PREVALENCE OF BACTEREMIA IN DAIRY CATTLE WITH ACUTE PUERPERAL METRITIS AND EVALUATION OF THE DISPOSITION OF AMPICILLIN TRIHYDRATE IN PLASMA, MILK, UTERINE TISSUE, AND LOCHIAL FLUID OF HEALTHY POST-PARTUM DAIRY CATTLE

by

BRENTON CHADWICK CREDILLE

(Under the Direction of Amelia Woolums and David Hurley)

ABSTRACT

The objectives of the studies presented herein were to investigate the prevalence of bacteremia in dairy cattle with acute puerperal metritis (APM), quantify the expression and activity of Indoleamine-2,3-Dioxygenase (IDO) in cattle with APM, and evaluate the pharmacokinetics of ampicillin trihydrate in healthy post-partum dairy cattle. The overarching goal of these studies was to better assess the systemic effects of APM on dairy cattle, particularly the effect of APM on the expression of pro and anti-inflammatory molecules and how frequently animals with APM become bacteremic. Should cattle with APM exhibit and immunosuppressive phenotype and bacteria regularly colonize the bloodstream in affected cattle, systemic antimicrobials could be better justified. In addition, knowledge of antimicrobial susceptibility of bacteria in the bloodstream would open doors to alternative therapeutic approaches and reduces the use of medically important antimicrobials, such as the cephalosporins, on dairy farms.

First, the prevalence of bacteremia in dairy cattle with APM was investigated in cattle with naturally occurring disease. This study showed that bacteremia was common, occurring in 17 of 32 post-partum dairy cattle, both healthy and with APM. Furthermore, cattle with hyperglobulinemia were 4.7 times more likely to develop bacteremia than cattle with lower serum globulin concentrations. In addition, cattle with basophils in the peripheral circulation were approximately 13 times less likely to be bacteremic than cattle in which basophils were not detected.

Second, the expression of pro-inflammatory cytokines, anti-inflammatory cytokines, and IDO mRNA in cattle with APM were evaluated with RT-qPCR. In addition, the activity of IDO in cattle with APM was evaluated with high performance liquid chromatography (HPLC). These studies showed that cattle with APM had significantly lower expression of IL-1β in PBMCs than healthy controls. Bacteremic cattle tended to have higher levels of expression of both IL-4 and IL-6, a finding consistent with a T_H2 polarization.

Lastly, the disposition of ampicillin trihydrate in plasma, milk, endometrial tissue, and lochial fluid using two dosing regimens in healthy post-partum dairy cattle was studied. Intramuscular administration of ampicillin trihydrate did not result in adverse effects in any of the twelve cattle evaluated and showed preferential accumulation in lochial fluid as compared to plasma, milk, and endometrial tissue.

INDEX WORDS: Bovine; Acute puerperal metritis; Pharmacokinetics;

Bacteremia; Indoleamine-2,3-Dioxygenase; Cytokine

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ATHENS, GEORGIA

2014

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DEDICATION

I would like to thank my parents, Cindy and Barry Credille, my wife Allison Credille, and my children, Collins and Brooks Credille, for their undying love and continued support. Mother and Father, you have always pushed to me to work hard and be the best that I can be, no matter the circumstances. Allison, you have been a foundation for me for the last 5 years. You are my best friend and partner in life. To Collins and Brooks, without you none of these accomplishments matter. You two are truly my greatest achievement and make all of this worthwhile.

ACKNOWLEDGEMENTS

I am forever grateful for the mentorship and support I have received from my major professors, Amelia Woolums and David Hurley. To my committee members, Drs. Steeve Giguère, Tom Robertson, and Michael Overton, thank you for all of your support and encouragement.

To my clinical mentors, Michelle Barton, Kelsey Hart, Steeve Giguère, Amelia Woolums, and Brett Tennent-Brown. Thank you for providing me with the skills, knowledge, and support to become an internist and a scientist.

To my resident-mates and friends, Lindsey Helms Boone, Erin McConachie, Lisa Fultz, Heidi Fishman, Jane Woodrow, and Amanda Bergren. Thank you for always supporting me and challenging me to be my best.

To Sarah Breidling and Londa Berghaus for their friendship and laboratory support. Without you, the last four years would not have been possible. Londa deserves a particular thank you for her guidance and patience.

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CHAPTER 1

INTRODUCTION

Acute puerperal metritis (APM) is one of the leading causes of infertility and decreased milk production in dairy cattle. Bacteremia has been documented in cattle with coliform mastitis, but has yet to be investigated in cattle with APM. Systemic antimicrobial agents are a mainstay of therapy for cattle with APM, with the 3rd generation cephalosporin drug ceftiofur being one of the more commonly administered compounds. However, recent restrictions placed on the use of cephalosporins in food-producing species and concerns about antimicrobial resistance in pathogens of human importance have necessitated the investigation of other antimicrobials for managing this disease. The studies presented herein were undertaken to further understand the systemic consequences of APM in dairy cattle and evaluate the disposition of ampicillin trihydrate in reproductive tissues and fluids of post-partum dairy cattle. This dissertation was designed to evaluate the systemic effects of APM on dairy cattle, particularly the effects of APM on cytokine gene expression and the prevalence of bacteremia. In addition, this dissertation sought to determine if ampicillin trihydrate is a functional therapeutic modality for treating cattle with APM.

Chapter 2 contains a review of the literature regarding APM in dairy cattle. Section I reviews the pathophysiology of APM and the immune responses of periparturient dairy cattle, acting both locally and systemically, to help the reader understand the factors related to the development of APM in cattle. Section II provides the reader with the current understanding on

the risk factors of APM in cattle and the effects of APM on milk production, reproductive performance, and culling. Section III describes the medical therapies currently available for cattle with APM and reviews antimicrobial pharmacodynamics. Section IV describes the significance of bacteremia in human and veterinary patients. Section V describes the concept of the compensatory anti-inflammatory response (CARS) in sepsis and the role of Indoleamine-2,3-Dioxygenase (IDO) in CARS in mice and humans. Finally, Section VI discusses the rationale and connections for the studies presented herein

Chapter 3 describes research undertaken to evaluate the prevalence of bacteremia in dairy cattle with APM. The purpose of this study was to document bacteremia in cattle with APM so as to provide an evidence based justification for the use of systemic antimicrobials in this population of cattle. If bacteremia is present then systemic antimicrobials are warranted in cattle with APM. However, if the bacteria isolated from the bloodstream of these animals are highly susceptible to currently available antimicrobial agents, then different therapeutic choices may be more suitable for treating these patients. This would allow us to preserve more medically important antimicrobials for sicker patients and prevent resistance in pathogens of human importance.

Chapter 4 describes research undertaken to evaluate the expression of pro and anti-inflammatory cytokine and IDO mRNA in peripheral blood mononuclear cells (PBMCs) from cattle with APM. In addition, the activity of IDO in serum was assessed using HPLC. Thus, the goals of the study were to evaluate whether cattle with APM exhibited a pro or anti-inflammatory immune bias. Current hypotheses derived from data from bacteremic human patients would suggest that impaired inflammatory responses rather than an over exuberant inflammatory response is responsible for a majority of the mortality seen in these individuals,

however little information is available on this phenomenon in cattle. Additionally, if evidence of an anti-inflammatory cytokine bias was found, the use of systemic antimicrobials could be further justified for use in this population of patients.

Chapter 5 describes research undertaken to investigate the disposition of ampicillin trihydrate in plasma, milk, endometrial tissue, and lochial fluid of healthy post-partum dairy cattle. Currently, ceftiofur, a 3rd generation cephalosporin, is one of the medications commonly used to treat cattle with APM. However, a recent order passed by the United States Food and Drug Administration prohibited the extra-label use of cephalosporins in major food-producing species because of concerns over the development of resistance in pathogens of human importance. Therefore, researching alternative antimicrobial agents is necessary to preserve the efficacy of the cephalosporins in both human and veterinary medicine. Thus, this study was undertaken to ensure that concentrations of ampicillin trihydrate in plasma and lochial fluid were above concentrations thought to be active against pathogens commonly isolated from the bloodstream and uterus of cattle with APM so as to provide an alternative antimicrobial for use in cattle with APM.

Chapter 6 summarizes the findings of the studies presented herein and discusses their clinical relevance. In addition, a discussion of future projects designed to evaluate the influence of bacteria and bacterial products on the local immune responses within the uterus will follow.

CHAPTER 2

LITERATURE REVIEW

SECTION I. INCIDENCE, ECONOMIC IMPACT, AND PATHOPHYSIOLOGY OF ACUTE PUERPERAL METRITIS

Incidence and economic impact of acute puerperal metritis

Dairy cattle are susceptible to numerous metabolic and infectious disorders in the immediate post-partum period. Acute puerperal metritis (APM), clinically defined as the presence of a fetid, watery, reddish-brown uterine discharge, an enlarged, flaccid uterus, and overt signs of systemic illness that may include fever, dehydration, and depression is one of the most commonly encountered infectious peri-parturient diseases in modern dairy practice.[1] Histologically, affected cattle demonstrate leukocytic infiltration of all uterine layers, uterine edema, myometrial degeneration, and mucosal congestion.[1] The median lactational risk for APM in North American dairy cattle has historically been approximately 10%.[2] However, more recent work has shown the incidence to be much higher with most modern dairy farms reporting a range of 18-35%.[3] While the ultimate outcomes of this disease are impaired reproductive performance, reduced milk production, increased culling risk and, in severe cases, death, the most significant consequence of APM is the economic losses absorbed by dairy producers.[4] Current estimates suggest that each case of APM costs producers $350-400.[4] Therefore, in a herd of 1,000 cattle with a median lactation incidence of APM approaching 20%, costs for this one disease alone can

exceed $70,000 per year. When applied to the United States dairy industry as a whole, APM represents a loss of $350,000,000-650,000,000 annually.[3]

Placental separation and uterine involution

Cattle have a placenta characterized by the presence of button-like structures known as cotyledons arising from the surface of the fetal chorionic membrane.[5] In conjunction with caruncular tissue that develops from the uterine tissue of the dam, placentomes are formed and provide nutritional support and waste removal for the developing fetus.[6] Following calving, the placenta and associated fluids are expelled from the reproductive tract. These processes involve the coordinated activity of the endocrine, immune, and muscular systems. Initially, rising plasma estradiol concentrations in the period immediately before calving cause the tissues of the placentomes to absorb fluid, swell, and soften.[6,7] This phenomenon subjects the placentomes to alternating periods of ischemia and hyperemia. During labor, pressures created by the calf and uterine contractions flatten the placentomes and, during the final stages of calf delivery, severing of the umbilical cord leads to tissue ischemia and separation of the cotyledons from the caruncles.[6,8] Generally, the fetal membranes and other tissue remnants are expelled during the 3rd stage of labor, a period of time that lasts 6-8 hours after delivery of the calf.

Traditionally, uterine involution is divided into 3 stages: I, II, and III.[7] Stage I is characterized by intense uterine contractions and vasoconstriction, processes that serve to decrease the physical size of the uterus. Within 24 hours of calving, the uterus will decrease in size by approximately by 50%.[9] During this time, white blood cells (WBCs) begin to accumulate within the uterine lumen, changing the color and consistency of the uterine discharge from a hemorrhagic nature to a thick, mucopurulent character.[9] In addition, under the influence

of estradiol from newly developed follicles, the uterus begins to regain normal muscular tone and the accumulated lochial fluids are expelled.[8]

Stage II of uterine involution is the period of desquamation.[7,9] Beginning 5 days after parturition, the superficial cells of the uterus begin to slough and the remaining tissues of the caruncular stalk begin to shrink. By day 14 post-partum, WBCs have emigrated into the uterine lumen in greater numbers to phagocytose the necrotic tissues and the caruncular stalk is almost completely gone.[9]

Stage III of involution is characterized by re-epithelialization of the endometrial surface, a process that takes place over a period of 1 week to 1 month.[7,9] This process occurs most rapidly in the intercaruncular areas and is slowest in the previously gravid horn.[9] It is during this time that the uterus begins to decrease to its normal size and the remaining lochial fluid becomes clear with only occasional flecks of purulent material.

Microbiology of the post-partum uterus and the relationship between bacteria and development of APM

The process of uterine involution is not sterile and studies have shown that a large proportion of cattle will have bacteria present in the uterine lumen after calving. One study demonstrated that > 93% of all uteri sampled within 2 weeks of calving are culture positive for various bacteria.[10,11] Thus, it is safe to assume that bacteria (pathogenic and non-pathogenic) colonize the uterus of virtually every cow after calving. Following delivery of the calf, the dilated cervix allows bacteria from the environment as well as the skin and feces of the dam to enter the uterus.[6] In the normal animal, the microflora of the uterine lumen fluctuates constantly over the first 7-8 weeks post-calving.[12] During this period, there are alternating periods of

contamination, clearance, and re-contamination. Bacterial colonization of the bovine uterus is generally polymicrobial and commonly isolated bacteria include *Staphylococcus* spp., *Streptococcus* spp., and *Bacillus* spp. and it is believed that these bacteria may represent normal microbial flora.[12] In fact, when these organisms are isolated from the reproductive tract of post-partum cattle, the risk of abnormal vaginal discharge and clinical APM is decreased.[13,14] However, when organisms such as *E. coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, or *Prevotella* spp are present, clinical disease often develops.[6] Similarly, studies from France have shown the proportion of animals culture positive for *Trueperella pyogenes* and Gram (-) anaerobes is significantly greater in animals with clinical disease than in healthy cattle.[15] Williams et al. found that when the aforementioned uterine pathogens were identified in cattle, the character of uterine discharge became more purulent and discharge became more fetid.[13] In addition, cattle with these organisms within the uterine lumen had more significant endometrial lesions and greater disease severity.[13] More recent work from the United Kingdom demonstrated that specific strains of *E. coli* are pathogenic for the endometrium in cattle.[16] These isolates of *E. coli* are typically more adherent and invasive to isolated bovine endometrial epithelial and stromal cells than isolates of *E. coli* obtained from other sites.[16] Moreover, when *E. coli* is isolated from the uterine lumen on day 1 after calving, the proportion of cattle that subsequently culture positive for *Trueperella pyogenes* and Gram (-) anaerobes and develop clinical disease is significantly greater than cattle in which *E. coli* is not isolated.[15] While *E. coli* seems to be quite important in the pathogenesis of APM, it is likely that organisms such as *Trueperella pyogenes* play a larger role in the clinical consequences of APM. Fertility is significantly impaired in cattle with *Trueperella pyogenes* isolated from the uterine lumen prior to 21 days post-partum.[14] It has also been established that *Trueperella pyogenes* secretes a potent pore-forming cytotoxin,

known as pyolysin, that causes endometrial cell death.[17] Furthermore, it is believed that *Trueperella pyogenes* is synergistic with *Fusobacterium necrophorum* and *Peptostreptococcus melaninogenicus*.[18] It is likely that *Trueperella pyogenes* produces a growth factor that allows Gram (-) anaerobes to persist and the Gram (-) anaerobes produce substances that inhibit the activity of leukocytes and further allow *Trueperella pyogenes* to overgrow and damage the uterine tissues.[18]

Role of immune dysfunction in development of acute puerperal metritis – An overview

While almost every post-partum cow will have some degree of uterine bacterial contamination, a much smaller proportion of animals will develop APM. As previously stated, colonization of the uterus with *E. coli* seemingly sets the stage for invasion of the uterus with other pathogenic bacteria. In addition, research would suggest *Trueperella pyogenes, Fusobacterium necrophorum* and *Peptostreptococcus melaninogenicus* have synergistic interactions once in the reproductive tract. Thus, it is clear that bacterial factors have an important role in the development of APM. However, numerous other factors contribute to the development of clinical disease in post-partum cattle. One of the best studied factors contributing to the development of APM is peri-parturient immune dysfunction. The uterus is an immunologically unique organ, as it must maintain normal immune responses to invading bacteria but prevent rejection of and remain tolerant to the developing fetus during pregnancy. It is likely that both local and systemically acting immune responses contribute to this phenomenon.

Systemic regulation of immune responses to the fetal allograft

Regulatory T cells (T_{regs}) are a population of $CD4^+$ T cells that regulate immune responses by suppressing inflammation and autoimmune reactions.[19] These cells are characterized by the expression of both CD4 and CD25 on the cell surface and intracellular expression of FOXP3.[19] In mice, the numbers of T_{regs} in the spleen, blood, and lymph nodes increase during pregnancy.[20] Furthermore, antibody mediated depletion of T_{regs} during pregnancy has been shown to decrease litter size in mice.[20] While comparatively little work has been done to evaluate T_{reg} function in cattle, Oliveira and Hansen demonstrated that the proportion of T cells positive for both CD4 and CD25 is significantly greater in the peripheral blood of pregnant cattle than in non-pregnant cattle.[21] The significance of this finding is still unknown.

Local regulation of immune responses to the fetal allograft

Immune cells are present in the uterus during both the normal estrous cycle and pregnancy. Vander Wielen and King showed that the number of lymphocytes in the uterus remain relatively unchanged through the estrous cycle of non-pregnant cattle.[22] However, in pregnant cattle, the number of lymphocytes in the uterine epithelium is significantly reduced.[22] Cobb and Watson found that cells positive for MHC class II were widely distributed through the non-pregnant uterus in cattle, with the largest populations being found in the stratum compactum.[23] They also found that lymphocytes could be found throughout the endometrium, with the largest population of $CD4^+$ cells found within the stratum spongiosum and the largest population of $CD8^+$ cells within the glandular and luminal epithelium.[23] Oliveira and Hansen demonstrated that macrophages are abundant within the endometrial stroma of pregnant cattle.[24]

Furthermore, some of these macrophages exhibit an M2 phenotype based on expression of genes associated with M2 macrophages in humans.[24] M2, or alternatively activated macrophages, are a population of macrophages characterized by their anti-inflammatory properties. Differentiation of macrophages to the M2 phenotype is stimulated by cytokines such as IL-4 and IL-13.[25] M2 macrophages have been implicated in tissue repair, malignancy, metabolism, and pregnancy.[26] Indeed, M2 macrophages have been shown to enhance clearance of apoptotic trophoblastic cells to reduce inflammation and promote allograft survival.[27] Thus, it is possible that the increased numbers of M2 macrophages seen in the uterus of pregnant cattle serve to promote survival of the fetal allograft by dampening inflammation.

