Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* isolates from clinical specimens of patients with nosocomial infection: are there unnoticed silent outbreaks?

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SUMMARY

Bacteriological and epidemiological studies were carried out on 90 isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) at Turgut Özal Medical Center of Inönü University, (Malatya/Turkey). MRSA isolates were obtained from patients with nosocomial infections. *Staphylococcus aureus* clinical isolates were collected between May 2004-May 2005. Isolates were tested for resistance to methicillin. Antimicrobial susceptibility testing and slime production evaluation was performed. Genotype studies were carried out by arbitrarily primed polymerase chain reaction (AP-PCR) and consequent cluster analysis. All of the isolates were mecA-positive in a PCR-based assay; all exhibited resistance to oxacillin, by agar dilution (MICs ≥4mg/L) and disc diffusion methods, and multiple antibiotics. Most MRSA isolates were collected in intensive care units. Of 90 samples, 53 were found to be unrelated to the others while the remaining 37 strains were either identical or closely related. Dendrogram analysis identified nine major clusters. These data support the opinion that MRSA are significant nosocomial pathogens in intensive care units and that resistant clones may be transmitted between patients. Molecular epidemiological tools are helpful for understanding transmission patterns and sources of infection, and are useful for measuring outcomes of intervention strategies implemented to reduce nosocomial MRSA.

KEY WORDS: Methicillin-resistant *Staphylococcus aureus*, nosocomial infection, molecular typing

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INTRODUCTION

Staphylococci are among the most important causes of hospital-acquired infections worldwide. Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an increasingly important pathogen leading to hospital-acquired infections (Waldvogel *et al.,* 2000, MMWR 1997, Aygen *et al.,* 2004). In addition to the associated major morbidity and mortality, MRSA infections are also transmissible. Transmission of MRSA infections occurs via direct person to person contact, usually spreading between patients through the colonized hands of healthcare workers. Invasive procedures, use of broad-spectrum antibiotics and therapeutic strategies which damage the mucosa and skin may lead to major outbreaks in hospitals (Wang *et al.,* 2001).

Resistance is primarily determined by production of an altered penicillin binding protein (PBP2a) which affects all beta-lactam antibiotics. Furthermore, MRSA strains are resistant to gentamicin, kanamycin and tobramycin (GR-MRSA), and to macrolides, tetracycline and fluoroquinolones. Thus, multiple resistance of *S.aureus* strains occur. The treatment of severe

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infections of MRSA is often a therapeutic challenge for physicians (Chambers *et al.,* 1997, Petinaki *et al.,* 2001).

Measures to prevent the spread of MRSA have been proposed for a long time, however, the frequency of hospital - acquired MRSA infections has continued to increase over the past two decades (Wang *et al.,* 2001, Ayliffe *et al.,* 1997). Strict microbiological monitoring and epidemiological investigation are essential for controlling MRSA infections in hospitals (TAKEDA *et al.*, 2000). In the last decade genetic analysis including Pulsed-Field Gel Electrophoresis (PFGE) and Arbitrarily primed Polymerase Chain Reaction (AP-PCR) have become the most common methods used to investigate the epidemiology of nosocomial pathogens (Raimundo *et al.,* 2002, Tekerekoglu *et al.,* 2004).

The aim of this study was to investigate epidemiological characteristics of nosocomial isolates of MRSA and analyse the clonal relationship of the isolates by AP-PCR. In addition, probable occurrence of unnoticed silent outbreaks was evaluated.

MATERIALS AND METHODS

Bacterial strains

Only the infections which occurred following a patient's admission to hospital were evaluated. The cultures were taken from patients at least 72 h after their admission to the hospital, or within 10 days of their discharge. Positive cultures were considered hospital acquired infections. A total of 90 MRSA isolates were obtained between 01.05.2004 and 01.05.05 from Turgut Özal Medical Center of Inonu University. Medical Center is a teaching hospital with 850 beds and nine intensive care units and two reanimation units. Isolates were obtained from clinical specimens including blood, catheters, tracheal aspirate, urine, wound and other samples. Duplicate isolates were excluded from collection. Samples collected at the intensive care units and inpatient clinics were analysed. Of the 90 MRSA strains isolated at our hospital, 32 were cultured from respiratory tract, 13 from blood, nine from urine, eight from invasive devices and 28 from wounds. The number of MRSA strains isolated in reanimation-intensive care units, surgery and internal medicine wards were 44, 30 and 16 respectively.

