TIAGO TOMAZI

Etiological and molecular profile of pathogens causing clinical mastitis, and antimicrobial use in dairy herds

Pirassununga

2017

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Thesis submitted to the Postgraduate Program in 2017 of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Department:

Nutrition and Animal Production

Area:

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Advisor:

Prof. Marcos Veiga dos Santos, Ph.D.

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CERTIFICADO

Certificamos que a proposta intitulada "Perfil de agentes etiológicos causadores de mastite clínica e uso de antimicrobianos em rebanhos leiteiros", protocolada sob o CEUA nº 2994060214, sob a responsabilidade de **Marcos Veiga dos Santos** *e equipe; Tiago Tomazi* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 13/10/2015.

We certify that the proposal "título em inglês", utilizing 5000 Bovines (5000 females), protocol number CEUA 2994060214, under the responsibility of **Marcos Veiga dos Santos** *and team; Tiago Tomazi* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/13/2015.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 03/2013 a 02/2014 Área: Nutrição E Produção Animal

Origem:							
Espécie:	Bovinos	sexo:	Fêmeas	idade:	a	N:	5000
Linhagem:	Holandesa, Gir, Jersey			Peso:	a		

Resumo: O presente projeto tem como objetivo principal determinar o perfil etiológico e a sensibilidade aos antimicrobianos dos patógenos causadores de mastite clínica e descrever os aspectos qualitativos e quantitativos relacionados ao uso de antimicrobianos para o tratamento da mastite em rebanhos leiteiros. O estudo será realizado pela condução de três experimentos. Serão selecionados 15 rebanhos leiteiros com estimativa média de 300 vacas em lactação para participação em um programa de monitoramento da mastite clínica desenvolvido em parceria com o Laboratório Qualileite da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (FMVZ/USP).

Local do experimento:

São Paulo, 05 de setembro de 2017

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

EVALUATION FORM

Author: TOMAZI, Tiago

Title: Etiological and molecular profile of pathogens causing clinical mastitis, and antimicrobial use in dairy herds

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Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

DEDICATÓRIA (DEDICATION)

Eu dedico essa tese a minha família, em especial aos meus pais Neuri e Eveli Tomazi.

Aqueles que nunca me deixaram faltar amor e carinho, me ensinando humildade, ética, respeito e dedicação ao trabalho.

Sem vocês nenhum dos meus êxitos seria possível.

Gostaria também de dedicar este trabalho à memória dos meus amados avós Alcides Tomazi e Elvira Josephina Ceppo Tomazi, os quais foram os primeiros a me ensinar o amor e o respeito pelos animais.

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Aos meus avós Alcides, Elvira, Alberto e Antonieta por me ensinar o "respeito", uma das virtudes mais belas de um ser humano.

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A todos que aqui não citei, mas que de alguma forma contribuíram para a realização do trabalho.

MEU SINCERO MUITO OBRIGADO!

EPÍGRAFE (EPIGRAPH)

"O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis." (José de Alencar)

RESUMO

TOMAZI, T. **Perfil etiológico e molecular de patógenos causadores de mastite clínica, e uso de antimicrobianos em rebanhos leiteiros.** [**Etiological and molecular profile of pathogens causing clinical mastitis, and antimicrobial use in dairy herds**]. 2017. 187 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Os objetivos gerais desta tese foram: (i) determinar o perfil etiológico e molecular da mastite clínica (MC) em 20 rebanhos leiteiros do Sudeste do Brasil; e, (ii) quantificar os antimicrobianos usados para tratamento da MC na população estudada. Para alcançar esses objetivos, quatro estudos foram realizados. No *Estudo 1*, foi caracterizada a frequência de patógenos causadores de MC e a gravidade das infecções nos rebanhos leiteiros. Além disso, foi determinada a taxa de incidência de mastite clínica (TIMC) e sua associação com as seguintes variáveis em nível de rebanho: contagem de células somáticas em leite de tanque (CCSLT), contagem bacteriana total em leite de tanque (CBTLT), tamanho (número de vacas em lactação), produção de leite, sistema de alojamento e estação do ano. A associação entre as variáveis em nível de rebanho e a TIMC foi determinada por dois grupos de modelos de regressão logística multivariada: um baseado na TIMC geral, e cinco baseados nos seguintes grupos específicos de patógenos: contagiosos, outros Gram-positivos, Gram-negativos, outros patógenos (composto de leveduras e *Prototheca* spp.), e cultura negativa. Um total de 5.957 casos de MC em nível de quarto mamário foi registrado e os patógenos mais prevalentes foram *Escherichia coli* (6,6% de todas as culturas), *Streptococcus uberis* (6,1%), e *Streptococcus agalactiae* (5,9%). A maioria dos casos de MC foi de gravidade leve (60,3%), enquanto 34,1% dos casos foram moderados e 5,6% foram graves. A TIMC geral foi de 9,7 casos por 10.000 quartos-dia em risco (QDR), e o único parâmetro em nível de rebanho associado com a TIMC geral foi a CCSLT, em que a TIMC mais alta foi observada em rebanhos com CCSLT >600.000 $\times 10^3$ células/mL. Nos modelos que avaliaram os grupos específicos de patógenos, a TIMC de patógenos contagiosos foi associada com a CCSLT, produção de leite e sistema de alojamento. Na avaliação de outros patógenos Gram-positivos, a TIMC foi maior na estação chuvosa de 2015 em comparação com as outras categorias referentes à estação do ano. Adicionalmente, para o modelo avaliando o grupo de patógenos Gram-negativos, a TIMC foi mais alta em rebanhos com CBTLT >30.000 $\times 10^3$ ufc/mL. O *Estudo 2* teve como objetivo caracterizar o

perfil de tratamento e o consumo de antimicrobianos em rebanhos leiteiros; e determinar a associação de uso de antimicrobianos (UAM) e as mesmas variáveis em nível de rebanho descritas no *Estudo 1*. Dados sobre as práticas terapêuticas e UAM foram obtidos de 19 rebanhos leiteiros durante um período de 12 meses por rebanho. A frequência de UAM para tratamento da MC foi quantificada mensalmente em unidades de doses definidas diárias (DDD) e expressa como incidência de tratamento antimicrobiano (ITA: número de DDD por 1.000 vacas em lactação-dia). A média de ITA mensal foi de 17,7 DDD por 1.000 vacas em lactação-dia (15,4 para compostos intramamários, e 2,2 para compostos sistêmicos). Entre os produtos intramamários, os aminoglicosídeos tiveram a ITA mais alta (11,7 DDD por 1.000 vacas em lactação-dia), enquanto que para os compostos administrados pela via sistêmica, as fluoroquinolonas (4,2 DDD por 1.000 vacas em lactação-dia) foram os antimicrobianos mais frequentemente usados. O tamanho do rebanho e CCSLT foram positivamente associados com a ITA. Além disso, a ITA foi mais alta em rebanhos com freestall do que em rebanhos com sistema tipo *compost barn*. No *Estudo* 3, determinou-se a filogenia de cepas de *E. coli* isoladas de casos de MC em vacas leiteiras, e a associação dos filogrupos mais frequentes com a susceptibilidade aos antimicrobianos. Um total de 100 isolados de *E. coli* identificados nos casos de MC descritos no *Estudo 1* foram categorizados de acordo com os grupos filogenéticos por meio de um método de PCR quadruplex; o perfil de susceptibilidade aos antimicrobianos também foi avaliado. A maioria dos isolados pertenceram ao grupo A (52%), seguido dos grupos B1 (38%), B2 (2%), C (4%), D (3%), e E (1%). Foram encontrados isolados resistentes para todos os antimicrobianos avaliados. De forma geral, mais de 96% dos isolados de *E. coli* foram resistentes a ampicilina, e mais de 23% foram resistentes a cefalotina, sulfadimetoxina ou tetraciclina. Altos níveis de resistência (>70%) foram encontrados também para eritromicina, oxacilina, penicilina e penicilina associada a novobiocina. Ao contrário, foi observado alta susceptibilidade ao ceftiofur (96.8%) entre os isolados de *E. coli*. Diferenças na susceptibilidade entre os grupos filogenéticos foi observada apenas para a cefalotina, em que os isolados de *E. coli* pertencentes ao filogrupo A foram inibidos em concentrações de antimicrobianas mais baixas que isolados pertencentes ao filogrupo B1. No *Estudo 4*, avaliou-se a diversidade genotípica entre isolados de *Strep. agalactiae* e *Strep. uberis* identificados em casos de MC em vacas leiteiras; adicionalmente, o estudo avaliou a associação dos genótipos agrupados de acordo com a similaridade genética com o perfil de susceptibilidade aos antimicrobianos. Os isolados foram genotipados por meio do método de amplificação randômica de DNA polimórfico (RAPD). Grande diversidade genotípica foi observada tanto para o *Strep. agalactiae* (45 subtipos de 89 isolados) quanto para *Strep. uberis* (56 subtipos de

89 isolados). Para a avaliação de susceptibilidade aos antimicrobianos, os subtipos de *Strep. agalactiae* foram agrupados em três clusters (Ia, Ib e II), enquanto que os subtipos de *Strep. uberis* foram agrupados em dois clusters (I e II) de acordo com a similaridade genética. De forma geral, os isolados de *Strep. agalactiae* apresentaram alta susceptibilidade à maioria dos antimicrobianos, exceto para tetraciclina e eritromicina. Diferenças na susceptibilidade aos antimicrobianos entre os clusters de *Strep. agalactiae* foram observadas para ampicilina, ceftiofur, eritromicina, pirlimicina, sulfadimetoxina e tetraciclina. Por outro lado, os isolados de *Strep. uberis* foram resistentes à maioria dos antimicrobianos, exceto para cefalotina e penicilina + novobiocina. Não foram encontradas diferenças entre os clusters para todos os antimicrobianos na análise de *Strep. uberis.* Em conclusão, os resultados desta tese indicaram alta TIMC nos rebanhos avaliados, e apesar de os patógenos ambientais serem a causa mais comum de MC nestes rebanhos, patógenos contagiosos como *Strep. agalactiae* e *Staph. aureus*, ainda são uma preocupação em alguns rebanhos do Brasil. Além disso, observaram-se altas frequências de UAM e de terapias não recomendadas em bula entre os rebanhos avaliados. O uso não judicioso de antimicrobianos pode se tornar um fator de risco para o desenvolvimento da resistência bacteriana aos antimicrobianos, o que foi inclusive observado para isolados pertencentes as três espécies bacterianas mais prevalentes nos casos de MC no nosso estudo (*E. coli, Strep. agalactiae* e *Strep. uberis*). Finalmente, pelo fato de algumas variáveis em nível de rebanho terem sido associadas com a TIMC e com o UAM em nosso estudo, é possível que hajam oportunidades para implementação de estratégias de manejo com o objetivo de melhorar o controle da MC em rebanhos leiteiros do sudeste do Brasil.

Palavras-chave: Uso de antimicrobianos. Susceptibilidade aos antimicrobianos. Diversidade genotípica. Taxa de incidência de mastite clínica. Epidemiologia molecular.

ABSTRACT

TOMAZI, T. **Etiological and molecular profile of pathogens causing clinical mastitis, and antimicrobial use in dairy herds. [Perfil etiológico e molecular de patógenos causadores de mastite clínica, e uso de antimicrobianos em rebanhos leiteiros]**. 2017. 187 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

The general objectives of this thesis were: (i) to determine the etiological and molecular profile of clinical mastitis (CM) in 20 dairy herds of Southeast, Brazil; and (ii) to quantify antimicrobial used for treatment of CM in the study population. To achieve this goals, four studies were performed. In the *Study 1*, we characterized the pathogen frequency and severity of CM in dairy herds. In addition, we determined the incidence rate of clinical mastitis (IRCM) and its association with the following herd-level descriptors: bulk milk somatic cell count (BMSCC), bulk milk total bacterial count (BMTBC), herd size (number of lactating cows), milk yield, housing system and season. The association between herd-level descriptors and IRCM were determined by two groups of mixed regression models: one based on the overall IRCM, and five based on the following specific-pathogen groups: contagious, other Gram-positive, Gram-negative, other (composed of yeast and *Prototheca* spp), and negative culture. A total of 5,957 quarter-cases of CM were recorded and the most frequently isolated pathogens were *Escherichia coli* (6.6% of total cultures), *Streptococcus uberis* (6.1%), and *Streptococcus agalactiae* (5.9%). The majority of CM cases were mild (60.3%), while 34.1% were moderate and 5.6% severe. Overall, the IRCM was 9.7 quarter-cases per 10,000 quarter-days at risk (QDAR), and the only herd-level parameter associated with overall IRCM was BMSCC, in which the highest IRCM was observed for herds with BMSCC >600.000 × 10^3 cells/mL. In the models evaluating the specific-pathogen groups, IRCM with isolation of major contagious pathogens was associated with BMSCC, milk yield and housing system. For the evaluation of other Gram-positive pathogens, the IRCM was higher in the rainy season of 2015 in comparison with the other seasonal categories. In addition, for the model evaluating the Gram-negative group, the IRCM was highest in herds with BMTBC >30 × 10^3 cfu/mL. The *Study 2* aimed to characterize the treatment profile and quantify the antimicrobial consumption for treatment of CM in dairy herds; and to determine the association of antimicrobial use (AMU) and the same herd-level descriptors as described in the *Study 1*. Data on treatment practices and AMU were

obtained from 19 dairy herds for a period of 12 months per herd. The AMU for treatment of CM was quantified monthly in units of defined daily dose (DDD) and expressed as antimicrobial treatment incidence (ATI; number of DDD per 1,000 lactating cows-day). The overall monthly mean ATI was 17.7 DDD per 1,000 lactating cow-days (15.4 for intramammary compounds, and 2.2 for systematically administered antimicrobials). Among intramammary drugs, aminoglycosides had the highest ATI (11.7 DDD per 1,000 lactating cow-days), while for systematically administrated antimicrobials, fluoroquinolones (4.2 DDD per 1,000 lactating cow-days) were the most frequently used antimicrobials. Herd size and BMSCC were positively associated with ATI. In addition, herd-level ATI was higher in freestall herds than in compost bedded-pack barns. In the *Study 3*, we determined the phylogeny of *E. coli* strains isolated from CM in dairy cows and the association of most frequent phylogroups with antimicrobial susceptibility. A total of 100 *E. coli* isolates recovered from CM cases described in the *Study 1* were categorized according to their phylogenetic group using a quadruplex PCR method; antimicrobial susceptibility pattern was also evaluated. Most isolates were assigned to phylogenetic group A (52%), followed by B1 (38%), B2 (2%), C (4%), D (3%), and E (1%). Resistant isolates were observed for all evaluated antimicrobials. Overall, more than 96% of *E. coli* isolates were resistant to ampicillin, and more than 23% were resistant to cephalothin, sulphadimethoxine or tetracycline. High levels of resistance (>70%) were also found to erythromycin, oxacillin, penicillin, penicillin associated with novobiocin, and pirlimycin. In contrary, high susceptibility was observed to ceftiofur (96.8%) among *E. coli* isolates. Difference in the antimicrobial susceptibility among phylogenetic groups was observed only for cephalothin, in which *E. coli* strains belonging to the phylogroup A were inhibited at lower antimicrobial concentrations than strains assigned to the phylogroup B1. In *Study 4*, we evaluated the genotypic diversity among *Strep. agalactiae* and *Strep. uberis* isolates recovered from CM in dairy cows; in addition, the study evaluated the association of genotypes clustered by genetic similarity with antimicrobial susceptibility pattern. Isolates were subtyped using randomly amplified polymorphic DNA (RAPD) analysis. A great genotypic diversity was found for both *Strep. agalactiae* (45 subtypes out of 89 isolates) and *Strep. uberis* (56 subtypes out of 88 isolates). For evaluation of antimicrobial susceptibility, subtypes of *Strep. agalactiae* were clustered into three groups (Ia, Ib and II), while *Strep. uberis* subtypes were clustered into two groups (I and II) according to their genetic similarity. Overall, *Strep. agalactiae* isolates showed high susceptibility to most antimicrobials, except to tetracycline and erythromycin. Differences in the antimicrobial susceptibility among clusters of *Strep. agalactiae* were observed for ampicillin, ceftiofur, erythromycin, pirlimycin, sulphadimethoxine and

tetracycline. In contrary, *Strep. uberis* isolates were categorized as resistant to most antimicrobials, except to cephalothin and penicillin+novobiocin. No differences were observed among clusters for all antimicrobials in the analysis of *Strep. uberis*. In conclusion, the results of this thesis indicated a high IRCM in the evaluated herds, and although environmental pathogens were the most common cause of CM in these herds, contagious pathogens such as *Strep. agalactiae* and *Staph. aureus*, are still a concern in some dairy herds of Brazil. Furthermore, high frequencies of AMU and off-label protocols were observed among the evaluated herds. The non-judicious use of antimicrobials can become a risk factor for the development of antimicrobial resistance, which was even observed for isolates belonging to the three most prevalent bacterial species identified from CM cases in our study (*E. coli*, *Strep. agalactiae* and *Strep. uberis*). Finally, because there were some herd-level descriptors associated with the IRCM and AMU in our study, there may be opportunity for management strategies aiming to improve the control of CM in dairy herds of southeastern Brazil.

Key-words: Antimicrobial use. Antimicrobial susceptibility. Genotypic diversity. Incidence rate of clinical mastitis. Molecular epidemiology.

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LIST OF ABREVIATIONS

AIC	Akaike information criterion
AMR	Antimicrobial resistance
AMU	Antimicrobial use
ATI	Antimicrobial treatment incidence
BHI	Brain heart infusion
BMSCC	Bulk milk somatic cell count
BMTBC	Bulk milk total bacterial count
CAMP	Christie, Atkins, Munch-Petersen test
CBPB	Compost-bedded pack barn
cfu	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CM	Clinical mastitis
CNS	Coagulase negative *Staphylococcus*
DDD	Defined daily doses
DIM	Days in milk
DNA	Deoxyribonucleic acid
IDF	International Dairy Federation
IMI	Intramammary infection
IRCM	Incidence rate of clinical mastitis
IU	International units
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MST	Minimum spanning tree
NMC	National Mastitis Council
PCR	Polimerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PYR	Pyrrolidonyl arylamidase test
QDAR	Quarter-days at risk

RAPD	Random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SCC	Somatic cell count
SD	Standard deviation
SEM	Standard error of mean
TBC	Total bacterial count
TSA	Trypticase soy agar

SUMMARY

Chapter 1

General introduction and objectives

1 GENERAL INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION AND JUSTIFICATION

Clinical mastitis (CM) is a common disease affecting dairy cattle and one of the most important causes of antimicrobial consumption in dairy herds (MITCHELL et al., 1998; SAINI et al., 2012a). Milk of cows with CM can have alterations in its physical, chemical, microbiological and sensorial characteristics, which makes it unsuitable for consumption (AULDIST; HUBBLE, 1998; SANTOS; MA; BARBANO, 2003). The monitoring of CM in dairy herds is important for estimation of losses associated with this disease, such as: early culling of lactating cows, medication costs, reduction of milk yield, and risk of death due mastitis (SANTOS and FONSECA, 2006).

The frequency of new cases of CM in dairy herds can be calculated as incidence rate and incidence risk (DOHOO; MARTIN; STRYHN, 2009). Briefly, incidence risk is calculated by dividing the number of new cases occurring over a given period by the total number of at-risk individuals at the beginning of the period. This measure assumes that all individuals identified at the beginning of the assessment were followed up for the entire monitoring period (DOHOO; MARTIN; STRYHN, 2009). Incidence risk is the most commonly used method for calculating incidence at the herd level and may be expressed as a percentage (e.g., the herd's incidence of CM in 2017 was 8%).

On the other hand, in observational studies it is common that the individuals under evaluation are not monitored for uniform periods. Some individuals may be followed for days, others for months, and others may be dropped from the study after a certain period. To consider the potential time variations in which a certain individual remained under evaluation, the calculation of the incidence rate is more suitable (DOHOO; MARTIN; STRYHN, 2009). For example, to calculate the incidence rate of clinical mastitis (IRCM) in a herd or region, the number of new cases of the disease occurring within a given period should be divided by the sum of time units (e.g., days or months) that each individual was not infected (period at risk). In this case, the unit of measure of IRCM is determined as the number of cases occurring in a population at risk over a given period of time (e.g., 2.5 cases of CM per 100 cow-days at risk).

The IRCM is an indicator of mammary gland health commonly used in epidemiological studies to evaluate the occurrence of new cases of the disease at the regional or national level.

The monitoring of IRCM allows to evaluate the occurrence of the disease over time, which helps in the early elaboration of prevention and treatment strategies. At the herd level, monitoring the IRCM allows farmers to have a view of their own situation compared to other farms, which may motivate them to implement specific control strategies.

Significant variations in the incidence of CM have been reported in several countries, ranging from 9% during the first three months of lactation of dairy cows in a study conducted in Australia (DANIEL et al., 1982) to 54.6 cases per 100 cows-year in British herds (WILESMITH; FRANCIS; WILSON, 1986). Differences in selection criteria, environmental conditions, facilities, season of the year, sampling methods, and criteria for defining CM cases are among the factors that contribute to the variation of the results between the studies (OLDE RIEKERINK et al., 2008).

The IRCM in a study conducted in England was 47 cases per 100 cows-year (BRADLEY et al., 2007), while in the Czech Republic (WOLFOVA; STIPKOVA; WOLF, 2006) and Ireland (MORE; CLEGG; O'GRADY, 2012) the incidence rate was approximately 55 cases per 100 cows-year. In Macedonia, the mean IRCM was 34.1 cases per 100 cows-year. Several studies have evaluated the IRCM in Canada, which reported the following results: 0.37 cases per cow-year (MEEK et al., 1986); 21.8 cases per 100 lactations (MCLAREN et al., 2006); 23 cases per 100 cows-year (OLDE-RIEKERINK et al., 2008); and 22 cases per 100 cows per year (REYHER et al., 2011). A study performed in the Netherlands reported that the IRCM reduced from 33.5 cases per 100 cows-year in 2005 to 28.1 cases per 100 cows-year in 2009 (LAM et al., 2013). In a more recent study, the IRCM reported in 227 herds in Netherlands was 32.5 cases per 100 cows-year (SANTMAN-BERENDS et al., 2015). Kivaria et al. (2007), evaluating small herds in Tanzania reported a quarter-level IRCM of 38.4 cases per 100 quarters-year. In the same study, the IRCM at the cow-level was 43.3 cases per 100 cows at risk-year.

Only one recent study characterizing CM in dairy herds in Brazil were found in the indexed literature, in which the mean annual incidence risk in primiparous cows was 27%, while for multiparous cows it was 31% (OLIVEIRA et al., 2015). Indices associated with the frequency of CM, such as prevalence, incidence rate, recurrence of cases, most prevalent etiological agents, and the association of such indices with herd-level descriptors were not found for dairy herds in Brazil. Data on the frequency of CM and a better understanding of descriptors associated with the occurrence of this disease in dairy herds can be used as reference for further studies, and for development of specific strategies of mastitis control and prevention.

The main causes of CM in dairy herds are bacteria, but this disease can also be caused by fungi, yeast and algae. In approximately 80% of bovine IMI, five species (*Escherichia coli, Streptococcus uberis, Staphylococcus aureus, Streptococcus dysgalatiae* and *Streptococcus agalactiae*) are responsible for the establishment of disease (BRADLEY, 2002). Furthermore, mastitis causing bacteria can have a variety of subtypes, which despite being genetically close-related, may present differences of virulence, pathogenicity, antimicrobial susceptibility and transmission profile (ZADOKS et al., 2011; RUEGG, 2012).

In the last decades, the molecular diagnosis of pathogens causing mastitis improved the understanding of the etiology and pathogenesis of IMI (ZADOKS; SCHUKKEN, 2006; ZADOKS et al., 2011). In addition, molecular typing methods enabled the better understanding of the transmission routes of most frequent bacteria causing mastitis. For example, herds with IMI caused by the same bacterial strain indicates that the pathogen transmission is mainly occurring by the contagious route (cow-to-cow); or could even suggest that the cows are getting infected by strains belonging to the same environmental reservoir. On the other hand, in herds presenting high genotypic diversity within species, it is more likely that different reservoirs in the environment are the sources for that pathogen (MUNOZ et al., 2007; CREMONESI et al., 2015).

Furthermore, with the advent of molecular typing methods, the classical distinction between microorganisms exclusively causing environmental or contagious mastitis has been increasingly questioned (SCHUKKEN, Y. et al., 2012). A study evaluating an outbreak caused by *Klebsiella* spp., which is classically defined as environmental pathogen, reported a homogeneous genotypic profile among strains in different cows identified with CM (MUNOZ et al., 2007). Similar results were observed in cases of CM caused by *Strep. uberis*, which is considered a pathogen that composes the environmental group of streptococci in the role of mastitis (ABUREEMA et al., 2014). Results of these studies suggest that even though the main reservoir of these bacteria species remains the environment, some strains could be also transmitted by the contagious route.

Several molecular techniques based on bacterial DNA, have been used in epidemiological studies for subtyping of mastitis pathogens, which includes pulsed-field gel electrophoresis (PFGE; RATO et al., 2013; ABUREEMA et al., 2014), RFLP-PCR (MCDONALD; FRY; DEIGHTON, 2005), ribotyping (PITKALA; KOORT; BJORKROTH, 2008) and multilocus sequence typing (MLST; CARVALHO-CASTRO et al, 2017). The random amplified polymorphic DNA (RAPD) analysis was also used for subtyping of *Strep. agalactiae* and *Strep. uberis* isolated from bovine mastitis (MARTINEZ et al., 2000;

WIELICZKO et al., 2002). Furthermore, a quadruplex PCR method targeting specific genes (*chuA, yjaA, arpA, trpA* and *TspE4.C2*) was published by Clermont et al. (2013) and have been used in several studies evaluating the phylogenetic distribution of *E. coli* causing IMI (SUOJALA et al., 2011; DOGAN et al., 2012; BLUM, SHLOMO E.; LEITNER, 2013). Regardless of the molecular method, genotyping studies are important for advancing the knowledge on epidemiological aspects of bacteria causing CM in dairy herds, especially in relation to transmission patterns (clonal or non-clonal), as well as for identification of herd-level conditions or descriptors associated with intraspecific strains. Furthermore, results on the genotypic diversity can be associated with the antimicrobial susceptibility, which can be used for monitoring resistance patterns among major pathogens causing CM, such as *E. coli*, *Strep. agalactiae*, and *Strep uberis*, and therefore, contributing with the improvement of therapeutic strategies.

Although management practices such as the use of adequate milking procedures, provide good nutrition, and the maintenance of the cows' comfort and hygiene, can assist in the control and prevention of IMI, these factors have little effect on eliminating intercurrent cases of CM (HOGAN, JOE; SMITH, 2012; ROBERSON, 2012). Therefore, the use of antimicrobials remains the most effective strategy for treatment of CM in dairy cows. However, the non-judicious use of antimicrobials for treatment of diseases in humans and animals is considered one of the main factors associated with the emergence of antimicrobial resistance (AMR; LEVY; MARSHALL, 2004). As consequence, there is a global interest of government institutions in the determination of antimicrobial use in food animals, because they are a potential source and disseminator of antimicrobial resistant bacteria (WHITE; MCDERMOTT, 2001; BENNEDSGAARD; KLAAS; VAARST, 2010). Furthermore, the availability of data on antimicrobial use can aid in interpreting patterns and trends of antimicrobial susceptibility among bacteria causing CM, and serve as a basis of risk assessment and evaluation of interventions for controlling of AMR. Data on antimicrobial use for treatment of bovine CM are, therefore, becoming increasingly important for development of policies to contain AMR (SAINI et al., 2012b).

Studies evaluating the use of antimicrobials for treatment of CM were conducted in several countries such as United States (SAWANT; SORDILLO; JAYARAO, 2005; POL; RUEGG, 2007; HILL, A. E. et al., 2009), Canada (SAINI et al., 2012b), Europe (GRAVE et al., 1999; GONZALEZ et al., 2010; STEVENS et al., 2016), and even in countries of South America (REDDING et al., 2014b). However, the quantification of antimicrobial formulations used for treatment of CM was not described in dairy herds in Brazil. Furthermore, there are few

studies that have evaluated the association between the frequency of antimicrobials used for treatment of CM and herd-level descriptors such as SCC, TBC, housing system, herd size and milk production (HILL, A. E. et al., 2009; SAINI et al., 2012b; STEVENS et al., 2016). Studies characterizing the antimicrobial use in dairy herds can benefit the dairy industry as they can be used as a basis for implementation of programs for the prudent use of antimicrobials, and consequently, for prevention of bacterial AMR.

1.2 GENERAL OBJECTIVES

The general objectives of this thesis were: (i) to determine the etiological and molecular profile of clinical mastitis in 20 dairy herds of Southeast, Brazil; and (ii) to quantify antimicrobial used for treatment of clinical mastitis in the study population.

1.3 SPECIFIC OBJECTIVES

(i) to characterize the pathogen frequency and severity of CM in 20 dairy herds in Southeastern Brazil;
(ii) to determine the overall IRCM at the quarter-level and its association with herd-level descriptors, such as herd size, average milk yield, bulk milk somatic cell count (BMSCC), bulk milk total bacterial count (BMTBC), housing system, and season;
(iii) to determine the IRCM within specific-pathogen groups and their association with descriptors at the herd-level, such as herd size, average milk yield, BMSCC, BMTBC, housing system, and season;
(iv) to characterize the treatment profile and quantify (overall and class-specific) the antimicrobial consumption for treatment of CM in 20 dairy herds of Southeast, Brazil;
(v) to determine the association of antimicrobial use for treatment of CM and the following herd-level descriptors: average milk yield, herd size, BMSCC, BMTBC, season and housing system.

(vi) to determine the phylogeny of *Escherichia coli* isolated from clinical mastitis in dairy cows and its association with the following variables: cow-level descriptors (days in milk, parity, position of affected quarter, and severity score of CM), housing type and season;

(vii) to determine and compare the antimicrobial susceptibility among the most frequent *Escherichia coli* phylogroups identified from clinical mastitis;

(viii) to genotypically characterize *Strep. agalactiae* and *Strep. uberis* strains recovered from cases of CM in dairy cows;

(ix) to determine the association of antimicrobial susceptibility and genotypes of *Strep. agalactiae* and *Strep. uberis.*

CHAPTER 2

Association of herd-level descriptors and incidence rate of clinical mastitis in 20 Brazilian dairy herds

2 ASSOCIATION OF HERD-LEVEL DESCRIPTORS AND INCIDENCE RATE OF CLINICAL MASTITIS IN 20 BRAZILIAN DAIRY HERDS

2.1 ABSTRACT

The objectives were to characterize the pathogen frequency and severity of clinical mastitis (CM) in 20 dairy herds in the Southeast of Brazil, and to determine the incidence rate of clinical mastitis (IRCM; overall and based on specific-pathogen groups) at the quarter-level and its association with descriptors at the herd-level. Data from 20 dairy herds that recorded CM cases for a period of 8 to 15 months from 2014 to 2016 were available for analysis. The association between herd-level descriptors and IRCM were determined by two groups of mixed regression models: one based on the overall IRCM, and five based on the following specific-pathogen groups: contagious, other Gram-positive, Gram-negative, other, and negative culture. The following herd-level descriptors were evaluated in the analysis: season (rainy 2014, dry 2014, rainy 2015, and dry 2015); herd size (≤100, 101-200, or ≥201 lactating cows); housing system (compost-bedded pack barn, free stall, paddocks); average daily milk yield per cow (≤20, 21-25, or ≥26 kg/d); bulk milk somatic cell count (BMSCC; ≤300, 301-600, or ≥601 × 10^3 cells/mL); and bulk milk total bacterial count (BMTBC; ≤30 or ≥31 × 10^3 cfu/mL). A total of 5,957 quarter-cases of CM were recorded, but only 4,212 had milk samples collected for culture. The most frequently isolated pathogens were *Escherichia coli* (6.6% of total cultures), *Streptococcus uberis* (6.1%), and *Streptococcus agalactiae* (5.9%). The majority of CM cases were mild (60.3%), while 34.1% were moderate and 5.6% severe. The frequency of severe CM cases was lower for those with a Gram-positive result (4.6%) compared to those with a Gram-negative result (11.4%). Overall monthly mean IRCM was 9.7 cases per 10,000 quarter-days at risk (QDAR). Herds with a geometric mean BMSCC ≥601 × 10^3 cell/mL had higher overall IRCM (16/10,000 QDAR) than those with BMSCC ≤600 × 10^3 cell/mL (≤7.7/10,000 QDAR). When the specific-pathogen groups were evaluated, for contagious pathogens, herds with higher BMSCC (≥601 × 10^3 cell/mL) also presented the highest IRCM (3.1/10,000 QDAR) when compared to those with lower BMSCC (≤0.8/10,000 QDAR). Additionally, within the contagious group, herds with average daily milk yield per cow between 21 and 25 kg/d had the highest IRCM (3.0/10,000 QDAR) in comparison to herds with average of ≤20 kg/d (≤0.7/10,000 QDAR) and ≥26 kg/d (≤0.5/10,000 QDAR). Cows housed in free-stalls

(1.2/10,000 QDAR) or compost-bedded pack barn (3.2/10,000 QDAR) systems presented the highest IRCM when compared to paddocks (0.5/10,000 QDAR). For the Gram-positive pathogen group, the IRCM was higher during the rainy season of 2015 (2.8/10,000 QDAR) compared with other seasonal categories (<1.8/10,000 QDAR). Furthermore, in the Gram-negative group, herds with BMTBC ≥31 × 10^3 cfu/mL had higher IRCM (1.8/10,000 QDAR) compared with herds with BMTBC ≤30 × 10^3 cfu/mL (0.9/10,000 QDAR). Although environmental pathogens were the most common cause of CM in this study, contagious pathogens are still a concern in dairy herds of Brazil. Additionally, as there were some herd-level descriptors associated with the IRCM, there may be opportunity for management strategies aiming to improve the control of CM in dairy herds.
Key-words: Dairy cattle. Pathogen distribution. Incidence of clinical mastitis. Brazil.

2.2 INTRODUCTION

Bovine mastitis has the highest incidence among diseases of dairy cattle and its clinical form is one of the major concerns for the dairy livestock industry. Clinical mastitis (**CM**) has been associated with treatment costs, milk discard, reduced milk production, increased mortality, early culling of lactating cows, and increased labor (HALASA et al., 2007). Milk from cows with CM presents visible physical alterations, as well as chemical, microbiological and sensory changes, which makes it unsuitable for human consumption. In addition, the change in milk quality reduces industrial performance and the shelf life of dairy products (BARBANO; MA; SANTOS, 2006).

The incidence rate and the etiological profile of CM may differ considerably between dairy herds from different countries and even between herds within a given country (OLDE RIEKERINK et al., 2008). Epidemiological studies have estimated the incidence rate of clinical mastitis (IRCM) in different regions of the world, including Europe (BRADLEY et al., 2007; VERBEKE et al., 2014; SANTMAN-BERENDS et al., 2015), North America (SARGEANT et al., 1998; OLDE RIEKERINK et al., 2008; OLIVEIRA; HULLAND; RUEGG, 2013), China (GAO et al., 2017), Australia (DANIEL et al., 1982), New Zealand (MCDOUGALL, 1999), and Tanzania (KIVARIA; NOORDHUIZEN; MSAMI, 2007). However, studies describing indicators of CM, such as the most prevalent causing pathogens, distribution of severity score and the IRCM are few in Brazil (OLIVEIRA et al., 2015). Additionally, to our knowledge, no

studies were conducted in Brazil associating the IRCM at the quarter-level and descriptors at the herd-level. Due to the etiological and epidemiological differences that occur between and within regions, results of epidemiological studies evaluating CM are useful for the development of specific strategies of control and prevention.

Clinical mastitis can be caused by a variety of microorganisms, which have different pathogenicity and frequency among dairy herds. However, these microorganisms can be broadly classified into two groups based on route of transmission: contagious and environmental (RUEGG, 2012). *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma* spp. have been reported as the most important contagious pathogens causing CM in dairy cows (KEEFE, G., 2012). On the other hand, environmental pathogens as Gram-negative bacteria (especially coliforms) and other Gram-positive microorganisms, such as environmental streptococci and minor pathogens (e.g., *Corynebacterium* spp. and CNS), can also be the cause of CM. In addition, other microorganisms, which are unlikely to respond to antimicrobial treatment, can also cause CM; this group includes non-bacterial pathogens (e.g., yeast and *Prototheca* spp.), and some bacterial species such as *Trueperella pyogenes* (ROBERSON, 2012).

Previous studies evaluating CM in other countries reported that specific characteristics at the herd-level influenced the distributions of pathogens causing CM among farms and regions (OLDE RIEKERINK et al., 2008; OLIVEIRA; HULLAND; RUEGG, 2013). Factors such as season, herd size, housing system, average milk yield per cow, bulk milk somatic cell count (BMSCC), and bulk milk total bacterial count (BMTBC) may be associated with both the pathogens causing CM and the IRCM in dairy herds. Studies evaluating the association of herd-level descriptors and the IRCM caused by specific groups of pathogens are few (VERBEKE et al., 2014), especially those evaluating Brazilian dairy herds where the etiology of CM may be different from that observed in countries with a more developed dairy industry (OLIVEIRA et al., 2015).

The aims of this study were to: (a) characterize the pathogen frequency and severity of CM in 20 dairy herds in Southeastern Brazil; (b) determine the overall IRCM at the quarter-level and its association with descriptors at the herd-level, such as herd size, average milk yield, BMSCC, BMTBC, housing system, and season; and (c) determine the IRCM within specific-pathogen groups and their association with the same descriptors at the herd-level.

2.3 MATERIAL AND METHODS

2.3.1 Selection of dairy herds

A convenience sample of 20 dairy herds (A-T) from Southeastern Brazil (15 from the State of São Paulo and 5 from the State of Minas Gerais), based on a client list of the Qualileite Lab (Mastitis and Milk Quality Research Laboratory at University of Sao Paulo, Brazil), were selected to participate of this study. Herds had to meet the following inclusion criteria: a) have conventional milking parlor with a mechanical milking system (vs. milking by hand); b) perform a milking routine that includes identification of CM in every cow (e.g., forestripping); c) perform monthly analysis of BMSCC and BMTBC in an official laboratory; d) have cow identification (e.g., ear tags); and e) have a recording system (e.g., notebooks, computerized spreadsheets or software system) able to provide information such as birth date, days in milk, parity, and mastitis information (e.g., diagnostic date, affected quarter and treatment protocol).

2.3.2 Clinical mastitis and severity definition

Before the beginning of the data collection, training on detection of CM, determination of severity scores and aseptic milk sample collection according to National Mastitis Council (**NMC)** guidelines (NMC, 2017) was reviewed with farm personnel from all herds. Kits containing gloves, gauze soaked in 70% ethanol and sterile tubes for milk sample collection were provided to each herd. The training was conducted to ensure data quality and to prevent sample contamination. In each farm, only one person was responsible to monitor the milk sample collection and the data recording about cow and clinical mastitis case (e.g., affected quarter and severity scores). The training consisted of a discussion of potential signs that may be observed in CM cases (e.g., changes in milk, udder or presence systemic symptoms). In addition, *in situ* demonstrations were performed when a cow occasionally had CM on the day of farm visit.

Clinical mastitis was identified at the quarter-level through fore-stripping by trained farm personnel and it was defined as a quarter with abnormal milk, accompanied or not by other

clinical signs, such as udder swelling, redness, heat, and pain (IDF, 1999). The severity of CM was recorded and defined as: **Mild** - changes only in the milk appearance, such as abnormal viscosity (watery appearance), color or consistency (presence blood, flakes or clots); **Moderate** - presence of abnormal milk accompanied by changes in the udder (hardening, swelling and/or redness); and, **Severe** - the combination of abnormal milk, with signs of inflammation in the udder and systemic signs (body temperature >39.5°C, lack of appetite, dehydration, weakness, depression; ROBERSON, 2012).

2.3.3 Milk samples and data collection

Milk sample collection was performed in each herd for a period of 8 to 15 months from March 2014 to January 2016. Milk sample vials were labeled with the cow identification number or name, affected mammary quarter, date of diagnosis, and severity score. If more than one quarter was identified with CM in the same cow, one vial per affected mammary quarter was collected. After collection, milk samples were frozen and sent in batches to the microbiology laboratory on ice packs for culture, or stored on the farm (at approximately -20ºC) until the university researchers could pick them up.

Visits by university researchers were performed every 14-30 days and the following herd level information was recorded: (1) the housing system used for lactating cows (at the first visit); (2) milk yield as a monthly average of daily milk production per cow; (3) monthly results of BMSCC and BMTBC; if a herd had more than one result of BMSCC or BMTBC in a given month, the arithmetic average of the results was used; (4) the number of dairy cows as an average of milking cows within a given month.

2.3.4 Microbiological identification

All frozen milk samples collected from cases of CM were submitted for microbiological culture according to procedures recommended by the NMC (2017) within 35 days after CM diagnosis. Once the samples were in the lab, they were processed within 5 days. Briefly, 0.01 mL of milk sample was plated on blood Agar (BBL-Becton Dickinson and Co., Le Point de

Claix, France) using a sterile loop, and incubated aerobically at 37°C. Phenotypic features were examined at 24 and 48 h after incubation and specific biochemical testing was performed in order to determine bacterial genus and/or species.

A milk sample was defined as negative if no colonies were observed on the streaking field of the blood Agar plate after 48 h of incubation. On the other hand, a milk sample was defined as positive if at least one colony of any pathogen (except for CNS and *Bacillus* spp.) was observed in the streaking field of the blood Agar plate. For CNS, 2 or more colonies isolated from a 0.01 mL milk sample were needed to establish presence of an intramammary infection (Dohoo et al., 2011). For *Bacillus* spp., an infection was defined as 5 or more colonies isolated from the streaked milk sample. Colonies were considered distinct based on morphological features, and if two distinct colonies were observed in the streaked field, the milk sample was defined as a mixed culture. A milk sample was defined as contaminated if more than 2 different colony types were present in the streaked field of the Agar plate (Cortinhas et al., 2016). If a contagious pathogen (e.g., *Staph. aureus* and *Strep. agalactiae*) were identified in the cultures with more than 1 different colony types, these pathogens were considered the cause of CM.