Hormonal regulation of immune responses to the fetal allograft

Many authors have long believed that progesterone is a potent immunosuppressant.[28] During pregnancy, concentrations of progesterone are quite high and this finding has led some authors to believe that progesterone may play a role in tolerance to the fetal allograft.[5] Indeed, studies have shown that progesterone prevents or delays the rejection of tissue graft responses in the rat.[29] Similarly, studies in sheep have shown that progesterone prolongs the survival of tissue xenografts in the uterus.[30] Progesterone treatment has been shown to decrease the numbers of lymphocytes within the endometrium of sheep, an effect mediated by a secretory product known as SERPIN14A.[31] SERPIN14A has been shown to block T cell proliferative responses, impair NK cell activity, and reduce antibody production in mice and sheep.[31] In addition to its effects on immune function, progesterone has an effect on antimicrobial responses in the uterus and can impair the elimination of bacteria experimentally introduced into the uterine lumen of gilts.[32] Studies in mice have shown that progesterone limits the expression of co-stimulatory molecules

and impairs the secretion of cytokines by uterine dendritic cells, factors that potentially impair the initiation and maintenance of immune responses to pathogenic microorganisms.[33]

Pathogen recognition and uterine immune responses

The mammalian immune system is comprised of two branches: innate and adaptive.[34] The innate immune system is the first line of host defense against pathogens and the effectors for the innate immune system are phagocytic cells (dendritic cells, macrophages, neutrophils), epithelial barriers, antimicrobial peptides, and the complement system.[34] The innate immune system is evolutionarily ancient and, in fact, various aspects of innate immune responses can be found in almost all animals and plants.[34] Central to the function of the innate immune system is pathogen recognition and differentiation of "danger" from normal physiologic events.[34] Innate immune responses generally lack the specificity of adaptive immune responses; however, this branch of the immune system can discriminate between healthy host tissues, microorganisms, and damaged tissue. Indeed, the innate immune system recognizes microorganisms and damaged cells via germ-line encoded pattern recognition receptors (PRRs) expressed on the surface and within the cytosol of numerous cells types.[35] The sensing of danger associated molecular patterns (DAMPs) by PRRs upregulates the expression of genes involved in inflammation. With upregulation of these genes comes increased activity of pro and anti-inflammatory cytokines, chemokines, antimicrobial proteins, and various interferons.[35] PRRs are characterized by their ability to recognize conserved molecular structures or metabolic products unique to microorganisms.[35] These microbial and tissue components are often referred to as DAMPs. In addition, these PRRs are expressed constitutively in the host and are not altered with stage of cell cycle. Finally, different PRRs recognized different DAMPs, have distinct patterns

of expression, and activate many signaling pathways. Generally, PRRs are divided into 4 families:[35]

1. Toll-like receptors (TLRs)
2. C-type lectins (CLRs)
3. RIG-I-like helicases (RLRs)
4. NOD-like receptors (NLRs)

The TLRs are perhaps the best-studied members of the PRR family. To date, 10 TLRs have been identified in cattle.[36] Different TLRs recognize different DAMPs expressed or produced by microorganisms or damaged cells (Table 1.1). Recognition of DAMPs by the TLRs results in upregulation of various genes regulating the expression of pro and anti-inflammatory cytokines.[35]

The RLR family is composed of RIG-I, MDA5, and LGP2. These PRRs are localized to the cytoplasm and primarily recognize the RNA of double stranded RNA viruses (Table 1.1). In addition, certain members of the RLR family can recognize double stranded RNA produced during the replication of single stranded RNA viruses.[37]

The NLR family of PRRs recognizes cytoplasmic pathogens. NOD1 and NOD 2, prototypical members of the NLR family, recognize peptidoglycan and other microbial products. In some cases, the TLRs and NLRs can act synergistically, particularly when the DAMP they recognize is similar.[37]

The CLRs are a family of membrane-bound receptors with a carbohydrate-binding domain. These receptors recognize carbohydrates on all classes of microorganisms. Generally CLRs

stimulate the expression of pro-inflammatory cytokine genes or inhibit TLR mediated immune responses.[37]

Activation of almost any of the PRRs results in the translocation of transcription factors such as NF-kB and AP-1 to the nucleus of mammalian cells.[34,37] Stimulation of PRRs results in the upregulation of expression of hundreds of genes. In addition to activation of genes encoding for cytokines, genes for antimicrobial peptides, cellular metabolism, and tissue repair are upregulated.[37]

The most important and best-described PRRs as it relates to the bovine uterus comprise the family of TLRs.[36] In normal, non-pregnant cattle endometrial cells express TLRs 1-10. In the post-partum uterus, TLRs 2, 3, 4, 6, and 9 are expressed in the greatest abundance with the greatest level of expression found in the caruncular endometrium.[36] Most, if not all, of the TLRs expressed by the bovine endometrium are functional, as endometrial cells have been found to secrete prostaglandin E_2 in response to various PAMPs.[36] In addition to the TLRs the cells of the bovine uterus express various antimicrobial peptides (AMP).[36] In particular, endometrial tissue has been found to express lingual AMP, tracheal AMP, and the β-defensins.[36]

Periparturient immunosuppression – Causes and consequences

It is well known that a majority of infectious diseases affecting dairy cattle occur within the first 60 days post-partum.[38] In fact, the risk of mastitis is greatest during the first 2-8 weeks of lactation with 25% and 60% of all cases occurring during this time period, respectively.[38] The process of parturition is associated with numerous physiologic changes. Many of these changes are associated with the onset of milk production and primarily include hypocalcemia and negative energy balance.[38] Along with these physiologic changes come alterations in normal

immune function. Kehrli, Nonnecke, and Roth performed some of the earliest studies evaluating immune function in the periparturient period.[39,40] In one study, investigations into the function of neutrophils during the weeks prior to and after parturition revealed significant reductions in random migration, phagocytosis, superoxide production, chemiluminesence, and iodination.[39] In a second study, lymphocyte responses to chemical mitogens was investigated and revealed significant impairment of lymphocyte blastogenic activity in response to phytohemagglutnin and concavalin-A.[40] Tan et al found that periparturient cattle have significantly lower expression of NOD-1 mRNA and NOD-1 protein in neutrophils than cattle in mid-lactation.[41] In addition, NOD-1 deficient neutrophils have reduced phagocytosis and ROS production compared to controls.[41] Seminal work by Kimura et al demonstrated significant declines in all T cell subsets in the days and weeks prior to calving.[42] They also demonstrated that the various T cell subsets do not return to normal until approximately 2 weeks after calving.[42] This same group also found that, if the mammary gland is surgically removed, the changes in lymphocyte subsets noted previously no longer occur.[43]

From these studies it appears that, during the immediate period before and after calving, significant changes in immune function occur and these changes are likely being driven by the stresses of lactation. Because cortisol concentrations are quite high after calving, most early investigations focused on the influence of cortisol on immune function. Burton et al discovered that dexamethasone and cortisol both down-regulate the expression of L-selectin on bovine neutrophils.[44] Konig et al found that while L-selectin is down regulated on neutrophils, migration into the uterus is not affected.[45] However, dexamethasone downregulates the production of reactive oxygen species (ROS) generated by neutrophils once in the uterus.[45] Madsen et al evaluated gene expression in neutrophils isolated from periparturient dairy cattle

and found that, when compared to cattle at other stages of production, periparturient cattle have lower expression of genes encoding cytochrome b, and rig/ribosomal protein S15, two genes essential for respiratory metabolism.[46] In addition, the expression of these two genes is correlated to serum progesterone concentrations.[46] Gilbert et al evaluated the effect of parity on neutrophil function and found that cows in their 4th or greater lactation have much greater impairment of neutrophil function than younger cattle.[47] Thus, it appears that hormonal changes occurring around the time of parturition, namely increased cortisol and estrogen and decreased progesterone, are driving certain changes in immune function. However, more recently, the influence of hypocalcemia and negative energy balance has also been studied. Indeed, Kimura et al found that hypocalcemia is associated with decreased intracellular concentrations of calcium within mononuclear cells.[48] In addition, the response of these cells to various stimuli is impaired greatly when compared to normocalcemic controls.[48] Ster et al evaluated the effect of serum NEFAs on functionality of immune cells and found that NEFAs significantly impair both lymphocyte proliferation and oxidative burst capacity in neutrophils.[49] Both Galvao and Hammon have studied the effect of NEFAs on neutrophils function and found that animals with uterine health disorders have much greater serum NEFAs, lower prepartum dry matter intake (DMI), and greater impairments in neutrophil activity when compared to healthy control cattle.[50,51] Galvao also noted that cattle with uterine health disorders have lower neutrophil glycogen content, a factor that may reduce cellular energy stores and contribute to impaired activity.[50] Contreras studied the effect of NEFAs on inflammatory responses in bovine endothelial cells and found that excessive concentrations of NEFAs increase the expression of adhesion molecules and cytokine mRNA.[52] Furthermore, the activity of cyclooxygenase 2 (COX2) is increased by NEFAs in a concentration dependent manner.[52] This same group

evaluated the expression of glucose transporters in monocytes of periparturient cattle and found significantly decreased expression of GLUT1, GLUT3, and GLUT4 in all animals studied.[53] In addition, this study found that both lipopolysaccharide (LPS) and TNF-α increase the expression of these same transporters.[53]

SECTION II. RISK FACTORS FOR ACUTE PUERPERAL METRITIS AND EFFECTS OF ACUTE PUERPERAL METRITIS ON DAIRY PRODUCTION

Risk factors for acute puerperal metritis

Significant factors contributing to APM include subclinical hypocalcemia and negative energy balance. Martinez et al found that the population risk for APM due to subclinical hypocalcemia is 91%.[54] Work by Guiliodori et al found that cattle experiencing dystocia, retained fetal membranes, and elevated pre-partum non-esterified fatty acid (NEFA) concentrations are 2.58 times more likely to develop APM than cattle without these issues.[55] Studies performed in Canada by Dubuc et al yielded similar results and found that cows with elevated pre-partum NEFA levels, cows that experienced dystocia, and cows with placental retention are more likely to develop APM.[56] From this work and work done by others, it appears that APM and other infectious disorders occurring in the immediate post-parturient period are mediated by a number of different factors. Of these, the metabolic status of the cow in the period prior to parturition seems to play the most significant role. Indeed, hypocalcemia and elevations in plasma NEFA concentration suppress the immune system and contribute to placental retention and impaired bacterial clearance.[48,50,51] Therefore, Huzzey et al evaluated the role of prepartum dry matter intake as a risk factor for APM. They found that cows that went on to develop APM consume less feed beginning 2 weeks prior to parturition than healthy cattle.[57] In this study, for

every 10-minute decrease in average daily feeding time, the odds of a cow developing APM increased by 1.72.[57] In addition, for every 1 kg decrease in dry matter feed intake, the odds of a cow developing APM increased 3 times.[57] Cows later diagnosed with APM also had fewer aggressive interactions at the feed bunk than cattle that remained healthy.[57] Clearly, the development of APM is preceded by changes occurring weeks before the disease is diagnosed. Most of these changes seem to result from social interactions and alterations in feed intake that lead to negative energy balance and disruptions in calcium homeostasis.

Effects of acute puerperal metritis on dairy cattle production

The goals of reproductive management within a dairy herd are to ensure cows are inseminated and conceive within a timely manner after calving.[4] Improved reproductive performance will lead to more revenue from increased milk production due to cows averaging fewer days in milk, increased revenues from the sale of excess calves, and increased value of cull cows.[4] While APM can certainly contribute to mortality on dairy farms, the mortality risk for affected cattle is typically less than 2%.[58] Thus, it's clear that the economic losses resulting from APM come from some other source. Indeed, cattle diagnosed with APM typically have more days open, a lower pregnancy rate, and lower milk production.[4,59] Overton and Fetrow developed an economic model to evaluate the costs of APM within a single dairy herd in California.[4] In this herd, cattle with APM were open on average 16 days longer and had a pregnancy rate 3-6 percentage points lower than that in healthy cattle.[4] In addition, culling risk for cattle with APM was 4.2% greater than the culling risk in other groups.[4] These same animals produced almost 5 lbs less milk per day over the first 120 days of lactation.[4] Dubuc et al demonstrated that cattle affected by APM produce 3.7 kg less milk at first test and 259 kg less milk over the course of a single lactation than unaffected herdmates.[59] Interestingly, cattle

diagnosed with both placental retention and APM produce over 1,000 kg less milk than healthy herdmates, suggesting an additive effect of the two disorders.[59] This same study also showed that cattle with APM have a first service pregnancy risk of 27.2% as compared to 35.3% in unaffected cattle.[59] In contrast to the work done by Overton and Fetrow, however, Dubuc found no relationship between APM and culling risk.[59] Giuliodori et al studied the effects of APM on milk production, reproductive performance and the efficacy of ceftiofur therapy in Argentinian cattle.[55] Their data showed that cattle with diagnosed with APM produce less milk (2,367 kg vs 2,647 kg) by 90 days in milk (DIM) and have a lower risk for pregnancy by 100 DIM than unaffected herdmates (adjusted odds ratio = 0.19).[55] Therapy with ceftiofur was associated with an increased pregnancy risk at insemination (adjusted odds ratio = 2.7) but not risk of clinical cure or milk yield.[55] Ribeiro et al evaluated the effects of APM on fertility of grazing dairy cattle.[60] In this study, approximately 5% of cattle were affected with APM and these animals had significantly lower pregnancy per artificial insemination (P/AI) than healthy herdmates (52.4% vs 63.9%).[60] In addition, cattle with APM were less likely to be cycling by 49 days post-partum.[60]

Mechanisms of decreased fertility in cattle with APM – Effects on ovarian function, oocyte development, and ovulation

It is well established that cattle with APM experience decreased fertility when compared to cattle not diagnosed with the disorder. The mechanisms for this are quite complex and still under intense investigation. From the work that has been done, it is likely that the causes of infertility are multifaceted and involve effects acting both locally and systemically. For example, cows with APM have slower growth of dominant follicles, reduced risk of ovulation, and lower blood concentrations of both estradiol and progesterone compared to healthy cattle.[61]

Furthermore, in cattle that do ovulate, the corpora lutea formed after ovulation are typically smaller and secrete less progesterone, a factor that may impair embryonic survival should fertilization occur.[62] Infusion of LPS into the systemic circulation reduces the secretion of GnRH from the hypothalamus and the secretin of LH from the pituitary gland, factors that may be responsible for the reduced risk of ovulation in cattle with APM.[61] Bromfield and Sheldon showed that, when bovine ovarian cortex cells are exposed to LPS, the primordial follicle pool is significantly reduced and this reduction in primordial follicles is associated with increased follicle atresia.[63] In addition, the effects of LPS were TLR4 dependent, as TLR4-deficient mice were unaffected by administration of LPS.[63] In a separate study, Bromfield and Sheldon demonstrated that the reduction in primordial follicle cells is likely due to arrested meiotic progression, a factor shown to be clearly related to LPS and the accumulation of inflammatory mediators within the granulosa cell.[64] Williams et al exposed bovine ovarian theca and granulosa cells to both LPS and TNF-α and evaluated steroid secretion *in vitro* and risk of ovulation *in vivo*.[62] They found that LPS significantly reduces granulosa cells estradiol secretion and TNF-α decreases the production of androstenedione and estradiol by both theca and granulosa cells.[62] In addition, fewer animals ovulated following intrauterine infusion of either LPS or TNF-α.[62] Further investigations into this phenomenon by this same group showed that the reduced secretion of estradiol by granulosa cells is primarily mediated by a reduction in aromatase activity and not cell death.[65]

Mechanisms of decreased fertility in cattle with APM – Embryo development, implantation, and pregnancy loss

In spite of the detrimental effects of APM on ovarian function, some affected animals do ovulate and, under certain circumstances, the ovulated oocyte can be fertilized. However, cattle

with APM show increased rates of embryonic death and pregnancy loss when compared to healthy controls. For example, when exposed to LPS prior to fertilization *in vitro*, bovine oocytes are less likely to develop to the blastocyst stage.[66] When co-cultured with neutrophils, *in vitro* produced bovine embryos display few, if any, developmental issues. However, when embryos are exposed to a cell-free uterine lavage solution obtained from cattle with experimentally induced endometritis, development is significantly impaired.[66] Thus, it is likely that cytokines and other inflammatory mediators cause the adverse effects on the developing embryos. When evaluated more closely, embryos obtained from animals with uterine inflammation have fewer trophoectoderm cells and, because trophoectoderm cells are the source of interferon tau, the signal for the establishment of pregnancy is lost.[66]

SECTION III. MEDICAL THERAPIES AVAILABLE FOR MANAGING ACUTE PUEPERAL METRITIS AND THE RELATIONSHIP BETWEEN ANTIMICROBIAL PHARMACODYNAMICS AND THERAPEUTIC SUCCESS

Medical therapies used in managing acute puerperal metritis

Systemic and intrauterine antimicrobials are the mainstay of therapy for cattle with APM. Currently, three antimicrobials are labeled by the United States Food and Drug Administration (FDA) for systemic use in cattle with APM. Oxytetracycline dihydrate, sold under the trade name Liquamycin LA 200®, is the oldest and least expensive of the available compounds still in use. It is labeled for therapy of acute metritis caused by susceptible strains of *Staphylococcus* spp. and *Streptococcus* spp. at a dose of 11 mg/kg once daily. However, because of the prolonged withdrawals (28 days meat and 4 days milk) and lack of data demonstrating efficacy in clinical trials, this product has fallen out of favor. Intrauterine infusions of tetracycline-based

products have been closely evaluated in recent years. For example, Goshen and Shpigel evaluated the effect of infusion of 5 grams of chlortetracycline into the uterus twice weekly for 2 weeks.[67] Cattle receiving this treatment produced 1,438 lbs more milk over the course of lactation and conceived 29 days sooner than untreated controls.[67] Furthermore, conception risk in treated cattle was 42.5% compared to 38.3% in clinically normal animals and 18% in untreated controls.[67] In addition, at current market prices, treated cattle would yield a net return to the producer of approximately $74.70/head, despite the prolonged milk withdrawal required with this therapeutic regimen (> 21 days).[68] Nevertheless, this treatment modality is not approved in the United States, making this protocol impractical for routine clinical use.

Ceftiofur, a 3rd generation cephalosporin, has become the gold-standard therapeutic agent for cattle with APM. Two preparations of ceftiofur, ceftiofur hydrochloride (Excenel®) and ceftiofur crystalline free acid (Excede®), are labeled for use in cattle with APM and have the advantages of short slaughter (3 and 13 days, respectively) and no milk withdrawal. In addition, the effects of ceftiofur on clinical cure and, to some extent, reproductive performance, have been thoroughly evaluated. Work by Chenault et al demonstrated that ceftiofur hydrochloride, when given at a dose of 2.2 mg/kg IM once daily for 5 days, is effective in reducing rectal temperature and improving the character of uterine discharge in cattle with APM.[69] Drillich et al compared the efficacy and economic efficiency of treating cattle diagnosed with APM with either ceftiofur hydrochloride or a combination of intrauterine and systemic antimicrobials.[70] Cattle treated with ceftiofur had rates of clinical cure and reproductive performance similar to cattle in the other groups.[70] Furthermore, economic analysis revealed that costs for the group treated with only ceftiofur were less, primarily as a result of reduced milk withholding periods.[70] Schmitt et al compared the clinical efficacy of ceftiofur hydrochloride to oxytetracycline in cattle with APM.[71]

Risk of clinical cure at day 7 was numerically higher in the ceftiofur group (64.8%) when compared to oxytetracycline treated cattle (58.1%), however the results were not statistically significant.[71] Between days 2-5 after treatment, ceftiofur treated cattle had significantly lower rectal temperatures than cattle treated with oxytetracycline.[71] Unfortunately, comparisons of milk production and reproductive performance between groups were not evaluated in this study. Ceftiofur crystalline free acid (CCFA) is labeled for treating APM and is given SQ at the base of ear at a dose of 6.6 mg/kg every 72 hours for two doses. McLaughlin et al evaluated the effect of two doses of CCFA on clinical cure risk in cows with APM and found that, when compared to a saline treated control, cows treated with CCFA are more likely to cure (74.3% vs 55.3%, respectively).[72] Giuliodori et al evaluated the effects of CCFA on clinical cure risk, milk yield, and reproductive performance and found that CCFA had no influence on clinical cure or milk yield but did increase risk of pregnancy at insemination (adjusted odd ratio = 2.688).[73]

Antimicrobial pharmacodynamics – Relationship to therapeutic success

For any disease caused or mediated by an infectious agent, the efficacy of antimicrobial therapy is dependent upon three factors:[74]

1. Susceptibility of the pathogen to the chosen antimicrobial
2. Characteristics of drug exposure necessary for optimum response
3. Concentrations of free drug at the site of infection.

This relationship, the interaction of systemic drug exposure and corresponding clinical effects, is termed the pharmacokinetic/pharmacodynamic relationship (PK/PD).[74] Here, pharmacokinetics is best defined as the handling of the drug by the host (i.e. what the body does to the drug) while pharmacodynamics is defined as the effect of drug on microorganisms over time (i.e. what the

drug does to the bug). It is the pharamacodynamic relationship between a specific antimicrobial and disease-causing microorganism that is the focus of this discussion.

