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MRSA genotypes in Turkey: Persistence over 10 years of a single clone of ST239

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Summary *Objectives:* Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant cause of life-threatening human infections. The clinical impact of MRSA is mounting, not only due to the ever-increasing prevalence but also due to the occurrence of new, community-acquired MRSA strains. The aim of this prospective, multi-centre study was to determine the prevalence and genetic relatedness of clinically relevant MRSA isolates, in Turkey.
Methods: During a 1-year period, data from 20 successive patients with invasive *S. aureus* infections were collected from eight university hospitals, geographically distributed over the six main regions of Turkey. Among these *S. aureus* isolates, the genetic association of MRSA isolates was investigated by pulsed-field gel electrophoresis (PFGE) and spa typing. A selected number of isolates were also analyzed by multilocus sequence typing (MLST). Furthermore, Panton Valentine leucocidin (PVL) genes were examined.
Results: In this study, the rate of methicillin resistance in *S. aureus* in patients with apparent infections (sepsis, meningitis, lung abscess or septic arthritis) ranged from 12 to 75% within the seven participating centres. Typing by pulsed-field gel electrophoresis and *spa* typing revealed

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the presence of 22 closely related genotypes. According to the PFGE and spa typing results, 53 out of 54 MRSA isolates were closely related. These isolates were of spa type t030 or a related spa type, contain an SCC mec type III element and belong to sequence type ST239. None of the isolates contained the PVL genes.
Conclusions: Despite the broad surface area of Turkey, a single predominant clone of ST239 circulates in hospitals in different regions and only few new types of MRSA were introduced over the past years. These results place Turkey in the epicenter of ST239 prevalence.
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Introduction

Staphylococcus aureus is a leading cause of both hospital acquired and community-onset infections, and a steady increase in methicillin resistance was reported in many regions of the world.[1] Factors contributing to the occurrence of MRSA infections are cross-transmission via the hands of healthcare workers and high selective pressure exerted by broad-spectrum antibiotic therapy. As a result of clonal spread, outbreaks of MRSA have been described in the hospital settings world-wide, and MRSA became endemic in many international healthcare settings.[2] Genotyping of MRSA is important in order to determine outbreaks, dissemination of virulent strains, and the understanding of its epidemiology. PVL is a cytotoxin produced by <5% of *S. aureus* in general and exhibits highly specific lytic activity against polymorphonuclear cells, monocytes, and macrophages in humans. PVL-positive MRSA is strongly associated with skin and soft tissue infections and severe community-onset pneumonia.[3–5]

Turkey being located at the Eurasia region, with a population of 74 million, is divided into seven regions (Fig. 1). MRSA is a well-known cause of infections in Turkey and several local studies showed an MRSA prevalence of 60–90% (highest in the ICU setting) and resistance rates of up-to 20% in community-acquired *S. aureus*.[6–9] As a result, MRSA infections are a major healthcare problem and a serious economic burden for Turkey. The aim of this prospective, multi-centre study was to determine the prevalence and genetic relatedness of clinically relevant MRSA isolates, in Turkey.

Materials and methods

Data collection

During a 1-year period (January 2005–January 2006), we asked for 20 successive *S. aureus* isolated from blood and other sterile fluids from eight university hospitals. These hospitals are geographically distributed over the six main regions of Turkey (Fig. 1). The demographic and clinical information, including age, sex, underlying diseases (diabetes mellitus, trauma, malignancy, immunosuppressive therapy), prior antibiotic usage, source of isolate (blood, pleura, synovia, cerebrospinal fluid), origin of isolate (community or hospital acquired) were recorded. MRSA isolates were classified as community-acquired if they were cultured within the first 48–72 h of hospitalization or in a community setting and if no healthcare-associated risk factors (recent hospitalization, surgery, etc.) were identified.[10] Chi-square test was used for statistical analysis and a p-value of <0.05 was accepted as statistically significant.

