Mechanisms of infection and immunity in the bovine female genital tract post partum
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Abstract
Infection of the female genital tract with bacteria after parturition is common in cattle and in humans. These infections lead to clinical diseases, known as puerperal fever or pelvic inflammatory disease in humans but often called metritis or endometritis in cattle. In cattle, metritis affects 20-40% of animals during the first few weeks after parturition. Clinical endometritis persists in about 20% of animals beyond three weeks postpartum and subclinical endometritis affects many other animals. Clinical disease is characterized by the presence of pus, usually detected in the vagina, and associated with infection of the uterus by Escherichia coli, Arcanobacterium pyogenes and anaerobic bacteria. The inflammatory response to infection is dependent on sensing of the microbes by innate immune receptors on endometrial epithelial and stromal cells, which drives an influx of neutrophils to clear the bacteria. However, the innate immune response also perturbs the endocrine function of the endometrium, at least in part by switching prostaglandin secretion from the F to the E series. In addition, cows with uterine disease have abnormal estrous cycles, associated with modulation of ovarian follicle and corpus luteum function. In summary, postpartum bacterial infections are common in dairy cattle and clinical uterine disease disrupts normal fertility by affecting not only endometrial function but also ovarian health.

Keywords: Cattle, uterus, metritis, endometritis, immunity, postpartum.

Introduction
Infection and microbial disease of the female genital tract is common in cattle and humans.1,2 In humans, postpartum bacterial infections cause puerperal fever or pelvic inflammatory disease (PID), whilst in cattle the terms metritis and endometritis are more commonly used. Sexually transmitted infections are also common where bulls are used in natural mating programs. In humans, sexually transmitted infections are widespread and can lead to PID, whereas postpartum infections, known as puerperal fever, are less common in the developed world. However, puerperal fever has a place in history because the studies of this disease lead to Semmelweis’s discovery of antisepsis.3 In the present review, we lay out our current understanding of infection and immunity in the female genital tract, particularly for the postpartum period.

Comparative aspects of uterine disease
Puerperal fever—infection of the uterus postpartum—was a common cause of maternal mortality for women in the developed world until the dawn of the twentieth century and presently is the cause of 75,000 maternal deaths every year, mostly in low-income countries.4,5 Ignaz Semmelweis observed that women attended by doctors had fatality rates for puerperal fever of 13 to 18% and this was greater than the 2% mortality for those women cared for by midwives.3 The high rate of mortality started when junior doctors began performing cadaver dissection, as part of their training, prior to assisting births in the maternity hospital. When the doctors were asked to disinfect their hands in antiseptic solutions before assisting births, the mortality rate fell to about 2%—down to the same level as the midwives. Later Semmelweis initiated the washing of medical instruments and the postpartum maternal mortality rate decreased to about 1%.3 Despite these clear results Semmelweis was shunned, and it took many years for the role of microbes in disease and the value of antisepsis to become widely accepted concepts. The advent of antimicrobials further reduced the impact of puerperal fever and now it is rare following normal labor in the USA.4,5

Puerperal fever is not the only disease associated with microbial infection of the female genital tract in women. Sexually transmitted infections are widespread with ~10% of USA women 16-24 years old having an active sexually transmitted infection and ~340 million new infections across the world each year.1 Sexually transmitted infections are a common cause of PID, which affects between 1 and 3% of women annually in the developed world.6,8 A frequent consequence of PID or sexually transmitted infections in humans is obstruction of the Fallopian tubes, which causes infertility.7 At the time of writing this review, Bob Edwards was awarded the 2010 Nobel Prize in
Physiology or Medicine for developing human in vitro fertilization (IVF), and IVF is commonly used to circumvent the infertility associated with obstruction of the Fallopian tubes. However, the role of uterine disease in relation to tubal obstruction in cattle is not clear. Immunity and inflammation not only have a role during infection but also when there is tissue damage. Two important conditions of the human uterus where this applies are menstruation and endometriosis, which are sterile processes associated with inflammation. Endometriosis is one of the most common health problems associated with lost days in employment, for example. Comprehensive reviews are available for the mechanisms and consequences of infection, immunity and inflammation in the human female genital tract.

