High Occurence of Methicillin Resistant *Staphylococcus sciuri* (MRSS) and First Detection of *mec*C from Broiler Flocks in Turkey

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ABSTRACT

In this study, twelve broiler farms were randomly sampled in three different cities (Adana, Hatay and Sakarya) in Turkey, to investigate the presence and genetic diversity of methicillin resistant coagulase-negative staphylococci (MRCoNS). Four houses for each broiler flock were selected for sampling and one hundred throat swabs from each house, from each broiler flock were collected and divided into five pools of 20 swabs. Overall, 63 methicillin resistant *S. sciuri* were isolated, and *mec*A was alone detected in 56 isolates, seven isolates were positive not only for *mec*A, but also for *mec*C. Most of the isolates (77.8%) were non-typeaple for SCC*mec* and ten isolates (15.9%) carried SCC*mec* type III. All isolates were resistant to cefoxitin and penicillin, and the frequency of the isolates for trimethoprim/sulfamethoxazole, clindamycin, erythromycin, tetracycline resistance ranged between 66.7% and 73%. The PFGE analysis revealed the presence of 47 distinct pulsotypes. The most common resistant genes detected were *lnu*A and *tet* genes. None of the isolates was positive for toxin and disinfectant resistance genes. The present study is the first reporting the presence of the *mec*C gene among CoNS in Turkey. The results of this study also indicated that broiler flocks were potential source of multi-resistant methicillin resistant *S. sciuri*, with a high diversity.

Key words: Antimicrobial Resistance; Broiler; *mec*A; *mec*C; *Staphylococcus sciuri.*

INTRODUCTION

Antimicrobial resistance among staphylococci is mediated by different mechanisms. One of the important resistance mechanisms is methicillin resistance, caused by an alternate penicillin-binding protein (PBP2a) encoded by the *mec*A gene, having low affinity to beta-lactams. The *mec*A gene is located on a mobile genetic element that is designated Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (1), based on the differences in the SCC*mec* structural organization, 13 major SCC*mec* types have been discovered and further divided into subtypes (2, 3). SCC*mec* element contains both the *mec* complex and cassette chromosom recombinase (*ccr)* genes. In 2011, Garcìa-Álvarez *et al*. (4) discovered a novel *mec* homologue sharing approximately 70% similarity to the *mec*A gene located in SCC*mec* type XI, referred as *mec*A_{LGA251}, and but later re-designated it as *mec*C. The first one is *mec*C allotypes have been reported, namely, *mec*C1 in *S. sciuri* (share 96.3% nucleotide similarity with *mec*C in *S. aureus* LGA251) was reported by Garcìa-Álvarez *et al*. (4). The second is *mec*C2 in *S. saprophyticus* (share 92.9% nucleotide similarity with *mec*C in *S. aureus* LGA251) was reported by Małyszko *et al*. (5), and third is *mec*C3 in *S. caeli* (share 93% nucleotide similarity with *mec*C in *S. aureus* LGA251) was reported by MacFadyen *et al*. (6). Very recently, Becker *et al*. (7) described plasmid encoded transferable *mecB* in *S. aureus,* similar to previously reported *mec*B genes in *Micrococcos caseolyticus* found as part of an SCC*mec* element on chromosome as well as on a plasmid (8, 9).

S. sciuri is commonly found on skin and mucous membranes of warm blooded animals as well as in the environment (10). Although it is often associated with opportunistic infections or considered as having the less pathogenicity,