Microbiological studies

Organism identification

The isolates were preliminarily identified at the participating hospitals as *S. aureus* on the basis of conventional diagnostic procedures. All MRSA isolates were sent for re-identification and genotyping analysis to The Netherlands Microbiology Laboratory of the Canisius-Wilhelmina

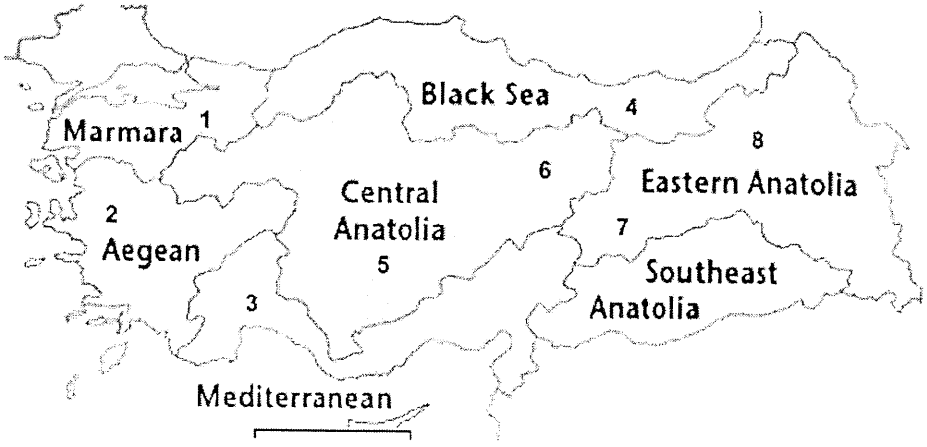


Figure 1 Regions of Turkey and participated centers. 1. Uludag University, 2. Ege University, 3. Akdeniz University, 4. Karadeniz technical University, 5. Erciyes University, 6. Cumhuriyet University, 7. Inonu University, 8. Ataturk University.

Hospital, Nijmegen. The identity of all MRSA isolates was confirmed by the presence of the *mecA gene* and the *S. aureus* specific Sa442 fragment as described earlier.[11]

Susceptibility testing

Susceptibility testing for methicillin was carried out according to the Clinical Laboratory Standards Institute (CLSI) guidelines using Oxacillin agar screen test whereby all MRSA isolates were spot inoculated onto Mueller–Hinton agar supplemented with 6 μg/ml oxacillin and 4% NaCl, from a 0.5 McFarland standard suspension. The plates were incubated at 35 °C for 24 h as recommended by the CLSI. If any growth (more than one colony) was detected, the isolate was considered oxacillin/methicillin resistant.[12]

PFGE analysis

MRSA isolates were typed by PFGE using standard procedures as described.[13] Digestion of the chromosomal DNA was performed using Sma I. Electrophoretically generated DNA profiles were digitized and analyzed using BioNumerics software. Cluster analysis was performed by the unweighted pair-group method on the basis of arithmetic averages (UPGMA). The resulting dendrogram was verified by inspection of the banding patterns by eye. Interpretation of the data was according to established guidelines.[13,14]

spa Typing

Amplification of the hypervariable X region of the spa gene was performed using recommended conditions.[15] The amplified fragments were sequenced on a MegaBACE 500 platform (GE Healthcare, Diegem, Belgium) using conditions recommended by the manufacturer. Obtained sequences were converted into spa types using the web-based spa server software.[16]

Multilocus sequence typing

A subset of isolates was subjected to the established multilocus sequence typing scheme as recommended (http://saureus.mlst.net).

SCC Mec typing

The type of staphylococcal cassette chromosome was determined according to Zhang et al.[17]

PVL gene detection by PCR

Presence of the PVL gene was determined using a real-time assay essentially as described by Johnsson et al.[18]

Results

During the study period 159 patients were included in the study. However one of the centers (center 8) collected only 19 consecutive MRSA isolates, and another center (center 2) did not send information about underlying diseases of the patients and prior antibiotic usage. Among 159 *S. aureus* isolates collected from eight centers, 68 isolates were phenotypically identified as MRSA. Of these isolates, 54 were confirmed to be true MRSA, by molecular methods. Ten isolates were methicillin-resistant non-aureus *staphylococci*, whereas four of them were methicillin-susceptible *S. aureus*. The overall prevalence of MRSA in seven centers was 33%, ranging from 12% (in center 3) to 75% (in center 5) (Table 1).

