | $P_{3a}$ | 10.200, 6.600, 5.000, 3.500, 2.200, 1.500 | 9 | AICU (7), IICU (2) |
| | $P_{3b}$ | 10.200, 6.500, 5.000, 3.500, 2.200, 1.500 | 4 | IICU (4) |
| $P_4$ | $P_4$ | 6.600, 4.300, 3.500, 2.400, 1.200 | 2 | THCSICU (2) |
| $P_5$ | $P_{5a}$ | 9.400, 3.500, 1.800 | 4 | IICU (4) |
| | $P_{5b}$ | 9.400, 3.500, 1.800, 1.200 | 2 | AICU (2) |

ICU - intensive care unit, AICU - anesthesia ICU, IICU - intern ICU,
THCSICU - thorax heart coronary surgical ICU, CICU - coronary ICU

**Table 8 -** Restriction endonuclease analysis of plasmids profiles, plasmid profiles and resistant patterns of methicillin resistant *staphylococcus aureus* strains isolated from Turkey.

| Wards (isolate no) | P1 | P2 | P3 | P4 | P5 | P1 | P2 | P3 | P4 | R1 | R2 | R3 | R4 | R5 | R6 | R7 | R8 | R9 | R10 | R11 | R12 | R13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AICU (23) | 16 | 4 | 11 | 11 | 2 | 2 | 7 | 13 | 11 | - | 1 | - | - | 5 | 2 | - | - | 2 | - | 5 | 5 | 13 |
| IICU (27)* | 6 | 13 | 2 | 2 | 4 | 4 | 6 | 13 | 2 | 1 | - | 1 | 4 | - | - | 1 | 1 | - | 1 | 1 | 9 | 8 |
| CICU (2) | - | 2 | - | - | - | - | - | 2 | - | - | - | - | - | - | - | - | - | - | - | - | - | 2 |
| THCSICU (9) | 6 | 1 | - | - | - | - | - | 9 | - | 1 | - | - | - | - | - | - | - | - | - | 1 | 7 | - |
| **Total (71)*** | **28** | **20** | **13** | **13** | **6** | **6** | **13** | **37** | **13** | **2** | **1** | **1** | **4** | **5** | **2** | **1** | **1** | **2** | **1** | **7** | **21** | **23** |

* - There was no plasmid in 2 isolates
ICU - intensive care unit, IICU - intern ICU, CICU - coronary ICU,
THCSICU - thorax heart coronary surgical ICU.

is probably related to changes in plasmid continent[10,20] interpretation of results may not be enough. However, changes in the zone sizes around the disks for 2 or more antibiotic disks must be observed before 2 isolates can be considered as different strains.[10] Antibiotic sensitivity tests are the least expensive method, and could be considered, especially in clinical laboratories as an initial screen to determine strain relatedness. The use of plasmid analysis has been helpful in analyzing the strain relationship in a number of MRSA outbreaks, and this analysis has been noted by a number of authors.[5,20-25] The usefulness of plasmid profiles to bacterial epidemiology depends upon the degree to which the requirements are satisfied; 1. The bacterial strains must contain plasmids. 2. Plasmids must be sufficiently diverse so that independent isolates does not likely to carry distinctly different plasmid. 3. Differences between plasmids must be distinguishable by the fragments that appear after restriction and after endonuclease digestion. 4. Methods for plasmid profiling must be rapid, inexpensive, reproducible and accessible to clinical laboratories. In our study we noticed that plasmid analysis correlates well with antibiotic sensitivity tests in most strains of methicillin-sensitive and resistant *S. aureus*. Two strains of MSSA, which we found multi-resistant to antibiotics, ($R_4$ in **Table 2**) contain the same plasmids in 22 (31%) MRSA strains ($P_{3a}$ in **Table 6**). On the other hand, these plasmids were different fragments from MRSA strains discovered by REAP, except for 8.800 and 1.200 kb fragments as they were the same in both MRSA and REAP. Three MSSA strains carried 2 small plasmids (1,600, 1,400 kb) that were found in 47 (66%) MRSA strains. Six (60%) of MSSA strains and 63 (88%) of MRSA strains contains the same sizes of plasmids (23,130 kb). At the same time by REAP, these 6 strains of MSSA contain the same fragments (8,800 kb) as that in 46(64%) and (4,000 kb) in 48 (67%) strains of MRSA and in this study we found that 8.800, 7.800, 5.400, 5.000 and 1.500 kb fragments of plasmids had been founded in the United States of America in a previous study of MRSA plasmids.[5] This data allows for the interpretation that these strains are transmitted

between personnel and patients, and these observations suggest that there are mechanisms at work, perhaps involving conjugation, transduction, transposition, phage mediated contact, and homologous recombination,[26-28] that are continuously generating new plasmid forms. In agreement with previous works,[22,27] we found that very few (2,8%) of the MRSA strains lacked plasmids, these findings assure the wide applicability of plasmid profiling to MRSA epidemiology.

We conclude that no single technique was clearly superior to others for typing MRSA strains, and the availability of plasmid analysis with antibiotic sensitivity tests on a routine bases may be helpful in characterizing isolates that cause outbreaks of MRSA. Restriction endonuclease analysis of plasmids tests is still more powerful epidemiological marker. For typing and gathering of epidemiological data, it is suggested that all 3 methods (antibiotic sensitivity tests, plasmid profile and REAP) which are cheap, practical and can easily interpreted should be employed together in clinical laboratories.

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