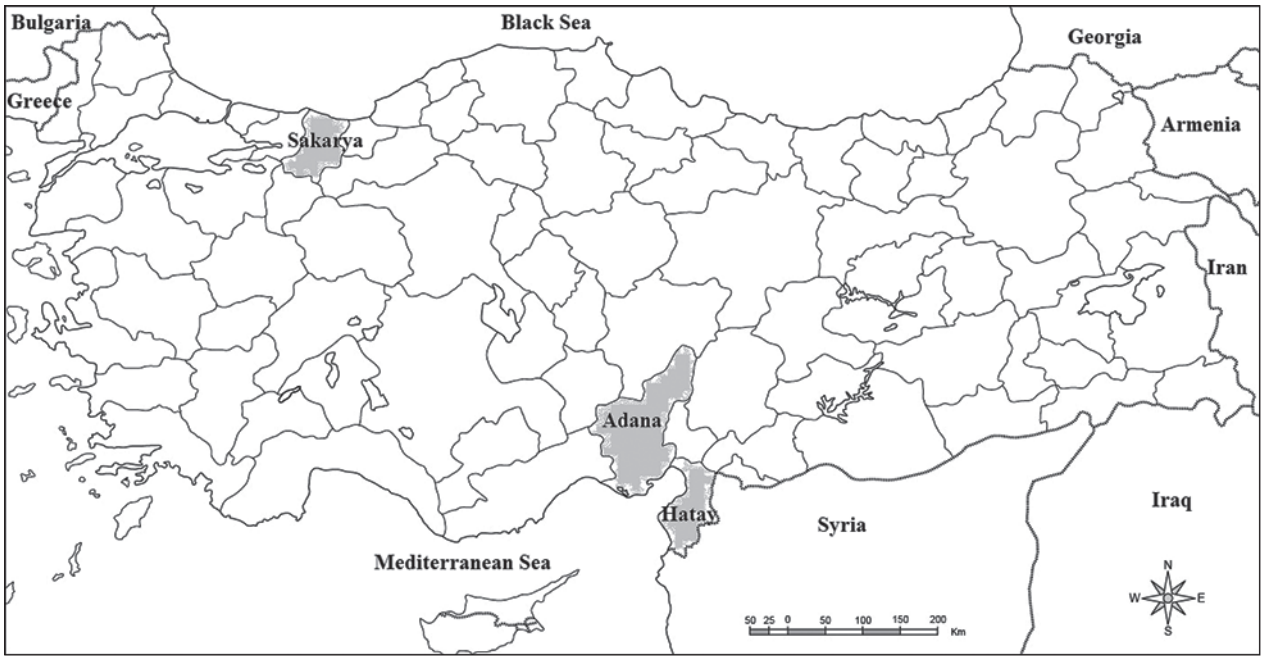


Figure 1: Map depicting the provinces from where the samples were taken

in the past decade, the clinical relevance of this agent has been increased due to their ability to develope resistance to different classes of antimicrobials (11, 12).

It has been reported that methicillin-resistant coagulase negative strains may act as a potential reservoir for the new SCC*mec* elements such as virulence and resistance genes for *S. aureus* (10). Pigs, cattle and broilers have been detected to be colonized with methicillin-resistant *S. sciuri* (MRSS) and carry the resistance and virulence genes similar to those found in *S. aureus* (13). Furthermore, the *mec*C gene carrying *S. sciuri* have been isolated from different animal species (14, 15).

The aim of present study was to determine the occurence of MRSS in broiler flocks and genetic diversity of the isolates by means of SCC*mec* typing and pulsed-field gel electrophoresis (PFGE). In addition, the potential status of MRSS as a reservoir of resistance and virulence genes for other staphylococci was assessed by antimicrobial susceptibility testing and detection of antimicrobial resistance and virulence genes.

MATERIALS AND METHODS

Ethical statement

The study was approved by the Animal Ethical Committee of Hatay Mustafa Kemal University 2018/9-4.

Sampling and isolation

In 2018, 12 broiler farms were randomly sampled in three different cities (Adana, Hatay and Sakarya), which are among the intensive broiler breeding regions of Turkey (Figure 1). Four houses for each broiler farm were selected for sampling and one hundred throat swabs from each house were collected and divided into five pools of 20 swabs. MRS was isolated by two step selective enrichment procedure followed plating to selective agar media as previously described by de Boer *et al*. (16). For the primary enrichment, pooled swabs were incubated in 20 ml Mueller Hinton Broth (Merck, Darmstadt, Germany) containing 6.5% NaCl at 37°C for 18 h. For the secondary enrichment, one ml of the primary enrichment broth was transferred to 9 ml Phenol Red Mannitol Broth (Merck, Darmstadt, Germany) with 5 mg/l ceftizoxime (Sigma-Aldrich, St Louis, MO, USA) and 75 mg/l aztreonam (Sigma-Aldrich, St Louis, MO, USA), and incubated for 18 h at 37°C. Subsequently, 10 µl of this culture was inoculated onto Baird-Parker agar (Merck, Darmstadt, Germany) [containing egg yolk tellurite, ceftizoxime (5 mg/l) and aztreonam (75 mg/l)], chromID™ MRSA and chromID™ MRSA Smart agar (bioMérieux, Marcy-l'Etoile, France), and incubated for 18 h at 37°C. Suspected colonies were purified on Blood agar.

