Vertical transmission of *Mycoplasma haemolamae* in alpacas (*Vicugna pacos*)

THESIS

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Abstract

Mycoplasma haemolamae is a parasite with tropism for the red blood cells of alpacas, llamas, and guanacos. Transmission of the parasite likely occurs via an insect vector although the vector has not been elucidated to date. Transmission via blood transfusion has been demonstrated experimentally. *In utero* infection has been suggested and later demonstrated in a limited number of cases (n≤5).

The purpose of this study was to 1) determine the frequency of vertical transmission of *Mycoplasma haemolamae* from dam to cria; 2) determine whether colostral transmission of *M. haemolamae* occurs; and 3) provide preliminary data on colostral *M. haemolamae* specific antibody from pregnant alpacas on a farm with known prevalence of infection. *Mycoplasma haemolamae* specific PCR was performed on blood and colostrum from pregnant alpacas and their cria (n=52 pairs). Indirect fluorescent antibody testing was performed on a subset (n=43) of these colostrum samples. Total immunoglobulin concentrations of colostrum and cria sera and *M. haemolamae* specific IgG (prior to and after ingesting colostrum) were determined by turbidometric immunoassay and indirect fluorescence antibody testing, respectively. Sixteen of 52 dams (30.7%) pre-partum and one of 52 cria post-partum (1.9%; prior to ingestion of colostrum) were PCR positive for *M. haemolamae*, while 36/52 dams (69%) and 51/52 cria (98%) tested negative for *M. haemolamae* by PCR. All 43 colostrum samples and 52 of 52 post colostrum cria blood samples (100%) were negative by PCR. The dam giving birth to the *M. haemolamae* PCR positive cria was PCR negative. Statistically, it was no more likely for a PCR

positive dam to give birth to a *M. haemolamae*, PCR positive cria (prior to colostrum ingestion) than a PCR negative dam ($p=0.3077$). *M. haemolamae* specific IgG was detectable in 22 of 43 (51%) of colostrum samples at a 1:10 dilution and 14 of the 22 positive 1:10 dilution samples (32.6% of the total samples) at a 1:100 dilution. There was no relationship between the PCR status of the dam and whether or not *M. haemolamae* specific antibodies were present in colostrum. Among the animals tested, *in utero* transmission of *M. haemolamae* was rare (1/52 pre-colostral alpaca cria), and all colostrum samples were negative for *M. haemolamae* by PCR. These data indicate that colostrum from positive dams is unlikely to harbor this parasite and therefore does not serve as a source of infection to newborn cria. Colostrum derived from both PCR positive and negative dams contained *M. haemolamae* specific antibodies. Our findings suggest that *M. haemolamae* specific antibodies may play a role in immunity to this hemoparasite; however, challenge studies are necessary to fully evaluate the role of *M. haemolamae* specific antibodies. Furthermore, antibody prevalence and detectable titers may provide different estimates than those available from current PCR based prevalence studies. Our findings also confirm that *M. haemolamae* isolates from geographically distinct regions do not differ significantly from each other.

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Fields of Study

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Chapter 1: Introduction

Mycoplasma haemolamae, a member of the hemoplasmas within the family *Mycoplasmataceae*, has the potential to impact the camelid industry by creating chronically infected individuals that show no signs of disease, or animals with chronic, insidious disease with signs of disease including weight loss and inappetance.¹ Finally, overt clinical signs of disease are generally observed in juvenile or geriatric, stressed or immunocompromised individuals and can include collapse or recumbency associated with anemia and occasionally hypoglycemia.¹⁻⁴ The widespread geographical distribution of the organism makes this blood parasite of interest for clinicians and researchers worldwide. Prevalence rates determined using PCR based assays vary between study and geographic regions with a range of 9.3-19.3% for camelids in South America, and a prevalence of 18.6% of the overall population with nearly 40% of tested herds having at least one positive animal in Switzerland.^{5,6}

Diagnosis was originally dependent on cytologic evaluation of blood smears, but more recent advances have produced molecular (PCR) based testing for *Mycoplasma haemolamae*. These PCR assays reportedly enable accurate identification of infected individuals regardless of the presence of clinical signs.^{2,5,7} PCR tests have also been used to evaluate the efficacy of treatment and to confirm the presence of the parasite in cases where immunosuppression or immunodeficiency, either as a primary process or secondary to concurrent disease, complicated diagnosis and treatment of the affected individual.^{2,5,7} Additionally, evaluating potential

genomic variations within the hyper-variable region of organisms from geographically distinct regions allows for improved understanding of the organism's potential for virulence as well as for improved PCR based diagnostics.

The emergence of clinical reports detailing potential spread from infected dams to naïve offspring has led to a desire to better understand possible transmission pathways.¹ It is believed that insect vectors are largely responsible for parasite transmission, although no studies have definitively identified a specific vector carrying *M. haemolamae* at this point. Experimentally, transfusion of *M. haemolamae* infected blood was found to transmit this organism resulting in parasitemia.² In that study, animals were not test positive by PCR until at 4 days post-transfusion, indicating that the timing of sample acquisition may be important for detecting and studying neonatal infection.^{1,2} Transplacental transmission has been suggested or demonstrated in a limited number of cases.^{1,3,4} There is insufficient data to evaluate whether colostral transmission is a potential mode of transmission.

Additionally, evaluation of the role of *Mycoplasma haemolamae* specific antibodies in parasite clearance, carrier status and especially the role of maternal antibodies in transfer of parasite from dam to offspring and neonatal infection, have not been defined. Understanding the biology of *Mycoplasma haemolamae*, the role of colostral antibodies in neonatal immunity is imperative for creation of meaningful management and husbandry practices that minimize the chance of the spread of infection or the passage of infectious organisms to naïve individuals. Furthermore, diagnostic testing modalities developed during the course of this research suggest a disconnect between PCR status of the dam, *Mycoplasma* specific antibody status of the dam on the ability to detect the organism, assess therapeutic interventions, and provide a baseline for ongoing research into transmission pathways. Eventually, *in vitro* cultivation of these

organisms will provide insight into the genome and proteome of this parasite which would provide improved diagnostic reagents and limit the use of *in vivo* animal experimentation.

Chapter 2: Literature Review

2.1 Defining Characteristics and Classification of *Mycoplasma haemolamae*

Mycoplasma haemolamae, a hemotropic mycoplasma formerly classified among the *Eperythrozoon spp.*, is an organism with tropism for the red blood cells (RBC) of llamas, alpacas, and guanacos. Like other organisms reclassified from the *Haemobartonella spp.* and *Eperythrozoon spp.* to the mycoplasmas, *M. haemolamae* is a small bacteria, generally < 1 micron (usually ~ 0.4 and 0.6 μm) in diameter, lacking a cell wall.^{8,9} The hemotropic mycoplasmas generally appear as coccoid, rod-shaped, or ring-shaped organisms that are found adherent to the periphery of erythrocytes, or in the background if slide evaluation is delayed and the organisms have detached and are free in the blood smear.^{2,3,8} The majority of hemoplasmas have been described as extracellular organisms although recent work demonstrates *Mycoplasma suis* invades erythrocytes via an endocytosis-like process.¹⁰ A similar intracellular phase of *M. haemolamae* has not been demonstrated to date.

Original classification of *Haemobartonella spp.* and *Eperythrozoon spp.* included these organisms as members of the *Anaplasmataceae* family in the order *Rickettsiales* on the basis of staining characteristics, obligate parasitic habitat, susceptibility to tetracycline antibiotics, and difficulty of *in vitro* cultivation.⁹ Phylogenetic testing based on DNA sequencing has reorganized these organisms, including *M. haemolamae*, to their current classification as members of the family *Mycoplasmataceae*.¹¹ All members of this family are evolutionarily related to the low-

G+C-content Gram positive bacteria where there are fewer G and C nucleotide bases than A and T bases similar to what is found in clostridial-type bacteria.

Classification of *M. haemolamae* shows some similarities to other more widely studied *Mycoplasma spp.* including *Mycoplasma suis*, *Mycoplasma wenyonii*, *Mycoplasma haemofelis*, and *Mycoplasma haemominutum* that have provided insights into describing, classifying, and developing testing modalities for the camelid-specific hemoparasite.^{3,8,11} Classification is based on DNA sequence analysis of the 16s small subunit ribosomal RNA (16s rRNA) gene from a variety of similar organisms which revealed inherent similarity to organisms already classified within the *Mycoplasma* genus.^{11,12}

The 16s rRNA gene is highly conserved among bacteria of genetically similar groups and has been used extensively in studies for phylogenetic classification purposes with regards to members of the *Mycoplasma* genus.^{13,14} Genetic similarity among the hemotropic mycoplasmas is most prominent between *M. haemolamae* and *M. suis* and *M. wenyonii* according to one study.¹¹ Sequence similarity between the hemosuis cluster (including *M. wenyonii*, *M. suis*, and *M. haemominutum*) and *M. haemolamae* was approximately 90%.

