Influence of pathogens causing clinical mastitis on reproductive variables of dairy cows

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ABSTRACT

In dairy cattle, mastitis is a disease of the mammary gland caused by pathogens such as bacteria, viruses, fungi, and algae. Mastitis causes economic losses to dairy farms as well as public health concerns. The reproductive efficiency of commercial dairy herds has important implications for the economic success of dairy operations and is strongly associated with the health status of cows. Mastitis has previously been linked with decreased fertility of dairy cows, but the effect of specific pathogens on the severity of fertility reduction is still unclear. In this study, cows diagnosed with mastitis caused by major pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Klebsiella* spp., *Mycoplasma* spp., and environmental *Streptococcus*) needed more artificial inseminations (AI) than did cows with mastitis caused by minor pathogens (coagulase-negative *Staphylococcus* and *Corynebacterium* spp.) and healthy cows. Cows diagnosed with mastitis, independent of what pathogen was causing mastitis, had more days open compared with non-mastitic cows. The percentage of cows that successfully established pregnancy at first AI was greater for the control group than for the major pathogens group but not significantly different from the minor pathogens group. Pregnancy loss was lower in the control group than in the major pathogens group; however, there was no difference compared with the minor pathogen group. Mastitis caused by gram-negative bacteria decreased the percentage of pregnancy per first AI and increased days open and pregnancy loss compared with the control group. Cows with mastitis caused by gram-positive bacteria also had increased days open compared with control cows. This study shows that different mastitis-

causing bacteria can affect the fertility of cows differently. Mastitis events caused by major pathogens and gram-negative bacteria were associated with the greatest decrease in reproductive efficiency.

Key words: bacteria, dairy cow, artificial insemination, mastitis

INTRODUCTION

Mastitis presents a significant challenge to global milk production, accounting for an annual loss of approximately \$2 billion to dairy farmers in the United States alone (Smith and Hogan, 2001). Major annual costs related to mastitis were (1) reduction of milk production (\$102/cow), (2) discarding of milk (\$24/cow), and (3) animal replacement (\$33/cow), bringing the total cost of mastitis to around \$159/cow per year (Smith and Hogan, 2001).

Mastitis is characterized by infections of the mammary gland that have multiple etiologies and are most frequently caused by bacteria (Deb et al., 2013), which trigger an inflammatory response (Fuenzalida et al., 2015). Mastitis can be classified as clinical or subclinical. Clinical mastitis involves abnormal milk production and may or may not present with udder alteration or clinical systemic signs (Pinzón-Sánchez and Ruegg, 2011). Subclinical mastitis causes mobilization of inflammatory cells to the udder without changes in milk or udder characteristics, resulting in increased SCC (de Freitas Guimarães et al., 2013).

Occurrence of clinical or subclinical mastitis can negatively affect reproduction (Lavon et al., 2011; Hudson et al., 2012). Cows with clinical mastitis require more AI events because they have a decreased conception rate (Barker et al., 1998; Santos et al., 2004). Furthermore, cows diagnosed with clinical mastitis have shown longer intervals between calving and first AI, greater pregnancy losses, more days open, decreased milk production, and decreased milk fat percentage compared

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with healthy cows (Boujenane et al., 2015; Kumar et al., 2017). Similarly, subclinical mastitis after AI has been associated with a decreased conception rate (Lavon et al., 2011; Hudson et al., 2012). Cows with SCC greater than 200×10^3 cells/mL had a 10% decrease in pregnancies per AI compared with nonmastitic cows (Bijker et al., 2015). Therefore, the aim of this study was to investigate the effect of clinical mastitis caused by different pathogens on reproductive variables following AI in dairy cows.

MATERIALS AND METHODS

The experiment was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, São Paulo State University, Brazil (permit no. 196/2016-CEUA).

