Journal of Infection and Public Health 12 (2019) 528–533

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Absence of the mecC gene in methicillin-resistant *Staphylococcus aureus* isolated from various clinical samples: The first multi-centered study in Turkey

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ARTICLE INFO

Article history:
Received 24 October 2018
Received in revised form 21 January 2019
Accepted 23 January 2019

Keywords:
Methicillin resistance
Staphylococcus aureus
mecC
mecA
Turkey

ABSTRACT

Background: *mecA* is a predefined gene causing methicillin resistance in *Staphylococcus aureus (S. aureus)* isolates; however, it has been shown that some methicillin-resistant *S. aureus* (MRSA) strains do not carry this gene. Recently, in isolates found to be MRSA-positive but *mecA*-negative, a new resistance gene called *mecC*, which is a homolog of *mecA*, has been reported. This study aimed to investigate the *mecC* and *mecA* genes in MRSA strains isolated from different geographic regions in Turkey.
Methods: The sample of the study consisted of 494 MRSA strains isolated from seven geographical regions in Turkey between 2013 and 2016. The strains were obtained from 17 centers, comprising 13 university hospitals, three education and research hospitals, and one state hospital. Methicillin resistance in *S. aureus* strains was determined using the agar disk diffusion method with a cefoxitin disk and the agar dilution method with oxacillin. The *mecC* and *mecA* genes in MRSA strains was investigated by Polymerase Chain Reaction (PCR).
Results: Of the MRSA strains investigated, 47.9% were isolated from intensive care units. Concerning sample type, 36.7% were detected in the respiratory tract (tracheal aspirate, sputum, etc.), 24.8% in blood, 18.7% in skin and soft tissues, 9.3% in nasal swabs, 5.4% in urine, 4.1% in ears, and 1% in sterile body fluid. Using PCR, *mecC* was not identified in any of the *S. aureus* strains isolated from different clinical microbiology laboratories. *mecA* gene positivity was found in 315 of the MRSA strains (63.8%). Staphylococcal Cassette Chromosome *mec* (SCC*mec*) type was identified in 232 strains (46.9%), of which 136 (58.7%) were type II, 75 (32.4%) were type IV, 12 (5.1%) were type IIIb, six (2.5%) were type I, and three (1.3%) were type III.

Abbreviations: *S. aureus*, *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*; PCR, polymerase chain reaction; SCC*mec*, staphylococcal cassette chromosome *mec*; PBP 2a, penicillin-binding protein 2a; *SCCs*, staphylococcal cassette chromosomes; CLSI, clinical and laboratory standards institute; PBS, phosphate buffer saline; SPSS, statistical package for the social sciences; TBE, tris-borate-EDTA buffer; MSSA, methicillin-susceptible *Staphylococcus aureus*; HA-MRSA, hospital-acquired MRSA; CA-MRSA, community-acquired MRSA.

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https://doi.org/10.1016/j.jiph.2019.01.063

Conclusion: This is the first multi-centered study to investigate MRSA strains isolated from different regions in Turkey. The *mecC* gene was not detected in any of the MRSA strains. We believe that this study will constitute an important basis for monitoring possible future changes.

Introduction

Staphylococcus aureus primarily causes skin and soft tissue infections, as well as other serious infections, such as pneumonia, osteomyelitis, meningitis, and endocarditis [1]. While *S. aureus* strains used to be only resistant to penicillin and its derivatives through the mechanism of β-lactamase production, they developed resistance to all β-lactam antibiotics as a result of the change in penicillin-binding protein 2a (PBP 2a) found in their genomes [2,3].

Methicillin-resistant *S. aureus* (MRSA) strains produce an altered penicillin-binding protein (PBP 2a) encoded by *mecA*, a gene located within a mobile genetic element called the Staphylococcal Chromosome Cassette *mec* (SCC*mec*) [4,5]. SCC*mec* elements are highly diverse in their structural organization and genetic content and have been classified into types from type I to type XIII [6,7].

