TREATMENT EFFICACY OF TRIMETHOPRIM SULFAMETHOXAZOLE, PENTOXIFYLLINE AND ALTRENOGEST IN EXPERIMENTALLY INDUCED EQUINE PLACENTITIS

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2009

To Stasia, who lights up my life with joy

ACKNOWLEDGMENTS

This work would never have been completed without the support and help of those around me, including my loving wife, who gave up nights on end while I wrote, my parents who served as editors and the faculty members in Large Animal Clinical Sciences. Particular thanks are due to Margo Macpherson for heading up my graduate committee, Chris Sanchez for her insightful editorial comments and Steeve Giguere for always making time to answer statistics questions when I was frustrated. In addition, I gratefully acknowledge the Grayson Jockey Club Research Foundation and Intervet Inc. for their financial support of this project.

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August 2009

Chair: Margo L. Macpherson
Major: Veterinary Medical Sciences

Successful treatment for equine placentitis remains elusive. The primary objective of this study was to determine if long-term treatment with trimethoprim sulfamethoxazole (TMS; antimicrobial), pentoxifylline (PTX; anti-inflammatory/anti-cytokine) and altrenogest (ALT; synthetic progestin) would improve pregnancy outcome in mares with experimentally-induced placentitis. We hypothesized that combined treatment with TMS, PTX and ALT would delay premature parturition in mares with experimentally-induced placentitis and improve neonatal viability.

Seventeen normal pregnant pony mares were enrolled in the study at 280-295 days of gestation. Placentitis was induced in all mares by intracervical inoculation of *Strep. equi* subsp. *zooepidemicus* . Five mares served as infected, untreated control animals (group UNTREAT). Twelve animals (group TREAT) were infected and administered TMS, PTX and ALT from the onset of clinical signs until delivery of a live foal or abortion. Blood samples were cultured from all foals and fetal stomach and thoracic contents were obtained for culture from dead fetuses at delivery. Uterine swabs were obtained for culture from mares within three hours of delivery.

Tissues were collected from all placentas and from non-viable fetuses for histopathologic examination.

More mares in group TREAT delivered live, viable foals than mares in group UNTREAT (10/12, 83% versus 0/5, 0%; $P < 0.05$). Mares in group TREAT maintained gestation longer after inoculation than those in group UNTREAT (31 ± 14 days versus 8 ± 5 d; $P < 0.05$). Fewer foals in group TREAT had positive blood cultures than those from group UNTREAT (1/12, 8% versus 4/5, 80%; $P < 0.05$). However, there was no difference between groups in the presence of uterine bacteria within 3 hours postpartum bacteria (8/12, 67% versus 5/5, 100%; $P>0.05$). Placentas from group TREAT tended to have fewer inflammatory lesions at the level of the cervical star than placentas from group UNTREAT (6/12, 50% versus 5/5, 100%, $P=0.07$).

These data suggest that this combined regimen can reduce the effects of infection and inflammation and improve neonatal outcome. Uterine bacteria were not reliably eradicated using this treatment.

CHAPTER 1
INTRODUCTION

Placentitis is a common infectious cause of abortion, premature delivery and neonatal mortality in the horse [1-5]. Placentitis occurs most frequently during late gestation, and can be caused by a bacterial, fungal or viral infection [1,3]. Of these, ascending bacterial infection through the cervix is the most common cause of placentitis [1-4]. In many cases, mares with ascending bacterial placentitis abort acutely without any recognized clinical indicators. However, foals born to mares with chronic placental infections may have an accelerated fetal maturation and may be more likely to survive [5]. The goal of treatment is to prevent acute abortion and maintain pregnancy long enough for fetal maturation to occur sufficiently for neonatal survival. To achieve this goal, it appears that a diagnosis of placentitis must be reached early in the course of disease and treatment must aggressively address the disease on a multifactorial level.

There are few studies critically evaluating either the treatment of equine placentitis or diagnostic methods to monitor treatment efficacy and predict preterm delivery during treatment. The largest body of relevant information in regard to premature delivery and intrauterine infection was derived from rodent and non-human primate models. Consequently, research from these animal models, as well as research in women experiencing preterm labor will be used for comparison.

CHAPTER 2
LITERATURE REVIEW

Pathophysiology of Premature Delivery in Mares with Placentitis

The most common cause of placentitis in mares is believed to be ascension of bacteria through the cervix [1,2,6]. The underlying mechanisms permitting bacterial invasion are not defined; however several anatomic conditions, such as cervical incompetency and poor perineal conformation have been implicated [2,7,8]. Both conditions result in the failure of a normal anatomic barrier to bacterial ascension. Poor perineal conformation may lead to pneumovagina or urovagina, facilitating bacterial ascension to the anterior vagina and resulting in irritation and inflammation of the cervix [7,8]. Cervical damage may prevent the complete occlusion of the cervix in the pregnant mare and allow further bacterial ascension through the cervix [9,10]. Once through the cervix, bacteria are believed to colonize the fetal membranes. Some bacteria may penetrate the fetal fluids, from which they may colonize the umbilicus and gain access to the fetus hematogenously or be inhaled or ingested by the fetus [6]. Bacterial invasion of the chorioallantoic membrane and allantoic fluid is thought to initiate an increased expression of proinflammatory mediators including IL-6, IL-8, PGE2 and PGF2α *in vivo* in mares [11]. In a separate study, McGlothlin and others demonstrated *in vivo* that experimental trans-cervical incoluation of the mare with *Streptococcus equi* subsp. *zooepidemicus (Strep. equi* subsp. *zooepidemicus)* bacteria resulted in an increase in the intensity and duration of uterine contractile activity and a loss of the normal diurnal rhythm of contractile events [12] which are characteristic to non-human primates [13,14], women [15] and mares [12].

It is likely that pathogens do not need to reach detectable levels to induce an inflammatory response, and inflammatory mediators may be released in the fetal circulation in the absence of fetal infection [16]. *In vitro* studies in other species have demonstrated that phospholipases

produced by bacteria initiate formation of arachidonic acid, a precursor of prostaglandin. Endotoxins cause proinflammatory cytokine release from inflammatory cells, placental cells and amniotic cells (*in vitro*), including interleukins 1, 6 and 8 (IL-1, IL-6, IL-8) and tumor necrosis factor α (TNFα) [17-20]. These, in turn, catalyze the conversion of arachidonic acid to prostaglandin E (PGE) and prostaglandin F2α (PGF2α) [20]. *In vitro* studies in rats have demonstrated that prostaglandins are associated with increased oxytocin receptor density and gap junction density ultimately resulting in coordinated myometrial contractions [21]. PGE has also been reported to further induce formation of prostaglandin precursors, resulting in a positive feed-back mechanism which further enhances uterine contractions [22,23].

In addition, some bacteria produce the enzymes sialidase or mucinase. These enzymes may weaken the protective mechanisms of the cervix and promote ascension of bacteria. Alternatively, bacteria may induce the matrix metalloproteinase (MMP) gene expression and breakdown of the extracellular matrix of fetal membranes, further enhancing the risk of preterm rupture of the fetal membranes, preterm labor and preterm delivery [24,25].

Compromise of the fetal membranes and fetus further alters maternal hormone levels. The equine placenta is part of an endocrine fetoplacental unit which results in the synthesis of multiple progestagens from fetal pregnenolone. Progestagens are selectively secreted into the fetal or maternal circulation [26] and are believed to be responsible for maintenance of pregnancy after 120-150 days of gestation. Serum progestagen concentrations in normal pregnant mares remain low through mid gestation, but begin to rise around 315 days of gestation. Progestagen concentrations increase during the final weeks of gestation, peaking 24-48 hours before parturition and then decreasing dramatically [26]. The prepartum rise in progestagens is associated clinically with mammary gland development, while the decline is concurrent with

increasing fetal cortisol concentrations [27]. It has been shown that mares with a chronic form of placentitis experience a premature rise in circulating concentrations of progestagens [27,28], while mares that abort acutely after bacterial infection experience a drop in circulating progestagens [28,29]. However, the regulation of the endocrine changes noted in both normal mares and mares with compromised pregnancies has not been elucidated to date [26]. It is possible that the drop in serum progestagen concentrations results in a relative decrease in the progestagen/estrogen ratio and induces parturition. In other species, gap junction formation [30], oxytocin receptor density [31] and prostaglandin production [32] have each been induced in endometrial cells by a relative decrease in the progesterone/ 17-β estradiol ratio.

While the mechanisms controlling placentitis are not fully described, it is likely that inflammatory mediators, including Il-6 and IL-8 play a role along with increased allantoic concentrations of prostaglandins and alterations of normal hormonal regulation. Further work is needed to fully understand the interactions ultimately resulting in fetal death or preterm delivery of an immature non-viable foal.

Diagnosis of Placentitis

A number of tests have been used to diagnose the source of pregnancy complications in mares and other species. They include clinical exam findings, ultrasonography, direct culture of the fetal membranes or fetal fluids, biochemical assays for markers of inflammation, and hormonal assays.

Clinical Signs

A presumptive diagnosis of placentitis is most commonly based on clinical signs consistent with impending, premature parturition. The most common presenting complaints for mares with placentitis are precocious mammary development, streaming of milk and vulvar discharge [1-3,33]. However, these clinical signs may not be apparent early in the course of disease. Other

tools, such as ultrasonography, are frequently combined with clinical signs to diagnose placentitis.

Ultrasonography

Transrectal ultrasonography has been evaluated for use as a screening test in equine placentitis. The normal thickness of the uteroplacental unit based on transrectal ultrasonographic evaluation is well-described [34-39]. In a study of nine light-breed horses, Renaudin and co-workers established normal measurements for the combined thickness of the uterus and placenta (CTUP) between four and twelve months of gestation. The mean measurement in this population plus two times the standard deviation (95% confidence interval) was less than 7 mm for mares up to 270 days of gestation, less than 8mm for mares between 271 and 300 days of gestation, less than 10 mm for mares between 301 and 330 days of gestation and less than 12 mm for mares greater than 330 days gestation [35,37]. Subsequently, this and other groups also demonstrated that mares with placentitis had abnormal increases in CTUP based on transrectal ultrasonographic measurement [36,38,39]. Kelleman and co-workers evaluated the use of transrectal ultrasonography of pony mares in an experimental model of placentitis. In this study, the CTUP also increased over time in normal pregnancies. Additionally, mares which were experimentally-infected with *Strep. equi* subsp. *zooepidemicus* had larger measures of CTUP on transrectal ultrasonographic examination than did non-infected mares. The authors concluded that transrectal ultrasonography was an effective means of diagnosing placentitis in this model [34].

Transabdominal is frequently used to evaluate fetal well-being and viability [40-43]. Reef and co-workers measured fetal parameters and fetal fluid characteristics, as well as uteroplacental thickness, using transabdominal ultrasonography. This group subsequently developed an equine biophysical profile that included six factors related to pregnancy outcome.

