Use of Systemic Antibiotics With or Without Topical Nasal Treatments to Clear *Mycoplasma ovipneumoniae* From Lambs

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Abstract

Mycoplasma ovipneumoniae (M. ovipneumoniae) is often carried asymptomatically in the nasal passages of domestic small ruminants worldwide and is commonly associated with chronic respiratory disease. Currently, no antibiotics are labeled for treatment of this bacterium in domestic sheep in the United States. The efficacy of systemic antibiotics with or without nasal flush treatments to clear *M. ovipneumoniae* in lambs was assessed. The study was conducted in two parts, Cohort I in 2021 and Cohort II in 2022. Yearling Suffolk lambs were identified as positive for *M. ovipneumoniae* via nasal swab PCR, randomly assigned to different treatments and a positive control group (n=6 animals per group). Pens were spaced 2.9 meters apart during the study. Treatments in Cohort I were as follows: oxytetracycline 20 mg/kg subcutaneously (SQ) once (OXO); oxytetracycline 10 mg/kg intramuscularly (IM) once daily for 5 d (OXD); oxytetracycline 10 mg/kg IM daily for 5 d with a dilute betadine nasal flush daily for 5 d (OXB); oxytetracycline 10 mg/kg IM daily for 5 d with a dilute chlorhexidine nasal flush daily for 5 d (OXC); and positive control receiving no treatment (POS). In Cohort II, treatments were as follows: lincomycin 5 mg/kg IM every 48 h for 3 doses (LIN); lincomycin 5 mg/kg IM every 48 h for 3 doses with dilute lincomycin nasal flush daily for 5 d (LIF); florfenicol 20 mg/kg IM every 48 h for 3 doses (FLO); florfenicol 20 mg/kg IM every 48 h for 3 doses with dilute florfenicol nasal flush daily for 5 d (FLF); oxytetracycline 10 mg/kg IM daily for 5 d (OXD); and positive control receiving no treatment (POS). Treatment efficacy was evaluated by nasal swab PCR obtained at days 7, 14, 21 and 28 post treatment. Statistical analysis was performed using non-linear mixed effects modeling in R. Response to treatment was evaluated by comparing mean PCR Ct values of each treatment to the Ct values of the POS groups over time, with Ct values classified as ‘Detected’ (Ct ≤36), ‘Indeterminate’ (Ct 36-40), or ‘Not Detected’ (Ct ≥40). In comparison to the Ct values of the POS groups, OXD was the only treatment found to increase (P = 0.003) Ct values overall, indicating a decrease in *M. ovipneumoniae* genomic material. OXD compared to POS was (P = 0.004) different on d 7, d 14 (P = 0.0005), and d 21 (P = 0.008), but not on d 28 (P = 0.52). Mean Ct values of OXD groups were 31.4 over the course of the study, while mean Ct values of POS groups were 25.3. Ct values of the OXD group decreased over time (d 7= 35.7, d 28=29.6). The OXB group was significant in increasing Ct value compared to the POS groups only on Day 7 (P = 0.034). All other groups

showed no detectable response to treatment in comparison to the POS groups overall, nor at any point in time. Although OXD increased Ct values when compared to POS, it did not induce complete response to treatment by achieving Ct values ≥40 in the majority of animals, nor were the Ct values maintained over time. We suspect the decreasing Ct values over the course of the study were related to the animals being re-infected with *M. ovipneumoniae* because of prolonged exposure to positive group members that did not respond to treatment. Failure to respond to treatment can likely be attributed to variations in *M. ovipneumoniae* strain type susceptibility and virulence. Complete response to treatment in the majority of the study animals is needed in order to recommend the antibiotic be used to treat animals with clinical disease. Further studies identifying the strain types present in a study cohort, and testing and segregating animals after treatment to prevent re-infection from a positive cohort is warranted.

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Dedication

To my family and friends, particularly Matt, for their love and support of my decision to go back to graduate school, and for their patience and encouragement while I devoted my time to my studies. I couldn't have done this without you.

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Chapter 1: Literature Review

Introduction

Respiratory disease related to *Mycoplasma ovipneumoniae (M. ovipneumoniae)* has been recognized in the family Caprinae worldwide (McAuliffe et al., 2003). Often associated with chronic cough, infected animals range from asymptomatic to developing severe polymicrobial pneumonia and death (Manlove et al., 2019). *M. ovipneumoniae* is often overlooked for its health impacts as a subclinical disease in sheep production systems causing reduced efficiency in weight gain and carcass yield in infected lambs (Besser et al., 2019), and chronic cough predisposing short tail docked lambs to rectal prolapse (Thomas, 2003). Characteristics of *Mycoplasma* spp make it difficult to culture, contribute to limited antibiotic options to treat this organism and without a vaccine available to prevent infection (Ziegler et al., 2014).

