ABSTRACT

VON DOLLEN, KAREN ANNA. Antibiotic Activity in Uterine Fluid. (Under the direction of Dr. Christopher Scott Bailey).

Bacterial infections of the uterus can have significant implications for a patient’s general wellbeing as well as reproductive future. Access by bacteria to the cranial reproductive tract is easily afforded when the cervix is open, such as during estrus or following parturition. While bacterial clearance from the uterus is generally effective in many animals, failure of clearance and/or the virulence factors of particular bacteria which have invaded offer an ideal opportunity for infection in this privileged location. Once a nidus of infection has been established within the uterus, most bacterial infections are associated with the presence of intrauterine fluid. This fluid not only provides an ideal growth medium for bacteria, but also may impact antibiotic efficacy due to inhospitable characteristics such as pH, protein content, and the presence of inflammatory debris including white blood cells. Antibiotics are a cornerstone of therapy across species, but rational selection of an antibiotic can be complicated by a number of factors. Among these are the difficulty in obtaining diagnostically relevant samples from the uterus in the case of small animals, the time-sensitive necessity to commence antibiotic therapy before in vitro culture and sensitivity results are returned, a distinct lack of information about antibiotic penetration into infectious uterine fluid, and uncertainty regarding antibiotic efficacy in this unique fluid environment. Furthermore, while in vitro microbiological analysis is considered the best method for antibiotic selection, the potential disconnect between in vitro results and in vivo efficacy in a clinical patient can mean antibiotic failure can occur despite a clinician’s best efforts to practice prudent antimicrobial stewardship. In an attempt to bridge the potential gap between in vitro results and efficacy in a clinical patient, we have undertaken work to explore the activity of
commonly utilized antibiotics within the environment of infected uterine fluid of bitches and mares. The activity of ciprofloxacin, an active metabolite of enrofloxacin, against *Escherichia coli* was evaluated in canine pyometra fluid at three dosage levels. Concentrations of 0.05 µg/mL and 1 µg/mL ciprofloxacin were bactericidal against both isolates of *E. coli* in Mueller Hinton Broth. Only the highest concentration (1 µg/mL) of ciprofloxacin inhibited bacterial proliferation in pyometra fluid. At that concentration, bacteriostatic activity was achieved against the clinical isolate at 8 hours, whereas a bactericidal effect was achieved at 24 hours. Similarly, the activity of ceftiofur and penicillin with gentamicin was assessed against *E. coli* and *Streptococcus equi* subspecies *zooepidemicus* in equine postpartum fluid. While treatment with ceftiofur was effective at reducing growth of *S. zooepidemicus* in equine postpartum uterine fluid, it did not reduce bacterial growth of *E. coli*. Treatment with procaine penicillin G with gentamicin achieved at least bacteriostatic activity against *E. coli* in both fluid types, and bactericidal activity against *S. zooepidemicus* in both fluid types. An *ex vivo* model was used to mimic the fluid environment of the uterus. In this model, representative autoclaved pyometritic fluid from bitches and lochia from mares was inoculated with a known quantity of bacteria, subjected to antibiotic treatment, and the number of bacteria which proliferated under each condition counted. As a control, samples of infected uterine fluid were paired with a matched sample of inoculated standard bacterial growth medium (Mueller Hinton Broth). We hypothesized that the milieu of infected uterine fluid would demonstrably hamper the activity of the antibiotics evaluated. This hypothesis was consistently supported as correct in both the canine and equine projects, suggesting that antibiotic selection should be made based not only on empiric or even culture/sensitivity results, but also with consideration of which antibiotics are able to maintain antimicrobial activity within infected uterine fluid.
DEDICATION

This is dedicated to those who believed in me every step of the way, supported me as I pursued nebulous dreams, and have been there to celebrate the transformation of those dreams into firm and promising reality. You know who you are, and I am the person that I am thanks to you (whether or not you’re reading this). Thank you for making it possible for me to become closer to an object of E.B. White’s admiration.

“…something is lost that has not been found, something's at stake that has not been won, something is started that has not been finished, something is dimly felt that has not been fully realized.”

— E.B. White
**BIOGRAPHY**

Karen Von Dollen was born and raised in Santa Barbara, California. She spent her childhood tussling with her siblings, playing the violin, and learning as much as she could about animals through her participation in 4-H. Wanting to experience seasons, she completed her undergraduate degree at Bryn Mawr College in Pennsylvania. There, she majored in chemistry with minors in mathematics and biology, played varsity lacrosse, and forged lifelong friendships in the bosom of sisterhood. From Pennsylvania, she traveled back to California to attend veterinary school at the University of California-Davis School of Veterinary Medicine, graduating in 2014. It was during this time at UC Davis that the earliest roots of her love for theriogenology began to take root. She completed internships at Alamo Pintado Equine Medical Center in California and Goulburn Valley Equine Hospital in Australia before beginning her theriogenology residency at the North Carolina State University College of Veterinary Medicine in 2016. She became a diplomate of the American College of Theriogenologists in 2018 and will transition to private practice as a clinician at Hagyard Equine Medical Institute in Lexington, Kentucky in 2019.
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“As one goes through life, one learns that if you don’t paddle your own canoe, you don’t move.”

— Katharine Hepburn

This is not to say that I could have done any of this alone, because I could not have. Rather, any success is due to the efforts of a great many coxswains and crewmates.

Particular thanks to:

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CHAPTER 1

Literature review

Introduction

Since the discovery of penicillin by Alexander Fleming in 1928, antibiotic usage has accelerated rapidly in both human and animal medicine. In parallel, microorganisms resistant to particular antimicrobials or classes of antibiotics have emerged. The increasing trend in the development of antimicrobial resistance emphasizes the critical importance of directed, rational, and prudent selection of antibiotics that are both appropriate for the etiologic agent at hand and effective in the animal or environment in which they are deployed.

Antibiotic activity can be affected by a multitude of factors. Antimicrobial stewardship and prudent selection of antibiotics dictate that choosing an antibiotic should ideally be based on in vitro culture and sensitivity results. There are two realities that make this ideal difficult to achieve in some situations. The first is that many disease processes require urgent intervention to halt disease progression and improve clinical outcome, which does not provide enough time for such results to be returned. The second is that even though in vitro results may indicate that a particular causative bacterium is susceptible to a tested antibiotic, this may or may not carry over to efficacy in a clinical patient.

One potential reason for this disconnect between diagnostic results and clinical outcome may be antibiotic failure in the intended site of action. Two frustrating clinical scenarios encountered by the comparative theriogenologist are pyometra in the bitch and postpartum metritis in the mare.
A common link between these two conditions is the presence of intrauterine fluid in association with infectious bacteria. Antibiotics provide the underpinning of therapy, along with supportive care and fluid evacuation from the uterus. The fluid is generally opaque white to red-brown in color, may have a foul odor, and has varying viscosity from patient to patient. The characteristics of this fluid (such as pH, protein content, and cellular count) have not been published in literature, and likely display considerable variation between cases. While the underlying provenance of this fluid is different in bitches and mares (a pathologic accumulation in the case of the bitch, an extension of physiologic lochia in the postpartum mare), its presence can cause vexation for the clinician in attempts to return the patient to reproductive function.

Once pathogenic bacteria have gained access to the uterus (most commonly through an open cervix during estrus or following parturition), the presence of uterine fluid may provide a hospitable environment for bacterial proliferation by providing a growth substrate as well as offering safe harbor from antibiotics. We hypothesized that the presence of purulent uterine fluid would suppress antibiotic activity of commonly utilized antimicrobials against common etiologic agents of pyometra in the bitch and postpartum metritis in the mare. The objective of our work described herein is to investigate this hypothesis in the hopes of spanning the void between \textit{in vitro} microbiological results and \textit{in vivo} antimicrobial efficacy in a clinical patient suffering from uterine bacterial disease.
Antibiotic use and characteristics

Antibiotics are widely used in the field of theriogenology. Common indications include bacterial endometritis in mares, prostatitis in dogs, pyometra in bitches, metritis in mares and bitches, and in the treatment of gestational disease with an underlying bacterial component (such as placentitis in mares and documented fetal compromise in bitches). Considerations for selection of an antibiotic include but are not limited to: drug levels reached at the intended site of action, collateral damage to a developing embryo or fetus in the case of pregnant females, efficacy against the targeted etiologic agent, and ease of administration. Broadly, antibiotics can be classified as time-dependent or concentration-dependent, based on what factor dictates the amount of bacterial killing they enact. Time-dependent antibiotics are those which are active against susceptible bacteria for the duration of time the drug concentrations in the patient exceed the minimum inhibitory concentration (MIC) of the bacterium. Concentration-dependent antibiotics are those which will exert greater antimicrobial action as their concentration at the site of action increases relative to the MIC.

The broad focus of our current research is the evaluation of antibiotic activity in uterine fluid, with the ultimate goal of directing antibiotic choice to select those drugs which are best suited for performance in the uterine environment. The specific characterization of uterine fluid (for this work in particular: postpartum fluid in mares and pyometritic fluid in bitches) has not been published in the literature, and therefore cannot be used – even hypothetically – to guide antibiotic selection.
In equine theriogenology, local administration of antibiotics for the treatment of bacterial infections in the uterus represents an attractive option due to the relative ease of access. For therapeutics which are safe for direct instillation into the uterus, high local tissue concentrations can be achieved at the site of intended action, while mitigating systemic side effects (such as antibiotic induced colitis).

In canine theriogenology, access to the uterus by nonsurgical methods necessitates the use of transcervical catheterization. The equipment to facilitate this procedure is not uncommon, but is generally limited to practices which have a robust reproductive caseload. The average general practice is not equipped with the equipment to accomplish this access. Therefore, systemic antibiotics are currently the frontline method of administration for treating bacterial infections of the canine uterus. Since pyometra and metritis both carry the risk of sepsis, this systemic administration confers the additional benefit of providing systemic antimicrobial coverage.

