

Prevalence and Molecular Mechanism of Macrolide and Lincosamide Resistance in Staphylococci Isolated from Subclinical Bovine Mastitis in Turkey

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ABSTRACT. Macrolide and lincosamide (ML) resistance and the related resistance genes of staphylococci were assessed from cases of bovine subclinical mastitis. Of the 104 *Staphylococcus aureus* and 62 coagulase negative staphylococcus (CoNS) isolates, 26 (25%) and 12 (19.4%) were resistant to ML, respectively. While constitutive ML resistance phenotype accounted for 15.4% (16/104) of *S. aureus* and 8.1% (5/62) of CoNS, inducible ML resistance phenotype accounted for 2.9% (3/104) of *S. aureus* and 3.2% (2/62) of CoNS. Among erythromycin-resistant isolates, single or various combination of different resistance genes were detected. The results of this study showed that ML resistance was prevalent among staphylococci from subclinical bovine mastitis cases in Hatay, Turkey. Therefore, a continuous surveillance is necessary to minimise the spread of antimicrobial-resistant staphylococci.

KEY WORDS: bovine subclinical mastitis, macrolide-lincosamide resistance, staphylococcus.

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Bovine mastitis is an important disease in the dairy industry worldwide with considerable economic losses. Although a wide variety of microorganism have been isolated from bovine mastitis cases, *Staphylococcus* spp. are the most frequently isolated [22]. Antimicrobials have been used not only for the treatment of mastitis but also for the prevention of new infections during the lactation and dry periods. Therefore, determining antimicrobial susceptibility of mastitis-causing pathogens is crucial to monitor the spread of resistant bacteria and guide in selecting the most effective antimicrobial agent/agents for therapy [16, 18, 22].

Although beta-lactam antibiotics have widely been used for the treatment of staphylococcal mastitis, macrolides and lincosamides (ML) are the choice of antibiotics when beta-lactam resistance occurs [2, 5, 10]. Previous studies have demonstrated that resistance to beta-lactam antibiotics is common in staphylococci causing bovine mastitis in Turkey. It has been reported that beta-lactam resistance among *S. aureus* isolates was 63.3% in Central Anatolia [7] and 95% in Aydın province [11] and among coagulase negative staphylococcus (CoNS) isolates was 58% in Kahramanmaraş [3] and 74% in Afyonkarahisar provinces [19].

ML have similar mechanism of antimicrobial activity despite differences in their chemical structure. Resistance to ML occurs by three primary mechanism, including ribosomal target modification by methylation or mutation, active efflux pumps and enzymatic inactivation. The most common resistance mechanism to ML antibiotics is mediated by target methylation. This can lead to cross resistance between the two antibiotics (ML resistance phenotype). ML resistance phenotype is either inducible (iML) or constitutive (cML). Strains with iML resistance phenotype are

resistant to 14- and 15- membered macrolides, and susceptible to 16- membered macrolides, while strains with cML resistance phenotype are resistant to all 14-, 15-, and 16-membered macrolides and lincosamides [12].

Erythromycin, clindamycin, lincomycin, spiramycin, tulathromycin, tylosin and tilmicosin are approved by Ministry of Agriculture for the treatment of animal diseases in Turkey. Therefore, it necessitates to check out the prevalence of the resistance to these agents by microorganisms related to bovine mastitis. This study was the first in which staphylococci isolated from subclinical bovine mastitis have been investigated for their ML resistance phenotype and the associated genotype in Turkey.

The aims of this study were (i) to evaluate the prevalence of ML resistance in 104 *S. aureus* and 62 CoNS (22 *S. epidermidis*, 15 *S. haemolyticus*, 13 *S. hyicus*, 8 *S. xylosus*, 4 *S. chromogenes*) isolates and (ii) to identify resistance genes associated with ML resistance. The strains tested in this study were isolated and identified from subclinical bovine mastitis cases during the years 2006 to 2008 in Hatay province of Turkey. Subclinical cases were determined based on California Mastitis Test (CMT).