Descriptions of *M. haemolamae* using light and electron microscopy have been performed and are consistent with descriptions of other hemotropic mycoplasmas.^{3,8} One study by Reagan et. al. described infection in four juvenile llamas using clinicopathologic evaluation as well as light and scanning electron microscopy.⁸ Hemograms revealed mild anemia with a weak or absent regenerative response.⁸ Evaluation of a blood smear revealed numerous coccoid- and ring-shaped basophilic organisms present on many of the red blood cells in 3 of the llamas and a few organisms present on a small number of red blood cells in the fourth llama.⁸ Scanning

electron microscopy demonstrated individual, pairs, and clusters of the organisms adherent to erythrocytes with little to no indentation at the site of attachment to the RBC membrane.

McLaughlin et. al. evaluated a herd of llamas over a period of several months.³ During the study period, 12 llamas were found to be parasitemic based on blood smear evaluation. The same characteristics defined the organisms in this herd as compared those described in a previous study.⁸ Hemograms showed mild to moderate anemia, often associated with the presence of the parasite. Values including RBC count, hemoglobin concentration, and packed cell volume (PCV) initially declined as disease progressed, and then gradually returned to normal during and after treatment. When present, hypoglycemia was often profound, and associated with severe parasitemia.³ Electron microscopy revealed morphologic features considered similar to those of *Eperythrozoon suis* (now *M. suis*) in swine. Treatment using parenteral and oral oxytetracycline yielded variable degrees of success such that some individuals were rapidly cleared of parasitemia, others were refractory to treatment, and some initially cleared infection only to recrudesce at a later time, presumably secondary to stress or concurrent disease impairing normal immunity.³ This study also investigated antibody titers to both *Anaplasma marginale* using a card agglutination test and *Eperythrozoon suis* (now *Mycoplasma suis*) using an indirect hemagglutination test. The card agglutination test showed no evidence of antibodies to *A. marginale*. Of the 84 individuals tested from the farm used in the study, 19 had titers to *M. suis* of 1:40 or greater. Of these, 5 were parasitemic, 2 became parasitemic 4 weeks later, and 12 were not observed to be parasitemic. Of the 65 seronegative llamas, 5 were parasitemic. The hemagglutination testing was expanded to include 1,753 samples from unrelated herds in several states. Of these 1,753 samples, 208 (11.9%) had a titer of 1:40 or greater.³

The hemotropic mycoplasmas have a vast geographic distribution and affect a wide range of species. Descriptions of clinical and subclinical infection have been described in North and South America, Europe, Asia, and Australia and in numerous domestic species including swine, cattle, cats, dogs, sheep, llamas and alpacas, and reindeer, as well as in a number of wildlife species including opossums, cervids, and sea lions.¹⁵⁻²⁰ Prevalence of infection in Chilean and Peruvian llamas and alpacas has been evaluated with a range between 9.26% - 19.3% amongst the individuals sampled at two sites.⁶ In Switzerland, PCR testing resulted in a population prevalence of 18.6%.⁵ Almost all individuals in both studies had no clinical signs of disease.

In general, hemoplasmas tend to be species specific, although *Mycoplasma ovis* has been transmitted from sheep to goats and *M. suis* has been identified in humans with close contact to infected swine.^{21,22} Studies have shown that *M. wenyonii* is not transmissible from cattle to splenectomized sheep, goats, or deer.^{23,24} No studies have shown transmission of *M. haemolamae* to species other than llamas and alpacas.

2.2 Clinical and Subclinical Disease

The majority of PCR positive infected individuals are asymptomatic representing a potential subclinical disease state in which individuals may serve as carriers of the parasite without overt clinical signs.² Clinical disease occurs most commonly in stressed, immunocompromised, or juvenile individuals, often secondary to concomitant infection or disease at or near the time of transport, weaning, or parturition.^{8,25-29} The most commonly noted signs of infection include fever, mild to marked anemia, depression, icterus, infertility, edema, poor growth rate, chronic weight loss, and mild to severe hypoglycemia.^{2,8,26,28,29}

Immunodeficiency was implicated in two case reports in which *M. haemolamae* infection was diagnosed during workup for nonspecific signs of illness including inappetance, fever, and decreased fecal output for one 14-month-old female llama and chronic weight loss in a 3.5-year-old female llama.^{26,27} For the juvenile llama, concurrent diseases included septicemic listeriosis, thrombocytopenia, and hepatopathy. It is likely that the stress associated with concurrent disease depressed the llama's otherwise functional immune system allowing for opportunistic parasitemia identifiable on a blood smear to develop.²⁷ Treatment of the underlying disease in addition to treatment for the circulating blood parasite was curative.²⁷ In the case of the adult llama, no other significant cause of disease was noted on physical and hematologic examinations aside from the presence of *M. haemolamae* on blood smear evaluation. In combination with a low serum IgG concentration, the authors concluded that parasitemia was secondary to immunodeficiency, similar to what is described for llamas with juvenile llama immunodeficiency syndrome (JLIDS).²⁶

2.3 Diagnosis

Clinical presentation can be suggestive of *M. haemolamae* infection, but packed cell volume (PCV), complete blood count (CBC), and serum biochemical analysis may be needed to determine the severity of disease. If clinical presentation and hemalogic testing suggest infection but parasitemia is not observed on cytologic evaluation of a blood smear, PCR testing may be required to confirm infection in the face of low levels of parasitemia. Crias infected early in life, born to dams not producing colostrum or some other reason for failure to passively acquire maternal immunity which present obtunded, seizing and respond to administration of glucose is strongly suggestive of *M. haemolamae* infection. Evaluation of the PCV, morphology and presence of parasites on the RBC in a blood smear is often strongly supportive of *M.*

haemolamae parasitemia. If infection is confirmed and treatment elected, ongoing monitoring of PCV values may be useful in monitoring response to therapy. Ongoing evaluation of PCV should occur every 2-3 weeks after initial presentation to ensure a continued regenerative response.

2.4 Treatment

Treatment for clinically affected individuals relies on the use of tetracycline antibiotics in combination with supportive care in the face of more severely debilitated individuals afflicted with severe anemia or profound hypoglycemia. Supportive treatment includes polyionic fluids containing dextrose in hypoglycemic patients and blood transfusions for individuals requiring intervention for anemia (generally for PCV < 10%). Cost of stabilization and hospitalization can be very high making prevention or early diagnosis desirable.

Empiric selection of oxytetracycline antibiotics for treatment of clinically affected parasitemic individuals occurred based on previous reports regarding treatment of other hemoplasmas using tetracycline antibiotics.³⁰⁻³⁶ In these reports, tetracycline antibiotics given parenterally or orally variably decreased parasite burden but frequently failed to completely clear the infection.³¹⁻³⁶ Treatment of parasitemic camelids with oxytetracycline antibiotics at a dose of 20-24 mg/kg intramuscularly or subcutaneously every 72 hours for 21 days was effective in clearing infection in some cases.³ Tetracycline antibiotics were also added to the water supply to provide an alternative method of treatment to prevent parenteral administration with 200 mg tetracycline/gallon of drinking water. The majority of individuals tested seronegative, but one llama remained parasitemic.³ Another study recommended treatment with oxytetracycline at a dose of 20 mg/kg subcutaneously every 72 hours as described in previous reports.^{2,3,26} The study demonstrated that treatment with 5 doses of parenteral oxytetracycline

did not clear PCR detectable infection faster than the control group that did not receive antimicrobial therapy.² When considering *M. haemolamae*, the utility of treatment seems to be useful for individuals that are clinically affected, have evidence of compromised immunity (either inherent immunodeficiency or immunosuppression due to concurrent disease), and that have marked parasitemia; however, the utility of treating subclinical carriers has been debated as a number of individuals remain chronic carriers regardless of route and duration of antibiotic therapy.² Potential side effects from prolonged administration of oxytetracycline can include decreased appetite, most likely associated with decreased number and activity of normal rumen flora. High dose administration of oxytetracycline has been linked to renal disease or failure in cattle suggesting that the lowest effective dose should be chosen whenever possible.