Gram staining method was performed for morphological characterization and identification of bacterial genus. Catalase test using hydrogen peroxide (3%) was used for differentiation between Gram-positive cocci (catalase-positive staphylococci) and catalase-negative cocci. Coagulase test using defibrinated rabbit plasma was performed to distinguish *Staph. aureus* from CNS. Streptococci were defined as esculin-positive (*Strep. uberis* or *Streptococcus* spp.) or esculin-negative (*Strep. agalactiae* or *Strep. dysgalactiae*). Christie, Atkins, Munch-Petersen test (**CAMP** test) was used to distinguish *Strep. agalactiae* from *Strep. dysgalactiae.* Bile esculin test and the pyrrolidonyl arylamidase test (**PYR** test; Probac do Brasil, São Paulo, Brazil) were used to differentiate isolates as *Streptococcus* spp., *Strep. uberis* or *Enterococcus* spp.

Gram-positive rods with negative reaction in the catalase test and with hemolysis after 48 hours of incubation were identified as *Trueperella pyogenes*. Gram-positive rods, with positive reaction in the catalase test were identified as *Bacillus* spp. or *Corynebacterium* spp., depending on the appearance of the colony in the optical microscopy and visual features on Agar. Yeast and *Prototheca* spp. were identified based on morphological features observed in optical microscopy.

All isolates were submitted for KOH (potassium hydroxide) test. Isolates with positive reaction in this test were suggestive of Gram-negative microorganisms, and then identified by colony morphology on MacConkey's Agar. In addition, the following biochemical

characteristics were evaluated for Gram-negative microorganisms: sucrose and glucose fermentation, hydrolysis of urea, gas production, motility capacity, indole production, H_2S production, L-tryptophan deaminase and lysine reaction.

2.3.5 Overall incidence rate of clinical mastitis

Farm personnel were instructed to record and collect milk samples from all cases of CM. The overall IRCM within herds (including all cases independent of the culture result) was calculated monthly as the number of occurred CM cases divided by the number of quarter-days at risk (**QDAR**) in each month and multiplied by 10,000 quarters at risk. The multiplication of the results by 10,000 quarters at risk was done because otherwise the final number (i.e., IRCM) would be too low to be presented, especially because the evaluation was performed at the quarter-level instead of at the cow-level. Clinical mastitis cases were considered new if there was at least 14 d between a previously diagnosed case and current case in the same quarter (LAM et al., 2013; SANTMAN-BERENDS et al., 2016); the interval between cases was counted from the diagnosis date of previous CM case until the date of the current CM diagnosis. The QDAR were calculated as the sum of days that each quarter remained healthy (or in milking), during a given month. The at-risk period for a mammary quarter started at the beginning of each month or at the date of calving, and ended at the end of the month, or at the day of CM diagnosis, or at the culling or drying-off date. Therefore, the following formula was used to calculate de IRCM:

$$\text{IRCM} = \left(\frac{\text{Number of quarter cases of CM within month}}{\text{QDAR}}\right) \times 10{,}000 \text{ quarters}$$

2.3.6 Incidence rate of clinical mastitis by specific-pathogen groups

The IRCM by specific-pathogen groups was calculated as the number of CM events with that culture result divided by the number of QDAR in each month and multiplied by 10,000 quarters. The at-risk period was calculated in the same manner as for the overall IRCM.

Negative cultures at 48 h of incubation or with isolation of a unique pathogen where summarized to create the following specific groups: (1) contagious (*Staph. aureus* and *Strep. agalactiae*); (2) other Gram-positive (environmental streptococci – *Strep. uberis*, *Strep. dysgalactiae* and *Strep.* group C; CNS and *Corynebacterium* spp.; *Enterococcus* spp.; and species of Gram-positive rods); (3) Gram-negative; (4) other pathogens (*Trueperella* spp., *Prototheca* spp. and yeast); and (5) negative culture.

2.3.7 Statistical analyses

Descriptive statistics of pathogen distribution and pathogen-specific severity were performed using the FREQ procedure of SAS 9.4 (SAS Inst. Inc., Cary NC). Pathogen distribution was evaluated at the quarter-level, such that a cow could contribute information on more than one quarter at any given time. However, the distribution of pathogen-specific severity was evaluated at the cow-level. Thus, if a cow had more than one infected mammary quarter with different severity scores, the higher score was considered in the evaluation.

The MEANS procedure of SAS 9.4 (SAS Inst. Inc., Cary NC) was used to describe characteristics at the herd-level (number of milking cows, average milk yield, BMSCC, BMTBC). Summary statistics were produced using the mean as a measure of central tendency, and standard deviation (**SD**) and standard error of mean (**SEM**) as measures of statistical dispersion, considering the total period that each herd was evaluated during the study. For BMSCC and BMTBC, the geometric means were used as a measure of central tendency based on the monthly average of the reports over the entire monitoring period in each herd. Therefore, the SD for BMSCC and BMTBC were used only for the overall arithmetic mean, considering all herds; the median was also computed for BMSCC and BMTBC. For any other descriptors (i.e., number of lactating cows and milk yield) arithmetic means were demonstrated.

The explanatory categorical variables used in statistical models were defined based on the distribution of data among herds using frequency histograms to assure adequate number of herds per category, as well as, considering the biologically relevant cut-offs. Therefore, the variables were categorized as: geometric mean of BMSCC (≤300, 301-600, or ≥601 × 10^3 cells/mL); geometric mean of BMTBC (≤30 or ≥31 × 10^3 cfu/mL); herd size (≤100, 101-200, or ≥201 milking cows); average daily milk yield per lactating cow (≤20, 21-25, or ≥26 kg), and housing system (compost-bedded pack barn, free stall, paddocks). The paddock housing system was characterized as an open area surrounded by fences or rails and without pasture for grazing.

Because of the potential differences in the seasons among the evaluated years (e.g., variation in the rainfall and in the temperatures), the variable season was categorized in four groups: (1) dry 2014; (2) rainy 2014; (3) dry 2015; and (4) rainy 2015. In Southeast region of Brazil, rainy season is characterized by the highest temperatures (summer), and the dry season has the lowest temperatures (winter; OLIVEIRA et al., 2015). Season categories were created associating the two characteristic seasons in Southeast of Brazil [rainy (October-March); or dry (April-September)], and the years that each herd were evaluated in the study (2014 or 2015-2016). One herd was monitored until the end of January 2016 and the IRCM recorded during this period was included in the category rainy 2015 of the variable season. Although the season is not a modifiable risk factor at the herd-level, we decided to include this variable in the data analysis because of its potential interaction with other variables such as housing system, BMSCC and BMTBC.

The associations between herd-level descriptors and the overall IRCM and IRCM of specific pathogen groups based on the microbiological identification were determined using six mixed effects regression models (PROC MIXED, SAS 9.4). All models contained time (month) as a repeated measure and herd as a random effect. Herd was selected as a random effect to account for clustering of unmeasured effects that are more likely to be similar within herds than between herds. Categorical explanatory variables were subjected to univariable analyses and variables with $P \leq 0.3$ were explored in a multivariable model. The multivariable analysis was performed using a manual backward stepwise selection and elimination procedure. Biologically relevant interactions were also evaluated and included (BMSCC*season, BTSCC*season, housing system*season). After each run, the variable with the highest *P*-value was excluded from the model until all variables had $P \leq 0.05$. Model fit was evaluated using the Akaike information criterion (AIC), where the lowest AIC was deemed the best model (Akaike, 1974).

Potential confounders were monitored by the change in the coefficient of a variable after removing another variable from the model. If the change of the estimates exceeded 25% or 0.1

when the value of the estimate was between −0.4 and 0.4, the variable was re-entered in the model. No variable was found as a potential confounder in the evaluation of the estimates after each run.

2.4 RESULTS

2.4.1 Herd characteristics

Descriptive results of the herds evaluated in this study are shown in Table 1. An average of 4,390 lactating cows was evaluated monthly in the selected herds from March 2014 to January 2016. Lactating cows were housed in three different housing systems: 10 paddocks (average total number of milking cows per month = 1,200; mean = 246; SD = 106), 5 free-stalls (average total number of milking cows per month = 2,587; mean = 647; SD = 417), and 5 compost bedded-pack barns (average total number of milking cows per month = 603; mean = 138; SD = 49). The overall mean of daily milk production per cow among herds was 22.7 kg (SD = 5.7; range = 15-35 kg). Most herds were composed by Holstein cows (n=15), while one herd had Jersey cattle, and four herds raised Gyr or Gyr × Holstein crossbreds (Girolando). The period of evaluation of CM within herds ranged from 8 to 15 months (mean = 12.6 months; SD = 1.3).

The overall geometric mean of BMSCC among herds was 557×10^3 cells/mL (median = 443×10^3 cells/mL; ranging from 167 to $1{,}713 \times 10^3$ cells/mL). The overall geometric mean BMTBC was 94×10^3 cfu/mL (median = 19×10^3 cfu/mL; ranging from 5 to 872×10^3 cfu/mL (Table 1). One herd (named here as N) did not provide the results of BMSCC and BMTBC and was excluded from the analyses of association between IRCM and the indicators of milk quality. However, the data from herd N were still used for the descriptive analyses (frequency of pathogens and severity of CM) and for the association of IRCM and other herd-level variables evaluated in this study (housing system, average milk yield, herd size and season).

Table 1 - Descriptive results of characteristics (mean and SD in parentheses) from a convenience sample of 20 dairy herds from Southwest, Brazil, evaluated for clinical mastitis characterization from March 2014 to January 2016

Herd	Months in study	Lactating cows	Milk yield kg/d[1]	BMSCC[2]	BMTBC[3]	Housing[4]
A	13	1470 (52)	34 (2.4)	401	12	FS
B	13	184 (25)	27 (5.9)	501	9	CB
C	12	68 (5)	18 (3.4)	167	21	P
D	13	165 (11)	29 (1.8)	443	20	FS
E	13	371 (24)	18 (2.3)	890	27	P
F	14	253 (13)	21 (2.6)	1220	62	P
G	8	77 (15)	16 (0.4)	908	414	P
H	12	71 (9)	22 (0.9)	368	5	P
I	12	167 (11)	29 (2.4)	632	18	CB
J	13	120 (10)	26 (1.7)	513	15	CB
K	14	313 (7)	35 (1.9)	261	15	FS
L	12	194 (7)	23 (1.7)	571	13	P
M	13	586 (17)	27 (1.0)	277	14	FS
N	15	55 (7)	22 (1.2)	-	-	FS
O	12	46 (3)	19 (2.4)	233	8	P
P	13	36 (1)	17 (1.0)	426	91	P
Q	12	75 (12)	22 (2.9)	1713	872	CB
R	12	22 (3)	15 (2.1)	515	42	P
S	12	55 (7)	22 (2.2)	280	104	P
T	13	46 (4)	16 (0.9)	263	19	CB
Overall	12.6 (1.3)	219 (318)	22.7 (5.7)	557 (387)	94 (210)	-

[1] Average of total daily milk produced over month divided by the average of total number of lactating cows during the period of study.
[2] Bulk Milk Somatic Cell Count - Geometric mean ($\times 10^3$ cells/mL) based on the monthly average of the reports over the entire monitoring period. The overall median for BMSCC was 443×10^3 cells/mL.
[3] Bulk Milk Total Bacterial Count - Geometric mean ($\times 10^3$ cfu/mL) based on the monthly average of the reports over the entire monitoring period. The overall median for BMTBC was 19×10^3 cfu/mL.
[4] Housing system - FS = free-stall, CB = compost-bedded pack barn, and P = paddocks.

2.4.2 Clinical mastitis occurrence and pathogen distribution

A total of 5,957 quarter-cases of CM were recorded during the study period. Among all reported cases, 418 (7.0%) were excluded from the analysis because CM occurred in the same quarter within 14 days after a previous case. In addition, 1,327 (22.3%) cases were recorded but milk samples were not submitted for microbiological culture because milk samples were not collected. In total, 4,212 (70.7%) cases were submitted to the laboratory and had culture results (Table 2). During the study period, 3,180 (57.5%) cows had only one case of CM, while 1,500 had 2 (27.1%), 534 had 3 (9.7%), 168 had 4 (3.0%), and 145 had ≥5 cases of CM (2.6%).

Based on microbiological culture results, 2,212 milk samples (52.5%) had growth of single bacterial species; 1,852 milk samples (44.0%) were negative after 48 hours of incubation; 129 (3.1%) were considered as contaminated (> 2 microorganisms); and 19 (0.4%) had mixed culture (two species of microorganisms). Out of cultures with positive results and isolation of a single species, 1,467 cases (66.3%) had isolation of Gram-positive bacteria, while 598 (27.0%) were Gram-negative, and 147 (6.7%) had isolation of non-bacterial microorganism (yeast or *Prototheca* spp.; Table 2). *Escherichia coli* (n=276; 46.2%) was the pathogen with the highest frequency of isolation in this study

In relation to the distribution of severity score, out of total quarter cases submitted for microbiological culture (n=4,212), 241 severity scores were excluded because they belonged to cows with CM in more than one quarter (i.e., only the more severe score was retained for evaluation). In addition, 147 cases were excluded because of lack of severity score in the records. Thus, the frequency of pathogen-specific severity was evaluated in 3,824 cases of CM. Of this total, 2,305 (60.3%) cases were classified as mild, 1,305 (34.1%) as moderate and 214 (5.6%) as severe (Table 2).

Table 2 - Culture results and pathogen-specific distribution of severity scores of clinical mastitis cases (n=4,212) occurring in dairy cows from 20 herds of Southwest, Brazil, evaluated from March 2014 to January 2016.

Microbiological culture	**Frequency n (%)**	**Severity[1] (%)**		
		Mild	**Moderate**	**Severe**
Total samples cultured	4,212 (100)	60.3	34.1	5.6
No growth	1,852 (44.0)	62.7	32.5	4.8
Contamination[2]	129 (3.1)	62.0	30.1	8.0
Mixed[3]	19 (0.4)	35.3	64.7	-
Single pathogen[4]	2,212 (52.5)	58.3	35.6	6.2
Gram-positive				
Streptococcus uberis	256 (17.4)	55.3	39.7	5.1
Streptococcus agalactiae	248 (16.9)	62.2	33.8	4.1
CNS[5]	242 (16.5)	68.2	26.9	4.9
Streptococcus dysgalactiae	207 (14.1)	58.8	36.6	4.6
Staphylococcus aureus	141 (9.6)	69.2	28.6	2.3
Corynebacterium spp.	136 (9.3)	70.4	27.0	2.6
Streptococcus spp.	126 (8.6)	63.7	28.3	8.0
Other Gram-positive[6]	111 (7.6)	59.4	35.4	5.2
Total[8]	1,467 (66.3)	62.8	32.6	4.6
Gram-negative				
Escherichia coli	276 (46.2)	41.9	46.0	12.1
Klebsiella spp.	110 (18.4)	45.1	43.1	11.8
Citrobacter spp.	63 (10.5)	41.1	50.0	8.9
Enterobacter spp.	34 (5.7)	43.8	50.0	6.3
Other Gram-negative[7]	115 (19.2)	46.7	41.0	12.4
Total[8]	598 (27.0)	43.5	45.1	11.4
Other microorganism				
Yeast	122 (83.0)	70.2	29.0	0.9
Prototheca spp.	25 (17.0)	86.4	13.6	-
Total[8]	147 (6.7)	72.8	26.5	0.7

[1](mild) only abnormal milk; (moderate) abnormal milk accompanied by visual inflammatory symptoms in the udder; and, (severe) abnormal milk, visual injury in the udder and systemic symptoms (increased body temperature, anorexia, dehydration, depression). The pathogen-specific frequency (%) of clinical mastitis severity was evaluated at the cow level (3,824 cases). A total of 241 cases were excluded because they were from cows with more than one infected mammary quarter; and for 147 cases, the severity score was not recorded by the farm personal.

[2] Isolation of three or more different pathogens.

[3] Isolation of two different pathogens.

[4] Cultures with isolation of only one species or group (i.e., coagulase-negative staphylococci).

[5] Coagulase-negative staphylococci.

[6] *Trueperella pyogenes* (n = 38), *Bacillus* spp. (n = 33), *Enterococcus* spp. (n = 32), *Nocardia* spp. (n = 8).

[7] *Proteus* spp. (n = 14), *Pseudomonas* spp. (n = 12), *Pasteurella* spp. (n = 10), *Serratia* spp. (n = 1), other Gram negative isolates not identified at the genus or species level by the tests (n = 78).

[8] Sum of isolated pathogens and relative frequency (%), and overall distribution of severity within groups. Only cultures with only one isolated species or group (CNS) were evaluated.

2.4.3 Overall IRCM and its association with descriptors at the herd-level

The monthly mean IRCM, for all recorded cases (i.e., those submitted and not submitted for microbiological culture; n=5,539) was 9.7 cases per 10,000 QDAR, ranging from 1.9 to 21.7 (Figure 1).

After univariate analysis, three variables where included in the multivariate model (BMSCC, BMTBC and season). However, after backward stepwise selection, BMSCC was the only covariate associated with the overall IRCM in this study (P = 0.008). Herds with geometric means of BMSCC ≥601 × 10^3 cell/mL had higher IRCM (16.0 cases per 10,000 QDAR) than herds with BMSCC ≤300 × 10^3 cell/mL (7.7) and herds with BMSCC between 301-600 × 10^3 cell/mL (7.5; Figure 2). There was no statistical difference (P = 0.95) among herds with BMSCC ≤300 × 10^3 cell/mL and herd with BMSCC between 301-600 × 10^3 cell/mL in relation to the overall IRCM.

Figure 1 - Distribution (overall and by specific microbiological groups) of the monthly average incidence rate of clinical mastitis (IRCM = number of cases per 10,000 quarter-days at risk) in 20 herds of Southwest, Brazil, evaluated from March 2014 to January 2016. Overall IRCM consisted of all reported cases of clinical mastitis (submitted and not submitted to bacteriology). The specific microbiological groups where: negative cultures – no bacterial growth after 48 h of incubation at 37ºC; Gram-positive pathogens - environmental streptococci (*Strep. uberis*, *Strep. dysgalactiae* and *Strep.* group C), minor pathogens (CNS and *Corynebacterium* spp.), *Enterococcus* spp., and species of Gram-positive rods; contagious pathogens - *Staph. aureus* and *Strep. agalactiae*; Gram-negative pathogen - all Gram-negative species; and other pathogens - *Trueperella* spp., *Prototheca* spp. and yeast. The range of the vertical axis in each graphic varies with the distributions of IRCM among groups. Standard error of means was used as measure of dispersion for the IRCM among herds.

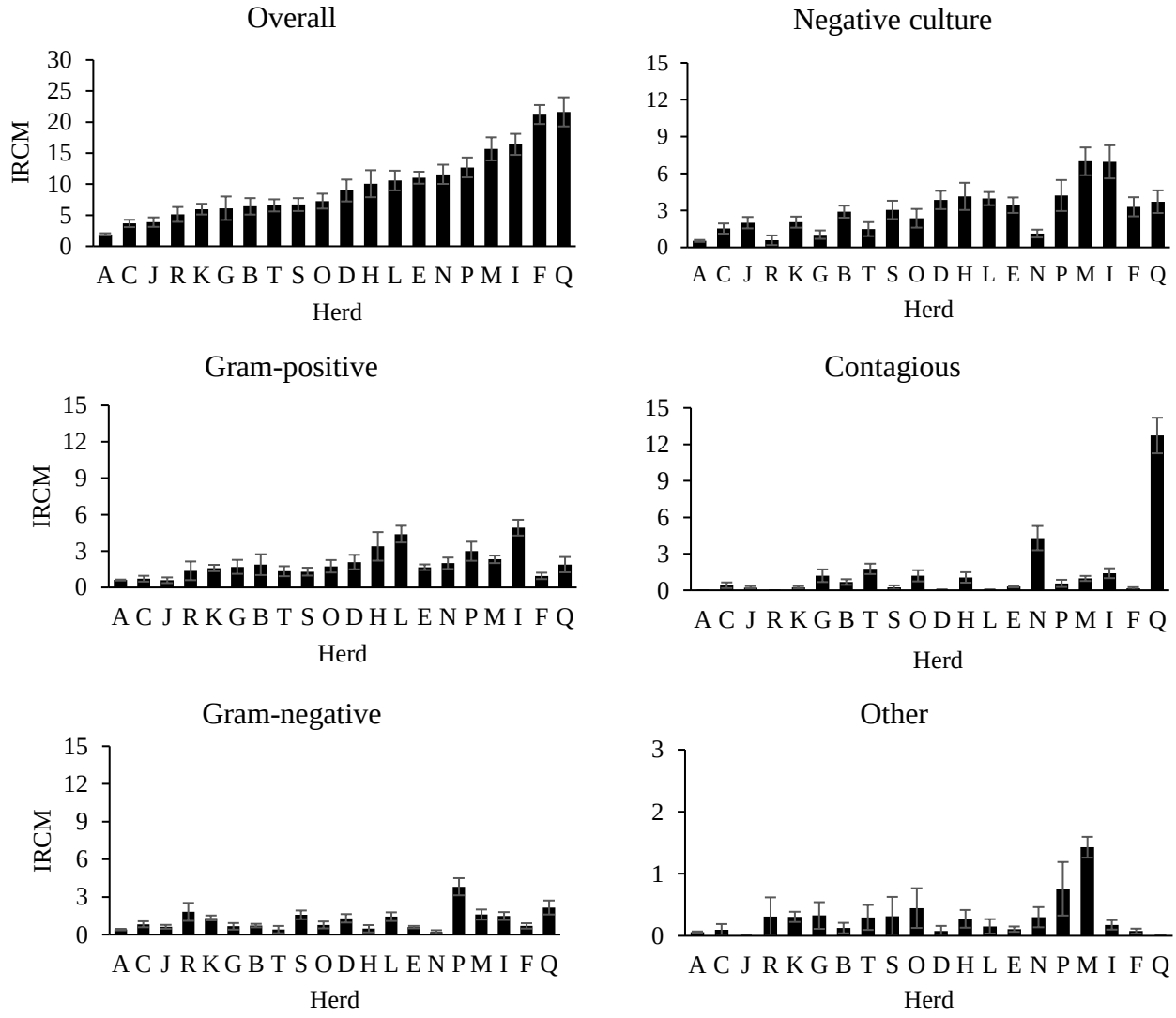
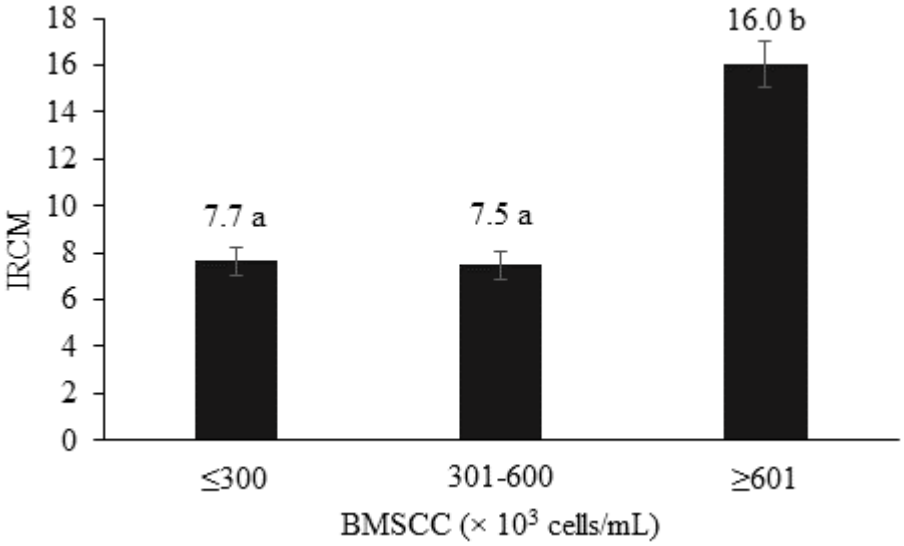


Figure 2 - Results of the multivariable regression model describing the association between overall incidence rate of clinical mastitis (IRCM; number of cases per 10,000 quarter-days at risk) and categories of bulk milk somatic cells count (BMSCC) in 20 herds of Southwest, Brazil, evaluated from March 2014 to January 2016 (means that are not sharing the same letter are statistically different; $P \leq 0.05$). Standard error of means was used as measure of dispersion for the IRCM among BMSCC categories.



2.4.4 Association of IRCM by specific-pathogen groups and other descriptors at the herd-level

A total of 4,068 cases of CM were clustered into the specific-pathogen groups according to the results of microbiological culture. Of these, 1,042 (25.6%) cases were caused by Gram-positive, 389 (9.6%) by contagious, 599 (14.7%) by Gram-negative, and 186 (4.6%) cases were caused by the group named as “other pathogens”. In addition, another 1,852 (45.5%) cases had no bacterial growth after 48 hour of microbiological culture.

The monthly mean IRCM (SD) estimated according to the specific-pathogen groups were: 3.0 (3.1) for negative cultures, 2.0 (2.2) for Gram-positive, 1.4 (3.2) for contagious, 1.2 (1.4) for Gram-negative, and 0.3 (0.7) cases per 10,000 QDAR for other pathogens (Figure 1).

The results from the mixed linear regression models evaluating the association of IRCM according to the specific pathogens groups and characteristics at the herd level are presented in

the Table 3. The IRCM caused by contagious pathogens was associated with the following variables: housing (P = 0.002), BMSCC (P = 0.02), and average daily milk yield per cow (P = 0.01). Herds with lactating cows housed in paddocks had lower IRCM (0.48 cases per 10,000 QDAR) than herds with free-stall (1.19 cases per 10,000 QDAR) or compost-bedded pack barn systems (3.2 cases per 10,000 QDAR). There was no statistical difference (P = 0.57) of IRCM caused by major contagious pathogens between herds with compost-bedded pack barn and free-stall systems.

Herds with geometric means of BMSCC ≥601 × 10^3 cell/mL had higher IRCM caused by major contagious pathogens (3.1 cases per 10,000 QDAR) than herds with BMSCC ≤300 × 10^3 cell/mL (0.8 cases per 10,000 QDAR) and herds with BMSCC between 301-600 × 10^3 cell/mL (0.3 cases per 10,000 QDAR). There was no statistical difference (P = 0.42) between herds with BMSCC ≤300 × 10^3 cell/mL and herds with BMSCC between 301-600 × 10^3 cell/mL in relation to the IRCM caused by contagious pathogens. In addition, herds with average daily milk yield per cow of 21-25 kg/d had higher IRCM (3.0 cases per 10,000 QDAR) compared to herds with average daily milk yield per cow ≤20 kg/d (≤0.7 cases per 10,000 QDAR) and ≥26 kg/d (≤0.5 cases per 10,000 QDAR). No statistical difference (P = 0.18) was observed between herds with average milk yield per cow of ≤20 and ≥26 kg/d in relation to the IRCM caused by contagious pathogens (Table 3).

In the model evaluating the group of Gram-positive pathogens, the IRCM was associated with season (P = 0.04), primarily due to the effect of the rainy 2015 season versus the other 3 seasons. In the rainy season of 2015 there were 2.8 cases per 10,000 QDAR and there was no difference of IRCM between the other three seasons: dry 2014 (1.7); rainy 2014 (1.6); and dry 2015 (1.8; Table 3).

Furthermore, in the evaluation of the IRCM caused by Gram-negative pathogens, BMTBC was the only significant effect (P = 0.031). Herds with BMTBC ≥31 × 10^3 cfu/mL had higher IRCM caused by Gram-negative pathogens (1.8 cases per 10,000 QDAR) than herds with BMTBC ≤30 × 10^3 cfu/mL (0.9 cases per 10,000 QDAR; Table 3).

Finally, no statistical association (P > 0.05) between the IRCM and the descriptors at the herd-level was observed when the models containing the groups of other pathogens and negative cultures were evaluated.

Table 3 - Results of four multivariable regression models describing the association between variables at the herd-level and the incidence rate of clinical mastitis (overall and by the specific-pathogen groups) of 20 dairy herds of Southwest, Brazil

Variable	Herds (n)	β[1]	SE	Mean[2]	SD[3]	*P*-value
Overall						
BMSCC[4]						0.008
≤300	6	-8.5	2.7	7.7a	5.5	
301-600	8	-8.3	2.5	7.5a	6.0	
≥601	5	Ref.		16.0b	8.0	
Contagious pathogens[5]						
Housing						0.002
Compost Barn	5	5.4	1.1	3.2a	5.2	
Free stall	5	6.2	1.7	1.2a	2.5	
Paddocks	10	Ref.		0.5b	1.0	
BMSCC[4]						0.02
≤300	6	-3.1	0.9	0.8a	1.2	
301-600	8	-2.4	0.9	0.3a	0.8	
≥601	5	Ref.		3.1b	5.5	
Milk Yield[6]						0.01
≤20	7	2.7	1.9	0.7b	1.2	
21-25	6	4.8	1.7	3.0a	5.1	
≥26	7	Ref.		0.5b	0.8	
Other Gram-positive[7]						
Season[8]						0.04
Dry 2014	18	-0.9	0.4	1.7a	2.0	
Rainy 2014	19	-1.0	0.4	1.6a	1.7	
Dry 2015	17	-1.1	0.5	1.8a	1.9	
Rainy 2015*	19	Ref.		2.8b	2.9	
Gram-negative[9]						
BMTBC[10]						0.03
≤30	13	-1.00	0.34	0.9a	0.98	
≥31	6	Ref.		1.8b	2.04	

[1]β = Regression coefficient.
[2]Means of incidence rate of clinical mastitis (number of cases per 10,000 quarter-days at risk). Means that are not sharing the same letter are statistically different ($P \leq 0.05$).
[3]Standard deviation related to the means of incidence rate of clinical mastitis.
[4]Bulk Milk Somatic Cell Count - Geometric mean ($\times 10^3$ cells/mL) based on the monthly average of the reports over the entire monitoring period.
[5]Group consisted by the major contagious pathogens: *Staph. aureus* and *Strep. agalactiae*.
[6]Average daily milk yield per cow (kg/d).
[7]Group formed by cases with isolation of Gram-positive bacteria, with exception of contagious pathogens and *Trueperella pyogenes*.
[8]Season categories were formed by the association of the two characteristic seasons in Southeast of Brazil [rainy (October-March); or dry (April-September); OLIVEIRA et al., 2015), and the years that each herd was evaluated in the study (2014 or 2015-2016).
[9]Group formed by cases with isolation of Gram-negative bacteria.

2.5 DISCUSSION

The overall monthly average IRCM was 9.7 cases per 10,000 QDAR. It is important to note that not all samples from CM cases were submitted for culture; however, in those that did, negative culture results represented the most common outcome (3.0 cases per 10,000 QDAR), followed by "Gram-positive pathogens" (2.0), "contagious" (1.4), "Gram-negative" (1.2), and "other pathogens" (0.3; Figure 1). Although the number of herds included in this study and their selection criteria (i.e., a convenience sample) does not allow us to make inferences at the country or even at the regional level, the characteristics of these herds are representative of the developing dairy supply chain in the region; moreover, these results can be used as reference for further epidemiological studies on CM in this country using a larger number of herds.

Only one study was found in the recent indexed literature describing the CM profile in Brazilian dairy herds (OLIVEIRA et al., 2015). However, the estimation of CM was expressed as incidence risk (or cumulative incidence) as opposed to rate as we did, and the evaluation was performed at the cow-level, not at the quarter-level as in the current study. Oliveira et al. (2015) reported an average incidence risk of CM in primiparous and multiparous cows of 27% and 31% per year, respectively. In our study, we evaluated incidence rate of CM (not risk) because diseases that can have long risk periods (e.g., mastitis) are often more accurately evaluated using the rate, which accounts for time at-risk (DOHOO et al., 2009).

Several other studies have investigated CM rate in dairy herds worldwide; however, most of these studies evaluated the at-risk period at the cow-level, even when the quarter was at risk for mastitis. Mammary quarters are considered anatomically independent from each other (Tucker, 1981), although recent studies support the hypothesis of the immunological interdependence of quarters based on the influence of infections on certain immune response parameters in contralateral uninfected quarters (BLAGITZ et al., 2015; PAIXAO et al., 2017). Therefore, the evaluation of IRCM at the quarter-level may be more accurate than evaluations at the cow-level. Additionally, the evaluation at the cow-level can result in bias because the cow can develop CM in more than one quarter, and therefore, the time at risk may be artificially decreased, which can potentially increase the reported CM rate. Studies performed in Canada reported an overall mean IRCM ranging from 22.0 cases per 100 cow-years (MEEK et al., 1986) to 37 cases per 100 cow-years (REYHER et al., 2011). In Europe, several studies also expressed the results at the cow-level with mean IRCM ranging from 20.1 cases per 100 cow-

years in France (BARNOUIN et al., 2005) to 55.0 cases per 100 cow-years in the Czech Republic (WOLFOVA; STIPKOVA; WOLF, 2006).

To the best of our knowledge, the current study is the first in which a quarter-level evaluation of the monthly IRCM was performed considering the QDAR instead of a cow-level estimate. Using the quarter-level evaluation, even if a cow had a case of CM in one mammary quarter, she was still considered at risk for developing CM in one of the other quarters. This method of calculation may prevent bias in the estimation of the IRCM as the quarters that did not have a case of CM were still summarized in the unit-time at risk in the IRCM formula.

In the current study, the average BMSCC was 557 × 10^3 cells/mL. Therefore, the most used cut-points for evaluation of BMSCC and milk quality (e.g., ≤200 vs. >200 × 10^3 cells/mL) were not followed because few farms had the average BMSCC under this threshold, and the herds were categorized as ≤300, 301-600, ≥601 × 10^3 cells/mL. The IRCM of herds with average BMSCC ≥601 × 10^3 cell/mL was more than two times higher than the IRCM of herds with a lower average BMSCC. Contrary to these findings, other studies reported no association of BMSCC and IRCM (BARKEMA et al., 1998; OLDE RIEKERINK et al., 2008), or even higher IRCM in herds with low BMSCC (≤150 × 10^3 cells/mL; ERSKINE et al., 1987). The differences of results between studies may be attributed to the fact that herds selected in the present study had higher average BMSCC than herds reported in previous studies (BARKEMA et al., 1998; OLDE RIEKERINK et al., 2008). Other factors that may account for differences of results among studies on the association of IRCM and BMSCC are the accuracy of CM diagnosis, the calculation of the outcome (e.g., risk or rate), differences of CM diagnosis between herds, and the difference in prevailing pathogens within herds and between studies (BARNOUIN et al., 2005). For example, the high frequency of isolation of contagious pathogens and environmental streptococci observed in our study may be associated to the high average BMSCC of the herds, as these groups of pathogens are associated with increased SCC in dairy cows compared to Gram-negative pathogens (RUEGG, 2012).

A similar relationship between BMSCC and IRCM was observed when evaluating the effect of major contagious pathogens on IRCM in our study. Herds with average BMSCC ≥601 × 10^3 cells/mL had 3 times higher IRCM than those with lower average BMSCC. In other studies, both *Strep. agalactiae* and *Staph. aureus* were associated with increase of SCC at the cow- and herd-level (WILSON; GONZALEZ; DAS, 1997; BARKEMA et al., 1999; DE HAAS et al., 2004). Twelve out of 20 herds evaluated in this study had *Strep. agalactiae*, including two herds (N and Q; Figure 1) in which this pathogen was isolated in 53% and 62% of the cultured milk samples, respectively. Furthermore, in herds with high frequency of isolation of

Strep. agalactiae (>5% of milk culture results), the average BMSCC (1.084×10^3 cell/mL) was 2.4 times higher than in herds with lower frequencies of isolation of this pathogen (458×10^3 cell/mL).

No associations between overall IRCM and other herd-level descriptors (i.e., herd size, BMTBC, milk yield, housing and season) were observed in the current study. Bates and Dohoo (2016) evaluated risk factors of CM in dairy cows from 30 days before and 90 days after calving and also reported no association between herd size and CM; however, it is important to note that the average herd size in that study (n = 666) was higher than in the current study. A recent study evaluating smaller herds from Netherlands (n=233 herds; average of 104 cows) did not report association of IRCM and herd size (SANTMAN-BERENDS et al., 2016). On the other hand, a study accounting for more than 70% of the United States dairy cow population reported that small herd size (30–99 cows) was associated with a greater within-herd prevalence of any given disease, including mastitis (HILL, A. E. et al., 2009).

To the best of our knowledge, no study evaluating housing type and IRCM has been reported in dairy herds of Brazil, where systems as paddocks are commonly used to house dairy cows. Although the association between overall IRCM and housing type was not observed in our study, the IRCM with isolation of contagious pathogens was higher in herds with compost-bedded pack barns and freestalls compared to herds where the cows were housed in paddocks. However, it is important to note that two herds, one with a compost-bedded pack system (herd Q; 4.3 quarter cases per QDAR) and the other with a freestall system (herd N; 12.7 quarter cases per QDAR) had the highest IRCM when contagious pathogens were evaluated (Figure 1). These results should be evaluated with caution, as a study with higher number of herds within each of the housing systems could provide different outcomes. In contrary to our results, other studies reported an association of overall IRCM and housing system. Olde-Riekerink et al. (2008) reported that cows housed in tie-stalls had higher IRCM than cows housed in freestalls. On the other hand, other studies reported that herds housing cows in loose-house system had higher IRCM than herds housed in freestalls and tie-stalls (PEELER et al., 2000; BARNOUIN et al., 2005). In those studies, the loose-house system was defined as an open yard with a shelter having common watering and feeding facilities, in which cows were kept untied.

Previous studies reported an association between CM and milk production, where higher production was positively associated with increased risk of CM (PEELER et al., 2000; BARNOUIN et al., 2005). Peeler et al. (2000), evaluating British dairy herds, reported higher IRCM in herds with an average lactation milk yield greater than 7.500 L/cow. Similar results

were observed in a study evaluating dairy herds in France, in which herds with an average milk yield >7.435 Kg (305-d) had higher IRCM than herds with lower average milk yield (Barnouin et al., 2005). In our study, although there was no association between overall IRCM and milk yield, herds with cows producing an average between 20 and 25 kg/d had the highest IRCM when contagious pathogens were considered; this association can be attributed to the fact that the two herds with high prevalence of *Strep. agalactiae* (N and Q) where included in the milk yield category of 20-25 kg/d. In addition, only 6 out of the 20 selected herds in our study had average milk yield over 25 Kg/cow/d (> 7.625 Kg when an average milk yield of 305 days was estimated); therefore, an increased number of herds could result in a different outcome. Other factors at the herd-level (e.g., housing system, period of evaluation, average number of lactations and DIM) could also influence the relationship between IRCM and milk yield.

There was no association between season and overall IRCM; however, there was an association between season and IRCM when "Gram-positive pathogens" were evaluated (higher IRCM during the rainy season of 2015). Oliveira et al. (2015) also evaluated dairy herds from Brazil and reported higher incidence risk of CM during the rainy season (October-March) than in the dry season (April-September), for both primiparous and multiparous cows. The rainy season in Southeastern Brazil comprises the months with the highest temperatures and environmental humidity, which is a combination that favors heat stress in dairy cows and may increases the risk of intramammary infections, especially those caused by environmental pathogens (Costa et al., 1998). The lack of association between 2014 rainy season and the IRCM in our study may be attributed to a drought that Brazil had undergone in 2014 (data from the INMET; Instituto Nacional de Meteorologia, Brazil; www.inmet.gov.br). The reduction of the environmental humidity in the rainy season of 2014, especially in paddock systems, may have reduced the microbial load in the environment, and consequently, reduced the risk of CM during this year. In addition, the evaluation of the herds over several years could result in different outcomes related to the association of season and IRCM in our study.

There was no association between overall IRCM and BMTBC in the current study, however herds with BMTBC $\geq 31 \times 10^3$ cfu/mL had higher Gram-negative IRCM than herds with lower BMTBC. The BMTBC is closely related to environmental factors as excess of humidity and organic matter (e.g., mud and feces) in the housing facilities, and improper cleanliness of milking parlor, which can result in poor cow and teats hygiene (HOGAN, JOE; SMITH, 2012). Both of these factors can increase the environmental load of Gram-negative microorganisms, especially coliforms, and thus, increase the risk of CM in dairy herds.

The frequency of isolation of pathogens observed in this study was similar to other studies, in which a higher frequency of Gram-positive pathogens was reported (VERBEKE et al., 2014; OLIVEIRA et al., 2015; CORTINHAS et al., 2016). Gram-positive organisms were the most commonly cultured pathogen group (66.3%), and this outcome is mostly related to the isolation of environmental streptococci and contagious pathogens. Environmental streptococci were the most frequent cause of CM in our study and similar results were observed in other studies (BRADLEY et al., 2007; VERBEKE et al., 2014). Among the environmental streptococci, *Strep. uberis* was the most isolated pathogen. Several environmental and anatomical sites of dairy cows have been reported as sources of *Strep. uberis*, including bedding, feedstuff, rumen, feces, vulva, nares and skin (BRAMLEY, 1982; KRUZE; BRAMLEY, 1982). In addition, recent studies have reported that cow-to-cow transmission of *Strep. uberis* can potentially occurre in dairy herds (DAVIES et al., 2016), which can increase the incidence of intramammary infection by this pathogen.

Contagious pathogens, such as *Strep. agalactiae* and *Staph. aureus* were also isolated frequently in this study. *Streptococcus agalactiae* was isolated in 12 out of the 20 herds selected in this study, while *Staph. aureus* was isolated in 14 herds. Oliveira et al. (2015), in another study evaluating CM occurring in dairy herds of Brazil, also reported a high frequency of isolation of *Strep. agalactiae* (aproximately 7.0% of the positive cultures). However, while *Staph. aureus* remains a significant cause of mastitis in some countries (OLDE RIEKERINK et al., 2008; KEANE et al., 2013), the prevalence of CM caused by *Strep. agalactiae* have been reduced in dairy farms by modern mastitis control programs. Ruegg (2012) described results of 11 studies on the distribution of pathogens causing CM and observed that isolation of *Strep. agalactiae* was reported in only 2 studies. In our study, another contagious pathogen that could be a cause of CM is *Mycoplasma* spp.; however, no specific method was used for identification of this pathogen, which may have contributed to the observed increase in the frequency of negative cultures. The adoption of specific management strategies such as use of post-milking teat desinfection, treatment of clinical cases, use of dry cow therapy, culling of chronically infected cows and periodic maintenance of milking equipment can result in reduction of intramammary infections caused by contagious pathogens (RUEGG, 2012).