For each factor a value of 0 or 2 was given, depending on whether the measurement fell within two standard deviations of the previously established means from normal mares: fetal heart rate (mean 75 ±7 bpm), fetal aortic diameter (22.78 ±2.15mm), maximal allantoic fluid depths (133.88 ±43.8mm), utero-placental contact (no separation noted), utero-placental thickness (13.8 ±2.3mm) and fetal activity [42,43]. These authors found that this biophysical profile had a high positive predictive value (a low score was indicative of fetal compromise) but did not have a high negative predictive value (a perfect score did not assure a positive outcome of pregnancy) [43]. Bucca and co-workers used transrectal and transabdominal ultrasonography to assess fetoplacental wellbeing in 150 uncomplicated pregnancies over 3 years from mid-gestation to term [40]. In this study, measurements of fetal parameters consistently fell within the limits previously established in the biophysical profile, further validating and expanding this technique in the diagnosis of placental disease [40]. Ultrasound examinations currently represent the best diagnostic tool available for the screening and diagnosis of placentitis in mares. However, these diagnostic tests are relatively insensitive and can only be used to diagnose grossly developed disease. In human obstetrics, similar ultrasonographic techniques represent the primary diagnostics used to evaluate fetoplacental well-being. Transvaginal ultrasound (TVU) examination and measurement of cervical length has been evaluated in numerous studies since the 1980s [44-47] and has been shown to be a sensitive screening tool (sensitivity 60-80%) for prediction of preterm labor and preterm birth in a population of high-risk patients [45]. In addition, transabdominal ultrasound examination is routinely recommended for women with late-term gestation (41 weeks) or any evidence of pregnancy complications, including preterm labor [48,49]. A biophysical profile with five primary criteria for fetal and placental well-being is well established in human obstetrical practice [50,51].

Bacterial Culture

Cervical cultures are not used as a routine screening-tool in mares, due to a concern that such a procedure could disrupt the barrier-function of the cervix. Disruption of the cervix or irritation of the cranial vagina and cervix could result in sufficient prostaglandin production to threaten the pregnancy. However, in mares with clinical and ultrasonographic evidence of placentitis, a culture of the cranial vagina, including any exudate visualized in the external os of the cervix may be useful in directing antibiotic treatment. The safety of this practice has not been tested experimentally in normal pregnant mares or mares with compromised pregnancies.

In women, bacterial culture has been explored as a screening tool for chorioamnionitis or intraamniotic infection. Surface cultures of the fetal membranes or the cranial vagina have not been shown to be effective as a screening tool for chorioamnionitis [52]. Cochrane systemic reviews in 2002 and 2007 concluded that there was no benefit in routine vaginal culture in otherwise low risk patients [53,54] to predict preterm labor. The Centers for Disease Control and Prevention and the American College of Obstetricians and Gynecologists do not recommend using universal bacterial cultures to screen asymptomatic women [55,56]. Currently, a culture of the cranial vagina is recommended in pregnant women before parturition to specifically screen for group B streptococci, which have been associated with increased maternal and neonatal morbidity at term [57-59]. Women that have positive vaginal growth of group B streptococci during pregnancy are given antibiotic therapy shortly before parturition to prevent systemic spread of the bacteria or contamination of the fetus during parturition [57-59]. A similar correlation between presence of vaginal/cervical bacteria has not been established in equine pregnancy.

Allantocentesis/Amniocentesis

Allantocentesis and amniocentesis are not commonly performed in the mare. Although allantocentesis has been performed successfully in a research setting [11,60-66], it is considered too invasive for clinical use. Several studies investigating the use of amniocentesis or allantocentesis demonstrated an increased pregnancy loss rate in mares after the procedure [60,63,66]. One study demonstrated mixing of amniotic and allantoic fluid over time in 50% of cases, using repeated ultrasound-guided amnio- and allantocentesis [60]. Several studies also found gross or histologic placental lesions of variable size in the ventral portion of the chorion and amnion when mares foaled or aborted [60,66]. As the mare has a diffuse epitheliochorial type of placentation and maintenance of pregnancy is dependent on the intimate connection between the chorioallantoic and the underlying endometrium [67], any procedure which would disrupt this dynamic cannot be considered safe. In addition, the environmental conditions of equine practice increase the risk of bacterial contamination of the fetal fluids by the procedure [66]. In women, culture of amniotic fluid derived by amniocentesis is the gold-standard for diagnosis of intraamniotic infection [68]. However, analysis of the available trials reveals that only 13% of amniocenteses performed in cases of preterm labor reveal intraamniotic infection [69]. This technique is also insensitive for the diagnosis of chorioamnionitis, a common risk-factor for preterm labor and fetal compromise [52] and the procedure itself is associated with potentially severe complications [70]. Fatal maternal sepsis was reported as a complication [71], as was umbilical vessel injury [72]. In addition, Romero and co-workers demonstrated a very short interval between amniocentesis and delivery (6 h), when intraamniotic infection was present [73]. This time-frame is insufficient for bacterial identification, further limiting the usefulness of this technique. Thus, amniocentesis for culture is rarely recommended as a diagnostic modality for women experiencing preterm labor [69].

Biochemical Assays

In mares, researchers have yet to investigate the usefulness of measuring proinflammatory cytokines to diagnose placentitis. LeBlanc and co-workers demonstrated increased levels of mRNA for IL-6 and Il-8 in allantoic tissue, but high levels of these cytokines were not identified in allantoic fluid in an experimental model of placentitis [11]. In addition, the difficulty of obtaining allantoic fluid samples diminishes the clinical usefulness of any biomarker obtained from these locations. In contrast, in women, the presence of elevated levels of proinflammatory cytokines (IL-6 and IL-8) in amniotic fluid, maternal serum and cervical fluid has been reported to be a reliable marker for chorioamnionitis and intraamniotic infection. Romero and co-workers found that high levels of IL-6 in amniotic fluid predict intraamniotic infection with 100% sensitivity and 83% specificity [74]. In a separate study, Kramer and co-workers showed that the presence of cytokines in amniotic fluid had a sensitivity of 87% and specificity of 89.5% for chorioamnionitis [75]. Strong correlations have been observed between IL-6 levels in cervical fluid and amniotic fluid [76], thereby cervical IL-6 levels may represent a relatively non-invasive means of diagnosis of chorioamnionitis.

Recently, Gravett and co-workers identified marker proteins in amniotic fluid and serum of women experiencing preterm labor through proteomic analysis of these body fluids. Calgranulin B, a member of the S100 calcium-binding protein family, and a novel 11-kDa proteolytic fragment of insulin-like growth factor binding protein 1 (IGFBP-1) were found to be elevated in serum and amniotic fluid of women experiencing preterm labor in comparison to normal pregnant women [77]. The authors concluded that these protein markers in blood were good indicators of placental disease. In a later study, the same group confirmed that these same marker proteins were also elevated in amniotic samples of rabbits with experimentally-induced

ascending intrauterine infection [78]. No studies have been performed in mares to date to analyze proteomic markers such as Calgranulin B in serum.

Hormonal Assays

In mares, fetoplacental hormones have been investigated to determine their potential as an indirect diagnostic for fetoplacental compromise or placentitis [26-29,80-94]. Fetoplacental progestagen production can be estimated by commercially available progesterone assays, and normal serum progesterone concentrations in maternal blood have been mapped throughout gestation for comparison [26,27]. In an uncompromised equine gestation, progestagen levels (based on test-specific cross-reactivity [26]) in maternal serum are between 10 and 16 ng/mL after approximately 200 days, gradually rise after 310 days to peak within 24-48 hours of parturition and decline rapidly thereafter [27,76]. In mares with compromised pregnancies, progestagen levels have been shown to follow one of two patterns: they may either drop precipitously before fetal demise or abortion [28,29,80] or they may be prematurely elevated [28,29,81-83]. This sudden change in progestagen levels may be a means of assessing the clinical condition of a mare in late gestation and potentially could be used as a predictor of outcome [84].

Estrogen is also a proposed marker of fetal compromise. Estrogen precursors are produced in very high quantities by the fetal gonads [85]. These precursors are subsequently metabolized to a variety of estrogenic compounds, including estrone sulfate, estradiol 17β, equilin and equilenin in the chorioallantoic membrane [86-88]. Total maternal serum estrogens in uncompromised pregnancy increase to variable peak between days 190 and 280 of gestation before falling to baseline values at term [89]. As maternal serum estrogen concentrations are dependent on gonadal function of the fetus and on placental function, estrogens may serve as a useful indicator of fetal well-being [90-92] However, a critical evaluation of estrone sulfate by

Santschi and co-workers in mares with medical conditions concluded that it was not an accurate indicator of fetal viability [29]. In addition, no difference in estrogen concentrations was seen between infected and non-infected mares in an experimental model of placentitis [28].

Serum relaxin has also been evaluated as a biochemical marker for placental insufficiency [93,94] However, this assay is not commercially available and has not been shown to be sufficiently specific, or to change early enough in the course of disease, to be a useful clinical diagnostic tool.

As yet, no acceptably reliable markers for placentitis or fetoplacental compromise have been discovered in the serum of mares. However, the recent identification of proteomic markers in the serum or amniotic fluid of infected primates and rabbits provides an interesting avenue for further research.

Diagnostic Modalities to Monitor Treatment-Effect

After initial diagnosis of disease, there is also a need for diagnostic tests to monitor changes in fetoplacental well-being and predict treatment outcome. Currently, equine clinicians are dependent on the resolution of clinical signs, such as mammary development. Serial ultrasound exams are used to monitor changes in the combined thickness of the uterus and placenta (CTUP), fetal heart-rate and fetal activity to evaluate fetal well-being and treatment response in mares with placentitis. Recently, Morris and co-workers proposed that serial progesterone assays may serve as a biochemical indicator to predict pregnancy outcome [84] however this study did not evaluate progestagen concentrations in treated mares. Likewise, other authors have proposed that estrone sulfate and relaxin might serve as indicators of fetal well-being [91,92] or placental insufficiency [93,94], yet neither hormone has been investigated in a clinical trial. In human medicine, ultrasonography and fetal cardiac monitoring also remain the most-used diagnostic tools for women experiencing pregnancy complications, including preterm

labor and premature preterm rupture of the fetal membranes. Studies using TVU after treatment for preterm labor found that an increase in cervical length was associated with an increase in incidence of term delivery, whereas cervical length < 25mm was associated with preterm delivery [45,95]. In another study, TVU for measurement of cervical length was evaluated after 17P-treatment [96]. This study found that progestin therapy attenuated the progressive cervical shortening which was otherwise seen in women after an episode of preterm labor. No biochemical assays are currently available to monitor women undergoing treatment for either chorioamnionitis or preterm labor.