2.5 Transmission

The mode of transmission has not been definitively identified, although a biting insect vector is suspected. Biting insects are vectors for other hemotropic mycoplasmas including *M. wenyonii* and *M. ovis*.¹⁹ Experimental infection of cats through exposure to fleas carrying *M. haemofelis* showed that transmission through the vector is possible although not inevitable.³⁷ Insect vectors also play a role in disease transmission in other blood-borne diseases including anaplasmosis and babesiosis in cattle, sheep, and goats.^{35,38,39} Testing to identify potential vectors involved in transmission of *M. haemolamae* lifecycle has not been investigated at this time. Identifying potential vectors may allow for the implementation of meaningful management or husbandry practices that prevent or decrease the prevalence or incidence of clinical disease.

Experimentally, transfusion of *M. haemolamae* infected blood was found to transmit this organism.² In that study, animals did not test positive by PCR until at least 4 days post-

transfusion, indicating that the timing of sample acquisition may be important for detecting and studying neonatal infection in ongoing studies.^{1,2}

Other modes of transmission that have been suggested or confirmed include vertical transmission from infected dams to neonatal crias (transplacental transmission) and colostral (transmammary) transmission of organisms. Initial descriptions of suspected *in utero* transmission included a case report of parasitemia in a one-day-old llama cria and a separate report of parasitemia in a 4-day-old alpaca cria.^{4,29} The llama cria presented for resection of an excessively long umbilical cord with no evidence of systemic disease. Complete blood count and IgG concentration were performed as part of a routine evaluation and revealed more than 50% of the erythrocytes having *M. haemolamae* organisms present on their periphery. The IgG concentration was 970 mg/dL indicating adequate transfer of passive immunity according to their laboratory reference values which were not given. The authors concluded that infection with such high numbers of organisms at so young an age pointed to transplacental transmission as the most likely means of infection.²⁹ The normal IgG concentration with concurrent infection suggests that the cria did not acquire antibodies specific to *M. haemolamae* from the ingested colostrum.

The alpaca cria, conversely, presented for profound weakness and inappetance.⁴ Complete blood count, serum biochemical analysis, venous blood gas, and serum IgG concentration were performed. The hemogram revealed mild anemia, and on evaluation of the blood smear an extremely large number of organisms were noted attached to the majority of erythrocytes with numerous free floating organisms concurrently present in the background. Cytologic identification of the organism led to submission of a blood sample from the cria and its dam for PCR confirmation of the organism as *M. haemolamae*. Serum IgG concentration was

12.3 g/L (normal reference range: >10.0 g/L) suggestive of adequate transfer of passive immunity.⁴ Consistent with infections characterized by marked parasitemia, the biochemical analysis showed profound hypoglycemia along with severe metabolic acidosis, hyperkalemia, and azotemia. Despite treatment of the underlying metabolic derangements and initial signs of improvement, the cria died. The blood for the cria and dam were evaluated for the presence of the 16s rRNA gene of *M. haemolamae* as described in a previous study.¹¹ Both dam and cria blood samples tested positive for *M. haemolamae*. Again, the short time interval between birth and the marked parasitemia in the cria suggested transplacental transmission as the most likely mode of infection, although transmammary and perinatal blood inoculation could not be ruled out.⁴ The presence of parasitemia despite adequate serum IgG suggests that the cria did not receive adequate *M. haemolamae* specific antibody from the dam remaining susceptible to parasitic infection.

More recently, a short case series described by Tornquist et. al. described vertical transmission from infected dams to their crias.¹ Five pregnant adult female alpacas tested positive for *M. haemolamae* infection by PCR assay. Blood samples were taken from their crias within 30 minutes of birth and before they suckled or had postpartum contact with the dam or another alpaca. Blood samples were additionally acquired at various timepoints for 4 of the crias until they reached 6 months of age. A colostrum sample was obtained from one dam. The PCR assay was completed as described in an earlier paper by the same author.² Three of the crias were negative by PCR assay as was the colostrum sample. The other two crias were positive immediately postpartum as well as after suckling colostrum; however, no specific analysis to determine *M. haemolamae* antibodies in serum or colostrum were performed. One of these crias remained positive until 6.5 months of age, the other was not retested. Perinatal

blood inoculation could not be ruled out as a means of transmission; however, in a previous study, Tornquist showed that the time between blood inoculation with *M. haemolamae* and the time of detectable parasitemia was 4 days using the same PCR test used in the more recent study.²

2.6 Testing Modalities

Testing for the presence of infective organism is useful for a variety of reasons. Most obviously, it allows for accurate diagnosis and appropriate treatment of clinically affected individuals. In addition, testing provides information regarding population prevalence, identifies subclinical carriers, allows for quantifiable measurement of the efficacy of treatments, and allows for ongoing research into the biology and means of transmission for the parasite.

The most simplistic test for infection with *M. haemolamae* is cytologic evaluation of a blood smear. The presence of organisms on blood smear, either attached to the periphery of erythrocytes or free in the background, is diagnostic for parasitemia and infection. Cytology is the least sensitive method of testing for infection with subclinical or treated animals that often have so few organisms circulating that they are not identified during cytologic examination.² It is important to remember, however, that parasitemia is not always directly correlated to clinical disease, although highly parasitized individuals have a higher propensity for clinical signs compared to less parasitized individuals.²

Polymerase chain reaction (PCR) testing has been developed as a more sensitive method of assessing parasite presence, burden, and success of treatment both for *M. haemolamae* as well as for other *Mycoplasma spp.*^{2,5,7,40-48} Tornquist et. al. developed conventional PCR using primers from the eubacterial 16s rRNA gene (forward primer: Fhf1 5'-ACG CGT CGA CAG AGT TTG ATC CTG GCT-3' and reverse primer: Rhf2 5'-CGC GGA TCC GCT ACC TTG TTA CGA CTT-3').²

The reaction contained 0.2 micromoles of each primer, 0.5 Units of Taq polymerase/microliter, 1.5 millimoles of MgCl₂, 0.2 millimole deoxynucleoside triphosphate mix, and 2 microliters of template. A negative control was included to control for potential contamination during sample preparation.² Automated thermal cycler settings were adapted from previously described settings for PCR testing using *M. haemofelis*.⁴³ Initially, the samples were incubated for 10 minutes at 94 degrees C followed by 32 cycles at 94 degrees C for 1 minute, 50 degrees C for 1 minute, and 72 degrees C for 2 minutes. A final elongation step at 72 degrees for 7 minutes was added, and the purified amplicon was sequenced.² The sequence obtained was 97% homologous to the published GenBank sequence of *M. haemolamae* (AF306346). Per Tornquist, et. al, species-specific primers were then developed from the hypervariable region of the *M. haemolamae* 16s rRNA gene (forward primer: 5'-TAG ATT TGA AAT AGT CTA AAT TAA-3' and reverse primer: 5'-AAT TAG TAC AAT CAC GAC AGA ATC-3').² Test samples were evaluated using the same DNA extraction kit, PCR conditions, and were compared to positive and negative controls, as well as to DNA samples from other *Mycoplasma spp* including *M. suis* and *M. haemofelis* to ensure specificity to *M. haemolamae*.² Sensitivity was tested using serial dilutions with a plasmid containing a known single gene copy number and the lower detection limit was 28 copies using the conventional PCR test described above.²

A separate research group developed a quantitative PCR test using real time PCR for future use in epidemiological and experimental studies.⁷ DNA from an organism suspected to be *M. haemolamae* was isolated, sequenced, and confirmed to be 99% homologous to the GenBank sequence (AF306346). Similar protocols were used to initially identify species-specific primers, and testing using serial dilutions was performed to determine the sensitivity of the real-time PCR assay. Specificity was achieved, and sensitivity was determined to be 1 copy in a 25

microliter reaction. When dilutions were run in tandem between the conventional PCR and real-time PCR, the real-time PCR assay had good agreement with the conventional assay in addition to showing one sample positive that had tested negative using conventional PCR.⁷ No relationship has been established in either study between copy number detected, severity of parasitemia, and presence of clinical disease.^{2,7}

2.7 Camelid Placentation

Diffuse epitheliochorial placentation, similar to that seen in swine and equids, prevents maternal and fetal blood from intermingling.⁴⁹⁻⁵⁵ The anatomy of the epitheliochorial placenta prevents the transfer of large molecules like antibodies from dam to fetus necessitating uptake via another route.⁵⁴ One study has shown that despite the diffuse epitheliochorial placentation, specialized areas of the maternofetal interface may impart microregional functions by which nutrition, hormone production, and molecular exchange are prevalent.⁵² At these sites, it is theoretically possible to have transplacental transfer of infectious organisms like *M. haemolamae*; however, it has not been shown whether an organism the size of *M. haemolamae* would be able to transfer across the placenta at one of these specialized zones.

