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The infectious disease epidemiologic triangle of bovine uterine diseases

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Abstract

Postpartum uterine diseases are important for animal welfare and economic reasons, causing cow discomfort, elimination from the herd and impaired reproductive performance. Metritis is characterized as an abnormally enlarged uterus and a fetid, watery, red-brown uterine discharge within 21 days after parturition. Endometritis is defined as inflammation of the endometrium after 21 days postpartum without systemic signs of illness, and can be considered the chronic stage of uterine inflammation. It has been reported that the metritis affects 10 to 20% of cows, and endometritis affects 5.3 to 52.6% of cows. Metritis affects the cow systemically, and has a negative impact on milk production and reproductive performance. Cows affected with endometritis are not systemically ill, and do not have their milk production altered; however, they have impaired reproductive performance. Metritis and endometritis are complex multifactorial diseases, and a wide range of factors contributes to their occurrence. They are often associated with mixed bacterial infection of the uterus, and the major pathogens associated with uterine diseases are *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum.* Events during the transition period related to negative energy balance and metabolic imbalance, mineral deficiencies, leading to immunosuppression are of great important during establishment of intrauterine bacterial infections. This, combined with endometrium trauma events during parturition (such as calving related problems), and environmental factors (poor hygiene at calving, housing type and calving season), increases the risk of metritis and endometritis.

Keywords: dairy cows, endometritis, metritis, reproduction, uterine diseases.

Introduction

The infectious disease epidemiologic triangle illustrates the interaction of epidemiologic factors that contribute to the outbreak of an infectious disease: the host, the pathogen or disease-causing organism, and the environment (Merrill, 2013). Metritis and endometritis are complex multifactorial diseases caused by mixed bacterial infection. During the past decades, several studies contributed to better understanding of the factors associated with the host, the pathogens, and the environment on how these factors influence the risk of uterine diseases. The objective of this review is to enumerate and discuss the published data on many factors that predispose to the development of uterine diseases in dairy cows.

Introduction to uterine diseases of dairy cows

Reproductive efficiency is a trait of great importance for the modern dairy industry, affecting the overall economic outcome of dairy enterprise. A healthy reproductive tract after parturition is essential for a satisfactory reproductive performance. Postpartum uterine diseases are important for animal welfare and economic reasons, causing cow discomfort, elimination from the herd and impaired reproductive performance. In North America, puerperal metritis affects 10 to 20% of cows (LeBlanc *et al.*, 2011), whereas the incidence of endometritis is approximately 28%, ranging from 5.3 to 52.6% (Dubuc *et al.*, 2010a; Cheong *et al.*, 2012).

Metritis is characterized as an abnormally enlarged uterus and a fetid, watery, red-brown uterine discharge within 21 days after parturition; however, the metritis incidence peaks within the first week post-partum. When metritis is associated with signs of systemic illness (decreased milk yield, dullness, or other signs of toxemia) and temperature >39.5°C, the appropriate term is puerperal metritis. Approximately half of the metritic cows are not diagnosed with fever (Benzaquen *et al.*, 2007; Martinez *et al.*, 2012; Lima *et al.*, 2014).

The effects of metritis on productivity are striking. Metritis has a detrimental effect on milk production during early lactation (Rajala and Grohn, 1998; Huzzey *et al.*, 2007; Giuliodori *et al.*, 2013), especially for multiparous cows (Dubuc *et al.*, 2011; Wittrock *et al.*, 2011). Metritis also contributes to reproductive failure, as cows diagnosed with metritis have decreased conception rate (Overton and Fetrow, 2008; Giuliodori *et al.*, 2013). Data regarding the effect of metritis on survivability are inconsistent; studies have reported no effect of metritis on culling rate (Rajala and Grohn, 1998; Dubuc *et al.*, 2011), whereas others observed that cows diagnosed with metritis are more likely to leave the herd than healthy cows (Linden *et al.*, 2009; Wittrock *et al.*, 2011). Wittrock *et al.* (2011) suggested that multiparous cows affected by metritis

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were at increased risk of being culled, primarily because of the detrimental effect of disease on milk production, rather than reproductive failure. Metritis is frequently treated with systemic antibiotic therapy. The antibiotics of choice to treat metritis are ceftiofur or penicillin (Smith *et al.*, 1998; Drillich *et al.*, 2001); however, alternative treatment with ampicillin had similar efficacy to ceftiofur (Drillich *et al.*, 2003; Lima *et al.*, 2014). The economic losses caused by each metritis case have been calculated at approximately US$329-386 due to antibiotic treatment and the detrimental effects of metritis on reproductive performance, milk production, and survivability (Drillich *et al.*, 2001; Overton and Fetrow, 2008).

