Phenotypic antimicrobial resistance profile of isolates causing clinical mastitis in dairy animals

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Abstract

Mastitis is the most frequent and costly disease of lactating animals and is associated with a significant reduction in milk yield, increased cost and culling. Early and specific antibiotic based treatment reduces the severity of the disease. Over the years the extensive use of antimicrobials has led to increase antimicrobial resistance. The present study was designed to investigate the prevalence of microorganisms responsible for mastitis and their antimicrobial resistance pattern. A total of 282 milk samples were collected from different animal species (sheep, cows and goats) with clinical mastitis. Antimicrobial resistance was evaluated for *Streptococcus* spp. and *Staphylococcus* spp. In cow samples *Streptococcus* spp. represented the most frequently isolated genus (33.84%), while *Staphylococcus* spp. was the most prevalent genus in sheep and goat samples (44.4 and 73.86%, respectively). Gentamicin and chloramphenicol were found to be the most effective drugs against the tested isolates, while the highest resistance rates were observed for amoxicillin, ampicillin, tetracycline, trimethoprim-sulfamethoxazole.

Introduction

Mastitis is an inflammation of the mammary gland (Fox *et al.*, 2001; Bradley, 2002) and is the most frequent and costly disease in dairy animals throughout the world (Gomes and Henriques, 2016). Mastitis causes decreased milk production and quality, decreased cheese yield, increased cost of treatment, labor and culling (Gomes and Henriques, 2016). The most frequent pathogen in small ruminant (Bergonier *et al.*, 2003) and cows (Fox *et al.*, 2001) is *Staphylococcus aureus* but mastitis is also caused by many other bacteria such us *Streptococcus* spp., *Escherichia coli*, *Pseudomonas* spp., and *Mycoplasma* spp. Currently, the use of antibiotics is the most common treatment (Gomes and Henriques, 2016) and β-lactams are the most frequently classes used for the treatment of mastitis. Additionally, mastitis therapy is commonly started before the results of antimicrobial susceptibility test of pathogens (Hendriksen *et al.*, 2008) representing one of the most important reason for treatment failure. Moreover, this antibacterial strategy has many disadvantages including a low cure rate, increasing the presence of antibiotics residues and occurrence of antimicrobial resistance (Minst *et al.*, 2012). Resistance to antibiotics may be acquired by spontaneously occurring genetic mutations and more commonly by the horizontal transfer of mobile DNA elements from a donor cell to another bacterial species (Chambers, 2001). Over the years, extensive use of antimicrobials has led to increasing resistant bacteria at alarming rate and has become a serious concern worldwide. In order to ensure suitable antibiotic therapy, the bacterial isolation and the evaluation of antibiotic susceptibility are essential. In addition, milk produced from animals with subclinical mastitis posed serious veterinary and epidemiology risk since its rich nutrient composition and neutral pH make it a good vehicle for the survival and growth of bacteria. Resistant bacteria may contaminate food products and they could be transmitted to humans through the food chain underlining the importance of pathogens surveillance. Hence monitoring pathogens and their antimicrobial resistance patterns are the need of the day. The aim of this study was to investigate the prevalence of microorganisms responsible for mastitis and their antimicrobial resistance in Catanzaro district (Italy).

Materials and Methods

Sampling

In 2016, a total of 282 milk samples were collected from animals (140, 110 and 32 from sheep, cows and goats respectively) with clinical mastitis bred in 125 dairy farms (60, 52 and 13 sheep, cows and goats dairy farms respectively) located in Southern Italy (Catanzaro, Calabria region). All the samples were obtained from routine submission of milk to the Institute for Experimental Veterinary Medicine of Southern Italy, Catanzaro. Samples were transported cooled to the laboratory and kept at 4°C before examination (within 24 h). From each sample, an amount of 10 ml was ten-fold diluted in 90 ml of a quarter-strength Ringer Solution (Oxoid Ltd., Hampshire, UK).

Isolation and identification

From each sample an amount of one mL was inoculated onto selective Baird-Parker, Columbia Blood and McConkey (Oxoid Ltd.) agar media for isolation of staphylococci, streptococci and coliform bacteria, respectively. Plates were incubated at 37°C per 24-48 hours. Colonies were presumptive identified based on morphology, hemolytic patterns and Gram strain. Confirmation was carried out using ID-GNB (BioMérieux, Marcy l'Etoile, France) cards for gram-negative bacteria and ID-GNP (BioMeriéux) cards for gram positive for Vitek 2 system (BioMérieux) following producer instructions.

