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***In vitro* antimicrobial susceptibility and genetic resistance determinants of *Streptococcus agalactiae* isolated from mastitic cows in Brazilian dairy herds**

Suscetibilidade *in vitro* a antimicrobianos e determinantes genéticos de resistência em *Streptococcus agalactiae* isolados de mastite em rebanhos bovinos brasileiros

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Abstract

Streptococcus agalactiae is one of the main causative agents of bovine mastitis and is associated with several economic losses for producers. Few studies have evaluated antimicrobial susceptibility and the prevalence of genetic resistance determinants among isolates of this bacterium from Brazilian dairy cattle. This work aimed to evaluate the frequency of the antimicrobial resistance genes *ermA*, *ermB*, *mefA*, *tetO*, *tetM*, *aphA3*, and *aad-6*, and *in vitro* susceptibility to the antimicrobials amikacin, erythromycin, clindamycin, tetracycline, gentamicin, penicillin, ceftiofur, and cefalotin, and the associations between resistance genotypes and phenotypes among 118 *S. agalactiae* isolates obtained from mastitic cows in Brazilian dairy herds. Of the resistance genes examined, *ermB* was found in 19 isolates (16.1%), *tetO* in 23 (19.5%), and *tetM* in 24 (20.3%). The genes *ermA*, *mefA*, *aphA3*, and *aad-6* were not identified. There was an association between the presence of genes *ermB*, *tetM*, and *tetO* and phenotypic resistance to erythromycin, clindamycin, and tetracycline. Rates of resistance to the tested antibiotics varied, as follows: erythromycin (19.5%), tetracycline (35.6%), gentamicin (9.3%), clindamycin (20.3%), penicillin (3.4%), and amikacin (38.1%); conversely, all isolates were susceptible to ceftiofur and cefalotin. Antimicrobial resistance testing facilitates the treatment decision process, allowing the most judicious choice of antibiotics. Moreover, it enables regional and temporal monitoring of the resistance dynamics of this pathogen of high importance to human and animal health.

Key words: Antimicrobial resistance genes. Bovine diseases. Bovine mastitis. GBS, MIC.

Resumo

Streptococcus agalactiae é um dos principais agentes causadores de mastite em bovinos e de consequentes

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perdas econômicas aos produtores. Poucos estudos que avaliaram a susceptibilidade a antimicrobianos e a presença de determinantes genéticos de resistência para este agente em bovinos leiteiros do Brasil. Este trabalho teve por objetivo avaliar a frequência dos genes de resistência a antimicrobianos *ermA*, *ermB*, *mefA*, *tetO*, *tetM*, *aphA3*, *aad-6*, bem como a susceptibilidade *in vitro* aos antimicrobianos amicacina, eritromicina, clindamicina, tetraciclina, gentamicina, penicilina, ceftiofur e cefalotina, e as associações entre genótipos e fenótipos de resistência em 118 isolados de *S. agalactiae* provenientes de casos de mastite em rebanhos bovinos brasileiros. Dentre os genes de resistência pesquisados, *ermB* foi encontrado em 19 isolados (16,1%), *tetO* em 23 (19,5%) e *tetM* em 24 (20,3%). Os genes *ermA*, *mefA*, *aphA3* e *aad6* não foram detectados. Verificou-se associação entre a presença dos genes *ermB*, *tetM* e *tetO* e os fenótipos de resistência à eritromicina, clindamicina e tetraciclina. Foram encontrados diferentes índices de resistência aos antibióticos testados: Eritromicina (19,5%), tetraciclina (35,6%), gentamicina (9,3%), clindamicina (20,3%), penicilina (3,4%) e amicacina (38,1%). Todos os isolados foram susceptíveis ceftiofur e cefalotina. Os testes de resistência aos antimicrobianos auxiliam na tomada de decisões relacionadas aos tratamentos, permitindo a escolha mais criteriosa dos antimicrobianos e o acompanhamento espaço-temporal da dinâmica de resistência para este patógeno tão relevante na saúde humana e animal.

Palavras-chave: CIM. Doenças de bovinos. GBS. Genes de resistência a antimicrobianos. Mastite bovina.

