Lancefield classification and antimicrobial resistance of hemolytic streptococci isolated from bovine mastitis

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Summary
Streptococcal species are known to be responsible for bovine mastitis. The aim of the present study was to determine antimicrobial drug resistance patterns of hemolytic streptococci distributed according to Lancefield serogrouping. *Streptococcus* sp. strains were isolated from 124 bovine milk samples from 31 cows with subclinical or clinical mastitis submitted to Mehmet Akif Ersoy University Faculty of Veterinary Medicine, Department of Microbiology Laboratory in Burdur province, Turkey from January 2015 to January 2017. A total of 63 *Streptococcus* sp. were isolated and the most frequently obtained isolates were classified as Lancefield's serogroup B (84.13%), the remaining isolates as serogroup F (15.87%). Out of 63 isolates, 53 (84.13%) showed beta-hemolytic activity whereas 10 (15.87%) alpha-hemolytic activity. Antimicrobial resistance was assessed by disk diffusion test against the most common antibiotics used in the field. Among the 63 *Streptococcus* sp. tested, the highest antimicrobial resistance patterns were observed for neomycin (95.24%), trimethoprim sulphamethoxazole (87.30%) and gentamicin (69.84%). None of the isolates showed resistance to amoxicillin-clavulanic acid, except for one serogroup F isolate. The resistance rates for the other antimicrobials ranged from 1.59% to 38.04%. A total of 50 isolates exibited multi-drug resistance to ≥ 3 antimicrobial agents tested. Overall, our results suggested that there is an urgent need to enhance awareness among the dairy farmers in choosing the appropriate drug for treating mastitis.

Introduction

Mastitis is one of the most frequent and multifactorial disease causing major losses in the dairy industries (Halasa *et al.* 2007, Vliegher *et al.* 2012). Economic losses mainly depend on early slaughter of cows (injured mammary parenchyma and decreased milk production) commercial devaluation of animals, microbiological diagnosis of pathogenic agent and use of in large amount of antibiotics without regard for resistance (Halasa *et al.* 2007, EMA 2017). The economic losses caused by bovine mastitis in Turkey reach to about 28 million dollars (Tekeli 2005).

Streptococcal species are known to be significant causative pathogens of bovine mastitis (Facklam 2002, Freney *et al.* 1992, Fortin *et al.* 2003, Leigh 1999). Streptococcal mastitis can be classified according to the source of infectious organisms, either contagious mastitis (*Streptococcus agalactiae*) or environmental mastitis (*Streptococcus uberis, Streptococcus dysgalactia, Streptococcus bovis, Streptococcus parauberis, Streptococcus equi* and *Streptococcus canis*) (Leigh 1999, Oliver *et al.* 1998). Although *S. agalactiae* was one of the main pathogens causing mastitis (Oliver *et al.* 1998, Keefe 1997), environmental streptococci have consistently been reported as a leading cause of both subclinical and clinical mastitis throughout the world (Phuektes *et al.* 2001, Teng *et al.* 1998).

Antimicrobial therapy is commonly used for mastitis for more than fifty years but an efficient, safe, and economical treatment is still lacking (Guérin-Faublée *et al.* 2002, Hendriksen *et al.* 2008). Proper use of drug, dairy husbandry, sanitation procedures and the stage of the disease are among the most common reasons for this situation (Keefe 1997). Thus, the emergence of antibiotic resistance of infectious agent during the bacteriological examination of milk samples is an important basis for the selection of the appropriate chemotherapeutic agents and reduction of the therapeutic failures (Schwarz *et al.* 2010). Although streptococci are generally sensitive to beta-lactams and macrolides (Hendriksen *et al.* 2008, Denamiel *et al.* 2005, Haenni *et al.* 2010, Kalmus *et al.* 2011, Tenhagen *et al.* 2006), the increasing resistance of streptococci to some antimicrobials as tetracyclines and macrolids with the broad spectrum commonly used in veterinary was reported (Aarestrup *et al.* 1998, Blowey *et al.* 1995a, Palmieri *et al.* 2011, Zhang *et al.* 2008).

The main objective of the current study was to determine the phenotypic antimicrobial resistance profiles of the hemolytic streptococci distributed according to Lancefield serogrouping.