Table 2 shows the clinical and demographic characteristics of the 134 patients (the information of the patients from center 8 was not included). Overall, 58% (78 of 134) of the patients were male. The median age was 54 (range less than 1–87 years). Diabetes mellitus was more common in patients with methicillin-susceptible *S. aureus* (MSSA) infections, whereas malignancy was more frequent in patients with MRSA infections ($p = 0.02$). Moreover, prior antibiotic usage was a major risk factor for the development of MRSA infections (OR = 5.53).

Most *S. aureus* isolates originated from blood cultures (92%); the others were from cerebrospinal fluid (2%), peritoneum (4%), pleura (1%) and synovia (1%), respectively (Table 3). In 93% of the cases with MRSA infection, the infection was nosocomially acquired, whereas this was the case in 65% of the patients with MSSA infections ($p = 0.009$).

Among the 54 MRSA isolates 4 different *spa* types were found. *spa* Type t030 was by far the predominant spa type present (89%). Two other *spa* types (t459 and t1459) contain a repeat sequence that could easily be derived from t030 by deletion of one or more repeat units. The presence of isolates with identical PFGE patterns but of spa type t030 and t459, supports this relationship (results not shown). Using PFGE analysis, the isolates could be grouped into 23 different genotypes, of which PFGE types A–V are closely related (Table 4) and also appear to be very similar to the Turkish MRSA isolates that were collected more than a decade ago.[19] None of the 54 isolates tested positive for the PVL gene. PFGE type Z was unrelated and was of a very different unrelated *spa* type (t189). One random isolate from each of the five most prevalent PFGE types was selected for multilocus sequence typing. All of them were of sequence type ST239. SCC Mec typing showed the presence of an SCC Mec type III element in all MRSA isolates.

Discussion

S. aureus is a major cause of life-threatening infections both in the hospital and community settings. EARSS data

Table 1 Prevalence of MRSA by centres.

Center (University)	MRSA *n*/total[a] (%)
1 (Uludag)	4/18 (20)
2 (Ege)	11/20 (55)
3 (Akdeniz)	2/17 (12)
4 (Karadeniz Technical)	4/20 (20)
5 (Erciyes)	15/20 (75)
6 (Cumhuriyet)	3/20 (15)
7 (Inonu)	3/19 (16)
8 (Atatürk)[b]	n.d.[b]

[a] while centres were asked to include 20 consecutive, invasive *S. aureus* isolates, number of included isolates may differ since some isolates were identified as coagulase-negative staphylococci at validation.

[b] n.d. = not done, centre contributed 19 MRSA isolates, whereas four of them were coagulase-negative staphylococci at validation, and three of them methicillin-susceptible *S. aureus* isolates.

Table 2 Demographic and clinical information of patients.

	MSSA patients ($n = 92$)	MRSA patients ($n = 42$)	Total ($n = 134$) [a]	p-Value
Age (median) (min–max)	53 (0–85)	58 (0–87)	54 (0–87)	n.s.[c]
Sex (male/female)	54/38	24/18	78/56	n.s.
Community-acquired	35%	7%	26%	0.009
Hospital-acquired	65%	93%	74%	
Underlying disease	($n = 83$)	($n = 31$)	($n = 114$)[b]	
Diabetes mellitus	21%	3%	16%	0.02
Trauma	4%	6%	4%	n.s.
Malignancy	16%	35%	21%	0.02
Immunosuppressive therapy	17%	10%	15%	n.s.
Prior antibiotic usage	19%	74%	34%	0.000

[a] The information of the patients' from center 8 was not included.
[b] The center 2 did not send information about the patients' underlying diseases and prior antibiotic usage.
[c] n.s. non-significant.

from South-Eastern European countries, as well as the results of Turkish single-centre reports, or studies limited to high-risk units, indicate a high prevalence of MRSA among patients with nosocomial infections in Turkey.[6–9] As well, in a last prevalence study from southern and eastern Mediterranean countries, the rate of methicillin resistance in *S. aureus* isolates from blood cultures was 40% in Turkey.[20] Also the aim of this study was to determine the prevalence and genetic relatedness of MRSA in all different regions of Turkey, using standardized validation methods.

The majority of *S. aureus* infections was hospital-acquired (74%) and the prevalence of MRSA in the seven participating centers varied from 12% to 75%. MRSA infections were significantly more frequently of nosocomial origin, compared to the proportion of infections caused by

Table 3 The source of *S. aureus* isolates.