Treatment of Placentitis

The goal for treatment of placentitis in mares is to arrest further placental damage by the infective agent and, if possible, to improve the function of the remaining normal placenta [33]. With few controlled studies in mares that evaluate drug penetration to the placenta or fetus, most treatment regimens for equine placentitis are empirical. Modeled after work in other species, treatment is generally designed to address the multifactorial nature of the disease.

Antibiotic Therapy

The first line of defense against placentitis is antimicrobial therapy. Antibiotic agents used to treat placentitis in mares include cephalosporins, tetracyclines, sulfonamides, trimethoprim, carboxypenicillins and penicillin plus betalactamase inhibitors. These drugs have good *in vitro* sensitivity against the most common organisms causing ascending placentitis, including *Strep. equi* subsp. *zooepidemicus* and *Escherichia coli* [97]. In addition, they have good *in vitro* inhibition of isolates of nocardioform bacteria from clinical placentitis cases [33].

Early prospective studies on treatment of equine placentitis focused on antibiotic therapy, yet initial work was inconclusive as to how well the commonly used antibiotics penetrated the pregnant uterus [65,98]. Sertich and Vaala determined maternal serum concentrations, amniotic

and allantoic fluid concentrations, and foal serum concentrations of penicillin G, gentamicin sulfate and trimethoprim sulfadiazine in a study of 11 periparturient mares. All antibiotics were detected in maternal blood. Trimethoprim sulfadiazine was detected in allantoic fluid and in foal serum at parturition, while penicillin and gentamicin were not detected reliably in either allantoic fluid or serum drawn from foals at time of parturition [65]. Santschi and Papich injected gentamicin into three mares within 60 minutes of parturition and subsequently assayed plasma of the newborn foals and amniotic fluid of one mare for the presence of the drug. They did not find measurable concentrations of gentamicin in any sample and concluded that the drug likely did not cross the placenta at term [98]. In contrast, two studies at the University of Florida established allantoic concentrations for penicillin G, gentamicin [62], trimethoprim sulfamethoxazole (TMS) and pentoxifylline (PTX) [64] using an *in vivo* microdialysis technique for continuous drug monitoring of the allantoic fluid after drug administration. In the first study, five normal pregnant pony mares and two mares with experimentally-induced bacterial placentitis were treated with standard doses of potassium penicillin, gentamicin and flunixin meglumine. Antibiotic concentrations in allantoic fluid and serum were determined in all samples using high-performance liquid chromatography (penicillin) and ELISA (gentamicin). In this study, penicillin and gentamicin were detected in allantoic fluid of normal mares and mares with experimentally-induced placentitis. Drug concentrations in allantoic fluid were furthermore found to exceed previously established minimum inhibitory concentrations (MIC) for susceptible organisms for at least 210 minutes [62]. Rebello and co-workers demonstrated the presence of TMS in allantoic fluid of both normal and experimentally-infected mares using the same techniques. Like gentamicin and penicillin, allantoic concentrations of TMS met or exceeded published MIC for *Strep. equi* subsp. *zooepidemicus* [64]. TMS is a broad-spectrum,

bacteriocidal antibiotic with good *in vitro* activity against common causative organisms of placentitis (*Strep. equi* subsp. *zooepidemicus, Escherichia coli,* nocardioform actinomycetes) [64,99,100]. It has been shown to have adequate *in vitro* efficacy (60-81%) against clinically observed strains of β hemolytic streptococci [101,102], which is the most common organism associated with ascending placentitis. Penicillin has a higher *in vitro* efficacy against clinically observed strains of β hemolytic streptococci than TMS (93-100%), but is not efficacious against other causative organisms of placentitis, including *Escherichia coli* and *Pseudomonas aeruginosa* [101,102]. Gentamicin has poor *in vitro* efficacy against gram positive bacteria, such as *Strep. equi* subsp. *zooepidemicus* or nocardioform organisms, but has adequate efficacy against clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa* (62-83%) [101,102]. Thus, either TMS, or penicillin and gentamicin in combination, are good choices to treat placentitis.

In a clinical trial examining the efficacy of TMS and PTX in the treatment of experimentally-induced placentitis, these drugs were identified in all placental and fetal tissues examined at the time of foaling, further confirming their ability to cross the placenta and enter the fetus *in utero* [100]. Additionally, treated mares tended to maintain pregnancy longer after infection than did untreated mares. However, there was no difference in fetal survivability after treatment, with one live foal in each group. Likewise, prophylactic antibiotic therapy alone has not resulted in a reduction of preterm delivery in women with high-risk pregnancies [25,103]. One possible reason for the failure of antibiotics to reduce rates of preterm birth is their inability to reduce chorioamnionitis [104]. Two recent trials evaluating placental samples after antibiotic treatment found no difference in histological chorioamnionitis between women with high-risk pregnancies who received prophylactic antibiotics or placebo treatment [105,106]. However,

antibiotic therapy is recommended in all women experiencing preterm labor of any cause to prevent neonatal infection with group B streptococci [58,59]. In addition, two clinical trials demonstrated that antibiotic administration in women with premature rupture of the fetal membranes resulted in prolongation of pregnancy, decreased neonatal morbidity and a reduced rate of maternal chorioamnionitis [107,108]. Thus, antibiotic therapy is recommended for this sub-population of women with high-risk pregnancies.

Anti-inflammatory Therapy

Inhibition of inflammatory processes that cause preterm labor represents a second major treatment goal in mares with placentitis. Two anti-inflammatory drugs have been investigated in equine studies.

Flunixin meglumine, a non-steroidal anti-inflammatory drug which specifically inhibits the conversion of arachidonic acid to prostaglandin is a component of many empirical treatment regimens for equine placentitis. In mares experimentally injected with endotoxin in early gestation (day 21-35), flunixin meglumine prevented prostaglandin synthesis and subsequent luteolysis [109], resulting in maintenance of pregnancy. However, flunixin meglumine was not effective at preventing cloprostenol-induced abortion between 80 and 150 days in a subsequent study by the same group [110]. In late gestation, flunixin meglumine has not been demonstrated to cross the placenta and its effects are unknown. In one study, flunixin meglumine was not detected in serum or allantoic fluid after microdialysis collection of samples [62]. It was detected in serum when blood was collected by venipuncture. The authors suspected that since the drug is highly protein bound to serum molecules, it might have been present in allantoic fluid, but unable to penetrate the microdialysis membrane [62]. To date, the use of flunixin meglumine has not been investigated in a treatment trial for placentitis as a stand-alone drug or in combination with other medications.

Pentoxifylline is an immune-modulator which can induce a dose-dependent reduction in TNFα [111,112] and IL-1 [111] by inhibition of membrane phosphodiesterase. These are potent proinflammatory mediators [113]. In non-human primates, TNFα, and other pro-inflammatory cytokines have been shown to rise within six hours after bacterial inoculation with β-hemolytic streptococci, followed by an increase in uterine contractility and subsequent preterm delivery [114]. Similar increases in uterine contractility have also been demonstrated in mares inoculated with *Streptococcus equi* subsp. *zooepidemicus* [20]. Pentoxifylline has been shown to block the pro-inflammatory actions of IL-1 and TNF on neutrophils *in vitro*, thereby potentially decreasing tissue damage caused by neutrophils [115]. In addition, pentoxifylline may have direct protective effects. It modulates platelet aggregation and improves tissue blood flow in the equine foot by increasing prostaglandin I2 (PGI2) production [111]. In critically ill septic human patients, pentoxifylline has been shown to improve tissue oxygenation by increasing oxygen transport and oxygen uptake [116]. It has also been shown to improve bacterial clearance and significantly decrease bacterial colonization of the lung and kidney in rabbits undergoing hemorrhage or endotoxemia [117]. In mares, Rebello and co-workers demonstrated that pentoxifylline reaches the allantoic fluid in normal pregnancy and in experimentally infected mares. In this study, allantoic concentrations of PTX mimicked serum concentrations, with peak levels of 1.3 μg/mL detected in both allantoic fluid and serum using microdialysis. These concentrations were lower for both serum and allantoic fluid when fluid was collected by microdialysis than in samples collected by venipuncture (1.3 μg/mL vs. 3.4 μg/mL) [63]. This difference may be a result of partial binding of pentoxifylline to plasma proteins. Graczyk and co-workers further demonstrated the presence of PTX in placental and fetal tissues at foaling,

confirming its ability to cross the placenta and reach the foal [100]. Therapeutic tissue concentrations have not been established for this drug in horses.

In women experiencing preterm labor, anti-inflammatory therapy is also considered a vital component of treatment. Data from rodents and non-human primates have shown that anti-inflammatory and immunomodulatory therapies suppress cytokine-induced contractions and may decrease uterine activity and aid in the prevention of preterm labor [118-120]. Sadowsky and co-workers used a model of preterm labor with rhesus-monkeys to show that both indomethacin and dexamethasone were effective at inhibiting cytokine-induced uterine activity [118,119]. Indomethacin, a nonselective cyclooxygenase (COX) inhibitor, effectively inhibited prostaglandin production and uterine contractility after infusion of recombinant IL-1ß despite a rapid increase of TNFα, IL-6, IL-8 and IL-1 [118]. Dexamethasone, a synthetic glucocorticoid, effectively inhibited an increase in IL-6 and uterine contractility after infusion of IL-1ß [119]. Recently, Gravett and co-workers demonstrated that the combination of immune-modulators with antibiotic therapy could effectively delay the onset of preterm labor [121]. Using a combination of ampicillin, dexamethasone and indomethacin, they successfully delayed preterm labor in non-human primates. They hypothesized that treatment with the combination of antibiotic and anti-inflammatory drugs was necessary to resolve intra-uterine infection and inflammation. After an intra-amniotic inoculation of group B *Streptococcus* species, the animals were monitored for uterine activity, infection and biochemical markers. Treatment with antibiotics alone resolved bacterial infection but did not reduce uterine activity, amniotic fluid cytokines or prostaglandin production. Animals treated with ampicillin, dexamethasone and indomethacin showed suppression of inflammatory cytokines, prostaglandins and uterine contractions. The duration to onset of labor was significantly prolonged in animals administered

the combined treatment [121]. This study was the first study to prospectively examine the effects of combined therapy in an experimental model of intraamniotic infection. The results strongly support the need for a multi-modal approach to the treatment of intrauterine infectious diseases, which includes, at a minimum, antibiotics and anti-inflammatory therapeutics. In the mare, TMS and PTX were not successful in increasing the length of gestation or improving neonatal viability after experimental induction of placentitis [100]. Further study is needed to investigate additional anti-inflammatory agents and antibiotics for this purpose.