Identification of the methicillin resistant *S. aureus* strains

Staphylococcus isolates were identified as *S. aureus* using a conventional biochemical test (Diagnostic microbiology), the sceptor system (Staphylococcus MIC/ID panel, Becton Dickinson and Company, USA) and API Staph reagent kit (Biomerieux, France). Antimicrobial susceptibilities of the isolates were investigated by a standardised disk-diffusion method performed on Mueller-Hinton agar by Kirby-Bauer disk diffusion method following the criteria of the Clinical Laboratory Standards Institute (CLSI). Furthermore the Sceptor system was used. Oxacillin disk diffusion method and oxacillin agar screen tests were employed for determination of methicillin resistance (CLSI, 2005). Methicillin resistance was confirmed by detection of mecA gene by polymerase chain reaction (PCR) (Murakami *et al.,* 1993). Slime production of the isolates was sought in Christensen medium using the tube method (Ay *et al.* 2002).

Molecular typing of the strains: arbitrarily primed polymerase chain reaction (AP-PCR)

Arbitrarily primed polymerase chain reaction (AP-PCR) was performed to investigate clonal relatedness among 90 MRSA isolates. DNA was extracted following the protocol of Welsh and McClelland (Welsh and McClelland, 1993). AP-PCR was performed with the core sequence phage M13 primer (5'-GAG GGT GGC GGT TCT-3') (Tekerekoglu *et al.,* 2004). The reaction mixture was amplified according to the following conditions: 2 cycles, each consisting of 5 min at 94ºC, 5 min at 40ºC, and 5 min at 72ºC; 40 cycles, each consisting of 1 min at 94ºC, 1 min at 40ºC, and 2 min at 72ºC. Amplification products were electrophoresed by 2% agarose gel and after staining were visualised and photographed under UV illumination.

Dendogram analysis

Comparison of DNA fingerprints was done with the GelCompar version 4.0 package (Applied Maths, Kourtrai, Belgium). Cluster analysis for

