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Antimicrobial profile of multidrug-resistant *Staphylococcus* spp. isolated from bovine mastitis cases in the northwest region of Paraná State, Brazil

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Bovine mastitis is the main cause of economic losses in dairy industry. The aim of this study was to determine the prevalence and antibiotic-resistance profile of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. isolated from bovine mastitis cases in the municipality of Altônia, northwest region of Paraná State, Brazil. Two hundred and forty-eight dairy cows were examined for the presence of mastitis. Milk samples from mastitis cases were collected and analyzed for the presence of *Staphylococcus* spp. according to the National Mastitis Council (NMC). The antimicrobial susceptibility of the isolates was also evaluated. The results suggest the presence of methicillin-resistant strains in high frequency (20.40%). Hence, the determination of antimicrobial resistance profile is essential to control mastitis in dairy cows. The use of antibiogram may also help to reduce the emergence of multidrug-resistant strains, since the choice of antibiotics will not be based on empirical methodology.

Key words: *Staphylococcus aureus*, coagulase-negative staphylococci, multidrug resistance, methicillin-resistance *Staphylococcus aureus*.

INTRODUCTION

Bovine mastitis is the main cause of economic losses in dairy industry due to reduced milk yield and increased expenditure on treatment of affected cows (Schroeder, 2012; Kateete et al., 2013). Among the bacterial species capable of causing this disease, *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. (CoNS) are the major etiological agents in many countries (Moon et al., 2007; Santos et al., 2007; Sawant et al., 2009; Taponen and Pyörälä, 2009; Guimarães et al., 2012; Jamali et al., 2014; Supré et al., 2014). Despite implementing control measures, it is difficult to eradicate mastitis in dairy cows because many strains are resistant to the antimicrobial drugs currently used (Güler et al., 2005; Jamali et al., 2014).

The bovine mastitis may be treated with a variety of antimicrobial classes, including penicillins, tetracyclines,

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lincosamides, macrolides and fluoroquinolones. However, the practice of incorporating antibiotics into feeds to prevent infectious diseases and the inadequate use of antibiotics in therapy allowed the emergence of multi-drug-resistant (MDR) strains (Jamali et al., 2014).

The isolation of MDR *S. aureus* and CoNS, including methicillin-resistant isolates, from bovine mastitis cases in Brazil and other countries has been reported with relatively high frequency (Zanette et al., 2010; Ferreira et al., 2007; Guimarães et al., 2012). Hence, the characterization of the antibiotic-resistance profile of these bacteria is crucial for a correct antibiotic choice and an effective mastitis treatment. The aim of this study was to determine the prevalence and antibiotic-resistance profile of *S. aureus* and CoNS isolated from bovine mastitis cases in the municipality of Altônia, northwest region of Paraná State, Brazil.

MATERIALS AND METHODS

Sample collection

The survey was conducted with 248 dairy cows from Holsteins, Jersey and Girolando breeds, belonging to 24 properties located in the municipality of Altônia, northwestern region of Paraná state, Brazil. The selection of a representative number of animals to study was performed from a list of 82 properties, totaling of 840 animals, using BioEstat program 5.0 (Ayres, 2007).

All the milk samples were collected from July 2009 to December 2010. The dairy cows were examined for the presence of mastitis both clinically and by the use of screening test California Mastitis Test (CMT). Milk samples were collected only from dairy cows with positive CMT. Approximately 30 mL of milk were collected from each mammary quarter in sterile flasks of 50 mL, according to the National Mastitis Council (NMC, 2004). The samples were kept at 4.0°C until processing at the Laboratory of Veterinary Preventive Medicine and Public Health at Paranaense University (UNIPAR). The microbiological analysis was performed using milk pools from the four mammary quarter samples of the same animal.

Bacterial isolation and identification

The milk samples were plated on blood agar and incubated at 37°C for 24 h for colony isolation. After this period, the isolates were identified according to the National Mastitis Council (NMC, 2004).

Antimicrobial susceptibility

The antimicrobial susceptibility tests were performed according to CLSI (2013). The disc diffusion method was performed using the following antibiotics: gentamicin (10 µg); ampicillin (10 µg), oxacilin (1 µg), penicillin (10 UI); cephalothin (30 µg), ceftiofur (30 µg); enrofloxacin (5 µg); trimethoprim-sulfamethoxazole (25 µg), clindamycin (2 µg), erythromycin (15 µg) and tetracycline (30 µg). The antibiotics were selected according to the classes available for mastitis treatment in Brazil.