Animals and Trial Period

This study was conducted on 5 commercial dairy herds with an average milk production of 30 kg/cow per day, SCC lower than 400×10^3 cells/mL, an active program of mastitis control, use of herd management software, and at least 200 cows in milk. A total of 833 lactating Holstein cows were enrolled in this trial. One herd was milked 3 times per day in a carousel-type milking parlor; cows were kept in cross-ventilated freestall barns and fed a TMR with ad libitum water access. The other 4 herds were milked twice per day in a herringbone parlor; cows were kept in freestall barns equipped with sprinklers and fans for cooling and fed a TMR with ad libitum water access. Farms were monitored for a period of 24 mo, and samples were collected by trained staff at the moment of clinical mastitis diagnosis during milking. All other cow-related data were recorded daily and stored on a monthly basis by the authors. Cows were classified based on whether they had been diagnosed with clinical mastitis. We enrolled in this study cows that had clinical mastitis in any time from parturition to pregnancy diagnosis. Cows diagnosed with mastitis were classified by pathogenicity of the agent isolated, as suggested by Schepers et al. (1997), and were grouped as follows: major pathogens group (mastitis by *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Klebsiella* spp., *Mycoplasma* spp., *Streptococcus uberis*, or *Strep. dysgalactiae*) and minor pathogens group (mastitis by CNS or *Corynebacterium* spp.). Cows were also divided according to the Gram staining classification as follows: gram-positive group (mastitis by *Staph. aureus*, CNS, *Strep. agalactiae*, *Strep. uberis*, or *Strep. dysgalactiae*) and gram-negative group (mastitis by *E. coli* or *Klebsiella*

spp.). Cows classified as control did not have mastitis diagnosed between calving and pregnancy. Cows in the control group had data and milk samples collected after 60 DIM until confirmation of pregnancy at around 60 d of gestation.

Microbiologic Culture

Milk samples from cows presenting clinical mastitis were collected for microbial isolation with assistance of the tamis or strip cup test (i.e., presence of blood, color change, or lumps). Milk samples from each mammary gland quarter diagnosed with mastitis were collected aseptically and transported to the laboratory under refrigeration (4–8°C). Microbiological cultures were carried out at the laboratory of Núcleo de Pesquisa em Mastites at the Department of Veterinary Hygiene and Public Health of Faculty of the Veterinary Medicine and Animal Science in Botucatu, São Paulo, Brazil. Milk samples (100 µL) were plated on bovine blood agar at 5% and MacConkey agar, kept at 37°C under aerobic conditions for 72 h, and observed for microbial growth every 24 h. Isolates of microorganisms were identified according to culture, morphology (Gram stain), and biochemistry characteristics (Quinn et al., 2011). Samples that had at least 3 colonies after 72 h of incubation were considered positive for mammary infection. Samples with more than 3 different colonies were considered contaminated following NMC (2004) recommendations.

The isolation of *Mycoplasma* spp. was performed using cultivation of milk in a Petri plate filled with Hayflick medium supplement (Merck Millipore, Darmstadt Germany), in accordance with the method described by Whitford et al. (1994). Incubation was conducted in a microaerophilic atmosphere of a CO₂ heating chamber, with subsequent observation every 2 d until d 15. Isolation of *Mycoplasma* spp. was carried out by visualization of culture plates under a stereomicroscope. The culture was positive when observed colonies appeared like fried eggs (with the central dark area representing the area of mycoplasmal downgrowth and the pale periphery indicating superficial growth of *Mycoplasma* on the agar) and films and a smear were formed as described by Pretto et al. (2001).

Reproductive Indices

Farms involved in the study used herd management software (DairyPlan C21, GEA, Düsseldorf, Germany) to store data, such as that for calving, health disorders, vaccinations, and time of AI. Reproduction data were collected by farm personnel and the veterinarian re-

sponsible for reproductive exams and were recorded on a daily or weekly basis depending on the standard operating procedures on each farm. Reproductive indices of each cow enrolled in the study included pregnancy per first AI, pregnancy loss (from 30 to 60 d of gestation), and days open.

Reproductive management used in the experiment was as follows: cows were monitored from 60 DIM using an automated activity monitor (HeatSeeker-TX, Boumatic Dairy Equipment, Madison, WI) for evaluation of cow movement and detection of estrus. Cows that had a relative increase in physical activity that surpassed a set threshold determined by the system were examined to confirm estrus and later were artificially inseminated. Pregnancy diagnosis was performed by rectal ultrasonography (Mindray 2200VET DP, Shenzhen Mindray Bio-Medical Electronics Co., Shenzhen, China) at 30 d after AI and then again at 60 d after AI for confirmation. Cows considered pregnant at 30 d post-AI and then not pregnant at d 60 of gestation were classified as having pregnancy loss. Metritis, retained placenta, and lameness were diagnosed by the farm veterinarian using the clinical criteria described by LeBlanc (2008) and Shearer et al. (2012). Milk production and SCC data were measured monthly. We used the data from the month before the diagnosis of mastitis, whereas for the control group the milk production and SCC data used were recorded in the farms' herd management software in the month before AI.