Mycoplasma ovipneumoniae in domestic sheep

Mycoplasma ovipneumoniae (M. ovipneumoniae) was first described in 1974 in domestic sheep in New Zealand (Clarke & Alley, 1974), causing chronic coughing and predisposing sheep to develop respiratory disease. This organism has since been identified in domestic goats, bighorn sheep, deer, elk, moose, mountain goats, and musk oxen (Besser et al., 2014, 2017; Handeland et al., 2014; Kamath et al., 2019). As in other *Mycoplasma spp.* afflicting the respiratory tract of livestock and humans, *M. ovipneumoniae* adheres to mucosal linings in the nasal passages and can cause damage to respiratory cilia, resulting in invasion and colonization of deeper tissues by *M. haemolytica* and *P. multocida* as the host is unable to maintain mucociliary clearance.

M. ovipneumoniae is transmitted between animals in close contact via aerosolized respiratory droplets. Adult animals can be asymptomatic carriers with no apparent clinical signs of disease. Domestic sheep lambs (or naïve adult animals) are exposed to *M. ovipneumoniae* through contact with infected adults in the flock that harbor the bacteria in their nasal passages (Brogden et al., 1988). Lambs initially experience colonization of the nasal passages, and then may develop respiratory disease progressing to pneumonia over the

course of 5-10 weeks (Nicholas et al., 2015). *M. ovipneumoniae* has been isolated from the nasal passages, cornual sinus, bronchi, and lungs of infected animals (Besser et al., 2017; Ionas et al., 1985). Therapy with antibiotics shows initial response, likely because of the treatment of secondary pathogens such as *M. haemolytica* and *P. multocida*, but relapses are common when *M. ovipneumoniae* is involved.

A 2011 study by the USDA National Animal Health Monitoring System (NAHMS) looked at sheep health and management on more than 400 sheep operations in 22 states representing 85.5% of the US sheep inventory (USDA, 2014). The study found 88% of all operations to have at least one animal PCR positive on a nasal swab for *M. ovipneumoniae*, with all flocks of range sheep having one or more positive animals. Individual infection rates are highly varied among herds, ranging from 1/10 to 10/10 in randomly sampled animals (Manlove et al., 2019). When comparing respiratory signs and growth rates of lambs in a *M. ovipneumoniae* negative flock to lambs born to *M. ovipneumoniae* positive dams, the lambs in the *M. ovipneumoniae* negative group had lower respiratory scores and better average daily gain and carcass quality than the *M. ovipneumoniae* exposed group (Besser et al., 2019). In the same study, lambs from both groups were housed separately and started in a feeding trial after weaning; lambs born to *M. ovipneumoniae* positive dams all tested positive for *M. ovipneumoniae* on PCR analysis of nasal swabs after the start of the feeding trial; however, lambs in the *M. ovipneumoniae* negative flock never tested positive for *M. ovipneumoniae* at any point during the trial. Multiple strains of *M. ovipneumoniae* have been identified in flocks of sheep (Harvey et al., 2007; Kamath et al., 2019), and multiple strains have been identified in single individuals (Lonas et al., 1991), supporting the high prevalence of *M. ovipnemoniae* positive individuals and flocks in sheep operations.

Mycoplasma bacteria

There are over 100 species of Mycoplasma bacteria, affecting both humans and animals (Murray et al., 2020). Ranging in size from 150-200 nm (Kashyap & Sarkar, 2010), Mycoplasma spp. are the smallest known bacteria, and lack a cell wall. Their small size and flexible cell membrane allow them to pass through 0.45 um pore filters. Mycoplasma bacteria also have a small genome (0.58-2.20 Mb) compared to other bacteria, resulting in

very limited metabolic and biosynthetic capabilities and necessitating parasitic dependence on specific host species and tissues to survive (Kashyap & Sarkar, 2010; Rottem, 2003). Though some Mycoplasmas are considered part of normal flora, such as in the human oral cavity, the majority are considered pathogenic even when the host is asymptomatic (Murray et al., 2020). The lack of a cell wall, small size, and slow growth in culture present a myriad of diagnostic and treatment challenges.

When enough bacteria are present, the mycoplasma cell membrane produces a polysaccharide biofilm, which protects the bacterial colony from the environment, antimicrobials, and the host's immune response (Murray et al., 2020; Rosengarten et al., 2000). Biofilms are an important virulence factor which enables *Mycoplasma* spp. to colonize tissues including the nasal passages, trachea, and urogenital tract. Mycoplasmas rarely invade tissues and survive through maintaining close contact with specific host cells. The ability to adhere to host cells is so critical that mycoplasmas have developed special membrane components called adhesins. Adhesins are necessary in initial colonization and in the progression of disease (Rottem, 2003). In *Mycoplasma pneumoniae (M. pneumoniae)*, a human respiratory pathogen, the P1 adhesin protein allows the bacteria to bind to cilia in the host's airways and destroy ciliated epithelial cells (Rosengarten et al., 2000). The loss of cilia in the upper respiratory tract reduces the host's ability to clear debris and other bacteria, resulting in colonization of the lower airways by secondary bacteria and development of pneumonia.