In 2005, Livestock-Associated MRSA (LA-MRSA) CC398 was detected for the first time in pigs and pig farmers. From this period onwards, it was determined that the pig population was a reservoir for LA-MRSA, but LA-MRSA was also found in a wide range animals such as chickens, horses, sheep, goats, calves, and dairy cattle [8–10]. In a study on cattle mastitis in the United Kingdom (UK), an oxacillin- and methicillin-resistant *S. aureus* isolate was phenotypically identified in a sample obtained from a milk tank [11]. However, it was determined that this isolate did not carry the *mecA* gene. The genomic sequence analysis of this isolate, LGA251, revealed that it carried a *mecA* homolog ($mecA_{LGA251}$) with approximately 69% similarity to the classical *mecA* gene, and this newly identified gene encoded a protein with approximately 63% similarity to the PBP 2a protein and was named *mecC* in 2012 [12].

mecC MRSA has been reported in a wide range of other host species, including livestock, wildlife and companion animals in many European countries. As in human isolates, those isolated from animals are associated more with the clonal complex 130 (CC130) and with ST425 to a lesser degree. Therefore, this lineage seems to have a broad host tropism [13]. Several studies have shown that *mecC*-positive MRSA is relatively common in dairy cattle, suggesting that cattle provide an infection reservoir and farmers in contact with dairy cattle may be at risk of acquiring these isolates [10,14,15]. Furthermore, researchers using whole-genome sequencing have reported evidence of zoonotic transmission of *mecC*-positive MRSA from cattle to humans [16]. In addition, MRSA carrying the *mecC* gene was found in sheep, which might be considered as another reservoir [15]. *mecC* MRSA is currently rare in humans, but there are interesting geographical differences in terms of prevalence; in particular, the latest increase in prevalence in Denmark underlines the need to monitor *mecC* MRSA [10,16]. However, to the best of our knowledge, there is no comprehensive study that examined the *mecC* gene in Turkey. Therefore, this study aimed to explore the presence of the *mecC* gene in MRSA strains isolated from different geographic regions in Turkey.

Materials and methods

Study area and bacterial isolates

There are seven geographical regions in Turkey. The sample of this study consisted of 494 MRSA strains isolated from all seven regions between 2013 and 2016. Of these strains, 120 were obtained from Central Anatolia, 111 from Black Sea, 102 from Marmara, 80 from Eastern Anatolia, 47 Aegean, 18 from South East Anatolia, and 16 from Mediterranean.

The study was carried out in Erzincan Binali Yıldırım University Mengücek Gazi Training and Research Hospital Microbiology Laboratory. The strains were transferred to our laboratory in cryobeads in accordance with cold chain transportation rules and stored at −80 °C until analysis. The cryobeads were thawed at room temperature before enrichment in tryptic soy broth and cultured onto a blood agar.

Antibiotic susceptibility testing

The methicillin resistance was determined using the Kirby-Bauer disk diffusion method with a cefoxitin disk (30 μg, Oxoid, UK) and the agar dilution method with oxacillin (Sigma-Aldrich, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) standards [17]. For the cefoxitin disk diffusion assay, a suspension of colonies was grown for 24 h and adjusted to give a turbidity equivalent to 0.5 McFarland standard. The bacterial suspension was spread over the surface of the Mueller–Hinton agar medium (Oxoid, UK) by swabbing, and cefoxitin disks were placed on the surface of the agar. The plates were incubated at 37 °C for 16–18 h; then, the zone diameters were measured, and the isolates with a zone diameter of ≤21 mm for cefoxitin were considered to be methicillin-resistant. For the oxacillin agar dilution assay, 0.5 McFarland standards were prepared from freshly grown bacterial cultures. This suspension was inoculated onto the Mueller Hinton agar medium (+4% NaCl) containing 6 μg/ml oxacillin and incubated at 37 °C for 24 h. Testing at temperatures above 35 °C may not detect MRSA. >1 colony growth was considered oxacillin-resistant [17].