A total of 44% of samples submitted to the microbiological culture in this study presented negative culture (no growth), which is similar to other studies evaluating milk samples from CM cases (OLDE RIEKERINK et al., 2008; PINZON-SANCHEZ; RUEGG, 2011; CORTINHAS et al., 2016). There are several factors that can influence a negative culture result: infections caused by bacteria that requires specific identification procedures (e.g.,

Mycoplasma spp.); unfavorable storage conditions of milk samples on the farm and during shipment to the laboratory (DINSMORE et al., 1992); and spontaneous clearance of the pathogen by the cows' immune system (SMITH; TODHUNTER; SCHOENBERGER, 1985). *Escherichia coli* was the most isolated species from CM in our study and it has been associated to culture-negative results in other studies (SMITH; HOGAN, 1993). Therefore, we can speculate that the high frequency of negative culture results in our study could be partially attributed to infections caused by *E. coli*, in which a spontaneous cure has occurred or due to freezing of milk samples before microbiological culture.

When the CM severity was evaluated, more than 94% of all cases submitted to microbiological culture were reported as mild to moderate. Similar distribution of the severity score was observed in a study evaluating CM in Flemish dairy herds, where 63.1% of the cases were reported as mild, 29.9% as moderate and 7% as severe (VERBEKE et al., 2014). In the late study, a higher frequency of Gram-positive pathogens, especially environmental streptococci, was reported in comparison to the current study. In contrary, higher frequencies of moderate (36.9%) and severe (15.3%) cases of CM were reported in another study with a high frequency of isolation of Gram-negative pathogens (OLIVEIRA; HULLAND; RUEGG, 2013). The higher frequency of mild cases of CM in our study may be associated to the high occurrence of cases with isolation of Gram-positive pathogens. Severe clinical mastitis cases were associated with Gram-negative pathogens, especially in herds with high prevalence of *E. coli* (OLIVEIRA; HULLAND; RUEGG, 2013; VERBEKE et al., 2014). In our study, when the CM severity was evaluated by group of pathogens, approximately 60% of the cases with isolation of Gram-negative pathogen presented moderate to severe scores. On the other hand, only 37.2% of the cases with isolation of Gram-positive pathogens and 27.2% of the group of other non-bacterial microorganisms presented CM with moderate to severe scores.

2.6 CONCLUSIONS

Overall, the IRCM was 9.7 quarter-cases per 10,000 QDAR, and the only herd-level parameter associated with overall IRCM was BMSCC. In the models evaluating the specific-pathogen groups, IRCM with isolation of major contagious pathogens was associated with BMSCC, milk yield and housing system. For the evaluation of other Gram-positive pathogens, the IRCM was higher in the rainy season of 2015 in comparison with the other seasonal

categories. In addition, for the model evaluating the Gram-negative group, the IRCM was highest in herds with BMTBC >30 × 10^3 cfu/mL. There was no association between herd-level descriptors and IRCM with isolation of "other pathogens" or with negative culture. Environmental bacteria, especially coliforms and environmental streptococci, were the most frequently isolated pathogens in our study. However, it seems that major contagious pathogens are still an important cause of CM in dairy herds Southeastern Brazil. More than 94% of the CM cases were mild or moderate; however, Gram-negative pathogens are more likely to cause moderate and severe CM cases than Gram-positive pathogens.

CHAPTER 3

Frequency of antimicrobial use for treatment of clinical mastitis and its association with herd-level descriptors on dairy herds

3 FREQUENCY OF ANTIMICROBIAL USE FOR TREATMENT OF CLINICAL MASTITIS AND ITS ASSOCIATION WITH HERD-LEVEL DESCRIPTORS ON DAIRY HERDS

3.1 ABSTRACT

The aims of this study were to: (a) characterize the treatment profile and quantify the antimicrobial consumption for treatment of clinical mastitis (CM) in dairy herds of Brazil; and, (b) to determine the association of antimicrobial use for treatment of CM and herd-level descriptors, such as herd size, average milk yield, bulk milk somatic cell count (BMSCC), bulk milk total bacterial count (BMTBC), season and housing type. Data on CM incidence and treatment practices were obtained from 19 dairy herds for a period of 12 months per herd. The CM treatment protocols were recorded for each case by the farm personnel using a form, which included information at the cow- and treatment-level. The antimicrobial use for treatment of CM was quantified monthly in units of defined daily dose (DDD) and expressed as antimicrobial treatment incidence (ATI; number of DDD per 1,000 lactating cows-day). Nonparametric tests were used to determine the associations between ATI and herd level descriptors. The overall monthly mean ATI was 17.7 DDD per 1,000 lactating cow-days (15.4 DDD per 1,000 lactating cow-days for intramammary compounds, and 2.2 DDD per 1,000 lactating cow-days for systematically administered antimicrobials). Among intramammary drugs, aminoglycosides had the highest ATI (11.7 DDD per 1,000 lactating cow-days), followed by a treatment with a combination of tetracycline, aminoglycoside and polypeptide (10.3 DDD per 1,000 lactating cow-days). For systematically administrated antimicrobials, fluoroquinolones (4.2 DDD per 1,000 lactating cow-days), fourth-generation cephalosporin (3.1 DDD per 1,000 lactating cow-days) and the combination of sulfonamide and pyrimidine (2.1 DDD per 1,000 cows per day) were the most frequently used antimicrobials. The use of combination therapy (i.e., association of intramammary and systematically administered antimicrobials) was reported for 64.3% of the treatments at the cow-level. Bulk milk somatic cell count and herd size were positively associated with ATI. In addition, herd-level ATI was higher in freestall herds than in compost bedded-pack barns.

Key-words: Antimicrobial consumption. Brazil. Clinical mastitis. Dairy cattle

3.2 INTRODUCTION

The non-judicious use of antimicrobials for treatment of infectious diseases in human and veterinary medicine has been reported as one of the main factors associated with the emergence of antimicrobial resistance (LEVY; MARSHALL, 2004). The increased antimicrobial resistance of microorganisms has become a public health issue, especially for diseases with the same etiology in both humans and animals (SARMAH; MEYER; BOXALL, 2006). Thus, there has been a global interest of government institutions in the prudent use of antimicrobials in food production animals, which include formulations administered for treatment of dairy cows (BENNEDSGAARD; KLAAS; VAARST, 2010).

Antibiotic therapy remains one of the main strategy for treatment of infectious diseases in dairy herds. Most of antimicrobials consumed in dairy herds were used for treatment of intramammary infections, especially for dry cow therapy and for treatment of clinical mastitis (MITCHELL et al., 1998; SAINI et al., 2012b). Antimicrobials are used for treatment of clinical mastitis (CM) aiming to eliminate the causing pathogen, for prevention of disease worsening (e.g., bacteremia), and for rapid return of marketable milk of affected cow (ROBERSON, 2012). However, in addition to its relationship with the emergence of bacterial resistance, the increased use of antimicrobials was positively correlated with the risk of antimicrobial residues in milk (ERSKINE; WAGNER; DEGRAVES, 2003).

Studies evaluating the frequency and quantity of antimicrobial use in dairy herds were performed in United States (POL; RUEGG, 2007a; HILL, A. E. et al., 2009), Canada (SAINI et al., 2012b), Europe (GONZALEZ et al., 2010; STEVENS et al., 2016), and in other Latin American countries (REDDING et al., 2014b). However, the characterization of antimicrobial use has not been described in dairy herds in Brazil, where specific antimicrobial formulations are legally commercialized for mastitis therapy, and the therapeutic strategies may be different from other countries. In addition, few studies have evaluated the association between the frequency of antimicrobial use for treatment of CM and herd-level descriptors such as bulk milk somatic cell count (BMSCC), bulk milk total bacterial count (BMTBC), housing type, herd size, and milk production (HILL, A. E. et al., 2009; SAINI et al., 2012b; STEVENS et al., 2016). Studies characterizing the antimicrobial use in dairy herds can benefit the dairy industry as they can be used as a basis for implementation of programs for the prudent use of antimicrobials, and consequently, for prevention of bacterial antimicrobial resistance.

The objectives of the present study were to: (a) characterize the treatment profile and quantify (overall and class-specific) the antimicrobial consumption for treatment of CM in dairy herds of Southeast, Brazil; and, (b) determine the association of antimicrobial use for treatment of CM and herd-level descriptors, such as herd size, average milk yield, BMSCC, BMTBC, season and housing type.

3.3 MATERIAL AND METHODS

3.3.1 Selection of dairy herds

A convenience sample of 20 dairy herds from Southeast, Brazil, based on a client list of Qualileite Lab (Milk Quality Research Laboratory at University of São Paulo, Brazil), and on the farmers' willingness to participate, were enrolled in this study. Herd selection criteria was described in detail in the chapter 2, in which the same dairy herds were evaluated on the determination of the incidence rate of clinical mastitis (IRCM) and its association with descriptors at the herd-level. As a post admission criterion, only herds that completed at least 12 months of data collection were included in the data analysis. One herd dropped out after 8 months of evaluation, and therefore, 19 herds were included in the final data analysis. Data collection was conducted from April 2014 to January 2016.

3.3.2 Antimicrobial usage

Data collection. Before the beginning of the data collection, a training on detection of CM was reviewed with farm personnel from all herds to ensure data quality. In addition, a form was presented to the farm personnel during the first visit, in which information at the cow- and treatment-level was recorded for all mastitis treatments. Treatment data that should be included in the form after diagnosis of each CM case were: identification of cow diagnosed with CM (number or name), affected mammary quarter(s), date of CM diagnosis, use of antimicrobial (yes or no), use of combination therapy (e.g., intramammary antimicrobials associated with

systematically administered products), commercial name(s) of antimicrobial(s), duration of treatment (days), number of administrations per day, and need for alteration of therapeutic protocol. Visits by university researchers were performed every 15-30 days for data collection, and the following herd-level information was recorded: (1) housing type used for lactating cows (at the first visit); (2) milk yield as a monthly average of daily milk production per cow; (3) monthly results of BMSCC and BMTBC; if a herd had more than one result of BMSCC or BMTBC in a given month, the arithmetic average of the results was used; and, (4) the number of dairy cows calculated as an average of milking cows within a given month.

Calculation of defined daily doses. The antimicrobial used for CM treatment in each herd was quantified in units of defined daily dose (DDD; GONZALEZ et al., 2010). For intramammary products the DDD (mg/cow-day) was defined as the total amount of active substance per injector multiplied by the number of administrations (Table 4). For estimation of DDD of systematically administered antimicrobials, the dose (mg) contained in a milliliter of a compound was multiplied by the on-label amount recommended in each administration considering a cow of 600 Kg of body weight (JENSEN; JACOBSEN; BAGER, 2004), and multiplied by the number of administrations per day (Table 5). When a range of doses was possible, the highest dose was used. For antimicrobials in which the dosage was expressed as international units (IU), the value was converted to milligrams as described by Gonzalez et al (2010), where 1 mg of compound corresponded to: 60 IU of bacitracin, 19,000 IU of colistin sulphate, 6,500 IU of polymyxin sulphate, 4,100 IU of spiramycin, 1,667 IU of penethamate hydroiodide, penicillin G sodium and penicillin G potassium, 1,000 IU of benzylpenicillin procaine, and 1,200 IU of benzylpenicillin benzathine. For combined compounds (e.g., more than one active substance in the same product), the total amount of each active ingredients (mg) contained in a specific product was used for estimation of DDD (SAINI et al., 2012).

Table 4 - Defined daily dose (DDD), daily application frequency, and amount of active substance present in the containers of intramammary antimicrobial compounds recorded for treatment of clinical mastitis in 19 dairy herds from Southwest, Brazil

Active substances	Antimicrobial class	A[1] (mg)	F[2] (n)	DDD[3] (mg)
Amoxicillin/Clavulanic acid	Penicillins	275	2	550
Ampicillin/Cloxacillin	Penicillins	275	2	550
Cephalexin/Neomycin	First-gen. cephalosporins/Aminoglycosides	200	2	400
Cephoperazone	Third-generation cephalosporins	250	1	250
Cefquinome sulfate	Fourth-generation cephalosporins	88	2	176
Ceftiofur hydrochloride	Third-generation cephalosporins	125	1	125
Ciprofloxacin	Fluoroquinolones	100	1	100
Gentamicin	Aminoglycosides	150	1	150
Oxytetracycline/Neomycin	Tetracyclines/Aminoglycosides	400	1	400
Spiramycin/Neomycin	Macrolides/Aminoglycosides	440.4	2	880.8
Sulfadiazine/Trimethoprim	Sulfonamides/Pyrimidines	240	2	480
Tetrac. + Neom. + Bacitr.[4]	Tetracycline/Aminoglycosides/Polypeptides	478	1	478

[1]Amount of antimicrobial contained in the antimicrobial compounds, obtained from the online version of the Brazilian Compendium of Veterinary Products (http://www.cpvs.com.br/cpvs). For combined compounds (e.g., more than one active substance in the same product), the total amount of active ingredients (mg) in a specific product was used for estimation of antimicrobial amount within containers.

[2]Frequency of application for the recorded antimicrobial compounds.

[3]Defined daily doses.

[4]Intramammary antimicrobial formulated with the combination of tetracycline, neomycin and bacitracin.

Table 5 - Defined daily dose (DDD), daily application frequency, and amount of active substance contained in the systematically administered antimicrobial compounds recorded for treatment of clinical mastitis in 19 dairy herds from Southwest, Brazil

Active substances	Antimicrobial class	Dose[1] (mg/Kg)	F[2] (n)	DDD[3] (mg)
Amoxicillin trihydrate	Penicillins	15	1	9000
Amoxicillin trihydrate/Potassium clavulanate	Penicillins	8.75	1	5250
BP procaine/BP benzathine/ BP sodium/DS sulfate[4]	Penicillins/Aminoglycosides	24.66	1	14796
BP procaine/BP benzathine/ DS sulfate[4]	Penicillins/Aminoglycosides	17.3	1	10380
BP procaine/BP potassium/Streptomycin sulfate[4]	Penicillins/Aminoglycosides	37.4	1	22440
BP procaine/DS sulfate[4]	Penicillins/Aminoglycosides	14	1	8400
Cefquinome sulfate	Fourth-generation cephalosporins	1	1	600
Ceftiofur hydrochloride	Third-generation cephalosporins	1.5	1	900
Enrofloxacin[5]	Fluoroquinolones	7.5	1	4500
Enrofloxacin[5]	Fluoroquinolones	2.5	1	1500
Florfenicol[6]	Amphenicols	20	1	12000
Florfenicol[6]	Amphenicols	40	1	24000
Gentamicin	Aminoglycosides	4	1	2400
Gentamicin/Amoxicillin	Aminoglycosides/Penicillins	11.4	1	6840
Norfloxacin	Fluoroquinolones	5	1	3000
Oxytetracycline	Tetracyclines	20	1	12000
Sulfadiazine/Trimethoprim	Sulfonamides/Pyrimidines	15	1	9000
Tylosin	Macrolides	10	1	6000

[1]Concentration of active substance contained in the antimicrobial compounds, obtained from the online version of the Brazilian Compendium of Veterinary Products (http://www.cpvs.com.br/cpvs). For combined compounds (e.g., more than one active substance in the same product), the total amount of active ingredients (mg) present in a specific product was used for estimation of dose.

[2]Frequency of application for the recorded antimicrobial compounds.

[3]Defined daily doses based in a cow with 600 Kg (Jensen et al., 2004).

[4]BP – Benzylpenicillin; DS – Dihydrostreptomycin.

[5]Antimicrobials compounds from the same class (Fluoroquinolones) and active substances (enrofloxacin), but with different recommended on label dosage (mg/Kg).

[6]Antimicrobials compounds from the same class (Amphenicols) and active substances (florfenicol), but with different recommended on label dosage (mg/Kg).

Antimicrobial treatment incidence. The consumption of antimicrobial for treatment of CM at the herd level was expressed as monthly antimicrobial treatment incidence (**ATI**; STEVENS et al., 2016). The ATI was calculated for each farm as the total amount of active substance (mg) used in each month, divided by the result of multiplication of DDD per month, number of lactating cows in the given month and number of days in the given month (i.e., 28, 30 or 31), which was multiplied by 1,000 cows per day:

$$\text{ATI} = \left(\frac{\text{Total amount of active substance (mg) used in a given month}}{[\text{DDD} \times \text{number of lactating cows within month} \times \text{days within month}]} \right) \times 1{,}000$$

The total amount of active substance for each class of antimicrobials was calculated by the sum of active substance (mg) used for all recorded treatments of CM; for each treatment, the total amount of active substance was calculated multiplying the DDD by the number of days in which the antimicrobial was administered. The monthly ATI was expressed as the number of DDD that was used for CM treatment per 1,000 lactating cows-day.

Antimicrobial stratification. Antimicrobials were classified according to the antimicrobial drug class and route of administration (i.e., intramammary and systematic; Tables 4 and 5).

3.3.3 Statistical analyses

Descriptive statistics of antimicrobial use (e.g., frequency of administration and ATI according to antimicrobial class) were performed using the FREQ procedure of SAS 9.4 (SAS Inst. Inc., Cary NC). The MEANS procedure of SAS 9.4 (SAS Inst. Inc., Cary NC) was used to describe characteristics at the herd-level (number of milking cows, average milk yield, BMSCC and BMTBC). Summary statistics were produced using the mean as a measure of central tendency, and standard deviation (**SD**) as a measure of statistical dispersion.

The ATI data were highly skewed; therefore, nonparametric tests were used to determine statistical differences in the frequencies of ATI for all herd-level descriptors. Correlations between overall ATI and continuous independent variables (i.e., herd size, average daily milk yield per cow, BMSCC and BMTBC) was estimated using the Spearman rank correlation coefficient (PROC CORR; SAS 9.4, SAS Inst. Inc., Cary NC). Both BMSCC and

BMTBC were recorded monthly at the herd-level and log-transformed (base 10) for normalization of the data distribution.

For the season descriptor, two categories were created based on the two characteristic seasons in Brazil (OLIVEIRA et al., 2015): dry (April-September) and rainy (October-March). Pairwise comparisons of overall ATI between season (i.e., dry and rainy) were done using the Wilcoxon rank-sum test (PROC NPAR1WAY; SAS 9.4, SAS Inst. Inc., Cary NC). Bonferroni adjustments were done using the MULTTEST procedure of SAS 9.4 (SAS Inst. Inc., Cary NC) for comparison of overall ATI between housing systems (compost-bedded pack barn, free stall, paddocks). The paddock housing system was characterized as an open area surrounded by fences or rails and without pasture for grazing. For all statistical analysis, a *P*-value <0.05 was considered statistically significant.

3.4 RESULTS

3.4.1 Herd characteristics

A total of 20 dairy herds were enrolled in this study, but only 19 met the post-admission criterion (i.e., complete 12 month of study accomplishment); one herd dropped out of the study after 8 months of evaluation. Therefore, an average of 4,306 lactating cows was evaluated monthly from April 2014 to January 2016. It is important to mention that there is no relation between the herd names in this study and the herd names presented in the in the study reported in chapter 2.

For those herds included in the final data analysis (i.e., herds A-S), the mean size of herds was 227 (SD = 322) lactating cows, ranging from 23 to 1,456. Lactating cows were housed in three different housing types: 9 paddocks (average of total milking cows per month= 1,123; mean = 125; SD = 116), 5 free-stalls (average of total milking cows per month = 2,577; mean = 515; SD = 507), and 5 compost bedded-pack barns (average of total milking cows per month = 607; mean = 121; SD = 56). The overall mean of daily milk production per cow among herds was 23.2 Kg (SD = 6.1; range = 15.3-35.3 kg).

One herd (named here as L) did not provide the results of BMSCC and BMTBC and was excluded from the analyses of association between ATI and those descriptors of milk

quality. However, the data from herd L was still included in the descriptive analyses of antimicrobial usage and in the association of ATI and other herd-level descriptors evaluated in this study (housing type, average milk yield, herd size and season). Therefore, the overall monthly geometric mean of BMSCC among herds was 537 × 10^3 cells/mL, ranging from 167 to 1,713 × 10^3 cells/mL. The overall monthly geometric mean BMTBC was 69 × 10^3 cfu/mL, ranging from 5 to 769 × 10^3 cfu/mL.

In relation to the CM occurrence, the overall monthly mean incidence rate of clinical mastitis during the data collection period was 9.8 quarter-cases per 10,000 quarter-days at risk (SD = 7.4), ranging from 1.9 to 21.7.

3.4.2 Descriptive results on the antimicrobial use

A total of 5,457 quarter-cases (5,020 cow-cases) of CM were recorded during the study period and 5,295 (97%) of those received antimicrobial treatment. The use of combination therapy (i.e., association of intramammary and systematically administered antimicrobials) was reported for 64.4% (n=3,231) of the treatments at the cow-level. A total of 612 (11.6%) treatment cases had alteration of the primary protocol during the therapy.

Twelve commercial intramammary compounds formulated with 10 antimicrobial classes (or combination of classes) had records of use over the entire study period (Table 4). Overall, third-generation cephalosporin was the most frequently used intramammary class (9,549 DDD), followed by antimicrobials with combination of tetracycline, aminoglycoside and polypeptide (8,501 DDD), and fourth-generation cephalosporin (5,024 DDD; Table 6). In relation to the frequency of administration, the antimicrobial with the combination of macrolide and aminoglycoside had the highest mean of administration per quarter-treatment (mean = 7.4; SD = 3.9), followed by penicillin combinations (mean = 6.9; SD = 2.6) and third-generation cephalosporin (mean = 6.9; SD = 5.9; Table 6).

For systematically administered treatments, 18 commercial products formulated with 9 antimicrobial classes (or combination of classes) had records of use over the study period (Table 5); antimicrobials belonging to the class of fluoroquinolones had the highest frequency of use (5,696 DDD), followed by fourth-generation cephalosporins (1,187 DDD) and penicillin-based products (968 DDD; Table 6). Compounds formulated with third-generation cephalosporins had the highest frequency of administration per treatment (mean = 4.2; SD = 2.6), followed by

fourth-generation cephalosporin (mean = 2.8; SD = 1.2) and fluoroquinolone-based compounds (mean = 2.7; SD = 1.7; Table 6).

Table 6 - Total number of defined daily doses (DDD) and frequency of administration of antimicrobial classes recorded for treatment of clinical mastitis in 19 dairy herds from Southwest, Brazil

Antimicrobial class	Total DDD[1] (n)	Administrations[2] (n)				
		Mean	SD[3]	Percentile		
				25th	50th	75th
Intramammary						
Third-generation cephalosporin	9,549	6.9	5.9	3	4	8
Tetracycline/Aminoglycoside/Polypeptide	8,501	3.8	1.5	3	4	4
Fourth-generation cephalosporin	5,024	5.9	2.6	4	5	8
Penicillin	3,438	6.9	2.6	6	6	8
First-gen. cephalosporin/Aminoglycoside	1,359	6.7	2.2	5	8	8
Macrolide/Aminoglycoside	1,110	7.4	3.9	3	8	10
Fluoroquinolone	913	5.1	1.7	4	5	6
Sulfonamide/Pyrimidine	433	6.1	2.0	4	6	8
Aminoglycoside	388	4.7	1.3	4	4	6
Tetracycline/Aminoglycoside	48	4.0	1.7	3	4	6
Systemic						
Fluoroquinolone	5,696	2.7	1.7	1	3	4
Fourth-generation cephalosporin	1,187	2.8	1.2	2	2	4
Penicillin combinations	968	2.1	1.2	1	2	3
Sulfonamide/Pyrimidine	616	1.9	0.9	1	2	2
Amphenicol	141	1.4	0.7	1	1	2
Third-generation cephalosporin	50	4.2	2.6	1	5	6
Tetracycline	42	2.1	0.9	1	2	3
Macrolide	29	2.2	1.0	1	3	3
Aminoglycoside	25	1.8	1.1	1	1	3

[1]Total number of defined daily doses (DDD); For systematically administered compounds, the DDD was based in a cow with 600 Kg (Jensen et al., 2004).

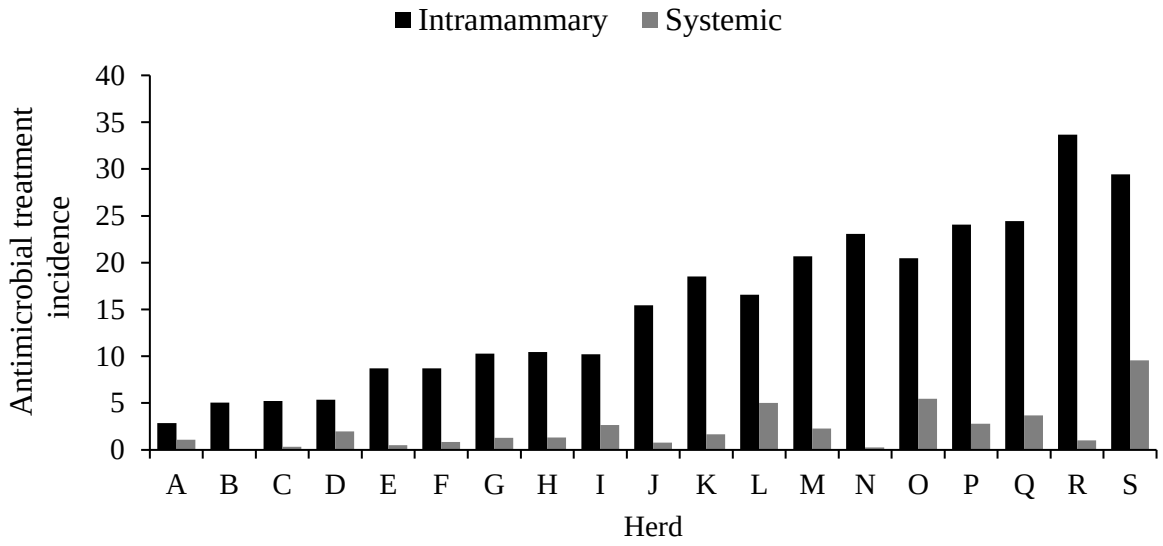
[2]Frequency (mean, minimum and maximum) of administrations of recorded antimicrobial classes used for treatment of clinical mastitis.

[3]Standard deviation.

3.4.3 Antimicrobial Treatment Incidence

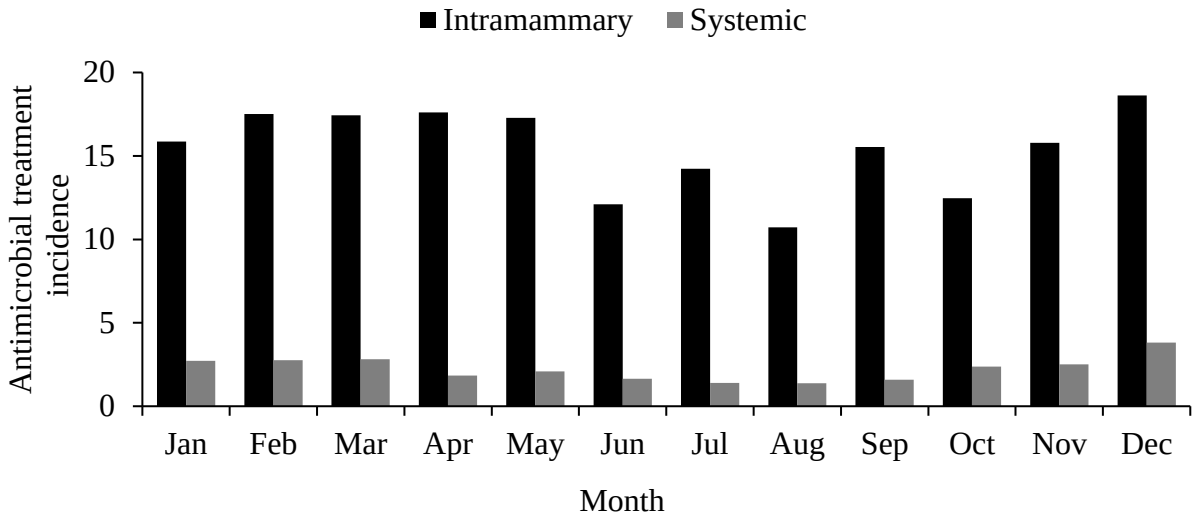
Overall consumption. The overall monthly mean ATI, regardless of antimicrobial class or route of administration, was 17.7 (SD = 3.0) DDD per 1,000 lactating cow-days. For the evaluation at the herd-level, the mean ATI ranged from 3.9 DDD per 1,000 lactating cow-days in the herd A to 39.0 DDD per 1,000 lactating cow-days in the herd S (Figure 3).

Figure 3 - Overall herd-level antimicrobial treatment incidence (DDD/1000 lactating cows-day) of intramammary and systematically administered compounds recorded for treatment of clinical mastitis in 19 dairy herds from Southwest, Brazil, from April 2014 to January 2016



Considering the evaluation performed at the monthly basis (regardless of herd), for intramammary compounds the overall monthly mean ATI was 15.4 (SD = 12.3) DDD per 1,000 lactating cow-days, ranging from 10.7 in August to 18.6 in December (Figure 4). For systematically administered compounds, the monthly mean ATI was 2.2 (SD = 3.2) DDD per 1,000 lactating cows-day, ranging from 1.4 in July to August to 3.8 in December (Figure 4).

Figure 4 - Monthly antimicrobial treatment incidence (DDD/1000 lactating cows-day) of intramammary and systematically administered compounds recorded for treatment of clinical mastitis in 19 dairy herds from Southwest, Brazil, from April 2014 to January 2016



Antimicrobial class. A total of 37,972.8 g of antimicrobials (based on the total amount of active substances) was used to treat CM in the 19 herds over the study period. Of this total, 8,944.8 g was administered by the intramammary route, while 29,028.0 g were systematically administered drugs (Table 7).

In relation to the distribution of use, for intramammary compounds, the drug with the combination of tetracycline, aminoglycoside and polypeptide had the highest distribution among the evaluated herds (n = 13; 68.4%), followed by antimicrobials of the classes of fourth- (n=12; 63.2%) and third-generation cephalosporin (n=11, 57.9%). For systematically administered products, compounds with combination of sulfonamide and pyrimidines (n=14; 73.7%) were the most distributed drugs among herds, followed by fluoroquinolone and penicillin-based products, both used in 13 herds (68.4%; Table 7).

Among intramammary drugs, aminoglycosides had the highest ATI (11.7 DDD per 1,000 lactating cow-days, SD = 7.9), followed by the combination of tetracyclines, aminoglycosides and polypeptides (10.3 DDD per 1,000 lactating cow-days, SD = 7.2), and by third-generation cephalosporins (9.9 DDD per 1,000 cows per day, SD = 9.1, Table 7). For systematically administered drugs, fluoroquinolone-based products had the highest ATI (4.2 DDD per 1,000 lactating cow-days, SD = 7.5), followed by the fourth-generation cephalosporin (3.1 DDD per 1,000 lactating cow-days, SD = 3.4), and the compound with the combination of sulfonamide and pyrimidine (2.1 DDD per 1,000 cows per day, SD = 2.9, Table 7).

Table 7 - Antimicrobial treatment incidence, total amount (mg) of antimicrobial used, and frequency of herds with reported use of antimicrobial classes (or combination of classes) administered by intramammary and systemic routes in 19 dairy herds from Southwest, Brazil

Antimicrobial class	Number of herds[1]	Total ATM (g)[2]	ATI[3]				
			Mean	SD	Percentile		
					25th	50th	75th
Intramammary							
Aminoglycoside	5	58.2	11.7	7.9	3.6	12.0	17.1
Tetracycline/Aminoglyc./Polypep.[4]	13	4,063.5	10.3	7.2	4.5	8.8	14.8
Third-generation cephalosporin	11	2,647.5	9.9	9.1	4.1	7.8	13.2
Macrolide/Aminoglycoside	5	488.8	7.9	11.3	1.2	2.2	8.8
Fourth-generation cephalosporin	12	258.9	5.5	4.8	1.7	3.6	8.2
Fluoroquinolone	6	90.9	5.0	5.3	0.7	2.3	10.6
Penicillin	9	942.1	4.2	3.6	1.8	3.4	5.3
First-gen. cephalosp./Aminoglyc.[5]	9	271.8	4.1	5.8	1.3	2.4	4.2
Sulfonamide/Pyrimidine	3	103.9	2.8	1.8	1.6	2.2	4.3
Tetracycline/Aminoglycoside	2	19.2	2.2	1.7	1.2	1.4	3.3
Overall[6]	19	8,944.8	15.4	12.3	6.1	12.3	22.1
Systemic							
Fluoroquinolone	13	10,362.0	4.2	7.5	0.6	1.3	4.3
Fourth-generation cephalosporin	9	712.2	3.1	3.4	0.6	1.7	4.5
Sulfonamide/Pyrimidine	14	5,544.0	2.1	2.9	0.6	1.2	2.6
Penicillin	13	8,766.0	1.7	2.3	0.4	0.8	2.1
Macrolide	2	174.0	1.3	1.3	0.4	0.7	2.8
Amphenicol	7	2,772.0	1.0	1.0	0.2	0.6	1.5
Tetracycline	5	504.0	0.7	0.7	0.2	0.5	0.9
Aminoglycoside	4	148.8	0.6	0.4	0.2	0.4	0.9
Third-generation cephalosporin	2	45.0	0.6	0.3	0.3	0.7	0.8
Overall[6]	19	29,028.0	2.2	3.2	0.4	1.1	2.8

[1]Number of herds using the specific antimicrobial drug class

[2]Total amount (mg) of active ingredients for each of the antimicrobial classes (or combination of classes) used during the study period.

[3]Antimicrobial treatment incidence reported as the number of defined daily doses per 1000 lactating cows-day.

[4]Intramammary drug formulated with the combination of tetracycline, neomycin and bacitracin.

[5]Intramammary drug formulated with the combination of a first-generation cephalosporin and an aminoglycoside.

Association of ATI and herd-level descriptors. Spearman correlation coefficients between ATI and herd-level descriptors are presented in Table 8. The variables herd size and BMSCC were positively correlated with herd-level overall ATI (P<0.0001; Table 8). On the other hand, the variables milk yield and BMTBC were not correlated with overall ATI. In addition, the herd-level ATI was not significantly different (P=0.29) between dry- (18.2 DDD per 1,000 lactating cow-days) and rainy season (21.2 DDD per 1,000 lactating cow-days).

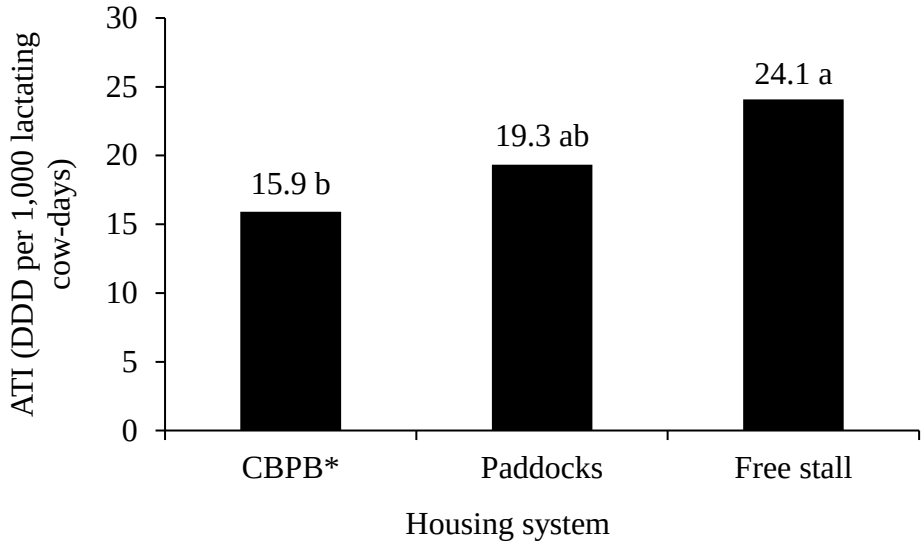
Table 8 - Spearman's correlations between monthly antimicrobial treatment incidence and herd level descriptors on 19 dairy herds from Southwest, Brazil, from April 2014 to January 2016

Variable[1]	n[2]	Mean	SD	Spearman's rho	*P*-value
Herd size	228	226.6	322.5	0.29	<.0001
BMSCC[3]	213	5.7	0.3	0.38	<.0001
BMTBC[3]	213	4.4	0.6	0.05	0.50
Milk yield[4]	228	23.2	6.1	0.07	0.28

[1]Continuous independent variables.
[2]Number of observations included in the correlation analysis.
[3]Log-transformed, base 10.
[4]Calculated as the average daily milk yield per cow (Kg/d).

When the differences in the antimicrobial use was evaluated between housing systems, the herd-level overall ATI was higher (P=0.009) for freestall herds (24.1 DDD per 1,000 lactating cow-days) than for compost bedded-pack barns (15.9 DDD per 1,000 lactating cow-days, Figure 5). There was no difference (P=0.09) between herds housing cows in freestalls in comparison to paddocks (19.3 DDD per 1,000 lactating cow-days). Likewise, there was no difference between the average herd-level overall ATI for compost bedded-pack barn in comparison to paddocks (P=0.22; Figure 5).

Figure 5 - Association of antimicrobial treatment incidence (ATI; number of DDD per 1,000 lactating cow-days) and housing type (compost bedded-pack barn [CBPB], paddocks and free stall) in 19 dairy herds from Southwest, Brazil, from April 2014 to January 2016



3.5 DISCUSSION

This is the first study quantifying the antimicrobial use for treatment of CM, and evaluating if descriptors at the herd-level could be associated with antimicrobial use in Brazilian dairy herds. The prospective evaluation performed in this study focused on a single disease, which allowed us to create a precise database in relation to the consumption of antimicrobials for CM therapy in dairy herds. The evaluation of antimicrobial consumption for treatment of CM can be used as a base of programs for the prudent use of antimicrobials in dairy herds.

Several methods have been used for quantification of antimicrobials in dairy herds, which includes cross-sectional surveys (POL; RUEGG, 2007a; REDDING et al., 2014a), wholesaler statistics (GRAVE et al., 1999), and inventory of empty antimicrobial containers (e.g., use of garbage can; SAINI et al., 2012; STEVENS et al., 2016). In the present study, the antimicrobial consumption was evaluated at the case-level by recording the specific treatment protocols used for each treated cow. Our method may have prevented the occurrence of recall and information bias, which are limitations likely to occur in studies using surveys; this method for antimicrobial quantification was possible because all herds enrolled in this study also participated in another epidemiological prospective study on characterization of CM (Chapter

2) conducted in parallel to this one. During the late study, microbiological culture for all cases of CM were made available freely to producers, which may have increased the farmers' compliance to the objectives of the present study.

The overall monthly mean ATI in our study, regardless of antimicrobial class or route of administration, was 17.7 DDD per 1,000 lactating cow-days, and a wide variation was observed between herds (3.9 to 39.0 DDD per 1,000 lactating cow-days). Other studies also reported a large variation between herds (POL; RUEGG, 2007a; STEVENS et al., 2016), which can be associated to factors at the herd-level, such as mastitis prevalence and incidence, frequency of pathogens causing intramammary infections, and different treatment protocols.

To the best of our knowledge, only one study calculated the ATI similarly as performed in our study (STEVENS et al., 2016); the study was conducted in Flanders, Belgium, and an overall mean ATI of 20.8 DDD per 1,000 cow-days was reported (6.3 DDD per 1,000 cow-days for intramammary compounds, and 7.4 DDD per 1,000 cow-days for compounds administered by systemic route). However, in that study it was not possible to differentiate whether the antimicrobials were used for treatment of clinical or subclinical mastitis, or whether the antimicrobials administered by systemic route were used for treatment of intramammary infections or for any other diseases. Anyway, the ATI of antimicrobials administered by intramammary route in that study (for treatment of clinical and/or subclinical mastitis) was more than two times lower than the ATI observed in our study (15.4 DDD per 1,000 cow-days). The higher frequency of antimicrobial use described in our study may be associated to differences between enrolled herds, such as treatment protocols and/or mastitis incidence, or even differences in the study design (e.g., length and methodology of data collection).

When antimicrobials were evaluated by classes, aminoglycoside had the highest ATI between intramammary compounds in our study, followed by the combination of tetracycline, aminoglycoside and polypeptide, and by third-generation cephalosporin. This results were different from findings of other studies evaluating the antimicrobial use in dairy herds. In the study of Stevens et al. (2016), the most frequently used antimicrobial class administered by the intramammary route was first-generation cephalosporin combined with aminoglycosides, followed by fourth-generation cephalosporins. In a Canadian study, penicillin combinations were the most frequently used intramammary class of antimicrobial (SAINI et al., 2012b). In another study, first-generation cephalosporin (i.e., cephapirin) and lincosamide (i.e., pirlimycin) were the most frequently used intramammary compounds for treatment of CM in conventional dairy herds of United States (POL; RUEGG, 2007a).

For compounds administered by systemic route, fluoroquinolones (especially enrofloxacin) was the antimicrobial class with the highest ATI in our study, followed by fourth-generation cephalosporins and the combination of sulfonamide and pyrimidine. Using a farm survey, a study conducted in US reported penicillin, tetracycline and third-generation cephalosporin (i.e., ceftiofur) as the three most frequently used systematically administered antimicrobials for treatment of CM (POL; RUEGG, 2007a). The comparison of our results with studies performed in other countries can be problematic as the treatment protocols are influenced by factors such as the incidence of CM, education of farm personnel, treatment costs, and the type and formulations of the antibacterial drugs approved for this indication in each country (GRAVE et al., 1999). For example, the use of fluoroquinolones and sulfonamides is not allowed for mastitis treatment in United States (ROBERSON, 2012), however its use is legally permitted in Brazil. Another factor limiting the comparison of results between studies is that the most recent evaluations of antimicrobial consumption in dairy herds have been based on the counting of antimicrobial packages disposed in garbage cans (SAINI et al., 2012b; STEVENS et al., 2016). The use of garbage cans may make it difficult to differentiate the indication of use of systematically administered antimicrobials.

Although our results showed a higher ATI for compounds administered by the intramammary route in comparison to systematically administered antimicrobials, the use of systemic compounds was 3.25 times higher when the antimicrobial use was quantified as the total amount (mg) of active ingredients. The administration of systemic antimicrobials associated with intramammary compounds is highly recommended for severe cases of CM (ROBERSON, 2012) because of the increased risk of bacteremia, especially in coliforms mastitis cases. Wenz et al. (2001) isolated bacteria from blood samples of 32% of cows with severe coliform mastitis. In addition, cows with coliform CM receiving intramuscular ceftiofur had lower risk of death or culling (13.8%) in comparison to cows that were not treated with the systematically administered antimicrobial (37%; Erskine et al., 2002). However, for treatment of mild and moderate CM, the administration of systemic antimicrobials is controversial and not usually necessary (ROBERSON, 2012). In our study, the use of systematically administered antimicrobials was reported for 65.2% of cows that had mild or moderate CM.