Endometritis is defined as inflammation of the endometrium after 21 days postpartum without systemic signs of illness, and can be considered the chronic stage of uterine inflammation. Endometritis has been classified as clinical or subclinical. Clinical endometritis is characterized by the presence of purulent or mucopurulent uterine exudates detectable in the vagina after 21 days postpartum (Sheldon *et al.*, 2006). Subclinical endometritis is defined as the inflammation of the endometrium determined by cytology of samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of purulent discharge in the vagina (Gilbert *et al.*, 2005). Although the definition of clinical endometritis is largely accepted and used by clinicians and researchers, a recent study challenged assumptions of this method of diagnosis, showing that cows with purulent vaginal discharge (PVD) did not always present endometrial inflammation; the nomenclature PVD has been proposed to properly represent what have been diagnosed in cases of clinical endometritis (Dubuc *et al.*, 2010a). However, in this literature review, we will use the terminology clinical endometritis. To define subclinical endometritis, various cutoff points of neutrophils in uterine cytology have been used, depending on stage of lactation that samples were collected. Increased cutoff points were used to define uterine inflammation in earlier stages of lactation. For instance, subclinical endometritis was defined as the presence of neutrophils in uterine cytology exceeding 18 and 10% relative to total cell count, for samples collected at 20 - 33 days and 34 - 47 days postpartum, respectively (Kasimanickam *et al.*, 2004). Others have used 5% of neutrophils in uterine cytology as the cutoff point used to define subclinical endometritis (Gilbert *et al.*, 2005; Lima *et al.*, 2013). Recent studies have been using the terminology cytological endometritis instead of subclinical endometritis; cytological endometritis is defined as the inflammation of the endometrium determined by cytology, regardless of the presence of clinical endometritis (Dubuc *et al.*, 2010a; Cheong *et al.*, 2012; Yasui *et al.*, 2014). Several diagnostic methods have been used to evaluate the reproductive tract infection and inflammation in dairy cows, such as vaginoscopy (Studer and Morrow, 1978; Barlund *et al.*, 2008; Westermann *et al*., 2010), the metrickeck device (McDougall *et al.*, 2007; Brick *et al.*, 2012; Machado *et al.*, 2015), ultrasonography of uterus and cervix (Senosy *et al.*, 2009; Brick *et al.*, 2012; Polat *et al.*, 2015), intrauterine bacterial culture (Studer and Morrow, 1978; Westermann *et al.*, 2010), uterine biopsy (Bonnett *et al.*, 1991; Meira Jr. *et al.*, 2012), reagent strips used to measure leukocyte esterase, protein and pH of uterine lavage samples (Cheong *et al.*, 2012), uterine lavage samples optical density (Machado *et al.*, 2012b), and cytology (Gilbert *et al.*, 2005; Dubuc *et al.*, 2010a; Lima *et al.*, 2013). To assess and evaluate the validation of each diagnostic method is beyond the objectives of this review, and has been intensively reviewed (de Boer *et al.*, 2014).

Differently from metritis, endometritis is not accompanied by systemic symptoms, being a disease contained within the uterus. Although it has been reported that endometritis does not directly impact milk production (Erb *et al.*, 1985; Dubuc *et al.*, 2011), others have shown that primiparous cows that produced more milk, and multimaprous cows that produces less milkin the first month of lactation were more likely to develop subclinical endometritis (Cheong *et al.*, 2011; Galvão *et al.*, 2010). However, endometritis impairs reproduction (Gilbert *et al.*, 2005; Dubuc *et al.*, 2010a; Machado *et al.*, 2015), and as a consequence has a negative economic impact on the modern dairy industry (Lee and Kim, 2007). It has been reported that clinical and subclinical endometritis reduce conception rate (Galvão *et al.*, 2009; Dubuc *et al.*, 2010a; Machado *et al.*, 2015), increase the calving-to-conception interval (Barlund *et al.*, 2008; Dubuc *et al.*, 2010a; Machado *et al.*, 2015), and increase embryonic mortality (Lima *et al.*, 2013; Machado *et al.*, 2015). To date, many endometritis therapy strategies have been evaluated with controversial efficacy, such as intrauterine administered chlorhexidine (Gilbert and Schwark, 1992), enzymes (Drillich *et al.*, 2005), hypertonic dextrose (Brick *et al.*, 2012; Machado *et al.*, 2015), and the systemic administration of PGF2α (LeBlanc *et al.*, 2002; Kasimanickam *et al.*, 2005; Lima *et al.*, 2013). Although the intrauterine infusion of cephalosporin has been reported to be efficacious to treat clinical endometritis (Runciman *et al.*, 2008; McDougall *et al.*, 2013), the use of intrauterine administered antibiotic is currently not approved in the

The host

Transition period, metabolic imbalance, mineral deficiency and immunosuppression

The transition period (defined as the period from 3 weeks before to 3 weeks after calving) is extremely challenging for the dairy cow (Drackley, 1999). As the time of calving approaches, the nutrient

requirements for fetal growth increase to maximum levels, whereas the dry matter intake (DMI) decreases approximately 20% (Bell, 1995). Around parturition, cows have to deal with nutritional changes, because their diet changes abruptly from being forage-based to concentrate-rich diets. They also dramatically alter their metabolism to supply the mammary gland with nutrients necessary for milk synthesis (Bell, 1995; Goff *et al.*, 2002). However, the nutrient requirements for milk synthesis during the first weeks of lactation exceeds nutrient intake. To support milk production, the cow has to mobilize her body reserves, leading to a condition of negative energy balance (NEB; Roche *et al.*, 2009). Dairy cows undergo a state of insulin resistance during early lactation, reducing glucose uptake by body tissues, helping to meet the nutrient demands for milk production during the first weeks of lactation (Bell, 1995). Combined with insulin resistance, a down-regulation in the liver growth hormone (GH) receptors have also been reported, which leads to a reduction in circulating insulin-like growth factor (IGF) and increased the circulating GH, resulting in increased lipolysis (Lucy *et al.*, 2001; Wathes *et al.*, 2009). At parturition, the blood progesterone level falls drastically, followed by a temporary increase of blood concentrations of estrogen and glucocorticoids, contributing to decreased DMI and mobilization of body fat reserves (Drackley *et al.*, 2005; Ingvartsen, 2006).