To further facilitate their ability to colonize mucosal surfaces, both *in vivo* and *in vitro* studies have found that mycoplasmas have developed mechanisms to either suppress or stimulate the host's immune response. Depending on the host species, mycoplasmas in their respective host can suppress or upregulate both B and T lymphocytes in a nonspecific manner and can stimulate secretion of pro-inflammatory cytokines including tumor necrosis factor (TNF-α), interleukins (IL)-1 and IL-6, by macrophages (Razin et al., 1998; Rottem, 2003). Some species of mycoplasmas are capable of penetrating or fusing their cell membrane with that of eukaryotic cells. The presence of mycoplasmas in prolonged close contact with host cells, combined with cytokine induction leading to chronic inflammation in host tissues, can result in long-term pathogenic changes in host mucosal tissues.

Mycoplasma pneumoniae in humans

Though many mycoplasmas infect humans, *M. pneumoniae* is a common respiratory pathogen capable of producing a range of respiratory disease symptoms in humans and is among the most well-studied of the mycoplasma species known to cause disease in humans or animals. In the United States, *M. pneumoniae* is a common cause of community-acquired pneumonia, though it is considered endemic worldwide (Kashyap & Sarkar, 2010; Murray et al., 2020). As discussed earlier, the major virulence factor is the P1 adhesin protein, which is critical in allowing the organism to adhere to cilia in the upper respiratory tract. Transmission is generally through contact with large, aerosolized droplets from a cough or sneeze in close quarters. Most adults are asymptomatic, with respiratory disease occurring more commonly in children and the elderly. Initial mild cough and pharyngitis can progress over several days to fever, wheezing, and bronchopneumonia. Extrapulmonary infections are uncommon, but typically involve the central nervous system and can be deadly (Murray et al., 2020; Rosales et al., 2017). Diagnosis is usually via PCR of throat swabs, as the turnaround time for culture is lengthy and deferring treatment while waiting for culture results could have a negative impact on patient outcome. The preferred treatment is a macrolide antibiotic; usually azithromycin is prescribed because of its relative lack of side effects in children (compared to tetracyclines or fluroquinolones) (Murray et al., 2020) and efficacy against *M. pneumoniae* both *in vitro* and *in vivo*.

Mycoplasma in livestock species

Mycoplasmas are responsible for significant respiratory, arthritic, and mammary disease in numerous livestock species. In cattle, *Mycoplasma bovis* (*M. bovis*) is a component of Bovine Respiratory Disease (BRD), keratoconjunctivitis (commonly referred to as pinkeye), and otitis media (inner ear infection) (Maunsell et al., 2011; Rosales et al., 2017). Transmission is similar to that of *M. pneumoniae*, where *M. bovis* is passed between hosts in close proximity through inhalation of large aerosolized respiratory droplets. *M. bovis* is of particular concern in dairy cattle as a cause of contagious mastitis resulting in chronic subclinical mastitis or mastitis that is not responsive to antibiotic therapy (Maunsell et al., 2011). In a dairy, *M. bovis* can be passed from udder to udder through contact with

contaminated milking equipment and bedding. Vertical transmission is also possible through feeding contaminated milk to dairy calves. The presence of *M. bovis* is not always associated with the development of clinical disease. Asymptomatic animals can be implicated in bringing this pathogen into both beef and dairy herds as well as maintaining the pathogen in these herds over time (Maunsell et al., 2011). Development of BRD in beef and dairy cattle at any stage of production is often associated with respiratory tract co-infections with *Manheimia haemolytica (M. haemolytica)* and *Pasteurella multocida (P. multocida)*, and the development of arthritis and or otitis media. Diagnosis is typically based on clinical signs but may include the use of milk culture or PCR of bronchoalveolar lavage samples in individual animals, and serology for antibody titers or PCR of bulk tank milk during herd biosecurity screenings. Treatment focuses on antibiotics to which mycoplasmas are susceptible (macrolides, tetracyclines, and fluoroquinolones) (Francoz et al., 2005) with special consideration to regulations regarding use in food animals, and meat and milk withdrawal times. In addition to costs associated with treatment and increased holding time to harvest, economic loss occurs in feedlots where beef cattle have poor weight gain because of *M. bovis* infection (Maunsell et al., 2011). Chronic mastitis because of *M. bovis* often results in decreased milk production and subsequent early culling of dairy cows related to poor performance.