Our study detected that off-label therapeutic protocols were extensively used for treatment of CM. In addition to the high frequency of observed combination therapy, for all the antimicrobial classes, the frequency of administrations per quarter treatment was higher than recommended in the drug label (i.e., extended therapy). For example, the mean number of administrations of third-generation cephalosporin was 6.9, even though the drug label of the

commercial product recommends only one, and if necessary, two intramammary infusions per treatment. Although combined and/or extended therapy has been reported as strategies for increasing the odds of cure of intramammary infection (TAPONEN et al., 2003; SWINKELS et al., 2013), both are off-label therapeutic protocols that increase the antimicrobial consumption. Because the non-prudent use of antimicrobial can increase the resistance of bacteria (MCEWEN; FEDORKA-CRAY, 2002), extra-label therapeutic protocols should be used only with veterinarian prescription and supervision. However, in Brazil antimicrobials used in veterinary medicine is still legally purchased without prescription, which could further facilitate the indiscriminate use of antimicrobials in dairy herds.

In our study, the overall ATI was positively associated with the BMSCC, although other studies did not report the same results. A Canadian study evaluating 89 dairy herds in 4 regions of Canada reported that the antimicrobial use rate was not significantly associated with average herd BMSCC (SAINI et al., 2012b). Likewise, another study did not find association between ATI and geometric mean BMSCC (STEVENS et al., 2016). In both studies, the number of enrolled herds was higher, and the overall average BMSCC were lower than observed in our study, which may have contributed with the differences between results. In addition, differences in the prevalence of pathogens causing mastitis can also influence the association between BMSCC and antimicrobial use. (BARKEMA et al., 1998) reported that CM caused by *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus agalactiae* were more frequent in herds with a high BMSCC. In comparison to other studies performed in regions with more developed dairy industry, such as countries in North America and Europe (OLIVEIRA; HULLAND; RUEGG, 2013; VERBEKE et al., 2014), our study had a higher frequency of CM caused these potential contagious pathogens (results showed in chapter 2), which may explain the positive association between ATI and BMSCC.

The present study also found that the ATI was positively associated with the herd size (i.e., number of lactating cows), and this result was probably affected by the herd frequency of CM. In a study accounting for more than 70% of the United States dairy cow population, the herd-level disease prevalence (including mastitis) tended to increase as herd size increased (HILL, A. E. et al., 2009). In another study, the use of third-generation cephalosporin was positively associated with herd size, although the overall use of antimicrobial was not associated with this variable (SAINI et al., 2012b). However, different results have been also reported in other studies. No association between overall antimicrobial use and herd size was observed in a Belgium study (STEVENS et al., 2016). In contrary to our results, a survey evaluating 168 small herds of Peru observed an increased incidence of treated diseases (including mastitis) in

smaller-sized herds (REDDING et al., 2014b). The authors described that this result could be attributed to the farm personnel education level; farmers from larger herds tend to have higher levels of education, and therefore they may use better management strategies for prevention of diseases than curative strategies only.

For the association between ATI and housing type, our study found that freestall herds had a higher mean ATI than herds housing cows in compost bedded-pack barns, however, no difference were observed between the other housing type comparisons (paddock *vs.* freestall, and paddock *vs.* compost bedded-pack barn). In contrary to our results, no association was observed in another study evaluating the association between antimicrobial use and housing type (freestall, tie-stall, compost bedded-pack barn), although some tendencies were observed for some antimicrobial classes (SAINI et al., 2012b). The comparison of our results with other studies can be difficult as factors such as herd management, incidence of CM, and therapeutic strategies may have a large variation among countries, or even, among herds within a region. In addition, our results should be evaluated cautiously, as a study with higher number of herds within each of the housing systems could provide different outcomes.

Our study did not found an association between season and ATI. Some studies have reported that season can influence the incidence of CM (OLDE RIEKERINK; BARKEMA; STRYHN, 2007; OLIVEIRA et al., 2015). In a recent study evaluating dairy herds in Brazil, the incidence risk of CM increased during the rainy season (i.e., October-March) in comparison to the dry season (i.e., April-September; OLIVEIRA et al., 2015). The increased CM incidence could also be associated with the increase of antimicrobial use during the rainy season. However, the lack of effect observed in our study may have been affected by the drought that the Southeast region of Brazil undergone in 2014 (INMET; Instituto Nacional de Meteorologia, Brazil; www.inmet.gov.br), especially considering that the data collection within herds was performed for only 12 months in our study.

The present study had some limitations as the low number of enrolled herds. Although the results on antimicrobial use in the 19 herds participating in this study does not allow us to make any inferences at the country, or even at the regional level, selected dairy herds are representative of the developing dairy supply chain, typical of Brazil. Furthermore, our study did not evaluate the antimicrobials used for treatment of CM during the dry period. As dairy cows can have CM during the dry period (SCHERPENZEEL et al., 2014), and consequently being treated during this phase, dry periods should be included in the estimation of antimicrobial use. Therefore, although some limitations should be considered, the results of our

study can be used as a reference and serve as a basis for further pharmaco-epidemiological studies.

3.6 CONCLUSIONS

The overall monthly mean ATI was 17.7 DDD per 1,000 lactating cow-days. For intramammary compounds, the overall monthly mean ATI was 15.4 DDD per 1,000 lactating cow-days, while for systematically administered antimicrobials the overall ATI was 2.2 DDD per 1,000 lactating cow-days. Twelve commercial intramammary compounds formulated with 10 antimicrobial classes (or combination of classes), and 18 systemic compounds formulated with 9 antimicrobial classes (or combination of classes) had records of use over the study period. Among intramammary drugs, aminoglycosides had the highest ATI, followed by a compound with the combination of tetracycline, aminoglycoside and polypeptide. For systematically administered drugs, fluoroquinolones, fourth-generation cephalosporin and the combination of sulfonamide and pyrimidine were the most frequently used antimicrobials for treatment of CM. A high frequency of off-label therapeutic protocols, especially because of combination and extended treatments was observed. The BMSCC and herd size were positively associated with ATI. Furthermore, herd-level ATI was higher for freestall herds than for compost bedded-pack barns. There was no difference in the ATI between freestall and paddocks herds, neither for the comparison of compost bedded-pack barn and paddocks.

Chapter 4

Antimicrobial susceptibility patterns of *Escherichia coli* phylogenetic groups isolated from bovine clinical mastitis

4 ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF *ESCHERICHIA COLI* PHYLOGENETIC GROUPS ISOLATED FROM BOVINE CLINICAL MASTITIS

4.1 ABSTRACT

This study aimed to determine the phylogenetic group of *Escherichia coli* strains isolated from clinical mastitis (CM) in dairy cows and its association with cow-level descriptors, housing style and season; and, to determine and compare the antimicrobial susceptibility among the most frequent *E. coli* phylogroups. A total of 100 isolates of *E. coli* from bovine CM cases were identified using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS). All isolates were subject to phylogenetic analysis using a quadruplex PCR method; in addition, the antimicrobial susceptibility of the isolates was determined using a commercial broth microdilution test composed of 10 antimicrobials. Chi-square test was used to determine if descriptors at the cow-level (DIM, parity, quarter position, CM severity), housing style and season were independent of the *E. coli* phylogenetic groups. The minimal inhibitory concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were calculated for each antimicrobial, and survival analysis was completed to verify the differences between the antimicrobial susceptibility among *E. coli* phylogroups. Most isolates were assigned to group A (52%), followed by B1 (38%), B2 (2%); C (4%); D (3%); and E (1%). The only evaluated descriptor associated with the phylogroups was parity, in which primiparous cows had a higher proportion of isolates assigned to the phylogroup A (84.6%) in comparison to phylogroup B1 (15.4%). No association was observed between the phylogenetic groups and any other evaluated descriptor. Resistant isolates were observed for all evaluated antimicrobials. Overall, more than 96% of isolates were resistant to ampicillin, and more than 23% were resistant to cephalothin, sulphadimethoxine or tetracycline. High levels of resistance (>70%) were also observed to erythromycin, oxacillin, penicillin, penicillin associated with novobiocin, and pirlimycin. In contrary, high susceptibility was observed to ceftiofur (96.8%). Based on the survival analysis, heterogeneous curve was observed only for cephalothin, in which strains belonging to the

phylogroup A were inhibited at lower MIC than strains assigned to the phylogroup B1. For the remaining antimicrobials, the curves comparing the antimicrobial susceptibility were homogeneous between phylogroups. In conclusion, most of *E. coli* isolates belonged to phylogroups A and B1, and primiparous cows had a greater number of isolates assigned to the phylogroup A. Finally, isolates belonging to phylogroup A were susceptible to cephalothin at lower concentration than isolates belonging to group B1.
Key-words: Antimicrobial susceptibility. Phylogeny. Intramammary infection. *Escherichia coli*.

4.2 INTRODUCTION

Mastitis is one of the most important disease affecting dairy cattle, and *Escherichia coli* is one of the main cause of this disease, especially in its clinical form (OLIVEIRA; HULLAND; RUEGG, 2013). *Escherichia coli* is a Gram-negative micro-organism, traditionally considered as an environmental pathogen that opportunistically invade the bovine udder via teat canal, causing tissue damage to the mammary gland (HOGAN, J.; LARRY SMITH, 2003). Although the main source of *E. coli* seems to be the environment, this pathogen was also associated with persistent intramammary infections with long-term effects in the mammary gland (BLUM; HELLER; LEITNER, 2014).

The genotypic profile of *E. coli* causing IMI is very heterogeneous, with a great genotypical diversity of strains isolated from mastitis. *Escherichia coli* species are mainly commensals, however pathogenic strains were also reported, which were clustered in different pathovars based on clinical data and specific virulence properties (KEMPF et al., 2016). Despite the great intraspecific diversity, *E. coli* can be classified into different phylogroups based on the presence/absence of specific genes, such as *chuA, yjaA, arpA, trpA* and *TspE4.C2* (CLERMONT; BONACORSI; BINGEN, 2000; CLERMONT et al., 2013).

Most studies describing the distribution of *E. coli* causing mastitis into phylogenetic groups were performed using the simple triplex PCR method that enables an *E. coli* isolate to be assigned to one of the phylogroups A, B1, B2 or D (CLERMONT; BONACORSI; BINGEN, 2000). Only few reports described the use of the improved quadruplex PCR method (KEMPF et al., 2016), which allows the identification of seven phylogenetic groups (A, B1, B2, C, D, E and F;

CLERMONT et al., 2013). Therefore, studies about the frequency of strains isolated from CM belonging to phylogroups C, E and F are scarce in the indexed literature (KEMPF et al., 2016). In addition, to our knowledge, studies describing the phylogeny of *E. coli* isolates in bovine CM has not been evaluated in Brazil. Identification of the phylogeny is important to better understand etiological characteristics of *E. coli*, such as route of transmission, pathogenicity, virulence factors, and antimicrobial susceptibility.

Most *E. coli* strains associated with bovine mastitis were reported to belong to phylogroups A and B1 (LIU et al., 2014; KEANE, 2016); in addition, virulence genes are more often detected in certain phylogroups than others (SUOJALA et al., 2011; LIU et al., 2014). Considering the potential diversity of genetic properties between phylogenetic groups, it can be hypothesized that certain phylogroups could be more/less frequent in dairy herds according to characteristics at the cow-level, such as parity or stage of lactation; or yet, with characteristics that may affect the occurrence of CM in the herd, such as housing system and season of the year. Furthermore, as *E. coli* can cause mastitis with clinical signs varying from mild (only changes in milk) to severe (presence of systemic symptoms); and considering that some virulence factors can be more present in certain phylogenetic group than others, it can be hypothesized that the phylogenetic group may have a relationship with the severity of CM.

Clinical mastitis caused by *E. coli* is mostly of short duration, and the cows' immune system is generally competent to eliminate the infection spontaneously (BURVENICH et al., 2003). Therefore, antimicrobial treatment could not be necessary for most *E. coli* CM cases. However, although the interest of use of management strategies for selective therapy such as on-farm culture is growing in dairy herds (LAGO et al., 2011), most of farms still does not identify the pathogen causing CM before treatment. Therefore, blanket antimicrobial therapy remains the main strategy for treatment of CM in dairy herds.

The abusive and non-judicious use of antimicrobials in dairy herds was associated with resistant pathogens, which has been reported as an important cause of fail in mastitis treatment (SUOJALA; KAARTINEN; PYORALA, 2013). As different *E. coli* phylogroups can have different genetic properties (e.g., resistance genes; LIU et al., 2014), it can be hypothesized that the susceptibility to antimicrobials can vary within phylogeny. The existence of a relationship between the phylogeny of *E. coli* causing mastitis and antimicrobial susceptibility still needs to be better elucidated. While studies reported an association of antimicrobial susceptibility and specific

phylogenetic groups (LIU et al., 2014), others did not find any association (KEANE, 2016). A better understanding about the susceptibility profile of *E. coli* causing CM at the phylogenetic group-level can improve the understanding of appropriate treatments of this disease in dairy herds.

The objectives of this study were to determine: (1) the frequency of phylogenetic groups of *Escherichia coli* strains isolated from clinical mastitis in dairy cows and its association with the following variables: cow-level descriptors (days in milk, parity, position of affected quarter, and severity score of CM), housing type and season; and, (2) the antimicrobial susceptibility among the most frequent *E. coli* phylogroups identified from CM cases.

4.3 MATERIAL AND METHODS

4.3.1 Bacterial strains and phylogeny characterization

Isolates identified as *Escherichia coli* during data collection described in chapter 2 were submitted for the following procedures: (1) re-inoculation of a single colony on trypticase soy agar (TSA; BD, Sparks, MD, USA); re-suspension of one loop of cultured bacteria in 2 mL vials containing brain heart infusion broth (BHI; Becton, Dickinson and Company; Le Pont De Claix, France) supplemented with 10% glycerin; and (3) cryopreservation of vials at -80ºC until further analysis. After field data collection, *Escherichia coli* was identified as the cause of 276 CM cases using the conventional phenotypic culturing method (NMC, 2017).

A total of 100 *E. coli* isolates out of 276 were randomly selected for phylotyping and analysis of antimicrobial susceptibility after meeting the following inclusion criteria: (a) be a new case (occurring in the same quarter of the same cow at least 14 days apart); (b) identified from cultures with identification of a single pathogen instead of mixed cultures; (c) have a pure growth (without contamination) in the re-cultivation performed before phenotyping. In addition, all isolates identified as *E. coli* by microbiological culturing were confirmed using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS), and only those with MALDI scores identification > 2.0 were randomly selected. Isolates were selected

aiming to include samples from all herds with identification of *E. coli* (i.e., at least one isolate per herd enrolled in the study); therefore, if a herd had few isolates they were selected without randomization.

4.3.2 MALDI-TOF MS

Sample preparation for MALDI-TOF MS was performed as previously described (TOMAZI et al., 2014). Briefly, a few (2 to 4) colonies from a fresh and pure overnight culture grown on blood agar at 37°C were suspended in 300 μL distilled water, to which 900 μL absolute ethanol was added. The resulting solution was homogenized and centrifuged at 13,000 × g for 2 min, and the supernatant was discarded. A solution of 70% formic acid (30 μL) was added to the pellet to lyse the bacterial cells and release their intracellular proteins, most critically, the ribosomal proteins. Subsequently, an equal volume of 100% acetonitrile was added to each tube, thus producing a bacterial extract in a 1:1 solution of 70% formic acid and acetonitrile. A final centrifugation step (13,000 × g for 2 min) was performed to separate bacterial cell debris from the supernatant containing the intracellular proteins used for the bacterial identification.

Samples were processed in an Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with flex control software (Bruker Daltonics) operated in linear mode and equipped with a 337-nm nitrogen laser. To prepare the MALDI target plate, 1 μL of each bacterial extract was spotted onto a 96-spot target plate (polished stainless steel, Bruker Daltonics) and dried at room temperature. The dried sample spot was overlaid with 1 μl of a matrix solution, consisting of α-cyano-4-hydroxy-cinnamic acid diluted in a solution of 50% acetonitrile and 2.5% trifluoroacetic acid. The Bruker bacterial test standard (BTS; Bruker Daltonics) was used for mass calibration and for instrument parameter optimization. Mass spectral data were collected within the m/z range of 2,000 to 20,000, and the data.

Biotyper 3.0 software (Bruker Daltonics) was used to process the raw spectra acquired by the Microflex. Data were analyzed using the built-in main spectra projection feature of the Biotyper software, which is a proprietary algorithm for spectral pattern matching that produces a logarithmic score from 0 to 3. The peak lists were compared with each entry in the Biotyper

database, which contained 3,995 references, using the standard parameters of the pattern-matching algorithm. The identification score cutoff values were applied to each measurement according to the manufacturer's instructions. Scores of ≥2 indicate species-level identification, scores of ≥1.7 but <2 indicate genus-level identification, and scores of <1.7 indicate that no reliable identification can be made.

4.3.3 DNA extraction

All isolates were cultured overnight on blood agar plates at 37°C under aerobic conditions to evaluate the purity of each isolate. The DNA extraction of *Escherichia coli* isolates were performed using an adaption of the method described by Fan et al. (1995). In our study, the phosphate buffered saline (pH 7.2) was substituted by Tris-EDTA buffer (TE; 10mM Tris HCL, 5mM EDTA, pH= 8,0). Briefly, cultured bacteria were re-inoculated in 2 mL of BHI broth and incubated overnight at 37ºC. After, 1 mL of the culture was transferred to 1.5 mL vial and centrifugated at 14,000 × g for 10 minutes. The supernatant was discarded and the pellet was washed twice with 200 μL of TE; during the washing procedure, the sample was centrifuged at 13,000 × g for 5 minutes. After this washing step, the supernatant was discarded and the pellet was re-suspended in 50 μL of TE. The cell suspension was heated directly at 95º C for 15 minutes in a dry block (MD-02N, Major Science, Saratoga, CA, USA), and then, immediately cooled on freezer at approximately -20º C for 30 minutes. Finally, the cell suspension was homogenized and centrifuged at 13,000 × g for 5 minutes and the supernatant (40 μL) containing the DNA was collected and stored at -80º C.

4.3.4 Phylogenetic group determination

The method described by Clermont et al. (2013) was used to classify strains into phylogenetic groups. The *E. coli* strains were tested for *chuA*, *yjaA*, TSPE4.C2, *arpA*, and *trpA* genes by PCR, which allows the classification of the strains into one of the following groups: A, B1, B2, C, D, E, and F. Primer sequences, gene target, and PCR product for the phylogroup assignment method are presented in Table 9.

Table 9 – Primer nucleotide sequences, gene target and sizes of PCR products used in the extended quadruplex phylo-typing method for classification of 100 *Escherichia coli* isolates from bovine clinical mastitis

PCR reaction	Target	Primer sequences	PCR product (bp)
Quadruplex	*chuA*	5′-ATGGTACCGGACGAACCAAC-3′ 5′-TGCCGCCAGTACCAAAGACA-3′	288
	yjaA	5′-CAAACGTGAAGTGTCAGGAG-3′ 5′-AATGCGTTCCTCAACCTGTG-3′	211
	TspE4.C2	5′-CACTATTCGTAAGGTCATCC-3′ 5′-AGTTTATCGCTGCGGGTCGC-3′	152
	arpA	5′-AACGCTATTCGCCAGCTTGC-3′ 5′-TCTCCCCATACCGTACGCTA-3′	400
Group E	*arpA*	5′-GATTCCATCTTGTCAAAATATGCC-3′ 5′-GAAAAGAAAAAGAATTCCCAAGAG-3′	301
Group C	*trpA*	5′-AGTTTTATGCCCAGTGCGAG-3′ 5′-TCTGCGCCGGTCACGCCC-3′	219

All PCR reactions were carried out in a 20 μL final volume that contained: 100 ng of DNA, 0.2 mM of each dNTP, 1x Green GoTaq™ Reaction Buffer (Promega), 2 U GoTaq™ DNA polymerase (Promega), and 1 μL of each primer; except for the primers of *arpA* gene in the quadruplex analysis, of which 2 μL was used. PCR reactions were performed under the following conditions: denaturation 4 min at 94°C; 30 cycles of 20 seconds at 94°C, and 20 s at 57°C (group E) or 59°C (quadruplex and group C), and 25 seconds at 72ºC; and a final extension step of 5 min at 72°C.

4.3.5 Agarose gel electrophoresis

Ten microliters of the amplified product were analyzed by electrophoresis in 1.5% agarose gel with 1X Tris-borate-EDTA (TBE) buffer stained with 0.1 μg/μL ethidium bromide. Images of gel were taken under ultraviolet light using a photo-documentation system. A marker of size of 100 bp was used to evaluate whether the specific bands were present and to compare the size of the bands generated in the electrophoresis.

4.3.6 Assignment of strains into the phylogroups

The *E. coli* isolates were assigned to one of the seven phylogroups (A, B1, B2, C, D, E, or F) based on the presence/absence of fragments of the genes in the order *arpA/chuA/yjaA/*TspE4.C2. Additional tests were necessary to differentiate strains of the groups C, D and E, as described in Table 10.

Table 10 - Quadruplex genotypes and steps required for assigning *Escherichia coli* isolates to phylogroups. Table adapted from the study of Clermont et al. (2013)

arpA	*chuA*	*yjaA*	TspE4.C2	Phylogroup	Next step
+	-	-	-	A	-
+	-	-	+	B1	-
-	+	-	-	F	-
-	+	+	-	B2	-
-	+	+	+	B2	-
-	+	-	+	B2	-
+	-	+	-	A or C	Screen using C-specific primers. If C+ then C, else A
+	+	-	-	D or E	Screen using E-specific primers. If E+ then E, else D
+	+	-	+	D or E	Screen using E-specific primers. If E+ then E, else D
+	+	+	-	E or Clade I	Screen using E-specific primers. If E- then clade I.

4.3.7 Antimicrobial susceptibility testing

The minimum inhibitory concentration of antimicrobials against the evaluated pathogens was tested using the mastitis panel of a commercial broth microdilution test (CMV1AMAF; Sensititre, TREK Diagnostics, Cleveland, OH), following guidelines of the Clinical and Laboratory Standards Institute (formerly NCCLS; CLSI, 2015). The antimicrobials agents and dilution ranges tested for each one of them are presented in Table 11.

Table 11 – Antimicrobial agents and their dilution ranges used in the susceptibility test performed in our study

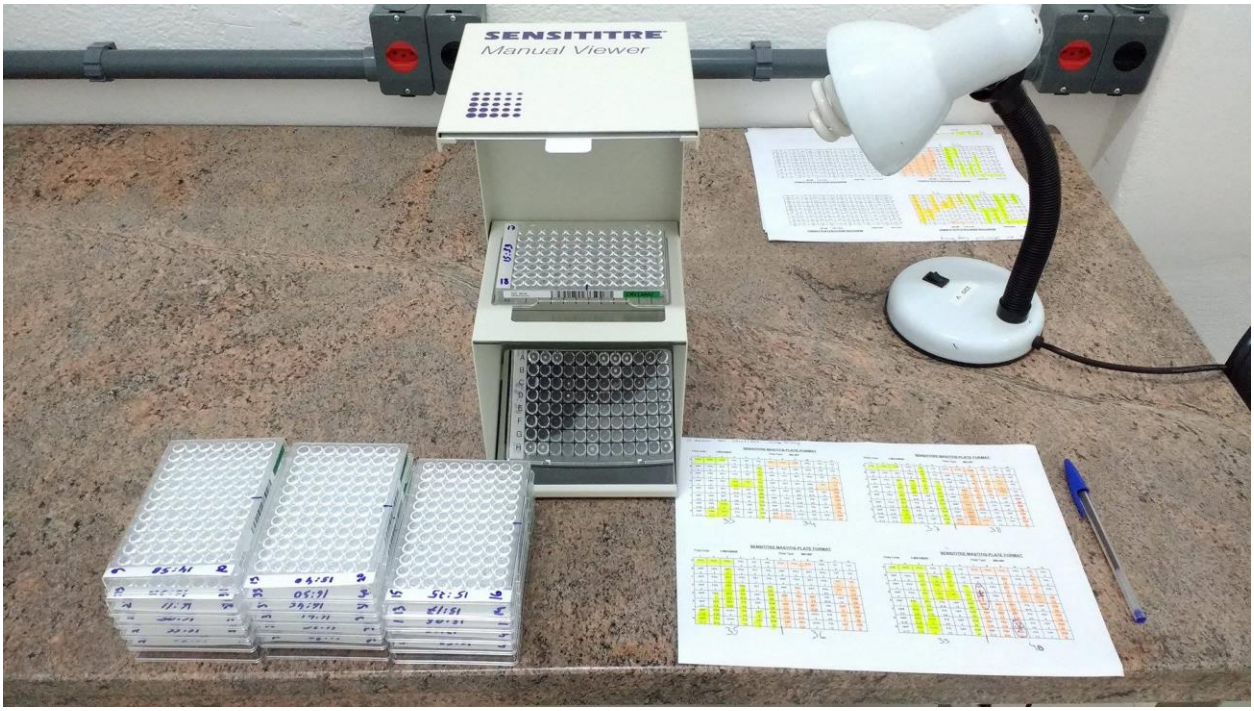
Antimicrobial agent	MIC dilution range (μg/mL)
Penicillin	0.12 - 8.0
Ampicillin	0.12 - 8.0
Oxacillin	2.0 - 4.0
Cephalothin	2.0 - 16.0
Ceftiofur	0.5 - 4.0
Penicillin + novobiocin	1.0/2.0 - 8.0/16.0
Erythromycin	0.25 - 4.0
Pirlimycin	0.5 - 4.0
Tetracycline	1.0 - 8.0
Sulphadimethoxine	32.0 - 256.0

Cryopreserved *E. coli* isolates were thawed, inoculated on blood Agar and incubated at 37ºC for 24 hours for evaluation of bacteria viability. After, a single and pure colony was re-inoculated on trypticase soy agar (TSA; BD, Sparks, MD, USA) and incubated at 37ºC for 24 hours. After incubation, the isolates were suspended in 0.9% saline solution using disposable sterile loops to approximate the density of a 0.5 McFarland standard. A DEN-1 McFarland Densitometer (Biosan, Riga, Latvia) was used for standardization of bacterial suspensions to the McFarland standard. Subsequently, 10 μL-aliquot of bacteria suspension was transferred into a tube containing 11 mL of Mueller-Hinton broth (pH = 7.3 ± 1; BD, Sparks, MD, USA) that was finally mixed on a vortex for approximately 10 seconds. The transferred aliquot was based on recommendations described on the manufacturer's guideline.

Sensititre® panels were reconstituted with the bacteria inoculum (50 μL per wheel) using a multicanal pipette with disposable tips. Panels were covered with an adhesive seal, and incubated at 35 ºC for approximately 18 hours. After incubation, results were read using the Sensititre® manual viewer (Figure 6). Growth appeared as turbidity or as a deposit of cells at the bottom of a well. The MIC was recorded as the lowest concentration of antimicrobic that inhibited visible growth. The growth control wells were read first, and if any of the control wells did not exhibit growth, the results were considered invalid, and the isolate was re-analyzed. The lowest concentrations inhibiting 50% (MIC_{50}) and 90% (MIC_{90}) of isolates were determined. For each

batch of microorganism tested, the NCCLS recommended quality control strains *Escherichia coli* ATCC 25922, and *Staph. aureus* ATCC 29213 were also evaluated.

Figure 6 – Sensititre® manual viewer used for reading the results of antimicrobial susceptibility testing and broth microdilution plates ready to be evaluated (post incubation).



4.3.8 Data analysis

Escherichia coli isolates were classified in categories based on their origin (i.e., quarter, cow and farm from which they were isolated), severity of CM and time of the year of CM diagnosis as following: DIM (≤100, 101-200, or ≥201 days after calving); parity (primiparous or multiparous); quarter position (front or hind); CM severity (**Mild** - changes only in the milk appearance; **Moderate** - presence of abnormal milk accompanied by changes in the udder; or, **Severe** - combination of abnormal milk, with signs of inflammation in the udder and systemic signs); season of CM diagnosis (**rainy** - October-March, or **dry** - April-September); and housing

system (compost-bedded pack barn, free stall, paddocks). The paddock housing system was characterized as an open area surrounded by fences or rails and without pasture for grazing.

The FREQ procedure of SAS 9.4 (SAS Inst. Inc., Cary NC) was used to determine the distribution of *E. coli* phylogenetic groups, and to perform χ^2 analysis to determine if descriptors at the cow-level (DIM, parity, quarter-position, CM severity), housing style and season were associated with phylogenetic groups. In each test, the phylogroups formed the row of the table and the descriptors categories formed the columns. Statistical significance was defined at $P \leq 0.05$.

The concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were calculated for each antimicrobial. Where interpretive cut-points for MIC of the evaluated antimicrobials have been established for *E. coli* (Clinical and Laboratory Standards Institute; CLSI, 2013; 2015), these were used to classify each isolate as susceptible, intermediate or resistant. Isolates identified as intermediate were interpreted as resistant.

Survival analysis using the PROC LIFETEST of SAS (SAS Inst. Inc., Cary NC) was completed to verify the differences between the antimicrobial susceptibility among *E. coli* phylogroups. The concentrations of antimicrobials contained in the commercial broth microdilution test were used as the "time" variable, and the inhibition of bacterial growth was used as the event. Isolates that presented growth at the highest tested concentration were censored by the statistical model. Kaplan-Meier survival curves of the phylogenetic groups were performed for each antimicrobial studied. The null hypothesis of no differences (homogeneity of survival curves) in the survivor functions of the strata (phylogenetic groups) was evaluated using Log-Rank and Wilcoxon tests. Statistical significance (i.e., heterogeneous survival curves) was defined at $P \leq 0.05$.

4.4 RESULTS

4.4.1 Descriptive data

All *E. coli* isolates were identified by MALDI-TOF MS from cases of CM in 19 dairy herds located in the States of São Paulo (n = 14) and Minas Gerais (n = 5). The herds' characteristics are described in the chapter 2 of this document.

A total of 179 *E. coli* isolates were cryopreserved during the etiological characterization of CM in dairy herds, previously described in chapter 2. Of these, 41 isolates were excluded because of one of the following reasons: MALDI escore <2.0 (n = 18); no growth during re-inoculation (n = 16); and contamination (n = 7). The other 38 isolates were not selected during randomization. For all selected isolates, analysis of antimicrobial susceptibility and phylotyping using the Clermont method were performed. The average MALDI score of selected isolates was 2.15 (SD = 0.08), which allowed us to confirm the identification of the isolates at the genus- and species level. All *E. coli* isolates were assigned to one of the phylogroups described by Clermont et al. (2013); however only 94 had results of antimicrobial susceptibility. Out of isolates that did not have MIC results (n = 6), 1 was contaminated during re-inoculation on blood Agar plate, and 5 had no growth in the positive control wheels of the Sensititre® panel.

4.4.2 Phylotyping profile and association with herd- and cow-level descriptors

All selected isolates were assigned to one of the Clermont phylogenetic groups. Most isolates were assigned to phylogroups A (n = 52; 52%) and B1 (n = 38; 38%). In addition, a total of 10 isolates (10%) belonged to one of the other phylogenetic groups, called here as less frequent groups: B2 (n = 2); C (n = 4); D (n = 3); and E (n = 1; Table 12). No isolate was assigned to the group F described by Clermont et al. (2013).

Phylogroup A was identified in 14 out of 19 (73.7%) herds, while the group B1 was identified in 15 (78.9%) herds (Table 12). Phylogeny groups identified at lower frequency were isolated from one (groups B2 and E), two (group D), and three herds (group C; Table 12).

Table 12 – Frequency of phylogenetic groups of *Escherichia coli* isolates (n = 100) identified from clinical mastitis in 19 dairy herds

Herd	Frequency of isolates assigned to the phylogenetic groups (n)						Overall herd
	A	B1	B2	C	D	E	
A	6	5	-	1	-	-	12
B	-	1	-	2	-	-	3
C	-	3	-	-	-	-	3
D	3	2	-	-	-	-	5
E	3	1	-	-	-	-	4
F	5	1	-	-	-	-	6
G	1	1	-	-	-	-	2
H	-	2	-	-	-	-	2
I	5	5	-	-	2	-	12
J	1	-	-	-	-	-	1
K	3	6	2	-	-	1	12
L	11	-	-	-	-	-	11
M	7	3	-	-	-	-	10
O	-	1	-	-	-	-	1
P	3	-	-	-	-	-	3
Q	2	3	-	1	-	-	6
R	1	-	-	-	-	-	1
S	1	3	-	-	1	-	5
T	-	1	-	-	-	-	1
Overall (group)	52	38	2	4	3	1	100

Association analysis between phylogroups and the descriptors related to the cow and herd were performed only for the most frequently identified phylogroups (A and B1). The only evaluated variable associated with the phylogroups was parity (χ^2 = 4.45; *P* = 0.03), where primiparous cows had a higher proportion of isolates assigned to phylogroup A (n = 11; 84.6%) in comparison to phylogroup B1 (n = 2; 15.4%). No association (*P* > 0.05) was observed between the phylogenetic groups and the other evaluated descriptors (i.e., housing type, season, DIM, quarter position, and CM severity; Table 13).

Table 13 – Association between phylogroups (A and B1) of *Escherichia coli* identified from clinical mastitis in 19 dairy herds and descriptors at the cow-level, housing type and season

Variable	Categories	Phylogenetic group				χ^2	*P*-value
		A		B1			
		n	%	n	%		
Housing (n = 90)	Freestall	19	54.3	16	45.7	2.94	0.23
	CBPB[1]	8	44.4	10	55.6		
	Paddocks	25	67.6	12	32.4		
Season (n = 90)	Rainy	42	60.9	27	39.1	1.16	0.28
	Dry	10	47.6	11	52.4		
DIM (n = 89)	0-100	14	58.3	10	41.7	0.30	0.86
	101-200	17	60.7	11	39.3		
	>200	20	54.1	17	46.0		
Parity (n = 88)	Primiparous	11	84.6	2	15.4	4.45	0.03
	Multiparous	40	53.3	35	46.7		
Quarter position (n = 90)	Front	21	51.2	20	48.8	1.33	0.25
	Hind	31	63.3	18	36.7		
CM severity (n = 85) [2]	Mild	24	66.7	12	33.3	3.20	0.20
	Moderate	22	61.1	14	38.9		
	Severe	5	38.5	8	61.5		

[1] CBPB – Compost-bedded pack barn

[2] CM severity - (Mild) changes only in the milk appearance; (Moderate) presence of abnormal milk accompanied by changes in the udder; or, (Severe) combination of abnormal milk, with signs of inflammation in the udder and systemic signs)

Association of the other identified phylogroups (B2, C, D and E) and the aforementioned variables were not evaluated because of the low frequency (n = 10) of isolates assigned to those groups; however, the distribution of isolates within the categories of the evaluated variables are presented in Table 14.

Table 14 – Distribution of less frequent phylogroups of *Escherichia coli* identified from clinical mastitis cases occurred in 19 dairy herds stratified by the variables at the cow-level, housing type and season

Variable	Categories	Phylogenetic groups			
		B2	C	D	E
Housing (n = 10)	Freestall	2	1	-	1
	CBPB[1]	-	3	2	-
	Paddocks	-	-	1	-
Season (n = 10)	Rainy	2	4	3	1
	Dry	-	-	-	-
DIM (n = 9)	0-100	-	1	1	-
	101-200	-	2	1	-
	>200	2	1	1	-
Parity (n=9)	Primiparous	-	2	1	-
	Multiparous	2	2	2	-
Quarter position (n = 10)	Front	2	-	-	1
	Hind	-	4	3	-
CM severity (n = 9) [2]	Mild	1	1	-	-
	Moderate	-	2	3	1
	Severe	1	-	-	-

[1] CBPB – Compost bedded pack barn

[2)] CM severity - (Mild) changes only in the milk appearance; (Moderate) presence of abnormal milk accompanied by changes in the udder; or, (Severe) combination of abnormal milk, with signs of inflammation in the udder and systemic signs)

4.4.3 Antimicrobial susceptibility testing

Overall

In total, 94 strains had results of MIC (Table 15). For ampicillin, the MIC_{50} was 2 μg/mL and the MIC_{90} was not possible to be indicated as more than 10% of isolates had growth at the highest antimicrobial concentration present in the panel. The strains were highly susceptible to ceftiofur, and both MIC_{50} and MIC_{90} were 0.5 μg/mL; only one isolate was not inhibited at the highest concentration of ceftiofur. For cephalothin, the overall MIC_{50} was 8 μg/mL, while the MIC_{90} was 16 μg/mL. For sulphadimethoxine, 76.6% of isolates were susceptible at ≤256 μg/mL (MIC_{50}), while 23.4% were not susceptible at the highest concentration contained in the test. The overall MIC_{50} for tetracycline was 4 μg/mL, while 29.8% of the isolates were not inhibited at the concentration present in the test.

There is no indication of use or susceptibility criteria for erythromycin, oxacillin, penicillin, penicillin/novobiocin, and pirlimycin for treatment of infections caused by *E. coli*. Therefore, more than 70% of strains were not inhibited by the highest antimicrobial concentration contained in the panel (Table 15)

Table 15 - Overall frequency distribution (%) of *Escherichia coli* isolates (n = 94) that had 50% (MIC50) and 90% (MIC90) of bacterial growth inhibited at each antimicrobial concentration. All isolates were identified from clinical mastitis cases occurred in 19 dairy herds of Southeast, Brazil

Antimicrobial	Frequency (%) of isolates at each indicated MIC (μg/mL)[1]												NI[2]	MIC_{50}[3]	MIC_{90}[4]
	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256			
Ampicillin	1.1	2.1	4.3	5.3	41.5	29.8	1.1						14.8	2	>8
Ceftiofur			92.6	2.1	2.1	2.1							1.1	0.5	0.5
Cephalothin					4.3	13.8	57.5	17.0					7.4	8	16
Erythromycin		1.1	2.1	3.2		1.1							92.6	>4	>4
Oxacillin					19.1	2.1							78.7	>4	>4
Penic/Novob.					1.1	1.1	27.7						70.2	>8/16	>8/16
Penicillin	1.1				1.1	2.1	2.1						93.6	>8	>8
Pirlimycin			6.4			2.1							91.5	>4	>4
Sulphadimet									25.5	34.0	12.8	4.3	23.4	64	>256
Tetracycline				6.4	18.1	41.5	4.3						29.8	4	>8

[1]The light gray shading represents the susceptible zone, and the darker gray shading represents the resistant zone. Results were interpreted according to the Clinical and Laboratory Standards Institute (2013; 2015). Interpretative criteria were based on human data (sulphadimethoxine and tetracycline), dogs' data (ampicillin), and bovine mastitis (ceftiofur). The resistant category included those isolates categorized as either intermediate or resistant. Antimicrobials without shading have no interpretive criteria available.

[2]NI = Not inhibited (growth at highest concentration tested).

[3]MIC (μg/mL) that inhibited 50% (MIC_{50}) of the isolates.

[4]MIC (μg/mL) that inhibited 90% (MIC_{90}) of the isolates.

Phylogenetic groups A and B1

The frequency distribution of MIC, the susceptibility results, and the MIC_{50} and MIC_{90} of each evaluated antimicrobial are shown in the Tables 16 and 17. The MIC_{50} of ampicillin for *E. coli* isolates of both phylogroups (A and B1) was 2 μg/mL. The MIC_{90} for the same antimicrobial was 4 μg/mL for isolates belonging to phylogroup A; however, the determination of MIC_{90} for isolates pertaining to phylogroup B1 was not possible because more than 10% of tested isolates were not inhibited at highest concentration of ampicillin present in the panel. In total, 10% of isolates assigned to phylogroup A and 14% of isolates belonging to phylogroup B1 were not inhibited at the highest concentration of ampicillin. Only 2% of isolates from phylogroup A, and 5.8% from the phylogroup B1 were considered as susceptible to ampicillin.

For ceftiofur, the MIC_{50} and MIC_{90} was observed at the lowest concentration (0.5 μg/mL) for both phylogenetic groups (A and B1). Only 2% (1 out of 50) of isolates from phylogroup A and 5.8% (2 out of 34) out from the phylogroup B1 were not susceptible to ceftiofur. The MIC of cephalothin that inhibited 50% (8 μg/mL) was also similar for phylogroups A and B1; however, the MIC_{90} was 16 μg/mL for isolates assigned to phylogroup A, and >16 μg/mL for isolates assigned to phylogroup B1. A total of 86% of isolates belonging to phylogroup A, and 61.8% of isolates from phylogroup B1 were susceptible to cephalothin.

The MIC of sulphadimethoxine to inhibit 50% of isolates from both phylogroups A and B1 was 64 μg/mL. On the other hand, the estimation of MIC_{90} for sulphadimethoxine was not possible because more than 10% of tested isolates were resistant to this antimicrobial at the highest concentration present in the commercial panel. A total of 80% of isolates assigned to the phylogroup A, and 76.5% of isolates from the phylogroup B1 were susceptible to sulphadimethoxine.

A total of 66% of isolates from phylogroup A, and 67.6% from phylogroup B1 were susceptible to tetracycline. The MIC_{50} was similar for both groups (4 μg/mL), while the MIC_{90} was greater than the highest antimicrobial concentration (8 μg/mL) contained in the panel.

For antimicrobials not recommended for treatment of *E. coli* infection, the isolates of both phylogroups A and B1 were resistant to the highest concentration contained in the panel in more than 70% of the tests for oxacillin and penicillin+novobiocin, and in more than 90% for erythromycin, penicillin and pirlimycin.