In production animal clinical practice, intrauterine antibiotics are also harnessed, albeit with additional regulatory considerations stemming from the Animal Medicinal Drug Use Clarification Act (AMDUCA) and the vital need to consider the consequences of antibiotic residues in food-producing animals. The use of intrauterine ceftiofur for the treatment of postparturient disorders in cattle was found to have a positive impact on a cow’s reproductive longevity in the herd, providing support for its efficacy in this species [1]. Gustafsson et al proposed the use of intrauterine antibiotics with efficacy in anaerobic conditions based on work investigating the intrauterine oxygen-reduction potential which revealed a significantly decreased oxygen-reduction potential in cows with experimental metritis as compared to normal
postpartum cows [2]. Cows with postpartum clinical metritis treated with intrauterine chlortetracycline were more productive members of the herd as assessed by milk yield and first service conception rates, an outcome which was not repeated for cows with retained fetal membranes (but no progression to metritis) [3]. The routine intrauterine infusion of gentamicin sulfate did not improve fertility in 233 clinically normal cows when bred at approximately 60 days in milk when administered shortly after insemination, which provided evidence to discourage administration of antibiotics in the absence of clinical signs [4]. Similarly, a single intrauterine infusion of ceftiofur in cattle at around 44 days in milk was not shown to confer significant benefit on the proportion of cows with subclinical endometritis during the transition period [5]. In food producing animals, antibiotic residues are an important consideration which does not influence antibiotic selection in companion animals. The presence of antibiotic residues have been confirmed in milk following intrauterine insemination, and must be considered when selecting an antibiotic and route of administration in these species [6, 7]. Following farrowing, the presence of vulval discharge for a period of time greater than six days was found to be associated with significantly reduced reproductive efficiency in sows [8]. This discharge was interpreted as clinical evidence of mastitis-metritis-agalactia (MMA) syndrome, which prompted intrauterine antibiotic infusion (determined by monthly surveillance of antibiotic sensitivity profiles performed on a farm-by-farm basis) [8].

It is well-documented that access for antibiotics to various organ systems in the body is variable, with the central nervous system providing a classic example of a privileged access point. When approaching a rational antibiotic choice to fight an infection in the central nervous system, it is vitally important to select a drug that possesses both antimicrobial efficacy against the causative
organism, and the ability to penetrate the blood-brain-barrier and maintain efficacy in the cerebral spinal fluid and nervous tissues. While literature to document the ability to achieve efficacy in the nervous system is abundant, the uterus is woefully neglected in veterinary literature. Therefore, this work was undertaken to begin to evaluate how antibiotic activity is altered in the face of the unique environment of the uterus. In particular, we explored ciprofloxacin’s activity in canine pyometritic fluid and the activity of ceftiofur, penicillin, and gentamicin in equine postpartum uterine fluid.

**Protein binding and tissue distribution**

That the binding of drugs by proteins leads to an inactivation of the drug’s intended activity is a documented and accepted fact, with support as early as the 1940s [9, 10, 11]. Only the unbound fraction of an antibiotic is microbiologically active. Binding of antibiotics by serum proteins shows evidence of being reversible, and therefore the degree of binding at any given time during a therapeutic time course and in varying environments is likely in flux [12]. Within a class of antibiotics, the particular type of serum and specific antibiotic itself can contribute to an extremely wide range of binding, over 100-fold for the penicillins in human serum alone [12]. In equine serum, 52-54% of penicillin G is bound by proteins as determined by ultrafiltration [13]. As the concentration of a drug increases, the proportion of unbound drug also increases [11, 14], so the concentration of active drug within the uterine environment would likely be different depending on how well the drug enters and stays within the lumen. This information is sparsely available, especially as relates to the bitch.
The pharmacokinetic profiles of enrofloxacin and ciprofloxacin in the dog were first reported in 1993 [15]. A large volume of distribution, more than that of total body water, supported a significant amount of tissue penetration [15]. While the spectrum of activity of the fluoroquinolones is primarily gram-negative, ciprofloxacin has demonstrated a wider spectrum as compared to other members of its class to encompass some gram-positive organisms [16, 17]. Another advantage of ciprofloxacin, as demonstrated in Zeiler’s work, is the ability to exert bactericidal activity against gram-negative bacteria while they were in stationary growth phase, which is not true for beta-lactam antibiotics [16]. When controlling for methods used to determine protein binding, enrofloxacin displayed higher protein binding (~35%) than ciprofloxacin (~18.5%), but both drugs were assessed as having relatively low protein binding in the dog [18]. However, this measurement of protein binding encompassed nonspecific proteins, and the proteinaceous variety of materials within infected fluid may contain variable amounts of albumin, α₁-acid glycoprotein, and lipoproteins, all of which may be influenced by the inflammatory state and site of action [18].

Ceftiofur, a third generation cephalosporin, is generally considered to be fairly resistant to β-lactamases and is a bactericidal, time-dependent antibiotic [19,20]. Ceftiofur enjoys widespread extracellular fluid distribution, but is not considered to pass well across the plasma membranes of cells [19]. However, inflammation of these membranes can allow therapeutic penetration. In a model of inflammation utilizing synovial injury, inflammation led to increased antibiotic concentrations, a finding which was attributed to increased blood perfusion secondary to inflammation [21]. Protein binding of some cephalosporins in horses is quite low, ranging from
~5-20% [22, 23]. Cephalosporins are recognized for their widespread distribution throughout the body, as well as effective penetration into tissue spaces and fluids [24].

*Antibiotic activity and pH*

Ciprofloxacin has been shown to display variable antibiotic activity in different media [25]. Acidic pH influenced a decrease of *in vitro* antibacterial activity of multiple fluoroquinolones, but ciprofloxacin maintained its bactericidal activity at a lower pH (6) than its classmates norfloxacin and nalidixic acid [16, 26]. This ability to adapt to varying pH environments may be, at least in part, due to the ability of ciprofloxacin to assume one of two pK values, allowing the surface charge of the molecule to change based on its surrounding pH [27].

Procaine penicillin G is a weak acid, with a pKa of 2.8, which could potentially lead to a lower concentration in an environment with an acidic pH [28]. While this was not seen in the infected tissue chamber model employed by Ensink and colleagues, it remains a pharmacologically reasonable outcome, which may have a different outcome in a different tissue environment (such as the uterus). An increase in the acidity of the growth medium was associated with an increase in the antibacterial activity of penicillin, which would be favorable in instances of infected tissue [29]. Gentamicin is a bactericidal, concentration-dependent antibiotic [30, 31]. The activity of gentamicin is negatively affected by low pH values, and its activity is greatest in an alkaline environment [32].
Ceftiofur is quickly metabolized to its active metabolite desfuroylceftiofur [33], a process which occurs more rapidly at alkaline versus acidic pH values [34]. In cattle, higher antibiotic activity of ceftiofur against *Pasteurella haemolytica* was observed in infected as compared to non-infected tissue chambers, suggesting that ceftiofur may be well-suited to working in an infected environment [35]. This increase in activity was likely due to the fact that the infected tissue chambers collected higher concentrations of ceftiofur as well as desfuroylceftiofur at all sampling intervals, which was attributed to binding of ceftiofur and desfuroylceftiofur to macromolecules including proteins which then served a depot effect as the reversible binding was undone over time [33, 35]. Extrapolation of these properties to other species should be done cautiously, as similar work has not been performed in the mare.

*Antibiotic interaction with neutrophils*

The antibiotic activity of ciprofloxacin was found to not be adversely affected by human pus in an *ex vivo* model [36]. The fluoroquinolones are widely lauded for their ability to accumulate within phagocytes [37]. Due to this quality, fluoroquinolones are able to hitchhike within white blood cells and be carried to sites of inflammation [37-39]. Cases of canine pyometra are characterized by the presence of purulent fluid within the uterus. Therefore, ciprofloxacin may be well-suited for performance in this environment.

The β-lactam antibiotics assessed in the current research (ceftiofur and penicillin G) have each been evaluated with regards to their performance in the face of neutrophils. Ceftiofur’s ability to perform within an infected environment was probed in a tissue cage model in ponies, with the
conclusion that this antibiotic was unable to eradicate *Staphylococcus aureus* within the infectious material and therefore should not be used as a sole local therapeutic agent [40]. As for penicillin’s interaction with neutrophils, penicillin G was not shown to concentrate in neutrophils in the work of Mandell and Coleman [41], which was a reinforcement of earlier work demonstrating the same finding [42, 43].

The chemotactic response of polymorphonuclear cells may be blunted by gentamicin [44, 45]. Aminoglycosides such as gentamicin must penetrate bacteria, and therefore the presence of a β-lactam antibiotic (which interferes with cell wall synthesis) can augment the antibacterial effects of gentamicin. Purulent debris binds aminoglycosides and leads to inactivation [46]. Aminoglycosides have low protein binding (<25%), are poorly soluble in lipids, and do not readily enter cells or penetrate cellular walls [46].

Keeping the above characteristics of antibiotics and their diverse capabilities as well as limitations in mind, the focus of the work which comprises the remainder of this document was the evaluation of the activity of commonly-used antibiotics in a model of uterine fluid. To represent the differences in clinical presentations between the species selected (canine and equine), the sources of these fluids were from clinical patients in scenarios frequently encountered in reproductive practice: pyometra in the bitch and postpartum bacterial infection in the mare. These two species and situations are widely divergent, with limited overlap in hormonal patterns, underlying etiologies, or treatment techniques. However, one critical commonality is the fact that uterine fluid and its peculiarities have been poorly characterized in veterinary literature. Fluid characteristics may have profound impacts on antibiotic efficacy, as
illustrated by the antibiotic profiles above. Whether an antibiotic is able to perform well against a
given bacterium in a controlled laboratory setting to generate a susceptibility profile may or may
not translate to similar performance in a clinical patient. This creates a potential chasm between
diagnostics and outcome, which we are attempting to bridge through this work.

Influence of steroid hormones on immune function

The hormonal environment of the patient, as determined by stage of estrous cyclicity, has a
critically influential role in the pathogenesis of disease due to its effects on the host’s immunity.
Some of the earliest work to support the augmentation of host immunity during etsrus occurred
during the 1920s, when an increase in lymphocytes was observed during estrus in the mare [47].
In contrast, impaired bactericidal activity in neutrophils was found under conditions of elevated
progesterone concentrations in the mare (as are seen during diestrus and pregnancy) [48]. More
recently, sophisticated molecular methods have been employed to deepen the understanding
surrounding these tenets of immune function and estrous cyclicity. Gene expression in the equine
uterus was recently explored using high-throughput RNA sequencing, which revealed the
upregulation of 1577 genes during estrus (including genes associated with immune function)
[49]. The same group had previously utilized the same methodology to compare gene expression
in the equine uterus following inoculation of Escherichia coli, concluding that there is a more
robust upregulation response (2422 genes c.f. 1476 genes) when inoculation occurred during
estrus versus diestrus [50]. In the bitch, peripheral blood mononuclear cells have been shown to
display blunted responses to E. coli under the influence of elevated serum progesterone,
providing further evidence for the dampened immune response seen during diestrus, as well as
an interesting piece of the puzzle in elucidating the pathogenesis of pyometra (most commonly caused by *E. coli*) [51]. An analogous uterine response to *E. coli* was demonstrated in gilts, in which inoculation of *E. coli* caused uterine disease (vaginal discharge ± positive culture) in only one of five gilts inoculated during standing heat, but in four of five gilts inoculated during diestrus [52].

