Postpartum uterine infection and endometritis in dairy cattle

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Abstract
After parturition, uterine involution, regeneration of the endometrium, return of ovarian cyclic activity, and the control of pathogenic bacteria in the uterus is required before cows are likely to conceive again. However, pathogenic bacteria often cause uterine disease in modern dairy cattle, leading to decreased productivity and reduced fertility. This review aims to provide an overview of postpartum uterine infection and disease in dairy cattle. Metritis and endometritis are the main postpartum clinical conditions; although, subclinical endometritis is an emerging issue. Postpartum uterine disease is associated with the isolation of *Escherichia coli*, *Trueperella pyogenes*, and anaerobic pathogenic bacteria. Sensing of bacteria or their pathogen-associated molecules, such as lipopolysaccharide, by the innate immune system generates inflammatory responses. Endometrial inflammation includes increased expression of complement, calgranulins, interleukins and acute phase proteins, as well as the chemotaxis of neutrophils and macrophages to the site of infection. Uterine disease is also characterised by tissue damage, including endometrial cytolysis caused by the cholesterol-dependent cytolysin, pyolysin. The responses to pathogens are energetically expensive, and depletion of the key cellular nutrients, glucose or glutamine, impairs inflammatory responses by endometrial tissues. For sustainable intensification of the dairy industry over the next 50 years, it is vital to understand why high-milk-yield cows are so susceptible to uterine pathology and develop new ways to prevent uterine disease.

Keywords: Cow, metritis, infertility, immunity, uterus, ovary.

Introduction
Bacterial infections of the endometrium that cause uterine disease are common in modern dairy cattle after parturition, and lead to decreased productivity and subfertility (Sheldon *et al.*, 2009). The rising incidence of postpartum metritis and endometritis over the last 50 years has generated interest in better understanding the characteristics of the diseases and the impact of the disease on animal health. There has also been a parallel increase in understanding of the mechanisms underlying uterine disease in dairy

cattle. Here we provide an overview of postpartum uterine infection and disease in dairy cattle.

Definitions of uterine diseases

The definitions of the various uterine diseases in the literature varied considerably until 2006, when a series of definitions were formulated, with consensus amongst about 20 international experts and referees prior to publication (Sheldon *et al.*, 2006). The initial definitions are now widely used, as set out in 2006 or with minor modifications (Sheldon *et al.*, 2009; de Boer *et al.*, 2014).

The two main postpartum clinical conditions are metritis and endometritis. Metritis is most common within 10 days of parturition, and is characterized by an enlarged uterus containing a watery red-brown fluid to viscous off-white purulent uterine discharge, which often has a fetid odour. The severity of metritis is categorized by the signs of the animal's health, from mild disease to toxaemia. The incidence of metritis varies between breed, country and herd, but in a study of the records from 97,318 cows in the USA, the lactation incidence of metritis, including retained placenta, was 21% (Zwald *et al.*, 2004). Clinical endometritis is defined as the presence of a purulent discharge detectable in the vagina 21 days or more post partum, or mucopurulent discharge detectable in the vagina after 26 days post partum. The incidence of clinical endometritis is around 10 to 20%, with variation between breed, country and herd; a typical study reported that 16.9% of 1,865 cows were affected in Canada (LeBlanc *et al.*, 2002). One of the determinants of the likelihood of uterine disease is the incidence of risk factors. These risk factors can be divided into factors that are associated with damage to the uterus, metabolic stress, or deficits in hygiene. Interestingly, the latter is the least important in the majority of epidemiological models that quantify the risk factors for uterine disease (Dubuc *et al.*, 2010; Potter *et al.*, 2010; Sheldon, 2014). The risk factors most frequently associated with uterine infection are those that likely lead to some trauma to the endometrium, including stillbirth, twins, male and beef-sire calves, dystocia, caesarean section operation, and retained placenta (Hussain *et al.*, 1990; Peeler *et al.*, 1994; Dubuc *et al.*, 2010; Potter *et al.*, 2010).

The diagnosis of metritis and clinical endometritis should include an inspection of the contents of the female genital tract by speculum or insertion of a clean-gloved-hand into the vagina (Sheldon, 2004; Sheldon *et al.*, 2006; de Boer *et al.*, 2014). Whilst somewhat invasive, examination of the vagina carries little risk of further microbial contamination of the uterus in postpartum dairy cattle (Sheldon *et al.*, 2002a). Manual examination of the vagina also facilitates collection of fluid from the vagina to evaluate the presence and odour of pus, which can be used to score the severity of disease and predict the likely success of treatment (Sheldon *et al.*, 2006). Vaginal examination also allows the operator to detect damage to the wall of the vagina and cervix, indicative of obstetric injuries, vaginitis, and cervicitis. However, as with any clinical examination, the

evaluation of uterine disease is subjective and there is inter- and intra-operator variation (Sannmann and Heuwieser, 2015).