DNA isolation

The isolates were subcultured on trypticase soya agar plates (bioMérieux, France) prior to DNA extraction. For DNA extraction, one colony was suspended on 500 μl of sterile phosphate buffer saline (PBS) (pH: 7.2). The bacterial cells were harvested by centrifugation at 3000 × *g* for 10 min, and the pellet was resuspended in 350 μl TE buffer [10 mm tris chloride, 1 mm EDTA (pH 8.0)] containing nystatin (100 μg/ml) (Sigma, St Louis, MO, USA) and incubated at 37 °C for 1 h, vortexing every 15 min. Then, 350 μl of 10% sodium dodecyl sulfate (SDS) containing proteinase K (100 μg/ml) (Vivantis Technologies, Malaysia) was added and incubated at 37 °C for 2 h, vortexing every 15 min. Next, DNA was extracted using the phenol/chloroform method as described by Sambrook and Russell [18]. The DNA was eluted in 100 μL of TE buffer [10 mM Tris chloride–1 mM EDTA (pH 8.0)], and stored at −20 °C until use.

*Multiplex PCR for SCC*mec *typing*

The cycling conditions and primers as described by Oliveira and de Lencastre [19] were used to detect the *mecA* gene and SCC*mec* types (I–IV) (Table 1). The primer sets used for the assignment of the *mecA* gene and SCC*mec* types are listed in Table 1. The multiplex PCR was performed in a 50 μl volume: 1 × PCR buffer; 200 μM (each) dNTP; 200 nM concentrations of primers KDP F1, KDP R1,

Table 1
Primers used in multiplex PCR [17].

Locus	Primer	Oligonucleotide sequence (5′-3′)	Location	Amplicon size (bp)	Specificity (SCC*mec* type)
A	CIF2 F2 CIF2 R2	5′-TTCGAGTTGCTGATGAAGAAGG −3′ 5′-ATTTACCACAAGGACTACCAGC-3′	18398-18419 18892-18871	495	I
B	KDP F1 KDP R1	5′-AATCATCTGCCATTGGTGATGC-3′ 5′-CGAATGAAGTGAAAGAAAGTGG-3′	10445-10467 10728-10707	284	II
C	MECI P2 MECI P3	5′-ATCAAGACTTGCATTCAGGC-3′ 5′-GCGGTTTCAATTCACTTGTC-3′	42428-42447 42636-42617	209	II, III
D	DCS F2 DCS R1	5′-CATCCTATGATAGCTTGGTC-3′ 5′-CTAAATCATAGCCATGACCG-3′	38011-37992 37670-37689	342	I, II, IV
E	RIF4 F3 RIF4 R9	5′-GTGATTGTTCGAGATATGTGG-3′ 5′-CGCTTTATCTGTATCTATCGC-3′	45587-45607 45829-45809	243	III
F	RIF5 F10 RIF5 R13	5′-TTCTTAAGTACACGCTGAATCG-3′ 5′-GTCACAGTAATTCCATCAATGC-3′	59573-59594 59986-59965	414	III
G	IS431 P4 pUB110 R1	5′-CAGGTCTCTTCAGATCTACG-3′ 5′-GAGCCATAAACACCAATAGCC-3′	49963-49982 50343-50323	381	
H	IS431 P4 pT181 R1	5′-CAGGTCTCTTCAGATCTACG-3′ 5′-GAAGAATGGGGAAAGCTTCAC-3′	29654-29673 29976-29956	303	
*mec*A	MECA P4 MECA P7	5′-TCCAGATTACAACTTCACCAGG-3′ 5′-CCACTTCATATCTTGTAACG-3′	1190-1211 1351-1332	162	Internal control

RIF4 F3, and RIF4 R9; 400 nM concentrations of primers CIF2 F2, CIF2 R2, MECI P2, MECI P3, RIF5 F10, RIF5 R13, pUB110 R1, and pT181 R1; 800 nM concentrations of primers DCS F2, DCS R2, MECA P4, MECA P7, and IS431 P4; 1.5 U of Taq DNA Polymerase (Vivantis Technologies, Malaysia); and approximately 5 ng of template DNA.