Transplacental transmission of infectious organisms has been documented in other species, at times leading to an immune response by the fetus.⁵⁶⁻⁶⁰ This transplacental transmission, followed by an immune response by the fetus, is well documented in certain pathogenic organisms affecting cattle (*Neospora caninum* and Bovine Viral Diarrhea Virus) and humans (*Toxoplasma gondii*).⁵⁶⁻⁶⁰ Transplacental transmission has also been described in horses associated with neonatal infection with *Theileria equi* and in cats associated with neonatal infection with *Mycoplasma haemofelis*.^{59,61} In these foals, clinical disease related to neonatal

infection seemed to be prevented by perinatal ingestion of colostrum containing antibodies to the organism.⁵⁹

2.8 Colostrum and Neonatal Immunity

Neonatal immunity in ruminants and camelids is almost entirely dependent on acquisition of maternal antibodies through colostrum ingestion within the first hours of life.^{49,50,62-65} Maternal antibodies are readily absorbed via the gastrointestinal tract providing passive acquisition of humoral immunity to the cria.⁶² While concentrations of IgG in normal camelid colostrum have been described, (ranges between 176-360 mg/mL), little information regarding *M. haemolamae* specific antibody is available.^{62,65} One study described serologic responses of llamas infected with *M. haemolamae* using an indirect hemagglutination test to *M. suis* antigen. PCR based testing methods were not available at that time.³ That seminal study estimated a seroprevalence for antibodies to the tested antigen of 12% among 1,753 animals from 4 different states; however, the presence of *M. haemolamae* specific antibodies in pregnant camelids, colostrum and crias was not evaluated.³

Colostrum transmission of infectious organisms, including caprine arthritis and encephalitis virus, as well as the parasitic nematode of foals, *Strongyloides westeri*, can lead to clinical disease in neonatal kids and foals.^{66,67} For some pathogens, management practices including pasteurization of milk and separation of neonates from adults can decrease the risk of passing infectious organisms to neonates.

Chapter 3: Vertical transmission of *Mycoplasma haemolamae* in alpacas (*Vicugna pacos*)

3.1 Hypothesis and Objectives

The purpose of this study was to determine whether *in utero* or colostral transmission of *M. haemolamae* is a potential route of infection for newborn crias. We hypothesized that *in utero* or colostral transmission of *M. haemolamae* leads to detectable infection in newborn crias as defined by a standard PCR assay. A second objective was to evaluate the presence of *M. haemolamae* specific antibodies in dams with known PCR status for this organism. We hypothesized that PCR positive dams produce higher concentrations of *M. haemolamae* specific antibody in colostrum when compared to PCR negative dams. To determine if these hypotheses were valid, we evaluated pregnant females and their newborn crias present on one farm with known prevalence of *M. haemolamae* infection (33.8%).

3.2 PCR Test Development and Validation

3.2.1 Clinical Case

The following case history represents the source of the parasite from which *M. haemolamae* DNA was amplified, sequenced and used for primer development for the reported PCR test used for the remainder of the study.

A 3-month-old female alpaca cria was presented for acute onset of seizures and ataxia first noted approximately 90 minutes prior to arrival at the hospital. The cria had been born subsequent to dystocia although the owner reported the cria was able to nurse from a bottle and serum IgG was adequate post colostrum ingestion. The morning of referral the cria was

found seizing, and was administered an unknown amount of dextrose and thiamine intravenously.

Upon presentation at the Ohio State University Hospital for Farm Animals, the cria was depressed and weak. Heart rate, respiratory rate, and temperature were noted to be within normal limits. Mucous membranes appeared injected with a capillary refill time (CRT) of 2 seconds. Anisocoria was noted, with the right pupil dilated more than the left. The left eye showed absent direct and consensual pupillary light reflexes.

Complete blood count revealed a PCV of 28% (24-35%) with a mild lymphocytosis. On cytologic evaluation, large numbers of 0.25-0.5 micron, basophilic, nonrefractile round bodies were present individually and in rows on the surface of red blood cells and in the background consistent with *M. haemolamae*. Biochemical profile abnormalities included calcium 11.4 mg/dL (normal 8.0-10.4 mg/dL), total serum protein 4.9 g/dL (normal 5.3-7.6 g/dL), globulin 1.9 g/dL (2.7-2.9 g/dL), anion gap 38 mEq/L (15-25 mEq/L), creatinine kinase 518 IU/L (normal 40-500 IU/L), glucose 18 mg/dL; (normal 100-132 mg/dL), and bicarbonate of 12 mmol/L (19-29 mmol/L). The seizures were attributed to profound hypoglycemia with concurrent metabolic acidosis. Muscle enzyme elevations were consistent with the history of seizures. The remaining EDTA blood sample submitted for CBC was processed for DNA extraction using DNeasy Qiagen kit and preparation of antigen slides for use in a *M. haemolamae* specific indirect fluorescent antibody test (IFAT). The cria was treated with intravenous fluids consisting of 0.45% sodium chloride with 2.5% dextrose and sodium bicarbonate (94 mEq) administered over 3 hours. Intravenous oxytetracycline (6 mg/kg) was administered twice daily for two days while hospitalized. The cria rapidly improved after fluids were initiated and began nursing aggressively. Repeated complete blood count 48 hours after presentation revealed fewer of the organisms as compared to the initial blood smear. She was discharged with instructions to

continue oxytetracycline administration with subcutaneous oxytetracycline (20 mg/kg) administered every other day for three more doses.

3.2.2 Initial Primer Development

PCR primers were selected from the hypervariable region of the sequenced 16s rRNA gene available from the National Library of Medicine, GenBank website using Vector NTI (version 11) (GenBank accession **JN214360**). The identified gene sequence was used to isolate two primer sequences: primer set II which amplifies a 219 bp sequence and primer set III which amplifies a 696 bp sequence that encompasses the amplified region of primer set II.

3.2.3 Primer Specificity

To evaluate primer set I specificity, DNA was extracted from bovine, ovine, equine, caprine, Dromedary camel, and llama anti-coagulated blood. DNA samples were run using the sample commercial DNA extraction kit and PCR cyclers settings used in the remainder of the study.

Primer set II was also run with the mammalian DNA samples used to determine primer set I specificity. To evaluate primer set II specificity to *M. haemolamae*, DNA samples were obtained from isolates of *Mycoplasma haemofelis* (Mhf), *Mycoplasma haemominutum* (Mhm), *Mycoplasma gallisepticum* (Mg), *Mycoplasma bovis* (Mb), and *Mycoplasma synoviae* (Ms) (DNA was provided by Dr. Jane E. Sykes, University of California Davis, CA, USA, (Mhf and Mhm); and Dr. Ziv Raviv, Dr. Amy Wetzel, The Ohio State University, Columbus, OH, USA (Mg, Mb, and Ms)). These samples were evaluated using the same PCR cycler settings used for the *M. haemolamae* samples.

3.2.4 Primer Sensitivity

To evaluate the sensitivity of primer set II, serial dilutions from 1:10 to 1:100,000 of the clinical cria and the *in utero* infected cria DNA samples were tested with primer set II. Likewise,

to evaluate the sensitivity of the PCR assay when using colostrum, a negative colostrum sample spiked with infected blood from the clinical case provided a means to determine the detection limits of the assay when using colostrum and provide a relative detection limit to the same known positive blood sample.

3.2.5 DNA Sequencing

To evaluate the application of the *M. haemolamae* PCR test against a geographically-diverse set of *M. haemolamae* isolates, *M. haemolamae* positive alpaca blood from the states of Ohio, Washington, Oklahoma, and Georgia^e were evaluated with primer set III. *Mycoplasma haemolamae* was amplified (696 bp) and DNA sequencing of this 696 bp product was performed to target a region of DNA encompassing the targeted region of Primer set II. Primer set III sequences are provided in Table 1.

DNA sequence analysis of DNA from these amplified samples were conducted using Vector NTI (version 11) and compared to sequences available through the National Library of Medicine, GenBank website. These DNA sequences were submitted to GenBank under the following accession numbers: **JN214356** (Oregon); **JN214357**; (Georgia); **JN214358** (Oklahoma); **FJ527244** (Ohio, clinical case); and **JN214359** (Ohio, *in utero* case). The alpaca sequence produced using primer set I is reported as GenBank accession **JN214360**.