The complex changes during the transition period lead to a state of metabolic imbalance, which results in exacerbated fat mobilization and body condition score loss during early lactation (Roche *et al.*, 2007). This reflected in elevated circulating concentration of non-esterified fatty acids (NEFA; Kunz *et al.*, 1985; Busato *et al.*, 2002). NEFA is an excellent source of energy for many body tissues and is also used for milk fat synthesis. However, when the liver meets its ATP needs, the uptake of NEFA to complete β-oxidation is diverted to β-hydroxybutyrate (BHBA) and other ketone bodies (Drackley, 1999). Additionally, when in high concentration, NEFA can be resterified into triglycerides and accumulate in the liver causing a condition known as fatty liver (Strang *et al.*, 1998).

The mechanisms by which all these factors associated with NEB and metabolic imbalance will contribute to a state of immunosuppression during the periparturient period that not yet fully understood. During this period, impairment of polymorphonuclear neutrophils (PMN) function and decreased blood concentration of immunoglobulins are observed (Kehrli *et al.*, 1989; Hoeben *et al.*, 2000; Colitti and Stefanon, 2006; Sordillo *et al.*, 2007; van Knegsel *et al.*, 2007; Herr *et al.*, 2011). Reduced DMI and elevated concentration of NEFA and BHBA are associated with immunosuppression during the transition period (Rukkwamsuk *et al.*, 1999; Hammon *et al.*, 2006; Graugnard *et al.*, 2012). *In vitro* studies have shown that bovine PMNs incubated with elevated concentration of NEFA have impaired function and viability (Lacetera *et al.*, 2004; Scalia *et al.*, 2006). High concentration of BHBA reduced bovine PMN capacity for chemotaxis, oxidative burst, and phagocytosis (Klucinski *et al.*, 1988; Hoeben *et al.*, 1997; Suriyasathaporn *et al.*, 1999). Recently, it was demonstrated that an induced hyperketonemia in cows disturbed the mammary gland immune response to lipopolysaccharide (LPS) challenge (Zarrin *et al.*, 2014). Cows undergoing severe NEB have decreased circulating concentration of IGF-1 during the early postpartum period (Lucy *et al.*, 2001; Wathes *et al.*, 2009). The bovine endometrium expresses the IGF system genes (Llewellyn *et al.*, 2008), which play a role in tissue repair, promoting proliferation and healing during uterine involution (Wathes *et al.*, 2011), and alters endometrial gene expression related to immune responses (Wathes *et al.*, 2009). Additionally, natural antibodies (NAb), an important component of the humoral branch of the innate immune system (Avrameas, 1991), have been reported to have a negative association with elevated serum NEFA concentrations (van Knegsel *et al.*, 2007, 2012).

The relationship between NEB, followed by metabolic imbalance during the periparturient period, and uterine diseases is well established in the current literature. For instance, feeding behavior and DMI has been associated with metritis and endometritis. Compared to healthy animals, cows diagnosed with metritis or endometritis had less feeding time and decreased DMI during the transition period (Urton *et al.*, 2005; Hammon *et al.*, 2006; Huzzey *et al.*, 2007), and had increased BCS loss during the dry period (Markusfeld *et al.*, 1997; Kim and Suh, 2003). The incidence of endometritis was increased for cows with low BCS at calving (Hoedemaker *et al.*, 2009; Dubuc *et al.*, 2010b), whereas overconditioned cows are at increased risk of developing metritis (Kaneene and Miller, 1995).

Although there are some minor discrepancies in the literature, generally, cows that develop metritis or endometritis have elevated circulating concentration of NEFA in the week preceding parturition, and elevated NEFA and BHBA serum concentration in the first week of lactation (Hammon *et al.*, 2006; Dubuc *et al.*, 2010b; Galvão *et al.*, 2010; Ospina *et al.*, 2010). These parameters have been explored as diagnostic tools to identify cows at high risk of developing uterine diseases, with satisfactory accuracy (Dubuc *et al.*, 2010b; Ospina *et al.*, 2010; Giuliodori *et al.*, 2013). Recently, it was reported that high prepartum IGF-1 was associated with reduced risk of developing metritis or other postpartum diseases (Piechotta *et al.*, 2012; Giuliodori *et al.*, 2013).