The minimal inhibitory concentration (MIC) values of 14- (erythromycin), 15- (tulathromycin) and 16- (spiramycin, tylosin and tilmicosin) membered macrolides and lincosamides (lincomycin and clindamycin) were determined using the broth microdilution method according to the guideline of Clinical and Laboratory Standards Institute (CLSI) [4]. *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used for quality control. All antibiotics were tested at the concentration range between 0.06 and 128 µg/ml. CLSI MIC breakpoints of erythromycin, lincomycin, clindamycin and tulathromycin are ≥8, ≥4, ≥4, and 32 µg/ml, respectively. Since standardized CLSI breakpoint for spiramycin, tilmicosin and tylosin are not available for staphylococci, the CLSI breakpoint of 32 µg/

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ml of *Mannheimia haemolytica* for tilmicosin, >32 µg/ml for spiramycin as recommended by SVARM [20], and 20 µg/ml for tylosin as recommended by VADS [24] were used in this study.

Determination of inducible ML resistance phenotype was performed in erythromycin resistant (ER-R) and clindamycin susceptible (CL-S) isolates as described by Fiebelkorn *et al.* [6] using erythromycin-clindamycin double disc (ECDD) test. Bacterial DNA was extracted by the method of Hesselbarth and Schwarz [8]. PCR assays for the resistance genes *ermA*, *ermB*, *ermC*, *msrA*, *mphC* and *lnuA* was performed as previously reported [9, 13–15].

The MIC values of ML for *S. aureus* and CoNS isolates are presented respectively in Tables 1 and 2. Among the 23 ER-R *S. aureus* isolates, 16 (15.4%) displayed cML resistance phenotype and 3 (2.9%) isolates displayed iML resistance phenotype. Four (17.4%) isolates displayed a erythromycin (E) resistance phenotype. Three (13.0%) isolates had an lincomycin (L) resistance phenotype only. Among the 12 ER-R CoNS, 5 (8.1%) isolates displayed cML resistance phenotype and two (3.2%) isolates displayed iML resistance phenotypes. Five (41.7%) isolates had an E resistance phenotype only.

No resistance genes was detected in isolates susceptible to both macrolides and lincosamides. Of the 23 ER-R *S. aureus* isolates, eighteen (78%) were positive for the *msrA*

gene, followed by *ermB* (12/23; 52.2%), *ermC* (7/23; 30.4%), *ermA* (4/23; 17.4%), *mphC* (7/23; 30.4%), *lnuA* (8/23; 34.8%). Three ER-R *S. aureus* isolates harboring the *msrA* gene only exhibited M resistant phenotype with an intermediate level of resistance to erythromycin (MIC=32 µg/ml). A single isolate harboring *mphC* and *lnuA* genes displayed a combined ML phenotype with resistance to erythromycin (MIC=64 µg/ml) and lincomycin (MIC=16 µg/ml). Three *lnuA* positive isolates were resistant to lincomycin (MIC=16–64 µg/ml), but remained susceptible to clindamycin (MIC=0.25–2 µg/ml). Of the 12 ER-R CoNS isolates, twelve (100%) were positive for the *msrA* gene, followed by *ermC* (3/12; 25%), *ermB* (3/12; 40%), *ermA* (1/12; 8.3%), *mphC* (2/12; 16.7%), *lnuA* (2/12; 16.7%). Five isolates with erythromycin MICs of 16–64 µg/ml harbored only the *msrA* gene. Remaining 7 isolates harbored the *msrA* gene in combination with *ermA*, *ermB*, *ermC*, *mphC* or *lnuA* genes and had erythromycin MICs of 32–>128 (Table 3).