3.2.6 Herd Prevalence Survey

All procedures involving animals and sample collection were approved by the Institutional Animal Care and Use Committee at The Ohio State University and the Clinical Trials Committee of the Veterinary Medical Center and were performed subsequent to obtaining the owner's consent. Samples were collected from approximately 140 alpacas housed on an alpaca breeding farm in southwestern Ohio starting in 2008 and continuing through the summer of 2009. Of the tested male alpacas, 32/90 were positive by PCR examination resulting in a

prevalence of 35.5%. The PCR prevalence in the females evaluated in this study prior to parturition was 30.8% (16/52 dams were PCR positive) and 36 of 52 dams were PCR negative (69.2%)

3.3 Materials and Methods

3.3.1 Sample Population

All procedures involving animals and sample collection were approved by the Institutional Animal Care and Use Committee at The Ohio State University and the Clinical Trials Committee of the Veterinary Medical Center and were performed after obtaining the owner's consent. Samples were collected from 67 dams and their crias housed on an alpaca breeding farm in southwestern Ohio between October 2009 and August 2010. During the years from 2005 - 2008, no whole blood transfusions were performed on any animals from this farm, while 5 plasma transfusions were performed. Two of these plasma transfusions were performed on crias admitted to our hospital and the remaining 3 were performed by the referring DVM on the farm. All plasma transfusions utilized commercial Llama plasma.

Each sample set contained a blood sample (EDTA anti-coagulated) from the dam at the time of parturition (A), a blood sample from the cria prior to ingestion of colostrum (B), a colostrum sample from the dam immediately after parturition (C), and a blood sample from the cria 48 to 72 hours after ingestion of colostrum (D). In addition, serum samples from the crias were obtained immediately before and 3 days post-colostrum ingestion to determine pre- and post-colostrum ingestion serum IgG concentrations. Initially, the pregnant dams were bled approximately 1 month prior to the anticipated date of their parturition to determine their *M. haemolamae* PCR status. All alpacas were closely monitored during parturition to ensure that the crias were not able to nurse prior to collection of a pre-colostral blood samples. Samples were placed in EDTA blood collection tubes and refrigerated at approximately 4° C prior to

analysis. Of the 67 dam-cria sets, data from 12 sets were not included as either pre-colostral (n = 8) or post-colostral cria blood samples (n=4) were not available. In addition, 3 sets were withdrawn due to failure to extract sufficient DNA or the presence of PCR inhibitors in the corresponding colostrum sample. Thus, PCR analysis was performed on blood from the remaining 52 complete sample sets. Serum immunoglobulin (IgG) data both prior to ingesting colostrum and 72 hours post-colostrum was available for all 52 crias included in this study. Sufficient volumes of colostrum for determination of colostral IgG, *M. haemolamae* and indirect fluorescent antibody testing (IFAT) were available for 43 of the 52 dam and cria pairs.

3.3.2 Preparation of IFAT slides

Blood from an *M. hemolamae* infected, 3-month-old female alpaca cria whose blood smear documented large numbers of 0.25-0.5 micrometer, basophilic, non-refractile round bodies present individually and in rows on the surface of red blood cells and in the background consistent with *M. haemolamae* was used for preparation of DNA as well as antigen slides for use in a *M. haemolamae* specific indirect fluorescent antibody test (IFAT). Blood from this cria was also shipped to a separate veterinary diagnostic laboratory (Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Oregon State University) for PCR analysis and confirmed to be *M. haemolamae*.

3.3.3 DNA Extraction

The blood samples from the dam-cria pairs were allowed to settle creating separated layers of red blood cells (RBC), buffy coat, and plasma. DNA extraction was performed using a commercial extraction system (DNeasy, Qiagen) with minor modifications to the extraction protocol. Samples of 50 µL of red blood cells were obtained by pipetting through the buffy coat and the removed RBCs were added to a 1.5 mL microcentrifugation tube containing 20 µL of proteinase K and 200 µL of Buffer AL. Viscosity of the colostrum samples made accurate

pipetting difficult; however, as close to 50 μ L as possible was used for DNA extraction. Several of the colostrum samples required 40 μ L of the proteinase K to facilitate protein digestion. The sample-buffer solution was pulse-mixed for 15 seconds and then placed in a mixing hybridization oven at 56° C for 20 minutes. The remainder of the kit instructions were completed without modification.

3.3.4 PCR Testing

For each reaction, 0.25 μ M of each primer, and 1 μ L of DNA template was added to 23 μ L of Platinum PCR SuperMix (Invitrogen). Negative controls consisted of all reagents without a DNA sample, and positive control consisted of all reagents with a previously confirmed PCR positive DNA sample extracted using the same commercial kit. Cycling conditions for primer set I started with an initial denaturation step of 94° C for 2 minutes followed by 30 cycles of 94° C for 30 seconds, 52° C for 45 seconds then 72° C for 2 minutes. The final cycle consisted of 94° C for 30 seconds and 52° C for 30 seconds with a final elongation at 72° C for 7 minutes. Cycling conditions for primer set II started with a denaturation step of 94° C for 2 minutes followed by 30 cycles of 94° C for 45 seconds, 52° C for 30 seconds then 72° C for 2 minutes. The final cycle consisted of 94° C for 30 seconds and 52° C for 30 seconds with a final elongation at 72° C for 7 minutes. The PCR products were evaluated by gel electrophoresis on 2% (w/v) agarose gel (NuSieve) in Tris-Borate-EDTA (TBE) stained with ethidium bromide and viewed by ultraviolet imaging. Products were sized with a 50-100 base pair DNA ladder.

Three different primer sets were used in this study (**Table 1**). To confirm that each sample contained amplifiable template DNA for PCR, primer set I (**Table 1**) was used as an internal control to amplify an alpaca-specific DNA sequence. We anticipated some genomic alpaca DNA carry-over (leukocytes, epithelial cells) from blood or colostrum to serve as this

control template. This primer pair was originally designed to target a small DNA fragment of a conserved alpaca gene evaluated as part of a different study.

Primer set II amplified a 415-bp fragment of *M. haemolamae* DNA, targeting the 16S ribosomal RNA (rRNA) gene. Primer set II was used for testing the dam and cria sample sets. Specificity and sensitivity of the primers were determined as described previously.

Primer set III amplified a 696-bp fragment of *M. haemolamae* DNA that encompasses the region targeted by Primer set II. To evaluate primer set III application to a geographically-diverse *M. haemolamae* isolates, *M. haemolamae* positive alpaca blood from the states of Washington, Oklahoma, and Georgia underwent testing with primer set III.

3.3.5 DNA Sequencing

DNA sequencing of the 696-bp PCR products were determined for *M. haemolamae* positive adult alpacas from 4 different states, Washington (WADDL, Pullman, WA, USA), Georgia (Dr. Alessandra Pellegrini-Masini, University of Georgia, Athens, GA, USA), Oklahoma (Shawnee Animal Hospital, Shawnee, OK, USA), and Ohio, including the 3-month old cria and the post-parturition positive cria. Primers targeting this region were 5'-ACG AGC AGT GAG GAA TTT TTC AC-3' and 5'-TGCACCACTGTCATACCGATACC -3', using the same cycling conditions as described for Primer set II. The target region for DNA sequencing encompassed the target region for Primer set II. Purification of the PCR products was performed using the QIAquick PCR purification kit (Qiagen). Purified PCR products were submitted for DNA sequencing to the Plant-Microbe Genomics Facility at the Ohio State University, Columbus, Ohio. The DNA sequence analysis was facilitated using Vector NTI and compared with published sequences available through the National Library of Medicine, GenBank website by BLAST analysis.⁶⁸ These DNA sequences were submitted to GenBank under the following accession numbers: JN214356 (Oregon); JN214357; (Georgia); JN214358 (Oklahoma); FJ527244 (Ohio, clinical case); and

JN214359 (Ohio, *in utero* case). The alpaca sequence produced using primer set I is reported as GenBank accession **JN214360**.

3.3.6 Immunoglobulin Concentrations

A camelid serum or plasma and colostral IgG turbidimetric assay (Value Diagnostics) was used to determine the concentration of IgG present in the pre- and post-colostrum samples (cria), and colostrum samples (dam). Serum IgG concentrations were recorded for each cria after colostrum ingestion at the 72 hour time point as part of this farm's routine preventive medicine program to ensure adequate uptake of maternal antibodies.