RESULTS

Among the 248 animals studied, 82 (33.0%) were positive for mastitis on CMT. Of these, 54/82 (65.00%) animals confirmed the presence of mastitis on the microbiological analysis of milk samples. The overall prevalence of mastitis among all the animals studied was 21.80% (54/248 animals). The microbiological tests identified 59 different isolates among 54 milk samples. In three samples, it was possible to identify two or more isolates from different bacterial species. The presence of a yeast isolate was found in one milk sample.

The most prevalent bacteria was *Staphylococcus aureus,* representing 29 out of 59 (49.10%) isolates, followed by CoNS with 14 (23.70%) isolates; *Streptococcus dysgalactiae* 3 (5.10%); coagulase-positive/VP-negative *Staphylococcus* spp. 3 (5.10%); *Corynebacterium* sp. 3 (5.10%); *Enterococcus faecalis* 2 (3.40%); and *Trueperella pyogenes*, *Enterococcus* sp., *Streptococcus* sp., *Streptococcus acidomihimus* and yeasts with one (1.70%) isolate each.

The antimicrobial susceptibility tests were performed only with the most common isolates of *S. aureus*, CoNS, *S. dysgalactiae* and coagulase-positive/VP-negative *Staphylococcus* spp. because of its importance in the mastitis epidemiology in Brazil. The most effective antibiotics were enrofloxacin with 93.85% of sensitivity, followed by streptomycin with 91.85% and gentamicin with 87.75%. The less effective antibiotics were penicillin with 67.35% of resistance, followed by ampicillin and tetracycline with 63.25 and 51.05%, respectively (Table 1). The antimicrobial susceptibility among each pathogen isolated is listed in Tables 2 and 3.

The analysis of oxacilin-resistance profile was 20.40% (10/49 isolates). Among the CoNS isolates, 35.70% (5/14 isolates) were oxacillin-resistant while 17.25% (5/29 isolates) of *S. aureus* isolates were-oxacillin-resistant. No oxacilin-resistance was observed among *S. dysgalactiae* and coagulase-positive/VP-negative *Staphylococcus* spp. isolates analyzed. All the oxacilin resistant isolates showed resistance to the other beta-lactams tested, including the cephalosporins (Table 4).

This study identified 12 (24.50%) isolates with multi-drug resistance profile. These strains were identified as *S. aureus* (7 isolates) and CoNS (5 isolates). The most effective antimicrobials against these bacteria were streptomycin and enrofloxacin, as compared to other antimicrobials tested (Table 4). Two MDR isolates showed resistance to all antibiotics tested.

DISCUSSION

The study was carried out to determine the antimicrobial resistance profile of *S. aureus* and CoNS strains isolated from milk samples from cows with bovine mastitis in the municipality of Altônia, northwest region of Paraná state, Brazil.

The prevalence of bovine mastitis was also assessed and revealed that 21.80% animals examined had

Table 1. Antimicrobial resistance profile of all isolates obtained from mastitis cases studied.

Antimicrobials	Resistance profile					
	Sensitive		Intermediate		Resistant	
	%	n	%	n	%	n
Penicillin	32.65	16	0.00	0	67.35	33
Ampicillin	34.70	17	2.05	1	63.25	31
Oxacilin	75.50	37	2.05	1	22.45	11
Streptomycin	91.85	45	0.00	0	8.15	4
Gentamicin	87.75	43	4.10	2	8.15	4
Cephalothin	79.60	39	0.00	0	20.40	10
Ceftiofur	77.55	38	0.00	0	22.45	11
Tetracycline	40.80	20	8.15	4	51.05	25
Enrofloxacin	93.85	46	2.05	1	4.10	2
Erythromycin	73.45	36	14.30	7	12.25	6
Clindamycin	73.45	36	4.10	2	22.45	11
Sulfa	65.30	32	6.10	3	28.60	14
Average	68.90%		3.55%		27.55%	

Table 2. Antimicrobial resistance profile of CoNS and coagulase-positive/VP-negative *Staphylococcus* spp. obtained from mastitis cases studied.

