A model of clinical endometritis in Holstein heifers using pathogenic *Escherichia coli* and *Trueperella pyogenes*.

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Interpretive Summary

A model of clinical endometritis in Holstein heifers using pathogenic *Escherichia coli* and *Trueperella pyogenes.* *Piersanti et al.* Clinical endometritis is common in postpartum dairy cows and is important because it reduces reproductive success, even following resolution of disease. However, it is difficult to disentangle mechanisms linking infertility with clinical endometritis because of multiple confounding factors, such as periparturient problems and metabolic stress. Here, we developed an experimental model of clinical endometritis in virgin Holstein heifers using pathogenic bacteria, which recapitulates the signs of disease, with an active infection of the uterus and vaginal discharge of pus. This model will allow exploration of the effects of uterine disease on fertility in a controlled animal model system.

ABSTRACT

Bacterial infection of the uterus causes clinical endometritis in 15 to 20% of postpartum dairy cows and reduces fertility, even after the resolution of disease. However, it is difficult to disentangle the mechanisms linking reduced fertility with endometritis because cows have multiple confounding postpartum conditions. The aim of the present experiment was to develop an *in vivo* model of clinical endometritis in Holstein heifers using pathogenic *Escherichia coli* and *Trueperella pyogenes*. Estrous cycles of heifers were synchronized using a 5-d Co-synch protocol, and subsequently received exogenous progesterone to elevate circulating progesterone at the time of uterine infusion. Endometrial scarification was performed before uterine infusion of live pathogenic *Escherichia coli* and *Trueperella pyogenes*, or sterile vehicle. Effects of infusion were evaluated by measuring rectal temperature, plasma haptoglobin, hematology, grading pus in the vaginal mucus, quantifying 16S rRNA in vaginal mucus, and transrectal ultrasonography. Bacterial infusion increased the median vaginal mucus to grade 2 by d 3 post-infusion, and to grade 3 from d 4 to 6 post-infusion. Control heifers maintained a median vaginal mucus grade ≤ 1 from d 1 to 6. Transrectal ultrasound revealed the accumulation of echogenic fluid in the uterus of heifers following bacterial infusion which was absent in control heifers. Total 16S rRNA in vaginal mucus was elevated in bacteria infused heifers compared to control heifers at d 5. Rectal temperature was increased in bacteria infused heifers. Plasma haptoglobin, general health, and appetite did not differ between groups. As indicated by increased vaginal mucus grade after bacterial infusion and absence of systemic signs of illness, this model successfully induced symptoms resembling clinical endometritis in virgin Holstein heifers. The model allows the isolation of effects of uterine disease on fertility from confounding factors that can occur during the postpartum period in dairy cows.

Keywords: animal model, dairy cow, clinical endometritis, inflammation, uterine infection.

INTRODUCTION

Clinical endometritis is characterized by inflammation resulting from bacterial infection of the endometrium that causes a purulent uterine discharge that is present 21 d or more postpartum. Endometritis has a lactation incidence of 15 to 20%, and is important because, even after resolution of the clinical signs, cows have delayed conception and are more likely to be culled for reproductive failure than cows without endometritis (LeBlanc et al., 2002, Gernand et al., 2012, Ribeiro et al., 2013). However, understanding how uterine disease affects fertility is complicated because endometritis is associated with other peripartum problems as well as metabolic challenges associated with the onset of lactation, which also impair fertility (LeBlanc, 2012, Ribeiro

et al., 2013). Developing an experimental model of clinical endometritis in heifers would help disentangle the mechanisms of how uterine disease impacts reproductive physiology.

Cows with clinical endometritis do not have systemic signs of disease, and diagnosis is based on the presence of a purulent uterine discharge detectable in the vagina 21 d or more postpartum (LeBlanc et al., 2002, Sheldon et al., 2006). The vaginal contents can be examined using a gloved hand, a Metricheck tool or a speculum, and the severity of clinical endometritis can be graded by the amount of pus in the mucus. There is debate about whether purulent vaginal discharge may also reflect cervicitis or vaginitis because not all cows with purulent vaginal discharge have infiltration of polymorphonuclear neutrophils detectable by endometrial cytology (Dubuc et al., 2010). Nevertheless, bacteria can be isolated from the uterus of cows with endometritis, and the presence of pus in the uterus or a slightly enlarged uterus can also be visualized using transrectal ultrasonography (Sheldon et al., 2002).

Clinical endometritis is associated with Gram-negative endometrial pathogenic *Escherichia coli*, Grampositive *Trueperella pyogenes*, and several anaerobes (Sheldon et al., 2002, Bicalho et al., 2012). Both *E. coli* and *T. pyogenes* can be detected in the uterus of cows that develop uterine disease as early as 1 to 3 DIM based on culture and PCR based technologies (Dohmen et al., 2000, Bicalho et al., 2012). Lipopolysaccharide (LPS) from Gram-negative bacteria and lipopeptides from Gram-positive bacteria activate the innate immune system in the endometrium, leading to inflammation and increased concentrations of circulating acute phase proteins (Sheldon et al., 2001, LeBlanc, 2012, Turner et al., 2014). Pus forms in the uterus when neutrophils phagocytize bacteria or endometrial cells damaged by cytolysins, such as the pore-forming toxin pyolysin secreted by *T. pyogenes* (Sheldon et al., 2010, Bicalho et al., 2012, Amos et al., 2014). A notable feature of the pathogenesis of endometritis is that the adhesion of *E. coli* to endometrial cells and the cytolysis caused by pyolysin is greatest in the stroma, which is exposed when the epithelium is lost during the peripartum period (Sheldon et al., 2010, Amos et al., 2014). Furthermore, peripartum problems that traumatize the endometrium, such as dystocia and retained fetal membranes, increase the risk of developing endometritis (Dubuc et al., 2010, Potter et al., 2010, Ribeiro et al., 2013).