In pigs, *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary agent of enzootic pneumonia, affecting the pork industry worldwide (Leal Zimmer et al., 2020; Maes et al., 2018). This chronic respiratory disease is a major source of economic loss in the pork industry and as such has been researched extensively with regards to vaccination and prevention strategies. Transmission is through prolonged close contact with an infected animal. Chronic, nonproductive cough is eventually followed by development of severe lung lesions and lung consolidation, as well as decreased average daily gain and increased days to market in finishing hogs (Maes et al., 2018). Secondary health problems including rectal prolapse can develop in infected animals. In commercial swine operations, efforts to establish and maintain a *M. hyopneumoniae* free herd are cost effective (Silva et al., 2019) when compared to the ongoing production losses related to *M. hyopneumoniae* in a positive herd. Strategies to attain negative herd status include herd management (all in/all out, segregated early weaning), sound biosecurity practices, screening of new pigs prior to arrival, blanket

antimicrobial treatment, and vaccination (Maes et al., 2018). Numerous commercial vaccine products are available. Response to vaccination is variable, but vaccination generally decreases both the severity and duration of disease in an individual.

Mycoplasma ovipneumoniae in bighorn sheep

Bighorn sheep were once widespread in North America, with substantial population declines coinciding with westward expansion and settlement in the 1800-1900's. Bronchopneumonia of bighorn sheep has played an ongoing role in disease outbreaks, population decline, and limiting population recovery and has been extensively debated and researched (Besser et al., 2008a, 2008b, 2014; Cassirer et al., 2018; Miller et al., 2012). *M. ovipneumoniae* has been identified as the underlying causative agent for polymicrobial pneumonia in bighorn sheep (Besser et al., 2008a) often involving *M. haemolytica* and *P. multocida* as secondary bacteria invading the lungs. *M. ovipneumoniae* produces significantly more severe disease in bighorn sheep than domestic sheep. On exposure to *M. ovipneumoniae*, naïve bighorn sheep adults and lambs develop severe epizootic pneumonia, with mortality rates in bighorn lambs ranging from 20-100% (Cassirer et al., 2018). Adults that recover from this disease episode can go on to become carriers of *M. ovipneumoniae* and pass the bacteria to lambs born in subsequent years, perpetuating the disease in a herd over time and limiting population recovery. Immunity to *M. ovipneumoniae* appears to be strain-specific (Cassirer et al., 2017), with recovered animals susceptible to subsequent infection by different strain types. In one study, genetic analysis of *M. ovipneumoniae* from 594 isolates from domestic sheep, domestic goats, bighorn sheep, and mountain goats showed high genetic diversity among *M. ovipneumoniae* strains in domestic sheep (Kamath et al., 2019). *M. ovipneumoniae* in bighorn sheep herds had low genetic diversity, and phylogenetic analysis of *M. ovipneumoniae* strains in bighorn sheep populations could be traced back to domestic sheep or domestic goat *M. ovipneumoniae* origin strains. These findings support the idea that *M. ovipneumoniae* strains in bighorn sheep are originally from domestic sheep and or goats, regardless of length of time circulating in bighorn populations, and that disease spillover events between domestic and wild populations are of ongoing concern. Reducing risk of disease related to *M. ovipneumoniae* in bighorn sheep is therefore intrinsically linked to controlling *M. ovipneumoniae* transmission from domestic sheep.

Laboratory diagnosis

Mycoplasmas are of great concern as a common contaminant in laboratory cell culture lines, causing interference with cellular growth and metabolism and necessitating disposal of the contaminated culture and disinfecting of the laboratory (Sung & Hawkins, 2020). Conversely, *Mycoplasma spp* are difficult to culture compared to other respiratory pathogens when using culture as a diagnostic tool. Because of their limited metabolic capabilities and dependence on host cells to meet their nutritional needs, mycoplasmas must be grown on specially enriched media that meet their nutritional needs *in vitro* and are at an appropriate pH (Parker et al., 2018). When cultured from a diagnostic sample, such as a nasopharyngeal swab, other bacteria often overgrow mycoplasmas because of the enhanced media and the slow growth rate and size of mycoplasma colonies compared to other bacteria. Culture for mycoplasmas is generally time consuming at 7-10 days of incubation. Sample collection and handling also impacts culture, and false negatives can occur if samples are not stored at the proper temperature or processed at the laboratory in a timely manner after being obtained. Ideally, samples should be kept at 4 ℃ and put into mycoplasma broth for transport (Ball et al., 2020), and inoculated in growth media within 48 h of collection, as prolonged storage regardless of refrigeration or freezing temperature decreases growth (Parker et al., 2018). The use of wooden shaft swabs or cotton tipped swabs is discouraged as these materials inhibit mycoplasma growth (Ball et al., 2020; Maunsell et al., 2011); plastic shafts and foam-tipped swabs are recommended instead.