Table 16 – Frequency distribution (%) of *Escherichia coli* isolates belonging to phylogenetic groups A (n = 50) and B1 (n = 34) that had 50% (MIC50) and 90% (MIC90) of bacterial growth inhibited at each antimicrobial concentration. All isolates were identified from clinical mastitis cases in 19 dairy herds of Southeast, Brazil

Antimicrobial	Group[1]	Frequency (%) of isolates at each indicated MIC (μg/mL)[2]												NI[3]	MIC_{50}[4]	MIC_{90}[4]
		0.12	0.25	0.5	1	2	4	8	16	32	64	128	256			
Ampicillin	A	-	2.0	-	8.0	44.0	36.0	-	-	-	-	-	-	10.0	2	4
	B1	2.9	2.9	8.8	2.9	35.3	29.4	2.9	-	-	-	-	-	14.7	2	>8
Ceftiofur	A	-	-	94.0	2.0	2.0	2.0	-	-	-	-	-	-	-	0.5	0.5
	B1	-	-	91.2	2.9	-	2.9	-	-	-	-	-	-	2.9	0.5	0.5
Cephalothin	A	-	-	-	-	8.0	20.0	58.0	10.0	-	-	-	-	4	8	16
	B1	-	-	-	-	-	2.9	58.8	26.5	-	-	-	-	11.8	8	>16
Erythromycin	A	-	2.0	2.0	4.0	-	-	-	-	-	-	-	-	92	>4	>4
	B1	-	-	-	2.9	-	-	-	-	-	-	-	-	97.1	>4	>4
Oxacillin	A	-	-	-	-	16.0	-	-	-	-	-	-	-	84.0	>4	>4
	B1	-	-	-	-	20.6	5.9	-	-	-	-	-	-	73.5	>4	>4
Penic+Novob.	A	-	-	-	-	2.0	-	26.0	-	-	-	-	-	72.0	>8/16	>8/16
	B1	-	-	-	-	-	-	29.4	-	-	-	-	-	70.6	>8/16	>8/16
Penicillin	A	-	-	-	-	2.0	4.0	2.0	-	-	-	-	-	92.0	>8	>8
	B1	2.9	-	-	-	-	-	2.9	-	-	-	-	-	94.1	>8	>8
Pirlimycin	A	-	-	4.0	-	-	2.0	-	-	-	-	-	-	94.0	>4	>4
	B1	-	-	5.9	-	-	2.9	-	-	-	-	-	-	91.2	>4	>4
Sulphadimethoxine	A	-	-	-	-	-	-	-	-	30.0	36.0	10.0	4.0	20.0	64	>256
	B1	-	-	-	-	-	-	-	-	20.6	38.2	14.7	2.9	23.5	64	>256
Tetracycline	A	-	-	-	8.0	20.0	38.0	4.0	-	-	-	-	-	30.0	4	>8
	B1	-	-	-	-	20.6	47.1	2.9	-	-	-	-	-	29.4	4	>8

[1] Phylogenetic groups identified according to Clermont et al. (2013).

[2] The light gray shading represents the susceptible zone, and the darker gray shading represents the resistant zone. Results were interpreted according to the Clinical and Laboratory Standards Institute (2013; 2015). Interpretative criteria were based on human data (sulphadimethoxine and tetracycline), dogs' data (ampicillin), and bovine mastitis (ceftiofur). The resistant category included those isolates categorized as either intermediate or resistant. Antimicrobials without shading have no interpretive criteria available.

[3] NI = Not inhibited (growth at highest concentration tested).

[4] MIC (μg/mL) that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates.

Table 17 – Frequency distribution (number of observations, and percentage in parentheses) of *Escherichia coli* phylotypes classified as susceptible (S) or resistant (R) according to the interpretation criteria described by the Clinical and Laboratory Standards Institute (CLSI, 2013; 2015)

Antimicrobial	CLSI criteria[1]	Antimicrobial				
		Ampicillin	Ceftiofur	Cephalothin	Sulphadim.	Tetracycline
A	S	1 (2.0)	49 (98.0)	43 (86.0)	40 (80.0)	33 (66.0)
	R	49 (98.0)	1 (2.0)	7 (14.0)	10 (20.0)	17 (34.0)
B1	S	2 (5.9)	32 (94.1)	21 (61.8)	26 (76.5)	23 (67.7)
	R	32 (94.1)	2 (5.9)	13 (38.2)	8 (23.5)	11 (32.3)
B2	S	-	2 (100)	2 (100)	2 (100)	-
	R	2 (100)	-	-	-	2 (100)
C	S	-	4 (100)	1 (25.0)	1 (25.0)	3 (75.0)
	R	4 (100)	-	3 (75.0)	3 (75.0)	1 (25.0)
D	S	-	3 (100)	3 (100)	2 (66.7)	2 (66.7)
	R	3 (100)	-	-	1 (33.3)	1 (33.3)
E	S	1 (100)	-	-	-	-
	R	-	1 (100)	1 (100)	1 (100)	1 (100)
Overall	S	4 (4.3)	90 (95.7)	70 (74.5)	71 (75.5)	61 (64.9)
	R	90 (95.7)	4 (4.3)	24 (25.5)	23 (24.5)	33 (35.1)

[1] S (Susceptible), R (Resistant); The resistant category included those isolates categorized as either intermediate or resistant

Phylogenetic groups B2, C, D and E

The frequency distribution of MIC, and the antimicrobial susceptibility results for the less frequent phylogroups (B2, C, D and E) are shown in Tables 18 and 19. The MIC_{50} and MIC_{90} were not calculated for these isolates because of the low identification frequency. None of the isolates belonging to less frequently identified phylogroups were susceptible to ampicillin. In contrary, all isolates were susceptible to ceftiofur, and 9 of the 10 isolates were susceptible at the lowest antimicrobial concentration. For cephalothin, two isolates were susceptible at 4 μg/mL, seven at 8 μg/mL, two at 16 μg/mL (considered as resistant), and 1 (phylogroup C) was resistant at the highest concentration. For both sulphadimethoxine and tetracycline, six isolates were considered susceptible and four resistant.

For antimicrobials with no susceptibility criteria (i.e., erythromycin, oxacillin, penicillin, penicillin/novobiocin, and pirlimycin), a high frequency of isolates (>60%) were resistant at the highest antimicrobial concentration contained in the commercial panel (Table 19).

Table 18 - Frequency distribution (%) of *Escherichia coli* belonging to phylogenetic groups B2 (n = 2), C (n = 4), D (n = 3) and E (n = 1) that were inhibited at each antimicrobial concentration. Only antimicrobials with susceptibility interpretative criteria for *E. coli* are presented. Isolates were identified from clinical mastitis cases in 19 dairy herds of Southeast, Brazil

Antimicrobial	Group[1]	Frequency (n) of isolates at each indicated MIC (μg/mL)[2]												NI[3]
		0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
Ampicillin	B2	-	-	-	-	2	-	-	-	-	-	-	-	-
	C	-	-	1	-	-	-	-	-	-	-	-	-	3
	D	-	-	-	-	2	-	-	-	-	-	-	-	1
	E	-	-	-	-	1	-	-	-	-	-	-	-	-
Ceftiofur	B2	-	-	2	-	-	-	-	-	-	-	-	-	-
	C	-	-	3	-	1	-	-	-	-	-	-	-	-
	D	-	-	3	-	-	-	-	-	-	-	-	-	-
	E	-	-	1	-	-	-	-	-	-	-	-	-	-
Cephalothin	B2	-	-	-	-	-	-	2	-	-	-	-	-	-
	C	-	-	-	-	-	1	-	2	-	-	-	-	1
	D	-	-	-	-	-	1	2	-	-	-	-	-	-
	E	-	-	-	-	-	-	1	-	-	-	-	-	-
Sulphadimethoxine	B2	-	-	-	-	-	-	-	-	2	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	1	-	3
	D	-	-	-	-	-	-	-	-	-	-	1	1	1
	E	-	-	-	-	-	-	-	-	-	1	-	-	-
Tetracycline	B2	-	-	-	-	-	-	-	-	-	-	-	-	2
	C	-	-	-	2	-	1	1	-	-	-	-	-	-
	D	-	-	-	-	-	2	-	-	-	-	-	-	1
	E	-	-	-	-	-	1	-	-	-	-	-	-	-

[1] Phylogenetic groups identified according to Clermont et al. (2013).

[2] The light gray shading represents the susceptible zone, and the darker gray shading represents the resistant zone. Results were interpreted according to the Clinical and Laboratory Standards Institute (2013; 2015). Interpretative criteria were based on human data (sulphadimethoxine and tetracycline), dogs' data (ampicillin), and bovine mastitis (ceftiofur). The resistant category included those isolates categorized as either intermediate or resistant.

[3] NI = Not inhibited (growth at highest concentration tested).

Table 19 - Frequency distribution (%) of *Escherichia coli* belonging to phylogenetic groups B2 (n = 2), C (n = 4), D (n = 3) and E (n = 1) that were inhibited at each antimicrobial concentration. Only antimicrobials without susceptibility interpretative criteria for *E. coli* are presented. Isolates were identified from clinical mastitis cases in 19 dairy herds of Southeast, Brazil

Antimicrobial	Group[1]	Frequency (n) of isolates at each indicated MIC (µg/mL)												NI[2]
		0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
Erythromycin	B2	-	-	-	-	-	-	-	-	-	-	-	-	2
	C	-	-	1	-	-	1	-	-	-	-	-	-	2
	D	-	-	-	-	-	-	-	-	-	-	-	-	3
	E	-	-	-	-	-	-	-	-	-	-	-	-	1
Oxacillin	B2	-	-	-	-	1	-	-	-	-	-	-	-	1
	C	-	-	-	-	2	-	-	-	-	-	-	-	2
	D	-	-	-	-	-	-	-	-	-	-	-	-	3
	E	-	-	-	-	-	-	-	-	-	-	-	-	1
Penicillin/Novobiocin	B2	-	-	-	-	-	-	-	-	-	-	-	-	2
	C	-	-	-	-	-	1	1	-	-	-	-	-	2
	D	-	-	-	-	-	-	2	-	-	-	-	-	1
	E	-	-	-	-	-	-	-	-	-	-	-	-	1
Penicillin	B2	-	-	-	-	-	-	-	-	-	-	-	-	2
	C	-	-	-	-	-	-	-	-	-	-	-	-	4
	D	-	-	-	-	-	-	-	-	-	-	-	-	3
	E	-	-	-	-	-	-	-	-	-	-	-	-	1
Pirlimycin	B2	-	-	-	-	-	-	-	-	-	-	-	-	2
	C	-	-	2	-	-	-	-	-	-	-	-	-	2
	D	-	-	-	-	-	-	-	-	-	-	-	-	3
	E	-	-	-	-	-	-	-	-	-	-	-	-	1

[1] Phylogenetic groups identified according to Clermont et al. (2013).
[2] NI = Not inhibited (growth at highest concentration tested).

4.4.4 Survival function analysis

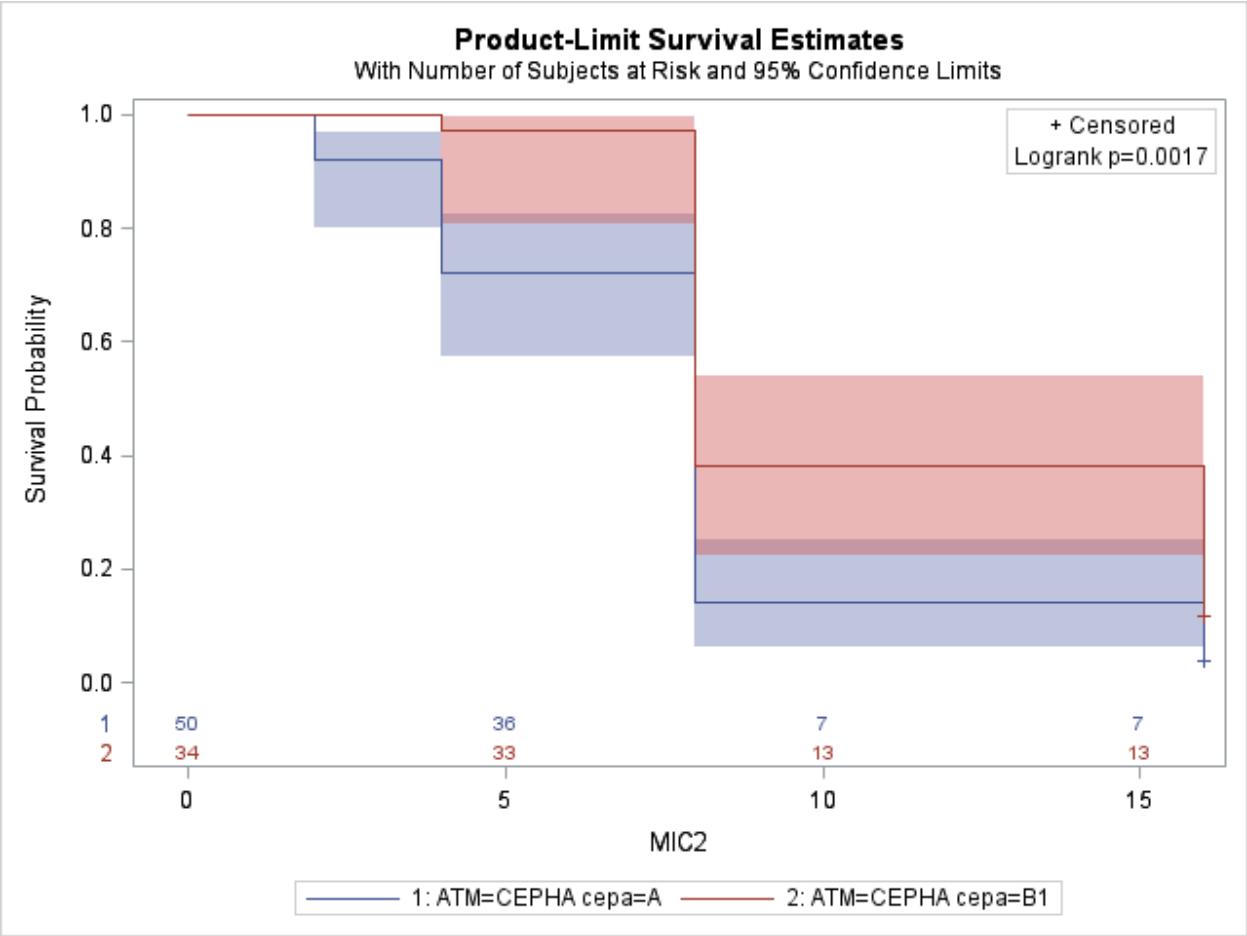
Kaplan-Meier survival curves were produced for each antimicrobial for comparison of susceptibility among the phylogenetic groups A and B1. Homogeneous survival curves were observed for all evaluated antimicrobials (Log-Rank > 0.25; Wilcoxon > 0.29), except for cephalothin (Log-Rank > 0.002; Wilcoxon > 0.0007; Table 20). Survival curves showed heterogeneity among phylogenetic groups for cephalothin, indicating that a lower concentration of this antimicrobial was needed to inhibit isolates of the phylogroup A in comparison with the phylogroup B1 (Figure 7).

Table 20 - Results of survival analysis comparing the antimicrobial susceptibility of *Escherichia coli* isolated from clinical mastitis cases identified in 19 dairy herds and stratified by the phylogeny groups A (n = 50) and B1 (n = 34)

Antimicrobial	Phylogenetic group	Censor[1]	Log-Rank[2]	Wilcoxon[3]
Ampicillin	A	5	0.92	0.93
	B1	5		
Ceftiofur	A	0	0.35	0.60
	B1	1		
Cephalothin	A	2	0.002	0.0007
	B1	4		
Erythromycin	A	46	0.33	0.33
	B1	33		
Oxacillin	A	42	0.25	0.29
	B1	25		
Penicillin/Novobiocin	A	36	0.93	0.94
	B1	24		
Penicillin	A	46	0.72	0.72
	B1	32		
Pirlimycin	A	47	0.62	0.62
	B1	31		
Sulphadimethoxine	A	10	0.49	0.39
	B1	8		
Tetracycline	A	15	0.73	0.65
	B1	10		

[1] Number of isolates censored (observed growth at the highest antimicrobial drug concentration of the used test).
[2] Log-Rank test for equality of strata at higher antimicrobial concentrations.
[3] Wilcoxon test for equality of strata at lower antimicrobial concentrations.

Figure 7 - Kaplan-Meier survival curves for 84 *Escherichia coli* isolated from CM in 19 dairy herds, according to cephalothin susceptibility testing and stratified by phylogenetic group A (n = 50; blue line) or phylogenetic group B1 (n = 34; pink line)



4.5 DISCUSSION

To our knowledge, this is the first study evaluating the phylogeny and antimicrobial susceptibility of *E. coli* isolated from bovine CM in Brazil. The distribution of *E. coli* isolated from CM into phylogenetic groups was completed for isolates identified in 19 dairy herds located in the states of São Paulo and Minas Gerais. The classification of *E. coli* causing IMI into phylogenetic groups can assist veterinary epidemiologists in further studies to better understand the pathogenicity mechanisms of this micro-organism during mastitis. Furthermore, better epidemiological understanding of *E. coli* causing mastitis may be beneficial for development of specific strategies for treatment and prevention of this pathogen in dairy herds.

In our study, 90% of *E. coli* isolates were assigned to phylogroups A (58%) and B1 (32%). A small proportion (10%) of isolates were assigned to phylogroups B2 (2.0%), C (4.0%), D (3.0%) and E (1.0%). In addition, no isolates were identified as belonging to group F. High frequency of phylogroups A and B1 were also observed in other studies evaluating the phylogeny of *E. coli* isolated from CM (GHANBARPOUR; OSWALD, 2010; LIU et al., 2014; KEANE, 2016). In a recent study conducted in Ireland, 54% of *E. coli* isolates belonged to phylogenetic group A, while 32% were assigned to phylogroup B1 (KEANE, 2016). Similarly, in a study conducted in dairy herds in Iran, 44.9% of *E. coli* isolates were assigned to phylogroup A and 35.6% to group B1 (GHANBARPOUR; OSWALD, 2010).

Phylogenetic groups A and B1 were chiefly associated with commensal strains of *E. coli* in dairy cows (HOUSER et al., 2008; SON; VAN KESSEL; KARNS, 2009), which may explain the highest prevalence of this groups among isolates recovered from bovine IMI in our and other studies (KEMPF et al., 2016). Results found in our study also suggest that the most of isolates are commensals, having the gastro-intestinal tract of dairy cows as the main source of infection. Thus, these pathogens could be better controlled with management practices (e.g., maintenance of clean and dry beds) aiming to reduce the risk of teat contamination with feces that may be accumulated in the farm facilities (HOGAN, JOE; SMITH, 2012)

Although phylogroups A and B1 are the most frequently reported in bovine mastitis, differences in distribution frequencies among groups were also reported. Suojala et al. (2011), evaluating the phylogeny and antimicrobial susceptibility of *E. coli* isolated from CM, reported

that the majority (82.6%) of isolates belonged to phylogroup A, followed by group D (11.1%), B1 (4.9%), and B2 (1.4%). In another study, 58.6% of the isolates belonged to phylogroup B1, 35.7% to group A, and 5.7% to group D; none of the isolates was found to belong to other groups. Factors such as diet, housing type, environmental hygiene, contamination with feces of human and animals of other species, and geographical location may be associated with the differences of *E. coli* genotypic diversity between studies (GORDON; COWLING, 2003).

Although the high prevalence of isolates belonging to phylogroups A and B1, other less frequently isolated phylogroups (B2, C, D and E) were also identified in our study, which indicate their potential to cause CM in dairy cows. The phylogroups B2 and D were also described in other reports evaluating *E. coli* isolated from IMI. Isolates belonging to group B2 had also low frequency (1.4%) in a Finnish study evaluating isolates recovered from CM (SUOJALA et al., 2011). Similarly, another study found that only 2 out of 30 *E. coli* isolates pertained to phylogroup B2 (DOGAN et al., 2012). On the other hand, phylogroup D was frequently reported in studies about mastitis, being described in 11.1% (second most frequent phylogroup) of isolates in the study of Suojala et al. (2011); in 16.4% of isolates recovered from CM in dairy herds of Iran (GHANBARPOUR; OSWALD, 2010); and in 14% of isolates in a Irish study (KEANE, 2016). Phylogenetic analyses have shown that virulent extra-intestinal strains isolated from humans were more likely to be members of phylogroups B2 or D than A or B1 (PICARD et al., 1999; JOHNSON; STELL, 2000).

Some phylogroups identified in our study were not described (phylogroup C) or had low frequency in other studies describing their relationship with IMI in dairy cows (phylogroup E; KEMPF et al, 2016). Because many phylogenetic studies are based on the initial rapid determination scheme purposed by Clermont et al. (2000), which only allows to distinguish isolates among four phylogenetic groups (A, B1, B2, and D), the frequency of phylogroup C and E may have been underestimated in previous studies. Using the quadruplex PCR method, it is possible to assign *E. coli* into eight phylogroups: seven belonging to *E. coli sensu stricto* (A, B1, B2, C, D, E, F), whereas the eighth is the *Escherichia* cryptic clade I (CLERMONT et al., 2013).

Mastitis-associated strains were found across five (A, B1, B2, D and E) out seven *E. coli sensu strictu* groups (KEMPF et al., 2016). However, to the best of our knowledge, this is the first study reporting *E. coli* of phylogroup C as the cause of CM. In other studies, strains belonging to phylogroup C were associated with intestinal and extra-intestinal infections in humans

(CLERMONT et al., 2011), and with fecal samples collected from diarrheal calves (SOUTO et al., 2017). Only four isolates were assigned to the phylogroup C in our study, and few isolates were assigned to the phylogroups B2, D and E. Therefore, further studies with a larger number of strains could better clarify whether these less frequently identified phylogroups in our study are only opportunistic pathogens, or important groups associated with IMI in dairy cows.

Parity was the only evaluated variable associated with the phylogeny of *E. coli* in our study. Primiparous cows were more likely to be infected by strains belonging to the group A than group B1. No biological explanation was found to explain this result; however, it could be attributed to factors at the cow-level such as better immune competence of primiparous cows against *E. coli* belonging to group B1 than group A; or the higher risk of teat contamination in primiparous cows with strains from group B1 than group A, especially in herds that segregate the cows based on parity. However, this result should be evaluated with caution as only 13 *E. coli* isolates were found in primiparous cows; studies with a larger sample size could provide a different outcome.

None of the other factors or descriptors evaluated in our study were associated with the phylogeny groups. As both phylogroups A and B1 have specific virulence factors (LIU et al., 2014), it could be speculated that differences in the mastitis severity could be attributed to the phylogenetic group; however, no differences among the phylogroups were observed in relation to the severity score of CM in our study. Likewise, another study found no association between phylogenetic groups (or virulence factors) with the clinical signs of acute CM cases (SUOJALA et al., 2011). Other studies also reported that severity of *E. coli* mastitis is not associated with any specific known virulence factor (WENZ et al., 2006; DYER et al., 2007). Furthermore, a review on *E. coli* CM reported that clinical signs are mainly determined by cow factors, rather than specific features of the bacterial strain (BURVENICH et al., 2003).

Escherichia coli isolates in our study were submitted for analysis of antimicrobial susceptibility for determination of the minimal concentration capable to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of isolates. The results of antimicrobial susceptibility revealed that a high proportion of *E. coli* isolates were considered resistant to most of antimicrobials tested. Overall, more than 96% of strains were resistant to ampicillin, and more than 23% were considered resistant to cephalothin, sulphadimethoxine or tetracycline. A direct comparison between studies cannot be easily performed, because several factors can affect the susceptibility results, such as: susceptibility testing methods (disc diffusion vs. broth dilution method); concentration of

antimicrobials used in MIC analysis, and differences in the interpretive criteria used for categorizing isolates as susceptible and resistant.

Only two studies were found reporting the same commercial panel as used in the present study for antimicrobial susceptibility testing of *E. coli* strains (RAJALA-SCHULTZ et al., 2004; HOE; RUEGG, 2005). In comparison to our study, the study of Rajala-Schultz et al. (2004) reported lower frequency of resistance to ampicillin (44.4% *vs.* 96.8% in our study), but higher resistance percentages to the rest of antimicrobial tested. Although a higher resistance to ampicillin observed in our study, the MIC_{50} was lower (2 μg/mL) in comparison to the aforementioned report (4 μg/mL). Lower MIC_{50} were also observed in our study for sulphadimethoxine (64 *vs.* >256 μg/mL) and tetracycline (4 *vs.* 8 μg/mL); similar MIC_{50} between studies were observed for ceftiofur (0.5 μg/mL) and cephalothin (8 μg/mL). Furthermore, our results showed lower MIC_{90} for ceftiofur (0.5 μg/mL) and cephalothin (16 μg/mL) than observed in the study of Rajala-Schultz et al. (2004), in which the MIC_{90} was 2.0 μg/mL for ceftiofur and >16 μg/mL for cephalothin. Hoe and Ruegg (2005) evaluating *in vitro* antimicrobial susceptibility of pathogens (including *E. coli*) isolated from cows with CM reported an overall susceptibility of *E. coli* similar to our results, however for ampicillin 91% of isolates were susceptible in the aforementioned study, while only 3.2% of isolates in our study was susceptible to this antimicrobial. The difference in the MIC results for ampicillin between studies is due to different interpretive criteria for classification of the isolates as susceptible or resistant; we used 0.25 μg/mL as cut-off (criteria used for dog's skin and soft tissue), while a higher limit (8.0 μg/mL) was used in the study of Hoe and Ruegg (2005), which was based on human criteria.

Results of MIC from other studies that used different susceptibility testing methods could only be partially compared to our study. Suojala et al. (2011) evaluated VetMIC™ microdilution method (SVA Uppsala, Sweden) at different ranges of antimicrobials, and reported the same MIC as observed in our study for ampicillin and ceftiofur, but lower MIC_{50} for tetraclycline (2.0 μg/mL). Tark et al. (2017) evaluated the antimicrobial susceptibility of 374 strains of *E. coli* isolated from bovine mastitic milk in South Korea from 2012 to 2015 using a different Sensititer® broth dilution panel (KRNV4F; Trek Diagnostic Systems, West Sussex, UK). Four antimicrobials were present in both studies (our and South Korean), and for three of them (ampicillin, ceftiofur and cephalothin), the MICs were similar; however, the MIC_{50} for tetracycline was lower than observed in our study (4 μg/mL). Differences in the MIC between studies may be related to the variability

in the frequency of antimicrobial use in dairy herds, genotypic diversity, and presence of antimicrobial resistance genes in the evaluated strains (SRINIVASAN et al., 2007).

High levels of antimicrobial resistance (>70%) were also observed for erythromycin, oxacillin, penicillin, penicillin associated with novobiocin, and pirlimycin. However, these antimicrobials are not recommended for treatment of Gram-negative bacteria, and no interpretive criteria are available for susceptibility analysis of *E. coli* strains, therefore, a high resistance was expected. Similar outcomes were observed in other studies (RAJALA-SCHULTZ et al., 2004; HOE; RUEGG, 2005).

Most of strains (96.8%) were susceptible to ceftiofur and similar results were reported in other studies. Bengtsson et al. (2009), reported that the concentration of 0.5 μg/mL of ceftiofur inhibited 96.3% of 163 *E. coli* strains isolated from acute bovine CM. In another study 100% of *E. coli* strains isolated from CM were susceptible to ceftiofur at a concentration of 0.5 μg/mL (THOMAS et al., 2015). Ceftiofur is a third-generation cephalosporin which acts by inhibiting bacterial cell wall synthesis of both Gram-negative and Gram-positive bacteria (SCHUKKEN et al., 2011). Since this antimicrobial has been marketed to be administered by the intramammary route, it has been used extensively for treatment of CM in dairy cattle (SAINI et al., 2012b; OLIVEIRA; RUEGG, 2014). Results of a study comparing the efficacy of two cephalosporins for treatment of nonsevere CM showed that cows treated with ceftiofur had a higher bacteriological cure for CM caused by Gram-negative (79%) than cows treated with cephapirin sodium (50%), which is a first-generation cephalosporin (SCHUKKEN et al., 2013).

Kaplan-Meier curves and survival analysis was used in our study to determine whether the phylogroups A and B1 had different MICs for the evaluated antimicrobials. In general, in survival analysis it is assumed that events may occur over a continuous scale, such as time (CORTINHAS et al., 2013). In our study, we assumed that the MIC determined by broth microdilution was the continuous variable, which allowed us to compare the shape of survival curves of susceptibility test. An important feature of this method is the possibility of inclusion in the analysis of those isolates that were not inhibited at the highest concentration.

Heterogeneous survival curve was observed in our study only for cephalothin, in which strains belonging to phylogroup A were inhibited at lower antimicrobial concentrations than strains assigned to the phylogroup B1. For the remaining antimicrobials, the curves comparing the antimicrobial susceptibility were homogeneous between phylogroups A and B1. Studies

evaluating the relationship of phylogenetic groups and susceptibility to antimicrobials are few in the indexed literature (LIU et al., 2014; KEANE, 2016). Furthermore, studies using survival analysis to compare the susceptibility of antimicrobials among phylogenetic groups were not found. A recent study conducted in Ireland also found a high prevalence of strains belonging to phylogroups A and B1, however, no relationship between antimicrobial resistance and phylogenetic group was observed. In another study, 70 *E. coli* isolates recovered from clinical and subclinical mastitis were characterized with respect to their phylogenetic group, virulence factors and antimicrobial susceptibility (LIU et al., 2014). The results of this study showed that 67.1% of isolates were resistant to streptomycin, and those from group B1 were more resistant to this antimicrobial than isolates from group A. The authors also reported that isolates belonging to phylogroup B1 contained more virulence genes than isolates of group A, which could be related with potential differences in antimicrobial resistance among phylogroups.

One limitation of our study was the low sample size for evaluation of descriptors at the cow- or herd-level and phylogenetic groups, especially for less frequent phylogroups. In addition, further studies evaluating the antimicrobial susceptibility and the presence of resistance genes for these less frequently identified phylogroups are needed to better understand their epidemiology and possible role in the etiology of mastitis. However, this is the first study evaluating the phylogeny and the susceptibility of *E. coli* strains isolated from CM in dairy herds of Brazil; the results observed in this report may be used as the reference for further studies evaluating the pharmaco-epidemiology and pathogenicity of *E. coli* causing IMI in dairy cows.

4.6 CONCLUSION

Escherichia coli isolates associated with CM mainly belonged to phylogenetic groups A and B1. Phylogenetic groups isolated less frequently included strains belonging to groups B2, C, D and E. Parity was the only cow-level descriptor associated with the phylogeny, with group A being more prevalent in primiparous cows than group B1. Resistance was observed for all evaluated antimicrobials; however, *E. coli* isolates were more susceptible to antimicrobials pertaining to the cephalosporin class. Finally, strains belonging to group A1 were inhibited at lower cephalothin concentration than strains assigned to phylogroup B1.

Chapter 5

Molecular characterization and antimicrobial susceptibility pattern of *Streptococcus agalactiae* and *Streptococcus uberis* isolated from clinical mastitis in dairy cattle

5 MOLECULAR CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF *STREPTOCOCCUS AGALACTIAE* AND *STREPTOCOCCUS UBERIS* ISOLATED FROM CLINICAL MASTITIS IN DAIRY CATTLE

5.1 ABSTRACT

The objectives of this study were to genotypically characterize *Strep. agalactiae* and *Strep. uberis* isolates recovered from cases of clinical mastitis (CM) in dairy cows; and to determine the association of antimicrobial susceptibility and genotypes of these *Streptococcus* species. A total of 89 isolates of each species identified from bovine CM cases were subtyped using randomly amplified polymorphic DNA (RAPD) analysis. In addition, the antimicrobial susceptibility of the isolates was determined using a commercial broth microdilution test composed of 10 antimicrobials. Descriptive analysis was used to report the frequency of subtypes and genotypic clusters within herd, housing system, season and CM severity scores. The minimal inhibitory concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were calculated for each antimicrobial, and survival analysis was completed to verify the differences between the antimicrobial susceptibility among genotypic clusters. Results of RAPD showed a great genotypic diversity for both *Strep. agalactiae* (45 RAPD-types) and *Strep. uberis* (56 RAPD-types). Subtypes of *Strep. agalactiae* were clustered into three groups (Ia, Ib and II), while *Strep. uberis* strains were clustered in two groups (I and II) according to their genetic similarity. A high similarity, based on the clusters created, was observed within- and between herds for both evaluated species. Overall, *Strep. agalactiae* showed high susceptibility to most antimicrobials, except to tetracycline and erythromycin. Differences in the antimicrobial susceptibility among clusters of *Strep. agalactiae* were observed for ampicillin, ceftiofur, erythromycin, pirlimycin, sulphadimethoxine and tetracycline. In contrary, *Strep. uberis* were categorized as resistant to most antimicrobials, except to cephalothin and penicillin+novobiocin. Although some differences in the MIC_{50} and MIC_{90} were found among genotypic clusters of *Strep. uberis*, homogeneous curves were observed among clusters for all antimicrobials in the survival analysis. In conclusion, *Strep.*

agalactiae is still highly susceptible to most antimicrobials, although differences in susceptibility were observed among genotypic clusters for some antimicrobial agents. On the other hand, *Strep. uberis* isolated from CM cases were resistant to most antimicrobials, and no differences were found in susceptibility among genotypic clusters.
Key-words: Antimicrobial susceptibility. Genotyping. Intramammary infection. *Streptococcus* spp.

5.2 INTRODUCTION

Streptococcus is one of the most important genus of bacteria in the mastitis epidemiology, which includes both contagious and environmental species with potential to cause clinical and subclinical IMI. In a previous study performed by our research group, *Strep. agalactiae* and *Strep. uberis* accounted for approximately 23% of CM cases with positive culture results (Chapter 2; Table 2).

Streptococcus agalactiae is a contagious pathogen which has the mammary gland as the main reservoir. The transmission of this pathogen occurs mainly from cow-to-cow via milking units, liners, milker's hands or towels of common use (KEEFE, 1997; KEEFE, G., 2012). As the consequence of implementation of specific control programs aiming to reduce contagious mastitis in dairy herds, the prevalence of *Strep. agalactiae* have become very rare in North America and some countries in Europe (PIEPERS et al., 2007; REYHER et al., 2011; OLIVEIRA; HULLAND; RUEGG, 2013). However, this pathogen is still an important cause of IMI in other regions, especially in countries of South America. For example, a study performed in Uruguay reported a cow-level prevalence of 11% of IMI caused by *Strep. agalactiae* (GIANNEECHINI et al., 2002). In Brazil, a study reported a herd-level prevalence of 60% (DUARTE et al., 2004), while in our study, it was the third most prevalent pathogen causing CM in 20 dairy herds (Chapter 2; table 2). Furthermore, a reemergence of *Strep. agalactiae* has been described in the Scandinavian countries, particularly Denmark (ZADOKS, R. N. et al., 2011; KATHOLM et al., 2012).

On the other hand, *Strep. uberis* is one of the most frequently isolated pathogens causing CM worldwide (RUEGG, 2012). This pathogen is considered as a major barrier to control of

bovine mastitis because its role in the epidemiology of this disease is not completed understood (ZADOKS, R. N. et al., 2003). While the environment seems to be the main reservoir of these pathogen, modern molecular analysis has provided evidences that the contagious transmission also occurs (RATO et al., 2008; ABUREEMA et al., 2014; LEELAHAPONGSATHON et al., 2016).

Molecular methods have been successfully used in studies aiming to understand better the epidemiology of *Streptococcus* spp. causing mastitis (ZADOKS, R. N. et al., 2011). Several DNA fingerprinting methods for the subtyping of *Strep. uberis* and *Strep. agalactiae* have been reported, which includes pulsed-field gel electrophoresis (PFGE) techniques (RATO et al., 2013; ABUREEMA et al., 2014), RFLP-PCR (MCDONALD; FRY; DEIGHTON, 2005), ribotyping (PITKALA; KOORT; BJORKROTH, 2008) and multilocus sequence typing (MLST; CARVALHO-CASTRO et al, 2017). In addition, random amplified polymorphic DNA (RAPD) PCR was used effectively in epidemiological studies evaluating the molecular characteristics of both *Strep. agalactiae* (MARTINEZ et al., 2000), and *Strep. uberis* (WIELICZKO et al., 2002; ZADOKS, R. N. et al., 2003). Some of the advantages of RAPD-PCR over other typing methods are the simplicity of procedure and low cost; while the major disadvantage is the inability to compare RAPD-types between studies, as the differentiation of strains is based on DNA fragment distribution on the agarose gel. However, this method has sufficient discriminatory power and acceptable reproducibility to characterize the genotypic diversity of *Strep. uberis* and *Strep. agalactiae* isolated from CM on different herds (ZADOKS et al., 2003).

Molecular epidemiology of *Strep. agalactiae* and *Strep. uberis* causing CM was reported in several countries (ZADOKS, R. N. et al., 2003; ABUREEMA et al., 2014; MAHMMOD et al., 2015; KACZOREK et al., 2017), but few studies addressed the molecular characterization of these species causing CM in dairy herds of Brazil (DUARTE et al., 2004; CARVALHO-CASTRO et al., 2017). Subtyping of bacteria can contribute to the epidemiological understanding of mastitis by the identification of particular strains and their specific characteristics, such as potential reservoirs, route of transmission, virulence, pathogenicity, and also, their relationship with conditions at the herd-level, such as housing system, management practices and season (ZADOKS et al., 2011). Therefore, as *Strep. uberis* and *Strep. agalactiae* are still important causes of IMI, studies evaluating their molecular characteristics can be useful in the development of programs for control and prevention of mastitis caused by these pathogens in dairy herds.

Although prevention strategies such as adoption of good milking procedures and increasing hygiene of cows and facilities can reduce the risk of new IMI caused by *Strep. agalactiae* and *Strep. uberis*, they do not eliminate these pathogens from the herd, especially if they are chronically infected cows. Therefore, the antimicrobial treatment of CM caused by these pathogens remains an important part of a mastitis control program (KEEFE, 2012). However, the non-judicious use of antimicrobials for treatment mastitis of dairy cattle have been associated with increased risk of antimicrobial resistance in both veterinary and human medicine (LEVY; MARSHALL, 2004; SARMAH; MEYER; BOXALL, 2006). Resistant strains of *Strep. uberis* and *Strep. agalactiae* causing bovine mastitis were reported in previous studies (RAJALA-SCHULTZ et al., 2004; DOGAN et al., 2005; SCHMITT-VAN DE LEEMPUT; ZADOKS, 2007). Besides the judicious use of antimicrobial for mastitis treatment, monitoring the antimicrobial susceptibility of these pathogens is also a component of the prevention strategies for reducing the development of resistant strains. The identification of strains of *Strep. uberis* and *Strep. agalactiae* causing IMI, and the determination of potentially antimicrobial resistance may help to improve the efficacy of CM therapy (KACZOREK et al., 2017). To our knowledge, few recent studies conducted in Brazil have reported the molecular epidemiology and antimicrobial susceptibility of *Strep. agalactiae* causing IMI (CARVALHO-CASTRO et al., 2017), and none has focused on the epidemiology and resistance profiles of *Strep. uberis.*

Therefore, the objectives of this study were to: (a) genotypically characterize *Strep. agalactiae* and *Strep. uberis* strains recovered from cases of CM in dairy cows; and, (b) determine the association of antimicrobial susceptibility and genotypes of these *Streptococcus* species.

5.3 MATERIAL AND METHODS

5.3.1 Bacterial isolates

During the field data collection described in chapter 2, *Streptococcus uberis* was identified in 256 cases of CM, while *Streptococcus agalactiae* was identified in 248 cases (Table 2). After

identification using microbiological culturing (NMC, 2017), the isolates were cryopreserved using the following procedures: (1) re-inoculation of a single colony on trypticase soy agar (TSA; BD, Sparks, MD, USA); re-suspension of one loop of cultured bacteria in 2 mL vials containing BHI (Becton, Dickinson and Company/BBL; Le Pont De Claix, France) supplemented with 10% glycerin; and (3) cryopreservation of vials at -80ºC until further analysis.

All isolates were cryopreserved, however, only 89 of each species were randomly preselected for analysis of genotypic diversity and antimicrobial susceptibility. This number of isolates was established because we aimed to analyze all isolates belonging to each species at the same time to avoid potential variation of the results that could happen if the isolates were segregated in more than one batch (GALLEGO; MARTINEZ, 1997); the agarose gel was produced with 96 wheels, but 6 of them were used for the DNA ladder (4 in each side and two in the center of the gel) and two were used for the positive and negative controls. Therefore, isolates were randomly selected after attending the following inclusion criteria: (a) be a new case (occurring in the same quarter of the same cow at least 14 days apart); (b) identified from cultures with identification of a single pathogen instead of mixed cultures; and (c) have a pure growth (without contamination) in the re-cultivation before further analysis. Because *Strep. uberis* identification by conventional culturing method could be subject to misidentification (WERNER et al., 2014), all *Strep. uberis* isolates were also identified using MALDI-TOF MS as described previously (Chapter 4). Only isolates with correct species identification and presenting MALDI scores greater than 2.0 were selected. *Streptococcus agalactiae* isolates were not submitted for MALDI-TOF MS because conventional microbiological culture could easily differentiate it from other species using the biochemical tests, such as presence of β-hemolysis, positive CAMP reaction, and negative reaction to sculling (NMC, 2017).

5.3.2 DNA extraction

All selected isolates were cultured overnight on blood agar plates at 37°C under aerobic conditions. Extraction of genomic DNA from pure cultures (i.e., no contamination) was performed with the Illustra bacteria genomicPrep mini spin kit (GE Healthcare, United Kingdom) following the manufacturer's guidelines.

5.3.3 RAPD typing: *Streptococcus agalactiae*

The amplification of the bacterial DNA was performed as described by Martinez et al. (2000), with some modifications. The PCR mixture consisted of 12.5 μL of Go Taq® Green Master Mix 2x (Promega), 1.25 μL of primer OPB-17 (5'-AGGGAACGA-3'; Exxtend Solução em Oligos, Campinas, Brazil), 5 μL of genomic DNA (20 ng/μL), and water q.s.p. to a final volume of 25 μL. The mixture was then submitted for thermocycling using the following program: one initial cycle of denaturation (5 minutes at 94ºC) in a DNA Thermal Cycler (Eppendorf Mastercycler Gradient, Hamburg, Germany); and 44 subsequent cycles consisting of denaturing at 94ºC for 30 seconds, annealing at 35ºC for 1 minute, and extension at 72ºC for 5 minutes. Ramp times were at 0.5ºC/s. A negative control, consisting of the same reaction mixture but with water instead of DNA, was included in the run. In addition, a positive control, with a template of DNA from a reference strain (*Strep. agalactiae* ATCC 13813), was also amplified.

