Preventive Veterinary Medicine 161 (2018) 9–18

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

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

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ARTICLE INFO

Keywords:
Dairy cattle
Incidence of clinical mastitis
Pathogen distribution

ABSTRACT

The objectives of this study were to characterize the pathogen frequency and severity of clinical mastitis (CM) in 20 dairy herds of southeastern Brazil; and to determine the incidence rate of clinical mastitis (IRCM; overall and based on specific-pathogen groups) based on quarter time at risk and its association with risk factors at the herd-level. Data were recorded in each herd for a period of 8 to 15 months. The association between herd-level risk factors 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 risk factors were evaluated: herd size, housing system, average daily milk yield per cow, bulk milk somatic cell count (BMSCC), and bulk milk total bacterial count (BMTBC). A total of 5957 quarter-cases of CM were recorded from 2637 cows, but only 4212 cases 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 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 $\geq 601 \times 10^3$ cell/mL had higher overall IRCM (16/10,000 QDAR) than those with BMSCC $\leq 600 \times 10^3$ cell/mL ($\leq$7.7/10,000 QDAR). When the specific-pathogen groups were evaluated, for contagious pathogens, variables housing (free-stalls or compost-bedded pack barns), BMSCC ($\geq 601 \times 10^3$ cells/mL), and average daily milk yield per cow (21 and 25 Kg/d) presented the highest IRCM. Furthermore, in Gram-negative group, herds with BMTBC $\geq 31 \times 10^3$ cfu/mL had higher IRCM compared with herds with BMTBC $\leq 30 \times 10^3$ cfu/mL. Although environmental pathogens were the most common cause of CM in this study, contagious pathogens (e.g., *Strep. agalactiae* and *Staph.* aureus) are still a concern in dairy herds of Brazil. Additionally, as there were some herd-level risk factors associated with the IRCM, there may be opportunity for management strategies aiming to improve the control of CM in dairy herds.

1. 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. Occurrence of 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 et al., 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 occurrence of CM in different regions of the world, including Europe (Bradley et al., 2007, Verbeke 2014; Santman-Berends et al., 2015), North America (Sargeant et al., 1998; Olde Riekerink et al., 2008; Oliveira et al., 2013), Australia (Daniel et al., 1982), New Zealand (McDougall, 1999), and Tanzania

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https://doi.org/10.1016/j.prevetmed.2018.10.007
Received 11 April 2018; Received in revised form 21 August 2018; Accepted 11 October 2018

(Kivaria et al., 2007). However, studies describing indicators of CM, such as the most prevalent causing pathogens and distribution of severity score are few in Brazil (Oliveira et al., 2015). Additionally, to the authors' knowledge, no prospective studies have been conducted to evaluate the frequency of CM estimated as incidence rate and considering the at-risk period at the quarter-level. A quarter-level evaluation (instead of a cow-level estimate) may be more accurate for evaluation of CM frequency, since non-affected quarters continue to contribute time at-risk as long as they remain healthy. Furthermore, due to the etiological and epidemiological differences that occur between and within regions, results of studies evaluating CM are useful for the development of specific strategies of control and prevention. This is even more important in countries with production systems similar to Brazil, where the dairy industry remains under development, and the establishment of large-scale milk quality programs are still extensively needed (Busanello et al., 2017).

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, 2012). With the use of specific management strategies for controlling contagious pathogens in the last decades, some of these microorganisms, especially *Strep. agalactiae*, are very rare as the cause of intramammary infections **(IMI)** in several countries (Ruegg, 2012). However, contagious pathogens continue to be a challenge in countries with a less developed milk production chain such as Brazil, where well-known mastitis control practices have not been generally adopted. 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 et al., 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 incidence rate of clinical mastitis (**IRCM**) in dairy herds. Studies evaluating the association of herd-level risk factors 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.