Serology can be used as a diagnostic aid or screening tool but is not definitive in diagnosis of active mycoplasma infections (Maes et al., 2018; Maunsell et al., 2011). Competitive enzyme-linked immunosorbent assay (cELISA) is used to evaluate serum for the presence of antibodies. Antibodies are found in serum after the host's adaptive immune system is exposed and responds to a pathogen. Antibodies can also be present in neonates through passive transfer in colostrum or across the placenta in gestation, or after immunization. Antibody level varies between animals and is not directly correlated with protective immunity (Besser et al., 2014; Ziegler et al., 2014). Domestic sheep have been found to have high amounts of *M. ovipneumoniae* in their nasal passages yet low antibody titers, suggesting an evolved tolerance of *M. ovipneumoniae* and the ability to shed the

organism without incurring high rates of disease (Cassirer et al., 2018). In a study evaluating a vaccine for *M. ovipneumoniae* in domestic sheep, inoculation with a large amount of antigen would stimulate significant antibody response in immunized domestic ewes, whose lambs acquired passive antibodies to *M. ovipneumoniae* as well (Ziegler et al., 2014).

Polymerase chain reaction (PCR) is the current standard for laboratory diagnosis of Mycoplasma spp. in humans and animals. Compared to traditional culture-based methods of detection, PCR is superior in sensitivity and specificity as well as efficiency. PCR can be used to detect genomic material from a specific target organism from a variety of samples, including mucosal swabs, fluid from a bronchoalveolar lavage, and tissues (Besser et al., 2017; Parker et al., 2018). Because PCR amplifies DNA, there must be genomic material from the target organism that is not degraded, but it does not have to be viable to grow as needed in culture. Real-time PCR (RT-PCR) uses cycle times (Ct) to quantify the amount of genomic material present in a sample. The greater the amount of genomic material present, the faster it is amplified in the assay and the lower the Ct to detect the presence of the target organism. Several RT-PCR methods to identify *M. ovipneumoniae* have been described (Ackerman et al., 2019; Besser et al., 2019; Manlove et al., 2019; Yang et al., 2014; Ziegler et al., 2014).

Vaccine use for Mycoplasma spp.

Vaccination for *M. ovipneumoniae* has been proposed as a method to decrease the incidence of disease in domestic sheep and reduce the risk of transmission from domestics to bighorn sheep. Mycoplasma spp. present a challenge for vaccine development as most vaccines prime the host's immune system to respond to a pathogen by recognizing antigenic material associated with that pathogen. The adhesin protein in the mycoplasma cell membrane is the antigen suitable for vaccine development, but variations in the adhesin protein among strains of mycoplasma alter both virulence and the ability of the host's immune system to recognize the antigen (Cassirer et al., 2017; Harvey et al., 2007; Razin et al., 1998). In pigs, many commercial vaccine products for *M. hyopneumoniae* have been developed with most products being a bactrin vaccine (Maes et al., 2018). Bactrin vaccines consist of inactivated, or killed, whole-cell bacteria paired with an adjuvant to increase duration of time at the injection site and enhance immune response to the antigen (Murray et

al., 2020). Vaccination of pigs for *M. hyopneumoniae* induces partial protection from development of disease and limits development of gross lung lesions but does not protect the host against colonization by *M. hyopneumoniae* (Haesebrouck et al., 2004).

A bactrin vaccine for *M. ovipneuoniae* in domestic sheep was explored by Ziegler et al. (2014), who demonstrated that a large amount of antigenic mass (250 ug) was needed for the animals to produce antibodies against *M. ovipneumoniae*. That portion of the study did not challenge the animals with *M. ovipneumoniae*, so protection in the face of a pathogen challenge could not be evaluated. Previous experiments in the study inoculated sheep with 50 ug of live *M. ovipneumoniae* without an adjuvant, and 50 ug of killed *M. ovipneumoniae* with an adjuvant. When challenged, the animals in both these groups had varying response in antibody production, and still experienced colonization of nasal passages by *M. ovipneumoniae*, similar to colonization by *M. hyopneumoniae* in pigs despite vaccination (Haesebrouck et al., 2004). Considering the inherent abilities of *Mycoplasma* spp. to evade detection by the host's immune system at mucosal surfaces and variation between strain types (Haesebrouck et al., 2004; Leal Zimmer et al., 2020; Razin et al., 1998), development of a vaccine that produces mucosal immunity to prevent colonization would be a logical yet challenging next step.

Antibiotic therapy

Mycoplasmas are inherently resistant to antibiotics that target the bacterial cell wall, including the β-lactams, glycopeptides, and phosphomycins. Mycoplasmas do not synthesize folic acid; consequently, sulfonamide antibiotics including trimethoprim sulfa are not an option for antibiotic therapy (Maunsell et al., 2011). Antibiotic classes active against *Mycoplasma* spp. include aminoglycosides, chloramphenicol, fluroquinolones, lincosamides, macrolides, and tetracyclines. A study evaluating the susceptibility of *M. ovipneumoniae* isolates from domestic sheep and domestic goats to nine different antibiotics *in vitro* showed all sheep and goat strains to be susceptible to enrofloxacin, ciprofloxacin, and tiamulin (Maksimović et al., 2020), with varying responses to other antibiotics depending on host species and strain type. This study used minimum inhibitory concentration, or MIC, to evaluate susceptibility. The MIC is the lowest concentration of antibiotic to have an inhibitory effect on a given microbe; MIC_{50} is the lowest concentration of antibiotic to inhibit

50% of the isolates, while MIC_{90} is the lowest concentration of antibiotic to inhibit 90% of the isolates.