Uterine disease is associated with prolonged luteal phases, slowed rate of growth of ovarian follicles postpartum, and impaired ovarian endocrine function (Opsomer et al., 2000, Sheldon et al., 2002). However, the mechanisms linking endometritis with reproduction are not fully established because fertility is also perturbed by many of the risk factors for endometritis, including peripartum problems and the inability to adapt to the shifts in metabolism to accommodate lactation (Chagas et al., 2007, LeBlanc, 2012). Animal models of clinical endometritis have been reported using mature cows infused with *T. pyogenes* (Rowson et al., 1953, Ayliffe and Noakes, 1982, Farin et al., 1989, Amos et al., 2014). However, an experimental model of endometritis in heifers would be attractive for exploring how uterine infection affects reproduction. Using heifers as the basis for the model circumvents many of the confounding effects of parturition and lactation observed in lactating cows. The aim of the present experiment was to develop a defined *in vivo* infection model of clinical endometritis in Holstein heifers using pathogenic *E. coli* and *T. pyogenes*.

MATERIALS AND METHODS

The University of Florida Institutional Animal Care and Use Committee approved all procedures with heifers under the protocol number 201508884. The experiment was conducted from June to October, 2017 at the University of Florida Dairy Unit.

Establishment of Uterine Infection in Heifers

Nine virgin Holstein heifers aged between 11 and 13 months were enrolled in the experiment. All animals were free of general health conditions and tested negative for *Brucella abortus*, *Neospora caninum*, and *Leptospirosa*. Heifers were vaccinated against bovine viral diarrhea, infectious bovine rinotracheitis, parainfluenza, bovine respiratory syncytial virus, and multiple serovars of Leptospira (Bovi-Shield Gold FP 5 VL5 HB; Zoetis, Parsippany NJ) and de-wormed using moxidectin (Cydectin; Bayer HealthCare, LLC, Animal Health Division, Shawnee Mission, KS). Heifers were fed a diet as TMR that was offered once a day in addition to pasture access and provided *ad libitum* access to water.

The experiment followed a randomized complete block design with heifer as the experimental unit. Heifers were blocked by age and weight, and randomly assigned to one of two infusion treatments: intra-uterine infusion of sterile vehicle medium alone (n = 5) or intra-uterine infusion of live bacteria (n = 4; details below).

Estrous cycles were synchronized using a modified 5-d Co-synch protocol (Lima et al., 2013). Briefly, heifers received 100 mg i.m. of GnRH (gonadorelin diacetate tetrahydrate; Ovacyst, Bayer) followed by 25 mg i.m. of $PGF_{2\alpha}$ (dinoprost tromethamine; Prostamate, Bayer) administered 5 and 6 d later (Figure 1A). Eight days following initial GnRH, heifers received a final dose of 100 mg of GnRH i.m. Starting on the day following the final GnRH, heifers received 200 mg i.m. of progesterone (P4) in corn oil (50 mg/mL; Sigma-Aldrich, St Louis MO) daily for 7 d. Exogenous P4 was administered to mimic diestrus and ensure elevated circulating P4 at the time of bacterial infusion.

Uterine infusion of treatments were performed 72 h following final GnRH administration and designated as d 0 relative to treatment (Figure 1A). Endometrial scarification was implemented before intra-uterine infusion to disrupt the endometrial epithelium. Heifers were restrained and received a caudal epidural injection of 60 mg of lidocaine hydrochloride 2% (Aspen Veterinary Resources, Greely CO). The perineum and vulva were cleaned and disinfected with povidone iodine followed by 70% ethanol, and a sterile metal scarification tool enclosed by a metal catheter covered in a sanitary sheath (IMV Technologies, Normandy, France) was introduced through the vagina and cervix. The scarification tool consisted of a stainless steel rod with a 39 mm long, 6 mm diameter threaded component on the tip, similar to a threaded bolt (Figure 1B). The scarification tool was manipulated through the cervix and into the body of the uterus by transrectal palpation. Once inside the body of the uterus, the sanitary sheath was retracted, and the scarification tool was placed in direct contact with the endometrium. The scarification tool was then rotated once to disrupt the endometrial lining before removal from the reproductive tract. Inspection of the scarification tool upon retraction confirmed tissue disruption by the presence of small pieces of tissue. Following endometrial scarification, a metal infusion catheter enclosed in a sanitary sheath was introduced transcervically into the body of the uterus. The sanitary sheath was retracted, and treatments were infused using 10 mL syringes. Bacterial infusion consisted of 10 mL of 4.64×10^7 CFU/mL of *E. coli* MS499, 10 mL of 3.38×10^7 CFU/mL *T. pyogenes* MS249 followed by 10 mL of sterile Luria-Bertani (LB) broth to flush the catheter (Goldstone et al., 2014a, Goldstone et al., 2014b). Vehicle infusion consisted of 30 mL of LB broth. Heifers were returned to pasture and monitored for clinical signs for 24 h. Animals did not receive any additional treatments or medication during the experimental period.

Bacterial Culture and Preparation of Inoculants

Escherichia coli MS499 and *T. pyogenes* MS249 were collected and isolated from cows with metritis and characterized previously (Goldstone et al., 2014a, Goldstone et al., 2014b).

Escherichia coli was cultured from frozen glycerol stocks on LB agar. The day before infusion, a single colony was picked from the plate and inoculated into LB broth containing 1% tryptone, 0.5% yeast extract and 1% sodium chloride. The culture was incubated overnight at 37°C with shaking at 200 rpm. Growth was monitored by measuring optical density at 600 nm (OD_{600} = 5.0). A final preparation of 4.64×10^7 CFU/mL *E. coli* was diluted in sterile LB broth and loaded into 10 mL syringes for infusion.