Introduction

Mastitis is the most common and costly infectious disease affecting dairy farms worldwide (KEEFE, 2012). This condition affects the quality and quantity of milk produced, and results in treatment-related expenses and losses due to milk disposal and replacement of chronically affected animals (RUEGG, 2012; SANTOS; FONSECA, 2007). It is a multifactorial disease involving environmental conditions, aspects inherent to the animals themselves, and infectious factors. Regarding this aspect, more than 140 different microorganisms have been implicated in its etiology (RIBEIRO et al., 2006).

The bacterium *Streptococcus agalactiae* is well adapted to the bovine mammary gland, and generally causes acute clinical diseases and persistent sub-clinical infections (HILLERTON et al., 2004). It is highly contagious and leads to mastitis associated with a very low rate of spontaneous cure. In Brazil, *S. agalactiae* is one of the main etiologic agents of bovine mastitis, with an estimated average prevalence in herds of 60% (KEEFE, 2012). Affected animals act as important reservoirs in the herd, and transmission predominantly occurs during the milking period (BAL et al., 2010).

Besides its role in bovine mastitis, *S. agalactiae* is an important public health concern. In humans, this microorganism colonizes the urogenital and gastrointestinal tracts, and may lead to sepsis, pneumonia, neonatal meningitis, mastitis in lactating women, and high mortality rates among immunocompromised individuals (JOHRI et al., 2006; MAIONE et al., 2005; ZADOKS et al., 2011). It is also an emergent pathogen in aquaculture, causing sepsis or meningoencephalitis among freshwater, marine, and estuarine fishes with high rates of morbidity and mortality (EVANS et al., 2002).

Since the introduction of penicillin in the 1940s, antimicrobials have been extensively used for bacterial infection control, both in human medicine and for the improvement of animal health and well-being (BARLOW, 2011). Therapeutic intervention has a central role in the control of bovine mastitis, leading to frequent antimicrobial use on farms (OLIVEIRA; RUEGG, 2014). However, the efficacy of such agents is compromised when resistance arises, a problem that affects animal health and has public health implications. Resistance is influenced by intensive and indiscriminate use of antimicrobials and the frequency with which mutations occur in

resistance genes (JOO et al., 2016; SCHWARZ et al., 2001).

Efficient, permanent prevention and early treatment are crucial goals for the adequate control of mastitis, as non-treated cases generally develop into a chronic condition, resulting in permanent losses (TENHAGEN et al., 2006). Intramammary infusions of high doses of one or more antibiotics, principally penicillins, cephalosporins, tetracyclines, aminoglycosides, and macrolides, are often used to treat bovine mastitis (KEEFE, 2012). Thus, determining resistance patterns and detecting the dissemination of genetic elements conferring resistance are important in aiding the treatment decision process.

Studies that contribute to our understanding of the susceptibility profiles of pathogenic agents responsible for dairy cow mastitis are therefore indispensable (ERSKINE et al., 2003). Such investigations allow continuous epidemiologic monitoring of bacterial antimicrobial susceptibility, and enable the detection of resistant strains. Notably, patterns of resistance to antimicrobials used for mastitis must be continuously monitored as they may vary over time, among countries, and even between different farms from a same region (VINTOV et al., 2003).

Little research has been conducted concerning the antimicrobial susceptibility profiles of *S. agalactiae* strains isolated from mastitis-affected cows in Brazilian herds. In the few studies performed, causative agent identification has been limited to the genus level and the disc diffusion method used, which, although internationally standardized, is more error-prone than the minimal inhibitory concentration (MIC) approaches (FERREIRA et al., 2010; OLIVEIRA et al., 2011).

The objective of this work was to evaluate *in vitro* antimicrobial susceptibility and the presence of genetic antibiotic resistance determinants among *S. agalactiae* strains isolated from mastitic cows in Brazilian dairy herds.

Materials and Methods

This study was conducted at the Laboratório de Microbiologia of the Departamento de Medicina Veterinária/DMV - Universidade Federal de Lavras/UFLA, Lavras, Brazil, using 118 *S. agalactiae* strains isolated from bovine mastitis cases in the Northeastern (n=10), Southeastern (n=95), and Southern (n=13) regions of Brazil. The isolates used had been stored at -70°C at the abovementioned laboratory, and were kindly made available by scientists affiliated to research and teaching institutions in the aforementioned regions.

