Presence of Antibiotic Resistance Genes in Staphylococci Isolated From Bovine Subclinical Mastitis

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SUMMARY

The prevalence of antibiotic resistance increases rapidly worldwide, and the primary culprit is represented by their widespread use. Subclinical mastitis is the leading cause of most antibiotic treatment, representing also one of the significant problems of bovine herd management. One of the main causes of subclinical mastitis is *Staphylococcus aureus*. Therefore, the determination of antibiotic resistance against *Staphylococcus aureus* is an essential step in the treatment of subclinical mastitis. The aim of this study was to identify antibiotic resistance genes in staphylococci obtained from cases of bovine subclinical mastitis in three provinces and the relationship between antibiotic resistance and ease of antibiotic availability (Burdur, Hatay and Van) in Turkey. In total, 283 isolates (Burdur, $n = 36$; Hatay, $n = 47$; Van, $n = 200$ isolates) were studied. The isolates were first identified as *Staphylococcus aureus* and/or non-*aureus* staphylococci (NAS) by conventional phenotypic methods, and the species was then confirmed by a multiplex polymerase chain reaction (PCR). A simplex PCR assay was performed to detect antibiotic resistance genes (*mecA*, *mecC*, *aacA-aphD*, *ermA*, *ermB*, *ermC*, *tetK*, *tetM* and *blaZ*). Among the isolates from all three provinces, the *blaZ* gene was the most prevalent antibiotic resistance gene, present in 43 out of 156 (28%) NAS isolates, 27 out of 127 (21%) *S. aureus* isolates and 25% of all the isolates. In contrast, tetM was the most prevalent gene in the Hatay isolates, detected in 64% of all isolates. The *mecA*-gene was present in 10% of the NAS, and in 3% of the *S. aureus* isolates. The *mecC* and *ermA* genes were not detected in any of the isolates. This shows that antimicrobial resistance, as determined by PCR, is common in *Staphylococcus* isolates from mastitis in Turkey, and warrants systematic treatment protocols as well as the implementation of preventative strategies to reduce antimicrobial usage.

KEY WORDS

Bovine mastitis; antimicrobial resistance; *Staphylococcus aureus*; prevalence.

INTRODUCTION

Bovine subclinical mastitis is one of the main challenges in dairy farms, which results in huge economic losses due to reduced milk production and increased culling rate[1]. Subclinical mastitis is characterized by alterations in the milk composition without clinical signs and more prevalent than clinical mastitis[2]. Various pathogens are involved in the pathogenesis of subclinical mastitis in dairy cattle. However, *Staphylococci* and especially *S. aureus* are the most common subclinical mastitis bacteria worldwide[3,4]. In recent years, the increasing importance of non-*aureus* staphylococci (NAS) in bovine mastitis has been emphasized[5].

In dairy farms, mastitis is the main reason for antibiotic use, and antimicrobial therapy is the primary tool for preventing and treating mastitis[6]. Various factors related to individual cow and animal management determine the success of bovine mastitis treatment. Metabolic diseases such as ketosis and hypocalcemia perturbed the success of mastitis treatment[7,8]. Also, the type of antimicrobial, duration of the application and route of administration, and the type of infective agent affect the success of the mastitis treatment[9]. In addition, antibiotic resistance greatly contributes to the failure of mastitis treatment. Misuse of antibiotics causes the emergence of the antimicrobial resistance of the bacteria and the entrance of the resistant bac-

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teria into the food chain, leading to public health concerns and low cure rates in mastitis cases in dairy cattle[6].

Methicillin-resistant *S. aureus* (MRSA) is an important cause of infections and disease worldwide and this resistance is encoded by the *mec*A gene[10]. The methicillin-resistant staphylococci are resistant to all penicillin groups[11]. In 2011, a novel SCCmec type XI element (*mecC)* was detected in *mecA*-negative MRSA isolates from milk samples from dairy cows in the UK and human samples in the UK, Denmark [12] and Ireland [13]. Penicillin is one of the widely used antibiotics for the treatment of bovine mastitis. The prevalence of penicillin-resistant bovine isolates varies from 10 to 70% according to geographic location [14]. The *blaZ* gene is mainly responsible for resistance to benzyl penicillin[15]. Erythromycin-resistant staphylococci are usually resistant to other macrolides, lincosamides, and type B streptogramin. *erm* genes have been found frequently in staphylococci[16]. The most common genes responsible for tetracycline resistance in Staphylococci are *tetM* and *tetK*[17]. The *aacA-aphD* gene is responsible for aminoglycoside resistance. This gene codes for a bifunctional enzyme and confers cross-resistance to clinically used aminoglycosides[18].