PCR was carried out in a DNA Thermal Cycler CFX 96 (Bio-Rad, Hercules, CA) with the following conditions: predenaturation for 4 min at 94 °C, 30 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min; post-extension for 4 min at 72 °C; and soaking at 4 °C. The PCR products were electrophoresed on 2% agarose gel in 0.5 × Tris-borate-EDTA (TBE) buffer (Vivantis Technologies, Malaysia) and stained with ethidium bromide (0.5 μg/ml). *S. aureus* ATCC 43300 was used as the positive control strain.

Detection of the mecC *gene*

For the detection of the *mecC* gene by PCR, we used the following primers described by Stegger et al. [20] as follows: 5′-GAA AAA AAG GCT TAG AAC GCC TC-3′ (forward) and 5′-GAA GAT CTT TTC CGT TTT CAG C-3′ (reverse). Amplification was performed with the following conditions: 15 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 59 °C, and 1 min at 72 °C, with a final 10 min elongation step at 72 °C. The PCR products were electrophoresed on 2% agarose gel in 0.5 × Tris-borate-EDTA (TBE) buffer (Vivantis Technologies, Malaysia) and stained with ethidium bromide (0.5 μg/ml). *S. aureus* NCTC 13552 was used as the positive control strain.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS, IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY) was used to evaluate the data. Continuous variables were summarized as means ± standard deviation, and categorical variables as n (%).

Results

The *S. aureus*-isolated patients were between the ages of 1 and 92, and the mean age was 54.8 ± 2.4 years. Of the strains, 269 (54.5%) were isolated from male patients and 225 (45.5%) from females.

Table 2
Distribution of *S. aureus* strains by clinic.

Clinic	Total (n/%)
Intensive care unit	237 (47.9)
Internal medicine	53 (10.8)
Pediatric service	40 (8.1)
Orthopedics	35 (7.0)
Otorhinolaryngology	23 (4.6)
Infectious diseases	21 (4.3)
Dermatology	17 (3.5)
Urology	16 (3.3)
Pulmonary diseases	16 (3.3)
Other	36 (7.2)
Total	494 (100)

Table 3
Distribution of *S. aureus* strains by type of sample.

Type of Sample	Total (n/%)
Respiratory (tracheal aspirate. sputum. etc.)	181 (36.7)
Blood	123 (24.8)
Skin and soft tissue	92 (18.7)
Nose	46 (9.3)
Urine	27 (5.4)
Ears	20 (4.1)
Sterile body fluid	5 (1.0)
Total	494 (100)

Fig. 2 and Table 2 present the distributions of the MRSA strains according to the geographical regions and clinics from which they were isolated, respectively. Most of the MRSA strains were isolated from intensive care units (47.9%), followed by internal medicine (10.8%), pediatrics (8.1%), and orthopedics and traumatology services (7%).

Concerning the type of samples, most MRSA strains were isolated from the respiratory tract (sputum, tracheal aspirate, bronchoalveolar lavage etc.), blood, skin and soft tissues. Table 3 presents the distribution of all MRSA strains by type of sample.

All isolates were reported to be phenotypically resistant to oxacillin and cefoxitin, and identified as MRSA by diagnostic laboratory analysis. In this study, the multiplex PCR did not reveal the presence of *mecC* gene in any of the 494 MRSA isolates obtained from all seven geographical regions in Turkey. The *mecA* gene was positive in 315 of the MRSA strains (63.8%) and the SCC*mec* type was