Optimal dosing of antimicrobial agents is dependent on both the pharmacokinetic and pharmacodynamics properties of a drug. Currently, the most widely utilized pharamacokinetic input is plasma drug concentration and the minimum inhibitory concentration (MIC) the primary pharmacodynamic input.[75] However, more researchers and clinicians have begun to realize the limitations of the MIC and now utilize the mutant prevention concentration (MPC) as the pharmacodynamic parameter of interest.[76]

Bacterial antimicrobial susceptibility is determined *in vitro* using one of several available tests. Disk diffusion, concentration-gradient agar dilution, and broth dilution (macro or micro) have all been used to evaluate susceptibility to antimicrobials.[75] Disk diffusion provides mostly qualitative information (susceptible, intermediate, resistant) while both the broth dilution and concentration-gradient agar diffusion tests provide qualitative (MIC) data. With these tests, the MIC is defined as the lowest concentration of antimicrobial that inhibits the growth of target bacteria. It is important to note that inhibition of bacterial growth rather than bacterial killing is the primary endpoint. The designation of a microorganism as susceptible or resistant is determined by comparing the organism's MIC to breakpoints established by the Clinical Laboratory Standards Institute (CLSI). Breakpoints, defined as the concentration above and below which specific bacterial isolates are characterized as susceptible, intermediate, or resistant, are determined by 3 criteria:[75]

1. Range of in vitro MICs of an antimicrobial for a representative population of specific bacterial pathogens

2. PK/PD parameters established on the basis of the relationship between drug concentrations and microbial susceptibility
3. Results of clinical trials in the target species

When these *in vitro* susceptibility tests are presented to the clinician or researcher, a pathogen will be designated as susceptible, intermediate, or resistant, designations defined as:[75]

- Susceptible: An infection caused by the specific isolate may be successfully treated with the recommended dosing regimen of an antimicrobial agent approved for that disease process and infecting microorganism
- Intermediate: An infection by the isolate can be treated at body sites where drugs are physiologically concentrated or when a high dosage can be used
- Resistant: An infecting isolate is not inhibited by typically achievable concentrations of a specific drug with a standard dosing regimen

It is important to note that clinical breakpoints are only relevant for specific bacteria, a specific drug, and a specific organ system. Thus, breakpoints established for ceftiofur against *Mannheimia haemolytica* in the respiratory tract are irrelevant when that organism is the cause of disease within another body system (mammary gland, uterus). In cattle, few antimicrobials have breakpoints established for specific diseases and, unfortunately, no antimicrobials have valid breakpoints established for APM. Generally, when species specific breakpoints are not available for a disease condition, breakpoints are adapted from humans or other domestic animal species. Therefore, the data obtained from these susceptibility tests must be interpreted with caution. In these situations, knowledge of an infecting organism's MIC combined with pharmacokinetic data

ideally describing the concentration of drug within the tissue of interest can assist in predicting efficacy.[75]

When evaluating antimicrobials, both the pharmacokinetic and pharmacodynamics properties of the drug of interest must be known to establish optimum doses and dosing intervals. Again, pharmacokinetics describes what the body does to the drug (absorption, distribution, metabolism, elimination) while pharmacodynamics addresses the relationship between drug concentration and antimicrobial activity. The most important parameter determining the efficacy of drugs within the β-lactam, tetracycline, and macrolide classes of antimicrobials is the time that the active drug concentration remains above the MIC of the infecting pathogen (T>MIC).[75] With these drugs increasing drug concentration more than 4-fold above the MIC will not alter the rate of microbial killing. With these drugs, it is the length of time that bacteria are exposed to concentrations above the MIC that determines efficacy (Table 1.2).[75] Antimicrobials such as the aminoglycosides and fluoroquinolones are classified as concentration-dependent and their rate of bacterial killing increases as the plasma concentration increases (Cmax/MIC) (Table 1.2). With these drugs maintaining concentrations above the MIC between doses is unnecessary and, in some cases, can be detrimental.[75] Finally, there are certain drugs that have characteristics of both time and concentration dependent drugs. For drugs such as rifampin, glycopeptides, certain macrolides, and some fluoroquinolones, the primary determinant of efficacy is the 24-hour plasma area under the curve (AUC) to MIC ratio (AUC_{0-24}/MIC) (Table 1.2).[75]

As previously stated, most infections occur in the tissues rather than plasma. Thus, it is logical that an antimicrobial reach the site of infection to be effective. The ability of a specific drug to penetrate extravascular sites is dependent on 4 factors:[77]

1. Extent of plasma and tissue protein binding
2. Molecular size
3. Lipid solubility
4. Blood flow at the site of infection.

In addition, certain sites in the body (central nervous system, prostate, eye) are further restricted by the presence of tight junctions between cells, a factor that further excludes active drug from tissues. For uterine infections caused by extracellular bacteria such as *E. coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella* spp, concentrations of antimicrobial within lochial fluid is likely a better determinant of efficacy than concentrations of drug in plasma or endometrial tissue.[78] Despite lacking CLSI breakpoints for the pathogens commonly associated with APM in cattle, numerous pharmacokinetic studies have evaluated the disposition of oxytetracyline, ceftiofur hydrochloride, and CCFA in plasma, uterine tissue, lochial fluid, and cotyledonary tissue of both clinically normal cattle and cattle with APM. In addition, several published studies have evaluated MICs of common uterine pathogens.[79-84]

Bretzlaff et al. performed 3 separate studies evaluating the disposition of oxytetracyline hydrochloride (OTC) in plasma, uterine tissue, and lochial fluid of healthy and diseased cattle. In the first study the disposition of OTC in the uterine tract of cattle the drug by two different routes (intramuscularly (IM) vs intrauterine (IU)) was investigated.[79] When given IM, OTC concentrations in endometrial tissue (0.43 µg/g) were numerically higher than OTC concentrations in plasma (0.05 µg/ml) 72 hours after dosing.[79] Concentrations of OTC in uterine secretions were sampled at 48 hours after administration and, similar to endometrial tissue, were numerically higher than plasma (0.57 g/ml vs 0.34 µg/ml, respectively).[79] IU administration of OTC led to high concentrations in endometrial tissue (> 4 µg/g) but no detectable levels of OTC

in plasma 72 hours after administration.[79] The second study evaluated the disposition of OTC in genital tissues of healthy post-partum cattle when the drug was given intravenously (IV) or IU. Cattle were given OTC a dose of 11 mg/kg as a constant IV infusion or at a dose of 5.5 mg/kg as a single IU infusion.[80] Similar to the previous study, IU infusion of OTC led to high concentrations in endometrial tissue (> 5 µg/g) at all sampling times.[80] However, concentrations of OTC in plasma, uterine wall, and ovarian tissue of all cattle was low, with the mean concentrations of OTC in these tissues of cows with metritis lower than that of healthy cattle.[80] Computer modeling demonstrated that, when OTC is given IV at a dose of 11 mg/kg twice daily, concentrations of drug in uterine tissues remain above 5 µg/g for the duration of the dosing interval.[80] In the final study, the disposition of OTC in uterine tissues and fluids of healthy and diseased post-partum cattle was investigated using an IV dosing strategy similar to the one used in the second study.[81] Concentrations of OTC in plasma and uterine tissue of healthy and diseased cattle were: plasma (4.95 and 5.23 µg/ml, respectively), uterine tissue (3.65 and 4.18, respectively), and ovarian tissue (4.57 and 4.53 µg/g).[81] Mean plasma to genital ratio of OTC in healthy and diseased cattle was 1.38 and 1.32, respectively.[81] Mean plasma to genital ratio of OTC in uterine tissue of healthy and diseased cattle was 1.38 and 1.31, respectively.[81] Thus, from these studies, it can be seen that systemic administration of OTC at a dose of 11 mg/kg given IV twice daily can achieve concentrations > 5 µg/ml in plasma and > 4 µg/g in uterine tissue of cattle with uterine disease. In addition, concentrations of OTC in tissues of animals were similar to concentration of OTC in plasma, suggesting distribution of OTC to the tissues from plasma.[79-81] Unfortunately, several studies have shown that resistance to OTC amongst bacteria involved in the development of APM is widespread. Malinowski et al found that approximately 63.7% of all *Trueperella pyogenes* isolates and 31% of all *E. coli* isolates

obtained from the uteri of cattle with APM are resistant to OTC.[85] Santos et al demonstrated OTC resistance in 53.7% of all *Trueperella pyogenes* isolated from clinical cases of APM.[86] In addition, Sheldon showed that the MIC_{90} for OTC against *E. coli* and *Trueperella pyogenes* is 32 µg/ml and 16 µg/ml, respectively, concentrations far above what can be achieved in any fluid or tissue at currently labeled dosing regimens.[87] Again, the tetracycline class of antimicrobials is generally considered to be time dependent and, because of that, the primary determinant of efficacy for this class of drugs would be the duration of time that free drugs concentration remains above the MIC of the infecting pathogen (T>MIC). Thus, based on the data presented, it is unlikely that OTC would be an effective therapy for cattle with APM as concentration of OTC in most fluids and tissues fail to reach the MIC_{90} of common uterine pathogens at both label and extralabel dosing regimens.

In addition to OTC, the pharmacokinetics and disposition of both ceftiofur hydrochloride and CCFA in plasma, uterine tissue, and lochial fluid have been evaluated in healthy and clinically ill post-partum cattle. Okker et al evaluated the disposition of ceftiofur hydrochloride in plasma, uterine tissue, and uterine secretions of lactating dairy cattle.[82] Ceftiofur hydrochloride was given at a dose of 1mg/kg SQ to healthy Holstein cattle within 24 hours of calving. Maximum plasma concentrations were 2.85 µg/ml 2 hours after administration.[82] In lochial fluid, maximum ceftiofur concentrations were 0.97 µg/ml 4 hours and declined to 0.22 µg/ml 24 hours after administration.[82] In uterine tissue, maximum ceftiofur concentrations were 2.23 µg/g and declined to 0.56 µg/g 24 hours after administration.[82] Drillich et al determined the concentrations of ceftiofur and its derivatives in serum, uterine tissue, cotyledonary tissue, and lochial fluid of cattle following fetal membrane retention.[83] In the aforementioned study, cattle were given ceftiofur hydrochloride at a dose of 1mg/kg SQ once daily for 3 consecutive days.

This group was able to demonstrate that mean concentrations of ceftiofur derivatives in each fluid and tissue remained greater than the MIC_{90} for common uterine pathogens reported by Sheldon et al.[83,87] Nevertheless, at each time, single samples were below the MIC_{90} of 0.5 µg/ml reported by Sheldon.[83] Witte et al evaluated the disposition of CCFA in serum, endometrial tissue, and lochial fluid of healthy post-partum cows after SQ administration of CCFA at a dose of 6.6 mg/kg to healthy post-partum cattle.[84] Five days following drug administration, concentrations of ceftiofur derivatives in plasma, (1.21 µg/ml), endometrial tissue (0.86 µg/g), and lochial fluid (0.96 ug/ml) were above the MIC_{90} for common uterine pathogens.[84] However, concentrations of ceftiofur derivatives in lochial fluid were quite variable and, at points beyond 72 hours following drug administration, numerous animals had concentrations well below the MIC_{90} of *E. coli.*[84] Therefore, from this study, it appears that administration of CCFA every 72 hours would be required to maintain concentrations above the MIC_{90} of common uterine pathogens in lochial fluid for the entirety of the dosing interval.

Currently, the use of numerous antibiotics is restricted in food animal veterinary patients due to the potential for the development of resistance in pathogens of human importance and the risk of adverse health events in humans consuming tainted products. In 2006, Tragesser et al found a herd-level association between the use of ceftiofur and the isolation of *E. coli* with reduced susceptibility to ceftriaxone.[88] In this study, herds in which ceftiofur was used were more likely to have *E. coli* with reduced ceftriaxone susceptibility (odds ratio = 25).[88] In April 2012, the United States Food and Drug Administration (FDA) passed an order restricting the extralabel use of cephalosporins in major food-producing species because of concern over the development of cross-resistance to ceftriaxone, a mainstay of therapy in adolescents with salmonellosis. In addition, the CDC recognized extended spectrum β-lactamase producing

bacteria as a major threat to public health. Recently, investigators identified *E. coli* containing the blaCMY-2 genetic element, a gene that encodes for ceftiofur resistance, from the feces of 38.1% of dairy cattle from farms where ceftiofur was frequently used.[89] Also, 13.0% of *Salmonella* spp. isolated from fecal samples of the same dairy cattle from these farms were positive for blaCMY-2.[89] Boyer and Singer evaluated the quantity of blaCMY-2 in dairy cattle treated with ceftiofur.[90] Significantly higher quantities of the blaCMY-2 gene were found in the feces of treated cattle. However, by day 1 post-treatment, levels of blaCMY-2 had decreased to pre-treatment levels.[90] Studies from feedlot cattle given CCFA at arrival have shown that administration of CCFA is associated with a transient increase in antimicrobial resistance in fecal coliforms.[91] In addition, resistance to ceftiofur was associated with cross-resistance to ampicillin, chloramphenicol, and tetracycline.[91] While most studies have focused on antimicrobial resistance in fecal coliforms in cattle given ceftiofur, some studies have shown that urine may be a more important contributor to antimicrobial resistance.[92] Ceftiofur and its metabolites are excreted primarily in urine (>75%). When cattle treated with ceftiofur urinate, the drug remains in soil for up to 3 weeks, particularly at cooler temperatures.[92] Ceftiofur-resistant *E. coli* in soil, when exposed to ceftiofur-containing urine, have an apparent survival advantage over other organisms, and persist for long periods of time (>2 months).[92] In addition, the resistant *E. coli* found in soil readily colonizes calves and other age groups of cattle by contact bedding, feed, and other fomites.[92] From this data it appears that antimicrobial resistant strains of bacteria of medical importance can be selected for by the use of antimicrobials on farms. Moreover, these bacteria may persist in the environment for prolonged periods of time, putting animals and humans at risk for colonization or infection. Therefore, we clearly need to evaluate antimicrobials with less importance in human health for use in cattle with APM.

SECTION IV. BACTEREMIA – SIGNIFICANCE AND CLINICAL CONSEQUENCES

Bacteremia is broadly defined as the presence of bacteria in the bloodstream. Studies in human medicine have identified bacteremia as a significant cause of morbidity and mortality in critically ill patients. The prompt identification of bacteremia and rapid institution of appropriate antimicrobial therapy significantly improves the outcome of affected human patients.[93] In those patients where antimicrobial therapy was delayed or inappropriate, a five-fold increase in mortality was noted.[93] In addition, evidence in septic human patients suggests that for every 1 hour that antimicrobial therapy is delayed, the risk of therapeutic failure increases by 11%.[94]

In veterinary medicine, particularly large animal practice, bacteremia is a common sequel to gastrointestinal disease and failure of passive transfer in both neonatal foals and calves.[95-98] In these groups of animals, bacteremia can often be found to have a significant impact on survival.[95,98] However, some studies have shown that bacteremia, particularly when associated with diarrhea in foals, has no effect on case outcome. For example, Frederick et al identified bacteremia in 49% of foals with diarrhea.[96] In the aforementioned study Gram (-) enteric bacteria were the most common bacteria isolated from the bloodstream.[96] Moreover, no association between bacteremia and survival could be found.[96] Hollis et al identified bacteremia in 50% of foals with diarrhea.[97] Similar to the study by Frederick et al, Gram (-) enteric bacteria were the most common bacteria identified and no association between the presence of bacteremia and survival could be found.[97] Until recently, bacteremia was thought to be an uncommon occurrence in adult large animal veterinary patients, particularly in equine medicine. However, Johns et al. identified bacteremia in a significant proportion of adult horses with colitis (9/31, 29%).[99] Horses > 1 year of age with diarrhea of less than 3 days of duration had blood collected aseptically from the jugular vein at admission and again 24 hours later. Eight horses

were culture positive on admission and 2 horses were culture positive on the second sample.[99] *Corynebacterium* spp. was the predominant isolate from the blood cultures (n=6). In this study, bacteremic horses were 12.7 times more likely to die or be euthanized than blood culture negative horses.[99] In addition, horses from which *Cornyebacterium* spp. was isolated were 25.3 times more likely not to survive than blood culture negative horses.[99] Thus, from this work it is clear that bacteremia does occur in adult horses and it is clear that bacteremia is associated with decreased survival. In cattle, bacteremia has been studied in dairy cattle with acute coliform mastitis (ACM) and calves with experimental bovine viral diarrhea virus (BVDV) infections. Reggiardo and Kaeberle documented the presence of bacteria in the bloodstream of calves experimentally inoculated with BVDV.[100] In this study, more than 85% of calves inoculated with BVDV were bacteremic within 5 days of infection.[100] The occurrence of bacteremia was highly correlated with the presence of lymphopenia and impaired responses of lymphocytes to mitogens. *Bacillus* spp. were the predominant isolates from the calves.[100] Non-inoculated calves and calves inoculated with bovine herpes virus-1 (BHV-1) had consistently negative cultures.[100] From this study it appears that certain aspects of the immune system, particularly lymphocytes, are essential in clearing the bloodstream of microorganisms that may invade the circulation. Nevertheless, similar to the results of Frederick et al, the presence of bacteremia had no influence of mortality in affected calves.[100] Cebra, Garry, and Dinsmore evaluated the physical exam findings and clinicopathologic data from 44 Holstein cows with ACM. Blood cultures were performed on 34 of 44 cows in the study and *E. coli* was isolated from the bloodstream of 32% (11/34) of cattle evaluated.[101] Bacteremic cows were sick longer prior to evaluation and had higher median nucleated cells counts, and more immature neutrophils forms in circulation than non-bacteremic cattle.[101] Survival in bacteremic animals was not different from survival in

non-bacteremic animals.[101] Despite the clear evidence that cattle with ACM can develop bacteremia, the study was criticized because animals were evaluated several days after ACM was first diagnosed. Therefore, Wenz et al repeated the work of Cebra et al and evaluated the prevalence of bacteremia in 144 dairy cattle with ACM on 6 farms.[102] Cattle were evaluated on the day of diagnosis and again 24-48 hours later. Blood was aseptically collected from the jugular vein at each time point and submitted for aerobic and anaerobic bacterial culture. Wenz and his group identified bacteremia in approximately 32% of adult dairy cattle with coliform mastitis.[102] In this study, the presence of bacteremia, particularly bloodborne infection with organisms such as *E. coli*, *Pasteurella multocida* and *Mannheimia haemolytica*, had a significant impact on cow survival, with 35% of animals from which these bacteria were isolated dying during the observation period.[102] This study also found that *Bacillus* spp., a genus of organisms historically considered to be a common skin contaminant, could be isolated from cattle with ACM more frequently than healthy cattle (15% vs 1.9%, respectively).[102] However, survival in animals from which *Bacillus* was isolated was not different from healthy controls.[102]

V. SEPSIS - THE COMPENSATORY ANTI-INFLAMMATORY RESPONSE SYNDROME (CARS) AND THE ROLE OF INDOLEAMINE-2,3-DIOXYGENASE (IDO)

Sepsis, defined as both the presence of infection or suspected infection and the systemic inflammatory response syndrome (SIRS) occurring as a result of this infection, has historically been considered to be a disease characterized by overwhelming inflammation.[103,104] However, these theories were based on studies performed in animals given extremely high doses of LPS or large numbers of virulent bacteria, models that do not always reflect the clinical picture. In addition, studies that used antagonists of inflammatory cytokines such as TNF-α or IL-1 as therapeutic agents showed an increased risk of mortality in certain groups.[104] Certainly, there are

subgroups of patients that benefit from aggressive anti-inflammatory therapy. However, the focus has shifted to immunosuppression as a contributor to the mortality associated with sepsis and the underlying mechanisms of this phenomenon.[104]

The compensatory anti-inflammatory response syndrome

As a response to the inflammation arising from tissue damage or bacterial invasion, the immune system attempts to balance the need for continued defense while preserving tissue integrity.[105] Indeed, concentrations of both pro and anti-inflammatory cytokines increase in the early stages of sepsis in people. Here, lymphocyte, hepatocyte, and endothelial cell apoptosis is increased.[105] In addition, the removal of neutrophils from the circulation is impaired while their function is decreased.[103] Moreover, monocytes from septic patients display increased expression of various inhibitors of NF-kB and have impaired responses to DAMPs. Monocytes also display reduced expression of MHC-II on their surface, a factor that impairs antigen presentation to adaptive immune cells.[106] In fact, failure to regain > 70% of surface MHC-II expression is a poor prognostic indicator.[106] Regulatory T cells are likely to play a role in sepsis-induced immunosuppression.[105] Treg cells have been defined as $CD4^+$, $CD25^+$, $FoxP3^{Hi}$ T lymphocytes.[107] Among these cells there are several subpopulations that function in different ways (Table 1.3). The basal T_{reg} state is the natural T_{reg} (or nT_{reg}) population. These cells are produced during T cell selection and activated to produce primarily contact dependent Granzyme B, TGF-beta or CD95 mediated cell lysis.[107] A second population that is induced (iT_{reg}) from naïve FoxP3 negative $CD4^+$ cells functions primarily through the actions of IL-10. Th3-iT_{reg} cells are also produced from CD25 and FoxP3 negative CD4 positive precursors and never express high levels of CD25 on their surface. They function by producing high levels of TGF-beta. All induced Treg cells have a requirement for IL-2 activation in their generation.[107]

A more controversial regulatory T cell is the CD39 positive and CD73 positive CD4 positive cells that generate adenosine from ATP and ADP through AMP. CD39 converts ATP and ADP to AMP and CD73 AMP to adenosine.[108,109] Adenosine blocks the function of lymphocytes, monocytes, macrophages and neutrophils through the binding of the A2a receptor. These cells appear to provide a circulating repository of memory cells that manage effector and regulatory responses to common and repeated threats.[108]

Several studies have shown that the proportion of T_{regs} is increased in the circulation of septic human patients.[110-112] Also, T_{regs} are known to stimulate the M2 pathway of macrophage activation and inhibit monocyte survival by increasing apoptosis.[113] However, the role for these cells in the pathophysiology of sepsis remains unclear. As a result of these diverse studies, it is clear that sepsis is a multifaceted syndrome with characteristics of both a pro and anti-inflammatory bias. It is unlikely that a single therapy will prove to be beneficial for all patients. However, a better understanding of the pathophysiology of the disease is necessary before definitive conclusions can be made.