All amplified products were electrophoresed at once in 1.5% agarose gel with 96 wells using Tris-borate-EDTA buffer (TBE; pH 8.3) at 150 V for 180 minutes. The agarose gel was stained with *SYBR Safe DNA Gel Stain* (1:10,000; Thermo Scientific®). Images of gel were taken under ultraviolet light using a photo-documentation system (Syngene, GeneGenius, Cambridge, United Kindom). Band sizes were determined by comparison to a 100-bp DNA ladder (Promega).

5.3.4 RAPD typing: *Streptococcus uberis*

Amplification of bacterial DNA using primer OPE-04 (5'-GTGACATGCC-3'; Exxtend Solução em Oligos, Campinas, Brasil) was done as described by Wieliczko et al. (2002), with some modifications: reactions contained 12.5 μL of Go Taq® Green Master Mix 2x (Promega), 1.25 μL of primer OPE-04, 5 μL of genomic DNA (20 ng/μL), and water q.s.p. to a final volume of 25 μL. The PCRs were performed in a DNA Thermal Cycler (Eppendorf Mastercycler Gradient, Hamburg, Germany). Cycling included an initial denaturation step at 94°C for 2 minutes, followed by 39 cycles at 94°C for 30 seconds; 33°C for 30 seconds; and 72°C for 2 minutes. Ramp times were at 0.5ºC/s.

All amplified products were electrophoresed at once in 2% agarose gel with 96 wells using TBE buffer (0.9 M Tris base, 0.09 M boric acid, 2.5 mM EDTA; pH 8.3) at 125 V for 55 min. The agarose gel was stained with *SYBR Safe DNA Gel Stain* (1:10,000; Thermo Scientific®). Images of gel were taken under ultraviolet light using a photo-documentation system (Syngene, GeneGenius, Cambridge, United Kindom). Band sizes were determined by comparison to a 100-bp DNA ladder (Promega).

5.3.5 Antimicrobial susceptibility testing

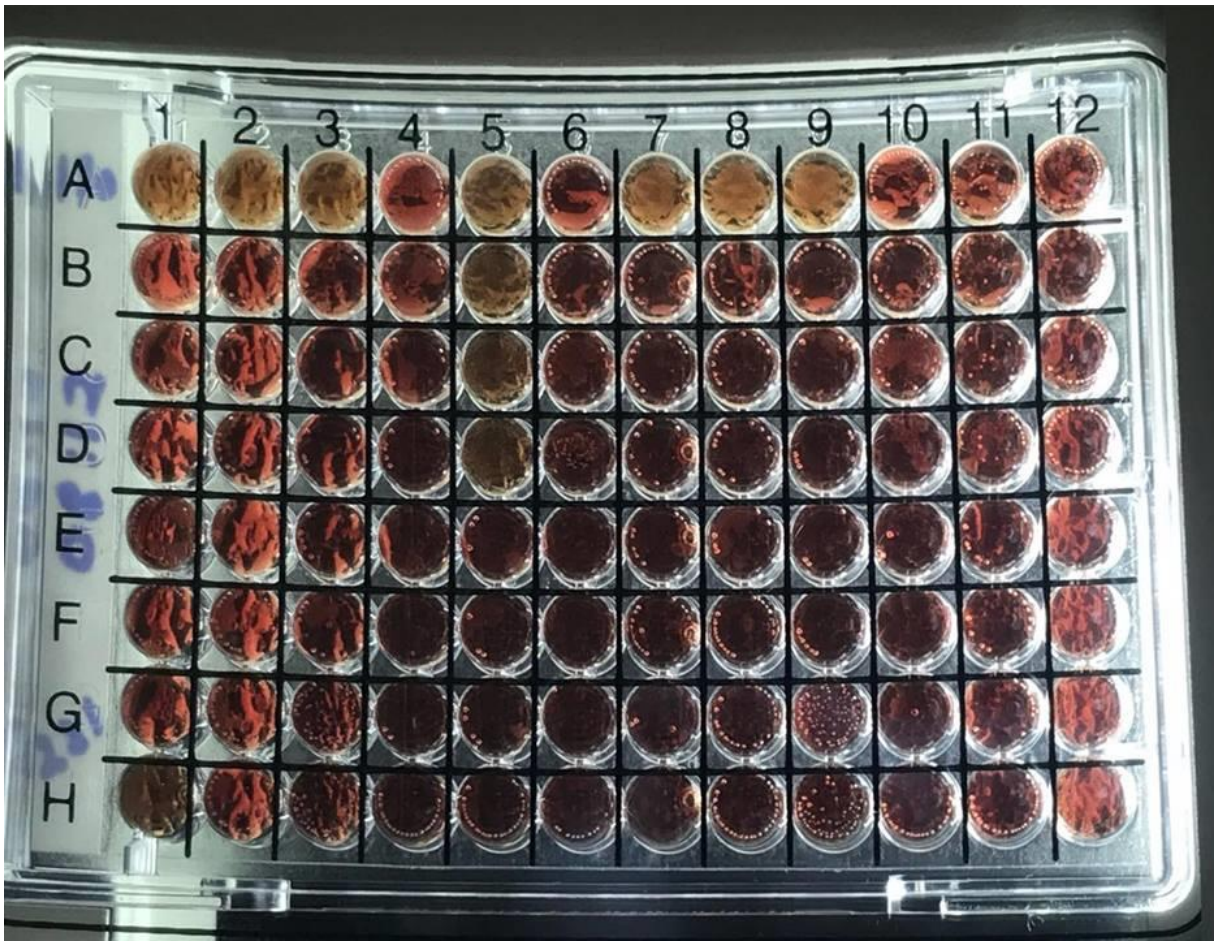
Antimicrobial susceptibility tests were performed in selected isolates using a mastitis panel of a commercial broth microdilution test (CMV1AMAF; Sensititre, TREK Diagnostics, Cleveland, OH), following guidelines of the Clinical and Laboratory Standards Institute (formerly NCCLS; CLSI, 2015). The antimicrobials agents and dilution ranges tested for each one of them were the same as described in chapter 4 (Table 11).

Briefly, cryopreserved isolates were thawed, inoculated on blood Agar and incubated at 37ºC for 24 hours for evaluation of bacteria viability. After, a single and pure colony was re-inoculated on trypticase soy agar (TSA; BD, Sparks, MD, USA) and incubated at 37ºC for 24 hours. After incubation, the isolates were suspended in 0.9% saline solution using disposable

sterile loops to approximate the density of a 0.5 McFarland standard. A DEN-1 McFarland Densitometer (Biosan, Riga, Latvia) was used for standardization of bacterial suspensions to the McFarland standard. Subsequently, 100 μL-aliquots of bacteria suspensions were transferred into a tube containing 11 mL of Mueller-Hinton broth (pH = 7.3 ± 1; BD, Sparks, MD, USA) supplemented with 5% of lysed horse blood, and the tube was mixed on a vortex for approximately 10 seconds.

Sensititre® panels were reconstituted with the bacteria inoculum (50 μL per wheel) using a multicanal pipette with disposable tips. Panels were covered with an adhesive seal, and incubated at 35 ºC for approximately 20-24 hours. After incubation, results were read using the Sensititre® manual viewer (Figure 8). Growth appeared as turbidity or as a deposit of cells at the bottom of a well. The MIC was recorded as the lowest concentration of the antimicrobial that inhibited visible growth. The growth control wells were read first, and if any of the control wells did not exhibit growth, the results were considered invalid. The lowest concentrations inhibiting 50 (MIC_{50}) and 90% (MIC_{90}) of isolated were determined. For each batch of microorganism tested, the NCCLS recommended quality control strains *Escherichia coli* ATCC 25922 and *Staph. aureus* ATCC 29213 were also evaluated.

Figure 8 – Reconstituted microdilution test for evaluation of antimicrobial susceptibility of *Streptoccus agalactiae* and *Streptococcus uberis* strains recovered from milk of dairy cows with clinical mastitis. Two isolates were analyzed per plate (one isolate was incubated from 1A to H6, and the other from A7 to H12). As expected, bacterial growth occurred in the control wells (A1, A2, A3 and A7, A8, A9), and for one of the isolates (on the left side of the plate), growth was observed in all wells containing tetracycline (A5, B5, C5 and D5), which demonstrates resistance of this isolate (*Strep. agalactiae*) to this antimicrobial.



5.3.6 Data analysis

The FREQ procedure of SAS 9.4 (SAS Inst. Inc., Cary NC) was used to determine the distribution of RAPD-types within herd, season, housing style, and severity score of CM; data were expressed as absolute numbers and percentages. For descriptive analysis, the bacterial isolates were classified in categories based on: herd from which the isolate was identified; CM

severity (**Mild** - changes only in the milk appearance; **Moderate** - presence of abnormal milk accompanied by changes in the udder; or, **Severe** - combination of abnormal milk, with signs of inflammation in the udder and systemic signs); season of CM diagnosis (**rainy** - October-March, or **dry** - April-September; OLIVEIRA et al., 2015); and housing system (compost-bedded pack barn, free stall, paddocks). The paddock housing system was characterized as an open area surrounded by fences or rails and without pasture for grazing.

For RAPD fingerprinting results, dendrograms and minimum spanning trees (MST) based on the intraspecific diversity and genetic relationship of *Streptococcus* spp. were constructed using BioNumerics software v. 6.6 (Applied Maths, Belgium). Dendrograms were generated by the unweighted pair group method with arithmetic mean (UPGMA) using the default configuration with both position tolerance and optimization of 1%. The Ward method was used for construction of the dendrogram based on the results of antimicrobial susceptibility and genetic diversity.

The MSTs were constructed based on a matrix of similarity distance between replicate genotypes, with the greatest distance between the branches, and with the isolate with the highest number of relationships in the center or node. The distance between the isolates was converted to a unit called distance bin size, in which the default is 1%. This means that two sequences with similarity of 100% - 99% will have a distance of 0, while two sequences with similarity 99% - 98% will have a distance of 1.

The antimicrobial susceptibility testing results were evaluated based on the minimal concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates. Where interpretive cut-points for MIC of the evaluated antimicrobials have been established for *Strep. agalactiae* and *Strep. uberis* (Clinical and Laboratory Standards Institute; CLSI, 2013; 2015), these were used to classify each isolate as susceptible, intermediate or resistant. Isolates identified as intermediate were interpreted as resistant.

Survival analysis using the PROC LIFETEST of SAS (SAS Inst. Inc., Cary NC) was completed to verify the differences between the antimicrobial susceptibility among genotypic clusters. The concentrations of antimicrobials contained in the commercial broth microdilution test were used as the “time” variable, and the inhibition of bacterial growth was used as the event. Isolates that presented growth at the highest tested concentration were censored by the statistical model. Kaplan-Meier survival curves of the RAPD-types clustered based on their genetic relationship were performed for each antimicrobial studied. The null hypothesis of no differences

(homogeneity of survival curves) in the survivor functions of the strata (RAPD-clusters) was evaluated using Log-Rank and Wilcoxon tests. Statistical significance (i.e., heterogeneous survival curves) was defined at $P \leq 0.05$.

5.4 RESULTS

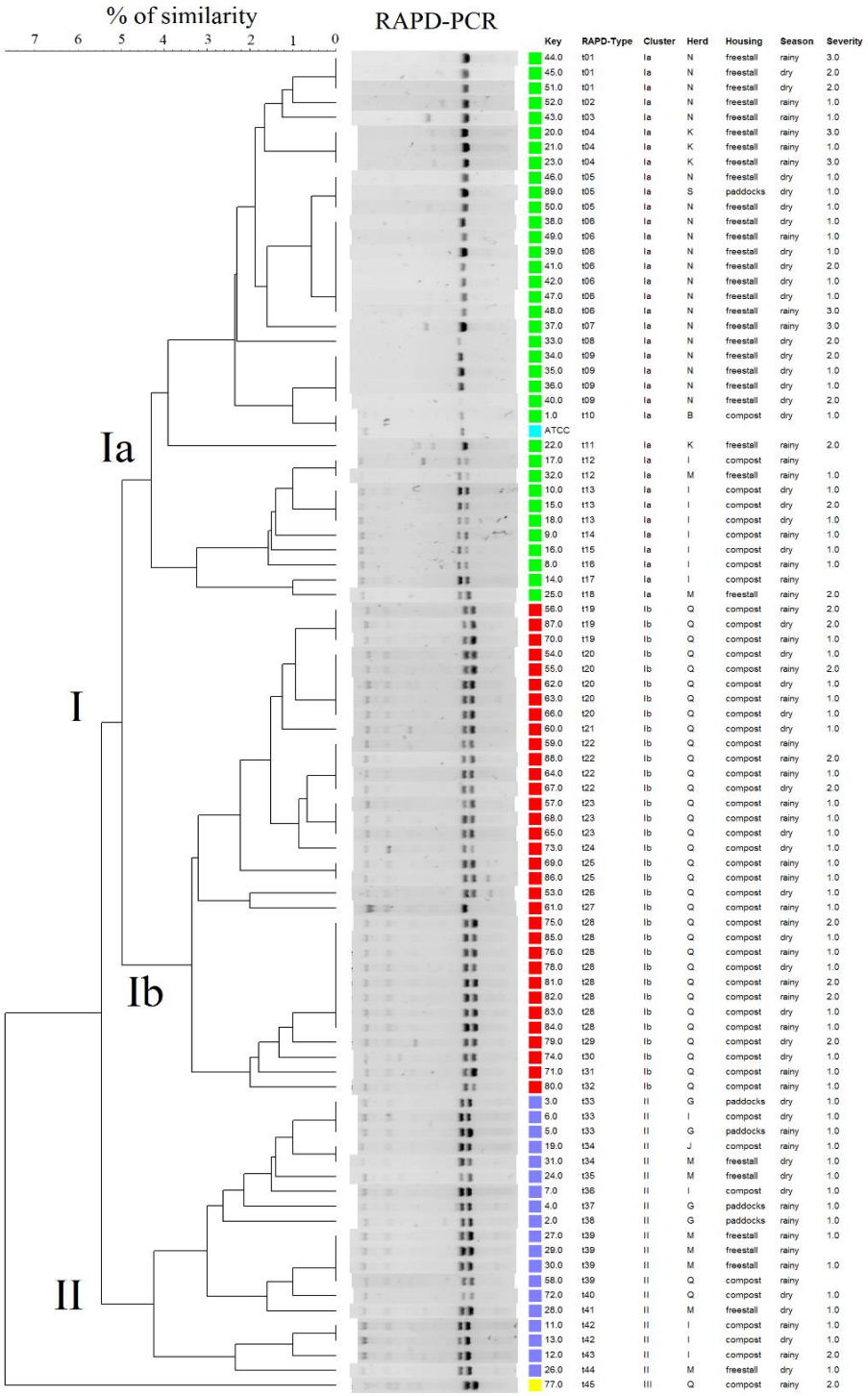
5.4.1 *Streptococcus agalactiae*

5.4.1.1 Descriptive results and RAPD typing

A total of 89 *Strep. agalactiae* isolates were recovered from milk of 62 dairy cows diagnosed with CM in nine dairy herds located in the states of São Paulo (n = 7; 79 isolates) and Minas Gerais (n = 2; 10 isolates). Five herds housed lactating cows in compost-bedded pack barns (CBPB), three in freestalls, and two in paddocks.

The genotypic diversity of *Strep. agalactiae* isolates was determined using the RAPD fingerprinting method. In total, 45 RAPD-types were identified in our study (Figure 9). Because of the high level of polymorphism observed among isolates, we clustered them into two genotype groups according to their similarity (clusters I and II; Figure 9). Cluster I was composed of 69 isolates (77.5%), while the cluster II had 19 isolates (21.3%). For statistical purposes, cluster I was divided into two subgroups (Ia and Ib) according to their genetic similarity within the cluster I; therefore, cluster Ia had 36 isolates, and cluster Ib had 33 isolates. In addition, one isolate (named here as t45; Figure 9) was not clustered into groups I and II because it had a lower level of similarity in comparison to other isolates.

Figure 9 – Dendrogram showing the genetic relationship between *Streptococcus agalactiae* isolates (n = 89) from bovine clinical mastitis, as estimated by clustering analysis of (RAPD-PCR) profiles, and its respective herd of origin, housing type, season and sever severity score. Three clusters were created based on the genetic relationship of isolates (Ia in green; Ib in red; and II in blue). The dendrogram was generated by the unweighted pair group method with arithmetic averages.



A total of 46 *Strep agalactiae* isolates were identified from 19 cows that had repeated CM cases; 11 cows had isolates from two cases, and 8 cows had isolates from three repeated cases of CM. Seven cows had CM caused by the same RAPD-type found in the first case, while 12 cows had CM caused by different RAPD-types. However, only two out of the 19 cows presenting repeated cases of CM had infections caused by isolates pertaining to a different cluster.

All *Strep. agalactiae* isolates belonging to cluster Ib (n = 33) were from the same herd (named here as Q, Table 21). Cluster Ia was the most prevalent and was identified in six out of nine herds. Cluster II was identified in 5 herds (Table 21). Herd N was the second herd with the highest number of isolates (n = 20), and all of them belonged to cluster Ia. A graphical illustration of the genetic relationship between isolates according to the herd of origin is presented in the Figure 10. The MST clearly shows the predominance of genotypically close-related types within herds.

Table 21 - Frequency of 89 *Streptococcus agalactiae* isolates from CM cases occurred in 9 dairy herds according to RAPD-clusters (Ia [n = 36], Ib [n = 33], and II [n = 19]), herd of origin, housing system, season and severity score of clinical mastitis

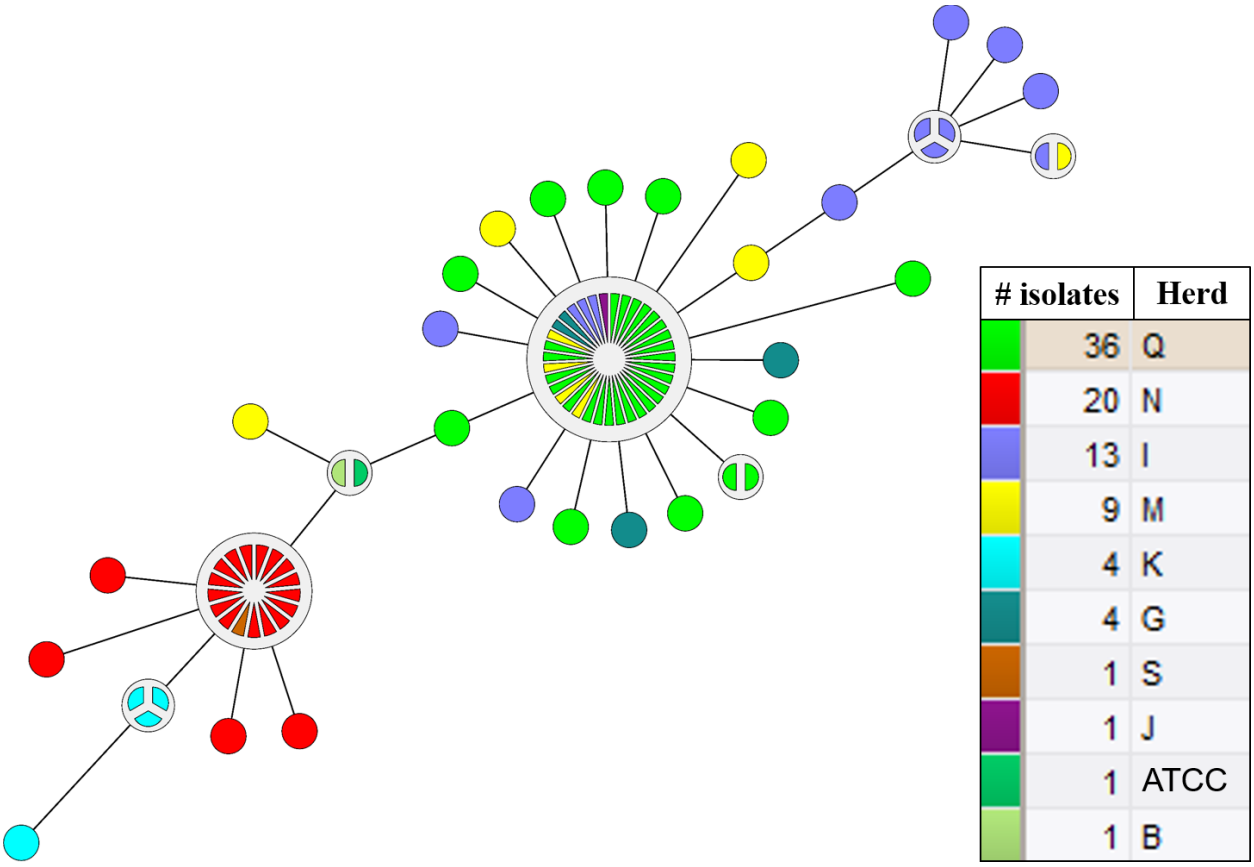
Variable	Categories	RAPD-clusters						t 45	
		Ia		Ib		II			
		n	%	n	%	n	%	n	%
Herd	B (n = 1)	1	100	-	-	-	-	-	-
	G (n = 4)	-	-	-	-	4	100	-	-
	I (n = 13)	8	61.6	-	-	5	38.4	-	-
	J (n = 1)	-	-	-	-	1	100	-	-
	K (n = 4)	4	100	-	-	-	-	-	-
	M (n = 9)	2	22.2	-	-	7	77.8	-	-
	N (n = 20)	20	100	-	-	-	-	-	-
	Q (n = 36)	-	-	33	91.7	2	5.6	1	2.7
	S (n = 1)	1	100	-	-	-	-	-	-
Housing	Freestall (n = 33)	26	78.8	-	-	7	21.2	-	-
	CBPB[1] (n = 51)	9	17.6	33	64.7	8	15.7	1	2.0
	Paddocks (n = 5)	1	20.0	-	-	4	80.0	-	-
Season[2]	Rainy (n = 46)	16	34.8	19	41.3	10	21.7	1	2.2
	Dry (n = 43)	20	46.5	14	32.6	9	20.9	-	-
CM severity[3]	Mild (n = 59)	20	33.9	23	39.0	16	27.1	-	-
	Moderate (n = 20)	9	45.0	9	45.0	1	5.0	1	5.0
	Severe (n = 5)	5	100.0	-	-	-	-	-	-

[1] CBPB – Compost bedded pack barn

[2] Rainy season (October-March); Dry season (April-September)

[3] CM severity - (Mild) changes only in the milk appearance; (Moderate) presence of abnormal milk accompanied by changes in the udder; or, (Severe) combination of abnormal milk, with signs of inflammation in the udder and systemic signs)

Figure 10 - Minimum spanning tree (MST) of 89 *Streptococcus agalactiae* isolates identified from clinical mastitis and stratified according to their herd of origin (n = 9). All isolates were analyzed by random amplification of polymorphic DNA (RAPD) PCR. A reference strain (ATCC 13813) was also included in the analysis as a positive control.



A total of 33 *Strep. agalactiae* isolates were identified from cows housed in freestals (26 belonged to cluster Ia, and 7 to cluster II), 51 from cows housed in CBPB (9 belonged to cluster Ia, 33 to cluster Ib, and, 8 to cluster II), and 5 isolates were identified from cows housed in paddock systems (1 isolate belonged to cluster Ia, and the other 4 were assigned to cluster II; Table 21).

In relation to the frequency of *Strep. agalactiae* isolates according to season, 46 (51.7%) isolates were identified from CM cases occurred in the rainy season. Of these, 16 (34.8%) belonged to cluster Ia, 19 (41.3%) to cluster Ib, and 10 (21.7%) to cluster II. For the isolates identified during the dry season (n = 43), 20 (46.5%) belonged to cluster Ia, 14 (32.6%) to cluster Ib, and 9 (20.9%) to cluster II (Table 21).

A total of 59 (70.2%) *Strep. agalactiae* isolates were recorded as causing mild CM cases, while 20 (23.8%) were associated with moderate, and 5 (6.0%) with severe CM cases. For those isolates identified from mild CM cases, 20 (33.9%) isolates belonged to cluster Ia, 23 (39.0%) to cluster Ib, and 16 (27.1%) to cluster II. For isolates identified in moderate CM cases, 9 (45.0%) belonged to cluster Ia, 9 (45.0%) to cluster Ib, and 1 (5.0%) to cluster II. The RAPD-type 45 was also isolated from a moderate case of CM. Finally, all isolates identified from severe cases (n = 5) belonged to cluster Ia (Table 21).

5.4.1.2 Antimicrobial susceptibility testing

Overall evaluation

A total of 87 (97.8%) *Strep. agalactiae* isolates had results of antimicrobial susceptibility (Figure 11); two isolates were excluded from data analysis because of contamination during microdilution testing. Antimicrobial susceptibility results of the RAPD-type “t45”, which was not clustered into groups I and II was not included in data analysis. For the other 86 isolates, overall results on the antimicrobial susceptibility are shown in Tables 22 and 23. Isolates had high susceptibility to ampicillin (98.3%), ceftiofur (97.7%), cephalothin (96.5%), oxacillin (96.5%), penicillin+novobiocin (100%), penicillin (97.7%), pirlimycin (83.7%) and sulphadimethoxine (98.3%). On the other hand, lower susceptibility results were observed for erythromycin (70.9%) and tetracycline (31.4%; Table 22 and 23).

Figure 11 – Dendrogram resulting from a computer-assisted analysis of the RAPD profiles of *Strep. agalactiae* isolates (n = 87) recovered from milk of cows with clinical mastitis, according to results of susceptibility to 10 antimicrobials. The interpretation criteria to categorize the RAPD-types as resistant (black boxes) or susceptible (without boxes) were based on recommendations of CLSI (2013; 2015)

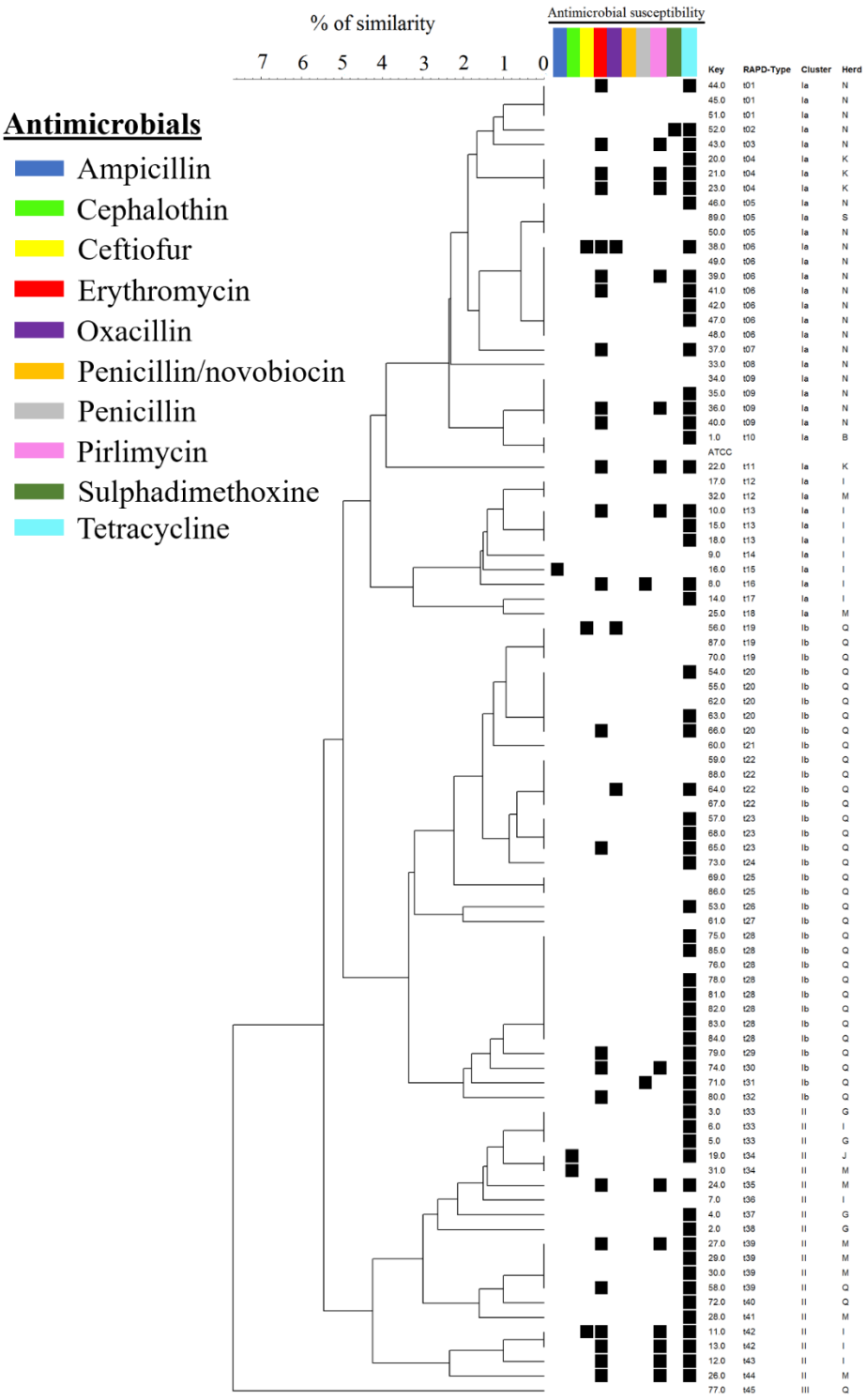


Table 22 – Frequency (overall and by RAPD-clusters) of *Streptococcus agalactiae* classified as susceptible (S) or resistant (R), according to the interpretation criteria described by the Clinical and Laboratory Standards Institute (CLSI, 2013; 2015)

Antimicrobial	CLSI criteria[1]	RAPD-clusters			Overall
		Ia (n = 35)	Ib (n = 32)	II (n = 19)	
Ampicillin	R	-	-	1 (5.3)	1 (1.7)
	S	35 (100)	32 (100)	18 (94.7)	85 (98.3)
Ceftiofur	R	2 (5.7)	-	-	2 (2.3)
	S	33 (94.3)	32 (100)	19 (100)	84 (97.7)
Cephalothin	R	-	2 (6.2)	1 (5.3)	3 (3.5)
	S	35 (100)	30 (93.8)	18 (94.7)	83 (96.5)
Erythromycin	R	1 (2.9)	22 (68.7)	2 (10.6)	25 (29.1)
	S	34 (97.1)	10 (31.3)	17 (89.4)	61 (70.9)
Oxacillin	R	-	2 (6.2)	1 (5.3)	3 (3.5)
	S	35 (100)	30 (93.8)	18 (94.7)	83 (96.5)
Penicillin+Novobiocin	R	-	-	-	-
	S	35 (100)	32 (100)	19 (100)	86 (100)
Penicillin	R	2 (5.7)	-	-	2 (2.3)
	S	33 (94.3)	32 (100)	19 (100)	84 (97.7)
Pirlimycin	R	-	14 (43.7)	-	14 (16.3)
	S	35 (100)	18 (56.3)	19 (100)	72 (83.7)
Sulphadimethoxine	R	1 (2.9)	-	-	1 (1.7)
	S	34 (97.1)	32 (100)	19 (100)	85 (98.3)
Tetracycline	R	26 (74.3)	30 (93.7)	3 (15.8)	59 (68.6)
	S	9 (25.7)	2 (6.3)	16 (84.2)	27 (31.4)

[1]S = susceptible; R = resistant

The results of MIC are presented in Table 23. For all antimicrobials, except for two (sulphadimethoxine and tetracycline), the MIC_{50} was observed at the lowest antimicrobial concentration contained in the microdilution test. The MIC_{50} for sulphadimethoxine was 64 μg/mL, while more than 50% of the isolates were not inhibited at the highest tetracycline concentration contained in the microdilution test. The antimicrobial concentration needed to inhibit 90% of all tested isolates were: ampicillin (0.25 μg/mL), ceftiofur (1 μg/mL), cephalothin (2 μg/mL), erythromycin (4 μg/mL), oxacillin (2 μg/mL), penicillin+novobiocin (1 μg/mL), penicillin (0.12 μg/mL), and suphadimethoxine (256 μg/mL). For both pirlimycin and tetracycline, determination of MIC_{90} was not possible because more than 10% of isolates had growth at the highest antimicrobial concentration present in the microdilution panel (Table 23).

Table 23 – Overall frequency (%) of *Streptococcus agalactiae* isolates (n = 86) that had 50% (MIC50) and 90% (MIC90) of bacterial growth inhibited at each antimicrobial concentration. All isolates were identified from clinical mastitis cases occurred in 9 dairy herds of Southeast, Brazil

Antimicrobial	Frequency (%) of isolates at each indicated MIC (μg/mL)[1]												NI[2]	MIC_{50}[3]	MIC_{90}[4]
	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256			
Ampicillin	67.8	31.1	1.1	-	-	-	-	-	-	-	-	-	-	0.12	0.25
Ceftiofur	-	-	87.5	9.2	1.1	1.1	-	-	-	-	-	-	1.1	0.5	1
Cephalothin	-	-	-	-	96.6	2.3	-	-	-	-	-	-	1.1	2	2
Erythromycin	-	71.3	5.7	5.7	3.5	4.6	-	-	-	-	-	-	9.2	0.25	4
Oxacillin	-	-	-	-	96.6	1.1	-	-	-	-	-	-	2.3	2	2
Penicillin+Novobiocin	-	-	-	100.0	-	-	-	-	-	-	-	-	-	1	1
Penicillin	97.7	2.3	-	-	-	-	-	-	-	-	-	-	-	0.12	0.12
Pirlimycin	-	-	82.9	1.1	-	1.1	-	-	-	-	-	-	14.9	0.5	>4
Sulphadimethoxine	-	-	-	-	-	-	-	-	23.0	46.0	23.0	6.9	1.1	64	256
Tetracycline	-	-	-	21.8	9.2	2.4	1.1	-	-	-	-	-	65.5	>8	>8

[1]The light gray shading represents the susceptible zone, and the darker gray shading represents the resistant zone. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2013; 2015). Interpretative criteria were based on human data (ampicillin, erythromycin, oxacillin, penicillin, sulphadimethoxine and tetracycline), dogs' data (cephalothin), and bovine mastitis (ceftiofur, penicillin/novobiocin and pirlimycin). The resistant category included those isolates categorized as either intermediate or resistant.

[2]NI = Not inhibited (growth at highest concentration tested).

[3]MIC (μg/mL) that inhibited 50% (MIC_{50}) of the isolates.

[4]MIC (μg/mL) that inhibited 90% (MIC_{90}) of the isolates.

Cluster evaluation

Different proportions between RAPD-clusters were observed in the classification of isolates as susceptible or resistant (i.e., only descriptive outcomes). For example, 97.1% of isolates belonging to cluster Ia and 89.4% of isolates belonging to cluster II were susceptible to erythromycin, while a lower proportion (31.3%) of isolates belonging to cluster Ib was susceptible to the same antimicrobial (Table 22). Furthermore, although 100% of isolates belonging to clusters Ia and II were classified as susceptible to pirlimycin, only 53.2% of isolates assigned to cluster Ib were susceptible to the same antimicrobial. For tetracycline, the susceptibility was 6.2% for isolates belonging to cluster Ib and 25.7% for cluster Ia; however, a higher proportion (84.3%) of susceptibility was observed for isolates belonging to cluster II (Table 22).

There was no difference in the MIC_{50} and MIC_{90} values among RAPD-clusters for cephalothin (2 μg/mL), oxacillin (2 μg/mL), penicillin+novobiocin (1 μg/mL) and penicillin (0.12 μg/mL; Table 24). However, differences in the MIC were observed for the other six antimicrobials evaluated. For example, the MIC_{90} of erythromycin for isolates belonging to cluster Ia was 0.25 μg/mL, which is the lowest concentration of this antimicrobial contained in the panel; however more than 10% of isolates belonging to clusters Ib were not inhibited at the highest antimicrobial concentration contained in the microdilution test. For pirlimycin, although 100% of isolates assigned to clusters Ia and II were inhibited at 0.5 μg/mL (lowest antimicrobial concentration), 40.6% of isolates belonging to cluster Ib were not inhibited at the highest antimicrobial concentration contained in the microdilution test (Table 24).

Table 24 - Frequency (%) of *Streptococcus agalactiae* isolates (n = 86) belonging to RAPD-clusters (Ia [n = 35], Ib [n = 32], and II [n = 19]) that had 50% (MIC50) and 90% (MIC90) of bacterial growth inhibited at each antimicrobial concentration. All isolates were identified from clinical mastitis cases occurred in 9 dairy herds of Southeast, Brazil

Antimicrobial	Cluster	Frequency (%) of isolates at each indicated MIC (μg/mL)[1]												NI[2]	MIC_{50}[3]	MIC_{90}[4]
		0.12	0.25	0.5	1	2	4	8	16	32	64	128	256			
Ampicillin	Ia	31.4	68.6	-	-	-	-	-	-	-	-	-	-	-	0.25	0.25
	Ib	100	-	-	-	-	-	-	-	-	-	-	-	-	0.12	0.12
	II	78.9	15.8	5.3	-	-	-	-	-	-	-	-	-	-	0.12	0.25
Ceftiofur	Ia	-	-	71.4	20.0	2.9	2.9	-	-	-	-	-	-	2.9	0.5	1.0
	Ib	-	-	100	-	-	-	-	-	-	-	-	-	-	0.5	0.5
	II	-	-	94.7	5.3	-	-	-	-	-	-	-	-	-	0.5	0.5
Cephalothin	Ia	-	-	-	-	100	-	-	-	-	-	-	-	-	2.0	2.0
	Ib	-	-	-	-	93.8	3.1	-	-	-	-	-	-	3.1	2.0	2.0
	II	-	-	-	-	94.7	5.3	-	-	-	-	-	-	-	2.0	2.0
Erythromycin	Ia	-	97.1	-	-	-	2.9	-	-	-	-	-	-	-	0.25	0.25
	Ib	-	31.2	12.5	12.5	9.4	9.4	-	-	-	-	-	-	25	1.0	>4
	II	-	89.5	5.3	5.2	-	-	-	-	-	-	-	-	-	0.25	0.50
Oxacillin	Ia	-	-	-	-	100	-	-	-	-	-	-	-	-	2.0	2.0
	Ib	-	-	-	-	93.8	-	-	-	-	-	-	-	6.2	2.0	2.0
	II	-	-	-	-	94.7	5.3	-	-	-	-	-	-	-	2.0	2.0

[1]The light gray shading represents the susceptible zone, and the darker gray shading represents the resistant zone. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2013; 2015). Interpretative criteria were based on human data (ampicillin, erythromycin, oxacillin, penicillin, sulphadimethoxine and tetracycline), dogs' data (cephalothin), and bovine mastitis (ceftiofur, penicillin/novobiocin and pirlimycin). The resistant category included those isolates categorized as either intermediate or resistant.
[2]NI = Not inhibited (growth at highest concentration tested).
[3]MIC (μg/mL) that inhibited 50% (MIC_{50}) of the isolates.
[4]MIC (μg/mL) that inhibited 90% (MIC_{90}) of the isolates.

Table 24 (cont.) - Frequency (%) of *Streptococcus agalactiae* isolates (n=86) belonging to RAPD-clusters (Ia [n = 35], Ib [n = 32], and II [n = 19]) that that had 50% (MIC_{50}) and 90% (MIC_{90}) of bacterial growth inhibited at each antimicrobial concentration. All isolates were identified from clinical mastitis cases occurred in 9 dairy herds of Southeast, Brazil

Antimicrobial	Cluster	Frequency (%) of isolates at each indicated MIC (μg/mL)[1]												NI[2]	MIC_{50}[3]	MIC_{90}[4]
		0.12	0.25	0.5	1	2	4	8	16	32	64	128	256			
Penic+Novob.	Ia	-	-	-	100	-	-	-	-	-	-	-	-	-	1.0	1.0
	Ib	-	-	-	100	-	-	-	-	-	-	-	-	-	1.0	1.0
	II	-	-	-	100	-	-	-	-	-	-	-	-	-	1.0	1.0
Penicillin	Ia	94.3	5.7	-	-	-	-	-	-	-	-	-	-	-	0.12	0.12
	Ib	100	-	-	-	-	-	-	-	-	-	-	-	-	0.12	0.12
	II	100	-	-	-	-	-	-	-	-	-	-	-	-	0.12	0.12
Pirlimycin	Ia	-	-	100	-	-	-	-	-	-	-	-	-	-	0.5	0.5
	Ib	-	-	53.2	3.1	-	3.1	-	-	-	-	-	-	40.6	0.5	>4
	II	-	-	100	-	-	-	-	-	-	-	-	-	-	0.5	0.5
Sulphadimet.	Ia	-	-	-	-	-	-	-	-	17.1	34.3	37.1	8.6	2.9	64	256
	Ib	-	-	-	-	-	-	-	-	34.3	56.3	6.3	3.1	-	64	64
	II	-	-	-	-	-	-	-	-	10.6	52.6	26.3	10.5	-	64	256
Tetracycline	Ia	-	-	-	17.1	8.6	-	2.9	-	-	-	-	-	71.4	>8	>8
	Ib	-	-	-	3.1	3.1	3.1	-	-	-	-	-	-	90.6	>8	>8
	II	-	-	-	63.2	21.1	5.3	-	-	-	-	-	-	10.5	1.0	>8

[1]The light gray shading represents the susceptible zone, and the darker gray shading represents the resistant zone. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2013; 2015). Interpretative criteria were based on human data (ampicillin, erythromycin, oxacillin, penicillin, sulphadimethoxine and tetracycline), dogs' data (cephalothin), and bovine mastitis (ceftiofur, penicillin/novobiocin and pirlimycin). The resistant category included those isolates categorized as either intermediate or resistant.

[2]NI = Not inhibited (growth at highest concentration tested).

[3]MIC (μg/mL) that inhibited 50% (MIC_{50}) of the isolates.

[4]MIC (μg/mL) that inhibited 90% (MIC_{90}) of the isolates.