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

2. Material and methods

The study was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science of University of São Paulo (registration code: CEUA 2994060214). All experimental procedures and the care of cows were in strict accordance with the rules issued by the Brazilian National Council for Control of Animal Experimentation (CONCEA; Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009).

2.1. 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), were selected based on a client list of the Qualileite Lab (Mastitis and Milk Quality Research Laboratory at University of Sao Paulo, Brazil) and willingness to participate in 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) have cow identification (e.g., ear tags); and d) 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). On the other hand, to encourage farmers' and herd personnel compliance with the objectives of the study, monthly results of microbiological culture were provided at no cost to the selected herds. In fact, the selected herds were interested in monitoring milk quality and mastitis data. In addition, for herds that completed the proposed data collection period, a detailed report with descriptive study results, such as frequency of CM (general and per group of pathogens) and distribution of severity scores, were presented to the farm personnel.

2.2. Clinical mastitis and severity definition

Before the beginning of the data collection, training on detection of CM and aseptic milk sample collection according to National Mastitis Council (**NMC**) guidelines (National Mastitis Council (NMC), 1999) was reviewed with farm personnel from all herds. In addition, clinical symptoms that may be observed in CM cases (e.g., changes in milk, udder or presence of systemic symptoms) were discussed during the training and farm visits. The training was conducted to ensure data quality and to prevent sample contamination. Kits containing gloves, gauze soaked in 70% ethanol and sterile tubes for milk sample collection were provided to each herd before the beginning of the study and throughout the data collection during the farm visits.

Clinical mastitis was identified at the quarter-level through forestripping 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 (International Dairy Federation (IDF), 1999). A case was 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 2016); the interval between cases was counted from the diagnosis date of previous CM case until the date of the current CM diagnosis. 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. 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. The sample collection period among the farms varied according to the willingness of the owners to remain in the study. Milk sample vials were labelled 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 -20 °C) 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.

2.4. Microbiological identification

All frozen milk samples collected from cases of CM were submitted for microbiological culture according to procedures recommended by the National Mastitis Council (NMC) (1999) 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 trypticase soy 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 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 agar plate. For CNS, 2 or more colonies isolated from a 0.01 mL milk sample were needed to establish presence of an IMI (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. 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 h 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.

2.5. 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. The QDAR were calculated as the sum of days that each quarter remained healthy (or in milking) during a given month, considering the number of lactating cows at the beginning of month and assuming that all cows had four functional quarters. 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. Dry cows were not included in the study. Therefore, the following formula was used to calculate de IRCM:

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

2.6. Incidence rate of clinical mastitis within specific-pathogen groups

The IRCM was evaluated by specific-pathogen group and 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.

The following pathogen groups were created based on final culture results at 48 h: (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.

2.7. 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 severity based on pathogen group 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 used in the models.

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 is defined as an open area surrounded by fences or rails without pasture for grazing.

Season categories were created associating 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 were evaluated in the study (2014 or 2015–2016). In Brazil, the rainy season is characterized by the highest temperatures (summer), and the dry season has the lowest temperatures (winter). 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 and included in the model as a fixed effect: (1) dry 2014; (2) rainy 2014; (3) dry 2015; and (4) rainy 2015. 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.

Six mixed effects regression models (PROC MIXED, SAS 9.4) were used to evaluate the associations between herd-level risk factors and the overall IRCM and IRCM blocked by microbiologically identified pathogen groups. All models included herd as a random effect with a variance component correlation structure, and used the Kenward-Roger degrees of freedom estimation. The residuals were checked for normality by including the 'residual' option in the model statement, and no departure from normality was observed. Categorical explanatory variables were subjected to univariable analyses and variables with $P \leq 0.3$ were explored in a multivariable model. The final multivariable model was reached after performing 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.

3. Results

3.1. Herd characteristics

Descriptive results of the 20 herds evaluated in this study are shown in Table 1. Lactating cows were housed in three different housing systems: 10 paddocks (mean = 246 lactating cows; SD = 106), 5 free-stalls (mean = 647 lactating cows; SD = 417), and 5 compost bedded-pack barns (mean = 138 lactating cows; 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 of Holstein cows (n = 15), while one herd had Jersey cattle, and four herds raised Gyr or Gyr × Holstein crossbreds (also called 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 1713 × 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).