Molecular tests

The *S. agalactiae* strains were grown on Trypticase soy agar (HiMedia®, Mumbai, India) supplemented with 5% ovine blood at 37°C for 24-48 hours. After this period, they were transferred to brain-heart infusion broth (Brain Heart Infusion) (Sigma-Aldrich®, Bengaluru, India) and cultured at 37°C for a further 24-48 hours. Genomic DNA was then extracted using a Wizard® Genomic DNA Purification Kit (Promega®, Madison, WI, USA), per the standard protocol for Gram-positive bacteria.

Antimicrobial resistance genes were detected using polymerase chain reaction (PCR) in a Peltier Thermal Cycler Multi-Purpose device (Biocycle®, Hangzhou, China), using conditions and primers previously described in the literature (Table 1). When necessary, optimization of these reactions was performed. We tested for the genes *ermA* and *ermB* (conferring cross-resistance to macrolides, lincosamides, and streptogramins), *mefA* (encoding an efflux pump resulting in macrolide resistance), *tetO* and *tetM* (causing tetracycline resistance by ribosomal protection), and *aphA3* and *aad-6* (enabling aminoglycoside resistance). To evaluate the reproducibility of PCR results, strains were re-tested after each run. Positive and negative controls were included during amplification of each gene. Reaction specificity was evaluated by product size and the presence of a single amplicon.

PCR products were separated by electrophoresis using 1× Tris-acetate-EDTA buffer (0.04 M Tris-acetate and 0.001 M EDTA) and a 1.5% agarose gel (Invitrogen Brasil, São Paulo, Brazil) stained with GelRed™ (Biotium®, Fremont, CA, USA). One hundred-base pair and 1-kb DNA ladders

(New England Biolabs® Inc., Ipswich, MA, USA) were also loaded onto each gel. Gel images were recorded in a transilluminator (L-Pix Chemi Photo Digitizer; Loccus Biotecnologia, Cotia, Brazil) for later analysis.

Table 1. Primers used in the detection of antimicrobial resistance genes in *S. agalactiae* isolated from mastitis in Brazilian dairy herds and size of PCR products.

Target Gene	Product Size	Sequence (5'-3')	References
<i>ermA</i>	640pb	F:5'-TCTAAAAAGCATGTAAAAGA-3' R:5'-CTTCGATAGTTTATTAATATTAGT-3'	(SUTCLIFFE et al., 1996)
<i>ermB</i>	640 pb	F:5'-GAAAAGRTACTCAACCAAATA-3' R:5'-AGTAACGGTACTTAAATTGTTTAC-3'	(SUTCLIFFE et al., 1996)
<i>mefA</i>	328 pb	F:5'-AGTATCATTAATCACTAGTGC-3' R:5'-TTCTTCTGGTACTAAAAGTGG-3'	(SUTCLIFFE et al., 1996)
<i>tetM</i>	359 pb	F:5'-GTGGAGTACTACATTTACGAG-3' R:5'-GAAGCGGATCACTATCTGAG-3'	(POYART et al., 1995)
<i>tetO</i>	538 pb	F:5'-GCGGAACATTGCATTGAGGG-3' R:5'-CTCTATGGACAACCCGACAGAAG-3'	(CLERMONT et al., 1997)
<i>aphA3</i>	848 pb	F:5'-GGGGTACCTTTAAATACTGTAG-3' R:5'-TCTGGATCCTAAAACAATTCATCC-3'	(POYART et al., 1995)
<i>aad-6</i>	978 pb	F:5'-AGAAGATGTAATAATATAG-3' R:5'-CTGTAATCACTGTTCCCGCCT-3'	(POYART-SALMERON et al., 1990)

MIC Tests

MIC assays, according to which isolates were classified as susceptible or resistant, were estimated using the microdilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008). Sterile microplates containing Mueller Hinton broth adjusted with the divalent cations Ca^{2+} and Mg^{2+} (CAMHB;

HiMedia®) and supplemented with 5% equine serum were used. The antibiotics amikacin, erythromycin, clindamycin, tetracycline, gentamicin, penicillin, ceftiofur, and cefalotin (Sigma-Aldrich®, St. Louis, MO, USA) were solubilized and diluted to test concentrations according to CLSI specifications (CLSI, 2008) (Table 2).