Center (University)	Source of *S. aureus* (n)				
	Blood	Peritoneum	Cerebrospinal fluid	Pleura	Synovia
1 (Uludag)					
MSSA	13	1			
MRSA	4				
2 (Ege)					
MSSA	9				
MRSA	9		1	1	
3 (Akdeniz)					
MSSA	15				
MRSA	2				
4 (Karadeniz Technical)					
MSSA	15			1	
MRSA	3	1			
5 (Erciyes)					
MSSA	4	1			
MRSA	12	1	2		
6 (Cumhuriyet)					
MSSA	17				
MRSA	2	1			
7 (Inonu)					
MSSA	15				1
MRSA	3				
Total	123	5	3	2	1

Table 4 Results of molecular typing.

PFGE types ($n = 23$)	Spa types ($n = 4$)
A (14), C(7), D(6), E(2), F(3), G(2), I(1), J(1), K(1), L(1), M(1), N(1), O(1), P(1), Q(1), R(1), S(1), T(1), U(1),V(1)	T030
A (1) B (3)	T459
H (1)	T1459
Z (1)	T189

methicillin-susceptible isolates (93% vs 65%). The prevalence of community-acquired (CA)-MRSA (7%) was slightly lower than the prevalence found in earlier, local studies.[9] Prior antibiotic usage was a major risk factor for the development of MRSA infections, probably due to promoting the selection of resistant strains.

The high prevalence of MRSA infections is an important healthcare burden, associated with increased mortality and morbidity. Effective infection control programs, including early detection of MRSA carriers, pre-emptive isolation, new decontamination regimens, restriction of certain antibiotic classes, environmental control and adequate hand hygiene are needed to control the spread of MRSA.[21]

PVL is an exoprotein of *S. aureus* that has been linked to furuncles, cutaneous abscesses, severe necrotic skin infections and severe necrotising pneumonia. None of the MRSA isolates of our study (>90% from patients with bacteremia) was PVL positive. While PVL is associated with an increased incidence of the above-mentioned *S. aureus* infections, the result is not surprising, since other investigators suggested that, PVL is not an important virulence factor in the pathogenesis of staphylococcal bacteremia.[18]

The 54 MRSA isolates typed in the present study originated from eight geographically well-separated hospitals in Turkey. We used spa-typing since it has several advantages in terms of speed, ease of use, ease of interpretation and database creation,[15,16,22] but furthermore applied PFGE since it – especially in isolates with a clonal background – has a higher discriminatory power.[23] Furthermore, PFGE is still considered to be the "gold standard" for MRSA strain typing. While we found 23 distinguishable PFGE pattern (A–V, Z), types A–V all appeared to be closely related to each other since most of these genotypes differed by a single band. All these isolates furthermore shared a common *spa* type (t030 or the closely related *spa* types t459 and t1459) and were of MLST sequence type ST239, indicating that 53 of the 54 isolates (98%) were clonally related. Consequently neither spa-typing nor MLST will be of use to investigate possible MRSA outbreak situations in Turkish hospitals.

ST239 is a major pandemic MRSA clone (http://saureus.mlst.org). The almost exclusive presence of this clone in this study puts Turkey in the epicenter of ST239 prevalence. In 1997, van Belkum et al.[19] found a single predominant circulating clone among 39 MRSA isolates obtained from seven Turkish hospitals located in two connected cities of central Turkey. Since no *spa* typing result or MLST data is available from this other collection, the only way of comparing our results to these other Turkish isolates is by visual comparison of the published PFGE patterns. Despite subtle genetic differences in the PFGE patterns, the isolates in the present study appear to be closely related to the isolates described by van Belkum and colleagues.[19] So, even after ten years, a single MRSA clone is predominantly present in Turkey and new clones seem to appear only sporadically.

To our knowledge, this is the first nation-wide multicenter molecular epidemiology study in Turkey. Although Istanbul, the biggest city of Turkey in Marmara Region, and one region (southeast) did not participate in the study, the prevalence of MRSA among invasive isolates of *S. aureus* is high. It might have been expected from the EARSS results of other countries in the SouthEast of Europe. Despite the extremely broad surface area of Turkey, the majority of hospital-acquired MRSA bacteremias is caused by a single MRSA clone.

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