TABLE 1 - *Comparison of epidemiologic and genotyping data.*

Patient No.	*Strain No.*	*Source of isolation*	*Date of isolation*	*Units*	*Genotype*	*Clinical relevance*	*Note*
4	5365	Tracheal asp.	25.05.03	R-ICU	4	+	ICU- intubation
5	5366	Tracheal asp.	25.05.03	R-ICU	4	+	ICU- intubation
13	Aug-16	Blood	09.08.03	ICU	4a	+	R-ICU <->ICU
25	Sep-253	Sputum	24.09.03	Neuorlogy	10	-	
26	Sep-205	Blood	29.09.03	CVS- ICU	10	+	ICU
37	Nov-164	Tracheal asp.	06.11.03	ICU	10	+	ICU-intubation
36	Nov-102	Catheter	13.11.03	Neurology	10	-	
38	Nov-193	Blood	23.11.03	ICU	10	+	ICU
35	Nov-47	Pleural asp.	06.11.03	ICU	10	+	ICU
79	Apr-401	Tracheal asp.	12.04.04	ICU	10	-	
76	Apr-61	Tracheal asp.	04.04.04	ICU	11	-	
32	Oct-326	Wound	30.10.03	Orthopedics	11	-	ICU <->
22	Sep-232	Catheter	18.09.03	Hematology	12	-	
31	Oct-311	Tracheal asp.	28.10.03	ICU	12	-	ICU–intubation
6	5413	Blood	10.06.03	R-ICU	12	-	
77	May-144	Tracheal asp.	05.04.04	ICU	13	+	Common date-ICU
78	May-148	Tracheal asp.	05.04.04	ICU	13	+	Common date-ICU
49	Jan-128	Wound	10.01.04	Orthopedics	15	+	ICU+operation
50	Jan-21	Wound	04.01.04	Orthopedics	15	+	ICU+operation
51	Jan-127	Tracheal asp.	10.01.04	R-ICU	15	+	ICU+intubation
53	Jan-116	Tracheal asp.	10.01.04	ICU	15	+	ICU+intubation
54	Jan-113	Tracheal asp.	10.01.04	ICU	15	+	ICU+intubation
11	5576	Wound	25.06.03	Orthopedics	17	-	Operation
15	Sep-92	Blood	09.09.03	ICU	17	+	ICU+operation
16	Sep-82	Wound	10.09.03	R-ICU	17	+	ICU+operation
17	Sep-125	thoracentesis fluid	15.09.03	ICU	17	+	ICU-intubation+operation
18	Sep-149	Wound	13.09.03	Orthopedics	17	-	Operation
86	Apr-422	Catheter	23.04.04	Nephrology	19	+	Common ward
87	Apr-427	Urine	23.04.04	Nephrology	19	+	Common ward
81	Apr-258	Wound	13.04.04	CVS	19	-	
68	Mar-63	Tracheal asp.	05.03.04	Pediatrics	26	-	
69	Mar-84	Tracheal asp.	07.03.04	Surgery	26	-	
71	Mar-313	Tracheal asp.	20.03.04	R-ICU	26	+	ICU
72	Mar-347	Peritoneal asp.	23.03.04	ICU	26	+	ICU
65	Feb-149	Wound	15.02.04	Surgery	26	+	ICU <->
66	Feb-154	Wound	15.02.04	Orthopedics	26	-	
67	Feb-128	Tracheal asp.	17.02.04	Surgery	26	+	ICU <->

<->: The entrance and exit ICU : Intensive care unit,R-ICU: Reanimation-ICU CVS: Cardiovascular surgery

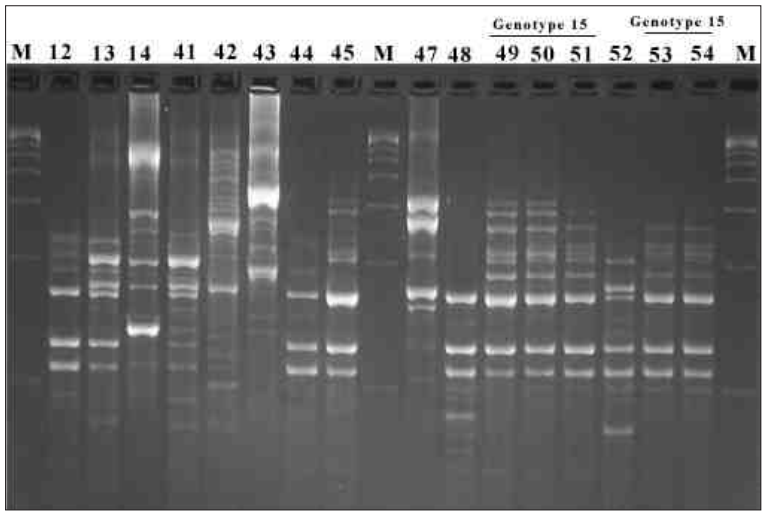

FIGURE 1 - *AP-PCR patterns of representative MRSA strains.*

AP-PCR was performed by calculation of the Dice similarity coefficient. According to this analysis, the isolates with a similarity coefficient equal or greater than 90% were evaluated as the same type, those having a similarity coefficient equal or greater than 70% and less than 90% were classified as a subtype, and the isolates showing a similarity coefficient less than 70% were identified as a different genotype (Ayan *et al.,* 2003).