Trueperella pyogenes MS249 was grown from frozen glycerol stocks on Trypticase Soy Blood agar and grown at 37°C for 48 h. The day before infusion, a single colony was selected and inoculated into Bacto Brain Heart Infusion broth (BHI; Fisher Scientific, Pittsburg, PA) supplemented with 5% fetal bovine serum (FBS; Fisher Scientific) and cultured overnight at 37°C with shaking at 200 rpm. Growth was monitored by measuring optical density at 600 nm (OD_{600} = 0.2). A final preparation of 3.38×10^7 CFU/mL *T. pyogenes* was diluted in sterile BHI and loaded into 10 mL syringes for infusion. Syringes were loaded with sterile LB broth for flushing catheters and vehicle infusions. Inoculants were transported to the farm on ice for infusion.

Blood Sampling and Hematology

Blood was collected from the coccygeal vessels into evacuated tubes (Vacutainer, Becton Dickson, Franklin Lakes NJ) containing sodium heparin for plasma separation or potassium EDTA for hematology. Blood was sampled every other day from d -2 to 18 relative to treatment. Blood was placed on ice until further

processing. Tubes containing heparin were centrifuged and plasma was collected, aliquoted, and stored at -20°C. Whole blood was transported to the laboratory on ice within 2 h of collection and used for hematology analysis performed using an automated hematology analyzer (ProCyte Dx Hematology Analyzer, IDEXX Laboratories, Westbrook ME). Hematology analysis included total and differential leukocyte counts (neutrophils, lymphocytes, and eosinophils), red blood cells, hematocrit and hemoglobin. Hematology was evaluated on d -1, 1, 3 and 7 relative to treatment (Figure 1A).

Plasma haptoglobin (Life Diagnostics, Inc., West Chester, PA) and P4 (DRG International, Inc., Springfield, NJ) were measured using commercially available ELISA according to the manufacturer's instructions. Plasma haptoglobin was evaluated on d 0, 7 and 13 relative to treatment, and P4 was evaluated on d -2, 1, 4, 11 and 17 relative to treatment (Figure 1A). The P4 ELISA is human specific and was validated for bovine plasma using spike-in/recovery performance based on actual and expected recovery of P4 supplied as standard with the kit. Intra-assay CV was calculated at 6.5%, while recovery of spike-in P4 was 89% to 101.8% of expected P4.

Examination and Grading of Vaginal Mucus, Transrectal Ultrasonography, and Rectal Temperature

Vaginal mucus was collected and examined using a clean Metricheck tool (Metricheck, Simcro, New Zealand). Vaginal mucus was scored as grade 0, no mucus or clear or translucent mucus; grade 1, mucus containing flecks of white or off-white pus; grade 2, mucus containing ≤ 50% white or off-white mucopurulent material; and grade 3, mucus containing > 50% purulent material (Sheldon et al., 2009). Evaluation of vaginal mucus was performed daily from d -1 to 7 relative to treatment (Figures 2A-C).

Transrectal ultrasonography with a linear 5.0 MHz probe (Aloka SSD-500, Hitachi Healthcare Americas, Twinsburg, OH) was performed to visualize fluid and pus in the uterus. Ultrasonography was performed every other day from d -1 to d 10 relative to treatment. Rectal temperature (AG-102 thermometer, AG-Medix, Mukwonago WI) was measured daily between 7 and 9 AM from d -11 to 10 relative to treatment. A rectal temperature of > 39.5°C was classified as fever.

Quantification of Total 16S rRNA Isolated From Vaginal Mucus

Vaginal mucus was stored in sterile bijou bottles at -20°C until processing. Total DNA was isolated from vaginal mucus using the DNeasy Power Soil Kit according to the manufacturer's instruction with modification (Qiagen, Hilden, Germany). Briefly, samples were thawed on ice and vortexed and 250 mg of vaginal mucus was used for DNA extraction using a bead beater tissue homogenizer (Precellys 24, Bertin Technologies SAS, France). The mucus was added to guanidine thiocyanate and homogenized with garnet particles using 3 bead beater cycles (30 s at 6000 × *g*, 60 s pause, 30 s at 6000 × *g*) with a 5 min incubation on ice between each cycle. Following homogenization, supernatants were applied to the DNeasy Power Soil spin columns for DNA purification.

Total 16S rRNA was quantified using the Femto Bacterial DNA Quantification Kit according to the manufacturer's instructions (Zymo Research, Irvine CA). Thermocycling conditions included initial denaturation at 95°C for 10 min, 40 cycles of amplification consisting of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. A total of 2 μL of extracted total DNA sample was applied to each PCR reaction. Quantification of 16S rRNA was analyzed based on a standard curve performed in parallel with mucus samples. Data for total 16S rRNA are described as nanograms of 16S rRNA per milligram of vaginal mucus. The extraction of 16S rRNA in mucus samples was validated using spike-in/recovery using of using known quantities of purified bacteria. Intra-assay CV was calculated at 0.3%, inter-assay CV was calculated at 2.2%, while recovery of 16S rRNA following extraction of bacteria spike-in pus was 100.5% of expected 16S rRNA content.

Statistical Analysis

All data were analyzed using SAS v. 9.4 (SAS Institute, Cary, NC). Vaginal mucus grade was reported as the median for each treatment group and analyzed using the GLIMMIX procedure following a Poisson

distribution. Cow within treatment was considered a random effect, and fixed effects of treatment and day were analyzed. Haptoglobin, P4, hematology and DNA concentration for the 16S rRNA gene were analyzed using the MIXED procedure of SAS and the models included the fixed effects of treatment (bacterial infusion), day (repeated measure), and their interaction. Heifer nested within treatment was considered as a random effect. First order autoregressive covariance structure AR (1) was used as the covariate structure. Values are reported as LSM ± SEM. Differences with $P \leq 0.05$ were considered statistically significant.