Progestin Therapy

In addition to antibiotic and anti-inflammatory treatments, synthetic progestins are common components of treatment regimens in both women and mares. Rationale for this therapy in preterm labor stems from the role progestins play in inhibiting formation of myometrial gap junctions, which facilitate uterine contractility. These findings were first demonstrated by Garfield and co-workers through *in vitro* studies of ovine endometrium [30]. Additional work suggested that administration of progestins also may decrease the number of uterine estrogen receptors [122]. More recent *in vitro* studies have shown that progesterone interferes with the binding of oxytocin to its receptor and inhibits prostaglandin secretion in ovine endometrium [123], and may inhibit MMP expression in term decidual cells of rodents [124]. In several species, (rats, sheep and rabbits) progesterone has been shown to decrease myometrial contractions *in- vitro* and *in-vivo* [125-127]. These effects have yet to be demonstrated in mares. An i*n vitro* study by Ousey and co-workers was not able to demonstrate a decrease in contractility in equine myometrial strips taken from mares in late-gestation when exposed to progesterone or a synthetic progestin [128]. However, altrenogest, a synthetic progestin, has been effective at preventing abortion in clinical trials involving early- and mid-gestation mares. In a study by Daels and co-workers, altrenogest was effective at promoting

pregnancy maintenance after intravenous infusion of *Salmonella typhimurium* endotoxins to mares between days 21 and 35 of gestation [129]. Endotoxin infusion resulted in a biphasic increase in serum prostaglandin concentrations and a reduction in plasma progesterone concentration to values less than 1 ng/ml within 24 hours in all mares. However, 13 of 14 mares treated with altrenogest maintained pregnancy throughout the treatment period. Mares treated with 44 mg of altrenogest, daily until day 70, maintained gestation to normal term and delivered live foals [129]. In a subsequent study, the same authors also demonstrated that altrenogest prevented cloprostenol-induced abortion at 80-150 days of gestation [130,131]. McKinnon and co-workers had similar results with altrenogest, but failed to prevent abortion with other progestins after prostaglandin-induced luteolysis in early gestation [132], while Vanderwall and co-workers were able to prevent abortion in cloprostenol-treated mares with both altrenogest and a compounded injectable progesterone formulation [133]. These studies support the hypothesis that progestins are effective replacements for ovarian progesterone and can maintain pregnancy under conditions that would otherwise cause abortion, such as endotoxin- and prostaglandin-induced luteolysis. Studies are limited investigating the usefulness of progestin therapy to prevent abortion in late gestation mares. Data from other species strongly support the use of progestin therapy to prevent preterm labor [30,122-127,134-141]. In women, clinical data clearly demonstrate that progestins are effective at preventing preterm labor [134-141]. A landmark, multi-center study confirmed the positive effect of 17α hydroxyprogesterone caproate (17P) treatment for women at risk for preterm labor [136]. Four hundred and sixty-three women (310 progestin treated, 153 placebo treated) were enrolled in the study at 16-20 weeks gestation. All patients had experienced preterm labor in previous pregnancies. Treatment with 17P significantly reduced the risk of delivery at less than 37 weeks gestation [136]. Several

subsequent studies have confirmed these results in women with a history of preterm labor [134,135,138,141]. The efficacy of 17P has also been demonstrated for the treatment of ongoing preterm labor in women. In one study, treatment with 17P was associated with a reduction in the degree of cervical shortening seen at 7 and 21 days after onset of therapy and a reduction in the risk of preterm delivery [96]. Progestin therapy is now a standard recommendation from the American College of Obstetrics and Gynecology for women experiencing preterm labor.

Tocolytic Therapy

Another therapeutic modality that has been used with some success in humans are tocolytics. Betamimetics [142] and other tocolytic agents, such as oxytocin antagonists [143-145] and magnesium sulfate [146] are widely used in the treatment of preterm labor in women. As yet, none of these has been conclusively shown to prevent preterm delivery in women or improve neonatal outcome [147,148]. In mares, limited studies have investigated the safety or efficacy of tocolytic agents. Palmer and co-workers investigated the effect of clenbuterol in term mares at multiple doses and were unable to inhibit parturition with this agent [149]. Further work is necessary to determine whether any tocolytic agent is effective at preventing parturition in the mare.

In conclusion, data suggest that no single therapeutic agent is effective at controlling intrauterine infection and inhibiting preterm delivery in either mares or women. Data from a large-scale field trial support the use of multimodal therapy to treat placentitis in mares [38]. Four hundred and fifty mares were examined for symptoms of placentitis over three years. Fifteen mares were clinically diagnosed with placentitis, based on the presence of vulvar discharge, mammary development or an increased CTUP on transrectal examination. The mares were maintained in a farm-environment and treated with a variety of regimens, including a broad-spectrum antibiotic, anti-inflammatory therapy, pentoxifylline and altrenogest. Mares were

treated from the time of diagnosis until delivery. Of the 15 mares diagnosed with placentitis, 13 maintained pregnancy until at least 310 days gestation and 12 delivered viable foals. These data, while uncontrolled, provide support for therapy including antibiotics, anti-inflammatory therapy and progestins to treat placentitis.

Based on data from the reviewed studies, the current study was designed to investigate the effects of a multimodal treatment approach in an experimental model of ascending placentitis. Oral medications commonly used for the treatment of placentitis in equine practice were administered to experimentally infected mares from the onset of clinical signs until delivery.

CHAPTER 3
OBJECTIVES AND HYPOTHESES

The objectives of the current study were to determine whether treatment with an oral regimen of trimethoprim sulfamethoxazole, pentoxifylline and altrenogest would 1) increase the length of pregnancy and 2) improve neonatal viability, and 3) to determine whether application of currently available diagnostic tests would accurately predict disease in mares with experimentally induced placentitis.

We hypothesized that 1) mares treated with TMS, PTX and ALT would maintain pregnancy longer than untreated mares, 2) that the resultant foals would be viable at parturition, and 3) that physical examination, transrectal and transabdominal ultrasonography and progesterone assays would be sensitive diagnostic indicators of disease post-inoculation.

CHAPTER 4
MATERIALS AND METHODS

Animals

Seventeen reproductively normal, pregnant pony mares were enrolled in the study at 280-295 days of gestation. Baseline inclusion data (normal systemic parameters; normal combined thickness of the uteroplacental unit [34-38], echogenicity of fetal fluids, fetal activity and heart rate within normal limits [40-43]) were recorded prior to experimentation. All mares were inoculated intracervically with *Strep. equi* subsp. *zooepidemicus* . Mares were divided randomly into two groups. Five mares served as infected, untreated control animals (group UNTREAT). Twelve animals were infected and administered trimethoprim sulfamethoxazole (TMS), altrenogest (ALT) and pentoxifylline (PTX) (group TREAT). Mares were maintained on pasture at the College of Veterinary Medicine, University of Florida and were supplemented with hay and concentrate. This project was approved by the Institutional Animal Care and Use Committee of the University of Florida.

Bacterial Inoculation

Between 280 and 295 days of gestation, mares were inoculated intracervically, with *S. zooepidemicus* obtained from a clinical isolate submitted to the Microbiology Laboratory at the University of Florida, College of Veterinary Medicine in 1999 [150]. The bacterial isolate was sensitive to TMS *in vitro*. The bacterial inoculum was approved by the Environmental Health and Safety Unit at the University of Florida and has been stored in cryovials containing Brucella broth with 10% glycerol and porous beads (Cryosaver®, Hardy Diagnostics, Santa Maria, CA) at minus 80ºC.

The day before inoculation of a mare, one cryovial was taken out of the -80ºC freezer and two blood agar plates (Remel Inc. Lenexa, KS) were struck with 1 bead each. After the initial

streak, a three-quadrant isolation method was used and the plates were incubated at 37ºC for a minimum of 18 hours. On the day of inoculation, a 10^7 CFU inoculate was made using the MacFarland standards for microbiology dilutions, which contain 0.05ml of 1% BaCL2 and 9.95mL 1% H2SO4 (McFarland Standard 0.5, Hardy Diagnostics, Santa Maria, CA). A bacterial concentration of $1.5x10^8$ CFU was fashioned by adding single colonies from the isolate made of the streptococcus until the turbidity matched the 0.5 McFarland Standard. To achieve the desired inoculate bacterial concentration of $1x10^7$ CFU, the solution was diluted to the 10th power using 0.9% sterile saline to make a final concentration of $1x10^7 – 1.5x10^7$ CFU *Strep equi* subsp. *zooepidemicus* bacteria. After all dilutions were made, 100µL of each dilution was struck on a blood agar plate (Remel Inc, Lenexa KS) for quality control analysis.

Mares were placed in stocks, their tails were wrapped and pulled laterally out of the field of work. The perineum was washed thoroughly with an iodine-based soap and dried. An inoculum of 1 x 10^7 *Strep equi* subsp. *zooepidemicus* organisms, diluted in 2 mL of saline, was deposited approximately 2.5 cm beyond the external os of the cervix using an artificial insemination pipette under digital guidance [100,150,151].

Mare Monitoring

Beginning the day of bacterial inoculation, a complete physical exam was performed on each mare in the morning and evening for the duration of the study.

Physical Exam

Systemic parameters: Systemic parameters (temperature, pulse, respiration, digital pulses, gut sounds, mucous membranes, attitude) were recorded until abortion or delivery of a live foal.

Vulvar discharge: Mares were monitored for presence of vulvar discharge and scored using the following system: 0 = no discharge; 1 = trace amount discharge at vulvar lips; 2 = slight amount discharge; 3 = moderate amount discharge; 4 = significant amount discharge.

Mammary gland development: Mammary gland development was scored as follows: 0= no development (flat glands); 1 = slight rounding of glands; 2 = moderate rounding of glands; 3 = glands developed but teats empty; 4 = glands developed with teats filled/waxed.

Transrectal Ultrasound

Using transrectal ultrasonography (Aloka 900® with 5-10MHz linear probe, Aloka CO, Ltd, Tokyo, Japan), the combined thickness of the uterus and placenta (CTUP) was monitored for signs of thickening or placental separation as evidence of placental disease. Established measures of CTUP for normal pregnancy were used as standards [34,36]. Baseline measures were recorded prior to bacterial inoculation of each mare. Beginning the day after inoculation, mares were examined, using transrectal ultrasonography, daily for seven days, and then three times weekly until abortion or delivery. In instances where separation of the chorioallantois from the endometrium was detected, this was noted and no CTUP was recorded. Allantoic and amniotic fluid character were also monitored during examinations. Fluid character was graded as: 0 =anechoic/black; 1 = hypoechoic/dark gray; 2 = echogenic/light gray; 3 = hyperechoic, non-shadowing/white.

Transabdominal Ultrasound

Mares were examined using transabdominal ultrasound (Aloka 900® with 2-5MHz curvilinear sector probe, Aloka Co, Ltd, Tokyo, Japan) to monitor fetal fluid character, fetal heart rate and evidence of placental separation [34,40,41]. A baseline examination was performed prior to bacterial inoculation. Beginning the day after inoculation, mares were examined, using transabdominal ultrasonography, daily for seven days, and then three times per

week until abortion or delivery of a foal. Fluid character was graded as for transrectal ultrasound evaluation.