Table 2. Antibiotic concentrations ($\mu\text{g/mL}$) used to determine Minimal Inhibitory Concentrations - MIC, in *S. agalactiae* isolated from mastitis in Brazilian dairy herds.

Antibiotic	Concentration ($\mu\text{g/mL}$)
Amikacin	256 - 0.5
Erythromycin	32 - 0.06
Tetracycline	128 - 0.25
Gentamicin	128 - 0.25
Penicillin	8 - 0.015
Ceftiofur	32 - 0.06
Clindamycin	16 - 0.03
Cefalotin	8 - 0.015

Strains were first cultured in Trypticase soy agar (HiMedia®) supplemented with 5% ovine blood at 37°C for 24-48 hours. Suspensions of each bacterial strain were then prepared in 0.9% saline solution and adjusted to a McFarland turbidity standard of 0.5, or 1.5×10^8 CFU/mL. Five microliters of bacterial suspension was added to each well of the microplate containing CAMHB and antibiotics at the appropriate concentrations. The plates were subsequently agitated and incubated for 24 hours at 37°C. All tests were performed in duplicate, using negative and positive controls for bacterial growth. After incubation, MICs (the lowest concentration of each antibiotic at which it was not possible to visually detect bacterial growth) were determined. Results were evaluated following CLSI standards (CLSI, 2008).

Associations between the presence/absence of antimicrobial resistance genes and phenotypic resistance to *erythromycin*, *clindamycin*, and *tetracycline* were statistically evaluated using SPSS 20.0 software (IBM Corp., Armonk, NY, USA) using Fisher's exact test at a 99% confidence level ($p \leq 0.01$).

Results

Based on the MICs measured, rates of resistance among the *S. agalactiae* isolates ($n=118$) varied between the antibiotics tested, as follows: *erythromycin* (19.5%), *tetracycline* (35.6%), *gentamicin* (9.3%), *clindamycin* (20.3%), *penicillin* (3.4%), and *amikacin* (38.1%); however, all isolates were susceptible to *ceftiofur* and *cefalotin*. MIC distributions are compiled in Table 3.

Table 3. Distribution of Minimal Inhibitory Concentrations for eight antimicrobials in *S. agalactiae* isolated from mastitis from Brazilian dairy herds.

CONC. (µg/mL)	>256	256	128	64	32	16	8	4	2	1	<0.5	T.R.	T.S
AMIKACIN	0	0	0	18	55	36	3	5	0	0	1	73	45
	R	R	R	R	I	S	S	S	S	S	S	38.10%	61.90%
CONC.(µg/mL)	> 128	128	64	32	16	8	4	2	1	0.5	<0.25	T.R.	T.S.
TETRACYCLINE	4	1	13	20	4	0	0	1	4	5	66	42	76
	R	R	R	R	R	R	I	S	S	S	S	35.60%	64.40%
GENTAMICIN	0	0	0	0	0	11	51	38	9	6	3	11	107
	R	R	R	R	R	I	S	S	S	S	S	9.30%	90.70%
CONC.(µg/mL)	>32	32	16	8	4	2	1	0.5	0.25	0.12	<0.06	T.R.	T.S.
ERYTHROMYCIN	17	0	0	1	0	0	2	3	0	2	93	23	95
	R	R	R	R	R	R	R	I	S	S	S	19.50%	80.50%
CEFTIOFUR	0	0	0	0	0	0	0	0	0	0	118	0	118
	R	R	R	R	I	S	S	S	S	S	S	0%	100%
CONC.(µg/mL)	> 16	16	8	4	2	1	0.5	0.25	0.12	0.06	<0.03	T. R.	T.S.
CLINDAMYCIN	20	0	0	3	0	1	1	2	0	18	73	24	94
	R	R	R	R	I	I	S	S	S	S	S	20.30%	79.70%
CONC.(µg/mL)	>8	8	4	2	1	0.5	0.3	0.12	0.06	0.03	< 0.015	T.R.	T.S.
PENICILLIN	0	0	0	0	1	2	1	8	28	38	40	4	114
	R	R	R	R	I	I	I	S	S	S	S	3.40%	96.60%
CEFALOTIN	0	0	0	0	0	3	82	29	4	0	0	0	118
	S	S	S	S	S	S	S	S	S	S	S	0%	100%

Highlighted cells represent resistant isolates, i.e. those with MIC \geq standard cut-off values for each antimicrobial. R-Resistant; I-Intermediate; S-Sensitive, T.R. Total of Resistants, T.S. Total of Sensitives, CONC. Concentration.