This metabolic imbalance experienced by cows during the transition period is thought to increase the production of reactive oxygen species (ROS). Combined with reduced anti-oxidant capacity during the

periparturient period, cows experience a condition called oxidative stress (Castillo *et al.*, 2005; Sordillo, 2005; Abuelo *et al.*, 2013). Additionally, the blood concentrations of some minerals, such as Ca, P, Zn, and Cu are affected with the onset of lactation, as the blood minerals are utilized by the mammary gland for milk production (Goff and Stabel, 1990; Xin *et al.*, 1993; Meglia *et al.*, 2001; Goff *et al.*, 2002). The immune system is also suppressed by the transient minerals deficiency experienced by cows in the weeks around parturition, especially hypocalcemia (Ducusin *et al.*, 2003; Martinez *et al.*, 2012, 2014). Associations between low blood Ca concentration around parturition and compromised neutrophil phagocytosis and oxidative burst activities have been reported (Kimura *et al.*, 2006; Martinez *et al.*, 2012). It was proposed that the impairment of phagocytosis and oxidative burst activities in cows undergoing hypocalcemia could be explained by the fast decline of cytosolic iCa^{2+} (Martinez *et al.*, 2014). Furthermore, low blood Se concentration has been associated with impaired neutrophil adhesion, migration, and killing ability (Ndiweni and Finch, 1995; Cebra *et al.*, 2003). Deficiency of Cu and Zn is also linked to impaired immunity (Shankar and Prasad, 1998; Spears and Weiss, 2008).

Decreased postpartum concentration of blood minerals is associated with uterine diseases (Martinez *et al.*, 2012; Bicalho *et al.*, 2014a). Hypocalcemia after parturition was associated with increased incidences of metritis and clinical endometritis (Martinez *et al.*, 2012; Bicalho *et al.*, 2014a), and decreased postpartum serum concentrations of P, Zn, Cu, Mo and Se were reported to be linked with metritis and clinical endometritis (Bicalho *et al.*, 2014a). Injectable supplementation with a product containing Cu, Se, Zn, and Mn during the dry period decreased the incidence of clinical endometritis, and the presence of known intrauterine pathogens, suggesting that some of these trace minerals could be playing a protective role in the postpartum intrauterine environment (Machado *et al.*, 2012c, 2013).

During the pregnancy, the immune function of the uterus is suppressed to avoid maternal immune responses against the allogeneic conceptus. This uterine immunosuppression is partially regulated by elevated concentration of progesterone during the pregnancy (Padua *et al.*, 2005). Maternal tolerance to the fetus is also possible because of inhibition of inflammatory responses mediated by T regulatory cells (Lee *et al.*, 1992; Aluvihare *et al.*, 2004). This, combined with the systemic immunosuppression faced by dairy cows during the transition period discussed earlier, makes the uterus very susceptible to diseases in the early postpartum period. Associations between metritis and endometritis, and suppressed periparturient immune system have been reported in several studies. Although the data regarding the association between PMN phagocytic activity and uterine diseases is inconsistent (Mateus *et al.*, 2002; Kim *et al.*, 2005; Machado *et al.*, 2013), associations between the killing ability of neutrophils are more consistent. It was observed that cows that developed metritis have neutrophils that produced less superoxide activity before parturition (Cai *et al.*, 1994), and that decreased blood PMN oxidative burst activity is associated with increased risk of developing endometritis (Mateus *et al.*, 2002). The peripheral PMN killing ability determined by myeloperoxidase activity and cytochrome c reduction activity is reduced on the day of calving in cows that developed metritis and subclinical endometritis (Hammon *et al.*, 2006). Energy status of blood PMN measured by PMN glycogen concentration was also associated with uterine disease; cows that developed metritis or subclinical endometritis have lesser blood PMN glycogen than healthy cows (Galvão *et al.*, 2010). Recently, it was suggested that decreased circulating NAb concentration is another factor that may contribute to the impairment of the innate immune system around parturition, increasing the risk of uterine diseases (Machado *et al.*, 2014a).

Physical factors and genetic parameters

There are several risk factors that contribute to postpartum uterine contamination or physical damage of the uterine tissue, such as retained placenta (RP), calving abnormalities (dystocia, twins, and stillbirth), angle of the vulva, and parity. Many of these factors, combined with metabolic health parameters, were used to build a model aiming to predict postpartum diseases, including metritis (Vergara *et al.*, 2014).

Several studies have shown that RP is one of the most important risk factors for metritis and endometritis in dairy cows (Erb *et al.*, 1985; Kaneene and Miller, 1995; Bruun *et al.*, 2002; Machado *et al.*, 2012b). Retained placenta contributes to development of uterine diseases because cows that have their fetal membranes retained are immunosuppressed, have more uterine tissue damage (Paisley *et al.*, 1986), and are more likely to allow bacterial growth in the uterine lumen (Paisley *et al.*, 1986; Machado *et al.*, 2012a).