5.4.1.3 Survival function analysis

Kaplan-Meier survival curves were produced for each antimicrobial for comparison of antimicrobial susceptibility among genotypic clusters. Homogeneous survival curves were observed for cephalothin (Log-Rank = 0.30; Wilcoxon = 0.34), oxacillin (Log-Rank = 0.21; Wilcoxon = 0.34), penicillin+novobiocin (Log-Rank = 1.0; Wilcoxon = 1.0), and penicillin (Log-Rank = 0.22; Wilcoxon = 0.22; Table 25). On the other hand, heterogeneous survival curves based on genotypic clusters were obtained for ampicillin (Log-Rank <0.0001; Wilcoxon <0.0001; Figure 12), ceftiofur (Log-Rank = 0.0001; Wilcoxon = 0.0001; Figure 13), erythromycin (Log-Rank <0.0001; Wilcoxon <0.0001; Figure 14), pirlimycin (Log-Rank <0.0001; Wilcoxon <0.0001; Figure 15), sulphadimethoxine (Log-Rank = 0.003; Wilcoxon <0.003; Figure 16), and tetracycline (Log-Rank <0.001; Wilcoxon <0.001; Figure 17; Table 25).

Table 25 – Results of survival analysis comparing the antimicrobial susceptibility of *Streptococcus agalactiae* isolated from clinical mastitis cases in 9 dairy herds and stratified by the RAPD-clusters Ia (n = 35), Ib (n = 32) and II (n = 19)

Antimicrobial	Log-Rank[1]	Wilcoxon[2]
Ampicillin	<0.0001	<0.0001
Ceftiofur	0.001	0.001
Cephalothin	0.30	0.34
Erythromycin	<0.0001	<0.0001
Oxacillin	0.21	0.34
Penicillin+Novobiocin	1.0	1.0
Penicillin	0.22	0.22
Pirlimycin	<0.0001	<0.0001
Sulphadimethoxine	0.003	0.003
Tetracycline	<0.0001	<0.0001

[1]Log-Rank test for equality of strata at higher antimicrobial concentrations.
[2]Wilcoxon test for equality of strata at lower antimicrobial concentrations.

Figure 12 - Kaplan-Meier survival curves for 86 *Streptococcus agalactiae* isolated from CM in 9 dairy herds, according to ampicillin susceptibility testing and stratified by RAPD-clusters (Ia [n = 35; pink line], Ib [n = 32; green line], and II [n = 19; blue line])

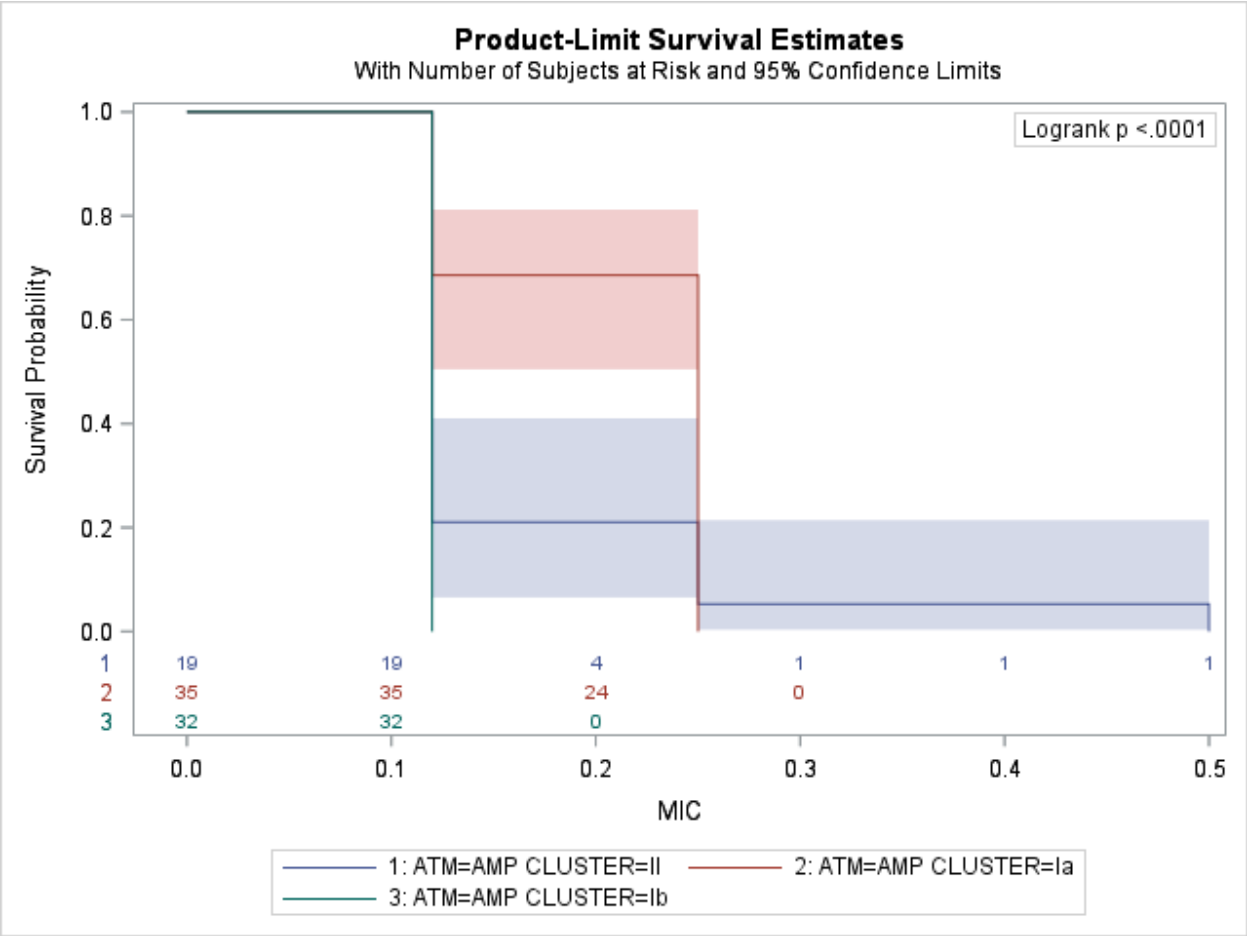


Figure 13 - Kaplan-Meier survival curves for 86 *Streptococcus agalactiae* isolated from CM in 9 dairy herds, according to ceftiofur susceptibility testing and stratified by RAPD-clusters (Ia [n = 35; pink line], Ib [n = 32; green line], and II [n = 19; blue line])

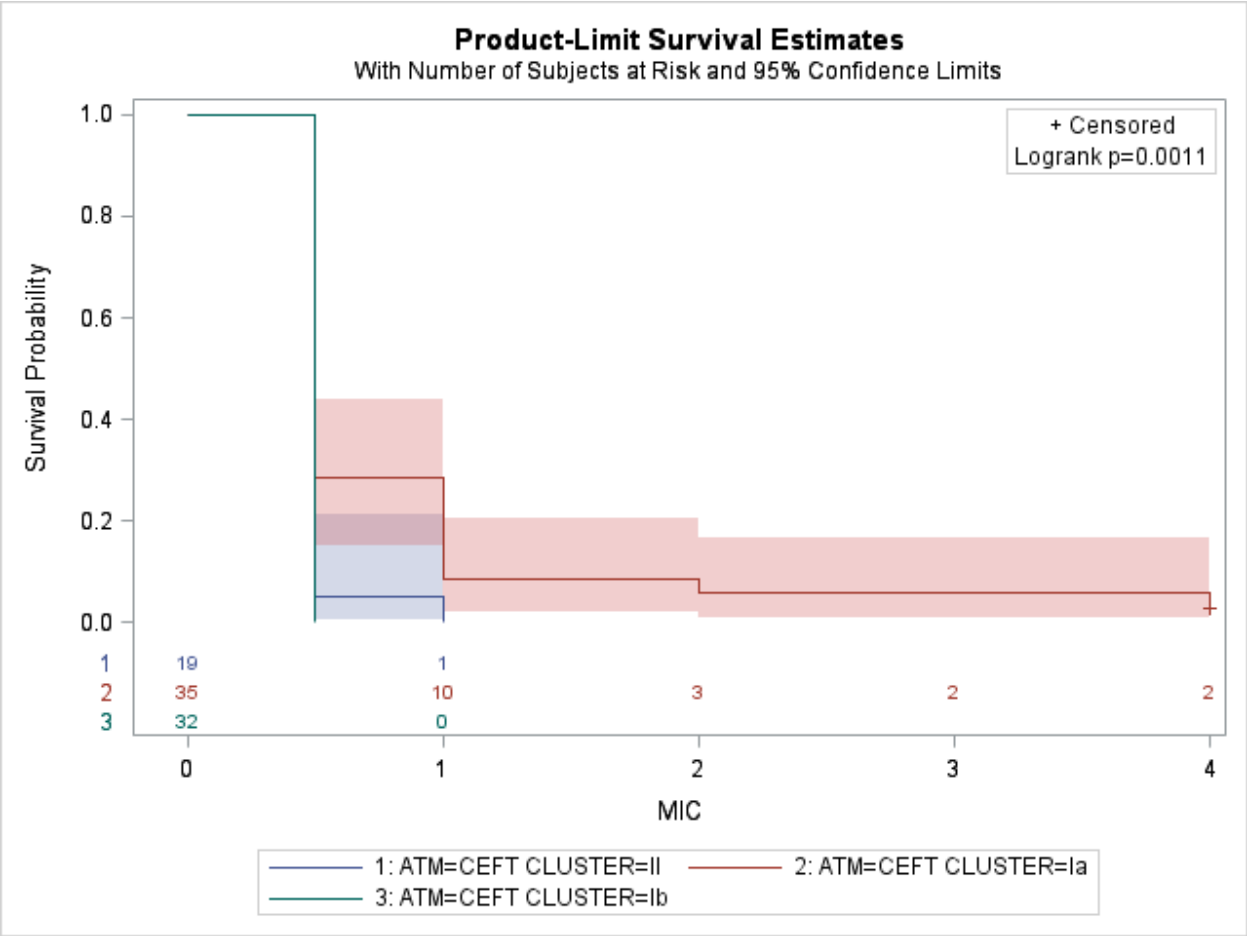


Figure 14 - Kaplan-Meier survival curves for 86 *Streptococcus agalactiae* isolated from CM in 9 dairy herds, according to erythromycin susceptibility testing and stratified by RAPD-clusters (Ia [n = 35; pink line], Ib [n = 32; green line], and II [n = 19; blue line]). Censored data are indicated on the right (cross)

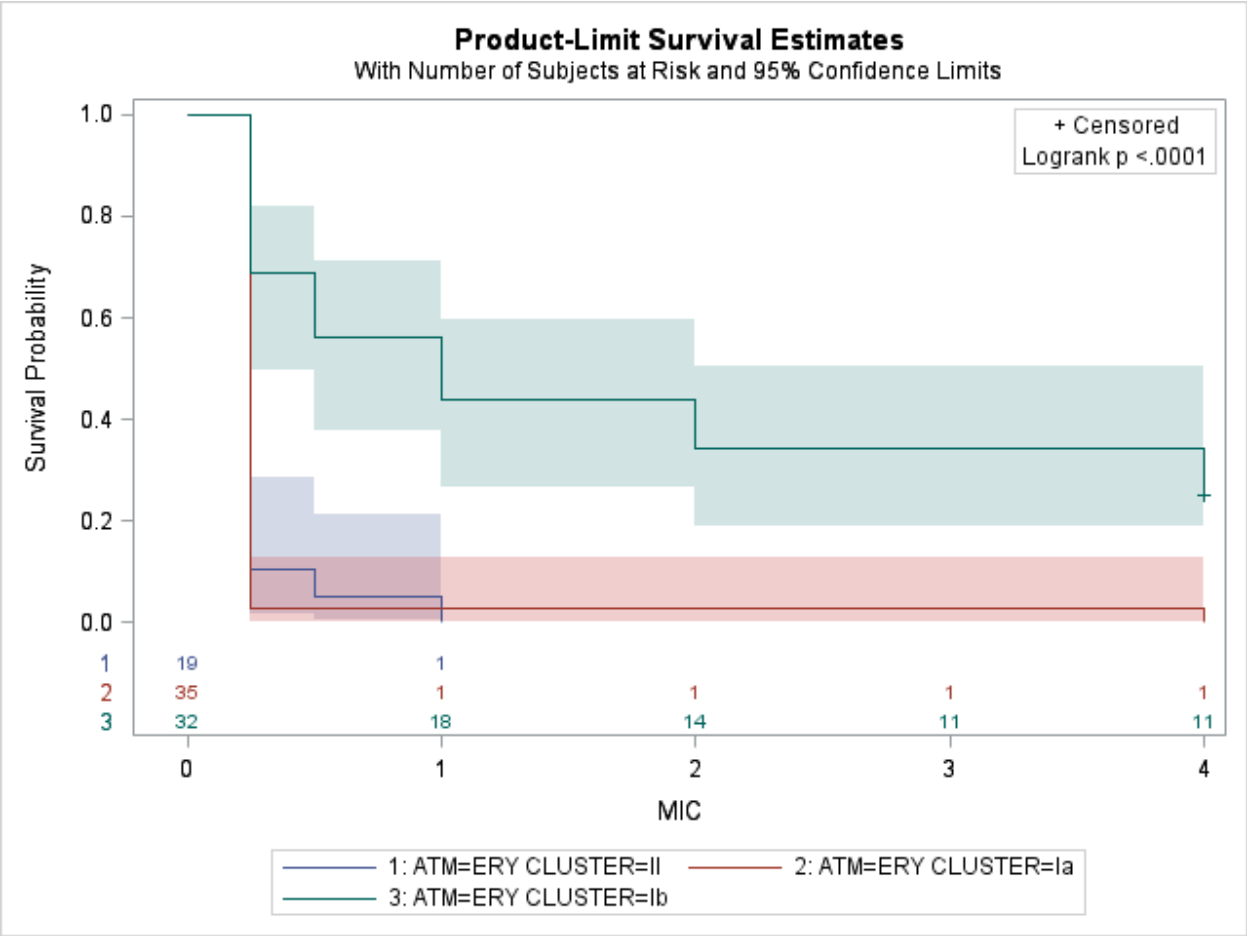


Figure 15 - Kaplan-Meier survival curves for 86 *Streptococcus agalactiae* isolated from CM in 9 dairy herds, according to pirlimycin susceptibility testing and stratified by RAPD-clusters (Ia [n = 35; pink line], Ib [n = 32; green line], and II [n = 19; blue line]. Censored data are indicated on the right (cross)

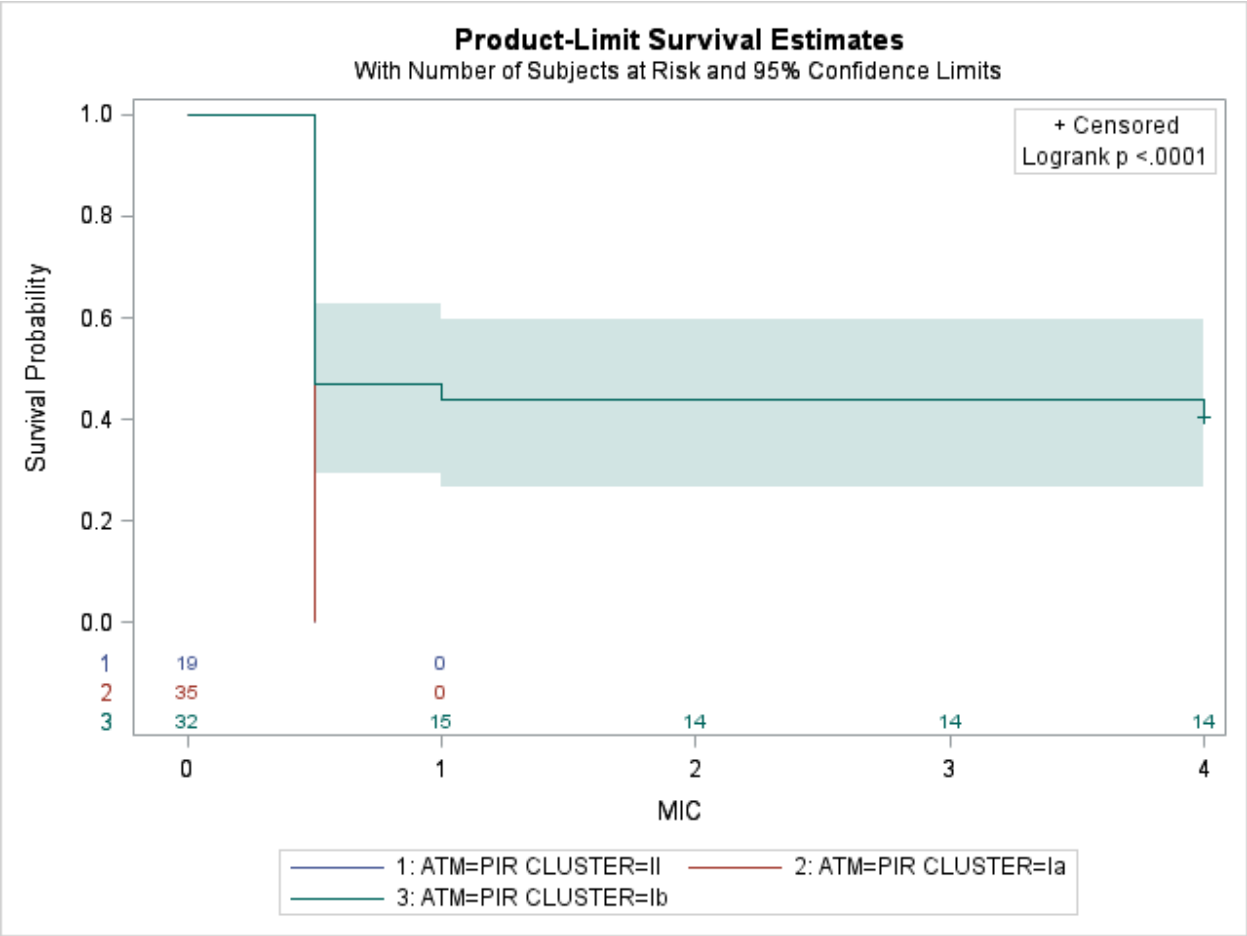


Figure 16 - Kaplan-Meier survival curves for 86 *Streptococcus agalactiae* isolated from CM in 9 dairy herds, according to sulphadimethoxine susceptibility testing and stratified by RAPD-clusters (Ia [n = 36; pink line], Ib [n = 33; green line], and II [n = 19; blue line]). Censored data are indicated on the right (cross)

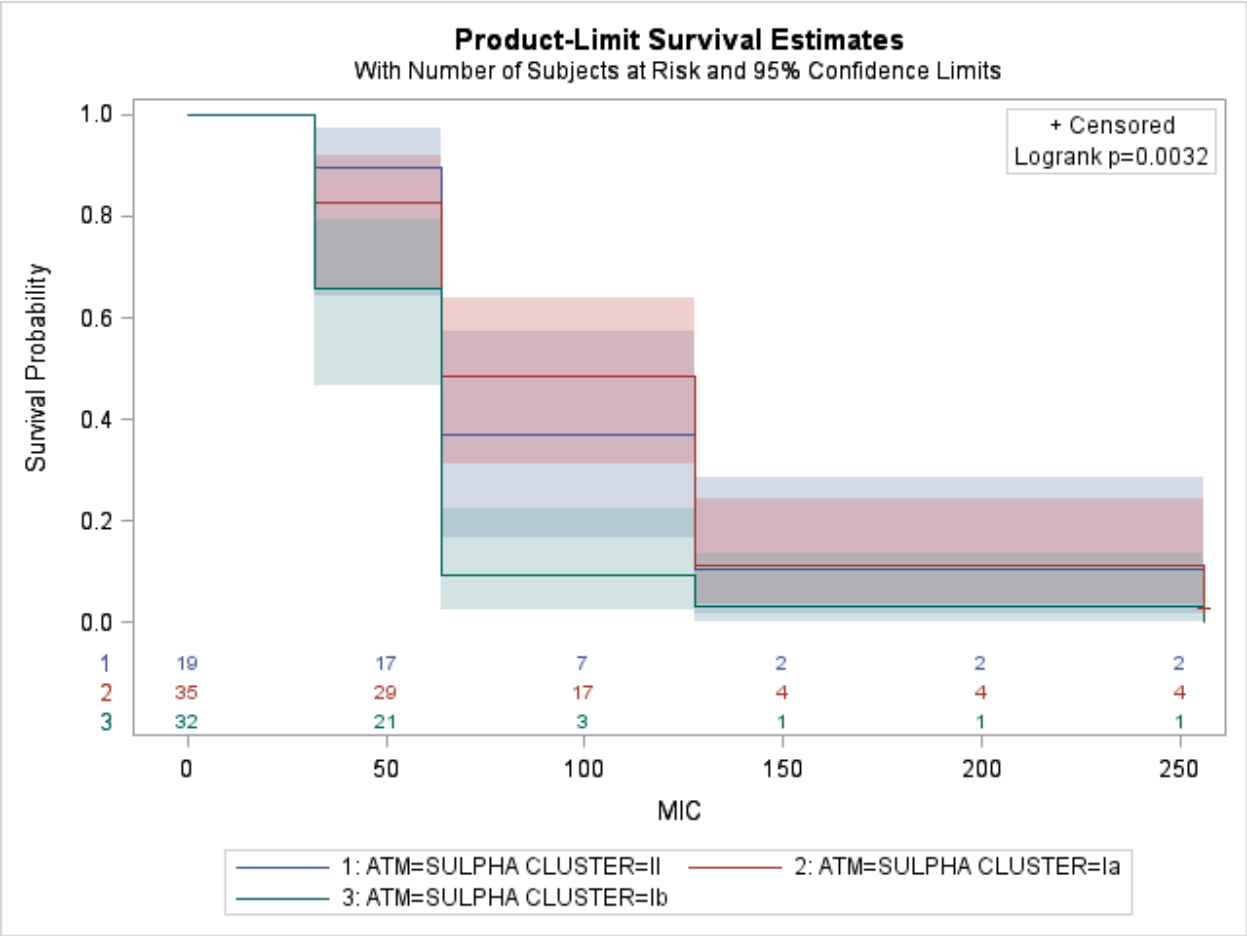
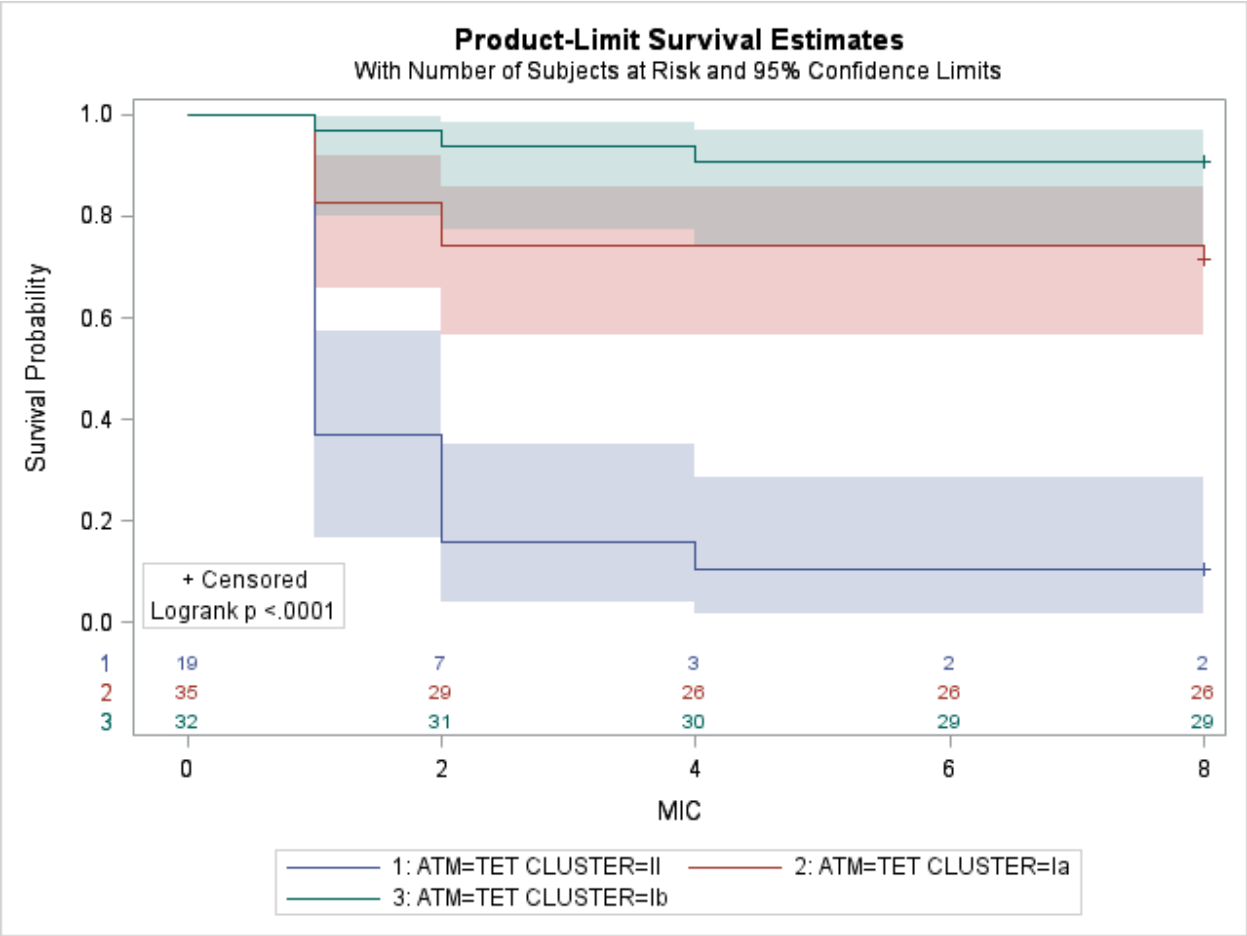


Figure 17 - Kaplan-Meier survival curves for 86 *Streptococcus agalactiae* isolated from CM in 9 dairy herds, according to tetracycline susceptibility testing and stratified by RAPD-clusters (Ia [n = 35; pink line], Ib [n = 32; green line], and II [n = 19; blue line]). Censored data are indicated on the right (cross)



5.4.2 *Streptococcus uberis*

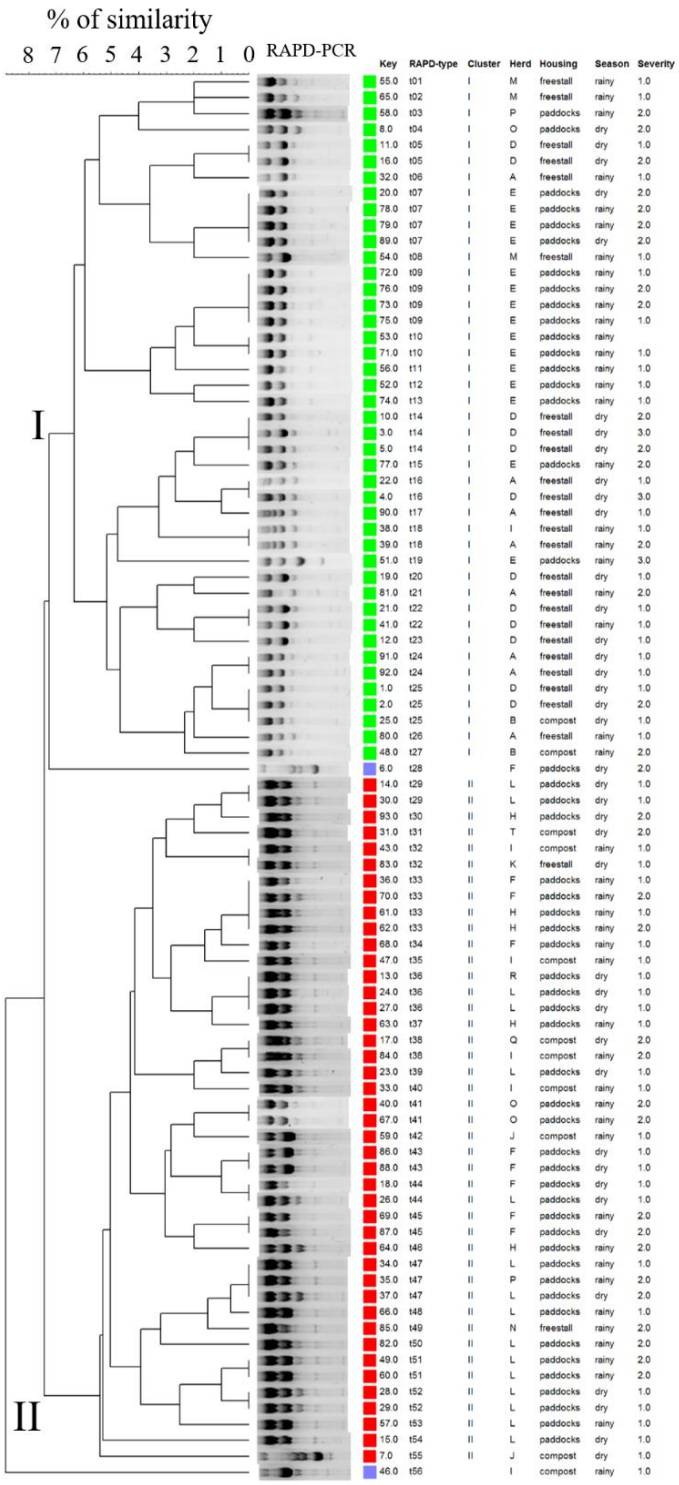
5.4.2.1 Descriptive results and RAPD typing

A total of 89 *Strep. uberis* isolates selected in this study were recovered from milk of 86 dairy cows with CM, which were distributed in 17 dairy herds located in the states of São Paulo (n = 12; 55 isolates) and Minas Gerais (n = 5; 34 isolates). One isolate was not amplified during the RAPD-PCR and was excluded from the study. Five herds housed their lactating cows in CBPB, seven in paddocks, and five in freestalls.

The genetic relationship among all RAPD patterns of *Strep. uberis* isolates is represented in the dendrogram shown in Figure 18. A high level of polymorphism was observed, as 56 RAPD-types were identified among the 88 isolates that were RAPD fingerprinted. All *Strep. uberis* isolates evaluated in our study shared more than 90% of similarity. Only two cows had isolates recovered from repeated cases of CM, and the same RAPD-type was observed only for one of them.

Because of the high level of polymorphism observed among isolates, and for statistical purposes, two clusters (I and II) were created based on the similarity of RAPD-types (Figure 18). Both clusters were composed of 43 isolates each. In addition, two isolates (named here as t28 and t46; Figure 18) were not assigned into clusters because it had a lower level of similarity in comparison to other isolates. Cluster I had 27 RAPD-types, and the two most frequent types (t) were t7 and t9, with only four isolates each. Cluster II was also composed of 27 RAPD-types, and the most frequent type was t33, with four isolates.

Figure 18 – Dendrogram showing Genetic relationship between *Streptococcus uberis* (n = 88) from bovine clinical mastitis, as estimated by clustering analysis of RAPD-PCR profiles, and its respective herd of origin, housing type, season and severity score. Two clusters were created based on the genetic relationship of isolates (cluster I in green; and cluster II in red). The dendrogram was generated by the unweighted pair group method with arithmetic averages.



A total of 61 (69.3%) *Strep. uberis* isolates were recovered from milk samples collected in 5 out of 17 evaluated herds. Except for two herds (O and P), a unique cluster was predominant among the isolates within herd (Table 26). A graphical illustration of the genetic relationship between isolates according to the herd of origin is presented in Figure 19. As we can see in the MST, isolates sharing the same color are mostly close to each other, which indicates the predominance of genotypically close-related strains within herd.

Most *Strep. uberis* isolates in this study were identified in dairy herds housing cows in paddocks (n = 51). Of these, 33.3% (n = 17) belonged to cluster I, while 64.7% (n = 33) belonged to cluster II. In addition, one isolate not included in any of the RAPD-clusters was also identified from a herd housing the lactating cows in paddocks. A total of 26 isolates were identified from cows housed in freestall, with 24 of them (92.3%) belonging to cluster I. Only 10 isolates were recovered from CM cases diagnosed in cows housed in CBPB (two belonged to cluster I, eight to cluster II, and one was identified as t46; Table 26).

Streptococcus uberis isolates selected in this study were well distributed among the rainy (n = 48; 54.5%) and dry seasons (n = 40; 45.5%). Likewise, the frequency of the isolates within the RAPD-clusters also had similar proportions among seasons. Finally, a total of 87 isolates had record of CM severity. Of these, 50 isolates (57.5%) were recorded as causing mild CM cases, while 34 (39.1%) were associated with moderate signs, and 3 (3.4%) with severe clinical signs. For those isolates identified from mild cases, 23 (46%) belonged to cluster I, 26 (52.0%) to cluster II, and one was the RAPD-type 46. For isolates identified from moderate CM cases, 16 (47.1%) belonged to cluster I, and 17 (50%) belonged to cluster II. The RAPD-type 6 was also isolated from a moderate case of CM. All isolates identified from severe cases (n = 3) belonged to cluster I (Table 26).

Table 26 - Frequency of 88 *Streptococcus uberis* isolated from CM cases of dairy cows distributed in 17 dairy herds according to RAPD-clusters, herd of origin, housing system, season and severity score of clinical mastitis

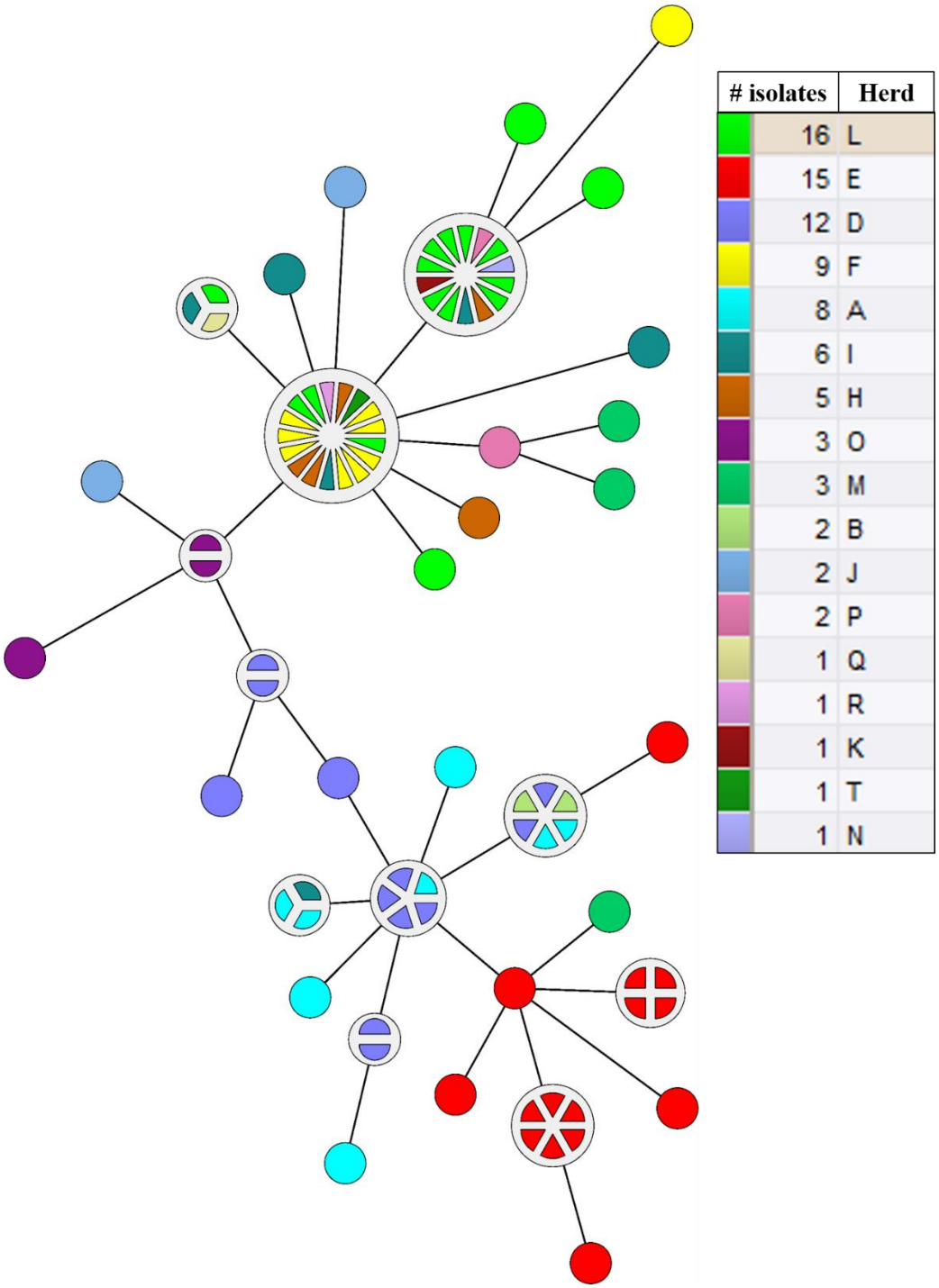
Variable	Categories	RAPD-clusters				RAPD-type 6		RAPD-type 46	
		I (n=43)		II (n = 43)					
		n	%	n	%	n	%	n	%
Herd	A (n = 9)	9	100	-	-	-	-	-	-
	B (n = 2)	2	100	-	-	-	-	-	-
	D (n = 12)	12	100	-	-	-	-	-	-
	E (n = 15)	15	100	-	-	-	-	-	-
	F (n = 9)	-	-	8	88.9	1	11.11	-	-
	H (n = 5)	-	-	5	100	-	-	-	-
	I (n = 5)	-	-	4	80	-	-	1	20
	J (n = 2)	-	-	2	100	-	-	-	-
	K (n = 1)	-	-	1	100	-	-	-	-
	L (n = 16)	-	-	16	100	-	-	-	-
	M (n = 3)	3	100	-	-	-	-	-	-
	N (n = 1)	-	-	1	100	-	-	-	-
	O (n = 3)	1	33.3	2	66.7	-	-	-	-
	P (n = 2)	1	50	1	50	-	-	-	-
	Q (n = 1)	-	-	1	100	-	-	-	-
	R (n = 1)	-	-	1	100	-	-	-	-
	T (n = 1)	-	-	1	100	-	-	-	-
Housing	Freestall (n = 26)	24	92.3	2	7.7	-	-	-	-
	CBPB[1] (n = 11)	2	18.2	8	72.7	-	-	1	9.1
	Paddocks (n = 51)	17	33.3	33	64.7	1	2.0	-	-
Season[2]	Rainy (n = 48)	24	50.0	23	47.9	-	-	1	2.1
	Dry (n = 40)	19	47.5	20	50.0	1	2.5	-	-
CM severity[3]	Mild (n = 50)	23	46.0	26	52.0	-	-	1	2.0
	Moderate (n = 34)	16	47.1	17	50.0	1	2.9	-	-
	Severe (n = 3)	3	100.0	-	-	-	-	-	-

[1] CBPB – Compost bedded pack barn

[2] Rainy season (October-March); Dry season (April-September)

[3] CM severity - (Mild) changes only in the milk appearance; (Moderate) presence of abnormal milk accompanied by changes in the udder; or, (Severe) combination of abnormal milk, with signs of inflammation in the udder and systemic signs)

Figure 19 – Minimum spanning tree (MST) of 88 *Streptococcus uberis* isolated from clinical mastitis and stratified according to their herd of origin (n = 17). All isolates were analyzed by random amplification of polymorphic DNA (RAPD) PCR



5.4.2.2 Antimicrobial susceptibility testing

Overall evaluation

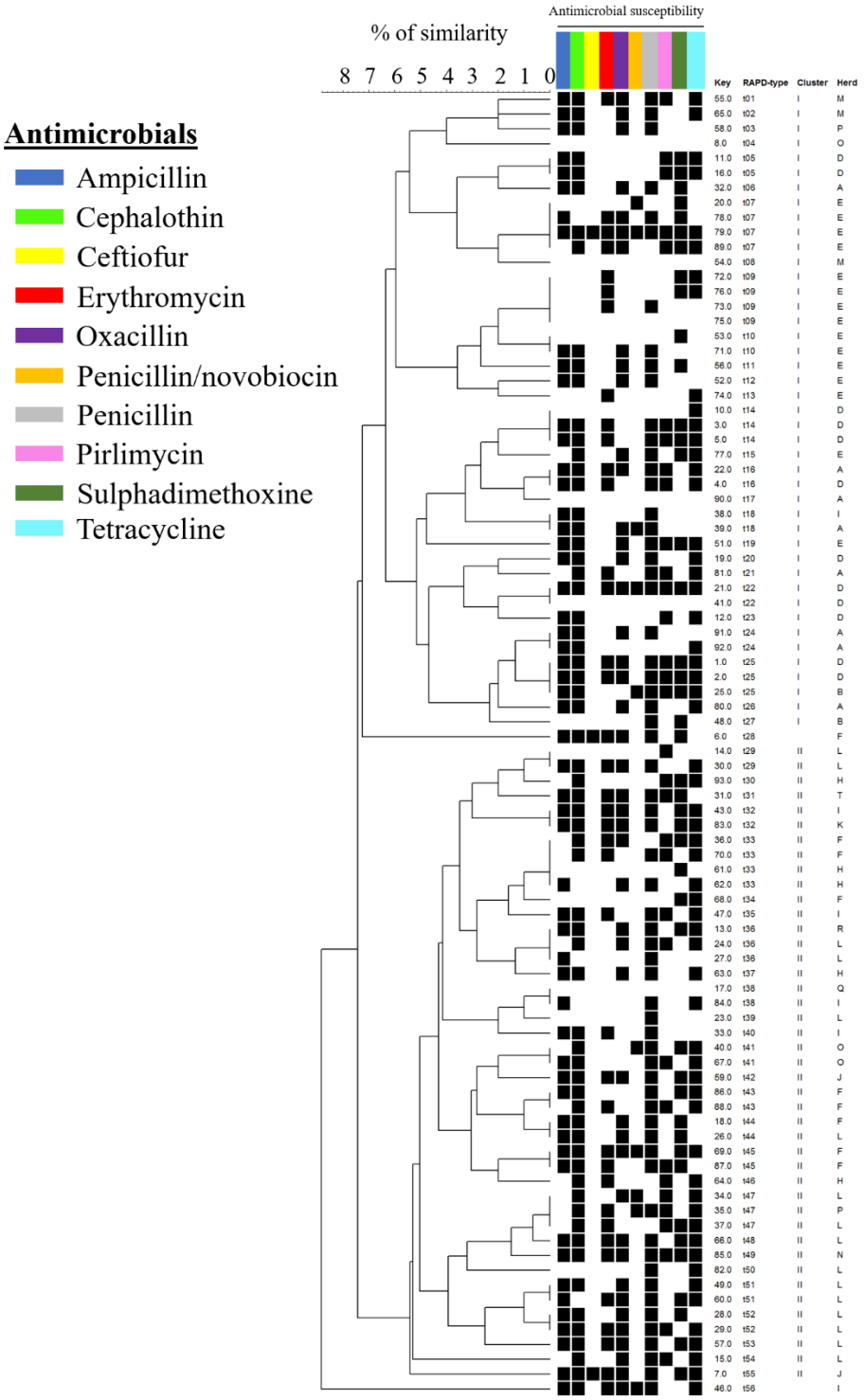
In total, 83 (94.3%) *Strep. uberis* isolates had results of antimicrobial susceptibility testing (Figure 20); three isolates were excluded from data analysis because of contamination, and two had no growth in the positive controls of the microdilution test. Isolates that were not clustered into groups I and II (t6 and t46) were not evaluated in the antimicrobial susceptibility data analysis. For the remained isolates (n = 81), overall results on the antimicrobial susceptibility are shown in Tables 27 and 28.