Materials and methods

Bacterial isolates

A total of 63 *Streptococcus* isolates from subclinical or clinical bovine mastitis cases in Burdur province of Turkey were included in the present study. The area is the crossing point of the Aegean, Central Anatolia and Mediterranean parts of Turkey. The isolates were obtained from 124 milk samples of 31 cows submitted to Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology Laboratory in Burdur from January 2015 to January 2017. Each of streptococci was isolated from an individual mammary gland of cow.

Isolation and identification

The milk samples (10 µl) were streaked on 5% defibrinated sheep blood agar and Edward's media (Oxoid, UK). The inoculated plates were incubated at 37 °C for 24-48 h aerobically. Suspected colonies were morphologically characterized and examined by Gram stain, catalase test, oxidase test, hemolytic activity, CAMP-reaction and esculin hydrolysis (Akan 2006, Barrow *et al.* 1993, Quinn *et al.* 2004).

Lancefield serogrouping

Serological grouping of isolates was performed with a commercial latex agglutination kit (STREP Test Kit, Plasmatec, UK) for the identification of streptococcal groups A, B, C, D, F and G. Streptococci were tested using the broth method described by the manufacturer. Isolates indicated the Lancefield group F were defined as *S. anginosus* group (also known as the *S. milleri* group) (Facklam 1977, Facklam 2002).

Antimicrobial susceptibility test

Antimicrobial susceptibility test was carried out by the disk diffusion method on Mueller-Hinton agar (Oxoid Ltd, Hampshire, UK) supplemented with 5% sheep blood according to the guidelines from Clinical and Laboratory Standards Institute (CLSI 2017). The following antibiotics commonly used in veterinary medicine were selected: amoxicillin (10 µg; AX, Oxoid, UK), amoxicillin clavulanic acid (30 µg; AMC, Oxoid, UK), cephaperazone (30 µg; CFP, Oxoid, UK), cephalexin (30 µg; CL, Oxoid, UK), ciprofloxacin (5 µg; CIP, Oxoid, UK), enrofloxacin (5 µg; ENR, Oxoid, UK), erythromycin (15 µ; E, Oxoid, UK), gentamicin (10 µg; CN, Oxoid, UK), lincomycin (10 µg; L, Oxoid, UK), neomycin (30 µg; N, Oxoid, UK), oxytetracycline (30 µg; OT, Oxoid, UK), penicillin (10 units; P, Oxoid, UK), trimethoprim sulphamethoxazole (25 µg; TS, Oxoid, UK). The results were obtained by measuring the diameter of the growth inhibition zone around the antibiotic disc for each isolated bacteria and recorded as sensitive, intermediate and resistant according to the interpretive standards of CLSI and antimicrobials manufacturers' instructions. Isolates displaying resistance to ≥ 3 antimicrobial agents tested were defined as exhibiting multi-drug resistance (MDR) (Schwarz *et al.* 2010, Tenover 2006).

Results

Isolation and identification

A total of 63 streptococci were characterised in this study. All isolates were aerobic, catalasi negative gram positivi cocci. They also demonstrated haemolytic activity. Beta-hemolysis (84.13%) was observed in 53 of isolates. Alpha hemolysis was instead evident in the remaining 10 isolates (15.87%). Out of 57 (90.47%) CAMP-positive isolates, 4 (6.34%) showed alpha and 53 (84.12%) beta hemolysis. Six alfa-haemolitic *Streptococcus* sp. exhibited an esculin-positive reaction when subcultured on Edward's media. According to serological analysis, of the 63 isolates, 53 (84.13%) were grouped as Lancefield's serogroup B streptococci (GBS) and 10 (15.87%) as Lancefield's serogroup F streptococci (GFS). CAMP and esculine hydrolysis results were summarized in Table I.

Antimicrobial resistance

Among the 63 *Streptococcus* sp. tested, the highest antimicrobial resistance patterns were observed for neomycin (95.24%), trimethoprim sulphamethoxazole (87.30%) and gentamicin (69.84%). Except for one GFS strain, none of the isolates showed resistance to amoxicillin-clavulanic acid. The resistance rates for the other antimicrobials

Table I. *CAMP and esculine hydrolysis results of hemolytic streptococci isolated from bovine mastitis and serogrouped according to Lancefield's group.*

	CAMP test (+)	Esculine hydrolysis (+)
Lancefield B, α-hemolytic	3 (4.76 %)	0
Lancefield B, β-hemolytic	50 (79.36 %)	0
Lancefield F, α-hemolytic	1 (1.58 %)	6 (9.52%)
Lancefield F, β-hemolytic	3 (4.76 %)	0
Total	**57 (90.47 %)**	**6 (9.52 %)**

were determined in varying rates from 1.59% to 38.04% (Table II). A total of 50 isolates including 40 (80%) GBS and 10 (20%) GFS, were defined as exibiting MDR (Table III).