These complementary outcomes (increased immunity during estrus, decreased immunity during diestrus) provide physiologically logical conditions for the goals of the female reproductive system at each stage of the cycle. During estrus, the female tract is assaulted by any number of foreign contaminants during copulation, and therefore her body must be primed to respond to these insults. Following ovulation and entry into diestrus, her tract must nimbly pivot to allow for potential reception of the semiallogeneic conceptus. While the suppressed immune backdrop afforded by progesterone during diestrus is favorable for procreation, it is necessarily also advantageous for bacteria.

Arguably as important as the hormonal levels themselves in determining downstream effects are the number, location, and availability of the respective hormone receptors. In comparing the hormonal levels and estrogen receptors of diestrus bitches, both clinically normal as well as affected by pyometra, bitches with pyometra had downregulated expression of estrogen receptors despite having the same steroid hormone profile as diestrus bitches without pyometra [53]. This suggests, but does not fully explore, the possibility that an underlying difference between estrogen receptors (with associated impacts on immune function) in the uteri of bitches which develop pyometra and those that do not may contribute to pathogenesis of this disease process.
Uterine involution and postpartum fluid in the mare

Within the first 12 hours postpartum, the mare’s uterus undergoes rapid involution, with the gravid horn decreasing in size to become around one and a half times the size of the nongravid horn [54]. While this initial decrease in size is facilitated by contraction of the myometrium and supportive ligaments, no marked decrease in tissue mass is appreciated during the immediate postpartum period. Starting at approximately three days postpartum, the weight of the uterus steadily declines as involution progresses, losing approximately five to seven kilograms within the first eight days after foaling [54]. The character of the effluent lavage fluid of the postpartum mare has been investigated in comparison to nonparturient diastral mares, with evidence to suggest that the secretory function of the endometrial glands (proteins and enzymes) is restored and normalized by the first postpartum diestrus [55].

The postpartum changes that occur in the equine uterus were evaluated with serial uterine biopsies throughout the first ten days following parturition by Gomez-Cuetara and colleagues [56]. In this work, 87 biopsy samples were obtained from 29 mares (seven primiparous, 22 multiparous) to characterize the histologic changes that occur in the uterus during this initial postpartum period. On the day of parturition, microcaruncles, with a cuboidal epithelium, were regularly distributed and present on the endometrial surface, along with eosinophilic debris. The uterine glands were dilated and contained dense eosinophilic contents. Vascular congestion, edema of the stratum spongiosum, and neutrophil migration into the microcaruncles and luminal epithelium was apparent. By the day after parturition, more cellular debris and neutrophils were present in the lumen of the uterus. The microcaruncles began to show evidence of degeneration
including increased cytoplasmic vacuolization and karyorrhexis [56]. Similarly, Jischa et al probed the postpartum period of nine pony mares who had experienced eutocia (two primiparous, seven multiparous) [57]. Endometrial biopsy samples were obtained within 24 hours of foaling, on the ninth day after foaling, and on the sixteenth day after foaling. These were evaluated on a cellular (histopathology) as well as molecular (qPCR analysis) basis. By day nine after parturition (and conserved at day 16), no evidence of lochial fluid or endometritis was seen in any of the study subjects [57]. On initial histologic examination of the postpartum uterus, microcaruncles were clearly evident in the stratum compactum. In contrast to the earlier Gomez-Cuetara work, the endometrium was populated by tall columnar epithelial cells (as opposed to cuboidal) in the majority of mares [57]. In explanation of this difference, the population of mares used by Gomez-Cuetara and colleagues was composed of horses, while that of Jischa et al was pony mares. While the possibility that there is a subtle difference in the postpartum involution process between horses and ponies was not definitively explored, it could provide an explanation for the difference observed. Furthermore, there were differences in sample preparation (the use of Bouin’s fixative in the Gomez-Cuetara work, as opposed to formalin in Jischa’s manuscript) which may have played a role in influencing differences seen. An abundance of neutrophils was apparent in the stratum compactum as well as infiltrating the epithelium and microcaruncles [56, 57]. Apoptosis of the microcaruncles and endometrial cells had already begun within the first 24 hours of delivery [56, 57]. This damage to the outermost layer of the endometrium may play a contributory role in the ease with which endotoxin can gain access to the bloodstream and lead to systemic endotoxemia and its associated cascade of negative sequelae. All microcaruncles had undergone apoptosis by day nine, although this work did not obtain samples between the first and ninth days postpartum, making this finding imprecise in terms of determining the exact
window of caruncular degeneration [57]. In concert with the above observations on the immediate postpartum period, purulent discharge can be a normal finding in the postpartum mare [58, 59].

In the initial postpartum period, neutrophils are found in large numbers in the endometrium as well as uterine fluid [57]. This predominance of neutrophils gives way to lymphocytes, macrophages, and siderocytes during the first nine days as the postpartum period progresses [57, 60]. Uncomplicated uterine involution proceeds rapidly, and by the end of the second week the endometrium has been restored to its pregravid state aside from mild inflammation and [57, 61]. Involution of microcaruncles was noted to occur both with and without the presence of lymphocytes and polymorphonuclear leukocytes [61], suggesting that inflammation is a common but not universal hallmark of involution.

The amount and type of microorganisms present within the postpartum reproductive tract have the opportunity to colonize the uterus whenever the vagina is accessed manually [58]. Once within the uterine lumen, these bacteria can proliferate rapidly and may contribute to complications secondary to retained fetal membranes in conjunction with the autolysis of the membranes [62]. The retention of fetal membranes in the mare leads to a delay in uterine involution [61]. In the clinically normal postpartum mare, *Streptococcus* sp. accounted for 14 of 29 uterine isolates, with *Escherichia* sp. representing a minority of isolates (2/29) [63]. Given that the positive culture of these organisms can be found in the normal postpartum mare, their isolation alone should not be an indication for antibiotic therapy in the absence of clinical signs. Recent work which presented the most common bacteria isolated from cases of postpartum
metritis in mares, along with their in vitro susceptibility patterns, was used as the foundation for our work evaluating antibiotic activity in postpartum uterine fluid [64]. This work guided the selection of the two bacteria evaluated (Escherichia coli and Streptococcus equi subspecies zooepidemicus), and also influenced the antibiotics chosen for treatment (penicillin with gentamicin and ceftiofur). We attempted to capture the milieu of the postpartum uterus by pooling uterine fluid from three postpartum mares. These mares had historically experienced eutocia and were presented alongside their clinically unwell foals. The samples were collected 12 hours, 72 hours, and 96 hours postpartum. This mixed sample was used as a growth medium to evaluate the performance of antibiotics, with the hope of providing evidence to guide first-line antibiotic therapy in postpartum mares.

Pyometra fluid in the bitch

Owners and veterinary providers of intact bitches should be prepared to manage pyometra, as approximately 20% of bitches will experience pyometra by 10 years of age [65]. While ovariohysterectomy is a treatment option that serves to remove the nidus of infection while also eliminating the risk of recurrence, it precludes future reproduction. For members of a breeding population, medical management of pyometra (encompassing fluid evacuation from the uterus, supportive care, and antibiotic therapy) is an alternative to surgery. Due to the urgency in commencing antibiotic therapy in this potentially life-threatening condition, antibiotics are routinely selected empirically based on the expected sensitivity pattern of the most common etiologic agent, E. coli. However, both the amount of active drug that reaches the uterus and its efficacy in this environment, are underreported in veterinary literature. While our work did not
explore the pharmacokinetics of antibiotics as they relate to the reproductive tract, we have endeavored to shed light on antibiotic activity within the fluid environment of the canine uterus affected by pyometra, in order to rationalize antimicrobial selection and improve patient outcomes.

Under the definition of “accumulation of pus in a confined space”, pyometra in the bitch can be approached as an abscess [66]. Similarly, at its etymological core and base definition, pyometra fits the definition of empyema as pus in a pre-existing body cavity (Merriam-Webster). The fluid known as pus, also known as purulent fluid or suppurative inflammation, is a conglomeration of leukocytes along with the byproducts of cellular and tissue necrosis and liquefaction [67]. Pus can form in the presence or absence of bacteria. It promotes an environment conducive to growth and proliferation of some types of bacteria and hinders the activity of antibiotics [67]. Defined characteristics of pus include a low pO$_2$ (mean 25 mm Hg), an abundance of amorphous necrotic tissue, polymorphonuclear cells, bacteria, low pH (range 6.2 - 7.2), and diminished redox potential [67].