RESULTS

Intra-Uterine Bacterial Infusion Increased Vaginal Mucus Grade

Vaginal discharge of pus was visually evident in heifers that were infused with live bacteria, but not in control heifers (Figure 2A). Vaginal mucus collected by Metricheck was graded and compared between treatments (Figure 2B-C). There was an increase ($P < 0.02$) in the vaginal mucus grade of heifers treated with bacteria (Figure 3). Heifers in both infusion groups had a median mucus grade of 0 prior to uterine infusion (Figure 3). Intra-uterine infusion of live bacteria resulted in a median mucus grade of 2 on d 3 relative to treatment, whereas control heifers maintained a median mucus grade of 0 (Figure 3). On d 4 to 6 relative to treatment, bacteria infused heifers had a median mucus grade of 3, whereas control heifers presented a median mucus grade ≤ 1. The presence of echogenic fluid in the uterus of bacterial infused heifers was confirmed by transrectal ultrasound, and control heifers had no evidence of increased fluid, or echogenic material present in the uterus (Figure 2D-E).

Intra-Uterine Bacterial Infusion Increased 16S rRNA Concentration in Vaginal Mucus

Total vaginal 16S rRNA was increased ($P < 0.05$) in bacteria infused heifers compared with control heifers on d 5 (Figure 4A). In addition, total vaginal 16S rRNA was greater ($P < 0.05$) on d 5 in heifers infused with bacteria compared to other sampled days (Figure 4A). There was no interaction between treatment and day relative to treatment ($P = 0.14$).

Effect of Uterine Bacterial Infusion on Hematology and Rectal Temperature

There was no effect of treatment on any hematological parameters measured (Table 1). There was an effect ($P < 0.05$) of day relative to treatment on the number of red blood cells (M/ μL), number of lymphocytes (K/μL), number of platelets (K/μL), percent of hematocrit and hemoglobin (g/dL). There was a tendency for an effect of treatment ($P = 0.08$) on rectal temperature with bacteria infused heifers showing elevated temperature on d 1 compared to control heifers (Table 1). Two bacteria infused heifers had rectal temperatures above 39.5°C on d 1 relative to treatment (40.0°C and 40.2°C). There were no overt signs of systemic disease observed in any heifers.

Effect of Bacterial Infusion on Circulating Concentrations of Haptoglobin and Progesterone in Plasma

Plasma concentration of haptoglobin did not differ ($P = 0.70$) between treatments and there was no effect of day ($P = 0.36$) or interaction between day and treatment ($P = 0.41$). A numerical 3.5-fold increase in haptoglobin concentration on d 13 relative to treatment was observed in bacteria infused heifers compared with control heifers (Figure 4B; $P > 0.05$).

Plasma concentrations of P4 increased in both treatment groups as a result of exogenous P4 supplementation (effect of day, $P < 0.001$); however, infusion with bacterial did not affect concentrations of P4 in plasma ($P > 0.05$, Figure 4C). The peak concentration was observed on d 4 relative to treatment and, once exogenous P4 supplementation was concluded on d 4, concentrations decreased in both treatments.

DISCUSSION

The present experiment developed an *in vivo* model of clinical endometritis in Holstein heifers using pathogenic *E. coli* and *T. pyogenes*. Heifers that received intra-uterine infusion of pathogenic bacteria developed purulent vaginal mucus, accumulation of echogenic fluid in the uterus, and increased bacterial load in vaginal

mucus. In parallel, bacteria infused heifers displayed no systemic signs of illness, such as altered hematology or general sickness. Control heifers did not display any of the clinical signs of clinical endometritis. Based on the criteria previously described for the disease, these results recapitulate the symptoms of clinical endometritis observed in the postpartum dairy cow (LeBlanc et al., 2002, Sheldon et al., 2006, Sheldon et al., 2009).

Previous experimental models of clinical endometritis have focused on the use of mature cows and intrauterine infusion of *T. pyogenes* (Rowson et al., 1953, Ayliffe and Noakes, 1982, Amos et al., 2014). These studies were capable of generating active infection of the uterus, evident by the presence of mucopurulent vaginal contents, using a single uterine pathogen. Although these models induce clinical symptoms of disease, it may be that the use of a second pathogen, *E. coli*, better reflects the molecular profile of endometritis to evaluate the consequences of disease on fertility. The dual pathogen model of Del Vecchio et al. (1992) utilized repeated infusions of both *E. coli* and *T. pyogenes* over the course of 3 d (Del Vecchio et al., 1992); however, the strain and pathogenicity of bacteria used may also be important. Strains used by others include β-hemolytic *E. coli* or *T. pyogenes* sourced from cows with severe endometritis (presumably from the uterus). Additionally, Farin et al., (1989) utilized intra-uterine infusion of *T. pyogenes* and the Gram-negative anaerobes, *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* (now *Prevotella melaninogenica*) to induce "pyometria" in lactating multiparous cows. The model describes the implementation of tissue damage using an intrauterine infusion of iodine solution, and intravenous administration of hCG to ensure elevated circulating P4, two factors recapitulated in our model but using different approaches. The model of Farin et al., (1989) also reports the consistent isolation and culture of uterine *T. pyogenes* during an extended window of active disease lasting up to 30 d post-infusion in some cows. The presence of *E. coli* was not reported in these studies, while isolation and culture of the anaerobes, *F. necrophorum* and *P. melaninogenica*, was inconsistent between cows with disease (Farin et al., 1989). Pathogenic strains of *E. coli* and *T. pyogenes* used in the current model were sourced from the uterus of cows with endometritis and have subsequently been sequenced (Goldstone et al., 2014a, Goldstone et al., 2014b), allowing a detailed understanding of virulence factors and host-pathogen interactions involved in disease. These two species of pathogens were specifically chosen to establish infection as they are readily detectable within days of parturition in the uterus of cows that develop spontaneous uterine disease (Dohmen et al., 2000, Bicalho et al., 2012). The pore forming toxin produced by *T. pyogenes* causes cellular damage to the endometrium during active infection, although little is known about its actions on ovarian function, especially after the clearance of disease. Conversely, LPS derived from Gram-negative bacteria is present in follicular fluid after the resolution of uterine disease. Indeed, studies have demonstrated that the presence of LPS negatively effects oocyte development and alters the follicular environment of the growing oocyte *in vitro* (Bromfield and Sheldon, 2011).