Of the resistance genes tested, *ermB* was found in 19 isolates (16.1%), *tetO* in 23 (19.5%), and *tetM* in 24 (20.3%). Genes *ermA*, *mefA*, *aphA3*, and *aad-6* were not detected in any of the isolates.

Associations between phenotypic resistance to *erythromycin*, clindamycin, and tetracycline and presence of the resistance genes *ermB*, *tetM*,

and *tetO* are reported in Table 4 ($p < 0.01$). Of the 19 *ermB*-positive isolates, 17 (89.5%) were phenotypically resistant to *erythromycin*; however, six *erythromycin*-resistant isolates did not carry this gene. Regarding clindamycin, 18 (94.7%) of the 19 *ermB*-positive isolates were phenotypically resistant, but *ermB* was not detected in six clindamycin-resistant isolates.

Table 4. Comparison of genotypic and phenotypic resistance to antibiotics in *S. agalactiae* isolated from mastitis in Brazilian dairy herds.

Antibiotics	Characteristics of Isolates					Association ^a
	Genes	F+/G+ ^b	F-/G- ^c	F+/G- ^d	F-/G+ ^e	p
Erythromycin	<i>ermB</i>	17	93	6	2	<0.001
Clindamycin	<i>ermB</i>	18	93	6	1	< 0.001
Tetracycline	<i>tetO</i>	21	74	21	2	< 0.001
	<i>tetM</i>	19	71	23	5	< 0.001
Amikacin	<i>aphA3/aad6</i>	0	73	45	0	< 0.001
Gentamicin	<i>aphA3/aad6</i>	0	107	11	0	< 0.001

^aAssociation between resistance phenotypes and resistance genes. Fisher's Exact Test (p<0.01)

^bNumber of isolates expressing the resistance phenotype for the named antibiotic (F+) that have the indicated gene (G+).

^cNumber of isolates not expressing the resistance phenotype for the named antibiotic (F-) that do not have the indicated gene (G-).

^dNumber of isolates expressing the resistance phenotype for the named antibiotic (F+) that do not have the indicated gene (G-).

^eNumber of isolates not expressing the resistance phenotype for the named antibiotic (F-) that have the indicated gene (G+).

Concerning the association between genes conferring tetracycline resistance and phenotypic resistance to this antimicrobial, 95.7 and 98.3% of *tetM*-positive and *tetO*-positive isolates, respectively, were phenotypically resistant. Four isolates lacking *tetM* and *tetO* were resistant to tetracycline. Ten tetracycline-susceptible isolates carried one or both of these genes, with three carrying both, two *tetO* alone, and five *tetM* alone. We also verified the association between carriage of *tetO* and *ermB*, given that 89.5% of *ermB*-positive strains carrying *tetO*.

Discussion

The implementation of programs for the control and prevention of bovine mastitis is of great importance considering the losses that this disease causes for producers and the industry as a whole. The measures comprising such programs include the early treatment of clinical cases and dry cow therapy. These actions are fundamental for effective mastitis control, since untreated cases generally become chronic, resulting in permanent losses (TENHAGEN et al., 2006). Considering this, evaluation of resistance patterns and the

dissemination of genetic elements conferring resistance is crucial in determining which antimicrobials may be used in the treatment of affected animals, as well as in monitoring the spatial and temporal dynamics of antimicrobial resistance.