The absence of pus in the postpartum genital tract does not mean that the tract is normal. The importance of subclinical endometritis has emerged over the last 15 years, with the realisation that cytological evidence of inflammation of the endometrium is associated with reduced fertility (Kasimanickam *et al.*, 2004; Gilbert *et al.*, 2005). The cause of subclinical endometritis is not yet clear, and may include resolving bacterial infections, immune-pathology without pathogenic bacteria, or even aberrations of postpartum tissue regeneration and repair. Subclinical endometritis is characterized by inflammation of the endometrium that results in a significant reduction in reproductive performance in the absence of signs of clinical endometritis. Subclinical disease is defined by the proportion of polymorphonuclear neutrophils (PMNs) exceeding operator-defined thresholds, usually about 5% of cells in samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of clinical endometritis, about 35 to 40 days post partum (Sheldon *et al.*, 2006; de Boer *et al.*, 2014).

Pyometra is characterized by the accumulation of purulent or mucopurulent material within the uterine lumen and distension of the uterus, in the presence of a closed cervix and an active corpus luteum. Postpartum pyometra is uncommon, with an incidence rate of less than 2%, and is thought to be caused by the growth of pathogenic bacteria within the uterine lumen after the formation of the first corpus luteum (Noakes *et al.*, 1990). Although there is functional closure of the cervix, the lumen is not always completely occluded and pus may occasionally discharge through the cervix into the vaginal lumen. Pyometra is sonographically characterised by mixed echodensity fluid in the uterine lumen with distension of the uterus, and a corpus luteum in an ovary (Sheldon *et al.*, 2006).

The postpartum period

Uterine disease reflects a disturbance of the normal postpartum period, which usually lasts about 40 days, and is defined as the time between parturition and completion of uterine involution (Sheldon, 2004). After parturition, four concomitant events need to be completed before cows are likely to be able to conceive again: uterine involution, regeneration of the endometrium, return of ovarian cyclic activity, and the control of pathogenic bacteria in the uterus is required before cows are likely to conceive again. (Sheldon, 2004; Sheldon *et al.*, 2006). Failure to resist the growth of pathogenic microbes in the endometrium commonly results in uterine disease.

Uterine involution

Involution is the term used to describe the physical reduction in size of the uterus and cervix after parturition. Involution is thought to be driven by uterine muscular contractions, turnover of the extracellular matrix, necrosis and sloughing of the uterine

caruncles, and regeneration of the endometrium (Gier and Marion, 1968). It is often difficult to insert a hand through the cervix 24 h after parturition, and it only admits two fingers by 96 h postpartum. By about 2 weeks post partum, the entire genital tract is palpable per rectum in normal animals; although, the previously gravid horn can still be identified because it is wider and longer than the previously non-gravid horn, and this difference is evident up to 4 weeks postpartum (Okano and Tomizuka, 1987; Tian and Noakes, 1991b; Risco *et al.*, 1994). In parallel with the changes in dimensions, the weight of the uterus decreases from about 9 kg at parturition to 1 kg by 30 days postpartum (Gier and Marion, 1968).

Uterine involution can be monitored by repeated estimation of the size of the uterus, using transrectal palpation or transrectal ultrasonography (Okano and Tomizuka, 1987; Sheldon *et al.*, 2000; Sheldon *et al.*, 2003). It should be noted that dimensions estimated by transrectal palpation are often about 1 to 2 cm greater than ultrasound measurements; presumably because operators include the thickness of the rectal wall when using transrectal palpation. The changes in uterine horn diameter are almost imperceptible by 4 weeks postpartum, and are probably complete by 6 weeks. In the literature, the time to completion of uterine involution is often reported, but this endpoint is difficult to estimate in clinical practice. On the other hand, factors that delay uterine involution are important because completion of involution is associated with fertility (Fonseca *et al.*, 1983). The factors that delay involution include dystocia, hypocalcaemia, retained placenta, metritis, and endometritis.

Regeneration of the endometrium

The epithelium of the endometrium is often damaged during parturition, the caruncular tissue sloughs as part of the physiological process of the puerperium, and there is considerable tissue remodelling during the postpartum period (Gier and Marion, 1968; Wagner and Hansel, 1969; Tian and Noakes, 1991a). It is thought that the endometrium takes 3 to 4 weeks to fully recover the normal tissue architecture, and it is assumed that a normal endometrium is important for fertility.