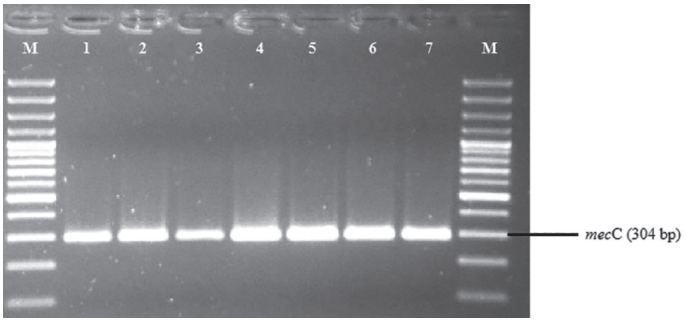


Figure 2: Agarose gel electrophoresis of *mec*C positive isolates

DNA isolation and identification of the isolates, *mec*A and/or *mec*C detection

DNA was extracted using commercial kit (Instagen Matrix, BioRad, France). Genus specific detection with single PCR (17), and species spesific identification were performed by two sets of multiplex PCR (18). *mec*A and *mec*C genes were investigated as previously described (19, 20), respectively. In addition, primers covering both *mec*A and *mec*LGA251 (=mec_u) designed from regions nearly identical to *mec*A from the sequence of the genome of MRSA LGA251 were also used (20).

Antibiotic susceptibility testing

Antimicrobial susceptibilities of the isolates were determined using disk diffusion methods in relation with Clinical Laboratory Standards Institute Guidelines (CLSI, 2012) (21) on Mueller Hinton Agar (Merck, Darmstadt, Germany), and following disks (Bioanalyse, Turkey) were used: penicillin (P, 10 U), cefoxitin (FOX, 30 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), erythromycin (E, 15 µg), clindamycin (DA, 2 µg), ciprofloxacin (CIP, 5 µg), gentamicin (CN, 10 µg), trimethoprim/sulfamethoxazole (SXT, 1.25 µg/23.75 µg), oxacillin (OXA, 1 µg), rifampicin (RA, 5 µg), quinopristin-dalfopristin (SYN, 15 µg), amoxicillin-clavulanic acid (AMC, 10/20 µg), vancomycin (VA, 30 µg), linezolid (LNZ, 30 µg) and tigecycline (TGC, 15 µg). The isolates found to be resistant to at least three or more different classes of antimicrobials were considered multi-drug resistant (MDR).

SCC*mec* typing

SCC*mec* types of the isolates were determined as described by McClure-Warnier *et al.* (22) and Kondo *et al.* (23).

Pulsed Field Gel Electrophoresis (PFGE)

Clonality of the isolates was determined using PFGE following the method suggested by Murchan *et al.* (24). PFGE analysis was performed using the restriction enzyme *Sma*I in the Molecular Microbiology Research and Application Laboratory, Ministry of Health, Public Health Agency of Turkey (Ankara, Turkey). The relatedness between the isolates was evaluated according to the criteria previously described by Tenover *et al.* (25).

Detection of antimicrobial and disinfectant resistance genes

All MRSS isolates were screened by PCR for the presence of the following genes: *bla*Z, *mec*A, *mec*C, *tet*K, *tet*M, *aac(6')-aph(2'')*, *aph(3')-IIIa, ant(4')-Ia, erm*A, *erm*C, *lnu*A, *qac*A/B and *smr* (QAC resistance genes) (17, 19, 26, 27, 28). Primers used in the study were given in Table 1.

Detection of toxin genes

Three set of multiplex PCR (mPCR) assays were used to detect the staphylococcal enterotoxins (SEs) and toxins (29, 30). The first (mPCR1) allowed detection of the following genes: *sea*, *seb*, *sec*, *sed* and *see* as reported by Mehrotra *et al.* (29); the second reaction (mPCR2) was performed to detect the *seg*, *seh*, *sei*, and *selj* genes (30). The third reaction (mPCR3) was used for the detection of *eta*, *etb* and *tst* genes (29).

RESULTS

Bacterial isolates

Overall 63 Methicillin Resistant Stphlococci were isolated, and identified as *S. sciuri* by PCR. Of 63 MRSS isolates, 56 were only positive for *mec*A and, 7 isolates carried both *mec*A and *mec*C (Figure 2). Distribution of MRSS isolates according to locations was as follow: 30 from Hatay, 26 from Adana, and 7 from Sakarya (Table 2). PCR covering both *mec*A and *mec*LGA251 genes gave positive results for all isolates. DNA sequence analysis of *mec*C carrying isolates showed high similarity (99.67%) with previously published sequence (Accession number: MK330620.1).