Species other than the cow have been used to develop models of uterine infection, including the sheep and mouse (Regassa et al., 2002, Sheldon and Roberts, 2010, Bromfield and Sheldon, 2013). However, when attempting to determine the mechanisms by which endometritis impacts fertility of the dairy cow, it is imperative to utilize the target species in question. Rodents are relatively inexpensive and convenient for such a model, but the reproductive physiology and immune function of the mouse is considerably different from those of the cow. Mice have been shown to be 1 million-fold less sensitive to LPS than humans (Seok et al., 2013). More appropriately, the human and bovine genomes share a high degree of amino acid sequence homology, limited species-specific orthologs, and a similar chromosomal organization; suggesting the bovine as a useful experimental model for human physiology (The Bovine Genome Sequencing and Analysis Consortium et al., 2009). In addition, bovine reproductive biology is closer to that of humans than mice; cows and humans are both monotocous with similar hormonal profiles over a 3 to 4 week ovarian cycle, compared to the polytocous mouse with a 4 d estrous cycle. The current model of endometritis may even serve as a tool to study the impacts of uterine infections on human fertility.

The use of the Holstein heifers in the present experiment was a deliberate choice in the generation of the experimental model presented. The long-term goal of this model is to study the mechanisms of endometritis mediated reproductive failure in the cow. As uterine disease occurs in the early postpartum period, a number of significant challenges can confound experimental investigation into the causes of endometritis-mediated infertility. Specifically, postpartum uterine damage, uterine involution, metabolic demands of lactation, negative nutrient balance, and additional postpartum illnesses which affect almost half of all postpartum cows (Ribeiro et

al., 2016). Retained placenta and dystocia are significant risk factors for the development of endometritis, and are both associated with damage to the endometrium (Dubuc et al., 2010). To recapitulate postpartum endometrial damage, we performed a scarification procedure to disrupt the endometrial epithelial layer at the time of bacterial infusion. This process of scarification may be critical to the establishment of the disease model to facilitate bacterial access to the underlying stroma. Previous work has reported differential susceptibility of epithelial and stromal endometrial cells to pathogenic *E. coli*, with pathogenic *E. coli* binding with stronger affinity to stromal cells, and purified LPS inducing a stronger inflammatory reaction in the endometrial stroma (Sheldon et al., 2010). A similar phenomenon of differential cellular susceptibility has been described in the response of endometrial epithelial and stromal cells to *T. pyogenes* pyolysin, in which stroma cells are considerably more sensitive to the cytoxic effects of pyolysin (Amos et al., 2014), suggesting the stroma is the target of pyolysin. These *in vitro* experiments, in combination with the risk factors associated with endometritis, suggest that endometrial scarification may be an important procedure in the establishment of clinical endometritis observed in the present model.

The administration of exogenous P4 was a calculated approach to modulate the immune function of heifers at the time of bacterial infusion. Rowson et al. (1953) reported that intra-uterine infusion of *T. pyogenes* (reported as *Corynebacterium pyogenes*) during the luteal phase of the estrous cycle resulted in "pyometritis"; however, no infection could be established when bacterial infusion was performed at estrus. These experiments were repeated using ovariectomized cows in conjunction with administration of exogenous P4 or estradiol. Only with the administration of exogenous P4 could pyometritis be achieved (Rowson et al., 1953). Since the 1950's, the immune modulating properties of P4 and estrogen have been reported in immune cells and the endometrium (reviewed in detail by (Wira et al., 2015)) with various studies demonstrating the immune-suppressive function of P4 within the endometrium of ruminants (Hawk et al., 1964, Del Vecchio et al., 1992, Hansen, 1998, Seals et al., 2002, Lewis, 2003). The ability of steroid hormones to modulate endometrial immune function have led to the practice of administering $PGF_{2\alpha}$ as a treatment for uterine infection to induce luteolysis in cows in diestrus, and elevate estrogen by stimulating a new follicular phase of the estrous cycle (Lewis, 1997); however this remains debated. In parallel, 55% of cows with metritis display extended luteal phases (Etherington et al., 1991, Opsomer et al., 2000). The extension of the luteal phase in cows with uterine disease can have consequences on the calving to conception interval, negatively impacting productivity. Exposure of endometrial cells to bacterial LPS *in vitro* switches prostaglandin synthesis from luteolytic $PGF_{2\alpha}$ to luteotrophic PGE_2 (Herath et al., 2006, Herath et al., 2009). This apparent LPS-mediated switch in endometrial secretion of luteotrophic PGE_2 may explain the extended luteal phase in some cows with uterine disease. In the current study we evaluated circulating P4 until d 18 post-infusion, and did not observe any effect of treatment at any time point. This result may be an influence of exogenous P4 administration for 7 consecutive days, or an artifact of the extensive manual manipulations performed on these heifers during the course of the study. Future studies using this model of induced infection should evaluate the duration of the luteal phase, monitoring follicular growth, ovulation, CL size, peak P4 and luteal phase duration.