Drug Administration

Drugs were administered to mares in group TREAT beginning with the first signs of disease (ultrasonographic evidence for increasing CTUP, placental separation, changes in fluid character, mammary gland development or vulvar discharge). TMS (Vintage Pharmaceuticals®, Huntsville AL; 30 mg/kg, PO, q 12 h), ALT (Regumate®, DPT Laboratories®, San Antonio TX; 0.88 mg/kg, PO, q 24h) and PTX (Apotex Inc®, Toronto ON Canada; 8.5 mg/kg, PO, q 12h) were administered to mares until abortion or delivery of a live foal. In order to approximate the therapeutic management of placentitis in a clinical setting, drugs were administered simultaneously, at doses consistent with those used clinically.

Serum Sampling of Mares

Serum samples were collected from mares for assay of progesterone. A baseline blood sample was obtained from mares prior to bacterial inoculation. Beginning the day after bacterial inoculation, blood samples were obtained from all mares once a day for one week. From the second week forward, blood samples were taken three times per week. Serum samples were stored in 500 µL aliquots at -80˚C until analysis.

Radioimmunoassay for Progesterone Concentrations

Quantification of serum progesterone levels was performed by a solid-phase radioimmunoassay kit (DPC Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA). The kit's standard calibrators yield a calibration curve with a range of 0.1-40 ng/mL progesterone. One hundred µL of equine serum were added to two, 12x75 mm tubes coated with progesterone antibodies. One mL of I-125 labeled progesterone tracer was added to each tube. The tubes were incubated with the I-125 tracer at room temperature for three hours. Following

incubation, the tracer was decanted and the tubes were analyzed for one minute on a gamma counter (Cobra 2, Packard Instruments, Meridian, CT USA). Progesterone levels were converted from counts per minute (CPM) to ng/mL using the calibration curve. The kit's intra-assay coefficient of variance (CV) was < 8.8, 4.9, 4.0, 3.6, 3.9, and 2.7% respectively, and the inter-assay CV's were <9.7, 7.1, 5.7, 3.9, 5.6, and 3.9% respectively. The kit's analytical sensitivity was 0.02 ng/mL.

Monitoring Mares for Impending Parturition

Mares were monitored twice daily for evidence of impending foaling (mammary gland enlargement, evidence of mammary secretions, vulvar softening, laxity of tendons or vulva). Once changes consistent with impending foaling were noted, mares were monitored by visual observation in the paddock every 2-3 hours. When evidence of parturition was noted (increased incidence of recumbency, restlessness, inappetence, straining to urinate, evidence of fluid from the vulva indicating rupture of the chorioallantois), mares were observed continuously through foaling and passage of the fetal membranes. Mares were allowed to foal normally unless assistance was deemed necessary (dystocia, premature separation of the fetal membranes). Mares with viable foals were allowed to bond in the postpartum period.

Management of Live Foals

All live foals immediately received a physical examination. Foals that were able to breathe without mechanical assistance, right themselves after birth, respond to nasal or ear stimulation, and which had good muscle tone were deemed viable. The neck was prepared aseptically to obtain blood for inoculation of a blood-culture bottle (BBL® SEPTI-CHECK™, Becton Dickinson, Sparks, MD), complete blood count (CBC), serum chemistry, and for serum cortisol assay (Immulite® 1000 Cortisol, Siemens healthcare Diagnostics, Inc, Llanberis, UK) in live foals. These data were used to determine the foal's health status and maturation of the pituitary

adrenal axis. The white blood cell count (WBC) was compared to established normal leukocyte counts for foals less than twelve hours old (6.9-14.4x10^3 cells/μL [152]). A neutrophil:lymphocyte ratio > 2 was considered indicative of fetal maturity [153]. Serum cortisol concentrations were compared to established normal values for foals delivered at term (120-140 ng/mL at approximately one hour postpartum, followed by a decrease to around 60 ng/mL by six hours postpartum [154]), and induced, preterm foals (8.4 +/-1.6 ng/mL at approximately one hour postpartum, with only moderate rises in the postpartum period [155]).

Minimum supportive care was provided to viable foals as needed. All viable foals were monitored frequently in the first 24 hours postpartum and twice daily physical exams for five to seven days post-foaling. All foals were administered antibiotics (Ceftiofur Sodium (Naxcel® Pfizer Animal Health Inc. New York, NY), 4mg/kg IM q 12h for 5-7 days; or Ampicillin (Generic, Webster Veterinary Supply Inc. Sterling, MA), 20mg/kg IV q8 h IV and Amikacin (Generic, Webster Veterinary Supply Inc. Sterling, MA) 25mg/kg IV q24h for 5-7 days). A nasogastric tube was placed for colostrum administration in viable foals which did not nurse within three hours after birth, or when the maternal colostrum was of poor quality. Foals that did not nurse readily received an indwelling nasogastric tube and supplemental feeding. Foals with mecomium impactions received a soapy-water enema.

Additional blood samples were taken from viable foals between 8 and 24 hours of birth and evaluated for presence of immunoglobulins (Snap® Equine Immunoglobulin test kit, Idexx Pharmaceutical Inc, Greensboro, NC) and cortisol concentrations (Immulite® 1000 Cortisol, Siemens Healthcare Diagnostics, Inc, Llanberis, UK). Blood immunoglobulin concentrations greater than 800 mg/dL at 8-24 hours post-foaling were considered indicative of adequate IgG transfer [154].

Management of Dead and Non-Viable Foals

Live-born foals which had clear evidence of immaturity or dysmaturity (soft haircoat, severe tendon laxity, inadequate muscle control to respond to stimulation, inability to achieve a sternal position) or which could not breathe independently were deemed non-viable. Non-viable foals were euthanized using an overdose of barbiturate (pentobarbital sodium and phenytoin in combination - Beuthanasia®, Schering-Plough Animal Health, Kenilworth NJ).

A necropsy was performed immediately on all euthanized and aborted foals. Blood was obtained aseptically by venipuncture from non-viable foals and by intracardiac puncture from foals that were dead at time of examination and inoculated into a blood-culture bottle (BBL® SEPTI-CHECK™, Becton Dickinson, Sparks, MD). A gastric aspirate and thoracic swab were obtained from all dead and euthanized foals for bacterial culture.

Histologic Tissue Analysis

Tissues were collected from fetal membranes for histopathologic analysis. The fetal membranes were weighed and evaluated grossly for completeness and abnormalities. Samples were procured from the pregnant horn, non-pregnant horn, uterine body, umbilicus and cervical star area of the fetal membranes. Any grossly abnormal area of the fetal membranes was also sampled.

A complete necropsy was performed on all dead and non-viable foals. Tissue samples of lung, liver, kidney, spleen, and adrenals were collected. Two samples were collected from each tissue/site in both the fetal membranes and fetus. One sample was preserved in formalin for histopathologic analysis and the second sample was placed in a small plastic bag and frozen at minus 80˚ C for possible future analysis.

Uterine Culture

A uterine swab was obtained for bacterial culture from all mares within three hours of foaling. The mare's tail was wrapped and pulled to the side, and the perineum was aseptically prepared. A McCullough® double-guarded uterine swab (HAR-VET™. Spring Valley, WI) was used, in routine fashion, to collect the uterine sample. All cultures were plated on three different media (blood agar, Columbia CNA with 5% sheep blood, and MacConkey agar, Remel Inc. Lenexa, KS) within 24 hours of collection using the streak plate method. The swab was directly rolled evenly over one quadrant of the plate. Then a sterile wire loop was used to spread the potential organisms over the rest of the plate using the 3 quadrant isolation method. The plates were placed in an incubator set at 37ºC for 24 to 48 hours. If bacteria were seen after 24 or 48 hours, the plates were then submitted to the microbiology lab (University of Florida, College of Veterinary Medicine, Gainesville, FL) for bacterial identification. Upon identification of the organism(s), antibiotic sensitivity was performed. If no growth was noted after 48 hours the plates were discarded. An organism was considered dominant when it represented ≥50% of the bacterial growth.

Data Analysis and Statistics

Results from dichotomous variables were reported as number of animals affected / number of animals in the group and as an affected percentage of the group. Results from continuous variables were reported as mean ± standard deviation.

All data-sets were evaluated for normality using a Shapiro-Wilk test. Dichotomous variables were analyzed using a Fisher's exact test or Wilkoxon Rank Sum test. Time to abortion/delivery was compared between groups using a student T test. CTUP and fetal heart-rate data from the day of baseline measurement, the day of clinical diagnosis and the last measurement taken before parturition were used for statistical analysis. Analysis was performed

using a two-way ANOVA with repeated measures. Progesterone data were displayed as daily change and analyzed for the first and last four data-points using a two-way ANOVA with repeated measures. White blood cell counts in neonatal foals were compared using ANOVA. The programs Statistix 8.1® (Statistix 8.1®, Analytical Software Inc, Tallahassee FL) and SigmaStat® (SigmaStat®, Systat Inc, Chicago IL) were used for all statistical analyses. Significance was assigned to all values $P < 0.05$.

CHAPTER 5
RESULTS

Pregnancy Outcome

Mares treated with SMZ, PTX and ALT carried pregnancies longer after bacterial inoculation (TREAT 31 ± 14 d, range 5-55 d; UNTREAT 8 ± 5 d, range 2-17 d; $P < 0.05$) (Figure 5-1). In addition, mares in group TREAT were more likely to deliver viable foals than mares in group UNTREAT (TREAT 10/12, 83%; UNTREAT 0/5, 0%; $P<0.05$) (Figure 5-2).

In group TREAT, two foals were non-viable. One fetus was aborted five days after bacterial inoculation, while the other fetus was delivered at term, but experienced premature separation of the fetal membranes at birth. The mare was found recumbent shortly after delivery. The fetus was encased in the chorioallantois and amnion and was non-viable.

All foals in group UNTREAT were non-viable. Two fetuses were dead at time of birth. Three foals were alive at birth, but had clear signs of immaturity and compromise, including silky hair-coats, floppy ears, sealed eyelids, failure to breathe independently and unresponsiveness to therapy. These foals were humanely euthanized.

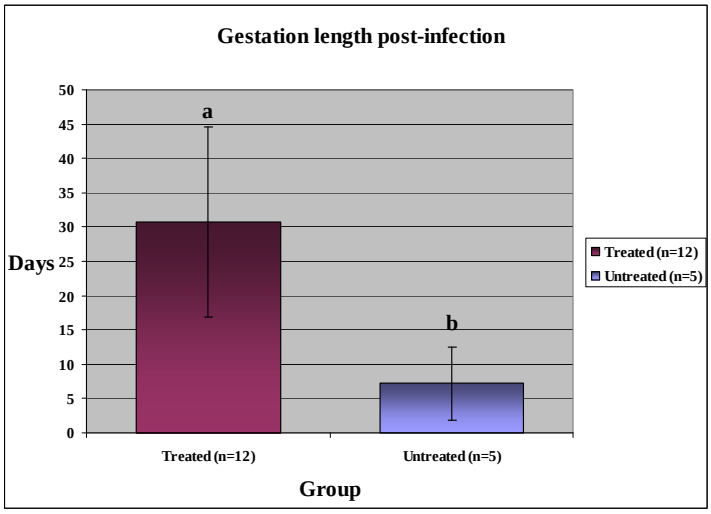

Figure 5-1. Gestational length after inoculation by group. Mares in group TREAT maintained gestation longer after inoculation than mares in group UNTREAT. Values with different letters differ ($P<0.05$).