Discussion

Milk production from cattle is 250.000 tons / a year. Only 20% of the product is processed in Burdur while the remaining 80% in the other provinces of Turkey (Elmaz *et al.* 2010). Decrease in milk yield due to mastitis is then a very important problem involving the whole country.

Staphyloccoccus sp. was referred to be the most frequent agent isolated in case of bovine mastitis world-wide (Turutoglu *et al.* 2002, Turutoglu *et al.* 2006, Shitandi *et al.* 2004, Gianneechini *et al.* 2002, Savasan *et al.* 2017, Tel *et al.* 2009, Minst *et al.* 2012). Over the last 20 years' period, the rate of streptococcal mastitis varied from 3.86% to 40% in different provinces of Turkey (Turutoglu *et al.* 2002, Tel *et al.* 2009, Ekin *et al.* 2011, Gurturk *et al.* 1998, Ak 2000, Ergun *et al.* 2004, Acar *et al.* 2012, Bal *et al.* 2010, Macun *et al.* 2011, Ikiz *et al.* 2013). As expected, in our study, GBS (84.12%) were the predominant serogroup and the rate of beta-hemolytic GBS (94.34%) was higher than alpha-hemolytic GBS (5.66%). However, the frequency of the GBS isolation was higher than that reported in the previous studies (Keefe 1997, Guérin-Faublée *et al.* 2002, Ekin *et al.* 2011, Akay *et al.* 1993). As already reported (Teixeira *et al.* 2003), also in this study GBS beta-hemolytic strains are most commonly identified than the alpha haemolytic ones.

To our knowledge the first case of bovine mastitis caused by GFS belonging the *S. anginosus* group (also known as the *S. milleri* group). Isolates of this group can demonstrate alpha, beta and gamma hemolysis patterns and be both CAMP and esculine-hydrolysis positive (Facklam 1977, Facklam 2002, Ruoff 1988, Spellerberg *et al.* 2015). We also found that some isolates have CAMP, esculine and beta or alpha hemolytic activity. *Streptococcus anginosus* group members have been implicated as etiologic agents in a variety of purulent infections (tissue abscesses, such as brain, dental and hepatic, occasional endocarditis and wound infections), but clinical significance still remains unclear (Gossling 1998, Whitworth 1990).

In this study, a wide diffusion of antibiotic resistance to most of antimicrobials tested was revealed even more concerning was the high prevalence and the MDR exibited by most of the isolated GBS and GFS. The generated data set allowed us to get better

Table II. *Antimicrobial resistance of 63 hemolytic streptococci from bovine mastitis cases.*

Antimicrobials	GBS (n = 53)			GFS (n = 10)			Total (n = 63)		
	S n (%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)
OT	31 (58.50)	7 (13.20)	15 (28.30)	1 (10)	2 (20)	7 (70)	32 (50.79)	9 (14.29)	22 (34.92)
L	34 (64.15)	3 (5.66)	16 (30.19)	7 (70)	0	3 (30)	41 (65.08)	3 (4.76)	19 (30.16)
N	3 (5.67)	0	50 (94.33)	0	0	10 (100)	3 (4.76)	0	60 (95.24)
CFP	47 (88.68)	4 (7.55)	2 (3.77)	8 (80)	1 (10)	1 (10)	55 (87.30)	5 (7.94)	3 (4.76)
TS	3 (5.67)	2 (3.77)	48 (90.56)	2 (20)	1 (10)	7 (70)	5 (7.94)	3 (4.76)	55 (87.30)
AMC	52 (98.11)	1 (1.89)	0	9 (90)	0	1 (10)	61 (96.82)	1 (1.59)	1 (1.59)
ENR	22 (41.51)	16 (30.19)	15 (28.30)	4 (40)	1 (10)	5 (50)	26 (41.27)	17 (26.98)	20 (31.75)
P	40 (75.48)	2 (3.77)	11 (20.75)	6 (60)	0	4 (40)	46 (73.02)	2 (3.17)	15 (23.81)
CN	14 (26.42)	0	39 (73.58)	5 (50)	0	5 (50)	19 (30.16)	0	44 (69.84)
AX	41 (77.36)	7 (13.21)	5 (9.43)	6 (60)	0	4 (40)	47 (74.60)	7 (11.11)	9 (14.29)
CL	38 (71.69)	7 (13.21)	8 (15.10)	6 (60)	0	4 (40)	44 (69.84)	7 (11.11)	12 (19.05)
CIP	24 (45.28)	8 (15.10)	21 (39.62)	5 (50)	2 (20)	3 (30)	29 (46.03)	10 (15.88)	24 (38.09)
E	35 (66.03)	10 (18.87)	8 (15.10)	3 (30)	3 (30)	4 (40)	38 (60.32)	13 (20.63)	12 (19.05)