Return of ovarian cyclic activity

Within a few days of parturition, circulating steroid hormone concentrations decrease to basal values, and there is an increase in plasma FSH concentration, with subsequent recurrent increases in FSH concentrations every 7 to 10 days (Crowe *et al.*, 1998; Duffy *et al.*, 2000). The first postpartum dominant follicle, with a diameter > 8 mm, is usually selected about 10 days after parturition. This dominant follicle may ovulate to form the first postpartum corpus luteum, the dominant follicle may undergo atresia with subsequent emergence of a second dominant follicle, or it may abnormally persist as an ovarian cyst (Savio *et al.*, 1990; Stagg *et al.*, 1995; Beam and Butler, 1997). The fate of the first postpartum dominant follicle depends on LH pulse frequency, and failure to ovulate is usually a consequence of inadequate LH pulse frequency and reduced ovarian

follicle estradiol (Beam and Butler, 1999; Duffy *et al.*, 2000; Cheong *et al.*, 2016). In dairy cattle, metabolic stress - most often negative energy balance - is the main cause of reduced LH pulse frequency, although a range of other factors can impact ovarian cyclic activity (Cheong *et al.*, 2016).

Microbes that cause uterine disease

Postpartum uterine disease is associated with the isolation of pathogenic bacteria, particularly *Escherichia coli, Trueperella pyogenes, Fusobacterium necrophorum, Prevotella* and *Bacteroides* (Elliott *et al.*, 1968; Griffin *et al.*, 1974; Huszenicza *et al.*, 1991; Noakes *et al.*, 1991). Indeed, *T. pyogenes, F. necrophorum* and *Prevotella* act synergistically to increase the likelihood and the severity of endometritis (Ruder *et al.*, 1981; Olson *et al.*, 1984). More recent studies using aerobic and anaerobic culture confirm the importance of *E. coli, T. pyogenes* and anaerobic bacteria (Dohmen *et al.*, 2000; Sheldon *et al.*, 2002b; Williams and Sheldon, 2003; Westermann *et al.*, 2010). Novel endometrial pathogenic *E. coli* have been isolated from animals with uterine disease (Sheldon *et al.*, 2010); and, *T. pyogenes* is associated with the severity of endometrial pathology and clinical disease (Bonnett *et al.*, 1991; Westermann *et al.*, 2010). The link between *T. pyogenes* and disease may be explained by the cholesterol-dependent cytolysin pyolysin (PLO) secreted by *T. pyogenes*, which causes cytolysis particularly of endometrial stromal cells (Amos *et al.*, 2014; Preta *et al.*, 2015).

The role of *E. coli* and *T. pyogenes* is highlighted by infusing *E. coli* and *T. pyogenes* into the uterus of naïve cows to create animal models of endometritis (Ayliffe and Noakes, 1982; Amos *et al.*, 2014). In addition, vaccines containing components of *E. coli, F. necrophorum* and/or *T. pyogenes* protect animals against postpartum uterine disease (Nolte *et al.*, 2001; Machado *et al.*, 2014). However, metagenomics techniques have found associations between uterine disease and bacteria that are not readily cultured by standard techniques (Machado *et al.*, 2012; Santos and Bicalho, 2012; Peng *et al.*, 2013; Knudsen *et al.*, 2015; Wagener *et al.*, 2015). Whilst some of the studies find *E. coli, T. pyogenes* and the expected anaerobic bacteria, others report finding *Bacteroidetes* and *Firmicutes*. There remains a gap in understanding how "uncultureable" bacteria contribute to the pathogenesis of uterine disease. A consistent finding among most microbiology studies is that anaerobic bacteria are more abundant in the diseased endometrium than in healthy uteri. Perhaps this is not surprising as the endometrium is a microaerophilic environment, with tissue damage likely reducing the oxygen tension further. Taken together the evidence is that *E. coli, T. pyogenes* and anaerobic bacteria are probably the main pathogens causing the clinical signs of postpartum uterine disease (Fig. 1).

One note of caution about our understanding of microbes in the endometrium is that recent evidence counters the traditional view that the uterus is sterile outside the postpartum period. There is evidence from studies using fluorescent probes for bacteria and from 16S ribosomal RNA gene sequencing, that there is a sparse microbiome in the uterus, even during pregnancy (Karstrup *et al.*, 2017; Moore *et al.*, 2017). The bacteria include *Trueperella, Fusobacteria and Prevotella* species, but the abundance of these bacteria is a small fraction of those present in animals with postpartum uterine disease. Whilst postpartum uterine bacteria may also derive from the vagina, skin and the environment, it is possible that the pathogenic bacteria present in the uterus before parturition grow and cause pathology after parturition.