RESULTS

All of the isolates of *S.aureus* were found to be positive by the *mec*A-specific PCR assay. Of the 90 isolates of *S.aureus*, 42 (47%) were positive for slime production according to Christensen assay. Twenty-two (60%) out of 37 genotypically related strains were found to be positive for slime production. None of the isolates tested revealed resistance to vancomycin. All of the isolates exhibited resistance to oxacillin and penicillin. Seventy-four (83%) of the isolates were resistant to erythromycin, 44 (49%) to clindamycin, 86 (96%) to tetracycline, 84 (93%) to rifampicin, 85 (95%) to gentamicin and 90 (100%) to ciprofloxacin. Genotyping features of the isolated strains were evaluated in combination with patient's epidemiological data (Table 1).

Results of AP-PCR and dendrogram analysis showed that 37 out of 90 isolates were found to be genetically related (Table 1). The isolates showed nine major genotyping patterns (Figure 2).

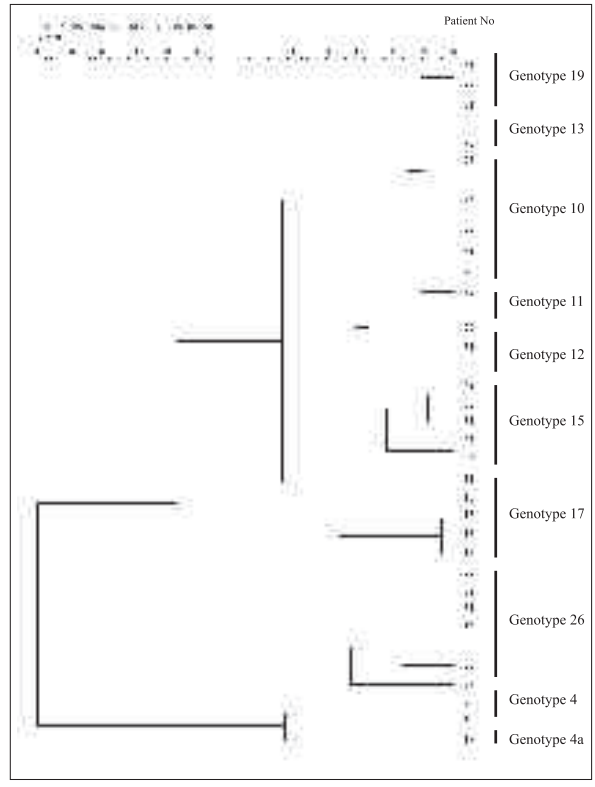

FIGURE 2 - *Nine major clusters identified by dendogram analysis of AP-PCR.*

DISCUSSION

PFGE is the most widely used molecular method in many hospitals and reference laboratories for typing MRSA, but it is time consuming and

expensive (Belkum *et al.,* 1995, Strande'n et. al. 2003). The MRSA isolates from patients suspected of having hospital acquired infections were evaluated retrospectively according to their epidemiological and phenotypical features. We were not informed for the occurence of any outbreak by clinicians. No signs of outbreak were obvious. Therefore we employed AP-PCR for retrospective screening purposes. Additionally all the strains were evaluated according to classic epidemiological data (subjection to same invasive applications, admission to the same clinic or intensive care unit, or recent admission dates to the clinics). Those having the same genotype with accompanying classical epidemiological relation were considered positive, whereas those being in the same genotype according to molecular typing method, but not correlated epidemiologically were interpreted as negative result.

Strains 4 and 5 were isolated from patients intubated simultaneously at the intensive care unit at the same time. Strain 13 closely related to these strains was also hospitalized at the reanimation intensive care unit at the same time as the others. We consider that this patient was probably subjected to intubation there and was infected. These data confirm both the epidemiological and genotypic correlations.

When the patients in genotype 10 with seven members were evaluated, it was observed that most of the patients had been admitted to the intensive care unit and were intubated in the same time period. The isolate from patient 79 was isolated at a different time and admitted to a different intensive care unit. Patients 25 and 36 had not been admitted to the intensive care units. However, both of these patients had been hospitalized in the neurology department. In other wards this strain had somehow found its way to the neurology department. Since the source was not identified, it was considered that there was no epidemiological correlation.