GBS = Group B streptococci; GFS = Group F streptococci; S = Sensitive; I = Intermediate; R = Resistant; OT = Oxytetracycline; L = Lincomycin; N = Neomycin; CFP = Cephaperazone; TS = Trimethoprim sulphamethoxazole; AMC = Amoxicillin clavulanic acid; ENR = Enrofloxacin; P = Penicillin; CN = Gentamicin; AX = Amoxicillin; CL = Cephalexin; CIP = Ciprofloxacin; E = Erythromycin.

Table III. *Antimicrobial resistance profiles of 50 multi-drug resistant* Streptococcus *sp. isolated from bovine mastitis.*

	Antimicrobials	GBS (n = 40)		GFS (n = 10)	
		n	%	n	%
Resistance to 3 antimicrobials	CL, CN, CIP	0	0	1	10
	N, TS, CN	7	17.5	0	0
	OT, N, TS	1	2.5	0	0
	CL, CIP, AX	1	2.5	0	0
Resistance to 4 antimicrobials	N, TS, ENR, CN	4	7.5	0	0
	TS, ENR, P, CN	1	2.5	0	0
	N, TS, P, CN	1	2.5	0	0
	N, TS, ENR, P	0	0	1	10
	OT, N, TS, CN	0	0	2	20
	OT, L, CN, CIP	1	2.5	0	0
	OT, L, N, E	1	2.5	0	0
	N, TS, CN, CIP	1	2.5	0	0
	N, TS, CL, CIP	1	2.5	0	0
	L, N, TS, CN	0	0	1	10
Resistance to 5 antimicrobials	N, TS, ENR, CN, CIP	1	2.5	0	0
	OT, TS, ENR, CN, CIP	1	2.5	0	0
	OT, N, TS, CN, E	4	10	0	0
	OT, N, TS, CL, CIP	1	2.5	0	0
	OT, N, TS, ENR, CIP	1	2.5	0	0
	OT, L, N, TS, E	1	2.5	1	10
Resistance to 6 antimicrobials	L, N, TS, ENR, CN, CIP	1	2.5	0	0
	OT, N, TS, CL, CN, CIP	1	2.5	0	0
Resistance to 7 antimicrobials	L, N, TS, ENR, CN, CIP, E	1	2.5	0	0
	OT, N, TS, ENR, CN, AX, E	0	0	1	10
	OT, L, N, TS, P, CN, CIP	2	5	0	0
	L, N, TS, CL, P, CN, CIP	1	2.5	0	0
Resistance to 8 antimicrobials	L, N, TS, ENR, P, CN, CIP, AX	1	2.5	0	0
	L, N, TS, ENR, P, CN, CIP, E	2	5	0	0
	OT, N, TS, CL, ENR, P, CN, CIP	1	2.5	0	0
	OT, CFP, N, TS, CL, CN, CL, E	1	2.5	0	0
Resistance to 9 antimicrobials	L, N, TS, CL, ENR, P, CN, CIP, AX	0	0	1	10
	OT, L, N, TS, CL, P, CN, CIP, AX	1	2.5	0	0
	L, N, AMC, CL, ENR, P, CIP, AX, P	0	0	1	10
Resistance to 10 antimicrobials	OT, L, CFP, N, CL, ENR, P, CN, AX, E	0	0	1	10