Statistical Analysis

Sample size was calculated using a single proportion (pregnancy per AI at 30 d of gestation) power analysis ($\alpha = 0.05/\beta = 0.20$) assuming a null hypothesis proportion of 35% (control) and an expected difference of 10 percentage units (25%; major). The variables pregnancy per first AI and pregnancy loss were analyzed by logistic regression using the GLIMMIX procedure of SAS (SAS Institute Inc., Cary, NC). Two models were used; one included the effects of the initial group of bacteria (major vs. minor), and other included Gram staining (positive or negative). Both models included the effects of farm, parity, metritis, lameness, retained placenta, milk production, SCC, and interactions. Effects of group of pathogens (major or minor and gram-positive or gram-negative) on hazard risk of pregnancy by 30 d after AI were analyzed using the PHREG procedure of SAS. The Cox proportional hazard regression models included days open as the outcome variable and group, Gram staining, farm, parity, metritis, lameness, retained placenta, and milk production as explanatory variables. Two models were used, one for group (major and minor) and other for Gram staining (positive and

negative). In this study, cows were used as random effect, and groups (pathogens), diseases (metritis, lameness, and retained placenta), milk production, and SCC were used as fixed effects. Milk production and SCC (log; linear SCC) for the test days used in the analysis of the respective groups were treated as continuous variables. Observations were right censored at culling or at 300 DIM if pregnancy had not been previously confirmed. Results are presented as means $\pm$ standard error of the mean and considered significantly different if $P < 0.05$ and a tendency if $0.05 < P < 0.10$. Significant effects were reported by least squares means. The nonsignificant variables were excluded from the model according to Wald's criterion ($P > 0.20$). The variables SCC and metritis were nonsignificant in all models and therefore excluded.

RESULTS

The study analyzed 2,519 milk samples from participating herds. No growth, contaminated growth, or pathogens not part of this investigation were observed in 1,958 samples. The final analysis comprised 273 cows in the control group, 191 in the minor pathogens group, and 369 in the major pathogens group. The minor pathogens group comprised 161 (84.3%) CNS spp. and 30 (15.7%) *Corynebacterium* spp. The major pathogens group comprised 204 (55.2%) environmental *Streptococcus* spp., 15 (4.1%) *Strep. agalactiae*, 94 (25.5%) *E. coli*, 29 (7.9%) *Klebsiella* spp., 15 (4%) *Staph. aureus*, and 12 (3.3%) *Mycoplasma* spp. The gram-negative group comprised 94 (76.4%) *E. coli* and 29 (23.6%) *Klebsiella* spp. The gram-positive group comprised 219 (55.4%) environmental *Streptococcus*, 161 (40.7%) CNS, and 15 (3.9%) *Staph. aureus*.

Pregnancy per first AI was greater ($P = 0.01$) in the control group than in the major pathogens group, but the minor pathogens group did not differ from either the control group or the major pathogens group (Table 1). Pregnancy per first AI was greater ($P = 0.004$) in the control group than in the gram-negative group ($P = 0.004$), but the gram-positive group did not differ from either the control group or the gram-negative group.

Pregnancy losses were greater ($P = 0.01$) in the major pathogens group than in the control group but did not differ between the minor pathogens group and either the control group or the major pathogens group (Table 1). Pregnancy losses were greater ($P = 0.003$) in the gram-negative group than in the control group and either gram-positive group, but no difference was observed between the gram-positive group and the control group (Table 1).

A significant difference ($P < 0.001$) was found for days open between control and mastitic cows (major

Table 1. Effects of mastitis caused by different groups of pathogens (control, minor, or major) or by Gram class (positive or negative) on pregnancy per first AI, pregnancy loss, and days open in dairy cows¹

Group ²	Pregnancy/first AI, %	Pregnancy loss, %	Days open, d
Control	32.6 ± 0.02 (89/273) ^{a,A}	12.8 ± 0.02 (35/273) ^{a,A}	129.5 ± 1.9 ^{a,A}
Minor	26.2 ± 0.03 (50/191) ^{ab}	16.7 ± 0.02 (32/191) ^{ab}	162.0 ± 4.1 ^b
Major	20.1 ± 0.02 (74/369) ^b	22.2 ± 0.02 (82/369) ^b	175.1 ± 3.7 ^c
Gram-positive	23.8 ± 0.02 (94/395) ^{AB}	17.2 ± 0.02 (68/395) ^A	172.7 ± 4.1 ^B
Gram-negative	15.4 ± 0.03 (19/123) ^B	30.1 ± 0.03 (37/123) ^B	191.1 ± 7.45 ^C

^{a-c}Means or percentages within a column followed by different lowercase letters are different ($P < 0.05$; control vs. major vs. minor).

^{A-C}Means or percentages within a column followed by different uppercase letters are different ($P < 0.05$; control vs. gram-negative vs. gram-positive).