**Hypotheses**

The number and diversity of factors on the part of both the antibiotic and the fluid environment complicate the unraveling of the particular components of each that contribute to efficacy or failure, individualities of patient and bacteria notwithstanding. We have undertaken two projects to begin to understand which antibiotics might be well-suited for use in the treatment of bacterial uterine infections in the postpartum mare and bitches with pyometra. An *ex vivo* model was
harnessed to compare antibiotic activity of commonly used antibiotics against select bacteria in standard bacterial growth media as compared to uterine fluid from representative patients. The results of the work completed thus far demonstrate an impact of uterine fluid on antibiotic activity, which should be considered when selecting an antibiotic for clinical use. It is our intention that these results will contribute to improved antibiotic selection to promote clinical efficacy while staving off antimicrobial resistance by utilizing drugs which are functional in the targeted environment.
REFERENCES


CHAPTER 2

Dose-dependent antibacterial activity of ciprofloxacin against *Escherichia coli* in a model of canine pyometra

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Abstract

The aging intact bitch is at substantial risk for developing pyometra, a potentially life-threatening condition with repercussions for future reproductive function. Antibiotic therapy is a hallmark of any treatment approach, generally targeted against *Escherichia coli*, the most common etiologic agent. Fluoroquinolones are commonly prescribed, as their spectrum of activity provides empiric coverage against this organism. The infected, purulent fluid which occupies the uterus provides a hospitable milieu for bacterial proliferation while simultaneously carrying the potential to hamper antibiotic efficacy. In this work, we have modeled the conundrum posed using an *ex vivo* system to mimic this environment and assess antibiotic activity of ciprofloxacin. Ciprofloxacin, an active metabolite of enrofloxacin, displayed dose-dependent antibacterial activity against two strains of *E. coli*. Antibiotic activity was decreased in pyometra fluid as compared to standard bacterial culture broth.
1. Introduction

Pyometra is a serious threat to the reproductive capacity and overall health of intact bitches, particularly as they age. In a colony of beagle bitches, 15.2% (25/165) of subjects experienced pyometra after the age of four [1], and approximately one in five bitches over the age of 10 years develop this condition, with nulliparous bitches at greater risk [2, 3]. Mortality ranges from 3%-10% [4, 5]. Reported incidence by breed is variable, with Bernese mountain dogs, great Danes, Leonbergers, Rottweilers, and Irish wolfhounds overrepresented in one large study, suggesting a potential genetic component to the etiology of this condition [6]. Broadly, treatment of pyometra consists of either medical management or ovariohysterectomy (OVH). For bitches that do not have value as breeding animals, OVH remains the recommended course of treatment as it removes the source of infection and eliminates the risk of recurrence.

For bitches with demonstrated worth as members of a breeding population or those which are not suitable anesthetic candidates, medical management represents an attractive alternative. In addition, pyometra in captive threatened species remains a concern, and improved medical therapy for treating pyometra has the potential to positively impact conservation efforts by retaining reproductive capacity in animals that might otherwise need to be surgically sterilized as part of treatment [7, 8]. Treatment entails antimicrobial therapy, supportive care, and removal of uterine fluid via ecobic drug administration, and/or transcervical lavage. Due to the time sensitive nature of commencing antibiotic therapy and the difficulty of obtaining samples from the bitch’s cranial reproductive tract for culture and sensitivity testing, antimicrobial therapy is usually selected empirically. Typically, an antibiotic with gram-negative coverage is selected...
based on the most common bacterial etiology, *Escherichia coli* (*E. coli*) [9]. In the authors’ experience, antibiotic failure is not uncommon and can result in clinical decomposition or recurrence of pyometra one to three weeks after medical therapy is discontinued. Treatment failure may result from selection of an inappropriate antibiotic which fails to address the pathologic organism, or from selection of an antibiotic to which the organism is resistant. An additional factor that can result in treatment failure may be inactivation of the chosen antibiotic in the fluid environment of the uterus. As a result, *in vivo* efficacy is not assured even when antibiotics are selected based on *in vitro* culture and sensitivity testing.

It is well described that antibiotic activity can be affected by environmental characteristics such as pH, protein content (with subsequent binding), and purulent material [10, 11, 12]. The particular impact of purulent uterine fluid on antibiotic activity in cases of canine pyometra has not been studied, resulting in the potential use of antibiotics that fail to work, or have reduced efficacy despite favorable *in vitro* sensitivity results. This may result in increased morbidity and mortality, disease recurrence, and increased antibiotic resistance [13]. In one recent study, ~50% of bacterial isolates from bitches affected by pyometra displayed multidrug resistance [14]. Experiments have been performed in our laboratory to probe the activity of multiple antimicrobials in the face of varying types of uterine fluid (including purulent fluid, lochia, and allantoic fluid) [15, 16, 17]. Of the tested drugs, ciprofloxacin, the primary active metabolite of enrofloxacin, showed the most promise for the treatment of canine pyometra. Enrofloxacin is a broad-spectrum, bactericidal antibiotic [18]. It is metabolized to ciprofloxacin through deethylation in the liver and has concentration dependent bactericidal action with a primarily gram-negative spectrum of activity.
In the current work, we characterized the organisms associated with 12 regional cases of pyometra and determined the sensitivity patterns of those organisms. We then compared activity of three concentrations of ciprofloxacin against two *E. coli* isolates in a model of antibiotic activity established in our laboratory. We hypothesized that larger doses of ciprofloxacin would demonstrate greater antibiotic activity, and that said activity would be diminished in the presence of pyometra fluid as compared to commercial a culture media.

2. Materials and Methods

2.1 Pyometra Fluid Sample Acquisition and Characterization

Veterinary clinics local to the NC State University College of Veterinary Medicine were contacted via phone and/or email to solicit samples of uterine fluid collected from bitches affected by pyometra. These samples (n=12) were collected at the time of ovariohysterectomy and stored in sterile plastic containers at -20°C. Samples were obtained between 2017 and 2019 and used for this experiment during the same time period. During the course of the experimental period, a small portion of each of twelve fluid samples was plated on Columbia agar with 5% sheep’s blood and incubated at 37°C and 5% CO₂. After 24 hours of growth, a single colony of the most predominant bacterial type was sub-plated on a second Columbia agar plate and incubated at 37°C and 5% CO₂ for an additional 24 hours prior to. Bacterial isolation and identification were performed in the Microbiology & Molecular Diagnostics Laboratory at the NC State University College of Veterinary Medicine according to standard operating procedures. Isolates were identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-
TOF) mass spectrometry. Antimicrobial susceptibility was assessed using a commercially available microbroth dilution platform (Sensititre, Thermo Fisher Scientific).

2.2 Study Overview

An *ex vivo* model previously developed in our laboratory was utilized to evaluate the activity of ciprofloxacin at three doses (0.015 µg/mL, 0.05 µg/mL, and 1 µg/mL) against two strains of *E. coli* in Mueller Hinton Broth (MHB, standard bacterial culture fluid) or autoclaved uterine fluid obtained from bitches affected with pyometra just prior to OVH. Bacterial growth (quantified by counting colony forming units, CFU) was compared across treatment groups to objectively evaluate antibiotic activity under the different conditions.

2.3 Media Preparation

Intrauterine fluid was obtained from four bitches treated at private practice veterinary hospitals local to NC State University. Each bitch had received a parenteral dose of antibiotics (cefazolin, enrofloxacin, ampicillin/sulbactam, or amoxicillin/clavulanic acid) immediately prior to anesthetic induction for OVH. Fluid was collected at the time of OVH into sterile plastic containers and stored at -20°C prior to being thawed for use in these experiments. High performance liquid chromatography (HPLC) was performed to check for the presence of antibiotics in the uterine fluid before its use in this project; no antibiotics were present in any uterine fluid sample assessed.
At the start of each of two experimental phases, the fluid from two bitches was thawed, pooled, and autoclaved at 121°C for 45 minutes to generate a sterile growth medium to mimic the intrauterine environment of a bitch affected by pyometra. Previous work in our laboratory has demonstrated that the autoclaving process does not impact bacterial growth or antibiotic activity in this model (unpublished data). Commercially acquired Mueller Hinton Broth (Teknova M5860) was used as the control growth medium.

2.4 Inoculum Preparation

A clinical strain of *E. coli* obtained from a case of canine pyometra (novel from the above four bitches) was isolated and used in both experimental periods. This culture was suspended in trypticase soy broth with 25% glycerol and stored at -80°C prior to use. The isolate was susceptible to enrofloxacin.

As a comparative reference, *Escherichia coli* ATCC 25922™ was used in parallel with the above clinical isolate, stored in an identical fashion. Susceptibility to enrofloxacin was confirmed at the start of the experiment.

At the start of each experimental period, a small aliquot of each bacterial sample was thawed and plated on Columbia agar with 5% sheep’s blood (Remel R01216) to verify purity. Two to three colonies from each plate were diluted in 5 mL MHB and cultured at 37°C and 5% CO₂ until they reached 0.5 McFarland based on nephelometry (Sensititre E1041). In order to determine the
starting amount of bacteria used at the time of inoculation, samples of each isolate were serially diluted and plated on Columbia agar with 5% sheep’s blood and the CFU of each strain counted.

2.5 Antimicrobial Preparation

Solutions of 0.001 mg/mL ciprofloxacin and 0.1 mg/mL ciprofloxacin were made from preservative free reference standard (Sigma-Aldrich 17850-5G-F) diluted in 0.1% trifluoric acid (Acros Organics 293811000 CAS 76-05-1).

Treatment doses of 0.015 µg/mL and 0.05 µg/mL were achieved by adding 30 µL and 100 µL, respectively, of the 0.001 mg/mL ciprofloxacin solution as prepared above to each 2-mL vial. Similarly, 20 µL of the 0.1 mg/mL ciprofloxacin solution were added to each treatment vial to provide a treatment dose of 1 µg/mL.

The lowest treatment dose (0.015 µg/mL) was selected based on the minimum inhibitory concentration (MIC) breakpoint for the greatest percentage of wild type *E. coli* isolates, as reported by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, [19]).

The 0.05 µg/mL and 1 µg/mL dosages were extrapolated from work documenting the pharmacokinetics of ciprofloxacin in the dog [20] and literature reporting the concentrations of ciprofloxacin in uterine tissue of the bitch after intravenous administration of enrofloxacin [21].
2.6 Time Kill Trial

Two milliliters of fluid medium (either MHB, n=41, or the previously autoclaved canine pyometra fluid, n=41) were added to sterile glass two-dram vials (experiment phase one, Animal Reproduction Systems 537-520/144), or 12 x 75 mm sterile polystyrene culture test tubes (experiment phase two, Fisher Healthcare 14-956-3C). Twelve samples (n=3 MHB, n=3 pyometra fluid, for each of two experimental phases) were capped and set aside as negative controls, with no additional additives. The remaining vials (n=35 MHB, n=35 pyometra fluid), were inoculated with one of two strains of *E. coli*. In the first phase, samples were inoculated with 1.272E+05 CFU/mL clinical isolate *E. coli* and 7.500E+04 CFU/mL *E. coli* ATCC 25922™. In the second phase, samples were inoculated with 1.496E+05 CFU/mL clinical isolate *E. coli* and 1.225E+05 CFU/mL *E. coli* ATCC 25922™. These inoculation amounts were achieved by adding 10 µL of the previously prepared 0.5 McFarland standard of each bacterial strain to each 2-mL sample.