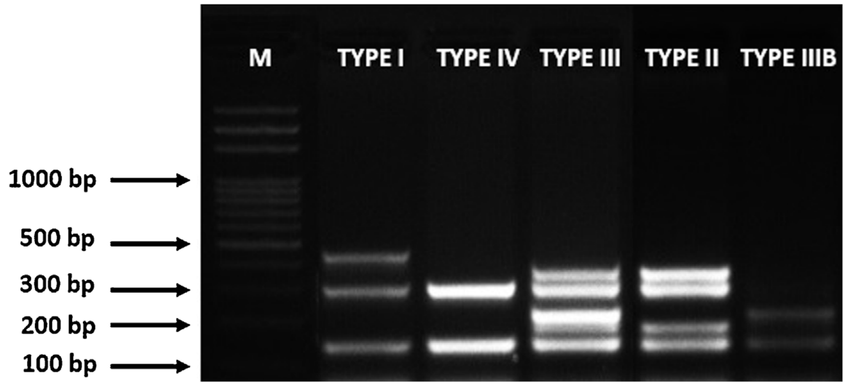

Fig. 1. Representative gel demonstrating the expected PCR products for SCC*mec* types. I–IV. Lanes M: DNA molecular size marker (VC 100 bp Plus DNA Ladder, Vivantis Technologies, Malaysia).

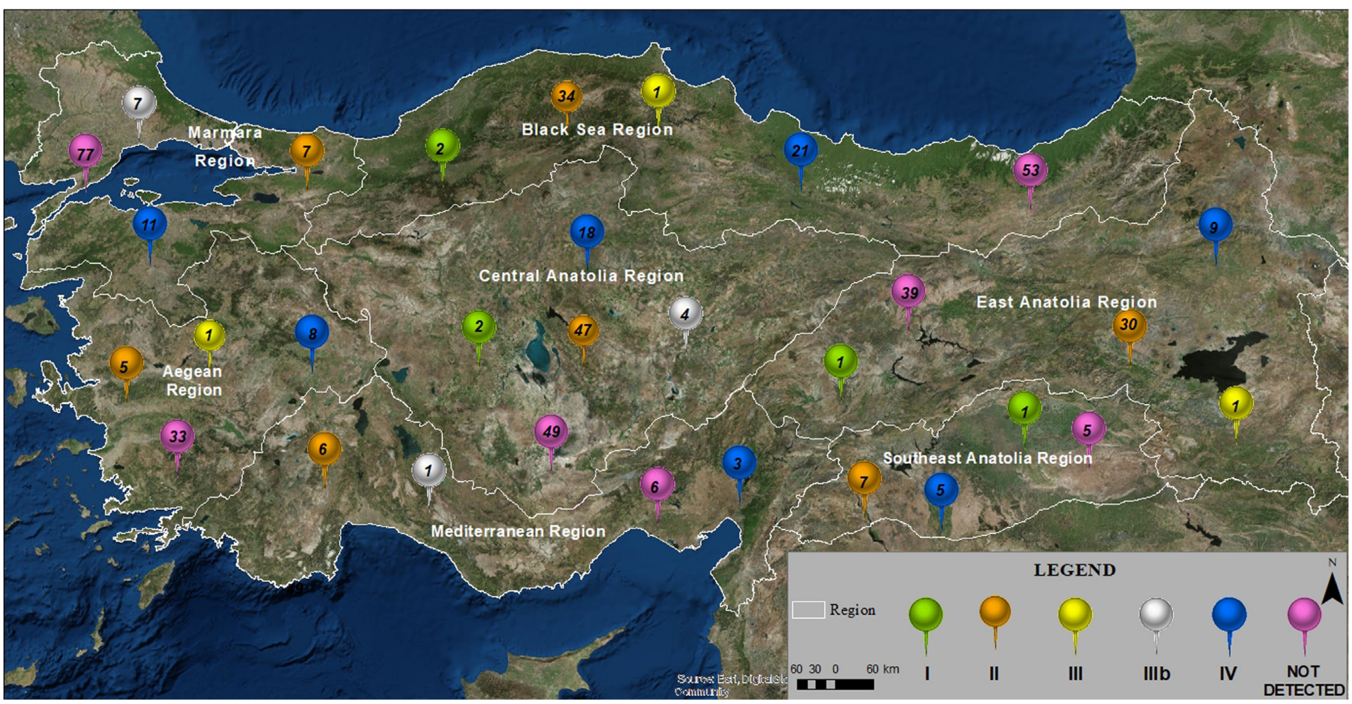

Fig. 2. Distribution of the investigated *S. aureus* strains and *SCCmec* types by geographical region.

identified in 232 strains (46.9%) as follows: type II in 136 (58.7%), type IV in 75 (32.4%), type IIIb in 12 (5.1%), type I in six (2.5%), and type III in three (1.3%) (Fig. 1). Fig. 2 presents the distribution of MRSA SCC*mec* types by geographical region.