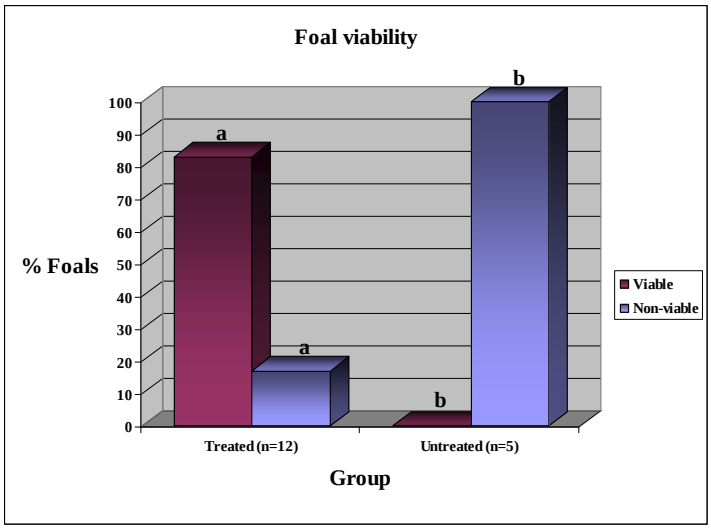

Figure 5-2. Viability of foals by group. Mares in group TREAT delivered more viable foals (10/12) than did mares in group UNTREAT (0/5). Values with different letters differ ($P<0.05$).

Peripartum Complications

Peripartum complications (dystocia and premature separation of the fetal membranes) occurred in both groups ($P>0.05$) (Table 5-1). No mares retained their fetal membranes beyond three hours postpartum in either experimental group.

Table 5-1. Incidence of peripartum complications between groups

Peripartum complications	TREAT	UNTREAT
Dystocia	1/12 (8%)	2/5 (40%)
Premature separation of the fetal membranes	2/12 (16%)	1/5 (20%)
Retained placenta >3 hours postpartum	0/12 (0%)	0/5 (0%)

Groups did not differ ($P>0.05$) in incidence of peripartum complications.

Fetal Viability/ Maturity

All viable foals stood without assistance within two hours of birth. Two foals in group TREAT required placement of nasogastric tubes for one-time feeding. Two foals required indwelling nasogastric tubes for feeding over 24-72 hours.

Serum Cortisol

Mean serum cortisol concentrations from nine foals in group TREAT at less than three hours age were consistent with published values [154] from mature neonatal foals (Table 5-2). Seven foals had cortisol levels above 60 ng/mL and three foals had cortisol levels between 21 and 52 ng/mL. One foal with cortisol concentrations below 60 ng/mL was clinically compromised (32 ng/mL) and one foal was normal on physical exam (52 ng/mL). Blood was not taken from one live foal which was more than three hours old because age is known to significantly affect serum cortisol concentrations in newborn foals. Blood was not taken from two non-viable foals in group TREAT since a second sample (8-24 hours) would not be available for comparison.

Mean serum cortisol concentrations from eight viable foals in group TREAT were consistent with published values from normal mature foals 8-24 hours postpartum [154] (Table 5-2).

White Blood Cell Count

Five of nine foals had WBC counts within published values for normal term foals [152] (Table 5-2). Two foals had WBC counts of 5.0-6.9x10^3 cells/μL and two foals had WBC counts which were lower than 5x10^3 cells/μL. Differential cell-counts were available for seven foals from group TREAT. The neutrophil to lymphocyte ratio was greater than two in six of seven foals form group TREAT (Table 5-2). A CBC was not performed on the two non-viable foals in group TREAT due to concerns of intravascular agglutination, which could damage the analyzer. For one live foal from group TREAT, the appropriate blood-sample was lost and no CBC was run.

Results for CBC were available from two non-viable foals in group UNTREAT (Table 5-2). In both cases, the total WBC was below published reference values. In addition, one foal

had a low neutrophil to lymphocyte ratio and cytologic evidence of neutrophil-toxicity. The foals were euthanized immediately before blood was drawn due to severe systemic distress. Statistical comparison between groups was not performed due to the low number of animals available in group UNTREAT.

Table 5-2. Neonatal cortisol concentrations and white blood cell count in foals from treated and untreated mares

Values	TREAT	UNTREAT
Cortisol: foaling	84.3 ± 54.7	N/A
Cortisol: 24h	23.1 ± 10.8	N/A
WBC >6900	5/9 (56%)	0/2 (0%)
Neutrophil/Lymphocyte ratio >2	6/7 (86%)	0/1 (0%)

Foals from group TREAT had hematologic findings consistent with mature term foals. Comparison between groups was not performed due to the low number of samples from group UNTREAT.

Blood Culture from Foals and Fetuses at Birth

Foals from group TREAT were less likely to have a positive blood culture than foals from group UNTREAT ($P<0.05$) (Table 5-3).

In group TREAT, one foal had pure growth of *Enterobacter cloacae*. In group UNTREAT, blood from two foals grew predominantly *S. equi* subsp. *zooepidemicus.* Two foals from group UNTREAT had pure growth of *Pseudomonas aeruginosa* and *Actinobacillus lignerisii*, respectively, on blood-culture (Table 5-3).

Table 5-3. Bacterial growth on blood culture from viable and nonviable foals

Bacteriologic findings	TREAT	UNTREAT
Bacterial growth	1/12 (8%) [a]	4/5 (80%) [b]
Strep. equi subsp. *zooepidemicus*	0/12 (0%)	2/5 (40%)
Other organisms	1/12 (8%)	2/5 (40%)

Foals from group TREAT were less likely to have positive blood cultures than foals from group UNTREAT. Values with different letters differ ($P<0.05$).

Tissue Culture from Non-Viable Fetuses

Samples of stomach contents and thoracic fluid were obtained from non-viable fetuses (TREAT: n =2; UNTREAT: n =5) during the necropsy exam. Bacterial growth was obtained

from one or both samples in all fetuses, regardless of group (Table 5-4). In group TREAT, *Strep. equi* subsp. *zooepidemicus* was recovered as the predominant organism from one fetus and *Enterobacter cloacae* was recovered as the predominant organism from one fetus. In group UNTREAT, *Strep. equi* subsp. *zooepidemicus* was recovered as the predominant organism from three fetuses. One fetus had predominant growth of *Pseudomonas aeruginosa* and one fetus had predominant growth of *Actinobacillus lignerisii* in group UNTREAT. Statistical comparison between groups was not performed due to the low number of non-viable fetuses in group TREAT.

Table 5-4. Bacterial growth from stomach and thoracic contents of nonviable foals

Bacteriologic findings	TREAT	UNTREAT
Bacterial growth:	2/2 (100%)	5/5 (100%)
Strep. equi subsp. *zooepidemicus* only	0/2 (0%)	2/5 (40%)
Mixed w/ dominant *Strep. equi* subsp. *zooepidemicus*	1/2 (50%)	1/5 (20%)
Other organisms	1/2 (50%)	2/5 (40%)

Samples from all nonviable foals had bacterial growth. Comparison between groups was not performed due to the low number of samples from group TREAT.

Histologic Tissue Analysis

Predominant histopathologic placental lesions (all mares) included funisitis and focal or focally extensive acute suppurative necrotizing placentitis in the region of the cervical star. There were no differences in the presence of placental lesions between groups ($P>0.05$). Mares in group TREAT tended ($P=0.07$) to have a lower incidence of placental lesions at the level of the cervical star than did mares in group UNTREAT (Table 5-5). Six mares from group TREAT had evidence of necrotizing suppurative placentitis at the level of the cervical star. Two additional mares in group TREAT had evidence of funisitis in the absence of chorionic inflammatory lesions. Placentas from four mares in group TREAT had no histologic inflammatory lesions at parturition. All mares in group UNTREAT had gross and histologic evidence of disease.

Bacterial colonization of lung alveoli was found in all non-viable fetuses, independent of group. Additional findings noted were pulmonary inflammatory changes and passive congestion of the liver, spleen, kidney and adrenal glands (Table 5-6). Due to the low number of non-viable animals in group TREAT, a statistical comparison between groups could not be made.

Table 5-5. Histopathalogic examination of placental tissues between groups

Histologic findings	TREAT	UNTREAT
Placentitis: All tissues	8/12 (67%)	5/5 (100%)
Placentitis: cervical star	6/12 (50%)	5/5 (100%)
Funisitis	5/12 (42%)	4/5 (80%)

Histologic findings were not different between groups (P>0.05). Histologic placentitis at the cervical star tended to be less common in group TREAT than group UNTREAT (P=0.07).

Table 5-6. Histopathalogic examination of tissues from non-viable fetuses

Histologic findings	TREAT	UNTREAT
Fetus: pulmonary bacteria	2/2 (100%)	5/5 (100%)
Fetus: pulmonary inflammation	2/2 (100%)	1/5 (20%)
Fetus: passive congestion (liver, kidney, spleen, adrenal)	2/2 (100%)	4/5 (80%)

Bacteria were found in the lungs of all non-viable fetuses. Comparison between groups was not performed due to the low number of samples from group TREAT.

Uterine Culture from Post-Foaling Mares

Positive uterine culture results were obtained from both treated and untreated mares and there were no differences between groups (P>0.05). In group TREAT, cultures from seven mares had growth of predominantly *Strep. equi* subsp. *zooepidemicus* and one mare had growth of predominantly *Enterobacter cloacae.* In group UNTREAT, five of five mares had positive uterine cultures with *Strep. equi* subsp. *zooepidemicus* as the predominant organism. Secondary organisms which were cultured from uterine swabs in the postpartum period included *Pasteurella multocida*, *Escherichia coli*, non-hemolytic and β-hemolytic staphylococci, *Enterobacter cloacae*, *Bacillus* sp. and other β-hemolytic streptococci.

Mare Monitoring

Physical Exam

There were no differences in physical exam findings between groups at any stage during the study. All mares had normal physical exam parameters (temperature, pulse, respiration) throughout the study period. Mammary gland development was not noted in any mare (independent of group) after bacterial inoculation and before development of vulvar discharge. Vulvar discharge was identified in 10/12 mares (83%) from group TREAT within 36 hours of inoculation. Two mares from group TREAT developed vulvar discharge later than 36 hours after bacterial inoculation (72 h and 96 h, respectively). All mares in group UNTREAT (5/5, 100%) showed evidence of vulvar discharge within 36 hours after bacterial inoculation.