Host defence against infections of the uterus

The host has a range of defences against microbial contamination of the uterus and infection of the endometrium. Whilst the animals' environment is heavily contaminated with bacteria, the vulva, vagina and cervix provide anatomical barriers to ascending infections, except during parturition (Fig. 1). Whether the resident flora of the vagina or the pH of the vagina might also compete with pathogens to limit disease is a contentious matter. However, there is a range of antimicrobial peptides, glycoproteins and mucins in the vagina, cervix and uterus, that counter bacterial contamination and restrain bacterial growth (Davies *et al.*, 2008; Chapwanya *et al.*, 2013; Kasimanickam *et al.*, 2014).

Of course, microbial invasion of the female genital tract is not unnoticed. Adaptive immune responses are evident, with increase abundance of antibodies (Dhaliwal *et al.*, 2001); which, concur with the ability to vaccinate against uterine pathogens (Nolte *et al.*, 2001; Machado *et al.*, 2014). A recent advance in knowledge has been about the role of innate immunity in the female genital tract (Fig. 1). Innate immunity depends on the binding of pathogen-associated molecular patterns from microbes to pattern recognition receptors in host cells. There is a range of pattern recognition receptors found in the plasma membrane or cytoplasm of mammalian hematopoietic cells. The two most widely investigated pattern recognition receptor families are the Toll-like receptors and components of the inflammasome (Moresco *et al.*, 2011; Lamkanfi and Dixit, 2014). The Toll-like receptors bind components of bacteria, such as lipopolysaccharide, lipopeptides and nucleotides, which leads to production of inflammatory mediators; typically interleukin (IL)-6 and IL-8. Similarly, pathogen-associated molecules that reach intracellular compartments activate the inflammasome. However, the inflammasome can also be activated by a range of generalized cell perturbations, including the ion fluxes that are associated with pore-forming toxins secreted by bacteria. Activation of the inflammasome typically leads to cleavage of pro-IL-1β and secretion of the mature form of IL-1β (Lamkanfi and Dixit, 2014). The Toll-like receptor system is present and active in the cells of the endometrium, both epithelium and stroma, as well as in bovine hematopoietic cells (Herath *et al.*, 2006; Cronin *et al.*,

2012; Turner *et al.*, 2014; Cronin *et al.*, 2016). However, endometrial cells secrete little IL-1β protein, and so inflammasome activity may be more important in hematopoietic cells.

The innate immune system provides a non-specific and rapid response to pathogens and damage. However, excessive inflammation leads to immunopathology or septic shock, and so innate immunity is carefully calibrated. A series of checks and balances are in place to scale inflammation to meet the level of microbial threat, and to limit inflammation when infections are cleared (Blander and Sander, 2012). One example in the bovine endometrium, is the role of STAT3 to regulate the secretion of IL-6 and IL-8 in stromal cells (Cronin *et al.*, 2016). Another example is the apical secretion of IL-6 and IL-8 from bovine endometrial epithelial cells, toward the invading pathogens in the uterine lumen and away from the underlying stromal cells (Healy *et al.*, 2015).

Beyond recognition of microbes, one of the features of infection is tissue damage, which in the endometrium is often caused by secretion of pyolysin by *T. pyogenes* (Amos *et al.*, 2014; Preta *et al.*, 2015). Damaged cells release damage-associated molecular patterns, such as nuclear and cytoplasmic molecules that are not normally encountered in the extra-cellular compartment (Kono and Rock, 2008). Some pattern recognition receptors, primarily in hematopoietic cells, sense damage-associated molecular patterns, leading to inflammatory responses. Damaged endometrial tissue cells, primed with LPS, produce the damage-associated molecular patterns, IL-1α, which is normally retained in the cytoplasm of healthy cells (Healy *et al.*, 2014). Furthermore, endometrial stromal cells express the receptor for IL-1 and generate inflammatory responses to IL-1α, including secretion of more IL-6 (Healy *et al.*, 2014).