Antimicrobial susceptibility testing

The most prevalent isolates were tested for their drug susceptibility using disc diffusion method against the following antibiotics: amoxicillin-clavulanate 20/10 µg; ampicillin 10 µg; cefalotin 30 µg; cefotaxime 30 µg; ceftiofur 30 µg; clindamycin 2 µg; chloramphenicol 30 µg; gentamicin 10 µg; enrofloxacin 5µg; erythromycin 15 µg; kanamycin 30 µg; oxacillin 1 µg; peni-

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cillin 10 U.I.; sulfisoxazole 250 µg; tetracycline 30 µg; trimethoprim-sulfamethoxazole 1.25/23 and tylosin 10 µg. Isolates were classified as resistant or susceptible towards the tested antimicrobials in accordance with breakpoints proposed by the Clinical and Laboratory Standards Institute (2007). Intermediate isolates were grouped with the resistant ones. Multidrug resistance was defined as resistance of 3 or more classes of antimicrobial agents (Schwarz *et al*., 2010).

Results

Out of 282 samples, 68 did not yield bacterial growth on culture, 52 were considered contaminated (>2 bacterial species yielded) and in 162 a single bacteria strain was isolated. Table 1 shows the number and frequency of microorganism isolated from milk samples according to animal species.

Microorganisms isolated from sheep samples were identify as *Staphylococcus aureus* (20.14%), *Staphylococcus chromogenes* (11.14%), *Staphylococcus epidermidis* (11.14%), *Staphylococcus warneri* (8.14%), *Enterobacter cloacae* (6.14%), *Kocuria rosea* (6.14%), *Staphylococcus simulans* (6.14%), *Streptococcus uberis* (5.14%), *Staphylococcus hominis* subsp. *hominis* (5.14), *Staphylococcus auricolaris* (4.14%).

The most prevalent isolates in cow samples were *Staphylococcus aureus* (24.62%), *Streptococcus agalactiae* (15.38%), *Streptococcus dysgalactiae* (12.31%), *Enterococcus avium* (10.77%), *Lactococcus* spp. (10.77%), *Aerococcus viridians* (9.23%), *Staphylococcus chromogenes* (4.62%) *Streptococcus equisimilis* (3.08%). From the goat samples the predominant bacteria isolated was *Staphylococcus aureus* (44.44%).

Staphylococcus spp. for sheep (69.31%) and goat (77%) samples and *Streptococcus* spp. for cow samples (33.84%) resulted the most prevalent bacterial genera.

A total of 121 samples resulted positive for *Staphylococcus* spp. and *Streptococcus* spp. and were tested for antibiotic resistance; the percentage of isolates resistant to tested antibiotics is reported in Table 2.

Gentamicin, oxacillin, cefotaxime were the most effective drugs (all the isolates were sensible) against *Staphylococcus* spp., whereas all of *Streptococcus* spp. showed to be sensitive to ceftiofur and chloramphenicol.

Staphylococcus spp. showed to be resistant to ampicillin (34% of isolates) followed by sulfisoxazole (25%) trimethoprim-sulfamethoxazole (18%), amoxicillin-clavulanate (15.62%), clindamycin (15.62%) tetracycline (9%). Some resistances were also observed against enrofloxacin, chloramphenicol, erythromycin (6%).

Most of *Streptococcus* spp. resulted resistant to tetracycline (57%); the 42% of *Streptococcus* spp. isolates resulted resistant to clindamycin and the 28% resulted resistant to ampicillin.

Only 4 isolates were susceptible to all the antibiotics tested. The 25% of bovine isolates and the 17% of ovine isolates were multiresistant. Multi drug resistance occurred most frequently among amoxicillin, tetracycline and trimethoprim-sulfamethoxazole, and in the β-lactam antibiotics class, a majority of isolates resistant to ampicillin were also resistant to amoxicillin-clavulanate.

Discussion

According to the previous paper (Contreras *et al.*, 2007), in our study the most representative bacteria genus isolated from sheep and goat mastitic milk was *Staphylococcus* spp. Unsurprisingly the most prevalent species was *Staphylococcus aureus* (20.14% in sheep and 44.44% in goat) whose presence in sheep and goat milk has been widely demonstrated in Italy: Cortimiglia *et al.* (2015) evidenced in bulk tank sheep's milk a *Staphylococcus aureus*

Table 1. Number and percentage of isolates from mastitic milk according to animal species.