Transrectal Ultrasonography

Baseline measurements for CTUP were within published reference ranges [35-39] in all mares (TREAT 5.8 ± 1.3 mm; UNTREAT 5.2 ± 1.1 mm).

On the day of clinical diagnosis of disease (presence of vulvar discharge), all mares had CTUP measurements within normal limits (<8mm). Placental separation determined by trasnrectal ultrasonography was the only abnormal finding. In group TREAT 1/11 mares (9%) had ultrasonographic evidence of placental separation. A CTUP could not be measured in one mare in group TREAT due to fetal positioning. In group UNTREAT, 2/5 mares (40%) had placental separation at the time of clinical diagnosis.

At the time of last examination (1-3 days before parturition), 10/12 mares (83%) in group TREAT had CTUP values within normal limits, while two mares had evidence of placental separation. In group UNTREAT, four mares had evidence of separation and the final mare had a CTUP value above published reference values for gestational age (>8mm at 296 days of gestation). Separation of the fetal membranes from the underlying endometrium, as detected by

transrectal ultrasound, occurred at a lower rate in group TREAT (2/12, 17%) than in group UNTREAT (4/5, 80%) during the course of the study ($P<0.05$).

Transabdominal Ultrasonography

Fetal heart rates were significantly higher than previously published values for horses [43] at baseline measurement (TREAT: 100 ± 10 bpm; UNTREAT :102 ± 16 bpm) but did not differ between groups. In group TREAT, there was a decrease in fetal heart rates seen over time with the last value preceding foaling significantly lower than the value at baseline (TREAT: 80 ± 10 bpm; UNTREAT: 89 ± 16 bpm). There was no difference between groups at any time ($P>0.05$).

Progesterone Concentrations

Baseline values for progesterone concentrations were highly variable between mares with no statistical difference between groups (Overall mares: 13.3 +/- 8.4 ng/mL, Range 4.9-33.7 ng/mL; TREAT: 11.0 +/- 6.7 ng/mL; UNTREAT: 18.4 +/-10.9 ng/mL). Statistical comparison of progesterone concentrations did not reveal an effect of time or an effect of treatment. However, when data were analyzed based on the change over time, there was a statistically significant difference between the average daily change in groups TREAT and UNTREAT over the first 5 days of the experiment (Figure 5-3).

For four of the mares in the UNTREAT group, as well as one mare in the TREAT group, the five measurements analyzed above include the period immediately preceding parturition. When the data were analyzed based on the last 4 assays before foaling or abortion no difference was detected between groups (Figure 5-4). All but one of the mares for which data were collected within one day of foaling experienced a decrease in progesterone values on the day preceding parturition when compared with 3-5 days preceding parturition.

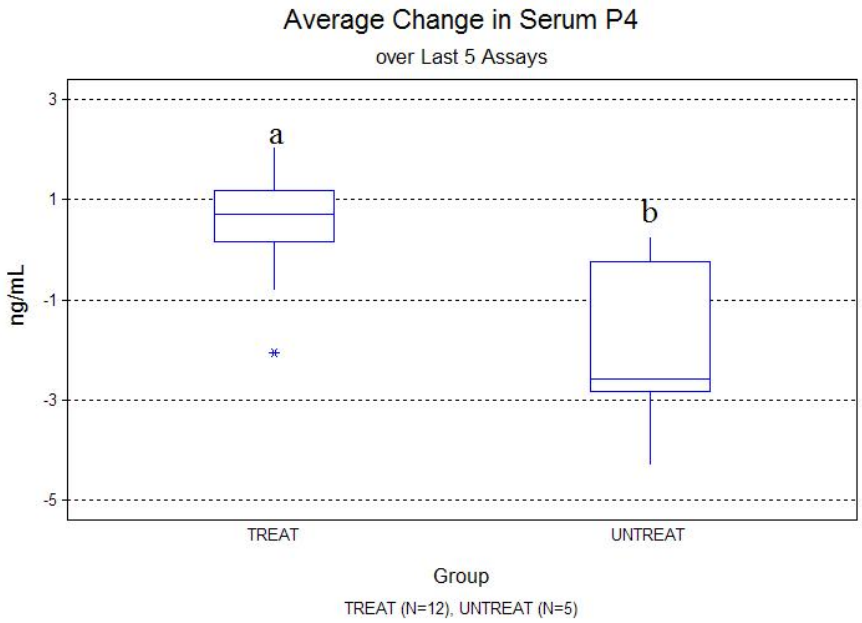

Figure 5-3. Mean daily change in serum progesterone over first 5 assays of study. Mean progesterone values in group TREAT increased 0.5 ±1.1ng/mL/day during the first five days of the study, while mean progesterone values in group UNTREAT decreased by 1.9 ±1.9 ng/mL/day during that time period. Values with different letters differ (P<0.05).

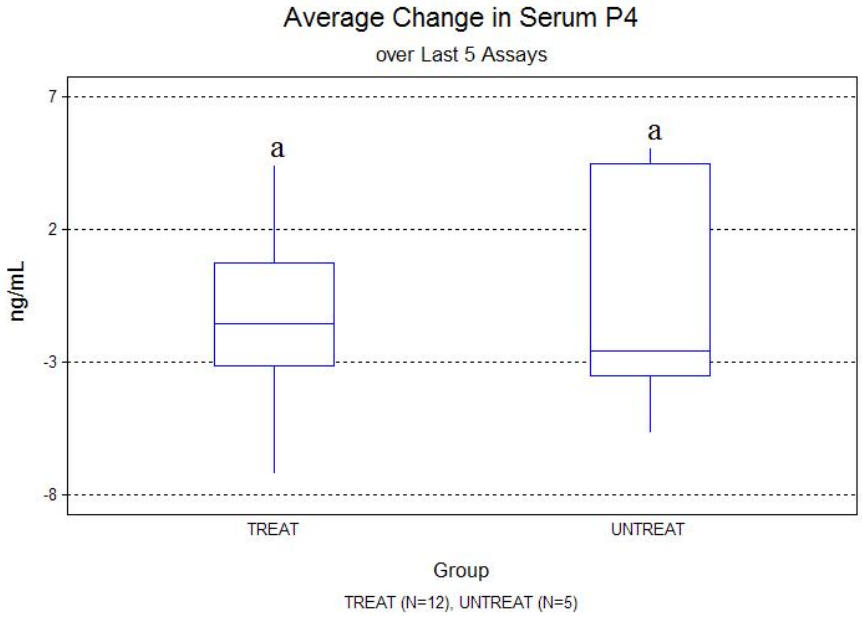

Figure 5-4. Mean change in serum progesterone over the last 4 assays of the study. There was no difference between groups in the mean change in progesterone values in the final 4-7 days of the study (TREAT -1.4 +/-3.2 ng/mL; CONT -0.4 +/-5.2 ng/mL). Values with different letters differ (P<0.05).

CHAPTER 6
DISCUSSION

Administration of trimethoprim sulfamethoxazole (TMS), pentoxifylline (PTX) and altrenogest (ALT) to mares with experimentally-induced placentitis resulted in dramatically improved gestation length and neonatal viability. No foals from group UNTREAT survived infection, whereas 10 of 12 foals in group TREAT were viable. Further, mean serum cortisol concentrations from foals in group TREAT were consistent with those of normal term foals. Five of nine foals from group TREAT had WBC counts within normal limits and seven of nine foals had WBC counts above $5x10^3$ cells/μL. Previous studies have shown that WBC counts greater than 5000 are a positive predictor of neonatal survivability [153]. Surviving foals also required minimal supportive care after foaling. These data support the conclusion that this treatment protocol can result in the birth of clinically mature foals after experimental induction of placentitis.

The treatment regimen selected for this study included an antibiotic, anti-inflammatory agent and a synthetic progestin. Oral medications were selected to mimic conditions in general practice. Oral treatment regimens are better tolerated by horses, and horse owners, for prolonged treatment than injectable medication. Drugs selected included trimethoprim sulfamethoxazole (TMS), pentoxifylline (PTX) and altrenogest (ALT). Trimethoprim sulfamethoxazole is a broad-spectrum, bacteriocidal antibiotic with good *in vitro* activity against common causative organisms of placentitis (*Strep. equi* subsp. *zooepidemicus, Escherichia coli,* nocardioform actinomycetes) [33,97,99]. It is known to penetrate the placenta and reach both the allantoic fluid and fetal tissues [64,65,100]. In the allantoic fluid, drug concentrations exceeded published MIC values for *Strep. equi* subsp. *zooepidemicus* [64]. Pentoxifylline was chosen for use in this study due to its' reported anti-inflammatory effects [111,112,115]. It has not been specifically

investigated for the treatment of pregnancy-related diseases, however a number of studies suggest that anti-inflammatory therapy is an important component of preventing preterm delivery [109,110,118-121]. Pentoxifylline has had a wide variety of potentially beneficial effects in multiple studies involving horses as well as other species. These include inhibition of pro-inflammatory cytokines, vasodilation in inflamed tissues and decreased bacterial attachment [111,112,115-117]. Altrenogest, a synthetic progestin, was added to the treatment protocol for tocolytic effects. It has previously been shown to prevent pregnancy loss in mares during early and mid gestation [128-133]. Its use to treat equine placentitis is further supported by literature from other species, which suggests that progesterone treatment may prevent or delay preterm delivery [122-127,134-141]. ALT has not previously been studied as a treatment for equine placentitis.

A multimodal treatment approach was selected to maximize the treatment response and pregnancy outcome in this study. This approach did not allow for identification of individual drug effects, but was chosen to aggressively address the pathophysiologic mechanisms of abortion, based on our current understanding of equine placentitis [11,20,156] and human preterm labor [16,52,114]. Data from a previous study have suggested that TMS and PTX, alone, were not effective at improving neonatal viability [100]. It is possible that these two drugs were insufficient to inhibit inflammation and prevent preterm delivery in mares with placentitis. The addition of altrenogest might provide additional inhibition of uterine prostaglandins [123,124] and prevent up-regulation of the uterine contractile mechanism as described in *in vitro* studies from other species [30,31,125-127]. Although a trial using altrenogest alone in this experimental model of disease might provide additional insight, this approach would not address the infectious origins of placentitis. Previous work by Ousey and co-workers suggested that

altrenogest alone was not effective in preventing preterm delivery in four high-risk pregnancies [83]. Further *in vitro* and *in vivo* studies are needed to determine the treatment-effect of synthetic progestins in the mare.