SCC*mec* typing

Most of the isolates were non-typeable (49, 77.8%), of which 10 were positive for type A *mec* complex and five were only

Table 1: Primers used in the study

Gene	Sequence 5'-3'	Size (pb)	Reference
blaZ	TAAGAGATTTGCCTATGCTT	675	Olsen *et al.* (2006)
	AATTCCTTCATTACACTCTTGG		
*mec*A	CCTAGTAAAGCTCCGGAA	314	Choi *et al.* (2003)
	CTAGTCCATTCGGTCCA		
aac(6')-aph(2'')	GAAGTACGCAGAAGAGA	491	
	ACATGGCAAGCTCTAGGA		
aph(3')-IIIa	AAATACCGCTGCGTA	242	
	CATACTCTTCCGAGCAA		
ant(4')-Ia	AATCGGTAGAAGCCCAA	135	
	GCACCTGCCATTGCTA		
*mec*C	GAAAAAAAGGCTTAGAACGCCTC	304	Stegger *et al.* (2012)
	GAAGATCTTTTCCGTTTTCAGC		
mec_u	ATTTGTCTKCCAGTTTC	728	Cuny et al. (2011)
	TCACCAGGTTCAACRCA		
*tet*K	GTAGCGACAATAGGTAATAGT	360	Strommenger *et al.* (2003)
	GTAGTGACAATAAACCTCCTA		
*tet*M	AGTGGAGCGATTACAGAA	158	
	CATATGTCCTGGCGTGTCTA		
*erm*A	AAGCGGTAAACCCCTCTGA	190	
	TTCGCAAATCCCTTCTCAAC		
*erm*C	AATCGTCAATTCCTGCATGT	299	
	TAATCGTGGAATACGGGTTTG		
*lnu*A	GGTGGCTGGGGGGTAGATGTATTAACTGG	323	Lina *et al.* (1999)
	GCTTCTTTTGAAATACATGGTATTTTTCGA		
*qac*A/B	ATGCCTTATATTTATTTAATAATAGCC	321	Noguchi *et al.* (1999)
	ATGCGATGTTCCGAAAATGTTTAAC		
smr	CTATGGCAATAGGAGATATGGTGT	417	
	CCACTACAGATTCTTCAGCTACATG		
sea	GGTTATCAATGTGCGGGTGG	102	Mehrotra *et al.* (2000)
	CGGCACTTTTTTCTCTTCGG		
seb	GTATGGTGGTGTAACTGAGC	164	
	CCAAATAGTGACGAGTTAGG		
sec	AGATGAAGTAGTTGATGTGTATGG	451	
	CACACTTTTAGAATCAACCG		
sed	CCAATAATAGGAGAAAATAAAAG	278	
	ATTGGTATTTTTTTTCGTTC		
see	AGGTTTTTTCACAGGTCATCC	209	
	CTTTTTTTTCTTCGGTCAATC		
eta	GCAGGTGTTGATTTAGCATT	93	Monday *et al.* (1999)
	AGATGTCCCTATTTTTGCTG		
etb	ACAAGCAAAAGAATACAGCG	226	
	GTTTTTGGCTGCTTCTCTTG		
tst	ACCCCTGTTCCCTTATCATC	326	
	TTTTCAGTATTTGTAACGCC		

Table 2: Distribution of MRSS isolates according to locations and SCC*mec* types

Location	The number of positive farms/ The number of farms sampled (%)	The number of positive pools/ The number of pools examined (%)	SCC*mec* III n (%)	SCC*mec* IVc n (%)	SCC*mec* V n (%)	NT* n (%)
Adana	5/5 (100)	26/100 (26)	8 (30.8)	–	2 (7.7)	16 (61.5)
Sakarya	3/4 (75)	7/80 (8.8)	–	–	–	7 (100)
Hatay	3/3 (100)	30/60 (50)	2 (6.7)	2 (6.7)	–	26 (86.7)

* NT, nontypeable

positive for *ccrAB*3, 34 did not carry both *ccr* and *mec* complex. Ten isolates were positive for SCC*mec* type III, two were positive for SCC*mec* type Vc, and two were positive for SCC*mec* type V (See Table 2).