3.2. Clinical mastitis occurrence and pathogen distribution

A total of 5957 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, 1327 (22.3%) cases were recorded but milk samples were not submitted for microbiological culture because milk samples were not collected. In total, 4212 (70.7%) cases from 2637 cows were submitted to the laboratory and had culture results (Table 2). During the study period, 2.637 (51.4%) cows had only one case of CM, while 558 had 2 (21.2%), 330 had 3 (12.5%), 174 had 4 (6.6%), and 219 had ≥5 cases of CM (8.3%).

In relation to the distribution of severity score, out of total quarter cases submitted for microbiological culture (n = 4212), 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 3824 cases of CM. Of this total, 2305 (60.3%) cases were classified as mild, 1305 (34.1%) as moderate and 214 (5.6%) as severe (Table 2).

3.3. Overall IRCM and its association with risk factors at the herd-level

The monthly mean IRCM, for all recorded cases (i.e., those submitted and not submitted for microbiological culture; n = 5539) was 9.7 cases per 10,000 QDAR, ranging from 1.9 to 21.7 (Fig. 1).

After univariate analysis, four variables where included in the multivariate model (BMSCC, BMTBC, milk yield and season). However, after backward stepwise selection, BMSCC ($P = 0.005$) and season ($P = 0.04$) were the only covariates associated with the overall IRCM in this study. 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; Table 3). 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. In addition, the IRCM during the rainy season of 2015 (11.8 cases per 10,000 QDAR) was higher than the IRCM of both dry and rainy seasons of 2014 (9.5 cases per 10,000 QDAR; Table 3).

3.4. Association of IRCM and risk factors at the herd-level blocked by specific pathogen groups

A total of 4068 cases of CM were used to evaluate the IRCM within specific-pathogen groups according to the results of microbiological culture. Of these, 1042 (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 1852 (45.5%) cases had no bacterial growth after 48 h 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,

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	Lactating cows	Milk yield kg/d[a]	BMSCC[b]	BMTBC[c]	H[d]	Period in the study	All CM cases	Cultured cases	At-risk period[e]
A	1470 (52)	34 (2.4)	401	12	FS	Jul/14 – Jul/15	442	379	2316179
B	184 (25)	27 (5.9)	501	9	CB	Apr/14 – Apr/15	179	178	294924
C	68 (5)	18 (3.4)	167	21	P	May/14 – Apr/15	36	35	98046
D	165 (11)	29 (1.8)	443	20	FS	Apr/14 – Apr/15	225	192	263349
E	371 (24)	18 (2.3)	890	27	P	Apr/14 – Apr/15	627	370	579785
F	253 (13)	21 (2.6)	1220	62	P	Mar/14 – Apr/15	874	212	421969
G	77 (15)	16 (0.4)	908	414	P	Apr/14 – Nov/14	43	40	80524
H	71 (9)	22 (0.9)	368	5	P	Feb/15 – Jan/16	98	90	105094
I	167 (11)	29 (2.4)	632	18	CB	May/14 – Apr/15	383	356	242686
J	120 (10)	26 (1.7)	513	15	CB	Apr/14 – Apr/15	72	69	195980
K	313 (7)	35 (1.9)	261	15	FS	Mar/14 – Apr/15	314	299	529317
L	194 (7)	23 (1.7)	571	13	P	May/14 – Apr/15	288	280	280679
M	586 (17)	27 (1.0)	277	14	FS	Dec/14 – Dec/15	1395	1208	914044
N	55 (7)	22 (1.2)	–	–	FS	Apr/14 – Jun/15	112	76	98617
O	46 (3)	19 (2.4)	233	8	P	Apr/14 – Mar/15	48	44	64723
P	36 (1)	17 (1.0)	426	91	P	Apr/14 – Apr/15	69	68	55528
Q	75 (12)	22 (2.9)	1713	872	CB	May/14 – Apr/15	220	212	107530
R	22 (3)	15 (2.1)	515	42	P	Apr/14 – Mar/15	16	13	32956
S	55 (7)	22 (2.2)	280	104	P	Jun/14 – May/15	52	50	80231
T	46 (4)	16 (0.9)	263	19	CB	Apr/14 – Apr/15	46	41	72402
Overall	219 (318)	22.7 (5.7)	557 (387)	94 (210)	–	Mar/14 – Jan/16	5539	4212	6834562