Calving related problems (dystocia, stillbirth, and twins) are also known to increase the risk of uterine diseases (Markusfeld, 1984; Benzaquen *et al.*, 2007; Potter *et al.*, 2010; Cheong *et al.*, 2011), by facilitating the access of bacteria into the uterine mucosa (Bicalho *et al.*, 2010), and by causing uterine tissue damage. Abnormal calving status were more likely to develop metritis (Benzaquen *et al.*, 2007; Giuliodori *et al.*, 2013) and clinical endometritis (Benzaquen *et al.*, 2007) than cows with normal calving. These calving related problems are also independent risk factors for uterine diseases. Independent effects of dystocia, twin parturition, and stillbirth on the incidence of metritis have been reported (Bruun *et al.*, 2002; Bicalho *et al.*, 2010; Dubuc *et al.*, 2010b), and clinical endometritis

(Potter *et al*, 2010; Dubuc *et al.*, 2010b; Prunner *et al.*, 2014). There are studies with conflicting results regarding the association between calving related problems and subclinical endometritis. Cheong *et al*. (2011) reported that these calving abnormalities were associated with subclinical endometritis, whereas others did not observe the same associations (Dubuc *et al.*, 2010b; Prunner *et al.*, 2014). Abortion and induced calving are also factors predisposing to uterine diseases (Kaneene and Miller, 1995; Bruun *et al.*, 2002).

Cows that give birth to males calves are more likely to have uterine contamination after parturition (Bicalho *et al.*, 2010), are more likely to have dystocia (Mee *et al.*, 2011), and stillbirth parturitions (Meyer *et al.*, 2001) than cows having female calves. However, to the best of our knowledge, there is no evidence that having male calves is a direct risk factor for metritis, but it was reported to be a risk factor for clinical endometritis (Potter *et al.*, 2010). The same association was not observed for subclinical endometritis (Cheong *et al.*, 2011).

There is a u-shaped association between parity and metritis; primiparous cows and cows in parity 3 or greater are more likely to develop metritis than cows in parity 2 (Markusfeld, 1984; Saloniemi *et al.*, 1986; Bruun *et al.*, 2002); however, others have not observed this u-shaped association, and simply reported that primiparous cows are more likely to develop metritis than multiparous counterparts (Dubuc *et al.*, 2010b; Machado *et al.*, 2012a). Primiparous are more likely to suffer uterine damage due to dystocia than older cows (Meyer *et al.*, 2001; Uematsu *et al.*, 2013). Similarly to metritis, parity is also a risk factor for endometritis; primiparous cows are more likely to develop clinical or subclinical endometritis than multiparous cows (Dubuc *et al.*, 2010b; Potter *et al.*, 2010; Cheong *et al.*, 2011).

Another cow-related factor that was found to increase the risk of uterine infection was the angle of the vulva (Potter *et al.*, 2010). A vulval angle $<70^{o}$ to the horizontal axis increases the risk of clinical endometritis; this conformation could allow fecal contamination of the vagina, allowing bacteria to access more easily the intrauterine lumen and cause infection.

It has been suggested that there is an involvement of genetic factors in the incidence of metritis, as the heritability of this disease was reported to be as high as 0.19 and 0.26 for primiparous and second lactation cows, respectively (Lin *et al.*, 1989). However, other studies have reported decreased heritability values for metritis, ranging from 0.02 to 0.07 (Lyons *et al.*, 1991; Van Dorp *et al.*, 1998; Zwald *et al.*, 2004a, b). Recent studies have investigated the association between single nucleotide polimorphisms (SNPs) occurring in bovine innate immune genes and uterine diseases (Galvão *et al.*, 2011; Pinedo *et al.*, 2013). Pinedo *et al.* (2013) reported weak associations between metritis, endometritis, and SNPs occurring in genes encoding the toll like receptors 2, 4, 6, and 9. Galvão *et al*. (2011) concluded that uterine health was not affected by the SNP at position +735 in the interleukin-8 receptor-α gene. Polimorphism in the leptin receptor gene was linked with increased metritis incidence (Oikonomou *et al.*, 2009). Although the sire predicted transmitting ability for milk production traits was associated with poorer reproductive performance, it was not linked with increased metritis susceptibility (Bicalho *et al.*, 2014b).

The environment

It is intuitive to think that poor hygiene in the maternity and calving area is a factor predisposing postpartum intrauterine contamination and development of uterine diseases. However, different studies present conflicting data to support its importance. The cleanliness of the perineal region at the time of parturition was associated with metritis (Schuenemann *et al.*, 2011). Herds using straw for calving pen bedding had decreased incidence of metritis (Kaneene and Miller, 1995) and subclinical endometritis (Cheong *et al.*, 2011) than herds using another material; straw could be considered a cleaner bedding material when compared to other materials, such as sand and sawdust. Pasture calvings were also associated with decreased metritis incidence, and the pasture could be also considered as an environment less congested with bacteria than a barn (Kaneene and Miller, 1995). However, other studies have found that poor hygiene is unrelated to uterine diseases; Potter *et al*. (2010) did not observe any association between clinical endometritis and markers of hygiene (fecal consistency score, cow cleanliness score, disinfection of calving equipment, and the wearing of gloves when assisting parturition). Additionally, the microflora of cows from two hygienically contrasting farms was not influenced by the environmental hygiene status; however, these findings should be interpreted with care, because this study was performed in only two herds and enrolled only 26 cows (Noakes *et al.*, 1991).