Innate immunity is an evolutionary ancient system and so it is not surprising that it is integrated with other cellular homeostatic and metabolic pathways (Kotas and Medzhitov, 2015). Dairy cattle are under metabolic stress after parturition, with reduced concentrations of nutrients and changes in metabolic hormones, including reduced abundance of glucose, glutamine and insulin-like growth factor 1 (Chagas *et al.*, 2007; Kerestes *et al.*, 2009). Negative energy balance may impair the inflammatory response and clearance of bacteria from the endometrium, leading to chronic endometritis (Esposito *et al.*, 2014). Certainly, the response to pathogen molecules is energetically expensive *in vivo* and *in vitro* (Turner *et al.*, 2016; Kvidera *et al.*, 2017). A striking example is that animals use > 1 kg of glucose in the first 6 h after challenge with LPS (Kvidera *et al.*, 2017). Furthermore, the depletion of the key cellular nutrients, glucose or glutamine, reduces inflammatory responses by endometrial tissues *in vitro* (Turner *et al.*, 2016; Noleto *et al.*, 2017). If metabolic stress compromises the ability of animals to respond sufficiently to pathogens, this may result in persistence of infections and chronic inflammation.

Impact of uterine disease on animal health and fertility

Clinical uterine disease has a marked impact on reproductive health in cattle, causing subfertility and infertility. In a meta-analysis of records from more than 10,000 animals, there was evidence that postpartum metritis caused subfertility by increasing the time to first insemination by 7.2 days, reducing conception rate to first insemination by 20%, and increasing the calving to conception interval by 18.6 days (Fourichon *et al.*, 2000). Similarly, clinical endometritis increased the interval to first insemination by 11 days, and delayed conception by 32 days, compared with animals that did not have endometritis (Borsberry and Dobson, 1989). Although less common than subfertility, uterine disease also cause infertility. Cows with clinical endometritis between 20 and 33 days post partum were 1.7 times more likely to be culled for reproductive failure than cows without endometritis (LeBlanc *et al.*, 2002).

Pathology in the endometrium is likely to be detrimental to fertilization and conception. In addition, extension of infection or inflammation to the oviduct likely disrupts the delicate balance of the immune systems that are required for fertilization (Marey *et al.*, 2016). However, an important observation for mechanisms that perturb fertility, is that postpartum uterine infection also impacts fertility after resolution of the clinical disease (Borsberry and Dobson, 1989). Several mechanisms may underlie the wider effects of uterine infection on fertility, beyond the tubular genital tract. First, there is evidence that bacterial infections disrupt the endocrine signalling in the hypothalamic-pituitary-gonadal axis, and the secretion of gonadotrophins (Karsch *et al.*, 2002). Secondly, uterine infections disrupt ovarian follicle growth and function, with smaller and less steroidogenic ovarian follicles (Sheldon *et al.*, 2002b). Finally, uterine infections may reduce oocyte quality, with increased rates of meiotic arrest and germinal vesicle breakdown failure (Bromfield and Sheldon, 2011). Oocyte development lasts about120 days, between the primordial follicle stage to ovulation of a cumulus-oocyte complex. Thus, in cows inseminated 60 to 120 days post partum, the oocytes that are ovulated may have been exposed to pathogen molecules and inflammatory mediators throughout the postpartum period, if the animal had uterine disease. Therefore, limiting uterine disease is not only important for the affected animals, but also for their offspring. Further discussion of the mechanisms linking uterine disease and reproductive biology are published elsewhere (Sheldon *et al.*, 2014; Bromfield *et al.*, 2015).

Outstanding questions

Whilst there is a clear understanding of the clinical aspects and implication of postpartum uterine disease, and some of the mechanisms of pathology, there are important outstanding questions. The most obvious question is why are modern high-milk-yield cows so susceptible to metritis and endometritis? Allied to this, is what can be done to prevent uterine disease? Answering these questions is vital for sustainable intensification of the dairy industry over the next 50 years.

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Figure legend

Figure 1. Schematic outline of factors contributing to postpartum uterine health. After parturition the anatomical barriers of the vulva, vagina and cervix are breached, introducing bacteria into the uterus, including pathogens, along with bacteria that constitute the uterine microbiome. However, tissue factors such as mucus, glycoproteins, the pH of the genital tract, and antimicrobial peptides help counter bacterial invasion. If bacteria or their pathogen-associated molecules, such as lipopolysaccharide (LPS), are sensed by the innate or adaptive immune systems then an inflammatory response ensues, including increased expression of complement, calgranulins and acute phase proteins, and chemotaxis of neutrophils and macrophages to the site of infection. As well as inflammation, uterine disease is characterised by tissue damage, including cytolysis caused by the cholesterol-dependent cytolysin, pyolysin (PLO).

Figure 1