[a] Average of total daily milk produced over month divided by the average of total number of lactating cows during the period of study.
[b] 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.
[c] 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.
[d] Housing system - FS = free-stall, CB = compost-bedded pack barn, and P = paddocks.
[e] At-risk period - Sum of days that quarters of lactating cows remained at risk (without presenting CM) in each herd throughout the study period.

and 0.3 (0.7) cases per 10,000 QDAR for other pathogens (Fig. 1). The results from the mixed linear regression models evaluating the association of IRCM within the specific pathogens groups and characteristics at the herd level are presented in the Table 4.

3.4.1. Gram positive group

The IRCM was associated with season ($P = 0.008$), primarily due to the effect of the rainy season of 2015 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 4).

3.4.2. Contagious pathogen group

Variables housing ($P = 0.0004$), BMSCC ($P = 0.01$), and average daily milk yield per cow ($P = 0.01$) were associated with IRCM. Herds with lactating cows housed in paddocks had lower IRCM (0.5 cases per 10,000 QDAR) than herds with free-stall (1.2 cases per 10,000 QDAR) or compost-bedded pack barn systems (3.2 cases per 10,000 QDAR); however, there was no statistical difference ($P = 0.57$) in IRCM between compost-bedded pack barn and free-stall systems. Herds with geometric means of BMSCC $\geq 601 \times 10^3$ cell/mL had higher IRCM (3.1 cases per 10,000 QDAR) than herds with BMSCC $\leq 300 \times 10^3$ cell/mL (0.8 cases per 10,000 QDAR) and herds with BMSCC between 301–600 $\times 10^3$ cell/mL (0.3 cases per 10,000 QDAR); however, there was no statistical difference ($P = 0.42$) between herds with BMSCC $\leq 300 \times 10^3$ cell/mL and herds with BMSCC between 301–600 $\times 10^3$ cell/mL. 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 ($P = 0.17$) was observed between herds with average milk yield per cow of ≤ 20 and ≥ 26 kg/d (Table 4).

3.4.3. Gram-negative group

BMTBC ($P = 0.04$) was the only variable associated with IRCM. Herds with BMTBC $\geq 31 \times 10^3$ cfu/mL had higher IRCM caused by Gram-negative pathogens (1.8 cases per 10,000 QDAR) than herds with BMTBC $\leq 30 \times 10^3$ cfu/mL (0.9 cases per 10,000 QDAR; Table 4).

3.4.4. Other pathogen group

There were no statistically significant associations ($P < 0.05$) between risk factors and IRCM.

3.4.5. Negative culture group

Season was associated with IRCM ($P = 0.05$), specifically, the rainy season of 2015 had higher IRCM (4.0 cases per 10,000 QDAR) than both dry (2.4 cases per 10,000 QDAR) and rainy (2.6 cases per 10,000 QDAR) seasons of 2014 (Table 4).