3.3.7 Immunoglobulin IFAT

Mycoplasma haemolamae-specific antibodies present in colostrum was evaluated by an indirect fluorescent antibody test (IFAT). The IFAT was performed using antigen prepared slides from red blood cells infected with *M. haemolamae* from the naturally infected blood smear positive cria described above. The blood from this cria contained numerous 0.25-0.5 micrometer, basophilic, non-refractile round bodies present individually and in rows on the surface of red blood cells consistent with the appearance of *M. haemolamae*. The blood was confirmed to be positive for *M. haemolamae* by PCR. The dam for this cria was also PCR positive although she demonstrated no signs of clinical infection.

The infected red blood cells were placed into the multi-wells slides (Cell Line Associates, Newfield, New Jersey), air-dried, fixed in methanol, and stored at -20°C until used. Colostrum samples were screened at a dilution of 1:10 and 1:100 in phosphate buffered saline (PBS). Briefly, 15 uL of diluted colostrum was added to each slide well, incubated in a humid chamber at 37°C for 45 minutes followed by three 5 minute PBS washes. A fluorescein-labeled, affinity purified goat anti-llama IgG heavy and light chain (Bethyl Laboratories, Inc. Montgomery, TX) were diluted 1:200 in PBS and added in 10-ul aliquots to each well, followed by an incubation

step in a humid chamber at 37°C for 45 minutes. After washing three times with PBS for 5 minutes, the slides were coverslipped with one drop of fluoromount G (Fluoroprobes, Birmingham, AL). Slides were read blinded independently by 2 readers (RLP and AEM) to verify the consistency of these results. The positive control consisted of the dam's colostrum from the *in utero M. haemolamae* infected cria. The negative control consisted of PBS in place of colostrum for the first step.

In addition, pre- and post-colostrum plasma from the pre-colostrum PCR positive cria was tested, using serial dilutions of plasma in PBS with the same protocol as used for the colostrum samples. These serum samples were evaluated with the IFAT to determine if antibodies to *M. haemolamae* could be identified that might corroborate clearance of the pre-colostral parasitemia. The cria demonstrated titers up to 1:8 from the precolostrum blood and up to 1:10 after ingesting colostrum.

3.3.8 Statistical Analysis

After tabulation of the data, descriptive statistics were performed to evaluate the PCR status of the dams and newborn crias. Two by two, contingency tables were constructed to evaluate the likelihood of PCR positive and negative dams giving birth to a PCR positive cria (prior to ingestion of colostrum) using the Fisher's exact test. In order to compare the presence of *M. haemolamae* specific antibodies in colostrum with PCR status of the dam, a 2 x 2 contingency table was constructed to test the hypothesis that PCR positive dams would have greater likelihood of having *M. haemolamae*-specific antibodies than PCR negative dams. These data were analyzed by Chi-square test for independence. Colostral IgG concentrations among dams of different ages (2-3 yrs, 4-5 years, and > 5 years) were compared using a one-way ANOVA. Colostral IgG concentrations were also compared to PCR positive and negative status of the dams using a one-way ANOVA.

3.4 Results

3.4.1 PCR Testing and DNA Extraction

Extracted DNA was successfully amplified from all individual samples included in the 4 sample sets analyzed for each dam-cria pair using primer set I. All dam, cria and colostrum samples when analyzed by PCR with primer set I produced a 219 bp fragment which was identical to that amplified by PCR of DNA from blood of the naturally infected cria. The DNA extracted from llama and camel blood also produced a 219 bp amplicon using primer set I; however, this primer set failed to produce amplicons when primer set I was tested with DNA obtained from equine, caprine, ovine, and bovine DNA. The 219 bp amplified alpaca sequence aligned to the *Bos taurus* Hereford assembly on chromosome 12 (GenBank NW_001493084.1) on the 5' side of the dedicator of cytokinesis 9-like gene, with 80% identity over 141 bp. It also aligned to one contig of the 2x Broad alpaca assembly, ARR0118852 with 96% identity over 193 bp.

Primer set II (*M. haemolamae* specific), amplified a 415-bp product using DNA extracted from a blood smear confirmed case of *M. haemolamae*, and no PCR product was identified when tested on DNA extracted from whole blood of a healthy adult alpaca testing negative multiple times for *M. haemolamae*. In this negative blood donor, test results were verified when blood from this animal was analyzed by a separate laboratory for *M. haemolamae* and was PCR negative. Primer set II failed to amplify DNA from the other mammalian species tested. Primer set II also failed to produce an amplicon when tested against a variety of different *Mycoplasma* species including, *M. haemofelis*, *M. haemoninutum*, *M. gallisepticum*, *M. bovis*, and *M. synoviae*.

The sensitivity of this PCR detection method, when the DNA extracted from blood of the clinically affected cria diluted 1:1,000 remained positive using primer set II but failed to amplify

this product at the 1:10,000 dilution. DNA extracted from blood from the cria born positive by PCR reaction for *M. haemolama* when diluted 100 fold provided the endpoint for PCR detection in this sample. The PCR assay was also capable of detecting the presence of 1 µL of infected blood when diluted 200 fold in a PCR negative colostrum sample (1:200 dilution of sample prior to DNA extraction).

In a pilot study, the *M. haemolamae* prevalence in a non-breeding male group housed on this farm (90 males; aged 1-7 years) was 35.5% with 32/90 males testing positive by PCR (Lakritz et al., 2008 unpublished). This study used primer set II and included a positive control (clinically affected cria) and negative control (no DNA template in reaction mixture). Considering both the males sampled in a pilot study and those females included in this prospective study, the overall PCR prevalence of *M. haemolamae* in this herd was 34% (48/142 tested by PCR of blood). The PCR prevalence in the females evaluated in this study prior to parturition was 30.8% (16/52 dams were PCR positive) and 36 of 52 dams were PCR negative (69.2%) (Table 2).

One cria tested positive by PCR at parturition prior to colostrum ingestion (1.9%); whereas, 51 of 52 crias were negative by PCR prior to colostrum ingestion (98.1%). (Table 2) The dam of the single PCR positive cria (3 years old), was blood PCR negative for *M. haemolamae*. The PCR status of the dam was not correlated to PCR status of the cria at birth (Fisher's exact test; $p=0.3077$). The pre-colostral, blood PCR positive cria, as well as all pre-colostral, blood PCR negative crias, were PCR negative 72 hours after ingesting colostrum. All 43 of the colostrum samples were PCR negative for *M. haemolamae* and the other *Mycoplasma* species parasites evaluated in this study.

DNA sequences obtained from the naturally infected, PCR positive cria, the cria that was PCR positive prior to ingesting colostrum and several of the PCR positive dam blood samples

were compared to *M. haemolamae* PCR positive samples obtained from different regions of the United States. These DNA sequences aligned with published *M. haemolamae* sequences, with minor sequence variations from a single nucleotide difference (GenBank JF495171) to 16 nucleotide differences (GenBank FN908077). Basic local alignment search tool (BLAST) of the DNA sequence from the naturally infected *M. haemolamae* blood-smear-positive 3 month old cria demonstrated 99% and 97% identity to GenBank JF495171 and FN908077 respectively, with a number of other published *M. haemolamae* GenBank sequences falling within this identity range.⁶³ Most importantly, none of the point differences occurred where primer set II anneals onto the target templates.

3.4.2 Immunoglobulin Testing

Mean serum, pre-colostrum IgG concentrations of the *M. haemolamae* PCR negative crias were 68.7 ± 19 mg/dL (range from 23-111 mg/dL). Mean serum, post-colostrum IgG concentrations of the *M. haemolamae* PCR negative cria were 1964 ± 813 mg/dL (range 120-2950 mg/dL (n=51)). The pre-colostrum blood sample IgG concentration from the *M. haemolamae* positive cria was 415 mg/dL, and following ingestion of colostrum it was 2213 mg/dL. There were no statistical differences in mean post-colostral IgG achieved by the crias after ingestion of colostrum when comparing crias born to PCR positive or negative dams ($p=0.790$).

Mean colostral IgG concentrations were 9,310 mg/dL (median: 9,856 mg/dL; range: 168-24,000 mg/dL). When colostral IgG concentrations were compared by age groups, average IgG concentrations in 3 year old dams were $8,006 \pm 3777$ mg/dL, average IgG concentrations within the 4 to 5 year olds range were $8,954 \pm 4916$ mg/dL, and IgG concentrations in dams > 5 years old had mean concentrations of $10,232.4 \pm 6455$ mg/dL (Table 3). The differences in the means of these 3 groups of females was not statistically different ($p = 0.600$). Furthermore, when

colostral IgG content was compared by the PCR status of the dam, no significant difference was noted with mean colostrum IgG concentrations in the PCR negative dams measuring 9517 ± 6000 mg/dL (range 168 – 24000 mg/dL) and PCR positive dams measuring 8730 ± 4610 mg/dL (range 608 – 15000 mg/dL) ($p=0.790$).