Information on the presence and distribution of antibiotic resistance genes can help developing effective treatment protocols and may therefore contribute to proper infection control in the dairy sector and possibly also in public health[19]. The aim of this study was therefore to describe the prevalence of antibiotic resistance genes in staphylococci obtained from bovine subclinical mastitis cases in three provinces (Van, Hatay and Burdur) in Turkey.

MATERIALS AND METHODS

Isolates

The animal material of the study consisted of 408 dairy cattle (Van=290, Hatay=58, Burdur=60). Milk samples were taken according to National Mastitis Council recommendations. A total of 283 *Staphylococcus* isolates from bovine subclinical mastitis were examined. All isolates were obtained from the culture collection of the Department of Microbiology laboratory of the Hatay Mustafa Kemal University, Hatay, Van Yuzuncu Yil University, Van and Burdur Mehmet Akif Ersoy University, Burdur, Turkey, from 2008 to 2013. All isolates were subjected to a multiplex PCR[20] to confirm they were non-*aureus* staphylococci species and/or *S. aureus*. Distributions of isolates for each province were shown in Table 1.

PCR Analyses

The phenol/chloroform extraction method was used for nucleic acid extraction according to Sambrook and Russel[21]. For the PCR analyses, DNA of the $MRSA_{LGA251}$ (a *mecC* positive MRSA isolate) was kindly provided by Dr. A. R. Larsen and Dr. H. E. Unnerstad and *mecA*, *blaZ*, *aacA-aphD*, *ermA*, *ermC*, *tetK* and *tetM* positive control DNA was kindly provided by Dr. R. Saidi. The details of the PCRs used in this study are shown in Table 2. All amplification reactions were prepared in a 25 µl volume containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 mM each dNTPs, 10 pmol oligonucleotide primer, 1 U Taq polymerase and 2 l template DNA. A pre-PCR step at 95°C for 5 min was applied. A total of 35 cycles were run at the following conditions: denaturation at 95°C for 30 sec, annealing (primer specific annealing temperatures are given in Table 2) for 60 sec, and extension at 72°C for 60 sec. The reaction was achieved with a final extension at 72 for 7 min. PCR products were visualised running 10 uL PCR products on a 1.5 % agarose gel with 0.125 mg/l ethidium bromide at 160 V for 30 min. Only clear, unambiguous and reproducible bands were recorded.

Table 1 - Distribution of staphylococci according to provinces.

Group	Number and %		
	Burdur (n=36)	Hatay (n=47)	Van (n=200)
NAS	23 (64%)	45 (96%)	88 (44%)
S. aureus	13 (36%)	2 (4%)	112 (56%)

Statistical Analyses

Differences in prevalence of antimicrobial resistance genes between NAS and *S. aureus* isolates and between the three provinces were tested in mixed logistic regression models, taking the clustering of isolates within farms in account as a random effect and modelling *S. aureus* versus NAS and the three provinces as fixed effects. The significance of the fixed effects was assessed by a likelihood-ratio test comparing nested models. The models were built in R version 3.4.0[27] using the glmer function in the lme4 package[28].

RESULTS

A total of 156 NAS isolates and 127 *S. aureus* isolates were cultured and tested for AMR genes. Table 3 summarizes the prevalence of resistance genes and their distribution over the 3 provinces for NAS and *S. aureus* isolates. In the present study, no antibiotic resistance genes were detected in 146 of the 283 (51.6%) isolates. The resistance profiles were shown in Table 4.

Overall, *blaZ* was the most prevalent gene in all isolates (28% in NAS and 21% in *S. aureus*). Most resistance genes showed similar prevalence both in NAS and in S. aureus, but *mecA* tended to be more prevalent in NAS (10%), than in *S. aureus* (3%) ($p = 0.07$), whereas *tetK* was significantly more prevalent in *S. aureus* (21%) than in NAS (10%) ($p = 0.05$). In NAS, the prevalence of *tetM* was higher than in *S. aureus*, but this was non-significant in the mixed model. In the Hatay province, *tetM* was the most prevalent gene and *tetM* was in this province significantly more prevalent than in the other two provinces ($p < 0.001$). The *mecC* gene and *ermA* gene were not detected in any of the isolates.