Positive control samples of MHB (n=5 clinical isolate, n=6 *Escherichia coli* ATCC 25922™) and pyometra fluid (n=5 clinical isolate, n=6 *Escherichia coli* ATCC 25922™) were then set aside, with no antibiotic added.

The remaining vials were treated with ciprofloxacin (n=5 clinical isolate, n=3 *Escherichia coli* ATCC 25922™, for each of the three treatment concentrations). Each sample was gently vortexed following the addition of antibiotics and placed into a 37°C incubator with 5% CO₂. After 8 hours of growth, 200 µL were aspirated from each sample vial,
and serially diluted 1:10 in 1X phosphate buffered saline (PBS; Fisher Scientific BP2438-4) in triplicate. Each dilution was then plated in duplicate on Columbia agar with 5% sheep’s blood culture plates in a modification of a time kill protocol per CLSI standards [22-24]. Plates were incubated at 37 °C and 5% CO₂ for approximately 14 hours to allow for distinguishable colony growth. After this growth period, 10-100 individually identifiable colonies were counted to quantify the amount of bacterial growth under each condition. This process was repeated after 24 hours of growth.

2.7 Data Analysis

For each treatment, the number of colonies per condition was averaged within each of three dilution replicates to determine the number of CFU/mL in each vial (n=5 per group for clinical isolate and n=3 for ATCC strain). Bacterial growth was compared across groups by determining the log change in CFU from inoculation. Because data were not distributed normally, the results were rank transformed prior to analysis using a split-plot two-way ANOVA with significance set at 0.05 (Statistix 10, Tallahassee FL). Bactericidal activity was defined as a ≥3 log decrease in CFU and bacteriostatic activity defined as a decrease in CFU from inoculation <3 log [25].
3. Results

In the twelve pyometra fluid samples acquired on a volunteer basis from private clinics in the greater Raleigh area, seven of the bacterial isolates were *E. coli* (58%). All of these isolates were sensitive to enrofloxacin. Two additional cases, of *Enterobacter aerogenes* and *Klebsiella oxytoca*, were also sensitive to enrofloxacin, making 75% of our bacterial isolates sensitive to enrofloxacin at industry accepted breakpoints [23, 24]. For the remainder of the isolates (*Pseudomonas aeruginosa, Streptococcus agalactiae*, and an unspecified Group G *Streptococcus sp.*, one of each bacterium), sensitivity to enrofloxacin was neither assessed nor reported in accordance with CLSI standards for veterinary pathogens [23, 24].

3.1 Clinical Isolate *Escherichia coli* (Figures 1 and 2)

The positive control samples for the clinical isolate of *E. coli* grew in both fluid types, with no difference in growth amount in MHB as compared to pyometra fluid. At 8 hours, there was a 3.53 log increase in bacterial CFU in MHB, and a 3.44 log increase in CFU in pyometra fluid. At 24 hours, there was a 3.89 log increase in CFU in MHB and a 4.05 log increase in CFU in pyometra fluid.

In Mueller Hinton Broth, the lowest treatment concentration of ciprofloxacin (0.015 µg/mL) resulted in bacteriostatic activity at both 8 and 24 hours, with a 0.40 and 0.15 log decrease in bacterial growth, respectively. At a treatment concentration of 0.05 µg/mL, ciprofloxacin was bactericidal against the clinical isolate at both timepoints, with a 3.04 log decrease in growth at 8
hours and a 4.99 log decrease at 24 hours. This bactericidal activity was maintained and augmented at the 1 µg/mL treatment concentration, with a 5.17 log decrease in bacterial growth from inoculation at both 8 and 24 hours.

In pyometra fluid, neither the lowest concentration (0.015 µg/mL) nor the 0.05 µg/mL concentration of ciprofloxacin were able to achieve even bacteriostatic activity against the clinical isolate of *E. coli*, with a 3.3-3.6 log increase in bacterial growth at both 8 and 24 hours. Only at the highest treatment concentration (1 µg/mL) was ciprofloxacin able to achieve antibacterial activity, with bacteriostatic activity (2.07 log decrease) at 8 hours and bactericidal activity (3.42 log decrease) at 24 hours.

### 3.2 *Escherichia coli* ATCC 25922™ (Figures 3 and 4)

The positive control samples for the reference isolate of *E. coli* grew in both fluid types, with no difference in growth amount in MHB as compared to pyometra fluid. At 8 hours, there was a 3.63 log increase in bacterial CFU in MHB, and a 3.49 log increase in CFU in pyometra fluid. At 24 hours, there was a 4.12 log increase in CFU in MHB and a 4.16 log increase in CFU in pyometra fluid.

In Mueller Hinton Broth, the lowest treatment concentration of ciprofloxacin (0.015 µg/mL) resulted in bactericidal activity at 8 hours (3.17 log decrease in bacterial growth) and nearly bactericidal activity at 24 hours (2.98 log decrease in bacterial growth). At treatment
concentrations of 0.05 µg/mL as well as 1 µg/mL at both 8 and 24 hours, ciprofloxacin achieved bactericidal activity, with a >5 log decrease in bacterial growth from inoculation.

In pyometra fluid, neither the lowest concentration (0.015 µg/mL) nor the 0.05 µg/mL concentration of ciprofloxacin were able to achieve even bacteriostatic activity against the clinical isolate of *E. coli*, with a 3.3-3.7 log increase in bacterial growth at both 8 and 24 hours. Only at the highest treatment concentration (1 µg/mL) was ciprofloxacin able to achieve antibacterial activity, with bacteriostatic activity at both 8 and 24 hours (2.04 and 2.74 log decrease in bacterial growth, respectively).

4. Discussion

Pyometra in the bitch represents a common and destructive condition. While surgical management can be extremely effective at removing the nidus of infection and eliminating the chance that disease will recur, it spells the end of a bitch’s reproductive career. Medical management, consisting of supportive care, clearance of infected fluid from the uterus, and broad-spectrum antibiotic therapy, offers the opportunity to preserve the reproductive potential of a genetically valuable bitch. Few advances have been made in the medical management of pyometra. Reported rates of recurrence of pyometra range from 14% to 86%, which may be due to treatment failure, ongoing endometrial pathology which facilitates the disease state, or persistence of bacterial infection [26-33] It is this last possibility which occupies the focus of our work. In work performed by Meyers-Wallen, uterine swabs were obtained from three bitches successfully treated using medical management which had returned to estrus and experienced
recurrence of pyometra on her next heat cycle. Each of these samples grew *E. coli* with a similar susceptibility pattern to the original isolate (also *E. coli* in the first instance of pyometra), suggesting that the uterus harbored a persistent population of *E. coli* despite stabilization of clinical signs and apparent resolution [27]. Similarly, reproductive failure in six out of 19 bitches, in which those bitches were bred following treatment with antibiotics and PGF$_{2\alpha}$, was attributed to “retention of a low-grade bacterial infection in the uterus” in one study [28]. In addition, emerging antimicrobial resistance and the need for increased antimicrobial stewardship justify the need for research advances in determining how to maximize antibiotic efficacy in clinical scenarios. Our current project aimed to characterize the effect of canine pyometra fluid on activity of a commonly used antibiotic, and to determine whether bactericidal effect could be achieved at physiologically obtainable antibiotic concentrations.

A laboratory model was used in this work, which inevitably had limitations: only a single clinical isolate of *E. coli* was evaluated, and pyometra fluid from only four bitches was used. Furthermore, the impact of patient metabolism on drug levels over a 24-hour period is not reflected in an *ex vivo* model. However, by using a fluid medium derived directly from the disease studied, and standardizing all other environmental conditions, we are able to specifically compare antibiotic activity across fluid conditions and antibiotic class. Thus, while no model can broadly encompass the myriad differences in bacteria and bitches, this model represents a first step to bridging the gap between *in vitro* susceptibility testing and *in vivo* antibiotic efficacy and may serve as a guide to clinical antibiotic choice in the future.
As test bacteria, we selected two strains of *E. coli*. For the purposes of standardization and comparison with other time-kill trials described in the literature, a laboratory strain (*Escherichia coli* ATCC 25922™) was selected. In addition to the reference strain, a clinical isolate from a case of pyometra submitted to our laboratory was chosen for use with the goal of capturing characteristics of *E. coli* that cause clinical pyometra. Across the literature, *E. coli* is overwhelmingly the most common isolate implicated in cases of canine pyometra, whereas either *Streptococcus sp.* or *Staphylococcus sp.* outnumbered *E. coli* in a small number of studies [28, 34-37]. In the twelve samples submitted to our project, *E. coli* was the most common isolate (7/12, 58%). The importance of using a clinical isolate is supported by work as early as the 1970s, which proposed that some strains of *E. coli* may display qualities that make them uniquely well suited to invade the uterus and cause disease. That work subsequently has been substantiated, including with genomic data identifying the mechanisms of some of these factors [38-45].