4. Discussion

In countries with a developing dairy industry such as Brazil, access to milk quality improvement programs, modern technologies used for dairy management (e.g., farm management software), and mastitis diagnostics (e.g., access to a laboratory for SCC and culture results) are not widely available to dairy producers (Busanello et al., 2017). Thus, research identifying areas to prioritize in milk quality improvement programs are needed for development of local dairy industry, and to our knowledge, there are no current prospective research studies evaluating clinical cases of mastitis in Brazil. Given the limited access to dairy management software and mastitis diagnostics by most dairy producers, we prioritize to perform the current research using a prospective study design, which would allow us to collect data (e.g., identification of affected cow and quarter, CM severity scores, and culture results) in a systematic and controlled fashion. Although the aforementioned reasons represent important benefits to a prospective study design, obvious limitations are the increased cost and time, when compared to a retrospective design for example. Thus, although the

Table 2
Culture results and pathogen-specific distribution of severity scores of clinical mastitis cases (n = 4212) occurring in dairy cows from 20 herds of Southwest, Brazil, evaluated from March 2014 to January 2016.

Microbiological culture	Frequency n (%)	Severity[a] (%)		
		Mild	Moderate	Severe
Total samples cultured	4212 (100)	60.3	34.1	5.6
No growth	1852 (44.0)	62.7	32.5	4.8
Contamination[b]	129 (3.1)	62.0	30.1	8.0
Mixed[c]	19 (0.4)	35.3	64.7	–
Single pathogen[d]	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[e]	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[f]	111 (7.6)	59.4	35.4	5.2
Total[h]	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[g]	115 (19.2)	46.7	41.0	12.4
Total[h]	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[h]	147 (6.7)	72.8	26.5	0.7

[a] (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 (3824 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.

[b] Isolation of three or more different pathogens.

[c] Isolation of two different pathogens.

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

[e] Coagulase-negative staphylococci.

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

[g] *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).

[h] 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.

number of herds included in this prospective 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 regions with a developing dairy industry. The high CM incidence associated with contagious pathogens and the elevated BMSCC levels observed in the current prospective study highlight the need of a large-scale mastitis control program in this country, which focus on contagious pathogens.

Descriptive data on herd characteristics and milk production systems are scarce in Brazil, which makes it difficult to compare the herds selected in our study with the average dairy herds from the same region (i.e., southeastern Brazil). Only one study evaluated a relatively high number of dairy herds (n = 474) in the southeastern region of Brazil (Busanello et al., 2017). Although some herd-level characteristics (e.g., milk yield per cow and BMSCC) were not reported in that study, the median herd size was quite similar to that found in the present study: 87 (range = 11-1,348 lactating cows in the study of Busanello et al. (2017) and 98 (range = 22-1,470 lactating cows) in our study. Furthermore, the number of lactating cows and milk yield for herds selected in our study were higher than the average of herds reported by the Brazilian Census of Agriculture (IBGE (Instituto Brasileiro de Geografia e Estatísticas), 2006), which estimated 1.35 million dairy herds in the country with an average of 30 lactating cows producing < 2000 kg of milk per year; almost all herds (99%) housed cows in extensive and semi-extensive systems. The census also reported that several herds (located mostly in the South and Southeast regions of Brazil) are comprised by Holstein cows producing > 4500 kg of milk per year. In our study, the average herd size was 246 lactating cows per herd and most herds were composed of Holstein cows (n = 15). Moreover, considering the overall mean of daily milk production per cow in our study (22.7 kg), we can suggest that the milk production of cows was much higher in our study compared to that reported in the Brazilian census (i.e., < 2000 kg/cow per year).

Due to the limited sample size, we acknowledge that there may not be enough power to detect small differences especially when variables were sub-divided into several categories (e.g., season). A post hoc analysis of power showed that 20 herds were needed to detect the difference when BMSCC was evaluated; however, this estimation was based on a large difference in estimates (Table 3), which was not seen with the other variables. As previously mentioned, a retrospective study may have resulted in a larger sample size thus allowing better generalizability, however, most Brazilian herds do not commonly record CM data (e.g., identification of affected cow and quarter, CM severity scores, and culture results). Therefore, a prospective study was done in order to have better control of data recording and for identification of confounding factors that could lead to information bias with the aim of strengthening data quality.