Vaginal mucopurelent content observed in heifers treated with intra-uterine infusion containing live bacteria could be associated with cervicitis or vaginitis as previously observed in spontaneously occurring cases in postpartum cows (Dubuc et al., 2010). Although the infusate was placed in the uterine horns, retrograde movement of the content could have inoculated the cervix and vagina. However, quantification of vaginal 16S rRNA the day after infusion on experiment d 1 revealed comparable total bacterial load between treatments. The presence of elevated 16S rRNA observed in the bacteria infused heifers 5 d following infusion suggests that mucopurulent vaginal discharge is derived from the infected uterus induced by infused bacteria, and not from environmental contamination. The accumulation of echogenic uterine fluid in bacteria-infused heifers supports these assumptions and suggests that any cervicitis or vaginitis would likely be the result of contamination or excessive manipulation, and would also be observed in vehicle infused controls. Interestingly however, hematological evaluation of heifers suggests a possible effect of the procedure of infusing material into the uterus itself. While we did not observe a treatment effect for any of the hematological parameters evaluated, we did see an effect of day on numbers of red blood cells, lymphocytes, and platelets, and the percent of hematocrit and hemoglobin. Consistently all of these parameters were elevated 24 h following infusion and steadily declined to pre-infusion levels by d 7. This observation may be indicative of the manual manipulation effecting systemic hematological parameters in the heifers.

Any experimental model of infection has limitations. Here, we propose using the described experimental model to define the mechanisms of endometritis-mediated subfertility. However, using heifers as the experimental unit limits our understanding of the reproductive potential of the individual animal, unlike using mature lactating cows that would normally have uterine disease. Additionally, the use of heifers revealed experimental limitations because of body size and ability to perform the manipulations in 12 to 13-mo old animals, indicated by the consistent day effect of some hematological parameters. Experimentation for the purposes of determining mechanisms of endometritis-mediated subfertility will require multiple, repeat manipulations which might be difficult in small frame heifers compared with mature cows. The longevity of the experimental model is also an important factor to determine in future cohorts of animals, it will be important to establish that the infection observed in the current cohort of heifers is reminiscent of the duration of spontaneous endometritis observed in lactating cows. Additionally, quantification of endometrial inflammation and microbial populations present as a result of experimentally induced infection will be critical to establish the validity of the model to study endometritis-mediated subfertility in the dairy cow.

CONCLUSIONS

We have successfully created a model of experimentally induced clinical endometritis in virgin Holstein heifers. This model will allow the investigation into the mechanisms of endometritis associated reproductive failure. The use of an experimental model of endometritis allows investigation into endometritis-mediated subfertility independent of confounding postpartum events common in lactating cows which may influence reproductive physiology. We utilized a combination of interventions to facilitate uterine infection, including disruption of the endometrial epithelial barrier, the supplementation of exogenous P4, and the infusion of uterine pathogenic bacteria. We conclude that our experimental model recapitulates clinical endometritis symptoms in virgin Holstein heifers.

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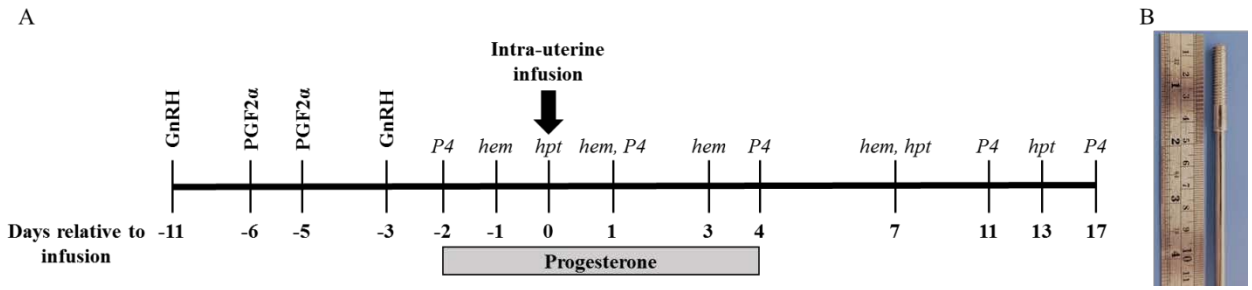

Figure 1. Requirements for establishment of experimental uterine infection. (A) Time line of experimental procedures employed during the experimental period. Gonadotropin releasing hormone (GnRH) and prostaglandin (PG) $F_{2\alpha}$ were used to synchronize estrous cycles in 9 virgin Holstein heifers before intra-uterine infusion of treatments. On d 0, intra-uterine infusion of either vehicle or live bacteria was performed. Vehicle infusion consisted of 30 mL of sterile Luria-Bertani broth. Bacteria infusion consisted of 10 mL of *E. coli* MS 499 (4.64×10^7 CFU/mL), 10 ml of *T. pyogenes* MS249 (3.38×10^7 CFU/mL) and 10 mL of sterile LuriaBertani broth. P4 was administered at 200 mg/d i.m. Days relative to treatment for evaluation of plasma haptoglobin (*hpt*), P4 (*P4*) and hematology (*hem*) are indicated in italics. (B) The tool utilized to enable scarification of the endometrium.