Antibiotic	*Staphylococcus* spp. negative coagulase						*Staphylococcus spp.* PC/NVP					
	Sensitive		Intermediate		Resistant		Sensitive		Intermediate		Resistant	
	%	n	%	n	%	n	%	n	%	n	%	n
Penicillin	42.85	6	0.00	0	57.15	8	33.50	1	0.00	0	66.50	2
Ampicillin	64.30	9	0.00	0	35.70	5	33.50	1	0.00	0	66.50	2
Oxacilin	64.30	9	0.00	0	35.70	5	100	3	0.00	0	0.00	0
Streptomycin	92.85	13	0.00	0	7.15	1	100	3	0.00	0	0.00	0
Gentamycin	78.55	11	7.15	1	14.30	2	100	3	0.00	0	0.00	0
Cephalothin	78.55	11	14.30	2	7.15	1	100	3	0.00	0	0.00	0
Ceftiofur	85.70	12	7.15	1	7.15	1	100	3	0.00	0	0.00	0
Tetracycline	35.70	5	7.15	1	57.15	8	66.50	2	33.50	1	0.00	0
Enrofloxacin	85.70	12	7.15	1	7.15	1	100	3	0.00	0	0.00	0
Erythromycin	64.30	9	14.30	2	21.40	3	100	3	0.00	0	0.00	0
Clindamycin	64.30	9	0.00	0	35.70	5	100	3	0.00	0	0.00	0
Sulfa	57.15	8	7.15	1	35.70	5	66.50	2	33.50	1	0.00	0

infection in their udders. This finding closely agrees with those found by other authors worldwide. (Salih et al., 2011; Tolosa et al., 2009 Piepers et al., 2007; Mello et al., 2012; Oliveira et al., 2009, 2011). Among the isolates analyzed in this study, *S. aureus*, CoNS, *S. dysgalactiae* and coagulase-positive/VP-negative *Staphylococcus* spp. were the most frequent, corroborating with the results found in other studies (Zanette et al., 2010; Ferreira et al., 2007 and Piepers et al., 2007; Silva et al., 2014).

The presence of mastitis in dairy cows requires the use of antibiotics to treat the animals. However, the inappropriate use of these drugs over the years allowed the emergence of multi-drug resistant bacteria (Silveira-Filho et al., 2014; Gaze et al., 2008; Thomson et al., 2008; Krause and Hendrick, 2011). This study showed that 27.55% of isolates were antibiotic-resistant among the *S. aureus,* CoNS, *S. dysgalactiae* and coagulase-positive/VP-negative *Staphylococcus* spp. isolated from bovine mastitis. Similar results were also found in different regions of the world (Szweda et al., 2014; Güller et al., 2005, Sawant et al., 2009).

The highest resistance rates were detected for the β-lactam antibiotics penicillin (67.35%) and ampicillin (63.25%). These results were expected because these

Table 3. Antimicrobial resistance profile of *Staphylococcus aureus* and *Streptococcus dysgalactiae* obtained from mastitis cases studied.

Antibiotic	*Staphylococcus. aureus*						*Streptococcus dysgalactiae*					
	Sensitive		Intermediate		Resistant		Sensitive		Intermediate		Resistant	
	%	n	%	n	%	n	%	n	%	n	%	n
Penicillin	24.10	7	0.00	0	75.90	22	0.00	0	66.50	2	33.50	1
Ampicillin	38.00	11	0.00	0	62.00	18	33.50	1	66.50	2	0.00	0
Oxacilin	79.30	23	3.45	1	17.25	5	0.00	0	100	3	0.00	0
Streptomycin	89.65	26	0.00	0	10.35	3	0.00	0	100	3	0.00	0
Gentamycin	89.65	26	3.45	1	6.90	2	0.00	0	100	3	0.00	0
Cephalothin	89.65	26	3.45	1	6.90	2	0.00	0	100	3	0.00	0
Ceftiofur	96.55	28	0.00	0	3.45	1	0.00	0	66.50	2	33.50	1
Tetracycline	41.35	12	3.45	1	55.20	16	33.33	1	33.33	1	33.33	1
Enrofloxacin	96.55	28	0.00	0	3.45	1	0.00	0	100	3	0.00	0
Erythromycin	72.40	21	17.25	5	10.35	3	0.00	0	100	3	0.00	0
Clindamycin	79.30	23	3.45	1	17.25	5	33.33	1	33.33	1	33.33	1
Sulfa	72.40	21	3.45	1	24.15	7	33.33	1	33.33	1	33.33	1

Table 4. Antimicrobial resistance profile of MDR *S. aureus* and CoNS isolated in this study.