Developing countries such as Brazil still have a high prevalence of poorly managed herds, which are characterized by high frequency of clinical and subclinical mastitis. A recent study evaluating 8285 test days from 517 dairy herds in Brazil (92% from southeast region) reported that almost half (46.4%) of the cows within herds were subclinically infected, and approximately 18% of the healthy cows developed new cases of subclinical mastitis every month (Busanello et al., 2017). Although we did not evaluate the frequency of subclinical mastitis in our study, the high observed mean of SCC (557×10^3 cells/mL) may indicate a high prevalence of subclinical mastitis within most herds. Thus, descriptive information about these herds is important because it may be difficult to make an accurate comparison of Brazilian dairy herds with studies conducted in countries with better overall mastitis control, where the mean SCC and the frequency of CM are significantly lower than observed in our study.

Our literature review found only one study describing the CM profile in Brazilian dairy herds (Oliveira et al., 2015). However, the estimation of CM frequency was expressed as incidence risk (or cumulative incidence) and performed at the cow level as opposed to rate and at the quarter level as was done 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).

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. 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 they recorded the mastitis occurrence at the quarter level. There is both biological and statistical support for evaluating the at-risk period at the quarter level. Mammary quarters are considered anatomically independent from each other (Tucker, 1981), although recent studies support the hypothesis of the immunological interdependence of

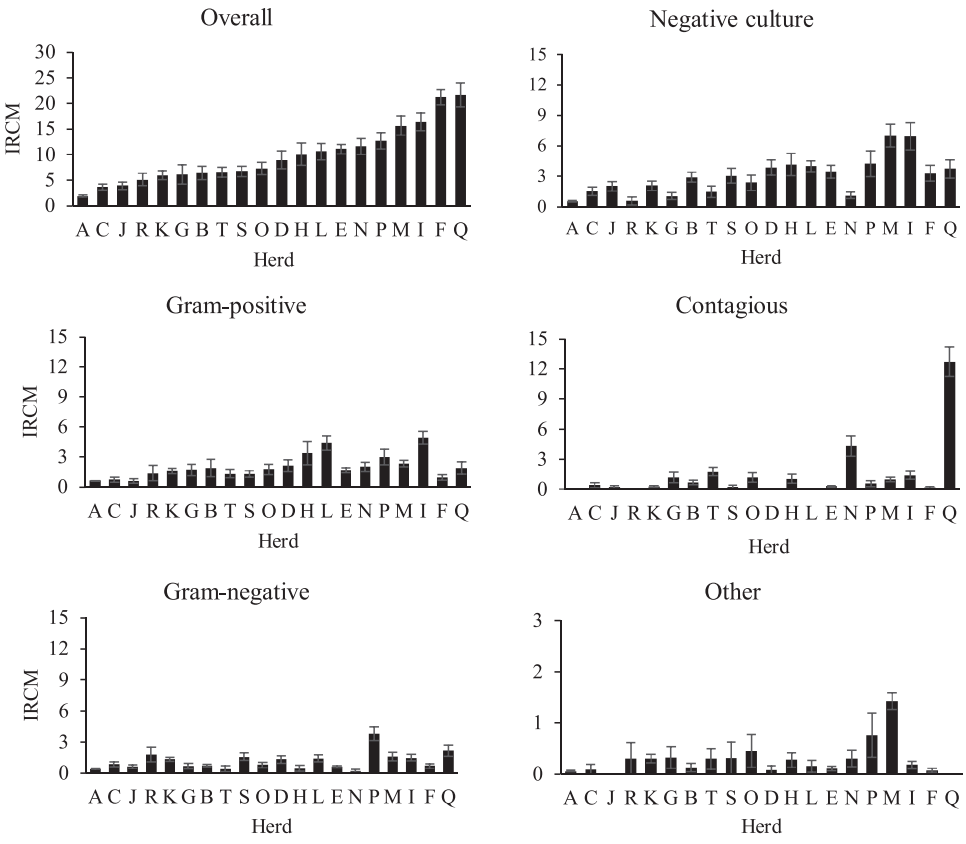

Fig. 1. Distribution (overall and by specific microbiological groups) of the monthly average incidence rate of clinical mastitis (IRCM = number of cases per 10,000 quarter-days at risk) in 20 herds of Southwest, Brazil, evaluated from March 2014 to January 2016. Overall IRCM consisted of all reported 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 37 °C; 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.