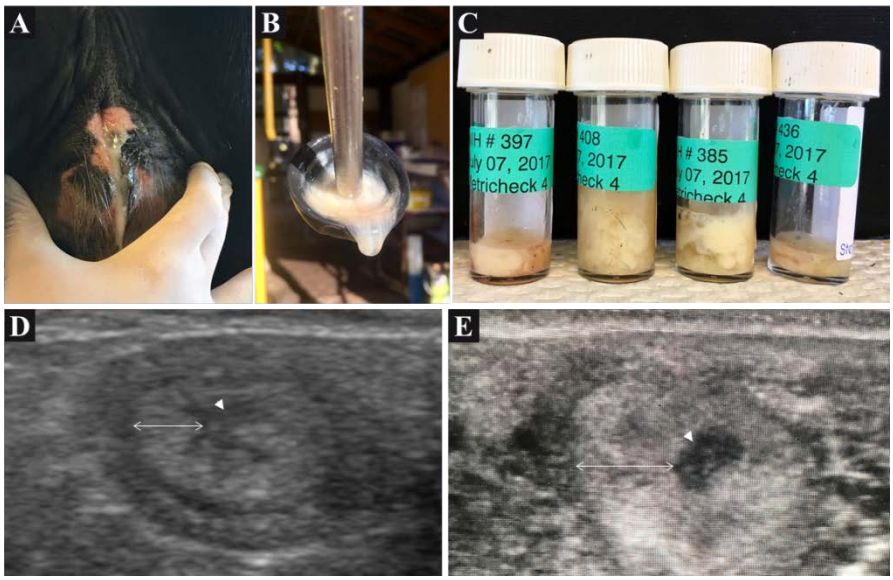

Figure 2. Clinical observations of induced uterine disease. (A) Vaginal mucus was visually confirmed in heifers receiving bacterial infusion. (B) Metricheck tool containing vaginal mucus of a bacteria infused heifer. (C) Examples of vaginal mucus samples collected from bacteria infused heifers using the Metricheck tool. (D and E) Representative ultrasound images of a transverse cross section of a uterine horn from a control (D) and bacteria infused (E) heifer. The arrow head denotes the uterine lumen; the double headed arrow denoted the uterine wall; scale bar represents 5 mm.

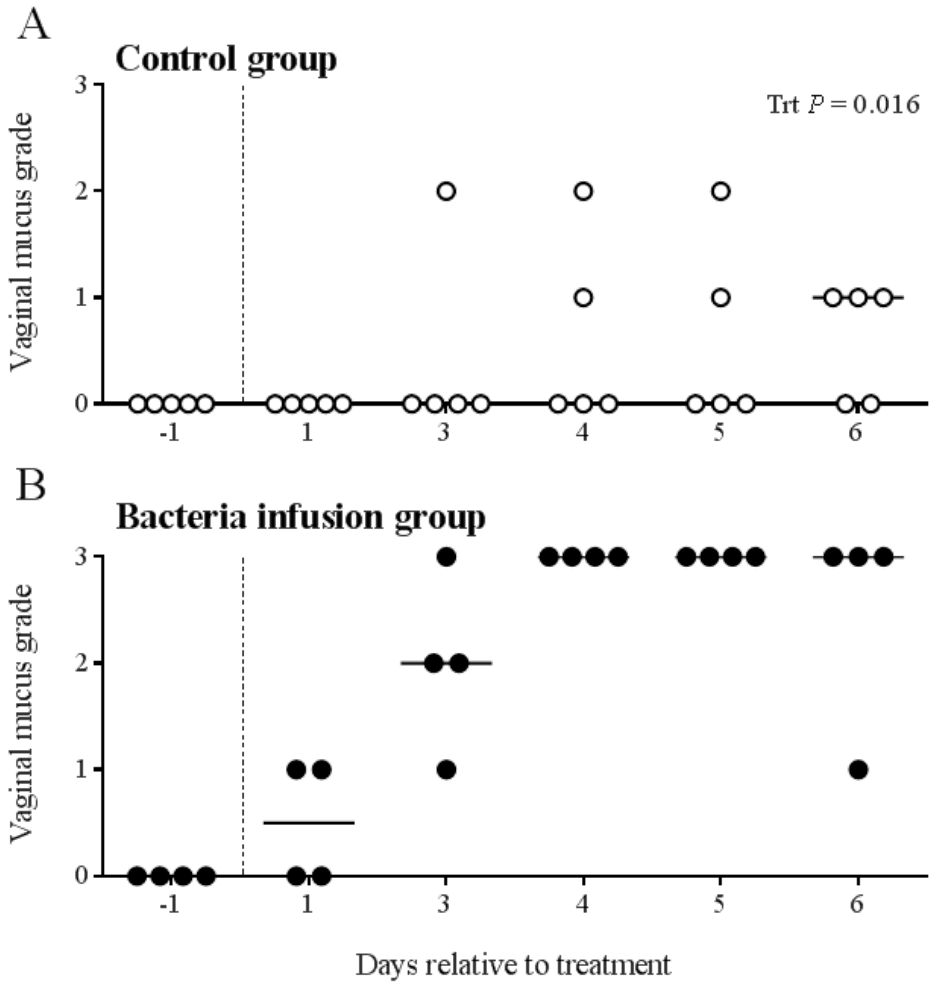

Figure 3. Vaginal mucus grade following intra-uterine infusion of bacteria. Vaginal mucus was collected using a Metricheck tool and graded according to Sheldon et al. (2009). Vaginal mucus was graded from 0 to 3 and each heifer is represented by a single circle. Control heifers are represented by open circles (A, ○), bacteria infused heifers are represented by filled circles (B, ●). The vertical dotted line denotes the day of treatment, solid horizontal lines indicate the median vaginal mucus score for the day of observation. Data were analyzed using the GLIMMIX procedure following a Poisson distribution to determine the effect of treatment (Trt).