The role of Indoleamine-2,3-Dioxygenase (IDO) in bacteremia and sepsis

Tryptophan is the least abundant amino acid in the human body. The majority of tryptophan metabolism occurs through a pathway known as the kynurenine pathway. Depletion of tryptophan through metabolism by IDO can suppress cellular proliferation, particularly proliferation of immune cells.[114] The rate-limiting enzyme in the kynurenine pathway of tryptophan catabolism is catalyzed by IDO. IDO is a heme-containing enzyme that catalyzes the oxidative cleavage of the indole ring of tryptophan.[114] Cleavage of the indole ring converts tryptophan to N-formylkynurenine.[115] The enzyme is expressed in a variety of tissues with the highest levels being found in the placenta, lung, intestine, and myeloid lineage cells (dendritic

cells, monocytes, macrophages, eosinophils).[114] IDO activity can also be found in epithelial cells, endothelial cells, and certain tumor cell lines. While the physiologic role of tryptophan depletion is multifaceted, the primary role likely lies in inhibition of pathogen growth. For example, starvation of tryptophan results in decreased proliferation of various species within the Chlamydia family, *Toxoplasma gondii*, and *Neospora caninum*[116]. Moreover, if tryptophan is replenished, pathogen growth can recommence. IDO is regulated by inflammatory cytokines (IFN-γ and TNF-α) and LPS. While the role of IDO in protection against intracellular pathogens is well known, its contribution to other aspects of physiology and disease are still poorly understood.[114] IDO has been shown to play a significant role in tolerance to the fetal allograft in pregnant mammals.[117,118] In mice, inhibition of IDO activity with 1-methyl tryptophan led to early pregnancy loss. IDO has been shown to promote the growth of tumor cells in various types of neoplasia.[119] Moreoever, inhibition of IDO activity with ethylene pyruvate was shown to inhibit outgrowth of melanoma cells in an *in vitro* study.[119] The study of IDO as a possible contributor to sepsis-induced immunosuppression is still in its infancy. However, some aspects of its contribution are known. For example, Chen et al found that IDO is an essential part of peripheral T_{reg} generation.[120] In addition, increased activity of IDO in dendritic cells can block clonal expansion of T cells in mice. Furthermore, concentrations of IDO were positively correlated to concentrations of IL-12 and negatively correlated to concentrations of IL-10.[120] Liu et al found that $CD8^+$ T cell cytotoxic activity could be impaired in the presence of IDO by inhibition of complex I in the electron transport chain.[121] Clinically, IDO activity has been used to predict mortality in bacteremic human patient.[122] In this single study, non-surviving patients were more likely than surviving patients to have higher indices of IDO activity. In addition, high IDO activity was found to remain as an independent risk factor for mortality even when

confounders were removed from the study.[122] Other studies using mouse models of LPS induced shock have shown that blockade of IDO will improve survival significantly.[123]

While much of the attention given to IDO in the field of sepsis has revolved around immunosuppression, newer studies have indicated that IDO and certain metabolites likely contribute to hypotension seen with septic shock.[124,125] Wang et al found that pharmacological inhibition of LPS induced IDO expression allowed for blood pressure to return to normal levels.[124] In vitro, kynurenine, the primary metabolite of the IDO pathway, was able to dilate porcine coronary arteries.[124] In septic human patients IDO activity was found to correlate with both mean arterial pressure and inotrope requirements.[125]

The evaluation of IDO as a contributor to diseases of cattle is in its infancy. Spekker et al found that IDO was required for inhibition of *N. caninum* in bovine fibroblasts and endothelial cells.[116] When tryptophan was added to culture media or the activity of IDO inhibited with 1-methyl tryptophan, parasite growth proceeded unimpeded.[116] Groebener et al. showed that IDO expression and activity were increased in the endometrium of cattle 18 days pregnant.[126] This expression seemed to be confined to endometrial stromal cells and was negatively correlated to the number of leukocytes found within the endometrium of pregnant animals.[126] Plain et al. found that IDO gene and protein expression were increased in monocytes infected with *Mycobacterium avium* subsp *paratuberculosis*.[127] In addition, levels of both the IDO gene and protein were increased in infected tissues harvested from sheep. Furthermore, IDO activity was associated with the onset of clinical signs in infected cattle and sheep.[127] To date, however, no studies have been performed to evaluate the expression of activity in cattle with other systemic illnesses.

VI. RATIONALE FOR PRESENTED STUDIES

Acute puerperal metritis is a common disorder with significant effects on dairy production. While risk factors for the disease have been well characterized, periparurient immunosuppression is likely one of the driving forces for the development of clinical disease. Systemic antimicrobials are a mainstay of therapy for cattle with APM and ceftiofur, a 3rd generation cephalosporin, is one of the drugs commonly used to treat clinically affected cattle. Emerging evidence would suggest that the use of ceftiofur in cattle may pose a risk to human health by selecting for resistant pathogens of medical importance. There is some data to suggest that other antimicrobials, both local and systemic, may be just as effective as ceftiofur. Our understanding of the effects of APM on the systemic health of affected cattle is minimal. Studies have shown that cattle with coliform mastitis can develop bacteremia and bacteremia impacts survival. In these animals invasion of the bloodstream with pathogenic gram (-) pathogens is common and systemic antimicrobials have been shown to improve survival. However, the prevalence of bacteremia and the state of the immune system in cattle with APM has yet to be investigated. If bacteria are found in the bloodstream of affected cattle and animals with APM demonstrate immunosuppression, systemic antimicrobial therapy is likely warranted. However, therapy with ceftiofur may not be needed, particularly if pathogens isolated from the bloodstream are highly susceptible. This would allow us to preserve drugs like ceftiofur for animals with more severe disease and, hopefully, reduce the emergence of resistant bacterial strains. Therefore, the more in-depth investigation of drugs such as ampicillin trihydrate as therapeutic agents for cattle with APM is warranted

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Table 2.1. PRRs, Cellular localization, and Ligands

PRR	Localization	Ligand
TLR		
TLR1	Plasma membrane	Triacyl lipoprotein
TLR2	Plasma membrane	Lipoprotein
TLR3	Endolysosome	dsRNA
TLR4	Plasma membrane	LPS
TLR5	Plasma membrane	Flagellin
TLR6	Plasma membrane	Diacyl lipoprotein
TLR7	Endolysosome	ssRNA
TLR9	Endolysosome	CpG-DNA
TLR10	Endolysosome	Unknown
TLR11	Plasma membrane	Profilin-like molecule
RLR		
RIG-I	Cytoplasm	Short dsRNA
MDA5	Cytoplasm	Long dsRNA
LGP2	Cytoplasm	Unknown
NLR		
NOD1	Cytoplasm	iE-DAP
NOD2	Cytoplasm	MDP
CLR		
Dectin-1	Plasma membrane	β-glucan
Dectin-2	Plasma membrane	β-glucan
MINCLE	Plasma membrane	SAP130

Table 2.2. Classifcation of antimicrobial agents based on their pharmacodynamics properties

Time (T>MIC)	**Concentration (Cmax/MIC)**	**Both (AUC/MIC)**
β-lactams	Aminoglycosides	Azalides
Tetracyclines	Fluoroquinolones	Fluoroquinolones
Macrolides	Metronidazole	Glycopeptides
Lincosamides		
Fenicols		

Table 2.3. Properties of Regulatory T Cells

Property	Natural Treg (nTreg)	Induced Treg (iTreg) - Tr1	Induced Treg (iTreg) - Th3
Development	Thymus	Periphery (MALT)	Periphery (MALT)
Phenotype	$CD4^{+}CD25^{+}CD127^{low}$	$CD4^{+}CD25^{-}$	$CD4^{+}CD25^{+}$ from $CD25^{-}$ precursors
Other Associated Markers	$CTLA\text{-}4^{+}GITR^{+}Foxp3^{+}$	$CD45RB^{low}Foxp3^{-}$	$CD25^{low\text{-}variable}CD45RB^{low}Foxp3^{+}$
Suppression	Contact-, Granzyme-B dependent, makes TGF beta	IL-10 mediated	TGF beta mediated
Target Cells	APC and Effector T Cells	Effector T Cells	Unknown
CD28 Involvement	Thymic development and maintenance in periphery	Unnecessary for development or function	Unnecessary for development or function
***in vivo* Role**	Suppression of autoreactive T cells	Mucosal immunity, inflammatory response	Mucosal immunity, inflammatory response
***in vitro* Expansion**	TCR/CD28 stimulation and IL-2	CD3, IL-10, Retinoic Acid	CD3, TGF beta

CHAPTER 3

PREVALENCE OF BACTEREMIA IN DAIRY CATTLE WITH ACUTE PUERPERAL METRITIS[1]

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To be submitted to *The Journal of Veterinary Internal Medicine*

ABSTRACT

OJBECTIVE: The objective of this study was to determine the prevalence of bacteremia in dairy cattle with acute puerperal metritis and determine clinicopathologic factors associated with bacteremia in this population of animals

MATERIALS AND METHODS: Cows were examined at the time of identification of APM. A complete blood count, serum biochemical analysis and bacteriologic culturing of blood and lochial fluid were performed on each animal at the time of diagnosis. The same samples were collected from healthy herdmates of a similar parity and days in milk. Blood culture results and clinicopathologic parameters were compared between groups. Conditional logistic regression was used to evaluate factors associated with APM, while multivariate logistic regression was used to evaluate factors associated with bacteremia.

RESULTS: Bacteremia occurred in 53.1% (9/17) of cattle with APM and 53.3% (8/15) controls. *Bacillus* spp. was the organism most commonly isolated from the bloodstream in cattle of both groups. Bacteremic cattle in both groups were significantly less likely to have basophils in the peripheral circulation and more likely to have higher serum globulin concentrations.

CONCLUSIONS AND CLINICAL RELEVANCE: Bacteremia is a common occurrence in post-partum dairy cattle. It is likely that bacterial colonization of the involuting uterus, peri-parturient immunosuppression, and systemic inflammation play a role in the high frequency of bacteremia. Further study is warranted to investigate the modes by which bacteria colonize the bloodstream in this population of animals and the significance of bacteremia on health and productivity of affected animals.

INTRODUCTION

Dairy cattle are susceptible to numerous disorders in the immediate post-partum period. Acute puerperal metritis (APM), defined as the presence of a fetid, watery uterine discharge, an enlarged, flaccid uterus, and overt signs of systemic illness that may include fever, dehydration, depression, and toxemia is one of the most commonly encountered infectious diseases in modern dairy practice.[1] APM occurs within the first 21 days of lactation and typically affects 20-30% of all cattle.[2] A recent economic analysis estimated that the average cost for a case of APM was $358, in spite of aggressive systemic antibiotic and supportive medical therapy.[3] Indeed, some estimates suggest that APM results in the loss of approximately $650,000,000 to the dairy industry yearly.[4] Thus, it is evident that APM has a significant economic impact on the dairy production and a better understanding of its pathophysiology is needed in order to develop new methods of prevention and treatment.

After calving, more than 90% of cattle experience some degree of contamination of the uterine lumen with bacteria.[5] Through the processes of uterine involution and normal immune function, most cattle clear this contamination and experience no complications. However, cattle with retained placenta, hypocalcemia, and significant negative energy balance fail to clear uterine contamination and develop APM.[2,6,7] While a variety of microorganisms may be isolated from the reproductive tract of both healthy post-parturient cattle and cattle with APM, *E. coli* and *Trueperella pyogenes* represent the bacteria most commonly associated with clinical disease.[4]

Bacteremia is defined as the presence of bacteria within the bloodstream and, until recently, was thought to be an uncommon occurrence in adult large animal veterinary patients.[8] However, researchers at the University of Pennsylvania identified bacteremia in a significant proportion of adult horses with colitis.[8] Furthermore, bacteremia has been documented in

approximately 32% of adult dairy cattle with coliform mastitis.[9] In cows with acute coliform mastitis, the presence of bacteremia, particularly bloodborne infection with organisms such as *E. coli*, *Pasteurella multocida* and *Mannheimia hemolytica*, had a significant impact on cow survival.[9]

Systemic antimicrobial therapy is the gold standard for treating cattle with APM. Currently, three antimicrobials are labeled for systemic use in cattle with clinical disease: oxytetracycline dihydrate (Liquamycin LA200), ceftiofur hydrochloride (Excenel RTU), and ceftiofur crystalline free acid (Excede). Studies have shown intrauterine infusion of these same antimicrobials is an effective therapy and, in some cases, improves performance beyond that of healthy herdmates.[10,11] However, if bacteremia was a common sequel to APM, administration of systemic antimicrobials would be a necessity and provide further reason for veterinarians to avoid extra-label use of the aforementioned drugs in affected cattle.

The purposes of the study reported here were to investigate the prevalence of bacteremia in dairy cattle with naturally occurring APM, determine if an association exists between the bacteria cultured from the bloodstream and those present in uterus and identify factors that may be of use in predicting occurrence of bacteremia in cattle with APM.

MATERIALS AND METHODS

Animals

Cows at 3 dairies in Northeast and Southwest Georgia that developed APM between September 2011 and November 2013 were eligible for inclusion in the study. During the study, there were approximately 9,000 Holstein and Holstein cross cows in lactation at the 3 dairies.

Cows were fed a total mixed ration (TMR) in groups based on production and were milked in a parlor 3 times daily. All cows were housed in drylot pens or freestall barns.

Inclusion criteria and data collection

Fresh cows at each dairy were examined daily by 1 of the authors (BCC). For inclusion in the study, cattle had to be enrolled in a fresh cow monitoring program. For the purposes of this study, APM was diagnosed if a cow was less than 10 days in milk, had a fetid, watery, reddish-brown uterine discharge, and systemic signs of illness that included at least one or more of the following: fever (rectal temperature > 103F), obtundation, toxemia (injected mucous membranes, tachycardia (heart rate > 84 beats per minute), or tachypnea {respiratory rate > 36 breaths per minute). Cows were evaluated only at the time of initial diagnosis. Any animals with evidence of other concurrent disease processes (mastitis, abomasal displacement, respiratory disease, diarrhea) were excluded from the study. Cows diagnosed with APM were treated according to on-farm protocols following evaluation and sample collection by study personnel. Treatment was not controlled in order to maximize study participation and evaluate the prevalence of bacteremia in dairy cattle with APM given current on-farm treatment protocols. Treatment varied between and within farms and included systemic antimicrobials, anti-inflammatories, prostaglandin, and oral electrolyte solutions. Healthy herdmates, as determined by a complete physical examination and of similar parity and days in milk (DIM) as cases, were enrolled as controls. Control cattle were monitored for the development of APM daily until day 10 after parturition. Any control that developed APM was removed from the study.

Hematologic testing

Blood samples were collected from the jugular vein at the time of diagnosis from both cattle with APM and controls. Samples anti-coagulated in EDTA were used for complete blood count (CBC) analysis. Samples collected into plain tubes without anticoagulant were used for serum chemistry analysis.

Bacteriologic culture

Lochial fluid was collected at the time of diagnosis. The tail was held to the side and the external genitalia were cleaned with 3 alternating applications of 4% chlorhexidine scrub and 70% isopropyl alcohol. A double-guarded culture swab[a] was passed into the uterus, a sample obtained, and the culture swab removed. The swab was capped and transported to the laboratory for processing on the day of collection. Swabs were cultured aerobically at 37°C for 48 h on sheep blood agar and MacConkey agar, and anaerobically for up to 7 days on pre-equilibrated sheep blood agar. Plates were evaluated for growth and findings recorded at each observation. Bacteria were identified on the basis of characteristics of the colony, morphology, Gram stain, hemolysis, and biochemical profile.

Blood was collected from the jugular vein of each cow with APM and each control cow on the day of diagnosis. The hair over the jugular vein was shaved using a battery-operated clipper with a #40 blade. The skin was disinfected with at least 3 alternating applications of a 4% chlorhexidine surgical scrub and 70% isopropyl alcohol. Thirty-five milliliters of blood was aseptically drawn from the vein into a 35-ml syringe through a 16-ga, 1-½ inch needle. Ten milliliters of blood was injected aseptically through a new 16-ga, 1-½ in needle into a 30 ml culture vial of 3.0% soybean casein digest broth containing 0.05% sodium polyanetholsulfonate[b]

and a 40 ml culture vial of 2.75% soybean-casein digest broth containing 0.035% sodium polyanetholsulfonate[c]. Samples were aerated through a filtered needle and incubated at 37°C in an atmosphere containing 10% CO_2. Samples were subcultured onto blood agar on days 0, 1, and 7 and incubated at 37°C in an atmosphere containing 10% CO_2. Plates were examined for growth and findings recorded at each observation.

Statistical analysis

Cattle were grouped by disease (control or APM) and blood culture (positive or negative) status. The proportion of cattle with bacteremia and the frequency with which specific bacteria were isolated from the uterus was compared between cattle with and without APM using McNemar's test for correlated proportions. Clinicopathologic data was compared between cattle with APM and healthy controls using a Wilcoxon signed-rank test, while clinicopathologic data was compared between bacteremic and non-bacteremic cattle using a Wilcoxon rank sum test. Data were reported as median and 10^{th} and 90^{th} percentiles. Likelihood ratios were calculated to investigate the role of farm in blood culture status. The association of clinicopathologic data with disease status was investigated using conditional logistic regression, while the association of clinicopathogic data with blood culture status was investigated using backward stepwise logistic regression. All variables were first screened using univariate logistic regression and all variables with a $P < 0.2$ were allowed to remain in the final logistic regression model. For the multivariate model, all variables with a $P < 0.05$ remained in the model. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. An OR greater than 1 corresponds to a positive association with APM or bacteremia and an OR of less than 1 corresponds to a negative association. The final logistic regression model fit was evaluated using the Hosmer-Lemeshow

Goodness-of- Fit test. The ability of the model to predict a given outcome was assessed by use of receiver operating characteristic (ROC) curve analysis. Statistical analysis was performed using commercially available statistical software.[d]

RESULTS

Uterine bacteriology

A total of 34 cows were enrolled in the study, 17 cases with APM and (50%) 17 controls (50%). Two controls were excluded from the study due to the development of APM during subsequent monitoring. *E. coli* and *Trueperella pyogenes* were the organisms most frequently isolated from the uterus of cattle with APM (Table 1). Other isolates included *Clostridium* spp., and combinations of Gram (+) and Gram (-) aerobes and anaerobes (Table 1). Cattle with APM were significantly more likely to have *E. coli*, *Trueperella pyogenes*, gram (+) aerobes, and gram (+) anaerobes isolated from the uterus than control cattle (Table 3.1).