In other livestock species, effectiveness of an antibiotic against respiratory pathogens is typically evaluated by response to treatment and resolution of clinical signs in naturally or experimentally infected animals. In a review of antimicrobial therapy in bovine respiratory disease in feedlots (DeDonder & Apley, 2015), respiratory disease cases were defined by elevated rectal temperature, clinical signs of respiratory disease including coughing, nasal discharge, and depression. Criteria for successful antimicrobial treatment, if defined, was based on need to re-treat the animal, and resolution of respiratory signs, depression, and rectal temperature. A study in Greece concluded that lincomycin was effective in treating *M. ovipneumoniae* in sheep based on response to treatment as indicated by the resolution of clinical signs (Skoufos et al., 2006) and absence of pathogenic organisms on culture of lung tissue at necropsy.

Use of antibiotics in minor species

The use of antibiotics in food-producing animals in the United States is regulated by the Center for Veterinary Medicine (CVM) within the Food and Drug Administration (FDA). Regulations are in place to reduce the risk of antibiotic residues in food-producing animals causing a harmful adverse effect on a human who consumes meat or milk products from that animal, and to reduce the development of antibiotic-resistant zoonotic bacteria. These specifications eliminate or severely restrict the use of chloramphenicol, enrofloxacin, and gentamicin in sheep in the United States. Use of other antibiotics in small ruminants, such as oxytetracycline, florfenicol, and lincomycin fall under the regulations of the Animal Medicinal Drug Use Clarification Act (AMDUCA). If a drug is not available with a label indicating use for a disease or condition in that species, a different drug labeled for use in that species must be selected. If there is not an effective drug labeled for use in that species, extra label drug use (Martin et al., 2018) requires the veterinarian to select an appropriate drug for the indication that is approved for use in other food animal species, and to set appropriate extended meat and milk withdrawal times.

Oxytetracycline

Tetracyclines are bacteriostatic and inhibit bacterial cell protein synthesis by reversibly binding to the 30S bacterial ribosomal subunit. This prevents binding of aminoacyl-tRNA in the mRNA ribosome complex. This does not kill the bacteria but renders them unable to grow or reproduce (Murray et al., 2020). Oxytetracycline has a broad spectrum of activity, including *Mycoplasma* spp. and is usually administered as an injection in livestock species because of increased bioavailability compared to oral administration. Long acting oxytetracycline formulations use a carrier to prolong the duration of the highly soluble oxytetracycline dihydrate salt at the site of injection and allow for slow release into the target tissues. Carriers used include polyethylene glycol, propylene glycol, 2-pyrrolidone (as used in LA-200 [Zoetis]), and povidone (Papich, 2015). Conventional oxytetracycline is labeled for use in cattle and pigs once daily for 2-3 doses, while the long-acting formula can be used in the same species at the same dose and interval or at a higher dose via a single treatment. When treated with a single dose of long acting oxytetracycline at 20 mg/kg body weight, cattle had therapeutic serum levels for 86.8 hours (Aguiar et al., 1987). Oxytetracycline is labeled for use to treat respiratory disease in cattle and pigs. When using a single dose of long acting oxytetracycline at 20 mg/kg in sheep, the Food Animal Residue Avoidance Databank (FARAD) recommends a withdrawal of 35 d for meat (Martin et al., 2018) (Table 1).

Florfenicol

Florfenicol is a bacteriostatic antibiotic which inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit. Florfenicol is highly lipophilic and able to treat intracellular bacteria (Papich, 2015). When comparing antibiotic susceptibility profiles of sheep and goat strains of *M. ovipneumoniae*, florfenicol has one of the highest MIC (Maksimović et al., 2020). It is available in combination with flunixin meglumine, a non-steroidal anti-inflammatory drug, and in this form is labeled for use to treat BRD associated with *M. bovis* in cattle. FARAD withdrawal recommendations for florfenicol in sheep are listed in Table 1.

Lincomycin

Lincomycin is a bacteriostatic lincosamide antibiotic, inhibiting bacterial protein synthesis by binding the 50S ribosomal subunit (Spížek & Řezanka, 2017). Lincomycin is labeled for use to treat mycoplasma pneumonia in pigs; dosage is 5 mg/kg once daily for 3-7 days as needed (Maes et al., 2020). A study used lincomycin to treat respiratory disease in sheep associated with *M. ovipneumoniae* at 5 mg/kg IM three times every 48 h (Skoufos et al., 2006). FARAD withdrawal recommendations are listed in Table 1.