According to studies conducted in Turkey, the prevalence of resistance to erythromycin among *S. aureus* [1, 11, 21] and CoNS varied from 3.6 to 14.2% and 20 to 21.9% [3, 11, 19]. In this study, a higher prevalence of resistance to erythromycin was detected in *S. aureus* (22.1%) isolates, but very similar prevalence rate was observed among CoNS (19.4%) isolates. This difference could be due to variable usage of

Table 1. The MIC distributions and resistance rates of *S. aureus* isolated from subclinical bovine mastitis cases

Antimicrobial agents	MIC (μg/ml)														Susceptible isolates ^{a)}	Intermediate isolates ^{a)}	Resistant isolates ^{a)}	MIC ₅₀ (μg/ml)	MIC ₉₀ (μg/ml)
	0.06≤	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32	64	128	>128						
Erythromycin	52	27	2	0	0	0	0	0	0	3	1	2	17	81 (77.9)	0 (0)	23 (22.1)	0.06	>128	
Lincomycin	0	10	48	19	3	2	0	0	6	2	1	0	13	77 (74.03)	5 (4.8)	22 (21.2)	0.25	>128	
Clindamycin	68	13	7	0	3	0	0	0	0	0	2	0	11	88 (84.6)	3 (2.9)	13 (12.5)	0.06	>128	
Tulathromycin	1	15	42	18	5	7	1	1	2	1	0	0	11	90 (86.5)	3 (2.9)	11 (10.6)	0.25	>128	
Spiramycin ^{c)}	0	1	0	13	54	18	4	1	0	0	0	1	12	91 (87.5)	N.A. ^{c)}	13 (12.5)	1	>128	
Tylosin ^{c)}	0	0	2	22	53	14	0	0	0	0	0	1	12	91 (87.5)	0 (0)	13 (12.5)	1	>128	
Tilmicosin	1	29	41	19	1	0	0	0	1	0	0	0	12	91 (87.5)	1 (1)	12 (11.5)	0.25	>128	

a) Numbers in parenthesis indicate the percentages. b) Thin vertical lines indicate breakpoint between susceptible and intermediate isolates. Thick vertical lines indicate the breakpoint between intermediate and resistant isolates. c) N.A., not applicable. No values representing the intermediate phenotype were shown in SVARM (2007).

Table 2. The MIC distributions and rates of CoNS isolated from subclinical bovine mastitis

Antimicrobial	MIC (μg/ml)														Susceptible	Intermediate	Resistant	MIC ₅₀	MIC ₉₀
resistance	0.06≤	0.125	0.25	0.5	1	2	4.0	8.0	16	32	64	128	>128	isolates ^{a)}	isolates ^{a)}	isolates ^{a)}	(μg/ml)	(μg/ml)	
Erythromycin	32	15	2	0	^{b)} 0	0	1	^{b)} 0	1	3	2	1	5	49 (79)	1 (1.6)	12 (19.4)	0.06	64	
Lincomycin	2	11	26	11	3	3	0	0	3	0	0	0	3	50 (80.6)	6 (9.7)	6 (9.7)	0.25	2	
Clindamycin	48	5	2	1	2	1	0	0	0	0	0	0	3	56 (90.3)	3 (4.8)	3 (4.8)	0.06	0.25	
Tulathromycin	3	6	17	24	6	3	0	0	1	0	0	0	2	59 (95.2)	1 (1.6)	2 (3.2)	0.5	1	
Spiramycin ^{c)}	0	0	2	7	33	12	4	1	0	0	0	0	3	59 (95.2)	N.A. ^{c)}	3 (4.8)	1	4	
Tylosin ^{c)}	2	0	2	8	33	10	3	1	0	0	0	0	3	58 (93.5)	1 (1.6)	3 (4.8)	1	4	
Tilmicosin	2	8	19	22	3	2	0	0	3	0	0	0	3	56 (90.3)	3 (4.8)	3 (4.8)	0.5	2	

a) Numbers in parenthesis indicate the percentages. b) Thin vertical lines indicate breakpoint between susceptible and intermediate isolates. Thick vertical lines indicate the breakpoint between intermediate and resistant isolates. c) N.A., not applicable. No values representing the intermediate phenotype were shown in SVARM (2007).