Mycoplasma haemolamae-specific IgG was detected in 22 of 43 (51%) of the colostrum samples by IFAT at the 1:10 dilution, and in 12 of 43 (28%) colostral samples at the 1:100 dilution. Twenty-one of 43 colostrum samples tested negative by IFAT at the 1:10 dilution. Of the 14 PCR positive dams for which sufficient colostrum was available to perform the IFAT, 7 (50%) were positive for *M. haemolamae* antibodies at the 1:10 dilution. Of those 7 samples, only 4 had antibody present at the 1:100 dilution. Of the 29 PCR negative dams for which sufficient colostrum was available for testing, 15 (52%) had antibodies present at the 1:10 dilution. Of those 15 samples, 10 (66%) also had evidence of antibodies at the 1:100 dilution (Table 4). The pre-colostrum PCR positive cria's titer was 1:8 and the corresponding post-colostrum was 1:10. When comparing PCR status of dam's blood to *M. haemolamae* specific IgG in the colostrum, there was no relationship between PCR status of the dam and *M. haemolamae* specific IgG ($p=0.9202$).

3.5 Discussion

This study evaluated many facets of PCR test development, primer validation, and test use for evaluation of vertical transmission of *M. haemolamae* in alpacas. The study specifically investigated the role of *in utero* or colostral transfer of *M. haemolamae* from dams to crias. Additionally, the study identified the presence and potential for transfer of antibodies specific for *Mycoplasma haemolamae* from dams to crias.

When developing a PCR test, validation of primer sequences is necessary to allow for determination of test sensitivity and specificity and accurate assessment of positive and negative results.

Primers targeting a segment of alpaca genomic DNA (primer set I) were selected to verify that sufficient DNA was recovered after extraction from all samples. Secondly, the use of these primers ensured that no PCR inhibitors were present that might alter PCR results. If any given sample did not test positive when using primer set I, that sample was excluded from analysis to prevent inadvertent inclusion of *M. haemolamae* negative tests that would bias results.

Primers targeting a segment of the 16s rRNA gene (primer sets II and III) were selected based on conservation of that region of the genomic sequence amongst *M. haemolamae* sequences provided by GenBank. Primer set III successfully amplified target sequences from *M. haemolamae* samples from a variety of geographic regions within the United States supporting the validity of using these primers for PCR testing. In addition, primers from primer set II did not create amplicons when run with DNA from the other *Mycoplasma spp.* that were tested. These results suggest that the primer set II sequences were adequately specific for ongoing testing purposes. Relative sensitivity of the assay was determined by performing serial dilutions of positive EDTA blood samples from both the original clinically affected cria and the *in utero* PCR positive cria. Samples from the clinically affected cria were positive to the 1:1,000 dilution, and samples from the *in utero* PCR positive cria was positive to the 1:100 dilution. Based on these results, the PCR primers and test are sensitive to identify subclinical infection in individuals that are not clinically parasitemic.

In anticipation of developing a multiplex assay, incorporating detection of both alpaca and *M. haemolamae* DNA, three sets of samples were tested using both primer sets, I and II, in

the same reaction using the cycling conditions of primer set II. Bands of approximately 219 base pairs consistent with genomic alpaca DNA were present for all samples and bands of approximately 415 base pairs consistent with *M. haemolamae*, were present for those samples that tested positive for *M. haemolamae*. These results suggest that a multiplex assay could be feasible in the future.

Previous studies have suggested prevalence of *M. haemolamae* between 9.26%-19.3% in South America and 18.6% in Switzerland.^{5,6} Comparatively, the prevalence of *M. haemolamae* infection for the herd evaluated in this study was approximately 35% with the herd female prevalence being 30.8%. Despite the higher incidence of *M. haemolamae* in the current study population, only one cria was born PCR positive prior to colostrum ingestion. This prevalence translates to a vertical transmission rate of <2%. The low transmission rate identified in this herd is much lower than that suggested by another recent study where 5 PCR positive females sampled prepartum gave birth to 2 PCR positive crias prior to ingestion of colostrum.¹ Assuming that those 5 females reflect the herd incidence in that study, vertical transmission could be more frequent on some farms as compared to the farm in this study. However, that prior study did not report results of testing performed on PCR-negative pre-partum females in the herd, so true herd transmission incidence may differ from that reported. Additionally, the retrospective nature of the study may have skewed results. Our results suggest that giving birth to a PCR positive cria is just as likely to be from PCR-negative dams as to PCR-positive dams. Possible explanations include clearance of detectable infection by the dam prior to parturition and PCR testing or inability of the PCR test to detect an extremely low level of circulating organism.

The majority of the dams evaluated in our study (69%) were PCR negative for *M. haemolamae* infection. As previously stated, the only PCR positive cria was born to one of these negative dams. Although it is possible that the positive test could have resulted from

contamination during PCR sample processing, it seems unlikely as the negative controls included in the assay were negative. All births were monitored to ensure that the crias could not ingest colostrum prior to acquisition of the pre-colostrum blood sample. Perinatal blood inoculation is an unlikely means of transmission as a previous study demonstrated a 4 day incubation period between experimental blood inoculation and PCR detectable infection. Additionally, the presence of elevated serum IgG concentration (415 mg/dL) supports the presence of *in utero* infection with an active immune response by the fetus. The elevated IgG concentration is much higher than reported for all other crias sampled on this farm (mean 68.7 ± 19 mg/dL) measuring more than two standard deviations from the mean of the normal crias in this study. Our study supports the findings of prior studies in which the mean IgG concentration expected in crias prior to colostrum ingestion should be 100 mg/dL or less.^{62,65} Fetal immune responses causing elevated IgG concentrations at birth have been documented in other species associated with transplacental transmission of pathogens like *Neospora caninum* and BVDV in cattle and *Toxoplasma gondii* in humans. As noted in other species, transplacental transmission of pathogens is of concern as it may increase the risk of clinical disease or provide a source of ongoing transmission to herdmates; however, in this study, transplacental transmission was uncommon and did not result in clinical disease in the single *in utero* infected PCR positive cria.

Colostrum samples tested as part of our study did not demonstrate the presence of *M. haemolamae*. These results suggest that colostral transmission is unlikely. Although PCR inhibitors in colostral samples cannot be ruled out as a potential cause for negative *M. haemolamae* results it seems unlikely to be a significant problem as the PCR test detected alpaca genomic DNA from all colostrum samples. Our findings confirm what was found in a previous study stating that colostral transmission of organisms is unlikely.¹ Potentially, blood-contaminated mammary secretions could transmit *M. haemolamae* to crias; however, blood

contamination of colostral samples was not noted in our study and has not been reported previously.

Our study suggests that colostral antibodies to *M. haemolamae* may play a crucial role in limiting infection in neonatal crias. Support for this assertion comes from data showing that the *in utero* infected PCR positive cria tested negative 72 hours after ingestion of colostrum. This finding suggests that maternal antibodies may allow for clearance of the organism in infected crias. This observation suggests clearance of the organism from the blood, at least to levels below the threshold for detection by this PCR assay. One potential explanation is that maternal factors transmitted to the cria within colostrum may serve to clear this organism. We evaluated whether *M. haemolamae* specific colostral IgG is present and could ostensibly function to reduce the number of parasites below the threshold of detection by PCR.

Evaluation of the presence of antibodies to *M. haemolamae* in colostrum was performed using an indirect fluorescence test. Results of this study demonstrated the presence of *M. haemolame*-specific antibodies within the colostrum from both PCR positive and negative dams. The results of this assay suggest that maternal factors (such as IgG) could modulate parasitemia in the cria. These findings are supported by prior work which demonstrated *M. haemolamae* specific antibodies (*Eperythrozoon weyonii* at that time) in the serum of a large number of camelids from a broad geographic area.³ The maternal IgG detected in our study could protect the cria by aiding removal of these parasites (e.g. by opsonization), thereby limiting clinical disease. Information defining the impacts of *M. haemolmae* specific antibodies could prove useful in understanding both the transmission of *M. haemolamae* and the clinical significance of *M. haemolamae* infection.^{1,3} Crias that have ingested colostrum with antibodies may be a decreased risk of developing clinical infection.