Table 27 - Frequency (overall and according to RAPD-clusters) of *Streptococcus uberis* (n = 81) classified as susceptible (S) or resistant (R) according to the interpretation criteria described by the Clinical and Laboratory Standards Institute (CLSI, 2013; 2015)

Antimicrobial	CLSI criteria[1]	Clusters		Overall
		I	II	
Ampicillin	R	27 (69.2)	26 (61.9)	53 (65.4)
	S	12 (30.8)	16 (38.1)	28 (34.6)
Ceftiofur	R	29 (74.4)	33 (78.6)	62 (76.5)
	S	10 (25.6)	9 (21.4)	19 (23.5)
Cephalothin	R	1 (2.6)	1 (2.4)	2 (2.5)
	S	38 (97.4)	41 (97.6)	79 (97.5)
Erythromycin	R	16 (41.0)	21 (50.0)	37 (45.7)
	S	23 (59.0)	21 (50.0)	44 (54.3)
Oxacillin	R	20 (51.3)	23 (54.8)	43 (53.1)
	S	19 (48.7)	19 (45.2)	38 (46.9)
Penicillin+novobiocin	R	5 (12.8)	4 (9.5)	9 (11.1)
	S	34 (87.2)	38 (90.5)	72 (88.9)
Penicillin	R	27 (69.2)	34 (81.0)	61 (75.3)
	S	12 (30.8)	8 (19.0)	20 (24.7)
Pirlimycin	R	16 (41.0)	17 (40.5)	33 (40.7)
	S	23 (59.0)	25 (59.5)	48 (59.3)
Sulphadimethoxine	R	20 (51.3)	22 (52.4)	42 (51.9)
	S	19 (48.7)	20 (47.6)	39 (48.1)
Tetracycline	R	25 (64.1)	32 (76.2)	57 (70.4)
	S	14 (35.9)	10 (23.8)	24 (29.6)

[1]S = susceptible; R = resistant

Figure 20 – Dendrogram resulting from a computer-assisted analysis of the RAPD profiles of Strep. uberis isolates (n = 83) recovered from milk of cows with clinical mastitis, according to results of susceptibility to 10 antimicrobials. The interpretation criteria to categorize the RAPD-types as resistant (black boxes) or susceptible (without boxes) were based on recommendations of CLSI (2013; 2015)



Differently of the antimicrobial susceptibility results observed for *Strep. agalactiae*, a high frequency of *Strep. uberis* isolates was categorized as resistant to most antimicrobials evaluated in our study: ampicillin (65.4%), ceftiofur (76.5%), erythromycin (45.7%), oxacillin (53.1%), penicillin (75.3%), pirlimycin (40.7%), sulphadimethoxine (51.9%), tetracycline (70.4%). On the other hand, *Strep. uberis* had high susceptibility only to two antimicrobials: cephalothin (97.5%), and penicillin/novobiocin (88.9%; Table 27 and 28, Figure 20).

The MIC_{50} for all *Strep. uberis* isolates, regardless of RAPD-type was: ampicillin (0.5 μg/mL), ceftiofur (4 μg/mL), cephalothin (2 μg/mL), erythromycin (0.25 μg/mL), oxacillin (4 μg/mL), penicillin+novobiocin (1 μg/mL), penicillin (0.5 μg/mL), and pirlimycin (1 μg/mL). More than 50% of *Strep. uberis* were not inhibited at the highest concentration of sulphadimethoxine and tetracycline contained in the test.

Overall MIC_{90} was 4 μg/mL for ampicillin and cephalothin, 2 μg/mL for penicillin+novobiocin, and 1 μg/mL for penicillin. Along with sulphadimethoxine and tetracycline, high proportions of resistant isolates were observed for pirlimycin (38.2%), ceftiofur (36.2%), erythromycin (27.7%), and oxacillin (19.3%; Table 28).

Table 28 – Overall frequency (%) of *Streptococcus uberis* isolates (n = 81) that had 50% (MIC50) and 90% (MIC90) of bacterial growth inhibited at each antimicrobial concentration. All isolates were identified from clinical mastitis cases occurred in 17 dairy herds of Southeast, Brazil

Antimicrobial	Frequency (%) of isolates at each indicated MIC (μg/mL)[1]												NI[2]	MIC_{50}[3]	MIC_{90}[4]
	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256			
Ampicillin	1.1	2.1	4.3	5.3	41.5	29.8	1.1	-	-	-	-	-	14.8	2	>8
Ceftiofur	-	-	92.6	2.1	2.1	2.1	-	-	-	-	-	-	1.1	0.5	0.5
Cephalothin	-	-	-	-	4.3	13.8	57.5	17.0	-	-	-	-	7.4	8	16
Erythromycin	-	1.1	2.1	3.2	-	1.1	-	-	-	-	-	-	92.6	>4	>4
Oxacillin	-	-	-	-	19.1	2.1	-	-	-	-	-	-	78.7	>4	>4
Penicillin+novobiocin	-	-	-	-	1.1	1.1	27.7	-	-	-	-	-	70.2	>8/16	>8/16
Penicillin	1.1	-	-	-	1.1	2.1	2.1	-	-	-	-	-	93.6	>8	>8
Pirlimycin	-	-	6.4	-	-	2.1	-	-	-	-	-	-	91.5	>4	>4
Sulphadimethoxine	-	-	-	-	-	-	-	-	25.5	34.0	12.8	4.3	23.4	64	>256
Tetracycline	-	-	-	6.4	18.1	41.5	4.3	-	-	-	-	-	29.8	4	>8

[1]The light gray shading represents the susceptible zone, and the darker gray shading represents the resistant zone. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2013; 2015). Interpretative criteria were based on human data (ampicillin, cephalothin, erythromycin, oxacillin, penicillin, sulphadimethoxine and tetracycline), and bovine mastitis (ceftiofur, penicillin+novobiocin and pirlimycin). The resistant category included those isolates categorized as either intermediate or resistant.
[2]NI = Not inhibited (growth at highest concentration tested).
[3]MIC (μg/mL) that inhibited 50% (MIC_{50}) of the isolates.
[4]MIC (μg/mL) that inhibited 90% (MIC_{90}) of the isolates.

Cluster evaluation

Results about the categorization as susceptible or resistant of *Strep. uberis* isolates stratified according to their clusters and are also described in Table 27. Overall, a high frequency of resistance to most antimicrobial was observed for isolates belonging to both cluster I and II. No major differences in the proportions of resistant isolates were observed between clusters in the descriptive evaluation. The greatest difference of antimicrobial resistance was found for penicillin, in which isolates belonging to cluster I were more resistant (11.8 percentage points) than isolates belonging to cluster II (Table 7).

The MIC distribution of the antimicrobial compounds for *Strep. uberis* stratified according to RAPD-clusters are shown in Table 29. Differences in the MIC_{50} among clusters were observed only for penicillin (cluster I = 0.5 μg/mL; cluster II = 0.25 μg/mL) and pirlimycin (cluster I = 2 μg/mL; cluster II = 0.5 μg/mL). Differences in the MIC_{90} were found for ampicillin (cluster I = >8 μg/mL; cluster II = 0.5 μg/mL), cephalothin (cluster I = 2 μg/mL; cluster II = 4 μg/mL), penicillin+novobiocin (cluster I = 2 μg/mL; cluster II = 1 μg/mL), and penicillin (cluster I = 0.5 μg/mL; cluster II = 1 μg/mL).

Table 29 - Frequency (%) of *Streptococcus uberis* isolates (n = 81) belonging to RAPD-clusters I (n = 39) and II (n = 42) that had 50% (MIC50) and 90% (MIC90) of bacterial growth inhibited at each antimicrobial concentration. All isolates were identified from clinical mastitis cases occurred in 17 dairy herds of Southeast, Brazil

Antimicrobial	Cluster	Frequency (%) of isolates at each indicated MIC (μg/mL)[1]												NI[2]	MIC_{50}[3]	MIC_{90}[4]
		0.12	0.25	0.5	1	2	4	8	16	32	64	128	256			
Ampicillin	I	23.1	7.7	48.7	5.1	-	-	2.6	-	-	-	-	-	12.8	0.5	>8
	II	14.3	23.8	54.8	-	-	2.4	-	-	-	-	-	-	4.7	0.5	0.5
Ceftiofur	I	-	-	12.8	10.3	2.6	43.6	-	-	-	-	-	-	30.7	4	>4
	II	-	-	4.8	-	16.7	38.1	-	-	-	-	-	-	40.4	4	>4
Cephalothin	I	-	-	-	-	92.3	5.1	-	-	-	-	-	-	2.6	2	2
	II	-	-	-	-	85.7	7.1	4.8	2.4	-	-	-	-	-	2	4
Erythromycin	I	-	59.0	12.8	2.6	5.1	-	-	-	-	-	-	-	20.5	0.25	>4
	II	-	50.0	4.8	7.1	2.4	2.4	-	-	-	-	-	-	33.3	0.25	>4
Oxacillin	I	-	-	-	-	48.7	38.5	-	-	-	-	-	-	12.8	4	>4
	II	-	-	-	-	45.2	33.3	-	-	-	-	-	-	21.5	4	>4
Penic+Novob.	I	-	-	-	87.2	5.1	-	-	-	-	-	-	-	2.6	1	2
	II	-	-	-	90.5	4.8	-	-	-	-	-	-	-	2.4	1	1
Penicillin	I	30.8	15.4	46.2	2.5	-	2.5	-	-	-	-	-	-	2.6	0.5	0.5
	II	19.1	35.7	33.3	9.5	2.4	-	-	-	-	-	-	-	-	0.25	1
Pirlimycin	I	-	-	43.6	5.1	10.3	2.6	-	-	-	-	-	-	38.4	2	>4
	II	-	-	50.0	4.8	4.8	-	-	-	-	-	-	-	40.4	0.5	>4
Sulphadimet.	I	-	-	-	-	-	-	-	-	20.5	2.6	12.8	12.8	51.3	>256	>256
	II	-	-	-	-	-	-	-	-	16.7	7.1	14.3	9.5	52.4	>256	>256
Tetracycline	I	-	-	-	7.7	28.2	12.8	-	-	-	-	-	-	51.3	>8	>8
	II	-	-	-	7.1	16.7	14.3	2.4	-	-	-	-	-	59.5	>8	>8

[1]The light gray shading represents the susceptible zone, and the darker gray shading represents the resistant zone. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2013; 2015). Interpretative criteria were based on human data (ampicillin, cephalothin, erythromycin, oxacillin, penicillin, sulphadimethoxine and tetracycline), and bovine mastitis (ceftiofur, penicillin+novobiocin and pirlimycin). The resistant category included those isolates categorized as either intermediate or resistant.

[2]NI = Not inhibited (growth at highest concentration tested).

[3]MIC (μg/mL) that inhibited 50% (MIC_{50}) of the isolates.

[4]MIC (μg/mL) that inhibited 90% (MIC_{90}) of the isolates.

5.4.2.3 Survival function analysis

Kaplan-Meier survival curves were produced for each antimicrobial for comparison of susceptibility among *Strep. uberis* RAPD-clusters. Homogeneous survival curves among clusters were observed for all antimicrobials evaluated: ampicillin (Log-Rank = 0.25; Wilcoxon =0.38), ceftiofur (Log-Rank = 0.31; Wilcoxon = 0.27), cephalothin (Log-Rank = 0.70; Wilcoxon = 0.35), erythromycin (Log-Rank = 0.18; Wilcoxon = 0.25), oxacillin (Log-Rank = 0.42; Wilcoxon = 0.53), penicillin+novobiocin (Log-Rank = 0.85; Wilcoxon = 0.66), penicillin (Log-Rank = 0.68; Wilcoxon = 0.88), pirlimycin (Log-Rank = 0.97; Wilcoxon = 0.78), sulphadimethoxine (Log-Rank = 0.95; Wilcoxon = 0.94), and tetracycline (Log-Rank = 0.41; Wilcoxon = 0.35; Table 30).

Table 30 – Results of survival analysis comparing the antimicrobial susceptibility of *Streptococcus uberis* (n = 81) isolated from clinical mastitis cases in 17 dairy herds and stratified by the RAPD-clusters I (n = 39) and II (n = 42)

Antimicrobial	Cluster	Censor[1]	Log-Rank[2]	Wilcoxon[3]
Ampicillin	I	5	0.25	0.38
	II	2		
Ceftiofur	I	12	0.31	0.27
	II	17		
Cephalothin	I	1	0.70	0.35
	II	0		
Erythromycin	I	8	0.18	0.25
	II	14		
Oxacillin	I	5	0.42	0.53
	II	9		
Penic+Novob.	I	1	0.85	0.66
	II	1		
Penicillin	I	1	0.68	0.88
	II	0		
Pirlimycin	I	15	0.97	0.78
	II	17		
Sulphadimet.	I	20	0.95	0.94
	II	22		
Tetracycline	I	20	0.41	0.35
	II	25		

[1] Number of isolates censored (observed growth at the highest antimicrobial drug concentration of the used test).
[2] Log-Rank test for equality of strata at higher antimicrobial concentrations.
[3] Wilcoxon test for equality of strata at lower antimicrobial concentrations.

5.5 DISCUSSION

5.5.1 *Streptococcus agalactiae*

A great intraspecific diversity (45 RAPD-types) of *Strep. agalactiae* causing CM was observed in this study when the tolerance of 1% in the genetic similarity among isolates was considered. Despite the high level of polymorphism, all isolates shared more than 92% of similarity. A study characterizing *Strep. agalactiae* isolated from bovine mastitis and human infections reported 58% of similarity among all isolates using the same typing method as used in this study (MARTINEZ et al.; 2000); however, after clustering the isolates had 70% of genetic similarity among them.

The high genetic similarity among *Strep. agalactiae* isolates in our study may be attributed to the contagious transmission of this pathogen in the mastitis epidemiology, which has the bovine udder as the only reasonable source of the organism in dairy herds (KEEFE; 1997). In our study, approximately 63% of all isolates were recovered from CM cases occurred in two herds (N and Q). Moreover, a significant relationship between herds and the RAPD-clusters was also observed. For all herds, especially those with a greater number of isolates, there was a predominance of strains belonging to the same cluster. For example, all isolates identified in herd N belonged to cluster named here as “Ia”, while 91.7% of isolates from herd Q belonged to cluster “Ib”.

Other studies also reported high genetic similarity of *Strep. agalactiae* causing IMI within herds, and even, within region of origin (YANG et al., 2013; MAHMMOD et al., 2015; CARVALHO-CASTRO et al., 2017). Using a multiplex-PCR assay for capsular polysaccharide (CPS) typing of *Strep. agalactiae* isolates identified from bovine IMI, a Brazilian study reported a great similarity between isolates within the geographical region from which the bacterial isolate was identified; for example, a high level of similarity was found in isolates from the state of Paraná (81.8% of isolates belonged to CPS type Ib), and similar results were observed for isolates from the state of Minas Gerais (74.1% of isolates belonged to CPS type III; CARVALHO-CASTRO et al., 2017). Another molecular study evaluating six Danish dairy herds with high prevalence of *Strep. agalactiae* reported the predominance of a single PFGE-type and sequence type (ST) within herd (MAHMMOD et al., 2015). Rato et al., (2013), using Pulsed-field gel electrophoresis (PFGE) and cluster analysis, evaluated 60 *Strep. agalactiae*

isolates from six dairy herds and found four major clonal clusters sharing 80% similarity or above.

The predominance of certain *Strep. agalactiae* strains, or even, clusters composed by close-related strains, is consistent with the contagious form of transmission of this pathogen. *Streptococcus agalactiae* are mainly transmitted due to insufficient hygiene in the milking parlor, which increases the risk of a healthy cow to come into contact with equipment, hands or towels contaminated by milk from an infected cow (KEEFE, G., 2012). Therefore, management practices aiming to reduce the contact of healthy quarters with contaminated milk, such as adequate milking routine, use of disposable gloves, use of post milking teat disinfectants, and treatment and segregation of infected cows, are recommended for the reduction of new IMI rate of *Strep. agalactiae* in dairy herds.

Differences in the frequency of strains within clusters among housing system were also observed in our study. Approximately 80% of isolates identified in herds housing cows in freestalls belonged to cluster Ia, while 65% of isolates identified in CBPB were assigned to cluster Ib. Moreover, 4 out of 5 isolates identified from herds housing cows in paddocks belonged to cluster II. However, because *Strep. agalactiae* is mainly transmitted during milking, it is likely that the similarity between isolates within housing system is more associated with other risk factors at the herd level, such as management practices related to the milking procedure and within-herd biosecurity, than with the type of housing facility.

Interesting results were also observed in the evaluation of antimicrobial susceptibility of *Strep. agalactiae* isolates. Among our isolates, the MIC_{50} was the lowest antimicrobial concentration contained in the microdilution test for eight out of 10 antimicrobials tested. High bacteria susceptibility was seen manly for β-lactams antimicrobials. On the other hand, the *Strep. agalactiae* isolates were less susceptible to other antimicrobials such as tetracycline (31.4%) and erythromycin (70.9%).

The overall resistance to erythromycin in our study was 29.1%, although difference in the susceptibility were observed among clusters. Comparison between results of studies evaluating antimicrobial susceptibility can be difficult because of differences in techniques used (disk-diffusion or broth dilution test), as well as, differences of interpretative criteria used for clinical categorization. However, resistance to erythromycin was also reported previously in isolates identified from bovine mastitis (DOGAN et al., 2005; DUARTE et al., 2005) and clinical isolates recovered from human diseases (UH et al., 2001). A Korean study evaluating 185 human clinical *Strep. agalactiae* isolates reported an increase of erythromycin resistance from zero in 1990 to 40% in 1998 (UH et al., 2001). Furthermore, Dogan et al., (2005) reported

27% of resistance to erythromycin in *Strep. agalactiae* isolates recovered from human diseases, but a lower frequency of resistance was identified for isolates recovered from bovine mastitis (3.6%). In general, the erythromycin resistance seems to be lower in cattle than human isolates, ranging from zero in a French study (GUÉRIN-FAUBLÉE et al., 2002) to 10.5% in a Brazilian report (DUARTE et al., 2004).

Our study showed significant differences among clusters in the antimicrobial susceptibility to erythromycin and pirlimycin, and this results were well illustrated in the Kaplan-Meier survival curves. Considering the categorization of isolates as susceptible or resistant, more than 89% of isolates belonging to clusters Ia and III were considered susceptible to erythromycin, while only 31% of isolates pertaining to cluster IIa were susceptible to the same antimicrobial. Similar results were found for pirlimycin, to which 100% of isolates belonging to clusters Ia and III were susceptible, while only 43.7% of isolates belonging to cluster Ib were resistant. Differences among genotypes were also reported in other studies. Duarte et al., (2005) evaluating isolates recovered from milk of dairy cows with clinical and subclinical mastitis reported that resistance to erythromycin was predominantly associated with isolates belonging to serotype II (50%) and III (22.2%). Resistance to pirlimycin was also reported for *Streptococcus* spp. isolated from cases of subclinical mastitis, which was described to be associated to the presence of *linB* gene, which encodes the resistance to lincosamides (RATO et al., 2013). Differences in antimicrobial susceptibility between studies, and among different genotypes (or clusters) within studies, seems to be related to genetic mechanisms of resistance. Resistance genes such as *lnu*(D), *emr*(A) and *emr*(B) were associated with antimicrobial resistance to macrolides and lincosamides of isolates recovered from cows with clinical and subclinical mastitis (DUARTE et al., 2004; KACZOREK et al., 2017). Therefore, it is reasonable to infer that isolates belonging to cluster Ib in our study may have genetic determinants associated with resistance to erythromycin.

Overall, approximately 70% of *Strep. agalactiae* isolates in our study were resistant to tetracycline, and heterogeneous curves were observed in the survival analysis comparing the tetracycline susceptibility among clusters. A total of 84.2% of isolates belonging to cluster II were susceptible to tetracycline, while only 25.7% and 6.3% of isolates pertaining to cluster Ia and Ib were susceptible to this antimicrobial. Likewise, differences in the tetracycline susceptibility among genotypes were also reported in a study from Brazil (DUARTE et al., 2005).

High prevalence of *Strep. agalactiae* resistant to tetracycline was also reported in other studies (DUARTE et al., 2004; RATO et al., 2013; KACZOREK et al., 2017). Tetracycline

resistance was described for 44.7% of isolates in a study evaluating *Strep. agalactiae* recovered from dairy cattle in Brazil (DUARTE et al., 2004), and similar results (44.7%) were observed in a study performed in Poland (KACZOREK et al., 2017). Rato et al. (2013) evaluated 60 *Strep. agalactiae* isolates recovered from milk of cows with subclinical mastitis and reported tetracycline resistance in 64.8% of the isolates, which is similar with the results of our study.

The low efficacy of tetracycline against *Strep. agalactiae* have been attributed to the excessive use of this antimicrobial in the past, especially when used as prophylactic agents or as a growth promoter (SPEER; SHOEMAKER; SALYERS, 1992; RATO et al., 2013). Furthermore, several tetracycline resistance-related genes were identified in *Strep. agalactiae* strains. Most tetracycline-resistant strains characterized in the study of Kaczorek et al (2017) had at least one of the five resistance genes evaluated in the experiment (*tet*O, *tet*L, *tet*M, *tet*K and *tet*S). Another study evaluating the resistance genes among *Strep. agalactiae* isolates recovered from bovine mastitis and human diseases also reported a high frequency of genes associated with tetracycline resistance (DUARTE et al., 2005); results from the same study showed that the frequencies of the genes associated with erythromycin and tetracycline resistance varied according to the origin of isolates. Therefore, considering that resistance genes can either be present or absent in specific genotypes, we could infer that the differences in the tetracycline susceptibility among clusters in our study could be related to the variation in the frequency of specific genetic determinants. However, although we found a genetic cluster with a relatively high proportion of isolates susceptible to tetracycline, this antimicrobial would be not the first choice for treatment of infections caused by *Strep. agalactiae* (DUARTE et al., 2005).

In contrary, β-lactams antimicrobials are still the first choice for treatment of infections caused by *Streptococcus* spp., and our results on *Strep. agalactiae* antimicrobial susceptibility testing reinforce this statement. Even the MIC_{90} of β-lactams antimicrobials in our study was revealed to be at lowest or second lowest antimicrobial concentration present in the test, which demonstrates the effectiveness of this antimicrobial against *Strep. agalactiae in vitro*. The minimal β-lactams resistance of *Strep*. *agalactiae* isolates found in our study agrees with the results reported by other (GUÉRIN-FAUBLÉE et al., 2002; KACZOREK et al., 2017). However, although the high susceptibility of *Strep. agalactiae* isolates to all antimicrobials pertaining to β-lactams class, heterogeneous survival curves were observed among clusters in our study for ampicillin and ceftiofur. This results suggests that some intraspecific genetic determinants may be involved with *Strep. agalactiae* susceptibility to these antimicrobials. Further experiments evaluating the genomic characteristics of *Strep. agalactiae* strains

evaluated in our study should be conducted to determine the distribution of antimicrobial resistance genes, which could better clarify the potential reasons for the differences in the antimicrobial susceptibility among strains found in this study.

5.5.2 *Streptococcus uberis*

Streptococcus uberis is ranked as one of the most important causes of CM in countries around the world, including USA (OLIVEIRA; HULLAND; RUEGG, 2013), Canada (OLDE RIEKERINK et al., 2008), Netherlands (BARKEMA et al., 1999), Belgium (VERBEKE et al., 2014), and Brazil (results presented in chapter 2). One of the possible reasons that may explain the high prevalence of this pathogen in dairy herds is its potential to be transmitted by both the contagious and environmental routes (ZADOKS, R. N. et al., 2003).

Streptococcus uberis isolates from our study were highly resistant to most of antimicrobials, including β-lactams. Over 50% of isolates were resistant to ampicillin, ceftiofur, and penicillin. These results were not in accordance with studies evaluating antimicrobial susceptibility of *Strep. uberis* causing IMI in dairy cattle (ROSSITTO et al., 2002; POL; RUEGG, 2007b; RATO, MÁRCIA G. et al., 2013; KACZOREK et al., 2017). Again, comparisons between studies evaluating antimicrobial susceptibility may not be easy, especially because of differences in the testing methods (disk diffusion or broth microdilution test), and in the cut-offs values used to categorize the pathogen as susceptible or resistant. In addition, limited historical MIC data is available on the antimicrobial susceptibility of mastitis pathogens, particularly for *Strep. uberis*.

It was found only three studies using the same method as we used for determination of antimicrobial susceptibility of *Strep. uberis* isolated from bovine IMI (ROSSITO et al., 2002; POL AND RUEGG, 2007; RAJALA-SCHULTZ 2004). Pol and Ruegg (2007) evaluating antimicrobial susceptibility of mastitis pathogens isolated from conventional and organic farms reported higher susceptibility of *Streptococcus* spp. (except *Strep. agalactiae*) to most antimicrobials (ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin, penicillin, and penicillin+novobiocin). Furthermore, results of both MIC_{50} and MIC_{90} reported by Pol and Ruegg (2007) were lower than those found in our study. However, the streptococci evaluated in that study were not differentiated into species, thus, reliable comparison could not be done with our results because other species such as *Strep. dysgalactiae* could bias the outcome.

Rossitto et al. (2002), evaluating the antimicrobial susceptibility patterns for *Strep. uberis* isolated from bovine mastitis in USA, also reported a higher susceptibility to most antimicrobials of isolates in comparison to our results, especially for β-lactams. However, similar frequencies of susceptibility were observed for cephalothin (97.2% vs. 97.5% in our study), tetracycline (27.1% vs. 29.6%), pirlimycin (60.9% vs. 59.3%) and erythromycin (51.9% vs. 54.3%). Sulphadimethoxine was the only antimicrobial with a higher susceptibility pattern in our study (29.6%) in comparison with the results found by Rossito and coworkers (1.5%). Moreover, the MICs found in our study were higher for all antimicrobials except for erythromycin and pirlimycin, in which the MIC_{90} was similar between studies (>4 μg/mL for erythromycin, and >8 μg/mL for pirlimycin); and for tetracycline and sulphadimethoxine, in the MIC_{50} and MIC_{90} in both studies exceeded the higher antimicrobial concentration contained in the test.

In accordance with our findings, a study comparing the antimicrobial susceptibility of mastitis pathogens between primiparous and multiparous cows reported high frequency of resistant *Streptococcus*. spp regardless parity (RAJALA-SCHULTZ et al., 2004). However, similarly as done in the study of Pol and Ruegg (2007), the streptococci isolates were not differentiated according to species. Nevertheless, in comparison to our results, the study of Rajala-Schultz and coworkers found even higher frequencies of resistant isolates to the following antimicrobials: ampicillin (77.3% vs. 65.4% in our study), cephalothin (63.3% vs. 2.5%), erythromycin (59.1% vs. 45.7%), penicillin (90.9% vs. 75.3%), pirlimycin (50% vs. 40.7%), and sulphadimethoxine (86.4% vs. 51.9%). On the other hand, lower frequencies of resistant isolates were observed for ceftiofur (63.6% vs 76.5% in our study), oxacillin (36.4% vs. 53.1%), penicillin+novobiocin (4.5% vs. 11.1%), and tetracycline (68.2% vs. 70.4%). Lower MIC_{50} was found in our study for all antimicrobials, except for penicillin+novobiocin and for pirlimycin, for which the values were the same among studies. In relation to MIC_{90}, our results were similar to those found by Rajala-Schultz and coworkers, except for ampicillin and penicillin+novobiocin, which the outcome was higher in our study, and for penicillin that was lower in our study.

Differences in the susceptibility patterns among studies may be related with the frequency of antimicrobial use in different farms, regions or countries. The excessive use of certain antimicrobials in different locations can play a role in the development of mechanisms of resistance among pathogens associated with mastitis. For example, most *Strep. uberis* evaluated in our study may have mechanisms of resistance against β-lactam antimicrobials such as production of inactivating enzymes (i.e., β-lactamases), and inhibition of release of autolytic

enzymes (HEESEMANN, 1993). Furthermore, similarly to results reported for *Strep. agalactiae*, the high resistance to erythromycin and pirlimycin could be associated with the presence of specific resistance genes. The *erm*B gene previously reported as a genetic mechanism of resistance of *Strep. uberis*, modifies the target site on the bacterial ribosome, thereby preventing the binding of the antibiotic and producing high-level resistance to both macrolides (i.e., erythromycin) and lincosamides (i.e., pirlimycin; SCHMITT-VAN DE LEEMPUT; ZADOKS, 2007).

Low susceptibility frequencies were also observed in our study for tetracycline and sulphadimethoxine, and this result may be attributed to the excessive use of these antimicrobials by the systemic route for treatment of infections in dairy cows and other species. In addition, genes of resistance to tetracycline, such as *tet*O and *tet*K are also associated with the *Strep. uberis* resistance patterns (RATO et al., 2013). Results of our study were in agreement with other reports that showed low susceptibility of environmental *Streptococcus* spp. to one or both of these antimicrobials (ROSSITTO et al., 2002; RAJALA-SCHULTZ et al., 2004; KACZOREK et al., 2017). Both tetracycline and sulfadimethoxine are antimicrobials that are frequently used to treat respiratory and other diseases in cattle by the systemic route, and neither of these two agents are recommended for treatment of CM, especially those without systemic signs (ROSSITTO et al., 2002; ROBERSON, 2012).

In our study, we also compared the antimicrobial susceptibility among clusters categorized according to the genetic similarity. Although some variation in the MIC values were observed among clusters, homogeneous survival curves were observed for all antimicrobials evaluated. These results suggest that the high genotypic diversity of *Strep. uberis* isolates evaluated in our study may have little association with antimicrobial susceptibility results. As next step in our research, an evaluation of the potential resistance mechanisms of these *Strep. uberis* strains should be carried out, especially because of the multiresistance pattern found among the isolates.

Multiple *Strep. uberis* subtypes were identified in each herd, although they were genetically close-related (belonged to the same cluster) and probably have the same ancestral strain. This resultt is in accordance with results of other reports (WIELICZKO et al., 2002; ZADOKS, R. N. et al., 2003; ABUREEMA et al., 2014), and is consistent with the hypothesis that the environment harbors a high diversity of *Strep. uberis* strains acting as a source of infection in dairy herds. Using pulsed-field gel electrophoresis (PFGE), an Australian study determined that the majority of recurrent episodes of CM occurring in dairy cows were caused by a new strain of *Strep. uberis*, indicating a greater probability that the environment was the

main reservoir of this pathogen, rather than mammary gland of infected cows (ABUREEMA et al., 2014). In our study, a predominance of isolates belonging to specific clusters was observed within housing system, especially in freestalls (92.3% of isolates belonged to cluster I) and CBPB (72.7% of isolates belonged to cluster II). Thus, the CM caused by *Strep uberis* evaluated in our study may occurred because of the exposure to the same environmental source, such as contaminated bedding in herds housing cows in freestalls or CBPB.

A high genetic similarity between isolates within herds was also observed in our study, even though they were not identified as the same strain. Therefore, the hypothesis of the occurrence of potential transmission of *Strep. uberis* from cow-to-cow cannot be ruled out. For example, eight RAPD-types were identified in the molecular analysis of 15 *Strep. uberis* isolates identified in herd E; of these, 53.3% belonged to RAPD-type 7 or RAPD-type 9, indicating that a contagious transmission of these subtypes may occurred in herd E. Contagious transmission of *Strep. uberis* have been reported in other studies (PHUEKTES et al., 2001; RATO et al., 2013), and this pathogen was even described as the cause of mastitis outbreaks (ZADOKS et al., 2001). Rato et al., (2008) in a study evaluating the molecular epidemiology of *Strep. uberis* isolated from cases of subclinical mastitis reported that three PFGE types accounted for almost half of the isolates evaluated; in addiction, these major types were herd specific, suggesting either cow-to-cow transmission or infection with isolates from the same environmental reservoirs. It is reasonable that herds with inadequate implementation of management practices for control of contagious pathogens may facilitate the transmission of *Strep. uberis* from cow-to-cows, even though it is considered an environmental pathogen (RATO et al., 2013). Thus, cows with chronic subclinical IMI caused by some specific *Strep. uberis* strains may serve as a source of infection to other cows.

A great frequency of closely related RAPD-types of *Strep. uberis* was observed on different herds in our study (Table 26). This result is consistent with other studies evaluating isolates from clinical and subclinical mastitis, although different molecular methods (e.g., PFGE) were used in those studies (DOUGLAS et al., 2000; RATO et al., 2008; ABUREEMA et al., 2014). The exactly reason for these findings are not clear; however, it is possible that these closely related strains are environmental, and thus, more adapted to specific geographical and within herd conditions than other strains (ABUREEMA et al., 2014).

All three isolates identified from dairy cows with severe CM in the present study belonged to cluster I and were found as close-related to each other in the molecular analysis. The predominance of closely related strains causing severe CM could be attributed to specific virulence factors of certain strains (i.e., ability to cause a great inflammatory reaction). Studies

have reported that some strains were more likely to cause CM than others (HILL, A. W., 1988; PHUEKTES et al., 2001). Zadocks et al. (2003) also reported a relationship between RAPD-type and the occurrence of CM, in which 8 of 10 infections with clinical signs were associated with a dominant strain type. Based on this results, is reasonable to speculate that some strains can be more virulent and cause IMI with more pronounced clinical signs that others.

Identification of the predominant strains causing infections in production animals, including those associated with mastitis in dairy cows, as well as periodic determination of antimicrobial resistance profile, can provide the opportunity to monitor and identify new antibiotic resistance mechanisms developing in a given geographical area or among particular groups of bacteria. However, one of the main limitation about the evaluation of antimicrobial susceptibility of clinical isolates from dairy cows is the lack of interpretative criteria specific for bovine, to categorize isolates as susceptible or resistant. Most cut-offs values used for evaluation of pathogens causing bovine mastitis are still based on other animal species or human interpretative criteria; specific criteria for pathogens causing mastitis are not available except for pirlimycin, penicillin+novobiocin, and ceftiofur (CLSI, 2013). Under this circumstances, the clinical outcome in bovine mastitis cannot be predicted with accuracy for most of antimicrobials by the susceptibility testing, which may be used only to determine which antibiotics not to use to avoid therapeutic failure. Microdilution test for evaluation of the minimum inhibitory concentration as performed in our study are suitable for resistance profile monitoring, which could also enable further studies to stablish a system to interpret the results of antimicrobial susceptibility testing for mastitis pathogens in dairy cattle.

5.6 CONCLUSION

High genotypic diversity was found for both *Strep. agalactiae* and *Strep. uberis*. However, after clustering a high genotypic similarity was observed within- and between herds for both *Streptococcus* species. Overall, *Strep. agalactiae* had high susceptibility to most antimicrobials, except to erythromycin and tetracycline. Differences in antimicrobial susceptibility among clusters of *Strep. agalactiae* isolates were observed for ampicillin, ceftiofur, erythromycin, pirlimycin, sulphadimethoxine and tetracycline. In contrary, *Strep. uberis* were categorized as resistant to most antimicrobials, except to cephalothin and penicillin+novobiocin. Although some differences in the MIC_{50} and MIC_{90} were found among genotypic clusters of *Strep. uberis*, homogeneous curves were observed among them for all antimicrobials in the survival analysis.

Chapter 6

Final considerations

6 FINAL CONSIDERATIONS

In chapter 2, we determined the incidence rate of clinical mastitis (IRCM) in 20 dairy herds located in the states of São Paulo and Minas Gerais, Brazil. Although we found a higher monthly average IRCM in comparison to most studies performed in other countries, a great variation in the disease frequency was observed among herds (1.9 to 21.7 cases per 10,000 QDAR). These results demonstrated that some herds have an excellent control of CM, while others continue to have the great challenge of reducing the occurrence of new cases of this disease. In relation to the frequency of pathogens causing CM, although most cases were caused by environmental micro-organisms such as *E. coli* and *Strep. uberis*, contagious pathogens, particularly *Strep. agalactiae* and *Staph. aureus*, remain an important cause of CM in some herds. Therefore, depending on the farm etiological scenario, mastitis control programs should focus on reducing both the environmental challenge (e.g., increasing the cows' hygiene), and the contact of healthy quarters with contagious pathogens, especially during milking.

Some herd-level descriptors were associated with the IRCM. For example, the IRCM (overall and caused by contagious pathogens) was highest in dairy herds with BMSCC >600.000 cells/mL. Moreover, the IRCM with isolation of Gram-negative pathogens was highest in herds with BMTBC >30.000 cfu/mL. Although it is difficult to make a cause-and-effect statement based on these results, we can infer that herds with high IRCM are more likely to provide lower quality milk to the dairy industry than herds with better control of CM.

In chapter 3 we characterized the CM treatment profile and quantified the antimicrobial used for therapy of this disease in dairy herds. A higher overall monthly antimicrobial treatment incidence (ATI; 17.7 DDD per 1,000 lactating cows-day) was observed in our study in comparison to other studies that used the same antimicrobial quantification method. Although our results showed a higher ATI for compounds administered by the intramammary route (15.4 DDD per 1,000 lactating cow-days) in comparison to systematically administered antimicrobials (while 2.2 DDD per 1,000 cow-days), the use of systemic compounds was 3.25 times higher when the antimicrobial use was quantified as the total amount (mg) of active ingredients. This result is mainly attributed to the high frequency of off-label protocols. The use of combination therapy was reported for 64.4% of the treatments at the cow-level, and the average number of administrations for most of antimicrobials was higher than recommended in the label. Furthermore, a great antimicrobial concentration is injected in the cattle by the systemic route in comparison to intramammary route.

Among intramammary drugs, aminoglycosides had the highest ATI, followed by a compound with a combination of tetracycline, aminoglycoside and polypeptide, and by third-generation cephalosporin. For systematically administrated antimicrobials, fluoroquinolones and fourth-generation cephalosporin were the most frequently used antimicrobials. Some of antimicrobials classes with high frequency of use in the herds evaluated in this study are classified by the World Health Organization (WHO) as critically important for human health, such as fluoroquinolones and third- and fourth-generation cephalosporins. These results raise a concern about the development of resistant bacterial strains that could potentially impair the therapy of infections in both humans and animals. Furthermore, because there were some herd-level descriptors associated with the IRCM and AMU in our study, there may be opportunity for management strategies aiming to improve the control of CM in dairy herds of southeastern Brazil.

Escherichia coli was the most frequent pathogen causing CM in our study, and in chapter 4, we determined the phylogeny of isolates pertaining to this species; we also evaluated the association of the most frequent phylogroups with antimicrobial susceptibility and with variables at the cow-level, housing system and season. Most *E. coli i*solates were assigned to phylogroups A and B1, and this result is in accordance with other studies evaluating *E. coli* isolates from bovine mastitis. However, other less frequent phylogroups were also identified in our study, including four isolates belonging to the phylogenetic group C. To our knowledge, isolates belonging to phylogroup C were not reported in previous studies evaluating the molecular epidemiology of *E. coli* from IMI.

Parity was the only variable associated with the phylogenetic group in this study, in which primiparous cows had a higher proportion of isolates assigned to phylogroup A in comparison to phylogroup B1. Although this was an interesting result, it must be evaluated with caution as only 13 isolates were recovered from primiparous cows in this study. In relation to antimicrobial susceptibility, resistant isolates were observed for all evaluated antimicrobials. Overall, *E. coli* isolates were greatly susceptible to ceftiofur (96.8%), and presented moderate susceptibility to cephalothin (75.6%), sulphadimethoxine (76.6%) and tetracycline (66%). Cephalothin was the only antimicrobial associated with the phylogenetic group, in which strains belonging to the phylogroup A were inhibited at lower antimicrobial concentrations than strains assigned to the phylogroup B1. Differences in the antimicrobial susceptibility among phylogroups may be attributed to the presence of specific resistance mechanisms of certain strains.

In chapter 5, a similar study as performed for *E. coli* was done for *Strep. uberis* and *Strep. agalactiae*, however, the isolates were genotyped using randomly amplified polymorphic DNA (RAPD) analysis. *Streptococcus uberis* and *Strep. agalactiae* accounted for approximately 23% of CM cases with positive culture results, and were the second and third pathogens most prevalent in our study, respectively. Results of RAPD-PCR showed a great genotypic diversity for both *Strep. agalactiae* and *Strep. uberis* and clusters were created based on their genetic similarity for comparative analysis of antimicrobial susceptibility. While *Strep. agalactiae* isolates showed high overall susceptibility to most antimicrobials, the opposite was observed for *Strep. uberis* isolates, which were highly resistant to most antimicrobials. Differences in the antimicrobial susceptibility among clusters of *Strep. agalactiae* were observed for several antimicrobials, indicating that genetic mechanisms of resistance may be involved with the differences among strains. In contrary, despite the higher antimicrobial resistance of *Strep. uberis* strains, no differences in the susceptibility was observed among strains clustered according to the genetic similarity.

Overall, our study provided etiological, molecular, and pharmaco-epidemiological data about CM in dairy herds of southeastern Brazil. Although the number of herds included in this study does not allow us to make inferences at the country or even at the regional level, the characteristics of these herds are representative of the developing dairy supply chain in the region; moreover, these results can be used as reference for further epidemiological studies on CM in Brazil. Furthermore, considering the high antimicrobial use, and the identification of resistant strains belonging to the most prevalent CM causing pathogens, programs on the judicious use of antimicrobials should be disseminated in dairy farms to prevent the potential spread of antimicrobial resistance in bacterial populations, which could cause serious implications for both animal and public health.

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