Discussion

MRSA, is a major pathogen associated with severe nosocomial infections due to its multidrug resistance that limits treatment options. In addition to being resistant to all β-lactams, MRSA can also show resistance to antimicrobials included in macrolide, quinolone, tetracycline, lincosamide and aminoglycoside groups [21]. Therefore, the diagnosis and detection of MRSA is necessary and important for the selection of appropriate treatment for patients [22].

MRSA isolates are detected by the disk diffusion method using a cefoxitin disk or the agar dilution method with oxacillin. Oxacillin maintains its activity during storage better than methicillin and is more likely to detect heteroresistant strains. However, cefoxitin is an even better inducer of the *mecA* gene, and tests using cefoxitin provide more reproducible and accurate results than those with oxacillin. In addition, CLSI, recommends the cefoxitin disk screen test. However, detecting the *mecA* gene by PCR is the gold standard method [17,23,24]. To date, 13 different SCC*mec* types have been defined [7]. The SCC*mec* type of hospital-acquired MRSA (HA-MRSA) differs from that of community-acquired MRSA (CA-MRSA) [25,26], with types I, II and III being mostly found in HA-MRSA and types IV and V in CA-MRSA [25–27].

Several studies conducted in Turkey showed that SCC*mec* type III was the most common. For example, Akoğlu et al. [28] reported SCC*mec* type III in 61.8%, type IIIb in 34.5%, and type IV in 2.7% of HA-MRSA strains. Kilic et al. [29] demonstrated that SCC*mec* type III was predominant (82.1%) in a tertiary care facility over a period of four years. In another epidemiological study conducted between 2006 and 2008, Bozdoğan et al. investigated the SCC*mec* types of 397 MRSA strains isolated from different geographical regions in Turkey and identified type III in 91.4% and type IV in 7.6% of the samples [30].

In this study, the SCC*mec* type of 232 MRSA strains was defined. Unlike many studies undertaken in Turkey, we found SCC*mec* type II to be the most common type. In previous studies, the SCC*mec* type of MRSA strains was shown to vary between countries, between different centers in the same country, or even in the same region over time. For example, of the MRSA strains isolated from Japan between 1979 and 1985, 53.6% were reported to be of SCC*mec* type IV, 22.7% type I, and 21.6% type II. However, after the 1990s, sequence type 5 (ST5)-SCC*mec* type II was found to be the dominant clinical MRSA strain in Japanese hospitals [31]. In the current study, 47.9% of the strains were isolated from intensive care units, 36.7% from respiratory samples, and 24.8% from blood samples. We consider that our results differing from those of other studies conducted in Turkey may be due to the differences in the study period and patient groups.

It was previously reported that in MRSA strains that did not carry the *mecA* gene, methicillin resistance was caused by the *mecC* gene [15]. *mecC* was first isolated from a bulk tank milk sample in southwest England [11]. The discovery of MRSA carrying the *mecC* gene has led to the investigation of the source and epidemiology of these isolates in many countries. In a previous study, *mecC* was identified in various samples obtained from 14 different local and wild animal species in 13 European countries [13]. Furthermore, *mecC*-positive *S. aureus* isolates were not only detected in animal species, but also in humans. Although these isolates are less common among humans, MRSA strains carrying the *mecC* gene may become problematic for public health since phenotypic and genotypic tests are not sufficient to properly detect this gene [32].

In a study conducted in Denmark between 2003 and 2011, the *mecC* positivity was reported to be 1.5%, and the authors noted an increase in *mecC*-positive samples from 1.9% in 2010 to 2.8% in 2011 [10]. In Germany, the presence of *mecC* was investigated in 1604 MRSA strains isolated in 2004 and 2005, and 1603 strains isolated in 2010 and 2011, and only one isolate (0.06%) was found to be *mecC*-positive for each period [33].