The combined treatment with these medications resulted in improved pregnancy outcomes, accompanied by a non-significant reduction of placental inflammatory lesions and a significant reduction in the risk of fetal bacteremia. Interestingly, however, bacterial clearance was not achieved from the uterus in all treated mares, despite long-term treatment of up to 33 days before parturition. These findings are consistent with the findings of Ensink and co-workers, who were unable to eliminate *Strep.equi* subsp. *zooepidemicus* or *Escherichia coli* from infected tissue chambers with TMS despite tissue levels which were effective *in vitro* [96,157-159]. In these studies, although treatment with TMS resulted in an initial decrease in bacterial numbers, they increased again before or shortly after antibiotics were discontinued, resulting in abscessation in all cases. Ensink and co-workers suggested that TMS would not be preferred for infections where bacteria reside in a lumen with fluid [157]. The duration to abscessation in untreated infected mares was not reported. However, abscessation occurred only after 10-42 days when treatment was initiated after inoculation, and after 19 days when treatment was initiated prophylactically and continued for 5 days. It is possible that treatment with TMS, which did not result in bacterial elimination, delayed abscess formation. Further, the efficacy of TMS in preventing bacterial growth *in vivo* in the horse has only been studied thus far in a tissue chamber, which is rapidly invaded by inflammatory cells, creating a highly complex environment. Further research is needed to determine the efficacy of TMS in preventing bacterial growth in other environments, such as the fetal fluids, as well as the efficacy of this antibiotic in preventing dissemination of bacteria to the fetus.

Further research also might identify additional therapeutic agents which would improve treatment outcome, including other anti-inflammatory agents and antibiotics. Penicillin G was more effective at achieving bacterial clearance in a tissue-chamber model than TMS [157]. The use of an antibiotic which could rapidly clear infectious organisms from the uterus might provide equally promising treatment outcomes with a shorter duration of treatment. Flunixin meglumine has a different mode of action than pentoxifylline and the combination of a non-steroidal anti-inflammatory drug and pentoxifylline might further improve treatment outcome, as suggested by Baskett and co-workers [112]. Although flunixin meglumine was not detected in allantoic fluid using *in vivo* microdialysis, it is not known whether it penetrates the placenta or would have therapeutic effects. Additional studies using direct allantocentesis or tissue analyses of fetal tissue would demonstrate whether flunixin meglumine reaches the allantoic fluid and fetus *in utero*. Furthermore, other anti-inflammatory medications also remain of interest in the treatment of equine placentitis. In non-human primates, indomethacin and dexamethasone have been effective at reducing cytokine-induced uterine activity [118,119], and Gravett and co-workers demonstrated a benefit of treatment with anti-inflammatory medication antibiotics in combination, compared to antibiotics alone [121].

In addition, further work is needed to clarify the mode of action and safety of each of the selected therapeutic agents. Although no complications were attributed to treatment in the current study, safety data are limited in the pregnant mare. These would be particularly important given the extended duration of treatment in mares with placentitis. A recent study by Neuhauser and co-workers suggested that ALT may be associated with an increased duration of parturition and neonatal morbidity when administered to normal periparturient mares [160]. Reports have linked the use of sulfonamides to folate deficiency in horses [161-163], including

one report of fetal toxicity in pregnant mares treated for equine protozoal myeloencephalopathy [163]. However, in pregnant women, sulfonamides have been studied extensively due to their usefulness in HIV patients, and in this population they are deemed safe during the second and third trimesters [164]. Further work is needed to demonstrate the safety of trimethoprim sulfamethoxazole or sulfadiazine in pregnant mares.

One aspect of this study that may have contributed to successful neonatal outcome from treated mares was the rapid onset of therapy after bacterial inoculation. The mares in this experiment were monitored carefully and treatment was initiated immediately after clinical signs (vulvar discharge) were noted, resulting in onset of treatment no more than four days of bacterial inoculation. This likely was a contributing factor for the successful treatment outcome in the current study and it highlights the need for sensitive screening tools for equine placentitis.

In this study, mucopurulent vulvar discharge was the most important clinical sign of placentitis. In most cases discharge production was scant and could have been missed without vigilant observation. Twice daily physical examinations of mares allowed for quick identification of vulvar discharge. Therefore, consistent monitoring of "at risk" mares (aged mares or mares with previous history of placentitis, poor perineal conformation or known urovagina) might facilitate earlier diagnosis of disease.

Surprisingly, mammary gland development was not an indicator of disease in this study. In addition, ultrasonographic changes in CTUP, fetal fluids or fetal heart rate did not appear as early indicators of disease. These findings contrast those in a practice setting, where precocious mammary development and milk production are most often the first clinical signs noted in placentitis [2,3,33]. Further, transrectal ultrasound examination of the caudal uterus is currently considered the most sensitive tool to either screen for disease or confirm placenitits in mares

with clinical signs consistent with pregnancy complications. One reason for differences in clinical presentation between mares with experimentally induced and naturally occurring placentitis may be that naturally occurring placentitis is more insidious in onset. It is unlikely that mares developing placentitis do so with an overwhelming inoculum of bacteria as is used in this experimental model. Rather, opportunistic bacteria likely colonize the caudal reproductive tract and then multiply [6,156]. As a consequence, mares may be more likely to develop subclinical disease which may be harder to detect in the early stages. To better approximate field conditions using the current model of placentitis, treatment-onset could be delayed until development of ultrasonographic changes. However, the large number of bacteria infused in the current experimental model makes this approach difficult. Many mares in the UNTREAT group of the current study aborted shortly after development of ultrasonographic changes. Thus, it is possible that a different mode of infection would need to be applied for these studies.

Alternatively, it is possible that other diagnostic tests, such as biochemical assays or hormonal assays would prove to be more sensitive than either physical exam or ultrasonography. The use of biochemical assays of serum or vaginal secretions would be less invasive and simpler to perform than ultrasound and therefore of great benefit in the diagnosis of equine placentitis. In other species, markers specific to intrauterine infection, such as Calgranulin B and IGFBP-1 have been identified in amniotic fluid, vaginal fluid and serum [77,78]. These have not been investigated in the horse, but warrant further study.

Due to the placental origin of many hormones during equine pregnancy, they have been investigated as diagnostic tests for placentitis in numerous studies [26-29,83-86,90-94]. Work using the same experimental model of equine placentitis as the current study suggested that serial assays for serum progesterone concentrations could be used in mares with clinical signs of

placentitis to predict the development of acute or chronic placentitis [28]. Stawicki and co-workers found that progesterone concentrations increased in the serum of mares that were infected but maintained pregnancy for more than seven days, while progesterone concentrations decreased in mares that aborted within seven days [28]. One aim of the current study was to determine whether this pattern would be found in treated and untreated mares after experimental induction of placentitis. In the current study, progesterone values were not useful as an early indicator of disease and no pattern in progesterone values was observed in either group in this study. To minimize the large variation in baseline values (13.3 +/- 8.4 ng/mL, range 4.9-33.7 ng/mL), serum progesterone values were analyzed for average changes over time and compared between treatment groups. Over the first five days of the study there was a significant difference in average daily change between groups, with an average daily increase in progesterone values in group TREAT and an average daily decrease in progesterone values in group UNTREAT. These findings are similar to those of previous studies in untreated mares [28,83], however the daily changes were very small compared to the overall variation between mares. A larger study on normal and infected mares is necessary to determine whether such changes would be clinically useful diagnose placentitis or monitor treatment effect after the initial diagnosis.

An ancillary finding of the current study was the variety of bacteria isolated from fetal tissues and uterine swabs after foaling. The inoculum used was confirmed as a pure culture of *Strep. equi* subsp. *zooepidemicus* prior to intracervical inoculation. All procedures were performed by one of two researchers under carefully controlled aseptic conditions. However, not all cultures were positive for *Strep. equi* subsp. *zooepidemicus* . *Enterobacter cloacae, Actinobacillus lignerisii and Pseudomonas aeruginosa* were each cultured from blood samples of one foal. Uterine cultures from six postpartum mares revealed at least one organism other

than *Strep. equi* subsp. *zooepidemicus ,* including *Pasteurella multocida*, *Escherichia coli*, non-hemolytic and β-hemolytic staphylococci, *Enterobacter cloacae*, *Bacillus* sp. and other β-hemolytic streptococci. These findings are consistent with those of a previous study using the same experimental model [6]. In that study, growth of *Strep. equi* subsp. *zooepidemicus* alone was found in 7 cases, *Strep. equi* subsp. *zooepidemicus* in combination with *Escherichia coli, Klebsiella* sp. and *Enterobacter* sp. was found in five cases and *Escherichia coli* alone was found in one case. It is possible that the additional organisms were introduced as contaminants during the inoculation procedure. More likely, the secondary bacteria may represent organisms present in the caudal reproductive tract as part of the normal vaginal flora which were able to ascend through the cervix as a result of the inoculation procedure or infection. The inoculum was placed in the cervix, not into the uterus in this experiment, thus any organisms present in the uterus at the time of foaling, including *Strep. equi* subsp. *zooepidemicus ,* migrated cranially from the cervix or vagina. The presence of multiple bacterial organisms in the uterus, including both gram positive and gram negative bacteria presents a challenge for antibiotic selection and necessitates broad spectrum antibiotic therapy, such as TMS or a combination of penicillin and gentamicin. The presence of multiple bacterial organisms in fetal samples underscores the importance of definitive diagnostic procedures in neonates to direct antimicrobial therapy, even when culture results from the mare implicate a specific organism.

In summary, using an experimental model of placentitis, mares administered TMS, PTX and ALT from the onset of clinical signs until delivery had longer gestational periods and more viable foals than untreated mares. This treatment regimen resulted in the birth of foals which had physical and hematologic characteristics consistent with mature foals and which required minimal supportive care in the postpartum period. The treatment regimen did not successfully

eliminate bacteria from the uterus of infected mares. However, a positive uterine culture did not correlate with neonatal viability or fetal bacteremia. This suggests that TMS may be effective at preventing fetal bacteremia and improving neonatal survivability, even when bacterial clearance from the uterus is not achieved Further work is needed to determine whether other antimicrobials would be more effective and to elucidate the role of individual therapeutics in the prevention of preterm delivery.

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BIOGRAPHICAL SKETCH

Dr. Bailey graduated from Kansas State University in 2003 with a Bachelor of Science in Animal Sciences and Industry and a Doctor of Veterinary Medicine. He subsequently completed an internship and worked as an associate veterinarian in Saratoga Springs, NY. In 2005, he returned to the university system to begin a clinical residency in theriogenology and a Master of Science program in Veterinary Medical Sciences at the University of Florida, College of Veterinary Medicine. After completion of the residency in 2008, he successfully became a member of the American College of Theriogenologists. He remained at the University of Florida to continue teaching veterinary students and complete the master's program. Dr. Bailey has a research interest in diseases of pregnancy and advanced reproductive techniques for the horse. He is also currently a member of the Veterinary Emergency Response Team at the University of Florida, with an interest in large animal technical rescue.