¹Pregnancy/first AI = cows diagnosed pregnant after first AI after calving; values are given as LSM ± SEM (no./no.). Pregnancy loss = cows diagnosed pregnant 30 d after AI and diagnosed nonpregnant at 60 d after AI; values are given as LSM ± SEM (no./no.). Days open = number of days between calving and new conception; values are given as LSM ± SEM.

²Control = cows with no mastitis events between calving and new pregnancy establishment. Minor = cows with mastitis caused by CNS or *Corynebacterium* spp. Major = cows with mastitis caused by *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Klebsiella* spp., *Mycoplasma* spp., *Streptococcus uberis*, or *Streptococcus dysgalactiae*. Gram-positive = cows with mastitis caused by *Staph. aureus*, CNS, *Strep. agalactiae*, *Strep. uberis*, or *Strep. dysgalactiae*. Gram-negative = cows with mastitis caused by *E. coli* or *Klebsiella* spp.

and minor) as well as between the minor and major pathogens groups ($P = 0.02$; Table 1; Figure 1). The hazard ratios of pregnancy were 1.92 ($P < 0.001$), 1.22 ($P = 0.02$), and 0.81 ($P = 0.02$) in the control, minor pathogens, and major pathogens groups, respectively. Days open were significantly lower in the control cows than in the mastitic cows, particularly cows in the gram-negative group, which had more days open than cows in the gram-positive group ($P < 0.001$; Table 1;

Figure 2). Pregnancy hazard ratio for cows with mastitis caused by gram-positive pathogens was 1.36 ($P = 0.004$), whereas for cows with mastitis caused by gram-negative pathogens it was 0.73 ($P = 0.004$).

DISCUSSION

This study aimed to assess the effect of clinical mastitis etiology on the reproductive performance of dairy

Figure 1. Survival curve of DIM to conception until 300 DIM for each group of cows. Control = cows with no mastitis events between calving and new pregnancy establishment. Minor = cows with mastitis caused by CNS or *Corynebacterium* spp. Major = cows with mastitis caused by *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Klebsiella* spp., *Mycoplasma* spp., *Streptococcus uberis*, or *Streptococcus dysgalactiae*. Letters (A–C) indicate that each individual survival curve was different ($P < 0.05$) from the other 2 survival curves.

Figure 2. Survival curve of DIM to conception until 300 DIM for Gram bacteria class. Control = cows with no mastitis events between calving and new pregnancy establishment. Gram-positive = cows with mastitis caused by *Staphylococcus aureus*, CNS, *Streptococcus agalactiae*, *Streptococcus uberis*, or *Streptococcus dysgalactiae*. Gram-negative = cows with mastitis caused by *Escherichia coli* or *Klebsiella* spp. Letters (A–C) indicate that each individual survival curve was different ($P < 0.05$) from the other 2 survival curves.

cows. It was observed that cows affected by mastitis caused by more pathogenic bacteria (major pathogens group) had poorer reproductive performance compared with cows in the control group and those with mastitis caused by less pathogenic bacteria (minor pathogens group). Similarly, cows affected by mastitis caused by gram-negative pathogens had poorer reproductive performance compared with those in the control and gram-positive groups. Another interesting finding was that the minor pathogens group and gram-positive group showed similar reproductive results, suggesting that pathogens causing mild clinical mastitis are able to disturb reproductive performance, but not as markedly as major pathogens and gram-negative bacteria.

Cows diagnosed with mastitis have previously been reported to have lesser pregnancy per first AI (Barker et al., 1998; Chebel et al., 2004), which agrees with the current study. The control group had a greater percentage of pregnancy per first AI compared with mastitic cows (major and minor groups together). The only significant difference was detected between the major and control groups, whereas the minor group was numerically intermediary between the major pathogen group and control groups but statistically not significant. These results support the hypothesis that mastitis causes negative effects in the reproductive performance of cows but with a noticeably greater influence from major pathogens. Previous reports (Soto et al., 2003; Hansen et al., 2004) showed that cows af-

ected by mastitis caused by gram-negative bacteria had poorer reproductive performance than cows affected by gram-positive bacteria; this result was not observed in this study for pregnancy per first AI despite a large numerical difference being observed. Other authors found similar results comparing mastitis before first AI caused by *E. coli* and *Streptococcus* (Lavon et al., 2019). A recent study showed that the pregnancy rates after embryo transfer were significantly affected by mastitis (Barbosa et al., 2018). The authors also found that major pathogens and gram-negative bacteria caused the greatest reduction in reproductive performance. In addition, they found that the minor pathogens and gram-positive groups (particularly the contagious sub-division, formed mainly by *Staph. aureus* and *Strep. agalactiae*) were similar to the control group in terms of reproductive performance (Barbosa et al., 2018), corroborating the data of this study. In this study there were no differences between the control and minor pathogens groups, which were formed by CNS and *Corynebacterium* spp., again supporting the findings of the previous authors.