Strain	Type	PEN	AMP	OXA	SXT	GEN	CEFA	CTF	TET	ENO	ERI	CLI	SUT
1	CoNS	R	R	R	S	I	R	R	R	I	R	R	R
6	CoNS	R	R	R	S	S	R	R	R	S	I	R	R
40	CoNS	R	R	R	R	R	R	R	R	R	R	R	R
48	CoNS	R	R	R	S	S	S	S	R	S	I	R	I
78B	CoNS	R	R	R	S	R	R	R	R	S	R	R	R
4	*S. aureus*	R	R	R	R	S	R	R	R	S	I	R	R
7	*S. aureus*	R	R	R	R	S	R	R	R	S	S	S	S
9B	*S. aureus*	R	R	I	S	S	S	S	R	S	S	R	R
19	*S. aureus*	R	R	R	S	I	R	R	R	S	S	S	S
24	*S. aureus*	R	R	S	S	S	S	S	R	S	R	S	R
44	*S. aureus*	R	R	R	S	R	R	R	R	S	R	R	R
63	*S. aureus*	R	R	R	R	R	R	R	R	R	R	R	R
Resistance (%)		100	100	83.30	33.30	33.30	75.00	75.00	100	16.65	50.00	75.00	75.00

PEN - Penicillin; AMP - Ampicillin; OXA - oxacilin; SXT - streptomycin; GEN - gentamicin; CEFA - cephalothin; CTF -ceftiofur; TET - tetracycline; ENO - enrofloxacin; ERI - erythromycin; CLI - clindamycin; SUT - trimethoprim-sulfamethoxazole

two antibiotics are widely used in mastitis treatment (McDougall et al., 2008; Guimarães et al., 2012; Güller et al., 2005). According to Vintov et al. (2003), the resistance to penicillin and other beta-lactams in bovines may vary from 10 to 70% depending on the region studied. Another antibiotic with high resistance rate was tetracycline with 55.05%. Similar results were found in Brazil, Guimarães et al. (2012) showed a resistance rate of 33.3% among Gram-positive strains isolated from bovines.

The most effective antibiotics were enrofloxacin, streptomycin and gentamicin. This high sensitivity was also demonstrated in other studies (Kaszanyitzky et al., 2004; Byarugaba, 2004). Despite these data, Freitas et al. (2005) reported high resistance level to gentamicin in CoNS isolated in regions where this antibiotic was used. These diverging results may occur due to farming conditions, handling and drug therapy programs to which animals are subjected.

According to Magiorakos et al. (2012), bacteria that exhibit resistance to three or more classes of antibiotics can be classified as multidrug-resistant (MDR) strains. In this study, it was possible to detect *S. aureus* (7 isolates) and CoNS (5 isolates) with these characteristics. Among these, 10 isolates showed resistance to oxacilin. It is important to emphasize that all oxacilin-resistant isolates were also resistant to other beta-lactams tested. According to Livermore et al. (2001), this resistance profile indicates the presence of methicillin-resistant *S. aureus* (MRSA) and

methicillin-resistant coagulase-negative staphylococci (MRCoNS).

The prevalence of MRSA and MRCoNS (20.40%) in this study was higher than those found in other studies. Vanderhaeghen et al. (2010) detected methicillin-resistant staphylococci (MRS) in 9.3% of clinical and subclinical mastitis cases in Belgian cows. Variations in findings may be attributable to different sample sizes and study locations.

The presence of MRSA and MRCoNS can complicate mastitis treatment because the resistance mechanism of these bacteria confers resistance to almost all types of β-lactam antibiotics available (Sawant et al., 2009). In addition to this, infections by MRS require rapid and accurate diagnosis for elimination at an early stage, because these strains can cause severe damage to infected sites and may be widespread in the environment (Lee et al., 2001, 2004). In most routine microbiological settings, the detection of methicillin resistance among staphylococcal isolates is based on phenotypic assays such as the disk diffusion test and MIC determination (Moon et al., 2007). Despite these data, the presence of MRS strains in this study need to be considered with caution, as molecular tests for *mecA* gene was not performed.

In addition to these data, presence of MRS strains among dairy cattle may represent a risk to human health due to risk of consuming contaminated food, mainly raw milk (Lee, 2003; Olivier et al., 2009).

Conclusion

The prevalence of MDR isolates causing mastitis was of concern. Hence, the determination of antimicrobial resistance profile is essential to control mastitis in dairy cows. The use of antibiogram may help to reduce the dissemination of MDR strains, since the choice of antibiotics will not be based on empirical therapy. More epidemiological and molecular studies are necessary to determine the current role of MDR strains in mastitis. These studies may help in the development of control programs to prevent the spread of these microorganisms in the environment.

Conflict of Interests

The authors have not declared any conflict of interests.

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