Table 3
Results of multivariable regression model describing the association between variables at the herd-level and the overall incidence rate of clinical mastitis of 20 dairy herds of Southwest, Brazil, evaluated from March 2014 to January 2016.

Variable	Herds (n)	β[a]	SE	Mean[b]	SD[c]	*P*-value
BMSCC[d]						0.005
≤300	6	−8.0	2.5	7.7a	5.5	
301-600	8	−8.2	2.4	7.5a	6.0	
≥601	5	Ref.		16.0b	8.0	
Season[e]						0.04
Dry 2014	18	−2.3	0.9	9.5a	7.4	
Rainy 2014	19	−2.4	1.0	9.5a	6.5	
Dry 2015	17	−1.4	1.1	10.4ab	7.4	
Rainy 2015	19	Ref.		11.8b	7.8	

Estimates of the variances components were $\hat{\sigma}^2_{herd} = 15.2$ and $\hat{\sigma}^2_{residual} = 24.5$.

[a] β = Regression coefficient.

[b] 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 ($P \leq 0.05$).

[c] Standard deviation related to the means of incidence rate of clinical mastitis.

[d] 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.

[e] 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).

quarters based on the influence of infections on certain immune response parameters in contralateral uninfected quarters (Blagitz et al., 2015, Paixao 2017). Therefore, the evaluation of IRCM at the quarter-level may be more accurate than evaluations at the cow-level. Additionally, the evaluation of time at-risk at the cow-level can result in bias because if the cow develops CM in more than one quarter, but she was no longer contributing time at-risk, the additional CM event will artificially increase the reported CM rate. Evaluating time at-risk at the quarter level allows accurate tracking because non-affected quarters continue to contribute time at-risk.

In the current study, the average BMSCC was 557×10^3 cells/mL based on monthly tests. Although more frequent bulk milk data may have provided a better estimate of the herd's milk quality, given both the observational nature of this study and current regulations for bulk tank testing, only monthly estimates available on farms were used. 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 $\times 10^3$ 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 $\times 10^3$ cells/mL; Erskine et al., 1987). As described previously, the comparison of our results with other studies evaluating well-managed herds with better indices of milk quality may be difficult, as the high mean SCC and IRCM observed in our study indicate failures of overall mastitis control in most enrolled herds. However, 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).

Table 4
Results of four multivariable regression models describing the association between variables at the herd-level and the incidence rate of clinical mastitis by the specific-pathogen groups of 20 dairy herds of Southwest, Brazil, evaluated from March 2014 to January 2016.

Variable	Herds (n)	β[a]	SE	Mean[b]	SD[c]	*P*-value
Contagious pathogens[d]						
Housing						0.0004
Compost Barn	5	5.3	1.1	3.2a	5.2	
Free stall	5	6.1	1.7	1.2a	2.5	
Paddocks	10	Ref.		0.5b	1.0	
BMSCC[e]						0.01
≤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[f]						0.004
≤20	7	2.7	1.9	0.7b	1.2	
21-25	6	4.7	1.7	3.0a	5.1	
≥26	7	Ref.		0.5b	0.8	
Other Gram-positive[g]						
Season[h]						0.008
Dry 2014	18	−0.9	0.3	1.7a	2.0	
Rainy 2014	19	−1.1	0.4	1.6a	1.7	
Dry 2015	17	−1.0	0.4	1.8a	1.9	
Rainy 2015	19	Ref.		2.8b	2.9	
Gram-negative[i]						
BMTBC[j]						0.04
≤30	13	−0.9	0.4	0.9a	0.98	
≥31	6	Ref.		1.8b	2.04	
Negative culture						
Season[h]						0.047
Dry 2014	18	−1.2	0.4	2.4a	2.9	
Rainy 2014	19	−1.1	0.5	2.6a	2.8	
Dry 2015	17	−1.0	0.6	3.2ab	2.9	
Rainy 2015	19	Ref.		4.0b	3.6	