Nasal flush

Biofilms are a characteristic adaptation of *Mycoplasma* spp to allow colonization of tissue surfaces (such as nasal passages and sinuses) and organs (within lung tissues and joint capsules). Biofilms formation creates a protective physical barrier to antibiotics and the host's immune system, allowing the bacteria to survive, persist and colonize in the host. In vitro studies of *M. pneumoniae* suggest that neither antibiotic therapy or innate immune response alone were capable of overcoming a mycoplasma infection involving a biofilm (Feng et al., 2021). Disruption of any biofilm formation in the nasal passages of livestock species, along with systemic antibiotic therapy, would plausibly be more effective in treating mycoplasma infections. Iodine and chlorhexidine based solutions are frequently used in humans and animals as flushes to disrupt biofilms in the oral cavity, in wound preparation, as a skin disinfectant before surgery, and are safe for use in exposed or healing tissues. Povidone and chlorhexidine were both successful in eliminating *Pseudomonas* biofilms *in vitro* (Hoekstra et al., 2017). Use of dilute antibiotics in topical application as a nasal flush has potential for disrupting *M. ovipneumoniae* biofilms in sheep. In an unpublished pilot study, systemic treatment of carrier ewes with injections of enrofloxacin, tildipirosin, gamithromycin, and tulathromycin failed to clear *M. ovipneumoniae*, however animals treated with a combination of systemic and intranasal enrofloxacin did clear *M. ovipneumoniae* (Besser et al, 2018 unpublished data). Unfortunately, use of enrofloxacin in sheep in this manner is considered illegal use of that antibiotic in food-producing species in the United States.

Chapter 2: Research

Materials and Methods

Animals, Treatments, and Experimental Design

All animals, treatments, and procedures were approved by the University of Idaho Animal Care and Use Committee (IACUC 2020-63).

Animals

This study was conducted in two timeframes, from December 2020- February 2021 (Cohort I), and from December 2021-February 2022 (Cohort II) at the University of Idaho (Moscow, ID). Thirty sheep were studied during Cohort I and thirty-six sheep were studied in Cohort II. All animals were sourced from the University of Idaho Sheep Center flock. Criteria for sheep study selection were: Suffolk breed lambs between 9-12 months of age; free from physical injury or illness; had not been treated with antibiotics for a period of 30 d prior to the start of the study; and all ewe lambs tested negative for pregnancy via BioPRYN (BioTracking, Moscow, ID) blood test at the time of selection. The thirty animals enrolled in Cohort I were chosen from available Suffolk ram and ewe lambs. The thirty-six animals enrolled in Cohort II were chosen from available Suffolk ewe lambs.

Once a pool of available animals meeting selection criteria was identified, the animals were screened for the presence of *M. ovipneumoniae* DNA via real time polymerase chain reaction (RT-PCR) using a deep nasal swab technique. In Cohort I, sixty available animals (thirty-four Suffolk ram lambs and twenty-six Suffolk ewe lambs) were swabbed two weeks prior to the start of the study, and swabs submitted to Washington State Animal Disease Diagnostic Laboratory (Pullman, WA) for analysis (Ziegler et al., 2014). Animals were categorized based on the cycle threshold (Ct) results: ‘Detected’ ($Ct \leq 36$), ‘Indeterminate’ ($Ct = 36\text{-}40$), or ‘Not Detected’ ($Ct \geq 40$) (Manlove et al., 2019). Thirty animals (twenty ram lambs and ten ewe lambs) were randomly selected from the pool of ‘Detected’ animals, and randomly assigned to treatment groups. To evenly distribute animals of each gender among

treatment groups, each treatment group in Cohort I included four ram lambs and two ewe lambs for a total of six animals per treatment group.

In Cohort II, sixty-two available Suffolk ewe lambs were nasal swabbed 2 wk prior to the start of the study and swabs analyzed via RT-PCR for *M. ovipneumoniae* at the University of Idaho using previously published methods (Manlove et al., 2019). Thirty-six animals were randomly selected from a pool of available 'Detected' animals and randomly assigned to treatment groups with a total of six animals per treatment group.

In both Cohort I and Cohort II, animals were housed in a pre-existing research barn facility on the University of Idaho campus. Animals were housed in groups of six, in corner pens separated from other groups by 2.9 meters. Pens had solid concrete walls on two sides, fenced on the remaining two sides with pipe corral, and concrete flooring bedded with straw. Each pen had a separate water source and feeder. Water was provided *ad libitum*. All animals were fed a total mixed ration consisting of alfalfa, grass hay, corn, and barley. Animals were fed twice daily and observed three times daily for any abnormal behavior including lethargy, cough, nasal or ocular discharge, diarrhea, and lameness. All animals were brought to the research barn for an acclimation period 1 wk prior to the start of the study.