PFGE analysis

PFGE analysis revealed 47 distinct PFGE types, clustering 13 PFGE group based on a smilarity coefficient of ≥80 (Figure 3).

Antibiotic Susceptibility Testing

All isolates were susceptible to vancomycin, linezolid and tigecycline, but resistant to cefoxitin and penicillin. Various rates of resistance were observed to trimethoprim/sulfamethoxazole (73%), clindamycin (73%), erythromycin (71.4%), tetracycline (66.7%), ciprofloxacin (42.9%), amoxicillin-clavulanic acid (30.2%), gentamicin (28.6%), quinopristin-dalfopristin (17.5%), rifampicin (4.8%) and chloramphenicol (3.2%).

PCR detection of antimicrobial resistance, disinfectant and virulence genes

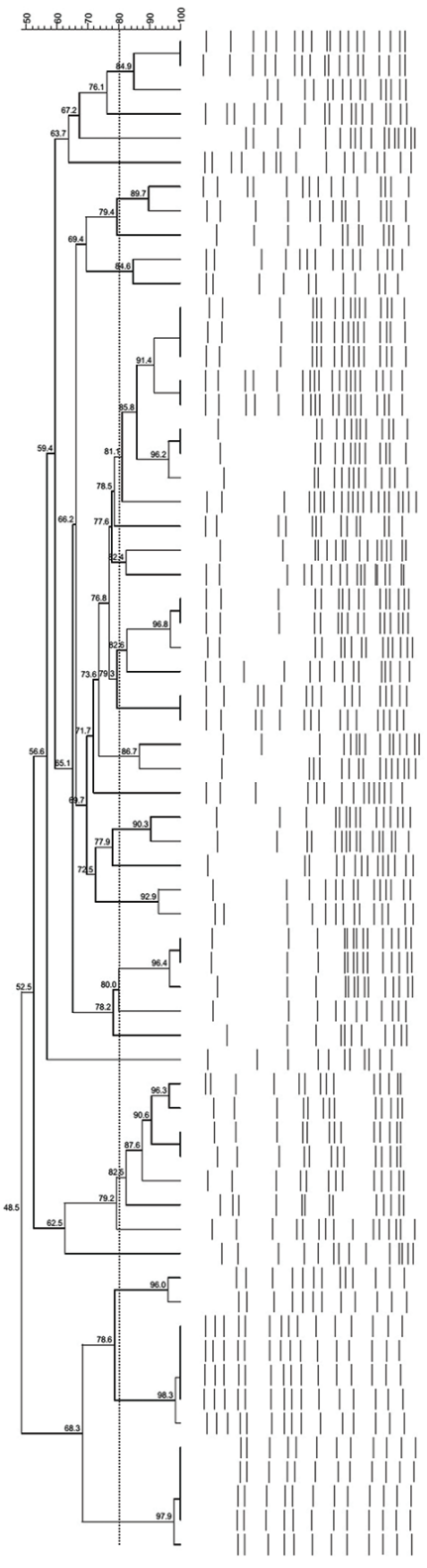
Among the 46 clindamycin resistant isolates, 40 carried *lnu*A gene. The *bla*Z gene was only detected in 20 (31.7%) of the isolates. Of the *erm* genes examined in 45 erythromycin resistant isolates, 11 (24.4%) carried *erm*C, and 2 (4.4%) harbored both *erm*A and *erm*C. Among the 42 tetracycline resistant isolates, *tet*K was detected in 16 (38.1%) isolates, *tet*M was detected in 14 (33.3%) isolates, and two isolates (4.8%) had both *tet*K and *tet*M. The *ant(4')-Ia* gene was detected in only 2 (11.1%) of the 18 isolates, but the other resistance genes was not detected. None of the isolates were positive for virulence and disinfectant resistance genes.

DISCUSSION

S. sciuri, has been considered as commensal species for many years (10), recently, however various rate of prevalence of MRSS have been reported in different healthy animal species including broilers (6, 13, 15, 31) and bovine mastitis cases as well (12). In this study, to the best of our knowledge, it was determined that broiler flocks were colonized with MRSS at high rates and which were characterized by molecular methods for the first time in three locations in Turkey. Sixty-three MRSS were isolated, leading to an overall prevalence of 27.5%. The prevalence found in this study was much higher when compared with a previous MRSS study conducted in Belgium, which reported a prevalence of 12.5% in broilers (13). However, another study was carried out in Belgium on a farm level presented the prevalence of MRSS as 34.2% in broiler farms and 29.8% in layer farms (31).