Individual housing in the maternity facility has been associated with increased risk of metritis (Kaneene and Miller, 1995). Additionally, housing was associated with incidence of subclinical endometritis (Cheong *et al.*, 2011; Prunner *et al.*, 2014). Herds housing early postpartum cows in freestall barns had decreased subclinical endometritis incidence than herds that housed their postpartum cows in bedded packs (Cheong *et al.*, 2011). Prunner *et al*. (2014) reported that tie stall systems were associated with decreased risk of subclinical endometritis when compared with stables with calving pens; however, housing system was not associated with clinical endometritis.

The incidence of metritis has been associated with calving season, but with little agreement on which season is a predisposing factor for uterine diseases (Erb and Martin, 1980; Markusfeld, 1984; Gröhn *et al.*, 1990;

Bruun *et al*., 2002). Markusfeld (1984) reported that cows calving during summer are more likely to be affected with metritis, whereas the incidence of metritis was associated with summer-fall (Erb and Martin, 1980), fall-winter (Gröhn *et al.*, 1990), or winter-spring calvings (Bruun *et al.*, 2002). Heat stress was also reported to be a predisposing factor for RP and consequently metritis (DuBois and Williams, 1980). These discrepancies could be explained by geographical and temporal differences among studies. However, more recent literature reported that season is unimportant for metritis (Dubuc *et al.*, 2010b), and endometritis (Dubuc *et al.*, 2010b; Prunner *et al.*, 2014). Perhaps the advances in management have minimized the detrimental effects of season on postpartum uterine health (Collier *et al.*, 2006).

The pathogens

Virtually all cows will have bacterial contamination in their uterine lumen after parturition (Foldi *et al.*, 2006; Santos and Bicalho, 2012). *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum* are considered the primary bacterial causes of uterine diseases (Miller *et al.*, 2007; Bicalho *et al.*, 2010; Santos *et al.*, 2011), but other pathogenic bacteria, such as, *Bacteroides spp*, *Ureaplasma spp*, *Staphylococcus spp*, *Helcococcus spp*, *Prevotella melaninogenicus* and *Streptococcus spp*. have also been associated with uterine diseases (Azawi *et al.*, 2008; Machado *et al.*, 2012c; Locatelli *et al.*, 2013). Although the etiology of uterine diseases is mainly attributed to bacterial infection, the bovine herpesvirus type 4 (BoHV-4) has been associated with poor postpartum uterine health, acting as a secondary pathogenic agent following bacteria (Monge *et al.*, 2006; Donofrio *et al.*, 2009; Chastant-Maillard, 2013).

Escherichia coli

Traditionally, *E. coli* has been described as the main pathogen initiating postpartum uterine infection and disease (Studer and Morrow, 1978; Bonnett *et al.*, 1991; Bicalho *et al.*, 2010; Sheldon *et al.*, 2010). It has been reported that uterine *E. coli* are merely opportunistic environmental bacteria, because none of the virulence factors evaluated in one study were associated with the probability of occurrence of uterine diseases (Silva *et al.*, 2009). Nevertheless, recent studies have characterized important virulence factors that enable *E. coli* to bind and invade the bovine endometrium, making significant advances to understand how *E. coli* plays a role in the pathogenesis of metritis and endometritis (Bicalho *et al.*, 2010; Sheldon *et al.*, 2010).

Silva *et al*. (2009) characterized the phenotype and genotype of 72 *E. coli* isolated from the uterus of metritic and non-metritic cows, and found that none of the 15 virulence factors evaluated were associated with metritis. Sheldon *et al*. (2010) investigated the presence of 17 virulence factors from 114 uterine *E. coli* isolated from 64 postpartum dairy cows and the only virulence factor associated with disease was *fyuA*. However, they found that *E. coli* isolated from cows with metritis were more capable of adhering and invading epithelial and stromal endometrial cells. In a larger scale study, Bicalho *et al*. (2010) explored 32 potential virulence factors, using 611 *E. coli* isolates from 374 cows housed in four different farms in New York State. It was found that six virulence factors common to extra-intestinal and entero-aggregative *E. coli* were associated with uterine diseases: *fimH*, *hlyA*, *cdt*, *kpsMII*, *ibeA*, and *astA*. The virulence factor FimH was the most prevalent and the most important for metritis and endometritis. The FimH protein is an *E. coli* type 1 pili adhesive protein that plays an important role in the adhesion to mannosides (Krogfelt *et al.*, 1990) and enables bacteria to colonize epithelial surfaces (Mooi and de Graaf, 1985). It is known that *E. coli* expressing the type 1 pili containing FimH causes urinary tract infection in humans (Kaper *et al.*, 2004), and it is critical for the ability of these *E. coli* to adhere to and colonize the bladder epithelium (Mulvey, 2002). In fact, it was demonstrated that FimH also mediates adhesion between endometrial pathogenic *E. coli* and the bovine uterine mucosa, because mannose treatment of *E. coli* decreased their ability to adhere to bovine endometrial cells *in vitro* (Sheldon *et al.*, 2010). Recently, an alternative prevention method for metritis using ultrapure mannose was tested, but intrauterine administration of 50 g of mannose in the first three days after parturition was ineffective to reduce bacterial contamination and prevent metritis (Machado *et al.*, 2012a).