Definitions of bovine uterine disease

Pelvic inflammatory disease in cattle is more usually called metritis, although the term for chronic disease—endometritis—is used in humans as well as cattle. The rates of diagnosis of uterine disease continues to increase, whilst common measures of fertility decline. There is an apparent association between increased milk production and reduced conception rates, although the mechanisms and the contribution of uterine disease to this issue are less clear. Of course it is not all bad news for reproductive health in cattle—the development of AI in the second half of the last century all but eliminated sexually transmitted infections in dairy cattle.

Metritis within a week of parturition commonly affects ~20% of animals with maximal herd rates for this clinical disease of 36% and 50%. Subsequently, 15% to 20% of cattle have clinical disease that persists beyond three weeks postpartum (endometritis) and about 30% have chronic inflammation of the uterus without clinical signs of uterine disease (subclinical endometritis). In cattle, we set out the definitions of disease in 2006 with minor revisions and a grading scheme for metritis in 2009. Animals are classified as having grade 1 metritis if they have an abnormally enlarged uterus and a purulent uterine discharge without any systemic signs of ill-health. Animals with additional signs of systemic illness such as decreased milk yield, dullness and fever >39.5°C, are classified as having grade 2 clinical metritis. Animals with signs of toxemia such as inappetance, cold extremities, depression and/or collapse are classified as grade 3 metritis.

Clinical endometritis is defined in cattle as the presence of a purulent discharge detectable in the vagina 21 days or more postpartum, or mucopurulent discharge detectable in the vagina after 26 days postpartum. A simple grading system based on the character of the vaginal mucus is readily used to evaluate cows with clinical endometritis. Vaginal mucus character is graded as 0 (clear or translucent mucus), 1 (mucus containing flecks of white or off-white pus), 2 (exudate containing <50% white or off-white mucopurulent material), or 3 (exudate containing ≥50% purulent material, usually white or yellow but occasionally sanguineous). The endometritis grade correlates with the presence of pathogenic bacteria associated with uterine disease and is prognostic for the likely outcome of treatment. However, there is a recent report that up to 38% of cows with pus in the vagina, diagnosed by a Metrichek device, do not have endometrial inflammation as determined by endometrial cytobrush. Further work using a gold standard test such as endometrial biopsy and histology will be needed to resolve the precision of the diagnostic methods.

Subclinical endometritis is defined by polymorphonuclear neutrophils exceeding 5-10% of cells in samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of signs of clinical endometritis about five weeks postpartum. The incidence of subclinical endometritis is dependent on the diagnostic cut-off used for the proportion of neutrophils in samples and the time postpartum, but is estimated to be on the order of 20 to 50% of animals. However, there remain many questions about the practicality of diagnosis of subclinical endometritis in the field; the dynamic nature of the diagnosis dependent on time postpartum and other factors such as estrous cycle stage; the mechanisms drawing neutrophils into the uterine lumen; and, how the problem should be treated. Another issue that has emerged from uterine cytology is the agreement between cytology and clinical signs. It appears that if animals are evaluated for ≥6% neutrophils in the uterus concurrently with ≥2 score of the vaginal mucus, some animals only have the clinical signs. The implications of this conundrum include the possibility that there are problems with the diagnostic criteria or that pus in the vagina reflects inflammation in the lower genital tract. However, irrespective of these questions, fertility was perturbed by any evidence of inflammation—whether it is ≥6% neutrophils in the uterus and/or ≥2 score of the vaginal mucus. Thus, whilst veterinarians need
to come to a consensus on diagnostic methods, the first step on the critical pathway for developing new treatments for uterine disease is to understand the mechanisms of infection, inflammation and immunity in the female genital tract.