	Sheep		Cows		Goat	
	Isolates	Samples, n (%)	Isolates	Samples, n (%)	Isolates	Samples, n (%)
Staphylococcus spp.	*Staphylococcus aureus*	19 (20.14)	*Staphylococcus aureus*	16 (24.62)	*Staphylococcus aureus*	4 (44.44)
	Staphilococcus chromogenes	10 (11.4)	*Staphylococcus warneri*	1 (1.54)	*Staphylococcus epidermidis*	1 (11.11)
	Staphylococcus epidermidis	10 (11.14)	*Staphylococcus chromogenes*	3 (4.62)	*Staphylococcus simulans*	1 (11.11)
	Staphilococcus warneri	7 (8.14)			*Staphylococcos capitis*	1 (11.11)
	Staphylococcus simulans.	5 (6.14)				
	S. hominis subsp hominis	4 (5.14)				
	Staphilococcus auricolaris	3 (4.14)				
	Staphylococcus haemolyticus	2 (3.14)				
	Staphilococcus caprae	2 (3.14)				
	Staphylococcus capitis	2 (3.14)				
	Staphylococcus intermedius	1 (2.14)				
	Total	65 (73.86)		20 (30.77)		7 (77.78)
Streptococcus spp.	*Streptococcus uberis*	4 (5.14)	*Streptococcus agalactiae*	10 (15.38)		
	Streptococcus dysgalactiae	1 (2.14)	*Streptococcus dysgalactiae*	8 (12.31)		
	Streptococcus ovis.	1 (2.14)	*Streptococcus equisimilis*	2 (3.08)		
	Streptococcus pluranimalium	1 (2.14)	*Streptococcus plurianimalium*	1 (1.54)		
	Streptococcus uberis	1 (1.54)				
	Total	7 (7.95)		22 (33.85)		
Other bacteria	*Enterobacter cloacae*	5 (6.14)	*Enterococcus avium*	7 (10.77)	*Klebsiella spp.*	1 (11.11)
	Kocuria rosea	5 (6.14)	*Lactococcus lactis*	7 (10.77)	*Alloiococcus otitis*	1 (11.11)
	Lactococcus lactis	2 (3.14)	*Aerococcus viridians*	6 (9.23)		
	Aerococcus viridans	*1 (2.14)*	*Aeromonas hydrophila*	*1 (1.54)*		
	Escherichia coli	1 (2.14)	*Pasteurella canis*	1 (1.54)		
	Kocuria kristinae	1 (2.14)	*Pseudomonas* spp.	1 (1.54)		
	Leuconostoc mesenteroides	1 (2.14)				
	Total	88 (100)		65 (100)		9 (100)

prevalence of 43%, Spanu *et al.* (2013) in bulk tank goat's milk showed a prevalence of 76%. On the other hand, the predominant bacterial genera isolated from cow samples was *Streptococcus* spp. (33.84%) out of these samples, 10 were identified as *Streptococcus agalactiae* and 8 as *Streptococcus dysgalactiae*; the first is most often associated with cows and well adapted to the mammary gland whereas *Streptococcus dysgalactiae* is an environmental pathogen (Minst *et al.*, 2012).

Our findings are in agreement with several other reports (Munsi *et al.*, 2016; Chaffer *et al.*, 1999; Minst *et al.*, 2012; Iqbal *et al.,* 2004) and are in contrast with other authors (Sumathi *et al*., 2008; Singh *et al*., 2016) who showed a higher prevalence of *Staphylococcus* spp. in cow mastitic milk. However in our work *Staphylococcus* spp. showed a high prevalence (30.77%) too; indeed, as shown in Table 1, the main representative isolate was *Staphylococcus aureus*. It is well known that *Staphylococcus aureus* is frequently isolated in bovine clinical or subclinical mastitis (Moroni *et al.*, 2006).

This high *Staphylococcus aureus* prevalence in sheep, cow and goat milk might pose a health risk to humans. It is well known that *Staphylococcus aureus* produces a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the microorganism. The staphylococcal enterotoxins are recognized agents of the staphylococcal food poisoning syndrome and may be involved in other types of infections with sequelae of shock in humans and animals (Akineden *et al.*, 2001). In addition, many traditional caprine and ovine milk products are not subjected to pasteurization representing a potential source of staphylococcal food poisoning (Merz *et al.*, 2016).