MIC values to antimicrobials are showed in Table 3. *Erythromycin* resistance has been documented worldwide in studies concerning *S. agalactiae* strains of human and bovine origin. In the United States and Europe, resistance rates of approximately 4-50% have been reported (DIPERSIO; DIPERSIO, 2006; DOGAN et al., 2005; GUÉRIN-FAUBLÉE et al., 2002; SADOWY et al., 2010), while in Brazil, estimates have been lower than 10% (CORRÊA et al., 2011; DUARTE et al., 2004, 2005; PALMEIRO et al., 2010). However, of the Brazilian bovine isolates examined by Pinto et al. (2013), 27.6% were resistant to this antibiotic, a higher value than that recorded in the present work (19.5%).

High rates of tetracycline resistance (60-90%) have been reported among both human and bovine *S. agalactiae* isolates (DOGAN et al., 2005; DUARTE et al., 2004; GAO et al., 2012; PINTO et al., 2013; RATO et al., 2013). In the current work, resistance to this antimicrobial was found to be approximately 35.6%. Such elevated resistance to tetracycline can

be explained by its indiscriminate use over many years to combat several animal diseases.

Cefazolin and cefoperazone resistance was not detected by Rato et al. (2013) in their analysis of 60 mastitis-associated *S. agalactiae* strains from Portugal. Similarly, in the present work, all isolates were susceptible to cefalotin and ceftiofur. These antimicrobials are first and third-generation cephalosporins, respectively, as are cefazolin and cefoperazone. Corroborating our results, Lindeman et al. (2013) also found high levels of ceftiofur and cefalotin susceptibility among *S. agalactiae* isolates from North American dairy cattle.

High penicillin susceptibility rates have been described in previous studies to *S. agalactiae* isolates from mastitic cows. In the present work, consistent with such investigations (GUÉRIN-FAUBLÉE et al., 2002; MINST et al., 2012; RATO et al., 2013), most isolates (96.6%) were susceptible to penicillin, indicating that this antibiotic is a viable option for the treatment of mastitis. In humans, penicillin is the recommended first-line treatment for *S. agalactiae* infections for all age groups, in addition to being administered prophylactically to pregnant women (BOLUKAOTO et al., 2015; VERANI et al., 2010).

Variable levels of resistance to gentamicin have been recorded among bovine *S. agalactiae* isolates. Gao et al. (2012), in a study of 55 *S. agalactiae* isolates collected from Chinese cows, reported that 29.4% were resistant to this antimicrobial. In contrast, all 85 strains of this bacterium recovered from Brazilian cattle in an investigation by Duarte et al. (2004) were found to be resistant. In addition, Rato et al. (2013) documented a resistance rate of 80.6% among 108 isolates collected from Portuguese cows. The results of the present study differ somewhat in this respect, given that only 9.3% of the tested strains were resistant to gentamicin, while 38.1% were resistant to amikacin, another aminoglycoside antibiotic.

The genetic resistance determinants of *S. agalactiae* identified in the present work (Table

4) have been widely documented in previous studies. The genes *ermA* and *ermB* determine cross-resistance to erythromycin and clindamycin. In this study, *ermB* was found in 16.1% of strains and its presence was associated with resistance to these antibiotics, whereas *ermA* was not detected. Prior investigations have described *ermB* as the most common *erm* gene in bovine *S. agalactiae* isolates, while *ermA* is more frequent among human strains (DUARTE et al., 2004; DUTRA et al., 2014). Six isolates showed phenotypic resistance to erythromycin and clindamycin despite not carrying *ermA* or *ermB*. This might be explained by the great variety of genetic mechanisms that may be associated with a single resistance phenotype. For instance, the isolates in question may harbor less frequently observed resistance determinants such as *ermC*, *ermF*, and/or *ermT*, all of which have been described in streptococci (ROBERTS, 2008).

The tetracycline resistance determinants *tetO* and *tetM* were present in the studied strains at very similar frequencies (19.5 and 20.3%, respectively), and both were associated with phenotypic resistance to tetracycline. These are the most commonly identified *tet* genes among both bovine and human *S. agalactiae* isolates (DOGAN et al., 2005; DUARTE et al., 2005; DUTRA et al., 2014; PINTO et al., 2014), while others determinants such as *tetS* and *tetK* being observed less frequently (DUARTE et al., 2004; RATO et al., 2013). Four isolates exhibiting phenotypic tetracycline resistance harbored neither *tetM* nor *tetO*, suggesting the presence of a different *tet* gene, such as *tetS* or *tetK*. Further investigation of these isolates is required to confirm this hypothesis. Carriage of both *tetO* and *tetM*, a phenomenon previously reported by Duarte et al. (2004), was observed in three isolates. However, 10 tetracycline-susceptible isolates carried one or both of these genes, with three carrying both, two *tetO* alone, and five *tetM* alone. The reasons that resistance genes are not always expressed need to be further evaluated. Possible explanations include promoter absence, weakness, or distance, and the

presence of mutations in the genes in question, as suggested by Gao et al. (2012). We found that 89.5% of strains carrying *ermB* also carried *tetO*, an association that has been previously documented.