**The etiology of clinical uterine disease in dairy cattle**

It is assumed that pathogenic bacteria do not reside in the uterus during pregnancy but after parturition the uterine lumen is almost always contaminated with a wide range of bacteria, which are readily cultivable by standard techniques.28-32 *Escherichia coli* and *Arcanobacterium pyogenes* are the most commonly isolated bacteria, followed by a range of anaerobic bacteria such as *Prevotella* spp., *Fusobacterium necrophorum*, and *F. nucleatum*.28,31,32 Infection of the uterus with *E. coli* appears to precede infection with other pathogenic bacteria or possibly bovine herpesvirus-4 (BoHV-4).29,30,33 Furthermore, there are specific strains of *E. coli* that possess a pathogenic potential for causing metritis in cattle, which we term endometrial pathogenic *E. coli* (EnPEC).34 These bacteria were first characterized in association with disease and their sensitivity to antimicrobials measured.28,35 We then further identified the bacteria using multi locus sequence typing (MLST) and found they differ from diarrheic and mastitis strains of *E. coli*. The EnPEC strains associated with pelvic inflammatory disease were most adherent for endometrial cells and, like most *E. coli*, expressed functional Type 1 fimbriae (FimH).34 However, the EnPEC were also invasive for endometrial cells, stimulated a host cell inflammatory response, and could replicate uterine disease *in vivo*.34 The implications of the discovery of EnPEC provides a paradigm shift for development of vaccines or biological therapeutics for pelvic inflammatory disease, which should specifically target EnPEC rather than other strains of *E. coli*.

Following the EnPEC infection, *A. pyogenes* and anaerobic bacteria cause the most severe endometrial lesions.36 The impact of the anaerobic bacteria has been harder to quantify but *Prevotella* and *Fusobacterium* species are common in animals with metritis and endometritis. Furthermore, *A. pyogenes*, *F. necrophorum* and *Prevotella* species appear to act synergistically to enhance the likelihood and severity of uterine disease.37,38 For example, *F. necrophorum* produces a leukotoxin, *P. melaninogenicus* produces a substance that inhibits phagocytosis, and *A. pyogenes* produces a growth factor for *F. necrophorum*. Unfortunately, antimicrobials that are most effective against anaerobic bacteria are either expensive or not licensed for food production animals.

Bovine herpesvirus 4 is the only virus consistently associated with uterine disease after parturition in cattle.39,40 Like other herpesviruses, BoHV-4 can establish latent infections in cattle, particularly in macrophages,41 and the viral infection is often identified concurrent with bacteria that cause uterine disease.29,42 The virus is highly tropic for endometrial cells, rapidly replicating and killing epithelial or stromal cells.41,44 The virus may be activated in endometrial cells by cellular pathways stimulated by prostaglandin E$_2$ (PGE) or by lipopolysaccharide (LPS).44 Conversely, the virus appears to activate the host gene promoter for interleukin 8 (IL-8) to stimulate the production of this chemokine,45 perhaps to attract more macrophages that the virus can then persistently infect.

Development of disease is dependent on the balance between host immunity and the pathogenicity of the bacteria. This balance can be tipped in favor of clinical endometritis by risk factors such as retained placenta, dystocia, large calves, twins, and stillbirth.46,47 Cow-level risk factors identified for subclinical endometritis include ketosis, acute metritis, and the interaction between parity and milk production, in which primiparous cows with higher milk production were at higher risk and multiparous cows with higher production were at lower risk for subclinical disease.25 The cleanliness of the animal or fecal contamination of the environment appeared to be less important risk factors for endometritis than retained placenta or dystocia in one study.48 However, in a larger study the herd level risk factors for subclinical endometritis did include environmental factors such as the bedding materials.25

**The mechanisms linking infection and infertility**

Understanding the mechanisms of disease is important for finding new therapeutic or prevention strategies. Furthermore, studying the mechanisms of infection, inflammation and immunity in uterine disease using cattle is biologically relevant because it is a common cause of infertility. Finally, cattle are a useful model to develop concepts that might be applied to humans.49
The initial defense of the mammalian endometrium against microbes is dependent on innate immune systems including pattern recognition receptors, complement, antimicrobial peptides, and acute phase proteins. Pattern recognition receptors on mammalian cells bind molecules specific to microbial organisms, often called pathogen associated molecular patterns (PAMPs) or microbial associated molecular patterns (MAMPs). The most studied group of receptors is the Toll-like receptors (TLRs), which are most often found in a broad range of immune cells. TLR1, TLR2, and TLR6 recognize bacterial lipids such as lipoteichoic acid (LTA) from gram-positive bacteria. TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids often from viruses. Lipopolysaccharide (endotoxin) is the cell wall component of gram-negative bacteria such as E. coli, which is bound to LPS-binding protein in plasma and recognized by TLR4 in complex with CD14 and MD-2. TLR5 binds flagellin; and TLR9 also recognizes bacterial DNA. Activation of TLRs initiates the production of pro-inflammatory cytokines and chemokines. The chemokines mobilize and activate immune cells. The influx of neutrophils into the uterus is particularly associated with metritis and endometritis. The endometrium from normal non-pregnant cattle expresses TLRs 1 to 10, and the endometrial epithelial and stromal cells express most TLRs. These TLRs appear to be functional as endometrial cells secreted PGE and inflammatory mediators in response to bacterial PAMPs. This LPS-induced PGE secretion by endometrial cells is also important for fertility because prostaglandins have multiple roles in endometrial function, and luteolysis is initiated by prostaglandin F2α (PGF2α) from oxytocin-stimulated epithelial cells.