DISCUSSION

In the present study, resistance genes related to four groups of antibiotics (penicillin, tetracycline, aminoglycoside, macrolide) were investigated in 283 *Staphylococcus* isolates from subclinical mastitis cases in three provinces in Turkey (Van, Hatay and Burdur). Table 5 summarises the distribution of antibiotic-resistance genes in staphylococci from bovine mastitis worldwide. The selected studies focused on genes studied in staphylococci related to bovine mastitis.

Table 2 - Properties of PCR assays used in the study.

Primer Name	Primer Sequences	Annealing Temperature ^{0}C	Amplicon size (bp)	Reference
mecALGA 251MultiFP	5'-GAAAAAAAGGCTTAGAACGCCTC-3'	59	138 bp	[22]
mecALGA 251MultiRP	5'-GAAGATCTTTTCCGTTTTCAGC-3'	59	138 bp	[22]
mecLGA 251 f *mecLGA 251 r*	5'-GCTCCTAATGCTAATGCA-3' 5'-TAAGCAATAATGACTACC-3'	50	304 bp	[23]
mecA1 *mecA2*	5'-CCTAGTAAAGCTCCGGAA-3' 5'-CTAGTCCATTCGGTCCA-3'	54	314 bp	[24]
blaZ1 *blaZ2*	5'-ACTTCAACACCTGCTGCTTTC-3' 5'-TGACCACTTTTATCAGCAACC-3'	56	173 bp	[25]
aacA-aphD 1 *aacA-aphD 2*	5'-TAA TCC AAG AGC AAT AAG GGC-3' 5'-GCC ACA CTA TCA TAA CCA CTA-3'	54	227 bp	[26]
ermA 1 *ermA 2*	5'-AAG CGG TAA ACC CCT CTG A-3' 5'-TTC GCA AAT CCC TTC TCA AC-3'	54	190 bp	[25]
ermC 1 *ermC 2*	5'-AAT CGT CAA TTC CTG CAT GT-3' 5'-TAA TCG TGG AAT ACG GGT TTG-3'	54	299 bp	[25]
tetK 1 *tetK 2*	5'-GTA GCG ACA ATA GGT AAT AGT-3' 5'-GTA GTG ACA ATA AAC CTC CTA-3'	54	360 bp	[25]
tetM 1	5'-AGT GGA GCG ATT ACA GAA-3'	54	158 bp	[25]
tetM 2	5'-CAT ATG TCC TGG CGT GTC TA-3'			

In the present study, *blaZ* gene prevalence was 28% and 21% in NAS and *S. aureus* respectively. Previous studies have reported that the *blaZ* gene was the most frequently detected resistance gene in staphylococci isolated from bovine mastitis cases. Frequencies of this gene ranged from 30% to 95% (Table 5).

The prevalence of the *mecA* gene was 10% in NAS and 3% in *S. aureus* in our study. Previous studies have reported that the *blaZ* gene was the most frequently detected resistance gene in staphylococci. Frequencies of this gene were found between 2% in China to 100% in Algeria (Table 5).

In the present study, the *mecC* gene was not detected in any of the isolates. Similar to this finding, the *mecC* gene in staphylococci was reported at a very low frequency (1 of 135 isolates, 0.7%) in Finland[37] and it was detected in only four (0.05%) isolates obtained from 8,757 milk samples in Sweden[38]. In studies conducted in the U.K., Paterson et al.[39] detected the *mecC* gene in 10 (2.15%) of 465 MRSA isolates obtained from dairy farms in England and Wales but did not detect the *mecC* gene in samples from 625 dairy farms in Scotland.

There are several factors affecting the prevalence of antibiotic resistance, including clinical misuse, low quality of available antibiotics, and ease of availability of antibiotics[41]. The cost and ease of availability of antibiotics are some of the main contributors to antibiotic resistance[40, 41]. The variations in the frequencies of the *blaZ*, *mecA* and *mecC* genes may be attributed to differences in the antibiotic usage policies of governments, ease of access to antibiotics and willingness of veterinarians and farmers about the use of antibiotics.