Ciprofloxacin was chosen for use in this *ex vivo* study design because it is the most active metabolite of enrofloxacin following deethylation of enrofloxacin in the liver, and thus may be more reflective of antibiotic activity *in vivo* than enrofloxacin. Enrofloxacin is commonly utilized in the treatment of pyometra and little bacterial resistance is reported in the literature. In a 2005 study investigating 80 isolates of *E. coli* from clinical cases of pyometra, 4% showed resistance to enrofloxacin [44]. Maity et al found that 100% of *E. coli* isolates from 43 cases of canine pyometra were sensitive to enrofloxacin and ciprofloxacin [35]. 90% (47/51) of canine pyometra *E. coli* isolates were sensitive to enrofloxacin and ciprofloxacin [40]. Low resistance to enrofloxacin was confirmed in recent work by Henriques et al and Chang et al, in which only 3%
of *E. coli* isolates from canine pyometra (n=29, [46]) were resistant to enrofloxacin, and 5.3% (n=114, [47]) of canine urinary tract isolates of *E. coli* resistant to each enrofloxacin and ciprofloxacin [46, 47]. 100% of *E. coli* isolates from canine pyometra (n=28) were sensitive to enrofloxacin [48]. Enrofloxacin was determined to be the most effective antibiotic against the largest percentage (59%) of bacterial isolates from 24 cases of canine pyometra [34]. The greatest percentage of isolates were sensitive to ciprofloxacin (77%, 69% sensitive to enrofloxacin) in a population of 20 canine pyometra cases [36]. In 25 *E. coli* isolates from cases of canine pyometra, 8% were resistant to ciprofloxacin, which was among the lowest percentages of resistance reported (aside from 0% resistance for norfloxacin, cefoxitin, and tobramycin) [14]. Likewise, in the twelve samples available to our laboratory for antibiotic susceptibility testing, all *E. coli* isolates were sensitive to enrofloxacin. In contrast, resistance to other commonly used antibiotics, including amoxicillin, ampicillin and cefazolin, was found in 58% of our samples (data not shown) and is also widely reported in the literature, with temporal and geographic variations evident in the results. A 2008 paper reported an increase in antimicrobial resistance to uropathogenic *E. coli* over the preceding five-year period [49]. In work reporting the antibiogram of bacterial etiologies in 20 cases of pyometra, gentamicin had the greatest sensitivity (85% of isolates sensitive), followed by enrofloxacin and ciprofloxacin (65% each) [50]. Given the nephrotoxic potential of aminoglycosides, and the concurrent kidney damage possible in cases of canine pyometra, gentamicin is not considered a first line therapy for pyometra in the bitch despite literature reports of its strong *in vitro* activity against *E. coli* isolates [51]. Furthermore, previous work in our laboratory has shown considerable inactivation of gentamicin in the face of uterine purulent material [15].
The tested antibiotic concentrations of 0.01, 0.05 and 1 µg/mL were selected with the intent of broadly bookending a range of therapeutically achievable antibiotic concentrations. While little is known about drug penetration of enrofloxacin to the uterine fluid of bitches with pyometra, our work was guided by published sensitivity breakpoints for *E. coli* [19], distribution of ciprofloxacin in plasma after oral enrofloxacin administration [20], and concentrations of ciprofloxacin in both canine and equine uterine tissue, amniotic fluid, and allantoic fluid [21, 52].

The results of the current work demonstrate considerable suppression of antibiotic activity in the presence of purulent fluid from a bitch with pyometra. While concentrations of 0.05 µg/mL and 1 µg/mL were bactericidal against both isolates of *E. coli* in Mueller Hinton Broth, only the highest concentration of ciprofloxacin inhibited bacterial proliferation in pyometra fluid. At that concentration, bacteriostatic activity was achieved against the clinical isolate at 8 hours, whereas a bactericidal effect was achieved at 24 hours. No concentration achieved bactericidal activity against the laboratory test strain at any time. Nevertheless, the suppression of bacterial growth demonstrated in both fluid types in our study supports the use of enrofloxacin or ciprofloxacin in the clinical treatment of pyometra. Despite the fluid effects, bacterial inhibition was achieved against both strains in both fluid types at drug concentrations reported in the uterus. Furthermore, the bactericidal activity of 1 µg/mL ciprofloxacin against the clinical isolate of *E. coli* in the environment of canine pyometra fluid may be of enormous benefit to improving long term clinical outcome and reproductive function in the face of *E. coli*’s unique ability to tenaciously colonize the canine reproductive tract [27, 28, 53].
In conclusion, we have demonstrated suppression of antibiotic activity for ciprofloxacin in purulent uterine fluid, which is overcome only at concentrations 100 times greater than the published *in vitro* MIC breakpoint for most *E. coli*. These findings highlight the need for detailed information regarding drug distribution to uterine fluid and similar sites of action, while also providing support for the use of this antibiotic in the treatment of clinical cases of pyometra if sufficient antibiotic concentrations can be achieved.

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Figure 1: Log bacterial growth clinical isolate *E. coli* at 8 hours. The shaded area denotes bacteriostatic versus bactericidal activity. Error bars indicate standard deviation from the mean. Different letters correspond to statistically significant differences (p<0.005). MHB=Mueller Hinton Broth (grey bars), PYO=pyometra fluid (black bars), ciprofloxacin treatment concentration listed in µg/mL; n=5 for all groups.
Figure 2: Log bacterial growth clinical isolate *E. coli* at 24 hours. The shaded area denotes bacteriostatic versus bactericidal activity. Error bars indicate standard deviation from the mean. Different letters correspond to statistically significant differences (p<0.005). MHB=Mueller Hinton Broth (grey bars), PYO=pyometra fluid (black bars), ciprofloxacin treatment concentration listed in µg/mL; n=5 for all groups.
Figure 3: Log bacterial growth *Escherichia coli* ATCC 25922™ at 8 hours. The shaded area denotes bacteriostatic versus bactericidal activity. Error bars indicate standard deviation from the mean. Different letters correspond to statistically significant differences (p<0.005). MHB=Mueller Hinton Broth (grey bars), PYO=pyometra fluid (black bars), ciprofloxacin treatment concentration listed in µg/mL; n=3 for all groups.
Figure 4: Log bacterial growth *Escherichia coli* ATCC 25922™ at 24 hours. The shaded area denotes bacteriostatic versus bactericidal activity. Error bars indicate standard deviation from the mean. Different letters correspond to statistically significant differences (p<0.05). MHB=Mueller Hinton Broth (grey bars), PYO=pyometra fluid (black bars), ciprofloxacin treatment concentration listed in µg/mL; n=3 for all groups.
REFERENCES


CHAPTER 3

Antimicrobial activity of ceftiofur and penicillin with gentamicin against *Escherichia coli* and *Streptococcus equi* subspecies *zooepidemicus* in an *ex vivo* model of equine postpartum uterine disease

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Abstract

The use of antimicrobials for the management of equine uterine disease is commonplace, with antibiotic selection generally based on empirical evidence or *in vitro* sensitivity results. However, the potential disconnect between these laboratory results and clinical efficacy in the mare raises concern for antibiotic failure and subsequent development of resistant organisms. In this work, we attempt to bridge this gap by employing an *ex vivo* model of the equine postpartum uterus to quantitatively evaluate the antimicrobial activity of two commonly used antibiotic treatments in the mare (ceftiofur and penicillin with gentamicin). The activity of both of these treatments was evaluated in two different fluid environments (standard bacterial culture broth and equine postpartum uterine fluid) against clinical isolates of *E. coli* and *S. zooepidemicus*. While treatment with ceftiofur was effective at reducing growth of *S. zooepidemicus* in equine postpartum uterine fluid, it did not reduce bacterial growth of *E. coli*. Treatment with procaine penicillin G with gentamicin achieved at least bacteriostatic activity against *E. coli* in both fluid
types, and bactericidal activity against *S. zooepidemicus* in both fluid types. The intrauterine infusion of procaine penicillin G with gentamicin in cases of postpartum uterine disease caused by *E. coli* or *S. zooepidemicus* is supported by the results of this work.

1. Introduction

Antibiotic use in the early postpartum period of mares is common, both for prophylaxis as well as for treatment of documented cases of bacterial uterine disease. Endometritis and metritis in the early postpartum period can have a profound impact on a mare’s future fertility, and prompt resolution of these disorders is paramount in maintaining her reproductive momentum as well as general health.

Uterine involution in the immediate postpartum period involves the production of lochia, a cellular fluid milieu, which can provide a hospitable environment for bacterial growth and inhibit the activity of antimicrobial agents. Antibiotic activity can be inhibited by purulent material, extremes in pH, and protein binding [1], [2], [3]; but, the effect of equine cellular uterine fluid on antibiotic activity of commonly utilized antimicrobial agents has not been studied. Because of a lack of data to guide treatment, antimicrobials may be ineffective, or have reduced activity despite good *in vitro* susceptibility test results. This lack of information regarding the unique environment of the postpartum uterus has resulted in the potential use of antibiotics that fail to work, or that have reduced activity despite good sensitivity *in vitro*. This may result in increased morbidity and mortality, disease recurrence, and increased antibiotic resistance [4].
The goal of this study was to investigate the antimicrobial activity of two antibiotic formulations: ceftiofur as a single agent, and penicillin combined with gentamicin, which are frequently prescribed for postpartum mares. We hypothesized that the fluid environment of the equine postpartum uterus would impact antibiotic activity as compared to activity in Mueller Hinton Broth. Our evaluation of activity was performed in standard bacterial culture media and in pooled equine lochial samples. Ceftiofur is a third-generation cephalosporin with *in vitro* activity against common etiologic agents of metritis and endometritis [5]. Penicillin with aminoglycoside infusions improved pregnancy rates in cycling mares [6]. These agents are recommended for intrauterine infusions by some practitioners, but antimicrobial activity of the combination has not been tested.

*Escherichia coli* (*E. coli*) and *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) were selected for this study because they are frequently isolated in cases of postpartum metritis [7]. Antimicrobial concentrations were selected based on pharmacokinetic data, or estimated concentration in a 60 mL intrauterine infusion.

2. Materials and Methods

2.1 Study Overview

A modified time kill trial according to Clinical Laboratory Standards Institute (CLSI) guidelines was performed to objectively characterize bacterial concentrations of *E. coli* and *S. zooepidemicus* in either standard growth medium (Mueller Hinton Broth) or uterine fluid.
obtained from clinically normal postpartum mares after exposure to each of the three antimicrobials at 8 and 24 hours [8], [9], [10].

An *ex vivo* model was employed to evaluate the activity of ceftiofur (0.5 µg/mL), and a compounded mixture of penicillin and gentamicin (2400 IU penicillin/mL with 0.2 mg/mL gentamicin) against two bacteria in either standard culture fluid, or clinically derived sterilized equine lochia. The study design yielded 40 different sample tubes, for which bacterial growth (quantified by counting colony forming units (CFU) of bacteria) was determined and compared.