SCC*mec* analysis revealed a high prevalence of non-typeable SCC*mec* type, accounting 77.8% of the isolates. This is in contrast with the findings of Nemeghaire *et al.* (13), who found that SCC*mec* type III was the most common type with nearly 60% of the isolates. However, similar observation was reported by Nemeghaire *et al.* (31), who found that most of the isolates (60%) carried a non-typeable SCC*mec* type. While the *mec* type A was detected in non-typeable SCC*mec* carrying isolates, these isolates were negative for *ccr* complex by PCR noted by Kondo *et al.* (23). However, detection of *ccr*A element by microarray was reported in most of non-typeable SCC*mec* carrying isolates, indicating high degree of genetic diversity in the *ccr* complex (13, 31). Urushibara *et al.* (32) suggested that *mec* gene complex and *ccr* genes are quite different in coagulase negative staphylococci (CoNS) and recombination may occur between different *ccr* complexes in nature.

*Sma*I restriction endonuclease based PFGE typing of *S. sciuri* isolates has been reported to have a highly discriminative method for investigating epidemiological proximity between *S. sciuri* isolates (13, 31). Indeed, in this study, high diversity of MRSS in broiler flocks revealing 47 distinct PFGE types clustering 13 PFGE group were identified among 63 isolates. This suggests that these isolates may



Isolate ID	Location	SCCmec	Resistance Phenotype	
2	C	IVc	P, FOX, TE	
11	C	IVc	P, FOX, E, TE, CN, DA, SYN	I
57	C	NT	P, FOX, TE, DA	
9	A	V	P, FOX, TE, STX, CIP	
34	A	V	P, FOX, TE, STX, CIP	
52	C	NT	P, FOX, CN, STX, DA	II
12	C	NT	P, FOX, E, TE, CN, STX, DA, CIP	
21	C	NT	P, FOX, E, TE, CN, STX, DA, CIP	
44	A	NT	P, FOX, E, DA	
3	B	NT	P, FOX, E, TE, C, STX, DA, SYN, CIP	
4	A	NT	P, FOX, SYN, SXT, DA, E, CIP, TE	
19	C	NT	P, FOX, AMC, E, TE, CN, STX, DA	
25	C	NT	P, FOX, E, CN, STX, DA, SYN	
40	C	NT	P, FOX, E, TE, STX, DA, CIP	
7	C	NT	P, FOX, STX	III
8	C	NT	P, FOX, TE, STX	
45	B	NT	P, FOX, STX	
53	C	NT	P, FOX, AMC, E, TE, DA, CIP	
14	C	NT	P, FOX, TE, STX, DA, RA	
22	A	NT	P, FOX, E, TE, STX	
5	C	NT	P, FOX, SXT, DA, TE	IV
56	A	NT	P, FOX, AMC, STX, DA	V
30	A	NT	P, FOX, E, TE, STX, DA	
27	A	NT	P, FOX, E, STX, DA, AMC	
28	A	III	P, FOX, AMC, E, STX	VI
49	A	III	P, FOX, AMC, E, STX, DA	
23	A	III	P, FOX, AMC, E, TE, CN, STX, DA, CIP	
16	A	NT	P, FOX, AMC, TE	VII
29	A	NT	P, FOX, STX	
20	A	NT	P, FOX, STX	VIII
24	A	NT	P, FOX, E, TE, STX, DA	
35	B	NT	P, FOX, E, TE, STX, DA	IX
50	A	NT	P, FOX, E, TE, STX, DA	X
51	A	NT	P, FOX, E, TE, CN, STX, DA, CIP	
36	A	NT	P, FOX, E, TE, STX, DA, CIP	
58	C	NT	P, FOX, AMC, E, TE, STX	
17	A	NT	P, FOX, AMC, E, STX, DA	
59	B	NT	P, FOX, E, TE, STX, DA	XI
60	B	NT	P, FOX, E, TE, STX, DA	
15	B	NT	P, FOX, E, CN, DA, CIP	
55	A	NT	P, FOX, E, C, STX	
46	A	NT	P, FOX, E, TE, STX, DA, CIP	XII
61	B	NT	P, FOX, E, TE, STX, DA	
31	C	NT	P, FOX, AMC, E, TE, CN, STX, DA, SYN	
37	C	NT	P, FOX, TE, SYN, SXT, DA, E	
38	C	NT	P, FOX	XIII
43	C	NT	P, FOX, TE, CN, DA	
62	C	NT	P, FOX, AMC, E, TE, DA, CIP	
18	C	NT	P, FOX, AMC, STX	
32	A	NT	P, FOX, E, TE, CN, STX, DA, CIP	
47	A	III	P, FOX, AMC, TE, CN, STX, CIP	XIV
54	A	III	P, FOX, E, TE, CN, STX, DA, SYN, CIP	XV
42	A	III	P, FOX, E, DA, CIP	
63	C	NT	P, FOX, E, TE, STX	
6	C	NT	P, FOX, E, TE, DA, CIP	
10	C	NT	P, FOX, E, TE, CN, STX, DA, SYN, CIP	
13	C	III	P, FOX, E, TE, CN, STX, DA, SYN, CIP, RA	
1	C	NT	P, FOX, AMC, E, TE, DA, CIP	XVI
26	C	NT	P, FOX, E, TE, DA, SYN, CIP	
33	A	III	P, FOX, AMC, E, DA, CIP	
41	A	NT	P, FOX, AMC, E, CN, DA, SYN, CIP	
48	C	III	P, FOX, AMC, E, STX, DA, CIP	
39	C	III	P, FOX, RA, AMC, E, TE, CN, DA, CIP	