In this study, the frequency of *tetM* was highest in the Hatay region (62% in NAS) when compared with other regions of Turkey. The high level of resistance in Hatay may be explained by the intensive and mostly off-label single intramuscular use of low-concentrate (30 mg/ml) commercial tetracycline non-justified administration by farmers, unregulated use of these products and ease of availability of these products in this region in the recent past[42]. Similar to the findings of this study in Hatay Province, Jamali et al.[30] detected *tetK* and *tetM* genes in 40% and 63% of staphylococci obtained from milk samples

Table 3 - Number and percentage of isolates carrying antibiotic resistance genes of 156 non-*aureus Staphylococcus* isolates and 127 *Staphylococcus aureus* isolates from 60 farms in three provinces in Turkey.

Target gene	Province							
	Non-*aureus Staphylococci*				*S. aureus*			
	Burdur (n = 23)	Hatay (n = 45)	Van (n = 88)	Total (n = 156)	Burdur (n = 13)	Hatay (n = 2)	Van (n = 112)	Total (n = 127)
blaZ	8 (35%)	11 (24%)	24 (27%)	43 (28%)	3 (23%)	0 (0%)	24 (21%)	27 (21%)
mecA	6 (26%)	3 (7%)	6 (7%)	15 (10%)	1 (8%)	0 (0%)	3 (3%)	4 (3%)
mecC	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ermA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ermC	1 (4%)	1 (2%)	1 (1%)	3 (2%)	0 (0%)	0 (0%)	6 (5%)	6 (5%)
tetK	5 (22%)	4 (9%)	7 (8%)	16 (10%)	1 (8%)	0 (0%)	26 (23%)	27 (21%)
tetM	2 (9%)	28 (62%)	5 (6%)	35 (22%)	0 (0%)	2 (100%)	5 (4%)	7 (6%)
aacA-aphD	4 (17%)	2 (4%)	0 (0%)	6 (4%)	1 (8%)	0 (0%)	0 (0%)	1 (1%)

Table 4 - Antibiotic Resistance Genes Profiles of the staphylococci according to provinces.

Antibiotic Resistance Profiles	Van (East)	Hatay (Middle)	Burdur (West)	Total
blaZ	30	3	6	39
blaZ+tetM	-	5	-	5
blaZ+tetK	14	1	1	16
blaZ+ermC	1	-	-	1
blaz+mecA+tetM	1	1	-	2
blaZ+ermC+tetK	2	-	-	2
blaZ+mecA+tetM+aacA-aphD	-	1	-	1
blaZ+mecA+tetK+aacA-aphD	-	-	4	4
blaZ+mecA+tetK+tetM	-	1	-	1
blaZ+mecA+ermC+tetK+aacA-aphD	-	1	-	1
mecA	5	-	1	6
mecA+tetM	3	-	-	3
mecA+ermC	-	-	1	1
mecA+tetK+aacA-aphD	-	-	1	1
tetM	5	26	2	33
tetK	16	-	-	16
tetK+tetM	-	1	-	1
ermC	2	-	-	2
ermC+tetM	1	-	-	1
ermC+tetK	1	-	-	1
Total isolates containing resistance genes	81 (40.5%)	40 (85.1%)	16 (44.4%)	137
total isolates not containing the resistance genes	119 (59.5%)	7 (14.9%)	20 (55.6%)	146
Total isolate numbers	200	47	36	283

with mastitis in Iran. Prevalence of *tetM* and *tetK* were reported from 0-100% in different countries worldwide (Table 5).
Jamali et al.[30] reported a 6.98% prevalence of *aacA-aphD* genes in *S. aureus* isolates from clinical mastitis samples in Iran. Similarly, the same genes were detected in only 2% of *S. aureus* isolates from clinical and subclinical mastitis cases in Northwest China[31]. In the present study, the prevalence of the *aacA-D* gene in NAS isolates from Burdur Province (14%) was higher than that in Hatay Province (4%). The *aacA-aphD* gene was not detected in any of the isolates (0%) from Van Province. In Burdur, there is a high level of antimicrobial resistance to aminoglycoside, which indicates extensive and misuse of this group of antibiotics compared to other provinces in Turkey. Prevalence of *aacA-aphD* was reported from 2-32% in different countries worldwide (Table 5).
In this study, the *ermA* gene, which mediates resistance to mac-

Table 5 - Prevalence of the antibiotic resistance genes in staphylococci in bovine mastitis in some similar studies worldwide[#].