The antimicrobial peptides (AMPs) are an ancient component of the immune system and the defensins family are particularly important for mucosal immunity. Bovine uterine tissue expresses lingual antimicrobial peptide (LAP), tracheal antimicrobial peptide (TAP), and β-defensins. Complement is also a likely ancient system with a role in countering uterine infection but little is known about this in cattle. Mucin-1 (MUC1) is an epithelial cell glycosylated transmembrane protein that may also have a role in microbial defense of the endometrium. MUC1 is expressed by epithelial cells of the bovine endometrium and expression was increased when the cells were treated with LPS. Finally, acute phase proteins are produced in the liver in response to circulating cytokines and peripheral plasma concentrations are increased during the first few weeks postpartum in cattle. Acute phase proteins such as LPS binding protein and haptoglobin have roles in immune defense and regulation of immunity.

Blood-derived neutrophils and monocytes are the main effector cells for removing bacteria from the uterus after calving. However, endocrine and metabolic changes around the time of parturition in cattle modulate neutrophil phagocytic function and gene expression. Further, blood neutrophils obtained from cows with endometritis are significantly less phagocytic. The process of transmigration into the uterine lumen also modulates neutrophil function; IL8-induced attraction of neutrophils into the uterine lumen increased the generation of reactive oxygen species by these cells. However, when neutrophils are in the uterine lumen their function is further modulated by soluble factors in lochia. Whereas lochia of healthy cows only moderately affected the function of neutrophils, the secretions of infected cows severely depressed the generation of reactive oxygen species.

Changes in hormone concentrations around the time of parturition may influence the risk of uterine infections, and at least the luteal phase or progesterone appear to increases the risk of uterine disease. Dietary energy balance also influences the course of the bovine puerperium, associated with changes in plasma and endometrial levels of insulin-like growth factor 1 (IGF-1). Indeed, “modern” dairy cows are selected for milk production, and the increasing capability to produce more milk is associated with decreased fertility. In the last few weeks before parturition and at the start of lactation dairy cows experience decreased dry matter intake (DMI), leading to mobilization of adipose tissue to support milk production. In consequence most lactating dairy cows experience a period of negative energy balance, which interferes with normal reproductive physiology. Decreased DMI and metabolic disturbances around calving are associated with suppression of immune function predisposing lactating cows to bacterial infection. Interestingly, reduced appetite up to two weeks before calving predicted which animals may develop metritis.

Ovarian function is also commonly perturbed in animals with postpartum infection. The oocyte and nurturing granulosa cells grow in a follicle micro-environment where disturbed metabolism can impact fertility. The negative energy balance of lactating dairy cows decreases the
frequency of luteinizing hormone (LH) pulses and is related to low blood concentrations of glucose, insulin and IGF-I. This reduces the production of estradiol by the dominant follicle, impairs oocyte quality and the development of embryos, and limits the production of progesterone by the corpus luteum. Furthermore, cows with uterine infections have slower growth of the first postpartum dominant follicle, lower peripheral plasma estradiol concentrations around the time of maximal follicle diameter, and in those animals that ovulate, peripheral plasma progesterone concentrations are lower after ovulation. These effects of uterine microbes on ovarian function could be caused by PAMPs or inflammatory mediators suppressing release of gonadotrophin releasing hormone (GnRH) and the pituitary secretion of LH. The follicular fluid of cattle with uterine inflammation also contains LPS and granulosa cells collected from growing or dominant follicles secreted less estradiol when treated with LPS. The effect of uterine disease on follicular function may be further enhanced by cytokines released by the endometrial cells because granulosa cell steroidogenesis is impaired by pro-inflammatory cytokines.