Cows in the major pathogens group had a greater percentage of pregnancy loss compared with cows in the control group. No significant difference was observed between the minor pathogens group and the other 2 groups. A difference between the control and major pathogens groups was expected, as udder infection has been shown to influence conception and preg-

nancy maintenance (Battaglia et al., 1997; Riollot et al., 2001; Hansen et al., 2004; Hockett et al., 2005). On the contrary, pathogens that caused less severe mastitis seemed to have no influence on pregnancy loss in cattle. The udder inflammatory reaction releases substances (e.g., interleukins, tumor necrosis factor- α , LPS, nitric oxide, and prostaglandins) into the bloodstream, which could lead to luteolysis or embryo death (Hansen et al., 2004). The gram-negative group had greater pregnancy loss compared with the control and gram-positive groups. Apparently, the LPS in the gram-negative bacteria causes a significant disturbance in embryo development, leading to an increase in pregnancy loss in cows with mastitis caused by gram-negative bacteria; this has also been observed by other authors (Bromfield et al., 2015; Ibrahim et al., 2015; Magata and Shimizu, 2017; Campos et al., 2018). No difference in pregnancy loss was observed for cows with subclinical mastitis after embryo transfer (Barbosa et al., 2018). This could be explained by the fact that the authors used 7-d-old embryos, which possibly avoided the effect of the inflammatory reaction on the oocytes and on the fertilization event. Another important point is that the authors worked with subclinical mastitis, which may have had a milder effect on the reproductive tract.

In this study, the days open periods were longer in the groups of cows with clinical mastitis compared with the control group. These findings are in accordance with literature that reported that mastitic cows had more days between calving and conception (Kumar et al., 2017). A significant difference was observed between the major and minor pathogens groups in pregnancy per first AI, indicating that the severity of mastitis could influence the number of days open. Supporting these findings, a few reports confirmed that mastitis causes reproductive impairments because of disturbances in oocyte and embryo development, decreased production of reproductive hormones, follicular growth disruption, deficiency of ovulation, and weaker estrous expression (Hockett et al., 2000, 2005; Soto et al., 2003; Herath et al., 2007; Williams et al., 2008). When different Gram status groups were evaluated, gram-negative bacteria were seen to cause an increase in days open compared with gram-positive bacteria. It was observed that gram-positive bacteria increased the number of days open compared with the control group. These findings support the theory that the LPS present in the gram-negative membrane bacteria causes relative disruption to reproductive tissues, leading to fertility problems (Hansen et al., 2004) and, consequently, more days open.

It is well known that the LPS from gram-negative bacteria infecting the mammary gland causes deleterious consequences for bovine fertility (Hansen et al., 2004).

However, the manner in which LPS affects reproduction is not completely known. Some authors have been studying this mechanism, trying to understand the underlying pathway. Lipopolysaccharide could delay oocyte development in cows (Soto et al., 2003), decrease the release of estradiol from granulosa cells (Williams et al., 2008), inhibit ovulation (Williams et al., 2008), lead to early apoptosis of oocytes (Zhao et al., 2019), affect oviducts promoting early embryo death (Ibrahim et al., 2015), delay cytoplasm maturation in oocytes causing embryonic developmental changes (Magata and Shimizu, 2017), cause problems in pregnancy maintenance (Bromfield et al., 2015; Campos et al., 2018), and reduce the primordial ovarian follicle pool to cause a long-term effect on fertility (Bromfield and Sheldon, 2013). These findings support the results discussed in this investigation. The effects of LPS mentioned above could be responsible for failure of the pregnancy per first AI as well as pregnancy loss and long-term effects on fertility, leading to more days open in cows with mastitis caused by gram-negative bacteria.

CONCLUSIONS

Cows that were not diagnosed with clinical mastitis had a higher rate of pregnancy per first AI and reduced pregnancy loss and days open compared with cows with mastitis. These results provide further evidence that pathogens causing clinical mastitis could lead to deleterious effects in reproductive performance of dairy cows, reinforcing the relevance of mastitis control programs in dairy herds. Different pathogens causing clinical mastitis exert different effects on reproduction, which further supports the concept that the bacteria have distinct ways of affecting reproductive variables. Cows diagnosed with mastitis caused by major pathogens and gram-negative bacteria suffered more substantial losses in reproductive performance.

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