S aureus	NAS	C / S	n	*% of isolates positive for resistance gene*								Country	References
				blaZ	*mecA*	*mecC*	*ermA*	*ermC*	*tetK*	*tetM*	*aacA-aphD*		
-	+	C	51	59	-	-	-	6	45	27	-	USA	[29]
+	-	C	43	86	12	-	9	40	40	63	7	Iran	[30]
+	-	C	35	60	-	-	-	9	51	31	-	USA	[29]
+	-	C	44	95	2	-	0	14	23	2	2	China	[31]
+	-	S	11	95	2	-	0	14	23	2	2	China	[31]
+	-	S	2	46	5	-	8	21	25	11	0	Turkey	[32]
+	-	S	28	82	36	-	0	7	11	0	32	China	[33]
+	+	S	21	43	100	-	0	0	0	100	0	Algeria	[34]
-	+	S	76	30	17	-	0	13	34	3	21	China	[33]
+	-	NR	52	-	-	-	4	29	15	26	-	Czechia	[35]
-	+	NR	170	80	14	-	0	3	-	-	-	Netherlands	[36]

[#]Values given as percentage between 3-11 columns, + and - values as given for specifying the study sampling group; C: Clinical; S: Subclinical; n: Number of isolates; NAS; Non-*aureus* Staphylococci; NR; No record.

rolides, was not found in any staphylococci, and the *ermC* gene was detected in only 3% of the isolates. The frequency of *ermC* in the isolates obtained from bovine mastitis cases in all three provinces was similar (Table 3).

The low frequency of *erm* genes detected in the isolates in the present study may be explained by the Turkish veterinary market, where macrolide antibiotic usage was not common in the past. Thus, only a few macrolide antibiotics were on the market, and the companies manufacturing these products had not adopted an aggressive marketing strategy targeting veterinarians or farmers directly. Prevalence of *ermA* and *ermC* was reported from 0% to 40% in different countries worldwide (Table 5).

Penicillin, penicillin plus streptomycin combinations and semisynthetic penicillin were the first antibiotics to be introduced for veterinary use in Turkey. In addition, penicillin and tetracycline were the cheapest and readily available antibiotics in Turkey. Intensive and misuse of these antibiotics may have triggered the current antibiotic resistance problem. The level of resistance for *blaZ* gene (31% in Burdur Province, 23% in Hatay Province and 24% in Van Province) was found to be positively correlated with the level of development in dairy farming in these regions. It was also determined that the higher resistance level was found for *aacA-aphD* gene in the more developed area of Western Turkey (Burdur, 14%) compared to Van, (0.0%), which is relatively less developed region of eastern Turkey. This might be explain with the misuse of antibiotics in in developed areas, though it is not a general rule. Due to their overuse, penicillin antibiotics in the veterinary market may have contributed to the development of resistance genes in Turkey.

CONCLUSION

The prevalence of antibiotic resistance increased from the East to the West of the country, particularly with regard to penicillin and gentamicin resistance. The prevalence of penicillin resistance in staphylococci was high. The prevalence of antibiotic resistance genes was associated with the ease of availability of antibiotics, together with easy access and low cost. On the other hand, the resistance to antibiotics that are less easily accessible and those that are not aggressively marketed by pharmaceutical companies was significantly lower.

Ethic approval

According to the legislation titled "Working Procedures and Ethics Committees of Animal Experiments" numbered 28914 published on February 15, 2014, in Turkey, the 8th article clearly suggested that milking and swabbing procedures are not subject to ethical committee approval.

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Author contributions

Zafer CANTEKIN: Conceptualization, Formal analysis, Investigation, **Gamze Ozge OZMEN**: Conceptualization, Formal analysis, data collection **Melek DEMIR:** Conceptualization, Formal analysis, data collection, **Zeynep YILMAZ ER:** Conceptualization, Formal analysis, data collection, **Kemal GURTURK:** Conceptualization, Formal analysis, Investigation, Data curation **Hasan SOLMAZ:** Conceptualization, Formal analysis, Investigation, Data curation **Ismail Hakkı EKIN:** Conceptualization, Formal analysis, Investigation, Data curation **Dilek OZTURK:** drafting manuscript, critical revisions, data collection, Writing original draft **Ahmet GOZER:** drafting manuscript, critical revisions, data collection, Writing original draft **Yasar ERGUN:** drafting manuscript, critical revisions, data collection, Writing original draft.

Conflict of interest

The authors declare that there is no conflict of interest.

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