It has been suggested that *E. coli* is important for metritis and endometritis in the first week postpartum, especially during the first three days after parturition, potentially inducing changes that will favor subsequent infection by other pathogens (Dohmen *et al.*, 2000; Bicalho *et al.*, 2012). However, its intrauterine presence after the first week postpartum is unimportant for disease and reproductive performance (Bicalho *et al.*, 2012; Machado *et al.*, 2012a, c; Sens and Heuwieser, 2013). Dohmen *et al*. (2000) suggested that the presence of *E. coli* and its endotoxin lipopolysaccharide (LPS) in lochia during the first two days postpartum leads to subsequent *T. pyogenes* infection at 14 days after calving. Similarly, Bicalho *et al*. (2012) found that cows tested positive for the intrauterine presence of the *E. coli* virulence factor FimH at 1-3 DIM were more likely to develop *F. necrophorum* intrauterine contamination at 8-10 DIM. The presence of *E. coli* in the early postpartum period was also associated with impaired reproductive performance (Bicalho *et al.*, 2012; Machado *et al.*, 2012a).

Fusobacterium necrophorum

The combination of anaerobic microorganisms' metabolism and oxygen consumption by PMNs fighting against the intrauterine infection in the first days postpartum decreases the intrauterine oxygen reductase potential, creating an anaerobic environment (El-Azab *et al.*, 1988). This will favor the growth of strict and facultative anaerobes, such as *F. necrophorum* and *T. pyogenes*, respectively. Several studies have identified *F. necrophorum* as an important etiological agent of uterine diseases (Ruder *et al.*, 1981; Noakes *et al.*, 1991; Dohmen *et al.*, 2000). Recent studies using molecular characterization of the intrauterine microbiota have reinforced this assumption. It was reported that *F. necrophorum* was the most prevalent bacteria in samples collected from cows affected with metritis, while being completely absent in samples from healthy cows (Santos *et al.*, 2011). Similarly, it was reported that the intrauterine presence of *F. necrophorum* at 8-10 DIM was associated with metritis (Bicalho *et al.*, 2012), and at 35 days postpartum is associated with clinical endometritis (Machado *et al.*, 2012c).

Fusobacterium necrophorum is a gram-negative, non-spore forming, rod-shaped anaerobe that produces butyric acid as a major product of fermentation (Nagaraja *et al.*, 2005). There are several virulence factors associated with toxicity, adhesion and aggregation that are implicated in the pathogenesis of *F. necrophorum* infections. However, leukotoxin (LKT) is considered the major virulence factor associated with infections in animals (Tan *et al.*, 1994; Narayanan *et al.*, 2002). It is known that LKT is highly toxic to bovine PMNs (Tan *et al.*, 1994), inducing apoptosis-mediated killing of them (Narayanan *et al.*, 2002); this toxicity is dose-dependent (Tan *et al.*, 1992). It is possible than LKT is acting in the uterus by weakening the intrauterine defensive line mediated by PMNs, impairing the ability of the innate immune system to eliminate bacterial infections from the uterus through phagocytosis. Recently, it was reported that the adhesion of *F. necrophorum* to endothelial bovine cells is mediated by outer membrane proteins (Kumar *et al.*, 2013), specifically, the virulence factor FomA (Kumar *et al.*, 2015).

Fusobacterium necrophorum and *T. pyogenes* are known to be synergistic microbes, causing numerous infections in cattle, such as liver, foot, lungs and mandibular abscesses, foot rot, summer mastitis, and calf diphtheria (Nagaraja *et al.*, 2005). This synergy is also observed in uterine diseases (Dohmen *et al.*, 2000; Bicalho *et al.*, 2012; Machado *et al.*, 2012c).

Trueperella pyogenes

Trueperella pyogenes, a Gram positive, non-motile, non-sporeforming, short, rod-shaped bacterium (Jost and Billington, 2005), is a common inhabitant of urogenital, gastrointestinal, and upper respiratory tracts of many animal species (Hagan *et al.*, 1988; Narayanan *et al.*, 1998; Carter and Wise, 2004). However, a physical or microbial insult to the host can lead to a variety of suppurative *T. pyogenes* infections; *T. pyogenes* is an opportunistic pathogen that acts in synergy with *F. necrophorum*, and is consistently associated with metritis and especially endometritis (Studer and Morrow, 1978; Bonnett and Martin, 1995; Williams *et al.*, 2005; Bicalho *et al.*, 2012; Machado *et al.*, 2012a, c).

Trueperlla pyogenes is equipped with several known and putative virulence factors that are important for its pathogenic potential. Its primary virulence factor, pyolysin (PLO), is a potent cholesterol-dependent cytolysin and is associated with the tissue damage caused by *T. pyogenes* infection (Jost and Billington, 2005; Amos *et al.*, 2014). It is known that *T. pyogenes* can provoke a cellular inflammatory response in the uterus, but the intact endometrium is protective against the tissue damage cause by PLO (Miller *et al.*, 2007; Amos *et al.*, 2014). It was demonstrated that the epithelial layer of the endometrium is protective against PLO because epithelial cells contain less cholesterol than stromal cells (Amos *et al.*, 2014). Therefore, it was suggested that *T. pyogenes* acts in the postpartum uterus as an opportunistic pathogen, causing disease once the epithelial layer is lost after parturition, that could have been a result of previous intrauterine infection and/or a traumatic event during parturition, such as dystocia and RP (Dohmen *et al.*, 2000; Bicalho *et al.*, 2012).