Table 3. Distribution of macrolide and lincosamide resistance genes among staphylococcal isolates

Phenotype	Genotype	<i>S. aureus</i> (n: 104)			CNS (n: 62)			Species
		cML	iML	Other type	cML	iML	Other type	
L	<i>lnuA</i>			3				
E	<i>msrA</i>			3			5	<i>S. haemolyticus</i> (2) <i>S. epidermidis</i> (2) <i>S. xylosus</i>
ML	<i>mphC, lnuA</i>			1				
cML	<i>ermB</i>	2						
cML	<i>ermB, mphC</i>	1						
cML	<i>ermB, lnuA</i>	1						
iML	<i>ermC, msrA</i>		3			1		<i>S. epidermidis</i>
cML	<i>ermC, msrA, mphC</i>					1		<i>S. haemolyticus</i>
cML	<i>ermA, ermC, msrA</i>	2						
cML	<i>ermC, mphC, msrA, lnuA</i>	1						
cML	<i>ermC, msrA, lnuA</i>	1						
cML	<i>ermA, ermB, msrA, lnuA</i>	2						
cML	<i>ermB, msrA, mphC, lnuA</i>	2						
cML	<i>ermC, msrA, mphC</i>	2			1			<i>S. epidermidis</i>
cML	<i>ermA, msrA, lnuA</i>				1			<i>S. hyicus</i>
cML	<i>ermB, msrA</i>	2			3			<i>S. xylosus</i> , <i>S. haemolyticus</i> <i>S. epidermidis</i>
None	—							<i>S. epidermidis</i> (17) <i>S. haemolyticus</i> (11) <i>S. hyicus</i> (12) <i>S. xylosus</i> (6) <i>S. chromogenes</i> (4)

L, lincomycin; E, erythromycin; ML, macrolide-lincosamide; cML, constitutive macrolide-lincosamide; iML, inducible macrolide-lincosamide.

erythromycin in each geographic region.

Incidence of iML resistance phenotype found in this study for *S. aureus* was lower than the prevalence (71.8%) of methicillin resistant *S. aureus* (MRSA) reported by Rich *et al.* [17] and prevalence (52.8%) of *S. aureus* strains from clinical bovine mastitis reported by Wang *et al.* [25]. However, low prevalence of iML for CoNS (9.7%) was also reported by Lühje and Schwarz [14]. Since low percentage of staphylococci with the iML resistance phenotype was found in this study, the use of clindamycin for the treatment of staphylococci induced bovine mastitis could be the choice of antibiotics. However, it could be useful to routinely screen bovine staphylococci for inducible resistance as resistance to clindamycin might develop as a result of over- or mis-use.

Isolates with erythromycin MICs of 128 or 128 µg/ml carried the constitutively expressed *erm* genes alone or in addition to other resistance genes. While *ermA*, *ermB*, *ermC* genes was found in *S. aureus* and CoNS isolates with cML resistance, only *ermC* gene was detected in iML resistance phenotype isolates. Recently, Türkyılmaz *et al.* [23] characterized 16 methicillin resistant *Staphylococcus aureus* (MRSA) from bovine milk in Aydın province, and found that all isolates were resistant to erythromycin and clindamycin. Among ER-R isolates, nine had *ermA*, seven had both *ermA* and *ermB* genes, but did not have *ermC* gene. These results imply that ER-R staphylococci from

subclinical bovine mastitis in Hatay, showed more variable profile of resistance genes.

All ER-R bovine *S. aureus* isolates with *erm* genes in this study showed complete cross-resistance to all antibiotics used in this study. This was also reported by Leclercq *et al.* [12] and Wang *et al.* [25]. However, one of two *ermB* carrying *S. aureus* isolates with cML resistance phenotype was entirely were susceptible to tilmicosin (MIC=0.5 µg/ml) and other isolate showed intermediate resistance to tilmicosin (MIC=16 µg/ml). Low level tilmicosin resistance can be attributed to difference in structural composition and the formation of more effective bond with 50S ribosomal subunit [25].

In conclusion, the results of this study indicate that the ML resistance was prevalent in staphylococci from subclinical bovine mastitis in Turkey and ML-associated resistance genes were present solely or in various combinations in these isolates, being *msrA*, *ermB* and *ermC* genes predominating. Therefore, prudent use of this antimicrobials and continuous surveillance should be established to prevent the emergence and spread of ML resistant staphylococci.

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