As compared to the PCR positive cria in our study that cleared its infection after ingesting colostrum, another recent study demonstrated that a PCR positive cria remained positive several months despite ingesting colostrum.¹ It is unknown whether *M. haemolamae* specific antibodies were present in the colostrum ingested by this cria, or if there was adequate uptake of colostrum by the cria as might be determined by a serum IgG concentration assay.

The presence of specific antibodies to *M. haemolamae* in colostrum was more prevalent in PCR negative dams which may indicate that individuals can develop relative immunity to the organism and that the antibodies may be passed to crias through colostrum. Our results demonstrated that the presence of *M. haemolamae* specific antibodies is not correlated with PCR based testing results in the dam's blood. This finding suggests a component of the dam's immune system is capable of reducing the number of parasites to below the threshold for detection, while producing antibodies specific to this organism which is compatible with prior reports.^{1,2} It is therefore possible that prior infection may provide the immunologic mechanisms necessary to clear infection and protect the newborn that acquires the organism early in life while colostral antibodies are still in circulation. In addition, since colostral *M. haemolamae* has not been detected, special management practices such as heat-treatment or pasteurization of colostrum are not likely to result in reductions in *M. haemolamae* transmission to the cria and may denature immunoglobulins that could provide protection.

The PCR based test does not provide sufficient information regarding the host-parasite interaction; specifically the risk of infection or the outcomes of the neonate that acquires infection. While we recruited a herd with what we proposed would have suitable infection prevalence, we only detected a single vertical transmission event. In fact, based upon our pilot PCR study, a sample size of 48 was predicted to be sufficient to detect differences in transmission from dam to cria. From this study, PCR-prevalence in the breeding animals in this

herd is 30.7%. However, antibody prevalence in colostrum from PCR-positive and PCR-negative dam's appears to be as high as 50%.

The presence of antibody specific to *M. haemolamae* may explain how clinical disease in neonatal crias could be prevented. Specific antibodies to *M. haemolamae* were noted in 50% of all evaluated colostrum samples indicating immunity to disease could be passed to these neonates. Specific antibodies were present in 50% of the dams regardless of their PCR test status. The presence of antibody in both PCR positive and negative dams indicates that infection may be acquired earlier in life and cleared prior to parturition. By passing these *M. haemolamae* specific antibodies to crias via colostrum, neonatal infection rates could be decreased. Challenge studies are required to definitively determine a protective role for colostrum anti-*M. haemolamae* immunoglobulin in naïve or infected individuals.

The IFAT we used to test for *M. haemolamae* specific antibodies in colostrum was also used to evaluate plasma samples. Compared to the colostrum samples, there was an increased level of non-specific fluorescence making results more difficult to read and interpret. Further evaluation of serial dilutions and titers was not pursued due to the difficulty in accurately reading the results. The colostrum samples were screened at 1:10 and 1:100 because of the limited availability of antigen slides. The dam's colostrum of the *in utero* infected cria was positive at 1:100 but negative at 1:500 thereby setting our screening dilutions.

One difficulty in interpreting IFAT results clinically is that positive results indicate that prior exposure to a specific antigen or organism has occurred (seroprevalence) but does not necessarily correlate to clinical disease or current infection.^{3,69} Although it has provided important information regarding the presence of colostrum antibodies, it is unlikely to be useful as a primary testing modality for determining disease status or likelihood of acquiring disease.

Ongoing monitoring of the crias in this study would provide interesting insight into the incidence of PCR test negative crias becoming positive later in life. Additionally, it would be interesting to note whether the *in utero* infected cria's PCR status would change at later timepoints. At 2 months of age the cria continued to be PCR negative, but further testing after maternal antibodies had completely waned would provide additional information regarding the role of *M. haemolamae* specific antibodies in developing immunity in crias.

Further study regarding transmission pathways focused on potential insect vectors will provide better understanding of the parasite's biology. Disease prevention strategies can best be developed once transmission pathways have been elucidated. At this point, we have confirmed that *in utero* transmission is uncommon but possible as a means of transmission of the organism. Secondly, our study shows that *M. haemolamae* specific antibodies may play an important role in preventing clinical disease in neonatal crias.

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Appendix A: Tables

Table 1 Primer sets, amplicon sizes and sequences used in this study.

Primer set	Size (bp)	Primer sequence
Set I Sense Set I Anti-sense	gDNA-219 bp*	5'-ATGCTTTTCTGTGTATGGTTATCTAGTG-3' 5'-CTAGTTTCCCAAGTTCATCTTTCTG- 3'
Set II Sense Set II Anti-sense	Mh-415 bp†	5'-ACG AGC AGT GAG GAA TTT TTC AC-3' 5'-TCA ATT ATG TCC CAG GTA CTC-3'
Set III Sense Set III Anti-sense	Mh-696 bp	5'-ACG AGC AGT GAG GAA TTT TTC AC-3' 5'-TGCACCACCTGTCATACCGATACC -3'

*gDNA-219 bp indicates amplification of genomic DNA of host cells as amplicon control. †Mh

indicates *Mycoplasma haemolamae* directed primers for the 16S, small subunit ribosomal RNA gene.

Table 2 Number of *Mycoplasma haemolamae* PCR positive and PCR negative dams, PCR positive and negative crias immediately after birth, PCR positive and negative colostrum samples and post-colostral testing of crias.

PCR Results	Dam	Cria		Cria
		Pre-colostrum	Colostrum	Post-colostrum
Positive	16 (30.8%)	1 (1.9%)	0	0
Negative	36 (69.2%)	51 (98.1%)	43(100%)	52 (100%)
Total	52	52	43	52

Table 3 Number and percentage of *Mycoplasma haemolamae* PCR positive and negative dams by age, colostral IgG concentrations by age and colostral *M. haemolamae* specific IgG by IFAT (at 1:10 and 1:100 dilutions). Colostrum samples from 43/52 dams were available for analysis due to limited volume of colostrum provided.

Age	Number Testing PCR Positive	Colostrum IgG Concentration (mg/dL)	Colostral <i>M. haemolamae</i> IFA 1:10	Colostral <i>M. haemolamae</i> IFA 1:100
2-3 yr	5/14 (35.7%)	8,006 ± 3,777	7/11 (63.6%)	2/11 (18.2%)
4-5 yr	7/18 (38.9%)	8,954 ± 4,916	8/16 (50.0%)	6/16 (37.5%)
>5 yr	4/20 (20.0%)	10,232 ± 6,454	7/16 (43.8%)	4/16 (25.0%)

Table 4 Colostral antibody presence and absence in *Mycoplasma haemolamae* PCR positive and negative dams included in this study. When grouped according to *M. haemolamae* status, IFAT evaluation demonstrated 50% of the dams had colostral IgG titers at 1:10, however, decreased to 29% at 1:100. Likewise, while 52% of the *Mycoplasma haemolamae* PCR negative dams possessed colostral IgG specific for *M. haemolamae* at 1:10 dilution, this number declined to 34% when evaluated at a 1:100.

Dam <i>Mycoplasma haemolamae</i> PCR Status	Colostral Antibody Titer			
	1:10		1:100	
	Antibody Detected	Antibody Not Detected	Antibody Detected	Antibody Not Detected
Positive (n=14)	7 (50%)	7 (50%)	4 (29%)	10 (71%)
Negative (n=29)	15 (52%)	14 (48%)	8 (28%)	21 (72%)
Total (n=43)	22 (51%)	21 (49%)	12 (28%)	31 (72%)

Appendix B: Figures

Figure 1: Ethidium bromide-stained agarose gel showing *M. haemolamae* specific polymerase chain reaction (PCR) products of approximately 415 base pairs, as sized by molecular size markers. The samples in this gel originated from the following animals: lanes 1, 4, 7: blood samples from alpacas positive for *M. haemolamae*; lanes 2, 3, 5, 6, 8, 9, 10: blood samples from alpacas negative for *M. haemolamae*; lane 11: PCR reaction negative control; lane 12: PCR reaction positive control.

Figure 2. Representative images of IFA slide wells showing the absence (left) and presence (right) of antibodies to *M. haemolamae* in colostrum from subjects included in this study. Colostral samples were diluted to 1:10 prior to placing onto IFA slide. Antibody binding was detected using a secondary, goat anti-llama-FITC conjugate antibody that was added to the slide wells to visualize antibody binding to RBC parasites by fluorescence. The image on the left demonstrates a negative control well from the animal tested in the right image. The image on the right indicates a positive test reponse demonstrating antibodies in the colostrum binding to parasites on the RBC membrane of the clinical crias RBC.