**Treatment of uterine disease**

A wide variety of therapies for uterine infection have been reported, including antibiotics administered systemically or locally, hormones such as estradiol and PGF$_{2\alpha}$, intrauterine infusions of antiseptics and supportive care including fluid therapy. The perceived success of therapy depends on the elimination of clinical signs and restoration of normal fertility. However, the choice of treatment often causes controversy among veterinarians, perhaps because of the lack of a precise diagnosis, different methods of classifying uterine infections, spontaneous resolution of disease in many animals, and few controlled trials. Early diagnosis and treatment of animals with metritis is important to control the severity of the disease and limit suffering. Implementation of fresh cow monitoring programs, with daily routine herd health checks to assess appetite, attitude, milk yield and rectal temperature of cows during the first ten days after calving provides an opportunity to identify affected cows. These animals can then receive early supportive therapy in order to maintain DMI during the transition from parturition to lactation. However, solely monitoring the rectal temperature of dairy cows to diagnose metritis is less reliable than including an examination of abnormal uterine discharge. In addition, extra attention needs to be paid to those animals with risk factors for metritis such as retained placenta, dystocia, stillbirths, twins and metabolic conditions such hypocalcaemia and ketosis. Severe, toxic metritis requires urgent and intensive veterinary treatment, whilst mild cases of metritis may require minimal intervention.

Treatment of metritis should aim to eliminate bacteria from the uterine cavity and endometrium without inhibiting uterine defense mechanisms. Metritis is most often treated with broad spectrum antibiotics administered for about three days by the parenteral and/or intra-uterine route, depending on the severity of the symptoms. However, because the tissues deeper than the endometrium are likely affected, the most important line of treatment for metritis is an appropriate parenteral antibiotic that achieves sufficient concentrations in the target tissues. The responsible use of antimicrobial agents in food-producing animals is a concern of many animal health regulatory bodies because of the potential for milk-residue violations, human health risks and development of antimicrobial resistance in bacteria. Bacteria collected from animals with uterine disease are susceptible to cephalosporin compounds. A third generation cephalosporin, ceftiofur, is approved in the USA for systemic administration to lactating dairy cows and the drug reaches all layers of the uterus, the lochia and blood. However, the effectiveness of ceftiofur administration postpartum varies between studies. Other cephalosporin compounds and broad-spectrum antibiotics are used for parenteral administration in cows with metritis, such as potentiated amoxicillin. Although oxytetracycline was widely used by veterinarians for the treatment of uterine infections, evidence for bacterial resistance to this antimicrobial and high minimum inhibitory concentrations indicate that oxytetracycline is unlikely to be the optimum treatment. Despite these data, many veterinarians use 2 to 5 g oxytetracycline administered by placing boluses into the uterine lumen for mild cases of metritis, and in one large study this was reported to be beneficial. Finally, non-steroidal anti-inflammatory agents may provide pain relief during metritis but the value of a single injection is limited when fertility outcomes were evaluated.
The rationale for the choice of treatment for clinical endometritis has been widely discussed and remains controversial with many conflicting studies. The commercial reality is that the choice is between no action, intrauterine administration of antimicrobials or antiseptics, and injection of PGF$_2\alpha$ or one of its analogs; the choice is often limited by country and commercial factors. Intrauterine antibiotics such as cephapirin appear to have acceptable clinical cure rates and benefits for subsequent fertility. Administration of PGF$_2\alpha$ is the treatment used by many veterinarians for cases of endometritis, particularly in animals where a corpus luteum is judged to be present. However, the efficacy and benefit of prostaglandin treatment for endometritis and the use of prostaglandin during the postpartum period varies considerably between studies. The new challenge is to identify the value of treatment of subclinical endometritis and which compounds should be used because the obvious approaches of PGF$_2\alpha$ and/or intrauterine antimicrobials are not particularly rewarding.

Summary

Postpartum infections of the female genital tract are an important cause of infertility in modern dairy cattle. The inflammatory response to uterine infection is dependent on sensing of the microbes by innate immune receptors on endometrial cells and the influx of neutrophils to clear the bacteria. However, these responses to the microbes disrupt not only endometrial function but also affect ovarian health. Although there are established treatments available to veterinarians, the emerging data on the mechanisms of disease may provide new insights to prevent disease or limit the impact on reproductive health.

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