The importance of monitoring genetic elements involved in tetracycline resistance has been emphasized in prior reports, as *tet* genes are often found in mobile genetic elements that also encode resistance to macrolides, lincosamides, and chloramphenicol (CHOPRA; ROBERTS, 2001). Associations between *tet* and *erm* genes, owing to their presence in the same mobile genetic element, have been described several times in relation to *Streptococcus pyogenes* and *Streptococcus pneumoniae*, but only rarely with respect to *S. agalactiae* (HAENNI et al., 2010; VARALDO et al., 2009), and their prevalence in this latter may be underestimated.

The *mefA* gene and the corresponding M phenotype (erythromycin resistance and clindamycin susceptibility) were not detected among the isolates tested. Similarly, neither Palmeiro et al. (2010) nor Rato et al. (2013) detected this gene or phenotype in their studies of *S. agalactiae* taken from bovine and human sources, respectively.

The genes *aphA3* and *aad-6*, which confer resistance to aminoglycosides, were not identified in the current work, consistent with the high rate of gentamicin susceptibility recorded. These genes have been reported in human and bovine *S. agalactiae* strains in other countries, associated with infections resistant to one or more aminoglycoside antimicrobials (GAO et al., 2012; POYART et al., 2003). To date, however, there have been no reports of these genes in bovine *S. agalactiae* strains isolated from Brazilian dairy herds.

In summary, the bovine *S. agalactiae* isolates studied in this work were heterogeneous with respect to the genetic resistance determinants and phenotypic resistance. The high rates of

resistance to tetracycline, clindamycin, and erythromycin observed suggest that the utility of these antimicrobial agents for the treatment of infected cows is limited, and imply that control and preventive measures, including restricted use of antibiotics, may not have been properly applied. On the other hand, the high levels of susceptibility to penicillin, ceftiofur, and cefalotin indicate that beta-lactams and cephalosporins are effective prophylactic and therapeutic options for control of bovine intramammary infections caused by this pathogen.

According to Myllys et al. (1994), antimicrobial resistance is an important factor in the introduction and dissemination of bacterial clones within a herd, with handling changes such as the implementation of systematic antibiotic treatment, stabling, and introduction of automatic milkers being closely associated, given their influence as selective forces on the pathogens responsible for mastitis.

A principal concern arising from increased antibiotic resistance among animal pathogens is that many of the organisms involved, including *S. agalactiae*, have zoonotic potential (FERREIRA et al., 2010; OLIVEIRA et al., 2011). Furthermore, resistance genes may be transferred horizontally to environmental microorganisms, those of the microbiota (MARTINEZ et al., 2009; WITTE, 2000), and even strictly human pathogens, limiting therapeutic options.

The present work contributes to our understanding of virulence, the dissemination of genetic resistance determinants, and antimicrobial susceptibility among *S. agalactiae* isolates associated with mastitis in Brazilian cattle. The results of this approach may aid the development of diagnostic, prophylactic, and therapeutic methods for pathologies caused by this bacterium, not only in cows, but also in humans and other affected species.

Conclusions

Susceptibility to the tested antimicrobials varied, with high rates of resistance to tetracycline, amikacin, erythromycin, and clindamycin and low rates of resistance to cephalosporins, penicillin, and gentamicin.

Most of the isolates tested carried at least one of the genes *ermB*, *tetO*, and *tetM*, however, *ermA*, *mefA*, *aphA3*, and *aad-6* were not detected. Associations were established between presence of the genes *ermB*, *tetM*, and *tetO* and phenotypic resistance to erythromycin, clindamycin, and tetracycline.

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