### 2.2 Media Preparation

Intrauterine fluid was obtained from three postpartum mares, which were presented as clinical cases to the North Carolina State, University College of Veterinary Medicine. All mares were presented within 36 hours of foaling and were clinically healthy. The transrectal ultrasound examination showed a greater volume of intrauterine fluid than would be expected. None of the three mares had received antibiotics prior to acquisition of fluid. This fluid was aliquoted in 50-mL sterile conical tubes and stored at -80°C until use. At the start of the experimental period, the fluid samples from the three mares were thawed, pooled, and autoclaved at 121°C for 45 minutes in order to produce a sterile growth medium representing the intrauterine postpartum environment. The autoclave process does not affect its ability to support bacterial growth as a medium or antibiotic activity *ex vivo* (unpublished data). Commercially prepared, sterile, Mueller Hinton Broth (Teknova M5860) was used as a standardized test media [9], [10].
2.3 Inoculum Preparation

Two bacterial isolates obtained from clinical cases presented to the NC State University, College of Veterinary Medicine, were used in this study. The *E. coli* isolate was procured from a clinical case of pyometra, and the *S. zooepidemicus* isolate from a postpartum mare that had aborted because of placentitis. The *E. coli* isolate’s MIC values were >8 µg/mL, 0.5 µg/mL, and 2.0 µg/mL for penicillin, ceftiofur, and gentamicin, respectively. The *S. zooepidemicus* isolate was sensitive to penicillin by Kirby Bauer testing. Bacteria were suspended in trypticase soy broth with 25% glycerol after identification and stored frozen at -80 °C. Just prior to use in this experiment, a small aliquot of each was thawed and plated on blood agar plates to ensure purity. Two to three colonies from each plate were then diluted in 10 mL Mueller Hinton Broth and cultured 37 °C and 5% CO₂ until the samples reached 0.5 McFarland (Sensititre E1041), based on nephelometry (approximately 8 hours for *E. coli* and 12 hours for *S. zooepidemicus*). Samples of each isolate were serially diluted and plated on Columbia agar with 5% sheep’s blood (Remel R01216) for quantification of bacteria used for inoculation.

2.4 Antimicrobial Preparation

A prepared solution of 0.1 mg/mL ceftiofur sodium was made from preservative free reference standard (Sigma-Aldrich 104010-37-9) diluted in 100% methanol.

A suspension of penicillin with gentamicin was made by mixing procaine penicillin G for injection (AgriLabs Agri-Cillin penicillin G procaine injectable suspension NADA 65-010) with
gentamicin sulfate for injection (Vedco GentaVed™ 100 NADA 200-037) to yield a final suspension concentration of 240,000 units/mL penicillin and 20 mg/mL gentamicin.

2.5 Time Kill Trial

Two milliliters of fluid growth medium (either Mueller Hinton Broth, n=20, or the previously autoclaved equine postpartum uterine fluid, n=20) were added to sterile 13 x 100 mm-borosilicate glass test tubes. Four tubes (n=2 Mueller Hinton Broth, n=2 postpartum fluid) were capped and set aside as negative controls, with no bacteria or antimicrobials added. The remaining 36 tubes were inoculated with either *E. coli* (n=18) or *S. zooepidemicus* (n=18). To accomplish this, 10 µL of each isolate at 0.5 McFarland were added to the 2-mL sample tubes (3.80E+05 CFU *E. coli*, for starting concentration 1.90E+05 CFU/mL, and 1.68E+05 CFU *S. zooepidemicus*, for starting concentration 8.4E+04 CFU/mL in each sample tube). Positive control samples for *S. zooepidemicus* and *E. coli* (n=3 Mueller Hinton Broth, n=3 postpartum fluid) consisting of inoculated fluid growth medium with no antimicrobial treatment were retained.

Immediately following inoculation, the remaining sample tubes (n=12 *S. zooepidemicus*, n=12 *E. coli*) were allocated to one of two antimicrobial treatment groups (ceftiofur or penicillin with gentamicin). Each antimicrobial treatment and fluid combination was cultured in triplicate.

The ceftiofur treatment samples (n=3 each of Mueller Hinton Broth/*S. zooepidemicus*, postpartum fluid/*S. zooepidemicus*, Mueller Hinton Broth/*E. coli*, and postpartum fluid/*E. coli*)
were treated with 10 µL of the above prepared solution to yield a treatment concentration of 0.5 µg/mL based on previous work documenting concentrations of ceftiofur expected to reach the uterus following systemic administration of ceftiofur [5].

The penicillin with gentamicin treatment samples (n=3 each of Mueller Hinton Broth/S. zooepidemicus, postpartum fluid/S. zooepidemicus, Mueller Hinton Broth/E. coli, and postpartum fluid/E. coli) were treated with 20 µL of the above prepared solution to yield a treatment concentration of 2400 units/mL penicillin and 0.2 mg/mL gentamicin. This concentration was based on the anticipated concentration in an intrauterine infusion volume of 60 mL, based on the volume of the equine uterus [11].

Following addition of antibiotics, each tube was gently vortexed, and the sample tubes placed into a 37 °C incubator with 5% CO₂ to promote bacterial growth. After 8 hours of growth, 200 µL were aspirated from each sample tube, and serially diluted 1:10 in 1X phosphate buffered saline (PBS; Fisher Scientific BP2438-4) in triplicate. Each dilution was then plated in duplicate on Columbia agar with 5% sheep’s blood culture plate in a modification of a time kill protocol per CLSI standards [8], [9], [10]. Plates were incubated at 37 °C and 5% CO₂ for 14 hours (E. coli) and 18 hours (S. zooepidemicus) to allow for distinguishable colony growth and 10 - 100 individually identifiable colonies were counted to quantify the amount of bacterial growth under each condition. This process was repeated after 24 hours of growth.
2.6 Data Analysis

The average number of colonies per dilution on each plate were tabulated and processed to determine the number of CFU/mL present under each treatment condition. The log change in bacterial growth was calculated based on the concentration of bacteria at the time of inoculation. The results were analyzed using Statistix 10 using a split-plot two-way ANOVA with significance level set at 0.05 (Statistix 10, Tallahassee FL). A ≥3 log decrease in CFU was defined as bactericidal activity and a <3 log decrease in CFU was defined as bacteriostatic activity [12].

3. Results

3.1 Escherichia coli (Figures 1 and 2)

The positive control samples for E. coli grew in both Mueller Hinton Broth and postpartum fluid. Peak growth was found at 8 hours, with 6.5 log and 4.2 log increase in bacterial numbers from inoculation in Mueller Hinton Broth and postpartum fluid respectively. At 24 hours, slightly fewer live bacteria were found compared to 8 hours, but numbers were still elevated from inoculation, with 4 log higher growth in both fluid types.

Treatment with ceftiofur inhibited growth of E coli at 8 hours in Mueller Hinton Broth, with a 0.4 log decrease in bacterial numbers from inoculation. In contrast, bacterial growth was not inhibited by ceftiofur in postpartum fluid at either time point, with 2.9 log and 3.3 log increases
in bacteria at 8 and 24 hours, respectively. Likewise, bacterial growth was not inhibited in Mueller Hinton Broth at 24 hours, with a 1.53 log increase in CFU.

The penicillin and gentamicin combination was bactericidal in Mueller Hinton Broth at 8 hours and in both fluid types at 24 hours, with a 5.3 log, 4 log, and 4 log decrease in numbers, respectively. Treatment with penicillin and gentamicin in postpartum fluid was bacteriostatic at 8 hours, with a 2.1 log decrease in bacteria.

3.2 *Streptococcus equi* subsp. *zooepidemicus* (Figures 3 and 4)

*Streptococcus zooepidemicus* grew in both media tested. In positive control samples at 8 hours, a 2.4 and 3.56 log increase from inoculation was documented in Mueller Hinton Broth and postpartum fluid, respectively. *S. zooepidemicus* continued to replicate in postpartum fluid, with a 3.87 log increase in bacteria from inoculation at 24 hours. However, in Mueller Hinton Broth, the concentration of *S. zooepidemicus* organisms at 24 hours was equivalent to the inoculum, reflecting a 3.87 log decrease in bacteria between 8 and 24 hour sampling times. All 24 hour Mueller Hinton Broth *S. zooepidemicus* samples were excluded from statistical analysis, because antibiotic activity could not reliably be differentiated from innate changes in growth pattern for these samples.

Treatment with ceftiofur was bacteriostatic in both fluid types, with a 2.1 log decrease and 0.7 log decrease in bacteria from inoculation in Mueller Hinton Broth and postpartum fluid,
respectively, at 8 hours. Bacterial growth was suppressed in postpartum fluid at 24 hours, with a 0.65 log decrease in bacteria compared to the inoculum.

Treatment using penicillin with gentamicin was bactericidal in both fluid types at both time-points, with a >4 log decrease in bacterial growth from inoculation in both Mueller Hinton Broth and postpartum fluid. With this treatment, no bacteria were cultured from any sample at any dilution.

4. Discussion

Our results in this work describe an effect of equine uterine fluid on antibacterial activity. Procaine penicillin G with gentamicin produced bactericidal activity against *E. coli* and *S. zooepidemicus* in both Mueller Hinton Broth and equine postpartum fluid, but ceftiofur did not. *Escherichia coli* and *Streptococcus* species (including *zooepidemicus*) are common pathogens cultured from uterine disease in the mare [7], [13], [14], [15]. In the population of cases evaluated by Ferrer and co-workers, 100% of *S. zooepidemicus* isolates were judged to be susceptible to ceftiofur and penicillin, while 96% of *E. coli* isolates were susceptible to ceftiofur (and 0% susceptible to penicillin) [7]. However, antibiotics can be affected by fluid characteristics such as volume, protein content, pH, and cellular content and, the *in vivo* efficacy of these antibiotics in postpartum lochia is unknown [1], [2], [3]. This report provides insight into the effect of equine postpartum uterine fluid on antimicrobial activity *ex vivo*. It may provide additional information for clinical antibiotic selection in cases of equine postpartum disease.
A gram-negative bacterium (*E. coli*), and a gram-positive bacterium (*S. equi* subspecies *zooepidemicus*) were chosen as representative etiologic agents which are commonly found in the equine uterus in cases of postpartum metritis, which is applicable to our choice of postpartum fluid as a comparative growth medium to Mueller Hinton Broth. Clinical isolates from equine uterine disease were specifically chosen over standard laboratory strains in order to better reflect the pathogenicity patterns of bacteria causing naturally-occurring disease. Unfortunately, specific virulence factors which promote equine uterine disease are not well-characterized and there are no laboratory strains available which could replicate them.

Ceftiofur has been proposed as a treatment for infectious endometritis in mares, with evidence to support that it reaches concentrations above MIC for *S. zooepidemicus* in endometrial tissue [5]. Under the conditions of our study, ceftiofur failed to inhibit growth of a clinical *E. coli* isolate and failed to achieve bactericidal activity against *S. zooepidemicus*. Complete suppression of ceftiofur activity against *E. coli* was attributed to the fluid environment of the postpartum equine uterus, because there was an increase in bacterial growth in samples of postpartum fluid compared to standard growth medium (Mueller Hinton Broth). This work agrees with Ensink et al, whereby ceftiofur’s antibiotic activity was impacted by the tissue environment [16]. Because the postpartum uterine environment is heavily contaminated and likely to contain both gram-positive and gram-negative organisms, ceftiofur should be used with caution in the postpartum mare.