Clinicopathologic values

Cattle with APM had significantly lower serum albumin concentration, albumin/globulin ratio, serum sodium, and total serum calcium concentration than control cattle, whereas plasma fibrinogen concentration, monocyte count, serum globulin concentration, and serum glucose concentration were significantly higher in cattle with APM than in controls (Table 3.2). No variables were significantly associated with APM in the final conditional logistic regression model.

Blood culture

Bacteria were isolated from the bloodstream of 53.1 % (9/17) of cattle with APM and 53.3% (8/15) of control cattle. No difference in the prevalence of bacteremia between groups was detected (P = 0.724). *Bacillus* spp was the organism most frequently isolated from the bloodstream of both control cattle (5/8, 62.5%) and cattle with APM (5/9, 55.6%) (Table 3.3). *Bacillus* spp was isolated in combination with *Trueperella pyogenes* in one cow with APM and in combination *Clostridium* spp. in another cow with APM.

Due to the lack of a statistically different prevalence of bacteremia between the two groups of cattle, the groups were combined for further analysis. There was a significant effect of farm on blood culture status. Cattle from farms 1 and 3 were significantly more likely to have a positive blood culture than cattle from farm 2 (LR = 7.7, P = 0.02). Cattle with bacteremia had significantly greater days in milk (DIM) and serum globulin concentration than non-bacteremic cattle, whereas basophil count and albumin/globulin ratio was significantly lower in bacteremic than non-bacteremic cattle (Table 3.4).

The only variables significantly associated with bacteremia in a multivariate logistic regression model (overall significance of the mode P = 0.0005) were the absence of basophils and serum globulin concentration (Table 3.5). The model correctly predicted 81.3% of cases. The area under the receiver operating characteristic curve for the ability of serum globulin concentration and absence of basophils to predict bacteremia was 0.850 (95% confidence interval, 0.679 to 0.951).

DISCUSSION

The findings of this study suggest that bacteremia is a common occurrence in both healthy post-partum dairy cattle and dairy cattle with APM, occurring in 53% of cattle in each group. The process of uterine involution is not sterile and, in one study, 93% of all uteri sampled within 2 weeks of calving were culture positive for various bacteria.[5] In addition to bacterial contamination of the uterine lumen after parturition, the surface cells of the endometrium slough and expose the deeper uterine layers. Indeed, the remnants of the maternal caruncle are necrotic by day 5 after calving and, by day 12 post-partum, a denuded endometrial surface with exposed blood vessels can be found.[12] It is well accepted that the epithelial barriers of the body's mucosal surfaces serve as a barrier to bacterial invasion of the deeper tissues and systemic circulation.[13] Bacteremic cattle were significantly later in lactation than non-bacteremic cattle (8 days vs 4.5 days, respectively). The longer period of time from parturition to diagnosis in these animals may put them at greater risk of bacteremia simply because of prolonged contact of a denuded endometrium with contaminating bacteria. Thus, it is possible that sloughing of the endometrial epithelium allows the bacteria that normally colonize the uterine lumen after calving to gain access to the systemic circulation prior to the beginning of reepithelialization.

Cattle diagnosed with APM had significantly lower serum albumin concentrations and significantly higher serum globulin concentrations than healthy controls. Similarly, bacteremic cattle in this study had significantly higher serum globulin concentrations and a trend towards lower serum albumin concentrations. Grunberg et al demonstrated a decline in plasma protein in dairy cattle immediately after calving.[14] Here, decreasing plasma globulin concentrations, likely resulting from uptake of IgG_1 by the mammary gland, rather than a decline in albumin caused the decrease in plasma protein. Other studies have found a decrease in serum albumin concentration

in periparutrient cattle and authors have suggested that expansion of plasma volume, impaired hepatic function secondary to lipid accumulation, or inflammatory disorders that downregulate hepatic albumin production may be responsible for these findings.[15-17] It is also possible that the decrease in serum albumin concentration in cattle with APM reflects loss of serum proteins into the uterine lumen due to tissue compromise. The increase in globulin concentration seen in both cattle with APM and bacteremic cattle in this study is likely to due to an increase in antigenic stimulation.

Farm played a role in the prevalence of bacteremia in this study. Cattle from 2 of the 3 farms enrolled in the study were significantly more likely to be bacteremic than cattle from the other farm. The reasons for this are not clear. The distance from these farms to the diagnostic lab were similar to the other. In addition, similar techniques were used to collect the blood cultures on each farm. One factor may be that cattle on the 2 farms from which bacteremic cattle were more frequently identified often diagnosed cattle with APM at a later time during the 10-day monitoring period.

The significance of identifying bacteremia in over half of all cattle sampled in this study is unknown. However, studies in humans and mice have shown that bacterial translocation from various organ systems to the mesenteric lymph nodes and mammary gland occurs during late pregnancy and early lactation.[18,19] It is believed that these bacteria may serve as a means to program the neonatal immune system to bacterial molecular patterns and ensure appropriate responses to pathogens and commensal organisms.[18,19] Clearly, more work needs to be done in this area before definitive conclusions can be reached.

Numerous studies have shown that peripaturient dairy cattle experience varying degrees of immunocompromise.[20-22] Cells of the innate and adaptive immune systems, particularly

members of the neutrophil, monocyte/macrophages, and circulating lymphocytes, have long been thought to be responsible for clearance of bacteria from the bloodstream. For example, Reggiardo and Kaeberle identified bacteremia in 85% of cattle experimentally infected with bovine viral diarrhea virus.[23] Here, bacteremia was closely associated with the number of circulating leukocytes, particularly the total lymphocyte population. In humans, neutropenia has been found to be a significant risk factor for bacteremia associated with gram (-) bacilli.[24] In this study, patients with neutropenia (< 500 neutrophils/μL) were 8.1 times more likely to be bacteremic than patients with > 500 neutrophils/μL.[24] Work from cattle with coliform mastitis would suggest that neutropenia is a contributor to bacteremia in that population of animals.[25] In this study, cattle with basophils present in the circulation were approximately 13 times less likely to be bacteremic than cattle without circulating basophils. Traditionally, basophils have been seen as contributors to allergic reactions and anti-parasitic defense.[13] However, recent evidence suggests that basophils might play a role in enhancement of immunological memory responses by enhancing B-cell proliferation and immunoglobulin production.[26] In addition, mice depleted of basophils and experimentally infected with *Streptoccous pneumoniae* were more likely to die than mice that were basophil replete.[26] It is clear, therefore, that cellular immune responses are important for defense against blood borne bacterial infection.

Bacillus spp. was the organism most frequently isolated from the bloodstream of cattle in both groups in this study. Traditionally, *Bacillus* spp has been thought to have a ubiquitous distribution in the environment and is often viewed as a contaminant of blood cultures from both humans and animals.[27-30] Nevertheless, Wenz et al identified *Bacillus* spp as the organism most frequently isolated from dairy cattle with coliform mastitis, even though it was not routinely isolated from milk of affected animals.[9] In fact, *Bacillus* spp could be isolated from the

bloodstream with approximately 8 times greater frequency in cattle with coliform mastitis than in controls. However, unlike cattle in which *E. coli*, *Salmonella* spp., or *Klebsiella pneumonia* could be isolated, animals with *Bacillus* spp. bacteremia were not at increased risk of mortality.[9] In addition, the frequency with which *Bacillus* spp. could be isolated was similar across disease severity groups. Members of the *Bacillus* genus, particularly *Bacillus licheniformis*, can frequently be isolated from the uterine lumen of cattle with and without uterine disease.[31] In fact, Williams et al showed that cattle from which *Bacillus licheniformis* could be isolated had greater acute phase protein responses than cattle from which this organism was not cultured.[31] Thus, *Bacillus* spp., under the right circumstances, can be a pathogen and stimulate significant inflammatory responses.

Studies from sheep and in vitro studies of bovine pulmonary endothelial cells have shown that endotoxin and inflammatory mediators can cause cellular damage severe enough to result in increased permeability and hydraulic conductance.[32,33] In addition, studies in mice have shown displacement of proteins associated with intercellular tight junctions during experimentally induced sepsis.[34] This altered expression and a disrupted mucosal barrier, as measured by radioactively labeled biotin permeability, accompanies disruption of tight junction proteins.[34] Furthermore, studies have also shown that cattle with APM have increased levels of LPS in the circulation when compared to health controls.[35] Therefore, bacteremia with *Bacillus* spp may reflect translocation of the bacteria from the uterine lumem or distant sites through compromised cellular barriers resulting from systemic inflammation or, as previously mentioned, a disrupted uterine epithelial barrier.

The results of this study demonstrate that bacteremia occurs in a large proportion of post-parturient dairy cattle, both healthy and with APM. While the cause of the high risk of

bacteremia is unclear, bacterial colonization of the involuting uterus, peri-parturient immunosuppression, and systemic inflammation may all play a role. In addition, novel data from humans and mice would suggest that bacteremia in the mother may serve to inoculate the neonatal gastrointestinal tract with bacteria. On the basis of the results of this study, bacteremia should be considered a common occurrence in post-parturient dairy cow.

FOOTNOTES

a. Double guarded culture swab, Jorgensen Laboratories, Loveland, CO
b. BD Bactec Plus Aerobic /F. BD Laboratories, Franklin Lakes, NJ
c. BD Bactec Lytic/10/Anaerobic/F. BD Laboratories, Franklin Lakes, NJ.
d. Stata, Version 12.1, StataCorp, LP, College Station, TX

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Table 3. 1. Bacterial isolates obtained from the uterus of clinically healthy cattle (n=15) and cattle with APM (n=17).

Organism	Group		P-value
	Control	APM	
E. coli	2	9	0.028
Trueperella pyogenes	6	15	0.008
Clostridium spp	0	4	0.104
Gram (+) aerobes	4	12	0.032
Gram (+) anaerobes	4	11	0.042
Gram (-) aerobes	1	3	0.603
Gram (-) anaerobes	8	10	1.000

Table 3.2. Comparison of hematologic and biochemical findings (median, 10^{th} and 90^{th} percentile) in clinically healthy cattle (n=15) and cattle with APM (n=17).

Parameter	Reference Range	Group		P-value
		Control	APM	
Hct (%)	24-46	29.3 (25.7 – 33.3)	27.6 (23.3 – 32)	0.191
Platelets (x 10^3/µL)	100-800	261 (156 – 561)	315 (183 – 535)	0.334
Fibrinogen (mg/dL)	100-600	300 (300 – 800)	800 (500 – 1100)	0.005
WBC (x 10^3/µL)	4-12	10.9 (7.20 – 17.9)	8.4 (6.2 – 12.5)	0.112
Segs (x 10^3/µL)	0.6-4	4.35 (1.11 – 10.5)	2.4 (1.4 – 7.8)	0.182
Bands (x 10^3/µL)	0-0.1	0 (0 – 0.65)	0.3 (0 – 0.6)	0.796
Lymphs (x 10^3/µL)	2.5-7.5	5 (2.57 – 10)	3.9 (1.8 – 7.3)	0.201
Monocytes (x 10^3/µL)	0-0.9	0.3 (0-1.44)	0.8 (0.2 – 1.6)	0.031
Eosinophils (x 10^3/µL)	0-2.4	0.12 (0 – 0.26)	0.1 (0 – 0.5)	0.565
Basophils (x 10^3/µL)	0-0.2	0 (0 – 0.12)	0.7 (0 – 0.1)	0.060
Creatinine (mg/dL)	1-1.8	0.6 (0.12 – 0.7)	0.6 (0.4 – 1)	0.040
Total Protein (g/dL)	6.4-9.5	6.6 (5.8 – 7.4)	6.7 (5.7 – 7.7)	0.320
Albumin (g/dL)	2.5-4.5	3.4 (2.8 - 3.8)	2.8 (2.3 – 3.5)	0.005
Globulin (g/dL)	2.6-6.5	3 (2.4 – 4.4)	3.7 (2.8 – 5.2)	0.010
A/G ratio	N/A	1.2 (0.67 – 1.2)	0.7 (0.4 – 1.3)	0.002
Glucose (mg/dL)	55-95	59 (48 – 70)	63 (31 – 81)	0.028
Sodium (mEq/L)	136-147	142 (139 – 147)	141 (136 – 145)	0.009
Potassium (mEq/L)	4-5	3.9 (3.3 – 4.4)	3.7 (3.4 – 4.2)	0.333
Chloride (mEq/L)	95-105	101 (96.4 – 103)	100 (94 – 104)	0.408
Bicarbonate (mEq/L)	20-30	27 (23.1 – 30)	28 (25 – 31)	0.243
Anion Gap (mEq/L)	13-20	20 (13-22.4)	18 (13 – 19)	0.056
Total Calcium (mg/dL)	7.6-10.2	9.2 (7.9 – 10.1)	8.5 (7.1 – 9.3)	0.003

Table 3.3. Bacterial isolates obtained from the bloodstream of clinically healthy cattle (n=15) and cattle with acute puerperal metritis (n=17)

Organism	**Group**	
	Control	APM
Bacillus spp	5	5
Trueperella pyogenes	0	1
Clostridium spp	0	1
Kytococcus sedentarius	1	0
Staphylococcus equoruum	1	0
Other	1	2

Other = Multiple organisms present or organism unable to be identified

Table 3.4. Comparison of hematologic and biochemical findings (median, 10^{th} and 90^{th} percentile) in bacteremic (n=17) and non-bacteremic cattle (n=15)

Parameter	Reference Range	Group		P-value
		Bacteremic	Non-Bacteremic	
Parity	N/A	1 (1-3)	2 (1-5)	0.25
DIM	N/A	8 (2.4 – 10)	4.5 (2-8)	0.01
Hct (%)	24-46	28.4 (24 – 31)	28 (24 – 35)	0.91
Platelets (x 10^3/µL)	100-800	270 (170 – 577)	341 (175 – 492)	0.55
Fibrinogen (mg/dL)	100-600	600 (370 -1100)	650 (370 – 930)	0.63
WBC (x 10^3/µL)	4-12	10.5 (6.8 – 12.5)	9.6 (6 – 17.5)	0.78
Segs (x 10^3/µL)	0.6-4	3.7 (1.1 – 7.9)	3.9 (1.4 – 10.5)	0.09
Bands (x 10^3/µL)	0-0.1	0 (0 – 0.7)	0 (0 – 0.28)	0.06
Lymphocytes (x 10^3/µL)	2.5-7.5	4.4 (2.4 – 7.7)	4.6 (2 – 10.3)	0.64
Monocytes (x 10^3/µL)	0-0.9	0.5 (0.1 – 1.4)	0.66 (0.1 – 1.6)	0.30
Eosinophils (x 10^3/µL)	0-2.4	0 (0 – 0.4)	0.1 (0 - .33)	0.47
Basophils (x 10^3/µL)	0-0.2	0 (0 – 0.1)	0.1 (0 – 0.2)	0.01
Creatinine (mg/dL)	1-1.8	0.7 (0.5 – 1)	0.7 (0.4 – 1)	0.62
Total Protein (g/dL)	6.4-9.5	6.9 (5.9 – 7.8)	6.5 (5.6 – 7.1)	0.01
Albumin (g/dL)	2.5-4.5	2.8 (2.3 – 3.6)	3.3 (2.7 – 3.8)	0.08
Globulin (g/dL)	2.6-6.5	4 (2.9 – 5.2)	3 (2.4 – 4.3)	0.01
A/G ratio	N/A	0.7 (0.4 – 1.2)	1.1 (0.6 – 1.5)	0.02
Glucose (mg/dL)	55-95	59 (23 – 66)	61 (31 – 82)	0.43
Sodium (mEq/L)	136-147	141 (137 – 146)	143 (137 – 147)	0.06
Potassium (mEq/L)	4-5	3.8 (3.4 – 4.4)	3.9 (3.4 – 4.2)	0.88
Chloride (mEq/L)	95-105	101 (96 – 104)	102 (94 – 104)	0.30
Bicarbonate (mEq/L)	20-30	27 (22 – 31)	29 (26 – 30)	0.25
Anion Gap (mEq/L)	13-20	19 (12 – 22)	18 (14 – 20)	0.35
Total Calcium (mg/dL)	7.6-10.2	8.9 (7.9 – 10.1)	8.5 (7.0 – 9.6)	0.09

Table 3.5. Result of multivariate logistic regression analysis of the variables associated with bacteremia in post-partum dairy cows.

Variable[†]	Coefficient	SE	P-value	OR	(95% CI)
Intercept	-4.790	0.074	0.001	N/A	N/A
Basophils	-2.581	1.142	0.023	0.076	0.008 to 0.711
Globulin	1.563	0.658	0.018	4.771	1.314 to 17.33

OR = odds ratio; CI = confidence interval; SE = standard error

CHAPTER 4

EXPRESSION OF INFLAMMATION-ASSOCIATED GENES IN CIRCULATING LEUKOCYTES AND ACTIVITY OF INDOLEAMINE-2,3-DIOXYGENASE IN DAIRY CATTLE WITH ACUTE PUERPERAL METRITIS[2]

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To be submitted to *The American Journal of Veterinary Research*

ABSTRACT

OJBECTIVE: To investigate whether expression of genes associated with inflammation and activity of Indoleamine-2,3-Dioxygenase (IDO) correlated with disease status and prevalence of bacteremia in post-partum dairy cattle

MATERIALS AND METHODS: Blood was collected from cattle with APM and control cattle matched by parity and days in milk. Leukocytes were isolated, diluted to a standard concentration, and frozen until RNA extraction. Expression of 6 genes associated with inflammation (TNF-α, IL-1β, IL-4, IL-6, IL-10, and IDO) was quantified by use of a real-time quantitative reverse transcription PCR assay. Serum was collected after centrifugation of whole blood and frozen until analysis by HPLC. Results were grouped by disease status and presence of bacteremia for comparison.

RESULTS: The relative expression of IL-1β in cattle with APM was significantly lower than that in controls. No significant difference was found in the relative expression of other genes tested. Serum levels of tryptophan and kynurenine were not significantly different between cattle with APM and controls. Activity of IDO was not significantly different between cattle with APM and controls. Serum levels of tryptophan, kynurenine, and IDO activity were not significantly different between bacteremic cattle and non-bacteremic cattle

CONCLUSIONS AND CLINICAL RELEVANCE: The expression of IL-1β was lower in cattle with APM. Otherwise, cytokine and IDO gene expression were not significantly different between cattle with APM and healthy controls, as well as bacteremic and non-bacteremic cattle. The activity of IDO in serum of cattle with APM and healthy controls, as well as bacteremic and non-bacteremic cattle, was similar. The lower levels of IL-1β expression in PBMCs of cattle

with APM suggest impaired inflammatory responses and may contribute to the development of the disease in this population of animals.