[a] β = Regression coefficient.
[b] 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 ($P \leq 0.05$).
[c] Standard deviation related to the means of incidence rate of clinical mastitis.
[d] Group consisted by the major contagious pathogens: *Staph. aureus* and *Strep. agalactiae*.
[e] 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.
[f] Average daily milk yield per cow (kg/d).
[g] Group formed by cases with isolation of Gram-positive bacteria, with exception of contagious pathogens and *Trueperella pyogenes*.
[h] 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).
[i] Group formed by cases with isolation of Gram-negative bacteria.
[j] 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.

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 et al., 1997, Barkema 1999; de Haas et al., 2004). Twelve out of 20 herds evaluated in this study had *Strep. agalactiae*, including two herds (N and Q; Fig. 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 (i.e., 666 lactating cows) 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), although the IRCM and average BMSCC of herds evaluated in that study were lower than observed in the present study. 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 et al., 2009).

To our knowledge, no study evaluating housing type and IRCM has been reported in dairy herds housing cows in similar conditions. A large subset of herds in this study include cows housed in outdoor paddocks, which although are representative of most of the cows in Brazil, are not readily included in research studies due to limitations in access to herd information (e.g., mastitis frequency). 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 free stalls compared to herds where the cows were housed in paddocks. However, it is important to mention that two herds, one with a compost-bedded pack system (herd Q; 4.3 quarter cases per QDAR) and the other with a free-stall system (herd N; 12.7 quarter cases per QDAR) had the highest IRCM when contagious pathogens were evaluated (Fig. 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 free stalls. On the other hand, other studies reported that herds housing cows in loose-house system had higher IRCM than herds housed in free stalls and tie-stalls (Peeler et al., 2000, Barnouin 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 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, individual SCC, average number of lactations and DIM) could also influence the relationship between IRCM and milk yield.

Season was associated with overall IRCM, and when "Gram-positive pathogens" and "negative culture" were evaluated. It is important to mention that we did not intend to make any inferences about the overall effect of season on the frequency of CM. However, we controlled the effect of season (dry versus rainy) during the period that each herd remained in the study by including this variable as fixed effect in the statistical regression models because environmental conditions are

known to affect the risk of CM, especially for cows in outdoor housing systems (i.e., paddocks). A longer duration of the study would be necessary to make more accurate conclusions about the overall association of IRCM and season.

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 South-eastern 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 and 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 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 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 and Bramley, 1982). In addition, recent studies have reported that cow-to-cow transmission of *Strep. uberis* can potentially occur 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* (approximately 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 disinfection, 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 and 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 et al., 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 and 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 et al., 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 et al., 2013, Verbeke 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.

5. Conclusion

Overall, the IRCM was 9.7 quarter-cases per 10,000 QDAR, and the only herd-level parameters associated with overall IRCM were BMSCC and season. 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 evaluations of other Gram-positive pathogens and negative cultures, 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 $> 30 \times 10^3$ cfu/mL. There was no association between herd-level risk factors and IRCM with isolation of "other pathogens". 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 South-eastern 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.

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo [FAPESP; grant numbers 2014/17411-6 and 2013/22286-6 (first author's scholarship)].

Acknowledgements

Sincere thanks to all the farmers for their motivation and for the accordance in participating in this study. We are also grateful to José Garcia Franchini and Lucineia Mestieri (Qualileite Milk Quality Laboratory, School of Veterinary Medicine and Animal Science, University of São Paulo, Pirassununga, Brazil) for their assistance with laboratory analysis.

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