Allen and coworkers observed that systemic absorption of penicillin from the uterus was enhanced when the endometrial tissue was irritated in some fashion, a state which would
presumably be present in a case of metritis [17]. Gentamicin is approved for intrauterine use in mares, and this use has been described in peer-reviewed veterinary literature, with drastically higher endometrial concentrations reached as compared to serum concentrations when administered via the intrauterine route [18], [19], [20]. High local tissue concentration supports the use of intrauterine versus systemic antibiotics. Further, our work demonstrated that a compounded formulation of procaine penicillin G and gentamicin was bactericidal against both organisms evaluated. These findings contrast with previous literature which suggested that penicillin was inactivated when mixed with gentamicin [21].

In Mueller Hinton Broth at 24 hours, the control samples of *S. zooepidemicus* did not grow. However, bacteria remained viable at the same time point in postpartum fluid. These findings confirm our preliminary data suggesting that this physiologic fluid represents a good growth-medium for bacteria and that autoclaving does not prevent bacterial growth. We conjectured that the decline in bacteria in Mueller Hinton Broth may have been the result of bacterial overgrowth and subsequent exhaustion of available nutrients in the finite volume of growth medium provided. All 24-hour samples of *S. zooepidemicus* in Mueller Hinton Broth were excluded from statistical analysis because an effect of antibiotic could not be differentiated from media-related die-off.

5. Conclusions

The results of this current work support the use of intrauterine procaine penicillin G with gentamicin in treating postpartum intrauterine infections caused by the most common etiologic
agents, *E. coli* or *S. zooepidemicus*. Ceftiofur reduced growth of *S. zooepidemicus* in equine postpartum uterine fluid, but not *E. coli*. In cases of mixed bacterial infection of *S. zooepidemicus* and *E. coli*, intrauterine infusion of penicillin with gentamicin provides antibiotic coverage against both bacteria.

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Declarations of interest: none
Figure 1: Log bacterial growth *E. coli* at 8 hours. Shaded area denotes bacteriostatic versus bactericidal activity. Error bars indicate standard deviation from the mean. Different letters correspond to statistically significant differences (p<0.05). n=3 for all groups. MHB=Mueller Hinton Broth (grey bars), PPF=postpartum fluid (black bars), CEFT=ceftiofur, PPG=penicillin with gentamicin.
Figure 2: Log bacterial growth *E. coli* at 24 hours. Shaded area denotes bacteriostatic versus bactericidal activity. Error bars indicate standard deviation from the mean. Different letters correspond to statistically significant differences (p<0.05). n=3 for all groups. MHB=Mueller Hinton Broth (grey bars), PPF=postpartum fluid (black bars), CEFT=ceftiofur, PPG=penicillin with gentamicin.
Figure 3: Log bacterial growth *S. zooepidemicus* at 8 hours. Shaded area denotes bacteriostatic versus bactericidal activity. Error bars indicate standard deviation from the mean. Different letters correspond to statistically significant differences (p<0.05). n=3 for all groups. MHB=Mueller Hinton Broth (grey bars), PPF=postpartum fluid (black bars), CEFT=ceftiofur, PPG=penicillin with gentamicin.
Figure 4: Log bacterial growth *S. zooepidemicus* at 24 hours. Shaded area denotes bacteriostatic versus bactericidal activity. Error bars indicate standard deviation from the mean. Different letters correspond to statistically significant differences (p<0.05). n=3 for all groups. PPF=postpartum fluid, CEFT=ceftiofur, PPG=penicillin with gentamicin.
REFERENCES


CHAPTER 4

Conclusions

The activity of commonly used antibiotics was consistently decreased in purulent uterine fluid samples from bitches and mares when compared to activity in standard bacterial growth medium (Mueller Hinton Broth). While extrapolation of this work on antibiotic activity in an ex vivo model should be made cautiously in applying these results to clinical patients, we believe these data have provided valuable insight into the nature of antibiotic activity in this unique environment which had been previously unreported. By comparing the activity of ciprofloxacin at three doses against E. coli in a standard bacterial growth medium (Mueller Hinton Broth) versus pyometra fluid obtained from clinical cases, we have demonstrated a decrease in antibacterial activity in pyometra fluid, and promising performance of ciprofloxacin in pyometra fluid at the highest dose tested. In the equine work, our findings supported the use of intrauterine procaine penicillin G combined with gentamicin to combat both E. coli and S. zooepidemicus in the postpartum mare, as their antibiotic activity was not lessened in the presence of equine lochial fluid.

In our laboratory, samples of pyometritic fluid were evaluated from 12 bitches (Table 1), with the pH, nucleated cell count, and total solids measured for each sample. These samples displayed a vast range of values for these parameters, with the tightest result for pH (5.99 ± 0.55). This aligns well with the tenet that infected fluids are typically acidic, and appropriately reflective of the disease state of pyometra. The nucleated cell count (194.6 ± 108.79 million cells/mL) and total solids (5.25 ± 5.24 g/dL) of the evaluated samples displayed a much broader range of
values. No associations between these three parameters were evident, with extrema of each parameter intermixed within the results when the data were sorted by parameter. Speculative explanations for this finding include duration of disease, individual bitch variation, and degree of uterine pathology. While no firm conclusions can be drawn from these data, they may serve as a useful springboard from which to base future work to more deeply delve into the defining qualities of pyometra fluid.

Similarly, samples of uterine fluid from four postpartum mares (three clinically within normal limits, one with retained fetal membranes) and one clinical case of pyometra were evaluated. As seen in the canine work, a broad range of results was obtained, with nucleated cell count (365 ± 303 million cells/mL), total solids (5.08 ± 2.6 g/dL), and pH (6.3 ± 0.95) spanning a large window of values (Table 2). When considering only the clinically normal postpartum mares, these results unfortunately did not generate an epiphany. The nucleated cell count (260.7 ± 267 million cells/mL), total solids (5.26 ± 3.3 g/dL), and pH (6.4 ± 1.3) were still representative of a wide range of results. This was attributed to the small sample size, as well as the broad window of postpartum interval they represented (less than one day to four days postpartum). Notably, there was an increase in total solids and nucleated cell count over time from the 12 hour postpartum sample to the 96 hour postpartum sample.

While it has been made clear that uterine fluid of bitches and mares is associated with suppression of antibiotic activity, the particular component(s) responsible for this outcome are not apparent from the current work and warrant further study.
Table 1: Characteristics of canine pyometra fluid (Von Dollen et al, 2019, unpublished data)

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Nucleated cell count (million cells/mL)</th>
<th>Total solids (g/dL)</th>
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<tbody>
<tr>
<td>A</td>
<td>5.64</td>
<td>60.5</td>
<td>2.6</td>
</tr>
<tr>
<td>B</td>
<td>5.63</td>
<td>66</td>
<td>5.2</td>
</tr>
<tr>
<td>C</td>
<td>6.54</td>
<td>14</td>
<td>4.3</td>
</tr>
<tr>
<td>D</td>
<td>5.43</td>
<td>414</td>
<td>-*</td>
</tr>
<tr>
<td>E</td>
<td>5.60</td>
<td>273.5</td>
<td>12.0</td>
</tr>
<tr>
<td>F</td>
<td>6.78</td>
<td>268.5</td>
<td>2.0</td>
</tr>
<tr>
<td>G</td>
<td>6.07</td>
<td>212.5</td>
<td>6.4</td>
</tr>
<tr>
<td>H</td>
<td>5.93</td>
<td>100</td>
<td>4.8</td>
</tr>
<tr>
<td>I</td>
<td>7.01</td>
<td>142.5</td>
<td>3.8</td>
</tr>
<tr>
<td>J</td>
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<td>202</td>
<td>5.2</td>
</tr>
<tr>
<td>K</td>
<td>5.34</td>
<td>206.5</td>
<td>4.4</td>
</tr>
<tr>
<td>L</td>
<td>6.23</td>
<td>32.5</td>
<td>7</td>
</tr>
</tbody>
</table>

Average: 5.99, 194.6, 5.25
Range: 5.34 - 7.01, 14 - 414, 2.0 - 12.0
Standard deviation: 0.55, 108.79, 5.24

Table 1: pH, nucleated cell count, and total solids for canine pyometra samples obtained at time of ovariohysterectomy. pH determined by GeneMate pH meter with A150418028 probe, cell count performed using hemacytometer with dilution in buffered formal saline, total solids determined by refractometer on supernatant of fluids after centrifugation at 3500 RPM for 20 minutes. Samples stored at -20 °C prior to thawing and aliquoting to obtain above results. *The viscosity of Sample D made total solids reading by refractometer impossible.
Table 2: Characteristics of equine uterine fluid (Von Dollen et al, 2019, unpublished data)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Clinical status</th>
<th>pH</th>
<th>Nucleated cell count (million cells/mL)</th>
<th>Total solids (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72h postpartum, no clinical abnormality</td>
<td>7.82</td>
<td>205.5</td>
<td>4.8</td>
</tr>
<tr>
<td>2</td>
<td>12h postpartum, no clinical abnormality</td>
<td>5.28</td>
<td>25</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>96h postpartum, no clinical abnormality</td>
<td>6.21</td>
<td>551.5</td>
<td>8.8</td>
</tr>
<tr>
<td>4</td>
<td>18h postpartum, retained fetal membranes</td>
<td>5.8</td>
<td>254</td>
<td>6.4</td>
</tr>
<tr>
<td>5</td>
<td>pyometra, pure growth <em>E. coli</em></td>
<td>6.38</td>
<td>787.5</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Average 6.30 364.7 5.08

Range 5.28 - 7.82 25 – 787.5 2.2 - 8.8

Standard deviation 0.95 302.76 2.62

Table 2: pH, nucleated cell count, and total solids for equine uterine fluid. pH determined by GeneMate pH meter with A150418028 probe, cell count performed using hemacytometer with dilution in buffered formal saline, total solids determined by refractometer on supernatant of fluids after centrifugation at 3500 RPM for 20 minutes. Samples stored at -20 °C prior to thawing and aliquoting to obtain above results.