Figure 3: Dendrogram based on PFGE *Sma*I restriction pattern analysis of 63 MRSS isolates. Similarity analysis was performed with Dice's coefficient, and clustering was done by using the unweighted-pair group method using average linkages (UPGMA) method. The vertical line shows the 80% similarity cut-off, whilst the pulsotypes are indicated by horizantal lines. Numbers colored in yellow indicate *mec*C positive isolates.

belong to different ecological MRSS populations. These results are similar to previous findings of Nemeghaire *et al*. (13) and Nemeghaire *et al*. (31), who reported presence of high diversity of MRSS from farm animals (pigs, cattle and broiler chickens) and healthy chickens.

Expectedly, all isolates were resistant to penicillin and cefoxitin by disc diffusion method, which is consistent with the use of cefoxitin supplemented media for MRSS isolation and the presence of *mec*A gene in all isolates. On the other hand, Nemeghair *et al*. (13) found that most of the MRSS strains isolated by using cefoxitin supplemented media were resistant to penicillins (98.6%), but a small percentage of them were susceptible with broth microdilution tests.

In different countries, antimicrobial resistance profiles may vary among bacterial populations depending on antimicrobial usage habits (33). MRSS isolates analyzed in this study showed high resistance to some antimicrobials tested. This resistance profiles was correlated with antimicrobials usage in veterinary field in Turkey. In addition, a high percentage (87.3%) of MDR profiles was present among the isolates. Similar observations were previously reported among MRSS isolates from farm animals and healthy chickens (13, 31).

In this study, a limited number of virulence genes were investigated in MRSS isolates and were found negative. When previous studies that investigate the presence of virulence genes in detail was examined, it was apparent that a limited number of MRSS isolates have been found to carry the virulence genes (6, 13, 31). However, continuous surveillance is needed to reveal the pathogenicity potential of MRSS isolates. On the other hand, MRSS isolates have been reported to be a reservoir for multiple determinants conferring resistance to clinically important antimicrobials. The genes mediating resistance are often found on mobile genetic elements (MGEs) such as plasmids [*bla*Z, *ant(4')-Ia*, *tet*K, *tet*L, *lnu*A, *erm*C] or transposons [*aac(6')-Ie-aph(2')-Ia*, *aph(3')-IIIa*, *tet*M, *erm*A, *erm*B], and even some of these resistance genes are co-localized on MGEs. The high prevalence of MRSS of multiple resistance determinants might facilitate the selection of resistant bacteria, and pose the risk for spread of resistance bacteria from animals to humans (33).

In conclusion, the study was the first to show high colonization of broiler flocks with MRSS in three locations in Turkey, and that some isolates carried *mec*C gene together with *mec*A gene. The findings of this study also indicate that MRSS could act as a reservoir for both *mec*A and *mec*C, as well as other antimicrobial resistance genes. In addition, further studies are needed to investigate potential reservoirs of *mec*C carrying staphylococcus species.

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