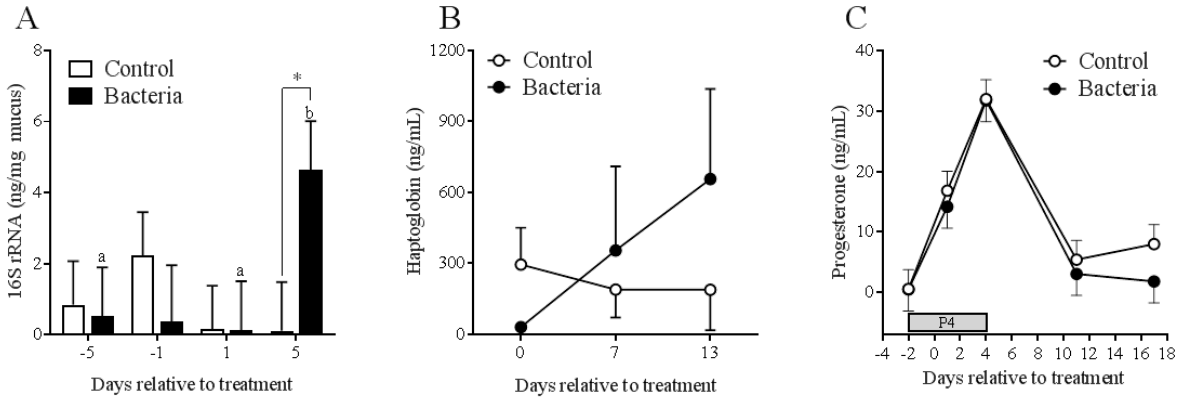

Figure 4. Effect of treatment on vaginal mucus 16S rRNA and circulating concentrations of haptoglobin and progesterone in plasma. (A) Total 16S rRNA quantification from vaginal mucus on d -5, -1, 1 and 5 relative to treatment in either control (open bars) or bacteria infused (solid bars) heifers. Total 16S rRNA was normalized to the weight of vaginal mucus processed for nucleic acid extraction (ng rRNA per mg mucus). Different superscript denote differences within the bacteria infused group; * denotes difference between treatment groups on a given day ($P < 0.05$). (B) Haptoglobin plasma concentration (ng/mL) was measured in control (○) and bacteria infused (●) heifers on d 0, 7 and 13 relative to treatment. (C) Progesterone plasma concentration (ng/mL) was measured in control (○) and bacteria infused (●) heifers on d -2, 1, 4, 11 and 15 relative to treatment. P4 denotes the period of exogenous administration of 200 mg/d of P4. All data are presented as LSM ± SEM.

Table 1. Effect of treatment on rectal temperature and hematology.

	1	38.7	39.5				
	3	38.3	38.7				
	7	38.5	38.5				
RBC (M/μL)	-1	6.78	6.36	0.31	0.3	<0.01	0.85
	1	7.79	7.03				
	3	7.37	6.75				
	7	7.17	6.70				
HCT (%)	-1	30.04	29.60	1.50	0.68	<0.01	0.81
	1	33.70	32.80				
	3	33.04	31.43				
	7	32.34	31.73				
HGB (g/dL)	-1	10.20	9.93	0.5[1]	0.65	<0.01	0.96
	1	11.24	10.98				
	3	11.00	10.55				
	7	10.80	10.43				
WBC (K/μL)	-1	12.12	11.72	0.85	0.65	0.45	0.86
	1	12.31	12.81				
	3	10.38	11.43				

[1] Data are presented as LSM with respective pooled SEM for the interaction between treatment and day.

Data quantified in whole blood included red blood cells count (RBC), hematocrit (HCT), hemoglobin concentration (HGB), white blood cells count (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS) and platelets (PLT) measured as the proportion of total cells (%) and number of cells per μL (K = 1,000; M = 1,000,000). [3]

Sample days relative to infusion (d -1, 1, 3 and 7).

	7	10.96	12.08				
NEU (%)	-1	30.86	22.33	3.47	0.34	0.34	0.28
	1	24.82	19.18				
	3	24.18	13.56				
	7	19.24	24.08				
NEU (K/µL)	-1	3.88	2.72	0.59	0.66	0.23	0.22
	1	3.06	2.58				
	3	2.53	1.42				
	7	1.94	3.19				
LYM (%)	-1	44.24	54.38	4.64	0.70	0.27	0.11
	1	50.12	58.35				
	3	50.98	51.23				
	7	60.80	52.70				
LYM (K/µL)	-1	5.20	6.25	0.43	0.29	0.59	0.51
	1	6.14	7.35				
	3	5.33	6.64				
	7	6.89	6.07				
MON (%)	-1	17.44	16.95	1.88	0.52	0.27	0.37
	1	18.32	14.93				
	3	18.00	26.60				
	7	14.32	17.18				
MON (K/µL)	-1	2.13	1.95	0.20	0.84	0.32	0.47
	1	2.28	2.18				
	3	1.83	1.84				
	7	1.59	2.11				
EOS (%)	-1	7.42	6.30	1.00	0.86	0.13	0.27
	1	6.68	5.50				
	3	6.80	9.18				
	7	5.01	6.03				
EOS (K/µL)	-1	0.91	0.80	0.15	0.85	0.14	0.20
	1	0.81	0.69				
	3	0.70	0.92				
	7	0.53	0.72				
PLT (K/µL)	-1	312.2	241.00	68.98	0.45	< 0.01	0.82
	1	380.00	261.00				
	3	318.20	221.00				
	7	146.20	117.75				

		Treatment			*P*-value[1]		
Variable[2]	Days[3]	Control	Bacteria	SEM			Trt × day
Temp (°C)		38.4	38.3	0.1			0.19
					TRT	Day	
	-[1]				0.08	0.01	

[1] TRT = effect of treatment (control vs. bacteria); Day = effect of day relative to treatment; TRT × day = interaction between TRT and Day.