INTRODUCTION

Dairy cattle are susceptible to numerous metabolic and infectious disorders in the immediate post-partum period. Acute puerperal metritis (APM), clinically defined as the presence of a fetid, watery, reddish-brown uterine discharge, an enlarged, flaccid uterus, and overt signs of systemic illness that may include fever, dehydration, and depression is one of the most commonly encountered infectious peri-parturient diseases in modern dairy practice, affecting 18-33% of all cattle that calve.[1,2] Economically, APM contributes to significant losses to the dairy industry. A recent analysis estimated that the average cost for a case of APM was $358, in spite of aggressive systemic antibiotic and supportive medical therapy.[3] Indeed, some estimates suggest that APM results in the loss of approximately $650,000,000 to the dairy industry yearly.[2]

Cattle that subsequently develop APM experience significantly greater degrees of peri-parturient immunosuppression than cattle that remain healthy. Indeed, cattle with APM have impaired neutrophil function as measured by chemotactic activity, phagocytosis, NOD activity, and bacterial killing capacity.[4,5] In one study, cattle with uterine health disorders had lower levels of pro-inflammatory cytokine gene expression than healthy cattle.[6] However, this study only evaluated the gene-expression profile of circulating monocytes, not peripheral blood mononuclear cells as a population. Other studies have shown impairment in lymphocyte function and alterations in circulating lymphocyte populations in the immediate periparturient period of all cattle, regardless of disease status.[7-9] Nevertheless, systemic inflammation may also be a factor in the pathophysiology of APM and studies have shown that cattle with APM have significantly higher concentrations of LPS in circulation than healthy cattle and that

inflammatory mediators produced during the course of the disease have a dramatic effect on pituitary and ovarian function, embryonic survival, and overall reproductive performance.[10]

Recent work in humans and mice has focused on the role of Indoleamine-2,3-Dioxygenase (IDO) in the systemic inflammatory response syndrome (SIRS) and sepsis. IDO is an intracellular enzyme that catabolizes tryptophan into kynurenine. IDO activity is associated with immunosuppression and immune attenuation. Clinically, IDO activity has been used to predict mortality in bacteremic human patients.[11] In this study, non-surviving patients were more likely than surviving patients to have higher indices of IDO activity.[11] In addition, high IDO activity was found to remain as an independent risk factor for mortality even when confounders were removed from the study.[11] Other studies using mouse models of LPS induced shock have shown that blockade of IDO improved survival significantly.[12] Little research has been performed to evaluate the role of IDO in diseases of cattle. It has been shown that IDO is required for *in vitro* inhibition of *N. caninum* in bovine fibroblasts and endothelial cells.[13] In addition, one study demonstrated that IDO expression and activity are increased in the endometrium of cattle 18 days pregnant when compared to non-pregnant controls.[14] Furthermore, it has been shown that IDO gene and protein expression is increased in monocytes infected with *Mycobacterium avium* subsp *paratuberculosis*, implicating the enzyme in contributing to the persistence of the organism within the animal.[15] Bacteremia has been shown to occur in approximately 32% of cattle with acute coliform mastitis and, the presence of bacteremia significantly affects survival.[16] In addition bacteremia was recently identified in 53% of healthy cattle and cattle with APM. However, to date, no one has evaluated the expression or activity of IDO in cattle with APM or bacteremia associated with parturition and uterine involution.

The purpose of the study reported here was to use real-time qRT-PCR and HPLC assays to quantify the expression of 6 genes encoding cytokines and IDO and to quantify the activity of IDO in serum of cattle with and without APM and bacteremia. It was hypothesized that gene expression and IDO activity would be associated with disease status and presence of bacteremia.

MATERIALS AND METHODS

Animals

Cows at 3 dairies in Northeast and Southwest Georgia that developed APM between September 2011 and November 2013 were eligible for inclusion in the study. During the study, there were approximately 9,000 Holstein and Holstein cross cows in lactation at the 3 dairies. Cows were fed a total mixed ration (TMR) in groups based on production and were milked in a parlor 3 times daily. All cows were housed in drylot pens or freestall barns.

Inclusion criteria and data collection

Fresh cows at each dairy were examined daily by 1 of the authors (BCC). For inclusion in the study, cattle had to be enrolled in a fresh cow monitoring program. For the purposes of this study, APM was diagnosed if a cow was less than 10 days in milk, had a fetid, watery, reddish-brown uterine discharge, and systemic signs of illness that included at least one or more of the following: fever (rectal temperature > 103F), obtundation, toxemia (injected mucous membranes, tachycardia (heart rate > 84 beats per minute), or tachypnea (respiratory rate > 36 breaths per minute). Cows were evaluated only at the time of initial diagnosis. Any animals with evidence of other concurrent disease processes (mastitis, abomasal displacement, respiratory disease, diarrhea) were excluded from the study. Healthy herdmates, as determined

by a complete physical examination, were matched to cattle with APM by parity and days in milk (DIM), and enrolled as controls. Control cattle were monitored for the development of APM daily until day 10 after parturition. Any control that subsequently developed APM was removed from the study.

Bacteriologic culture of blood

Blood samples were aseptically collected from the jugular vein at the time of diagnosis from both cattle with APM and controls and submitted for aerobic and anaerobic blood culture. For each cow, the hair over the jugular vein was shaved using a battery-operated clipper with a #40 blade. The skin was disinfected with at least 3 alternating applications of a 4% chlorhexidine surgical scrub and 70% isopropyl alcohol. Thirty-five milliliters of blood was aseptically drawn from the vein into a 35-ml syringe through a 16-ga, 1-½ inch needle. Ten milliliters of blood was injected aseptically through a new 16-ga, 1-½ in needle into a 30 ml culture vial of 3.0% soybean casein digest broth containing 0.05% sodium polyanetholsulfonate[b] and a 40 ml culture vial of 2.75% soybean-casein digest broth containing 0.035% sodium polyanetholsulfonate[c]. Samples were aerated through a filtered needle and incubated at 37°C in an atmosphere containing 10% CO_2. Samples were subcultured onto blood agar on days 0, 1, and 7 and incubated at 37°C in an atmosphere containing 10% CO_2. Plates were examined for growth and recorded at each observation.

Blood collection, leukocyte isolation, and RNA extraction

Sixty milliliters of blood was obtained from the jugular vein of each cow via venipuncture with a sterile 16-gauge hypodermic needle and a sterile syringe containing 1.5 mL of 0.1 M EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated by single-step density separation. Briefly, needles (18 gauge) were attached to sterile 60-mL syringes for use as controlled layering devices. These were placed at a slant into the mouth of 50-mL centrifuge tubes containing 20 mL of a single-density gradient (1.077 g/mL)[a]. Whole blood was placed slowly in the syringes in 30-mL aliquots, allowing the blood to form layers over the density gradient. Tubes were centrifuged at 2,220 rpm for 45 minutes. The mononuclear cell layer was removed from the interface between the plasma and density gradient. PBMCs were pelleted by centrifugation at 1,800 rpm for 15 minutes. The supernatant was discarded and contaminating erythrocytes removed by hypotonic lysis. The cells were centrifuged twice at 1,800 rpm for 10 minutes and washed in sterile PBS solution. The supernatant was discarded, the leukocytes suspended in 10 mL of PBS solution and a 50-μL aliquot of this cell suspension was transferred to a vial containing 450 μL of a 0.04% solution of trypan blue dye[b] for viability assessment on the basis of dye exclusion by leukocytes. Viable leukocytes were quantified microscopically by use of a hemacytometer and suspended at a concentration of 2×10^7 cells/mL in sterile PBS. One-milliliter aliquots of this leukocyte suspension were transferred to sterile microcentrifuge tubes. Tubes were immediately centrifuged at 14,000 rpm for 1 minute. The supernatant was discarded, and the microcentrifuge tubes containing the pelleted cells were stored at -80°C until RNA extraction. Total RNA was extracted from thawed leukocyte samples by use of a commercial kit[c] in accordance with the manufacturer's protocol with the addition of a

deoxyribonuclease I[d] digestion step to remove residual genomic DNA. Assessment of RNA concentration and quality was performed by spectrophotometry[e].

Primer design and real-time qRT-PCR assay conditions

Expression of 6 genes associated with systemic inflammatory responses was targeted for quantification. These 6 genes encoded TNF-α, IL-1β, IL-4, IL-6, IL-10, and IDO. Gene sequences for these targets were identified and downloaded from the GenBank nucleotide sequence database. Optimal primers were designed by use of a commercial software system[f] and synthesized (Table 4.1). Full validation of the SYBR green real-time qRT-PCR assay for the genes was performed with RNA isolated from LPS-stimulated bovine leukocytes.

Complementary DNA strands were synthesized from extracted RNA samples by use of a commercial kit[g] and thermal cycler[h]. The real-time qRT-PCR assay was performed by use of SYBR green nucleic acid stain[i] in a sequence detection system[j]. Sample denaturation was conduction at 95 C for 15 seconds followed by a combined annealing and extension phase at 60 C for 60 seconds. A commercial 18S ribosomal RNA kit[k] was used as an endogenous control template. The results of the real-time qRT-PCR assay for each sample were provided as the relative expression ratio as described by Pfafll:[17]

$$\text{ratio} = (E_{target})^{\Delta CTtarget\ (control\text{-}sample)} / (E_{ref})^{\Delta CTref(control\text{-}sample)}$$

where E_{target} is the real-time PCR efficicency of the target gene transcript, E_{ref} is the real-time PCR efficiency of the reference gene transcript, ΔCT_{target} is the CT deviation of control-sample of the target gene transcript, and ΔCT_{ref} is the CT deviation of control – sample of the reference gene transcript. The qRT-PCR assay was performed in triplicate for each cDNA sample aliquot.

Determination of Indoleamine-2,3-Dioxygenase activity in bovine serum

Serum concentrations of kynurenine, tryptophan, and the kynurenine/tryptophan ratio were determined by high performance liquid chromatography (HPLC) analysis using a standard curve as described previously.[18] Briefly, 100 µl of serum was diluted with 100 µl of 50 µmol/L 3-nitro-l-tyrosine, and, after the addition of 25 µl of 2 mol/L tricholoroacetic acid, the reaction vials were mixed. Samples were centrifuged at 12,000 x *g* for 6 minutes at room temperature to precipitate and separate proteins. Supernatants (180 µl) were transferred to microsampling vials and placed in screw-cap vials for autosampling. 25 µl of sample was injected onto a C18 reverse phase column with a flow rate of 0.9 mL/min. Kynurenine and 3-nitro-l-tyrosine were measured by UV detection at 360 nm. Tryptophan was measured by fluorescence detection at an excitation wavelength of 286 nm and an emission wavelength of 366 nm. The concentrations of components were calculated according to peak heights and were compared with 3-nitro-l-tyrosine as the internal standard.

Statistical analysis

Cattle were grouped by disease status (APM vs control) and presence or absence of bacteremia. For relative gene expression, data were tested for normality using the Kolmogorov-Smirnov test and log transformed prior to analysis. Relative gene expression was compared between control cattle and cattle with APM using a paired t-test. Relative gene expression was compared between bacteremic and non-bacteremic cattle by use of student's t-test. Serum tryptophan, kynurenine, and kynurenine/tryptophan ratio were compared between healthy controls and cattle with APM by use of the Wilcoxon signed-rank test. Serum tryptophan, kynurenine, and kynurenine/tryptophan ratio were compared between bacteremic and non-

bacteremic cattle by use of the Wilcoxon rank sum test. A P-value of < 0.05 was considered significant for all variables tested. All analyses were conducted by the use of a commercially available statistical software program[1].

RESULTS

Cytokine and IDO mRNA Expression in Peripheral Blood Mononuclear Cells

The relative expression of IL-1β in cattle with APM was significantly lower than that in controls (Table 4.2, Figure 4.1). No significant difference was found in the relative expression of other genes tested (Table 4.2, Figure 4.1). The relative expression of both IL-4 and IL-6 in bacteremic cattle tended to be higher than that in non-bacteremic cattle, however, these values did not reach predefined levels of significance (Table 4.3, Figure 4.2). No significant difference in relative expression of other genes tested (Table 4.3, Figure 4.2)

IDO Activity in Serum

Serum concentrations of tryptophan and kynurenine were not significantly different between cattle with APM and controls (Table 4.4). Activity of IDO, as determined by the serum kyn/trp ratio, was not significantly different between cattle with APM and controls. Serum concentrations of tryptophan and kynurenine were not significantly different between bacteremic cattle and non-bacteremic cattle (Table 4.5). Activity of IDO in serum was not significantly different between bacteremic cattle and non-bacteremic cattle (Table 4.5).

DISCUSSION

Several studies have documented alterations in peripheral leukocyte populations and function in periparturient dairy cattle.[4,5,7,8] These changes have been implicated in the high risk of infectious diseases such as APM and mastitis encountered during the immediate post-calving period. Here, we evaluated the inflammatory cytokine and IDO response in cattle with APM and cattle with bacteremia in an attempt to improve our understanding of the role of peripaturient immunosuppression on the subsequent development of APM in dairy cattle. In addition, we sought to better characterize the consequences of APM and bacteremia on the systemic inflammatory response in affected cattle. In this study, expression of several pro- and anti-inflammatory cytokines and IDO was evaluated in peripheral blood mononuclear cells isolated from healthy and diseased cattle. Overall, cattle with APM had significantly lower levels of IL-1β expression than healthy controls. In addition, bacteremic cattle tended to have higher levels of IL-4 and IL-6 expression than non-bacteremic cattle, although the relative levels of expression did not reach statistical significance.

Galvao et al found that cattle that subsequently developed APM had higher levels of expression of TNF-α, IL-1β, and IL-6 in isolated monocytes than cattle that remained healthy.[6] Here, blood was collected 12-24 hours after calving rather than at the time of diagnosis and it was hypothesized that negative energy balance or excessive tissue trauma may have activated the immune system. In this same study, cattle that subsequently developed APM had significantly lower levels of TNF-α expression in *E. coli* stimulated monocytes.[6] It is possible that the lower levels of IL-1β expression seen in PBMCs in cattle with APM in this study could result from alterations in immune responsiveness secondary to negative energy balance, hormonal changes, a compensatory anti-inflammatory response, or immune exhaustion. Indeed, the compensatory

anti-inflammatory response syndrome (CARS) has become a topic of intense investigation in septic human patients.[19] CARS develops as a response to inflammation arising from tissue damage or bacterial invasion. The immune system attempts to balance the need for continued defense while preserving tissue integrity.[20] In CARS, lymphocyte, hepatocyte, and endothelial cell apoptosis is increased.[21] In addition, the removal of neutrophils from the circulation is impaired while their function is decreased.[22,23] Moreover, monocytes from septic patients display increased expression of inhibitors of NF-kB and have impaired responses to danger associated molecular patterns (DAMPs).[20,24] Monocytes also display reduced expression of MHC-II on their surface, a factor that impairs antigen presentation to adaptive immune cells.[25] In fact, failure to regain > 70% of surface MHC-II expression is a poor prognostic indicator.[20,25] A recent study in septic human patients showed that, while plasma concentrations pro-inflammatory compounds such as TNF-α, IL-1β, and IL-6 are initially high in affected individuals, concentrations of each cytokine decreased significantly over time.[26] In addition, the expression of MHC II on the surface of antigen presenting cells and the proportion of both $CD4^+$and $CD8^+$ cells in circulation decreased during the monitoring period.[26] Therefore, it is possible that animals with APM, while initially exhibiting profiles of excessive immune activation, would be more likely to develop disease due to further impairment in the immune system brought about by inflammation induced impairment of both local and systemic inflammatory responses.

In this study, there were no significant differences in relative gene expression detected between groups for IDO or any of the cytokines tested. Nevertheless, there was a trend for higher levels of expression of both IL-4 and IL-6 in bacteremic cattle. It is possible that, with a larger group of cattle, that these differences would have been significant. Traditionally, IL-4 has been considered one of the prototypical cytokines of the T_H2 immune response, a pattern of T-

cell differentiation thought to be an important component of allergic and anti-parasitic responses in mammals.[27] In addition, T_H2-based immune responses promote the development of alternatively activated macrophages, suppress interferon-γ mediated classical macrophage activation, and inhibit defense against intracellular microbes.[27] Additionally, T_H2 based immune responses play an important role in protection of mucosal surfaces by driving mucous production and mucosal antibody production.[28] Newer research would suggest, however, that IL-4 plays a role in B-cell activation and acts synergistically with CD40 to drive clonal expansion of B-cells that precedes immunoglobulin production.[29] IL-6 is one of the prototypic pro-inflammatory cytokines and is responsible for B-cell differentiation, T-cell growth and differentiation, induction of acute phase protein synthesis, and induction of the febrile response.[27] Interestingly, IL-6 seems to work synergistically with IL-4 to drive T-cell differentiation to the T_H2 subtype.[30,31] Given the findings of this study, it is possible that bacteremic cattle are being driven towards a T_H2-biased immune response for reasons unknown. A study with larger numbers of animals will be needed to confirm these results.

IDO has received significant attention for its role in immunomodulation.[32] In cattle, little work has been performed to evaluate the role of IDO in both normal and pathologic states. While studies would suggest that IDO plays a role in protection against intracellular pathogens such as *Neospora caninum*, other studies have found that IDO may allow persistence of *Mycobacterium avium* subsp *paratuberculosis* within mononuclear cells of both cattle and sheep.[13,15] In addition, IDO may have a role in recognition of pregnancy and promoting tolerance to the fetal allograft.[14] Activity of IDO in bacteremic human patients has been shown to be significantly greater than that of blood-culture negative patients and IDO has been shown to be a predictor of survival in these patients.[11] In addition, inhibition of IDO in a mouse model of sepsis

was shown to significantly improve survival when compared to controls.[12] In this study, no differences were found between IDO gene expression nor IDO activity in any group of animals. Therefore, it appears as though IDO does not have a role in either APM or bacteremia associated with parturition or uterine involution.

In conclusion, the results of this study would suggest that cattle with APM may be driven towards impaired pro-inflammatory responses, as witnessed by significantly lower expression of IL-1β in isolated peripheral blood mononuclear cells from affected cattle. In addition, although not statistically significant, bacteremic cattle tended to have higher levels of expression of both IL-4 and IL-6, a finding that suggests a bias towards a T_H2 immune response. Future studies that evaluate the cytokine gene expression of cattle with APM over time and with larger numbers of animals are warranted to confirm these findings and better characterize the systemic immune response of cattle with APM.

FOOTNOTES

a. Histopaque-1077, Sigma-Aldrich, St. Louis, MO
b. Trypan blue, Sigma-Aldrich, St. Louis, MO
c. RNeasy, QIAGEN, Inc, Germantown, MD
d. TURBO DNase, Applied Biosystems Inc, Foster City, CA
e. ND-1000 UV-Vis Spectrophotometer, NanoDrop Technologies, Wilmington, DE
f. Primer Express Software, Applied Biosystems, Inc, Foster City, CA
g. High Capacity cDNA Reverse Transcription Kit, Applied Biosystems Inc, Foster City, CA
h. Mastercycler Gradient, Eppendorf, Westbury, NY

i. SYBR Green PCR Master Mix, Applied Biosystems, Inc, Foster City, CA

j. 7900HT Fast Real-Time PCR System, Applied Biosystems, Inc, Foster City, CA

k. 18S rRNA Taqman, Applied Biosystems, Inc, Foster City, CA

l. Stata, Version 12, StataCorp, LP, College Station, TX

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Table 4.1. Primer sequences for genes of interest

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	Accession number
TNF-α	GGCTCCAAGCATCCAATTTAA	CAGTCCTTGATGGTGGTTGGT	NM_173966
IL-1β	AGCATCCTTTCATTCATCTTTGAAG	GGGTGCGTCACACAGAAACTC	M37211
IL-4	CAACGAGAAAGAATTCATGC	CAAGTCCGCACCAGGAATTTG	P30367
IL-6	GGGCTCCCATGATTGTGGTA	GTGTGCCCAGTGGACAGGTT	BC123577
IL-10	ACTTTAAGGGTTACCTGGGTTG	GAAAGCGATGACAGCGCCGC	U00799
IDO	CGAATATACTTGTCTGGTTGG	GGAGAACATCAAAGCACTG	NM_001101866

Table 4.2. Comparison of relative gene expression (log_{10}) as assessed by RT-qPCR in control cattle and cattle with APM. Data are expressed as mean ± SD.

	Group		
Gene	**APM**	**Control**	**P-value**
TNF-α	3.2 ± 1.4	3.3 ± 1.9	0.815
IL-1β	5.1 ± 1.9	6.9 ± 1.7	0.016
IL-4	4.4 ± 3.4	5.5 ± 3.4	0.327
IL-6	4.1 ± 2.9	4.8 ± 2.9	0.436
IL-10	3.6 ±2.4	4.7 ± 2.1	0.288
IDO	3.9 ± 2.9	5.3 ± 1.8	0.182

Table 4.3. Comparison of relative gene expression (log_{10}) as assessed by RT-qPCR in bacteremic and non-bacteremic cattle. Data are expressed as mean ± SD.