Trueperella pyogenes also expresses a number of surface-exposed proteins, such as fimbriae, neuraminidases, and extracellular matrix-binding proteins, which are involved in adherence and mucosal colonization (Jost and Billington, 2005; Pietrocola *et al.*, 2007; Santos *et al.*, 2010; Machado and Bicalho, 2014). Although there were no associations between virulence factors and uterine diseases in one study (Silva *et al.*, 2008), others reported that virulence factor encoded by the gene *fimA* was associated with metritis (Santos *et al.*, 2010) and clinical endometritis (Bicalho *et al.*, 2012).

Other pathogens

A wide variety of other bacteria has been associated with postpartum uterine health of dairy cows. However, there are no details on their roles on the pathogenesis of metritis and endometritis. It was reported that *Bacteroides spp.* contributes to clinical endometritis, acting in synergy with *T. pyogenes* and *F. necrophorum* (Dohmen *et al.*, 1995; Machado *et al.*, 2012c). *Prevotella melaninogenica* was consistently isolated from diseased bovine uterus (Olson *et al.*, 1984), and its intrauterine relative abundance in the 7th week postpartum was increased for cows affected with clinical endometritis (Machado *et al.*, 2012c).

Non-hemolytic *Streptococcus spp.* and *Mannheimia haemolytica* were associated with the fetid mucus odor, a characteristic sign of uterine infection (Williams *et al.*, 2005). The intrauterine presence of *Streptococcus uberis* on the third day of lactation was reported to be highly associated with the risk of clinical endometritis (Wagener *et al.*, 2014). By the use of a metagenomic technique, *Helcococcus spp* was described to be associated with clinical endometritis (Machado *et al.*, 2012c); *Helcococcus kunzii* and *Helcococcus ovis* were isolated from metritic uterus of dairy cows (Locatelli *et al.*, 2013), suggesting that these species may play a role in the pathogenesis of uterine diseases. Furthermore, *Ureaplasma spp* was highly prevalent in the uterus of cows affected with clinical endometritis (Machado *et al.*, 2012c); *Ureaplasma diversum* has been associated with granular vulvitis, endometritis and reproductive failure (Doig *et al.*, 1980; Kreplin *et al.*, 1987). *Staphylococcus spp.* is another bacterium that has been previously associated with poor uterine health and impaired reproduction (Paisley *et al.*, 1986; Machado *et al.*, 2012c).

The BoHV-4 is the only virus that has been consistently associated with uterine infection of dairy cows (Parks and Kendrick, 1973; Monge *et al.*, 2006; Donofrio *et al.*, 2009, 2010; Chastant-Maillard, 2013; Jacca *et al.*, 2013). It was described that BoHV-4 can cause latent infection in bovine macrophages (Donofrio and van Santen, 2001), and are tropic for bovine endometrial epithelial and stromal cells, replicating and leading to non-apoptotic cell death (Donofrio *et al.*, 2007; Jacca *et al.*, 2013). The endometrium can respond to the BoHV-4 presence with an inflammatory response, overexpressing pro-inflammatory cytokines IL-8 and TNF-α (Donofrio *et al.*, 2010; Jacca *et al.*, 2013). It has been suggested that BoHV-4 acts in cooperation with bacterial infection to cause disease in the uterus of dairy cows (Donofrio *et al.*, 2008).

Conclusion

Metritis and endometritis are highly prevalent in postpartum dairy cows and both diseases have a negative impact in the modern dairy enterprise. They are complex multifactorial diseases, and a wide range of factors contributes to their occurrence. They are often associated with mixed bacterial infection of the uterus, and the major pathogens associated with uterine diseases are *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum* These infections are more likely to develop under some conditions related the host and to the environment. Environmental factors that can predispose metritis and endometritis are poor hygiene at calving, housing type and calving season. Events during the transition period related to negative energy balance and metabolic imbalance, mineral deficiencies, leading to immunosuppression are also of great important during establishment of intrauterine bacterial infections. This, combined with endometrium trauma events during parturition, such as calving related problems, increases the risk of metritis and endometritis. To understand all these factors, and their relationship and interactions, is key to implementing management practices to mitigate the risk of disease, and to develop new strategies to treat and prevent metritis and endometritis. Recently, encouraging preliminary results regarding the effectiveness of multivalent vaccines containing components of *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum* were published (Machado *et al.*, 2014b). It was reported that boosting the host immune system by systemically immunizing late pregnant heifers against cellular components and important virulence factors of these pathogens reduced the incidence of puerperal metritis. However, more research is needed to advance the knowledge on the pathogenesis of uterine diseases, and to develop better strategies to ameliorate immunosuppression during the transition period of dairy cows.

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