In studies conducted in various countries, *mecC* was not detected in 102 MRSA strains isolated from wounded military personnel in the United States of America (USA) between 2009 and 2011 [34], 34 MRSA strains isolated from a dental clinic in Egypt [35], and any of the 500 *S. aureus* isolates collected in the UK in 2012 and 2013 [36].

In Turkey, only one study was found to have investigated the *mec*C gene in MRSA strains isolated from humans. In that study, Kılıç et al. [37] isolated 1177 MSSA (Methicillin-Susceptible *S. aureus*) and 523 MRSA strains isolated from various clinical samples in a hospital between 2007 and 2014, and reported that *mecC* was not present in any of the samples.

Similarly, in the current study that explored the presence of the *mecC* gene in a selected number of strains collected from all geographical regions in Turkey, we did not identify any MRSA isolate that carried this gene.

Recently, during routine MRSA screening in an *S. aureus* isolate that tested negative for *mecA* and *mecC* but thought to be methicillin resistant, Becker et al. discovered a plasmid carrying the *mecB* gene. The isolate had been obtained from the nasal-throat swab of a 67-year-old cardiology patient with no sign of infection. A comparative analysis of the *mec*B DNA of *S. aureus* revealed that *Macroccocus caseolyticus* had 100% sequence identity with the reported *mecB* gene, and thus belonged to the same allotype. The *mecB* homolog of *S. aureus* shows a 60% nucleotide sequence similarity to the originally identified *mecA* gene of *S. aureus* N315. As in the *mecA* and *mecC* genes, *mecB* in *S. aureus* results in methicillin resistance, and therefore the strains it carries should be accurately identified as MRSA, rather than as MSSA. This can be achieved using antibiotic susceptibility testing. However, the PCR method with *mecB*-specific primers should also be undertaken for the accurate identification of MRSA strains [38,39].

The limitation of the current study was that the presence of *mecA* and *mecC* genes was not detected in the 179 isolates that were found to have phenotypic methicillin resistance. The absence of the detection of *mecA* and *mecC* genes in these isolates may be related to the PCR method used or the presence of a different *mec* gene (*mecB*).

In conclusion, certain questions remain unanswered about the *mecC* gene, concerning the origin of the *mec* homolog, the actual distribution and prevalence in animal populations and human beings, and the real zoonotic potential of the staphylococcal isolates harboring *mecC*. Our study was the first multi-centered study that investigated the presence of the *mecC* gene in MRSA strains isolated from all geographical regions in Turkey. To date, no MRSA strain isolated from human beings in Turkey has been reported to carry the *mecC* gene. However, considering that MRSA isolates with the *mecC* gene were found to have rapidly spread across Europe after the identification of farm animals as reservoirs, it is very likely that this gene will also be detected in Turkey in the near future. We believe that this study will provide an important basis for monitoring possible future changes. We also consider that there is a need for further studies to investigate the types of *mecA* in MRSA strains.

Funding

No funding sources.

Competing interests

None declared.

Ethical approval

This study was approved by the Clinical Research Ethics Board of Erzincan Binali Yildirim University, Faculty of Medicine (No: 01/04, Date: 06.02.2015).

Authors' contributions

The conception and design of the study: AC. Collected isolates: AC, MA, BG, FK, MGK, SY, MP, BG, ACC, FBB, IHC, MK, SA, TO. Acquisition of data: AC, MA, BG. Analysis and interpretation of data: AC, MA. Critical revision of the manuscript for important intellectual content and final approval of the manuscript: AC, MA. All authors read and approved the final manuscript.

Acknowledgements

This study was financially supported by the Scientific Research and Project Unit of Erzincan Binali Yildirim University (Project No: SAG-A-240215-0121). This research was presented as a poster in the 4th National Clinical Microbiology Congress in Antalya, Turkey, 2017.

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