Lactococcus lactis has also been evidenced in 7 cow samples: normally used as a starter strain in several foods such as cheese, it has been linked, in recent years, to bovine mastitis cases (Wyder *et al.*, 2011; Romero *et al.*, 2011).

Enterococcus avium, as previously reported by Nam *et al.* (2009), has been evidenced in cow mastitic milk samples.

Our findings revealed a wide diffusion of antibiotic resistance to most of antimicrobials tested; even more concerning was the high prevalence of resistance and multiresistant isolates for those antimicrobials normally used for mastitis treatment such as β-lactams. It is well known that β-lactams (penicillins and cephalosporins) are widely used for intramammary treatment of bovine mastitis. Similar results were reported for cow milk by Rajala-Schultz *et al.* (2004) and Bhatt *et al.* (2011) and in other studies carried out in Italy (Moroni *et al.*, 2006). Only oxacillin showed high activity against staphylococci.

Cephalosporins showed greater antistaphylococcal and antistreptococcal activities than other β-lactams such as amoxicillin, accordingly to Moroni *et al.* (2006) who evidenced a good activity against *Staphylococcus aureus* for cephalosporins of first generation and third generation (cephalonium, cefoperazone). Rajala-Schultz *et al.* (2004) reported that 63% of streptococci were resistant to cephalothin; in our study only 14% of *Streptococcus* spp. resulted resistant to cephalothin. Furthermore, cefotaxime and ceftiofur resulted highly efficient against staphylococci and streptococci respectively.

Lincosamides are commonly used for therapy of staphylococcal infection and are frequently used for the treatment of bovine mastitis (Wang *et al.*, 2008). In the present study, clindamycin, showed low activity against *Staphylococcus* spp. and resulted more efficient against *Streptococcus* spp.

Additionally, *Staphylococcus* spp. resulted highly sensitive to gentamicin followed by enrofloxacin and erythromycin. This study also revealed chloramphenicol to be the most effective drug against streptococci. Similar antibiogram patterns were also reported by Bhatt *et al.* (2011), Iqbal *et al.* (2004) and Sumathi *et al.* (2008). This latter proved gentamicin to be the drug of choice against clinical mastitis in dairy cattle.

An interesting observation is that isolates had high resistance and multi resistance for tetracycline and trimethoprim-sulfamethoxazole as reported in other studies (Rajala-Schultz *et al.,* 2004), although these molecules are only occasionally used for treating mastitis. Prescott (2000) suggests that sulfa-resistance is often carried along with tetracycline resistance in plasmids.

Although our results are in accordance with previous research, we have to considerer that comparison between different studies is difficult due to different susceptibility testing methods and different interpretative criteria being used to categorize isolates as susceptible or resistant. Antimicrobial test is useful to detect the most efficient drugs to contrast bacterial grow even though several factors can influence the overall susceptibility pattern of mastitis pathogens (Oliver *et al.*, 2011).

Table 2. Antimicrobial resistance pattern of *Streptococcus* spp. and *Staphylococcus* spp. isolated from mastic milk samples.

Antibiotics	Resistance (%)	
	Streptococcus spp.	*Staphylococcus* spp.
β-lactam antibiotics		
Ampicillin	28	34
Amoxicillin-clavulanate	nt	15.62
Penicillin	14	nt
Oxacillin	nt	0
Cefalotin	14	6
Cefotaxime	nt	0
Ceftiofur	0	nt
Aminoglycosides		
Gentamicin	2	0
Kanamycin	nt	3
Lincosamides		
Clindamycin	42	15.62
Fluoroquinolone		
Enrofloxacin	14	6
Macrolide		
Tylosin	28.57	nt
Erythromycin	14	6
Chloramphenicol	0	6
Tetracycline	57	9
Trimethoprim-sulfamethoxazole	28	18
Sulfisoxazole	nt	25

nt, not tested.

Conclusions

The study showed that *Streptococcus* spp. and *Staphylococcus* spp. are the most frequent bacteria found in mastitic milk of

cows and sheep/goats respectively. Of all samples, the main representative isolate turned out to be *Staphylococcus aureus*. Ceftiofur and chloramphenicol resulted the most effective antibiotics against streptococci, whereas isolates showed high resistance for tetracycline and clindamycin. Staphylococci resulted highly sensitive to gentamicin, cefotaxime, oxacillin and resistant to ampicillin and sulfisoxazole. The 25% of bovine isolates and the 17% of ovine isolates were multiresistant.

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