	Group		
Gene	**Bacteremic**	**Non-bacteremic**	**P-value**
TNF-α	3.4 ± 1.5	2.5 ± 1.4	0.147
IL-1β	5.8 ± 2.2	5.0 ± 2.3	0.381
IL-4	5.4 ± 3.3	3.3 ± 2.4	0.079
IL-6	4.8 ± 2.8	3.0 ± 2.3	0.093
IL-10	3.9 ± 2.5	2.7 ± 1.9	0.203
IDO	4.2 ± 2.9	3.2 ± 2.3	0.317

Table 4.4. Serum tryptophan (trp), kynurenine (kyr), and kynurenine/tryptophan ratio (kyn/trp) (median, 10^{th} and 90^{th} percentile) in cattle with and without acute puerperal metritis.

	Group		
Paramter	**APM**	**Control**	**P-value**
Trp (μmol/L)	12.7 (5.8-18.9)	17.6 (7.4-30.7)	0.285
Kyn (μmol/L)	2.6 (1.3–5.8)	4.3 (2.2-6.8)	0.241
Kyn/Trp	0.22 (0.12-0.32)	0.23 (0.15-0.55)	0.333

Table 4.5. Serum tryptophan (trp), kynurenine (kyr), and kynurenine/tryptophan ratio (kyn/trp) (median, 10^{th} and 90^{th} percentile) in bacteremic and non-bacteremic cattle.

	Group		
Paramter	**Bacteremic**	**Non-bacteremic**	**P-value**
Trp (μmol/L)	13.2 (7.7-30.7)	15.4 (5.8-21.6)	0.956
Kyn (μmol/L)	3.8 (1.5-6.9)	3.6 (1.5-4.9)	0.298
Kyn/Trp	0.23 (0.15-0.53)	0.22 (0.12-0.47)	0.622

Figure 4.1. Box and whisker plots showing relative expression of cytokine and IDO genes from peripheral blood mononuclear cells isolated from cattle with acute puerperal metritis (APM) and control (Con) cattle.

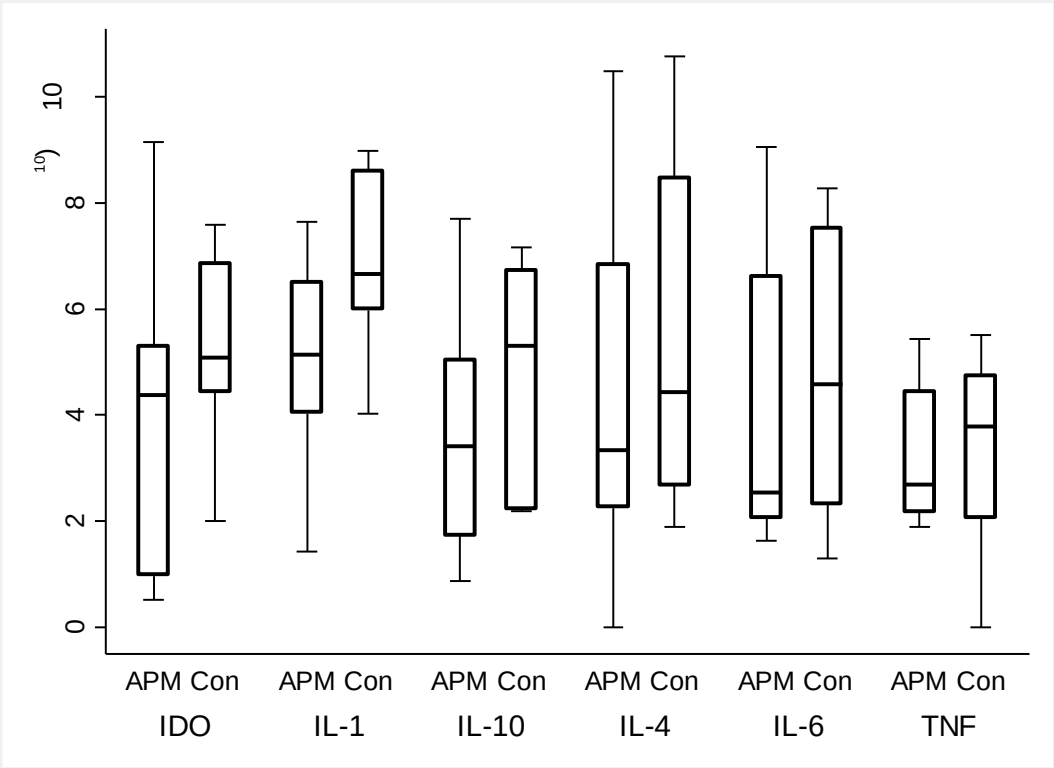
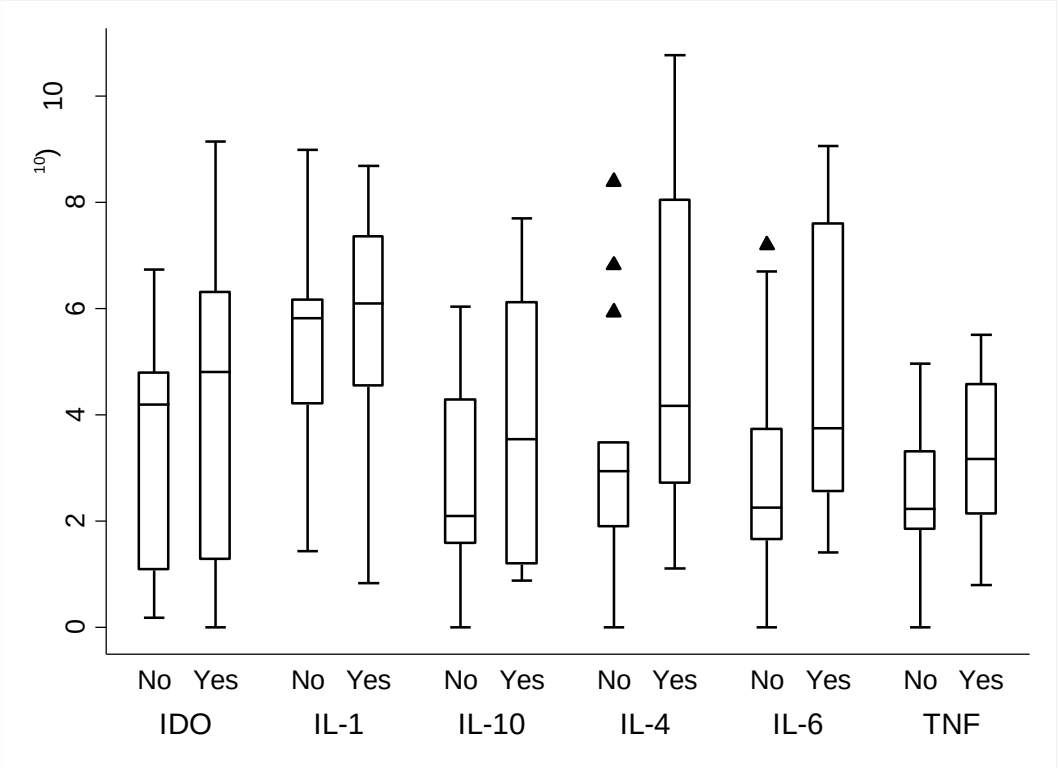


Figure 4.2. Box and whisker plots showing relative expression of cytokine and IDO genes from peripheral blood mononuclear cells isolated from bacteremic (Yes) and non-bacteremic (No) cattle.



CHAPTER 5

DISPOSITION OF AMPICILLIN TRIHYDRATE IN PLASMA, MILK, ENDOMETRIAL TISSUE, AND LOCHIAL FLUID IN HEALTHY POST-PARTUM DAIRY CATTLE[3]

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To be submitted to *The Journal of Veterinary Pharmacology and Therapeutics*

ABSTRACT

OJBECTIVE: The objective of this study was to determine the disposition of ampicillin in plasma, uterine tissue, lochial fluid, and milk of post-partum dairy cattle.

MATERIALS AND METHODS: Ampicillin trihydrate was administered intramuscularly (IM) at a dose of 11 mg/kg of body weight every 24 h (n=6, total of 3 doses) or every 12 h (n=6, total of 5 doses) for 3 days. Concentrations of ampicillin were measured in plasma, uterine tissue, lochial fluid, and milk using HPLC with ultraviolet absorption.

RESULTS: Quantifiable ampicillin concentrations were found in plasma, milk, and lochial fluid of all cattle within 30 min, 4 h, and 4 h of administration of ampicillin trihydrate, respectively. There was no significant effect of dosing interval (every 12 versus every 24 h) and no significant interactions between dosing interval and sampling site on the pharmacokinetic variable measured or calculated. Median peak ampicillin concentration at steady state was significantly higher in lochial fluid (5.27 µg/mL after q 24 h dosing) than in other sample types and significantly higher in plasma (3.11 µg/mL) than in milk (0.49 µg/mL) or endometrial tissue (1.55 µg/mL). CONCLUSIONS AND CLINICAL RELEVANCE: Ampicillin trihydrate administered once daily by the IM route at the label dose of 11 mg/kg of body weight achieves therapeutic concentrations in the milk, lochial fluid, and endometrial tissue of healthy post-partum dairy cattle. Twice daily administration does not provide any advantages over once daily dosing.

INTRODUCTION

Dairy cattle are susceptible to numerous infectious and metabolic disorders in the immediate post-parturient period. Acute puerperal metritis (APM) is one of the most common clinical conditions seen in modern dairy cattle, affecting 18.3-33.5% of all cattle that calve.[1] Consequences of APM include reduced milk production, impaired reproductive performance, increased culling risk and, in severe cases, death. The economic impact of APM has been studied extensively and current estimates suggest that the disease can cost individual producers $358 per case and the United States dairy industry $650,000,000 over the course of a single year.[2,3]

After calving, more than 90% of cattle experience some degree of contamination of the uterine lumen with bacteria.[3] Through the processes of uterine involution and normal immune function, most cattle clear this contamination and experience no complications. However, cattle with retained placenta, hypocalcemia, and significant negative energy balance fail to clear uterine contamination and develop APM.[3] While a variety of microorganisms may be isolated from the reproductive tract of both healthy post-parturient cattle and cattle with APM, *E. coli* and *Trueperella pyogenes* represent the bacteria most commonly associated with clinical disease.[1]

Antimicrobials are a mainstay of therapy for cattle with APM. Currently, three antimicrobials are labeled for systemic use in cattle with APM: oxytetracycline dihydrate (Liquamycin LA200), ceftiofur hydrochloride (Excenel RTU), and ceftiofur crystalline free acid (Excede). The United States Food and Drug Administration (FDA) recently passed an order prohibiting extra-label use of cephalosporins in major food producing species because of concerns over the development of resistance to similar 3rd generation cephalosporin compounds commonly used in critically ill human patients. While the use of these two ceftiofur based medications is allowed for cattle with APM, it is prudent to consider and investigate the use of

other antimicrobials for cattle with this important clinical disease to preserve their efficacy in both critically ill veterinary and human patients.

Ampicillin trihydrate is an aminobenzyl penicillin labeled for the therapy of infections in cattle and calves caused by susceptible strains of *Aerobacter* spp, *Klebsiella* spp, *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, and *Pasteurella* spp.[4] In addition, the activity of ampicillin against anaerobes and some organisms encountered in cattle with APM is, in some cases, broader than that of ceftiofur.[4] A recent clinical trial comparing ampicillin sodium to ceftiofur hydrochloride in cattle with APM demonstrated comparable clinical efficacy between the two compounds, suggesting that ampicillin trihydrate may be a useful alternative to currently available antimicrobials used to treat APM.[5] In addition, economic modeling has shown that, when used at current label dose and dosing frequency, ampicillin trihydrate costs less than ceftiofur hydrochloride when treating APM.[2] Furthermore, ampicillin has much less value as a first line antimicrobial in critically ill veterinary and human patients, allowing veterinarians, producers, and other healthcare providers to preserve later generation cephalosporins for use in more severely affected individuals.

While emerging evidence would suggest that ampicillin trihydrate would have value as a therapeutic agent in dairy cattle with APM, the disposition of the drug in plasma, uterine tissue and lochial fluid has not been evaluated in post-parturient dairy cattle, precluding the development of rational dosing regimens for this compound. Thus, the objectives of the study reported here were to determine the disposition of ampicillin trihydrate in plasma, uterine tissue, lochial fluid, and milk following once and twice daily intramuscular administration to healthy post-partum dairy cattle.

MATERIALS AND METHODS

Animals

12 Holstein and Holstein cross cattle between 2 and 6 years of age and weighing between 364 and 654 kg were selected for this study. All animals calved within 24 hours of enrollment in the study. The cattle were considered healthy on the basis of physical examination, complete blood count, and plasma biochemical profile. The cattle were milked 3 times daily and housed in a free-stall barn for the duration of the study. All procedures were approved by the Clinical Research Committee of the University of Georgia.

Study Design and Sample Collection

Ampicillin trihydrate in its market formulation[a] was administered at a dose of 11 mg/kg of body weight every 24 hours for 3 days via the intramuscular route in the cervical musculature of 6 cattle and at a dose of 11 mg/kg of body weight every 12 hours for 3 days via the intramuscular route in the cervical musculature of a second group of 6 cattle. No more than 10 ml of suspension was deposited in any one site and each injection site was separated by 3 inches. Blood samples for plasma separation were obtained from the jugular vein via direct venipuncture at 30 minutes and at 1, 2, 4, 6, 8, 12, 24, 48, 48.5, 49, 50, 52, 56, 58, 60, and 72 hours after initial drug administration in the 24 h dosing interval group and at 30 minutes, 1, 2, 4, 6, 8, 12, 48, 48.5, 49, 50, 52, 56, 58, and 60 hours after initial drug administration in the 12 h dosing interval group.

Lochial fluid and milk were collected at 2, 4, 8, 12, 24, 50, 52, 56, 60, and 72 hours after initial drug administration in the 24 h dosing interval and at 2, 4, 8, 12, 50, 52, 56, and 60 hours after initial drug administration in the 12 h dosing interval. Uterine biopsy was performed in all

cattle in both dosing internals at 50, 52, 56, 60, and 72 hours after initial drug administration. All samples were stored at -80°C until analysis.

Collection of milk, lochial fluid and endometrial tissue

Each teat end was cleaned by wiping with 70% isopropyl alcohol prior to sampling. The foremilk was removed from each quarter of the mammary gland and 1 mL of milk from each quarter was collected into 4 mL plastic tubes. The animal's tail was held to the side and the external genitalia cleaned with alternating applications of 4% chlorhexidine and 70% isopropyl alcohol. A gloved hand was inserted into the uterine lumen and 4 mL of lochial fluid was collected. Endometrial tissue (approximately 4 g) was collected using the Hauptner equine endometrial biopsy instrument.[b]

Measurement of ampicillin concentrations

The concentration of ampicillin in plasma, milk, uterine tissue and lochial fluid was measured using a validated analytical procedure based on modifications of a method reported previously by Nelis and coworkers.[6] For plasma and milk samples, aliquots (1 mL) were transferred into clean glass tubes containing 0.05 mL of cephalexin (20 µg/mL) as internal standard. Milk samples were mixed with 2 mL of 0.067M K_2HPO_4 (pH 4.0), vortex mixed and centrifuged for 20 min at 2200 × g (room temperature). The supernatant from milk extracts was removed and the extraction repeated and the supernatant fractions were combined. Extracts of milk and plasma samples were transferred onto C-18 SPE columns that were preconditioned with 2 mL each of methanol, water and 0.067M phosphate buffer. Columns were rinsed with 0.067M phosphate buffer (2 mL) prior to analyte elution with methanol (2 mL for plasma and 2.5 mL of

milk extract). Methanolic extracts were dried under nitrogen stream at 40°C, reconstituted in 1 mL of phosphate buffer and passed through 0.45 micron syringe filter prior to analysis.

Lochial fluid and endometrial tissue samples were prepared in a different manner in order to remove interfering peaks. In brief, samples were weighed and approximately 1 g was placed in 10 mL of acetonitrile:water mixture (90:10) and homogenized with a Polytron tissue digester (30 sec at setting 20) prior to centrifugation (30 min at 2500 × g). Aliquots of supernatant were transferred to clear plastic tubes and mixed with hexane (10 mL) by vigorous shaking for 1 min prior to centrifugation for 30 min at 2500 × g. The hexane layer (top) was discarded and the lower layer was transferred to a clean glass tube and dried under nitrogen at 50°C prior to reconstitution and filtering as described above. The concentration of ampicillin in all sample extracts was determined by high-performance liquid chromatography with ultraviolet absorption (210 nm). Ampicillin and internal standard were separated using an ODS Hypersil C-18 (5 μm) analytical column (4.6 x 250 mm, Thermofisher) and a multi-step gradient with mobile phases containing 94:6 (0-35 min), 50:50 (35-45 min) and 94:6 (45-75 min) mixtures of 0.067M phosphate buffer (pH 4): acetonitrile at a flow rate of 1.0 mL/min. Peak areas were compared to a standard curve for ampicillin (0.025 – 10.0 mcg/mL, 8 non-zero concentrations) that exhibited good linearity (R^2 values above 0.98 and a RSD < 3.0% at 1 μg/mL. The limit of detection (LOD) was 0.05 μg/mL for plasma and milk and 0.10 μg/mL for lochial and endometrial samples. The lower limit of quantification (LLOQ) was 0.25 μg/mL in all matrices as determined by guidelines set forth in Guidance for Industry #145 (Bioanalytical Method Validation) from the FDA Center for Drug Evaluation and Research.

Pharmacokinetic analysis

For each cow, plasma, milk, lochial fluid, and endometrial tissue median ampicillin trihydrate concentration vs. time data were analyzed based on noncompartmental pharmacokinetics using commercial software.[c] For plasma samples, the rate constant of the terminal phase (λ_z) was determined by linear regression of the terminal phase of the logarithmic plasma concentration versus time curve using a minimum of 3 data points. Half-life of the terminal phase ($t_{½\lambda z}$) was calculated as ln 2 divided by λ_z. The area under the concentration-time curve (AUC) was calculated using the trapezoidal rule. For each animal, maximum concentration (C_{max}), time to maximum concentration (T_{max}), and concentration at the last sampling time (C_{last}) were calculated for each type of sample.

Statistical analysis

Normality of the data and equality of variances were assessed using the Shapiro-Wilk and Levene's tests, respectively. Variables that did not meet the assumptions for parametric testing were rank-transformed prior to analysis. A two-way ANOVA with one factor repetition was used to assess the effect of sampling type (plasma, milk, lochial fluid, endometrial tissue), dosing interval (12 h vs 24 h) and interactions between sample type and dosing interval each on measured and calculated pharmacokinetic variable. When applicable, multiple pairwise comparisons were made using the Holm-Sidak method. Differences were considered significant at $P < 0.05$. All analyses were conducted by the use of a commercially available statistical software program.[d]

RESULTS

No adverse effects were noted in any cow during the course of the study. Quantifiable ampicillin concentrations were found in plasma, milk, and lochial fluid of all cattle within 30 min, 4 h, and 4 h of administration of ampicillin trihydrate, respectively. There was no significant effect of dosing interval (q 12 versus q 24 h) and no significant interactions between dosing interval and sampling site on C_{max}, T_{max}, AUC_{0-t}, $t_{1/2}$, or C_{last} (Table 5.1) after administration of the first or last dose of ampicillin trihydrate. The plasma concentration versus time profile for both dosing intervals is presented in Figure 5.1. After administration of the first dose, there was a significant effect of sample type on C_{max} (P = 0.004), AUC_{0-t} (P = 0.007), and C_{last} (P = 0.017). Median C_{max}, AUC_{0-t}, and C_{last} were significantly higher in lochial fluid than in plasma or milk (Table 5.1).