GBS = Group B streptococci; GFS = Group F streptococci; OT = Oxytetracycline; L = Lincomycin; N = Neomycin; CFP = Cephaperazone; TS = Trimethoprim sulphamethoxazole; AMC = Amoxicillin clavulanic acid; ENR = Enrofloxacin; P = Penicillin; CN = Gentamicin; AX = Amoxicillin; CL = Cephalexin; CIP = Ciprofloxacin; E = Erythromycin.

insights in to the antibiotic resistance of hemolytic streptococci. The highest resistance to neomycin (95.24%), trimethoprim sulphamethoxazole (87.30%) and gentamicin (69.84%) was not surprising for *Streptococcus* sp. as it has an intrinsic resistance to aminoglycosides and sulphonamides (Swedberg *et al.* 1998). On the other hand, except for one GFS isolate, all isolates showed high susceptibility to the amoxicillin-clavulanic acid. Similar antibiogram patterns were observed by Ikiz and colleagues (Ikiz *et al.* 2013). Tel and colleagues (Tel *et al.* 2009) reported high gentamicin resistance (78.3%) as well. Conversely, the high resistance rate to penicillin (23.81%) and amoxicillin (14.29%) was in contrast to what observed by other researchers who reported lower resistance rates (Tenhagen *et al.* 2006, Ekin *et al.* 2011). Beta-lactam antibiotics were widely used in cows for the treatment and prevention of diseases; a high rate of resistance to these antibiotics was therefore not unexpected. On the other hand, it has stated that the cephalexin (first generation cephalosporins) and cephaperazone (third generation cephalosporins) have greater anti-streptococcal activities than other beta-lactams (Ceniti *et al.* 2017). Also in this study a lower resistance to cephalexin (19.05%) and cephaperazone (4.76%) was observed (Rügsegger *et al.* 2014, Zhang *et al.* 2018).

Increasing resistance of streptococci to commonly used broad-spectrum antimicrobials including tetracyclines (up to > 90%) and macrolides (up to > 70%) has been reported worldwide since the 1980s (Aarestrup *et al.* 1998, Blowey *et al.* 1995a, Palmieri *et al.* 2011, Zhang *et al.* 2008). In the present study, the isolated GFS and GBS showed similar resistance rates (30%) against lincomycin, whereas the isolated GFS were found to be highly resistant to oxytetracycline (70%) and erythromycin (40%). These results were similar to what has been previously described in other countries (Kalmus *et al.* 2011, Petrovski *et al.* 2006). Although fluoroquinolones are considered among the most effective drugs, increasing the risk of quinolone-resistant bacteria should not to be ignored (Lopez *et al.* 2015). In this study, the resistance rates to enrofloxacin and ciprofloxacin were high in both GBS (28.30% and 39.62%) and GFS (50% and 30%). This was in agreement with other reports in Turkey that streptococci have variable resistance to fluoroquinolones (Acar *et al.* 2012, Macun *et al.* 2011).

MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Schwarz *et al.* 2010, Tenover 2006). In this study, multiple resistances to three or more than three antimicrobial agents tested were observed. Of the 63 *Streptococcus* isolates, 50 (79.36%) were found as MDR. MDR rates of GBS and GFS isolates were 75.47% (40/53) and 100% (10/10), respectively. Both, the isolated GBS and GFS were particularly resistant to gentamicin, oxytetracycline, trimethoprim sulphamethoxazole and neomycin. Meanwhile, the isolated GBS and GFS were resistant to ≥ 3 antimicrobials in varying rates from 2.5 to 17.5% and from 10 to 20%, respectively. Nevertheless, one alpha-hemolytic GFS strain showed resistance against 10 antimicrobials. As observed in other countries, our results indicated the presence of *Streptococcus* sp. isolates with high level of MDR (Nam *et al.* 2009, Ding *et al.* 2016).

Conclusions

Streptococcal mastitis is a common cause of economic loss in dairy herds in Turkey as well as throughout the world. The economic losses could be irreversible because of late and improper diagnosis of the main etiological agent. There is an urgent need to enhance awareness among the dairy farmers in choosing the appropriate drug for treating mastitis. This should be done keeping in mind the emergence of MDR strains. In addition to treatment, control measures should be improved and put in place in accordance with contagious transmission and environmental exposure.

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