No epidemiological correlation was found between patients infected with strains belonging to AP-PCR genotype 11 and 12. Strains 77 and 78, showing genotype 13 were isolated from two samples of tracheal aspirate at the same intensive care unit simultaneously. It was thought that the contamination occurred between the patients by various ways.

Genotype 15 and closely related 15a were isolated from the patients from various wards within a close period of time. However all of the patients had been admitted for some time to the intensive care unit and were intubated. It appears that the source of infection may be the tracheal aspiration device. A correlation between tracheal aspiration and intubation can be established.

The same is true for the isolates from patients 11, 15 and 16 which yielded genotype 17. These patients had been operated and admitted to the intensive care unit. Patient 18 being in this genotype had been operated but not admitted to the intensive care unit.

Epidemiological correlation of this strain was not found. In other words the source was not identified.

Isolates 86, 87, genotype 19, were isolated from the patients at the same ward and at the same time and epidemiological correlation between these strains was confirmed. No epidemiological correlation was found between these strains and the genotypically related patient 81.

The isolates with the profile of Genotype 26 formed one of the largest clusters.

Epidemiological relation was found in patients 71, 72, 65 and 67 of this cluster. Patients 71 and 72 had been admitted to the same intensive care unit. Patients 65 and 67 had been in the intensive care unit for a short time of period. No epidemiological correlation was found between patients 66 and 69 despite the fact that they were isolated at the same time. In other words, the infection source or transmitter is not certain.

As shown in the table, most of the strains considered as the cause of the hospital infections in our hospital were related to the intensive care unit. The patients admitted to the intensive care and reanimation units comprised 5-10% of total patients hospitalized.

However 25% of all hospital infections occurred in intensive care units.

The rate of hospital infections acquired from the intensive care units were five to ten times higher than the other clinics (Trilla, 1994). Causes of higher frequency of infections in intensive care units are as follows: diabetes mellitus, immunocompromisation, invasive procedures, prolonged duration of hospitalization, shock, coma, use of broad spectrum antibiotics, surgical operations, causative diseases and complications. In

addition, in developing countries, an increased number of patients per healthcare staff and the resulting inadequacy in execution of infection control measures cause hospital infections brought about by resistant pathogens (Goldmann *et al.,* 1997). Craven *et al.* investigated the possible causes of frequent occurrence of hospital infections in patients admitted to the intensive care units and they found a significant correlation between the progress of the hospital infections and 23 variables (Craven *et al.,* 1988). Therefore every intensive care unit must have its own infection control programme directed towards infection control, prevention and surveillance (Ayliffe *et al.,* 1999).

Epidemiologically unrelated strains exist within the genetically correlated genotypes and the sources cannot be determined. We think that if the environmental and personnel searching were conducted on the same date, dramatic results could arise. But the strains we detected are not the ones sent to our laboratory from other clinics. They are not the strains arising from an outbreak either. They are the resistant strains that we had stocked in our archives. It is possible that unnoticed and self limited small outbreaks can occur in hospitals.

In the absence of an outbreak notification and without the detection of MRSA carriage among the hospital staff, the appearance of such clustering among strains indicates that transmission among epidemiologically unrelated patients might have occurred through the hospital staff. Since this is a retrospective study, necessary measures could not be taken promptly.

MRSA isolates from patients hospitalized at departments with a high risk of infection should be tested by molecular epidemiological methods synchronously. Thus, the source of infection should be detected by taking samples from the environment and the staff, the outbreak should be notified promptly and necessary infection control measures should be implemented.

In conclusion in order to implement infection control measures at departments at high risk of nososcomial infections and outbreaks, surveillance studies should be conducted. Detection of microorganisms forming the flora (detection of the colonising microorganisms) and their resistance patterns, and genotyping of the resistant strains are also indicated.

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