The AUC extrapolated to infinity after administration of the first dose was not significantly higher than the AUC_{0-t} after administration of the last dose, indicating that steady state plasma concentrations were reached. After administration of the last dose, there was a significant effect of sample type on C_{max} (P = <0.001), T_{max} (P = 0.031), AUC_{0-t} (P = <0.001), and C_{last} (P = 0.016). Median C_{max} was significantly higher in lochial fluid than in other sample types and significantly higher in plasma than in milk or endometrial tissue (Table 5.1). Median AUC_{0-t} was significantly higher in lochial fluid than in other sample types (Table 5.1). Median T_{max} was significantly higher for endometrial tissue than for plasma and median C_{last} was significantly higher for lochial fluid than for endometrial tissue (Table 5.1).

DISCUSSION

Because ampicillin is active against many bacterial pathogens associated with APM and because a recent data indicate that ampicillin trihydrate is effective for the treatment of APM we

investigated the disposition of ampicillin trihydrate in plasma, uterine tissue, and lochial fluid of post-partum dairy cattle. Median peak plasma concentrations obtained in the present study (1.66 to 2.61 μg/mL) were lower than those previously reported after intramuscular administration of ampicillin trihydrate at a dose of 7.7 mg/kg to calves (3.7 μg/mL) but elimination half-life was similar (3.7 h).[7] Median peak milk concentrations obtained in the present study (1.63 to 2.12 μg/mL) were higher than those reported after intramuscular administration of an oil based suspension of ampicillin trihydrate at a dose of 12.5 mg/kg to cows with normal (0.03 μg/mL) and mastitic (0.2 μg/mL) milk.[8] The bioavailability of intramuscular ampicillin trihydrate was not evaluated in the present study. In calves, the bioavailability of ampicillin trihydrate after intramuscular administration is nearly 100%.[7]

Development of an optimal dosing regimen for a specific antimicrobial is dependent on both the pharmacokinetic and the pharmacodynamic parameters of the drug being used. The most important factor determining the efficacy of β-lactam antimicrobials such as ampicillin trihydrate is the amount of time that concentrations of the drug exceed the MIC (minimum inhibitory concentration) against given pathogen.[9] The goal of therapy with time dependent antimicrobials is to maintain concentrations of drug above the MIC of the infecting organism for the entirety of the dosing interval.[9] Increasing the dose of antimicrobial administered and shortening the duration of time between doses are methods that are used to prolong the duration of time that concentrations of drug remains above the MIC of infecting pathogens. Therefore, it is conceivable that shortening the dosing interval of ampicillin trihydrate to every 12 hours could potentially increase the duration of time that ampicillin concentrations are above the MIC of potentially pathogenic bacteria. However, in the present study, administration of ampicillin

trihydrate every 12 h, as compared to every 24 h, did not have any significant effect on the pharmacokinetic variables assessed.

Traditionally, most pharmacokinetic/pharmacodynamic studies evaluating antimicrobials have evaluated concentrations of the drug in plasma as an indicator of potential efficacy. While drug concentration in plasma is clearly a driving force for penetration to the site of infection, the actual drug-concentration time profile at the site of infection may be quite different from that of plasma.[10] The rate and extent of drug penetration into most sites outside the vascular space are also determined by the drug's molecular charge and size, lipid solubility, extent of plasma protein binding, and by blood flow at the site of infection.[11] The difference between total plasma concentration and free tissue concentrations can be substantial, particularly when drug protein binding is high. However, only the free fraction of the antimicrobial in the fluids at the target site is ultimately responsible for therapeutic success.[11] In the uterus, a lipid barrier exists between the vascular compartment and lochial fluid. In the present study, median C_{max} of ampicillin was significantly greater in lochial fluid than in plasma for both the q 12 (75.7 vs 1.66 µg/mL) and q 24 h (55.7 vs 2.61 µg/mL) dosing regimens. In addition, concentrations of ampicillin in lochial fluid in the present study were considerably above the MIC that inhibits 90% of the isolates of *T. pyogenes* (0.25 µg/mL) and *E. coli* (8.0 µg/mL) cultured from cattle (Figure 5.2).[12-14]

In the present study, ampicillin trihydrate was found to concentrate in lochial fluid as compared to plasma with a mean ratio of maximum lochial fluid to plasma ampicillin trihydrate concentration of 5.3. Currently, both ceftiofur hydrochloride and ceftiofur crystalline free acid (CCFA) are labeled for parenteral treatment of cattle with APM. Previous work evaluating ceftiofur hydrochloride has found that, while ceftiofur and its derivatives achieve therapeutic concentrations in both plasma and lochial fluid, the mean ratio of maximum lochial fluid to

plasma ceftiofur concentrations is lower (0.34), suggesting that ceftiofur hydrochloride does not preferentially accumulate in lochial fluid.[15] Studies evaluating CCFA have yielded similar results and found that the maximum lochial fluid to plasma concentration ratio of CCFA was 0.79, a finding that also suggests lack of preferential accumulation in lochial fluid.[16] The preferential accumulation of ampicillin trihydrate in lochial fluid as compared to plasma is important as antimicrobial accumulation at the site of infection is a primary determinant of efficacy and further supports the potential for ampicillin trihydrate as a therapeutic agent in cattle with APM.

Based on the results of the present study, ampicillin trihydrate administered once daily by the IM route at the label dose of 11 mg/kg of body weight achieves therapeutic concentrations in the milk, lochial fluid, and endometrial tissue of healthy post-partum dairy cattle. In addition, administration of ampicillin trihydrate at the label dose once daily is equivalent to twice daily dosing and allows veterinarians to avoid using an extra-label dosing interval that might result in residues in meat and milk of treated cattle. While the use of ampicillin trihydrate to treat cattle with acute puerperal metritis is extralabel, this drug may serve as an alternative to currently available antimicrobials should they become unavailable or ineffective. Further work is needed to characterize the activity of ampicillin against common uterine pathogens of cattle and establish susceptibility breakpoints to better guide therapy.

FOOTNOTES

a. Polyflex injectable suspension, Boehringer-Ingelheim Vetmedica, Inc, St. Joseph, MO
b. Hauptner endometrial biopsy instrument, Jorgensen Laboratories, Loveland, CO
c. PK Solutions 2.0; Summit Research Services, Montrose, CO
d. SigmaPlot 12, Systat Software, Inc, San Jose, CA

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Table 5.1 – Plasma, Milk, Lochial Fluid, and Endometrial tissue pharmacokinetic variables (median and range) after IM administration of ampicillin trihydrate to cows at a dose of 11 mg/kg every 24 h (n=6) or every 12 h (n=6) for 3 days.

Variable	Plasma	Milk	Lochial Fluid	Endometrial Tissue
First dose				
C_{max} (µg/mL)	1.66 (0.91-2.14)[a] 2.61 (0.44-4.5)[a]	1.63 (0.91-3.98)[a] 2.12 (0.93-3.27)[a]	75.7 (3.63-382)[b] 55.7 (6.40-313)[b]	N/A
T_{max} (h)	6 (0.5-8) 4 (2-12)	5 (2-12) 6 (2-24)	3 (2-24) 6 (2-12)	N/A
AUC_{0-t} (µg•hr/mL)	33 (19-88.7)[a] 32.1 (3.8-62.4)[a]	12.4 (7.9-34.4)[a] 31.7 (3.1-46.5)[a]	423 (29.6-2710)[b] 343 (117.3-1510)[b]	N/A
C_{last} (µg/mL)	0.23 (0-0.64)[a] 0 (0-0.34)[a]	1 (0.18-3.98)[a] 1.08 (0-2.57)[a]	13.40 (0-200.6)[b] 6.97 (1-99.03)[b]	N/A
Last dose				
C_{max} (µg/mL)	3.07 (1.67-4.7)[a] 3.11 (2.2-5.4)[a]	0.78 (.68-1.7)[c] 0.49 (0-1.48)[c]	76.7 (29.9-153)[b] 5.27 (2.4-129)[b]	1.25 (0-1.9)[c] 1.55 (0-13.8)[c]
T_{max} (h)	1 (1-4)[a] 3 (1-4)[a]	3 (2-8)[a,b] 6 (2.0-24)[a,b]	4 (2-12)[a,b] 8 (2-24)[a,b]	10 (2-24)[b] 5 (2-12)[b]
AUC_{0-t} (µg•hr/mL)	19.5 (7.6-65.1)[a] 55.6 (24.1-106.3)[a]	21.2 (0.4-56.5)[a] 5.35 (0-53.9)[a]	557 (436-4250)[b] 152 (24.9-5031)[b]	9.8 (0.5-63.5)[a] 35.6 (0.5-183)[a]
C_{last} (µg/mL)	0.64 (0.22-1.34)[a,b] 0.60 (0.13-1.50)[a,b]	0.96 (0.04-1.36)[a,b] 0.24 (0-1.09)[a,b]	16.6 (0-41.7)[a] 2.95 (1.8-29.6)[a]	0 (0-5.1)[b] 0 (0.40-1.1)[b]
$t_{1/2}$ (h)	2.60 (1.21-14.9) 6.36 (4.41-11.7)	N/A	N/A	N/A

$t_{1/2}$ = terminal half-life; AUC_{0-t} = Area under the plasma concentration versus time curve from time 0 to the last quantifiable time point; T_{max} = Time to maximum concentration; C_{max} = Maximum concentration; C_{last} = Last quantifiable concentration. [a,b]Different letters within a row indicate statistically significant differences between sample types ($P < 0.05$).

Figure 5.1. Mean (+ SD) plasma ampicillin concentration (µg/mL) after IM administration of ampicillin trihydrate at a dose of 11 mg/kg every 24 h (n=6) or every 12 h (n=6) for 3 days . Plasma was collected after administration of the first (time 0) and last (48 h) dose.

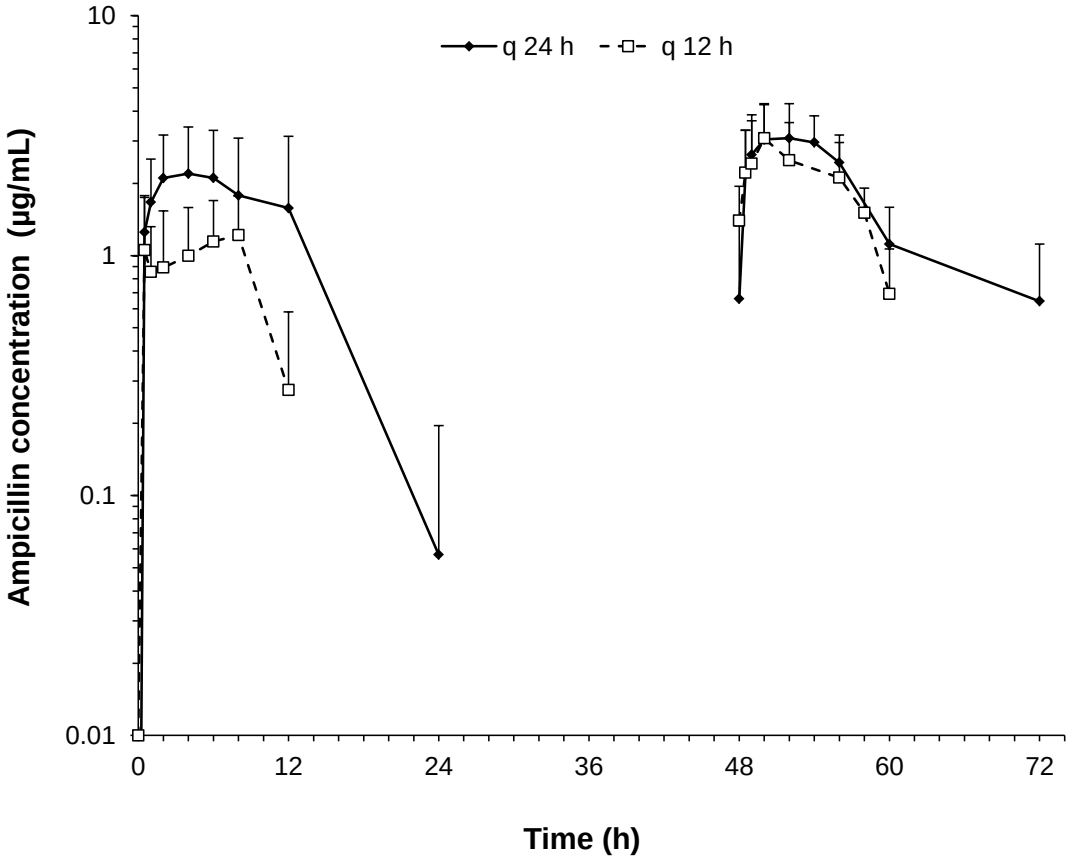
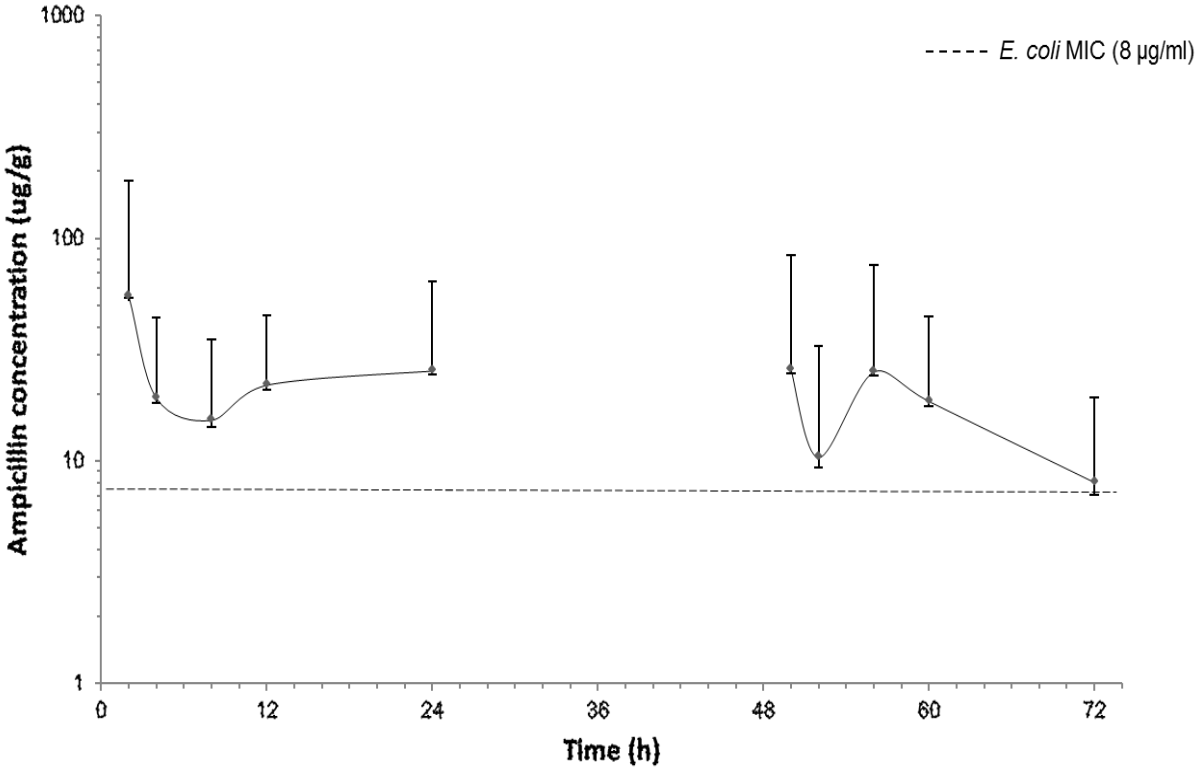


Figure 5.2. Mean (+ SD) lochial fluid ampicillin concentration (µg/g) after IM administration of ampicillin trihydrate at a dose of 11 mg/kg every 24 h for 3 days. Lochial fluid was collected after administration of the first (time 0) and last (48 h) dose. The dotted horizontal line represents the MIC_{90} of *E. coli* isolates cultured from various sites in cattle.



CHAPTER 6

CONCLUSION

Acute puerperal metritis (APM) is one of the leading causes of infertility and decreased milk production in dairy cattle. Bacteremia has been documented in cattle with coliform mastitis, but has yet to be investigated in cattle with APM. Systemic antimicrobial agents are a mainstay of therapy for cattle with APM, with the 3rd generation cephalosporin drug ceftiofur being one of the more commonly administered compounds. However, recent restrictions placed on the use of cephalosporins in food-producing species and concerns about antimicrobial resistance in pathogens of human importance have necessitated the investigation of other antimicrobials for managing this disease. The studies presented herein were undertaken to further understand the systemic consequences of APM in dairy cattle and evaluate the disposition of ampicillin trihydrate in reproductive tissues and fluids of post-partum dairy cattle. This dissertation was designed to evaluate the systemic effects of APM on dairy cattle, particularly the role of immunosuppression and bacteremia. In addition, this dissertation sought to determine if ampicillin trihydrate is a functional therapeutic modality for treating cattle with APM.

Chapter 3 evaluated the prevalence of bacteremia in dairy cattle with acute puerperal metritis. Suprisingly, approximately half of both controls and cattle with APM were bacteremic. This is a novel finding; as such a high prevalence of bacteremia would not be expected in clinically normal cattle. This findings lends credence to the possibility that bacteremia may be a normal occurrence in periparturient cattle, particularly during the course of uterine involution.

To strengthen the clinical relevancy of this study, factors associated with bacteremia that are easily detectable on physical examination should be assessed. This would provide information that could be utilized by practitioners in a field setting that could assist in making therapeutic decisions. In addition, enrolling a larger number of animals to increase the statistical power of the information obtained would be beneficial.

Chapter 4 evaluated the expression of inflammation-associated genes in peripheral blood mononuclear cells and the serum activity of Indoleamine-2,3-Dioxygenase in dairy cattle with and without acute puerperal metritis. The results of this study showed that cattle with acute puerperal metritis have significantly lower levels of IL-1β expression in PBMCs than healthy controls. In addition, bacteremic catle tended to have higher levels of both IL-4 and IL-6 expression than non-bacteremic cattle. This finding would suggest that cattle with APM may have impaired pro-inflammatory responses that place them at greater risk of developing disease. In addition, bacteremic cattle have a tendency towards a T_H2 T-cell differentiation.

To improve the clinical relevancy of this study, a larger number of animals should have been enrolled and a larger number of genes evaluated. In addition, rather than evaluating just gene expression, techniques such as ELISAs could have been used to evaluate the actual production of these compounds. In addition, PBMCs isolated from cattle in the study could have been stimulated with either whole bacteria or LPS to evaluate their response to inflammatory stimulate *in vitro.* These techniques would have given use better characteriziation of the ability of the immune system to responds to stimuli and assess overall immunocompetence.

Chapter 5 evaluated the disposition of ampicillin trihydrate in plasma, uterine tissue, lochial fluid, and milk of healthy post-partum dairy cattle. The results of this study showed that ampicillin trihydrate preferentially accumulates in lochial fluid as compared to plasma. In

addition, once daily dosing was statistically similar to twice daily dosing when evaluating time above the MIC of common uterine pathogens. No adverse reactions were noted in any animal of this study.

A major flaw in this study is the large amount of variation in ampicillin concentrations seen in both lochial fluid and endometrial tissue samples. No standard HPLC assays for the measurement of ampicillin in these fluids are in use and the methods used were developed for the project. In addition, the nature of lochial fluid and its varying characteristics make extracting the compound of interest difficult and could contribute to the variability seen. To improve the clinical relevance of this study, developing susceptibility breakpoints for ampicillin against common uterine pathogens is warranted.

Further work should be directed towards better characterizing the immune response in dairy cattle with APM by collecting samples chronologically after calving. This would allow for dynamic changes in gene expression and cytokine activity to be monitored more closely and provide the researcher with a more sensitive tool to assess the immune system. Furthermore, exposing these cells to stimuli and assessing their reaction to these stimuli would provide more information about immune reactivity. In addition, developing antimicrobial susceptibility breakpoints for common uterine pathogens would help veterinarians more effectively manage systemic antimicrobial therapy for cattle with APM.