Study Timeline

In both Cohort I and Cohort II of the study, treatments were initiated following the week-long acclimation period. Treatments started on a Monday (d 1 of treatment) and, if continued, were administered at 24- or 48-h intervals through Friday (d 5 of treatment), which was the conclusion of the treatment timeframe. Sampling occurred starting 6 d after the conclusion of the treatment timeframe (d 6 post-treatment) and followed the sampling timeline described below.

Treatment

In Cohort I, groups received treatment as follows: Group 1 (OXO): oxytetracycline (Liquamycin LA-200, Zoetis Animal Health, Parsippany, NJ) 20 mg/kg SQ one time only d 1; Group 2 (OXD): oxytetracycline 10 mg/kg IM once daily on Days 1-5 for a total of five doses; Group 3 (OXB): oxytetracycline 10 mg/kg IM once daily on Days 1-5 for a total of

five doses, in addition, a nasal flush consisting of betadine (2% mixed with 0.9% sodium chloride [Vetivex 0.9% Sodium Chloride, Dechra Pharmaceuticals, Overland Park, KS] to a 100ml volume) once daily on Days 1-5 for a total of five nasal flushes; Group 4 (OXC): oxytetracycline 10 mg/kg IM once daily on Days 1-5 for a total of five doses, in addition, a nasal flush of chlorhexidine (2% mixed with 0.9% sodium chloride to a 100mL volume) once daily on Days 1-5 for a total of five nasal flushes; Group 5 (POS): positive control, animals received no treatment.

In Cohort II, groups received treatment as follows: Group 1 (LIN): lincomycin (Lincomix 300, Zoetis Animal Health, Parsippany, NJ) 5 mg/kg IM every 48 hours (Days 1, 3, and 5) for a total of three doses; Group 2 (LIF): lincomycin 5 mg/kg IM every 48 hours (Days 1, 3, and 5) for a total of three doses, in addition, a nasal flush consisting of lincomycin 0.5 mg/kg (mixed with 0.9% sodium chloride to a volume of 100mL) once daily on Days 1-5 for a total of five nasal flushes; Group 3 (FLO): florfenicol (Nuflor, Merck Animal Health, Madison, NJ) 20 mg/kg IM every 48 hours (Days 1, 3, and 5) for a total of three doses; Group 4 (FLF): florfenicol 20 mg/kg IM every 48 hours (Days 1, 3, and 5) for a total of three doses, in addition, a nasal flush consisting of florfenicol 2 mg/kg (mixed with 0.9% sodium chloride to a volume of 100mL) once daily on Days 1-5 for a total of five nasal flushes; Group 5 (OXD): oxytetracycline 10 mg/kg IM once daily on Days 1-5 for a total of five doses; and Group 6 (POS): positive control, animals received no treatment.

Each animal was weighed on the first day of the acclimation period. That weight was used to calculate antibiotic and nasal flush dosages. All injections were administered using a 20-gauge, 1-inch hypodermic needle (Monoject Hypodermic Veterinary Needle, Cardinal Health, Dublin, OH) and either a 3cc or 6cc luer lock syringe (Monoject Syringe, Cardinal Health, Dublin, OH). Intramuscular injections were administered in the cervical muscle. Nasal flushes were administered with a large volume low pressure human sinus irrigation bottle (NeilMed Sinus Rinse, NeilMed Pharmaceuticals, Santa Rosa, CA). Animals were restrained using a halter to hold the head below the point of the shoulder with the nostrils angled down to facilitate drainage of the flush solution and reduce the likelihood of aspiration. Drug withdrawal for each antibiotic was requested from FARAD (Table 1).

Sample Collection

On d 6, 13, 20, and 27 post-treatment, all animals in both Cohort I and Cohort II of the study underwent a physical examination. Weight (kg), heart rate, respiratory rate, and temperature (℃) were recorded. Blood samples were obtained from each animal via jugular venipuncture. The blood samples were collected into a sterile, 10 mL draw glass blood collection tube with no additive (BD Vacutainer Blood Collection Tube, Becton Dickson Co., Franklin Lakes, NJ), and a sterile, 10 mL draw glass blood collection tube containing EDTA (BD Vacutainer Blood Collection Tube, Becton Dickson Co.). Blood in the EDTA tube was immediately inverted 15-20 times to ensure mixing and prevent clot formation, while blood in the tube with no additive was kept upright and allowed to clot. All blood samples were processed within 4 h of sample collection. A complete blood count (CBC) was run on each animal using 0.5 mL of EDTA blood (VETSCAN HM5 Hematology Analyzer, Zoetis Animal Health). The remaining EDTA blood was centrifuged at 500 x RPM for 5 min at room temperature; 1.0 mL of plasma was pipetted into a labeled microcentrifuge tube and stored at -20 ℃ for future analysis. The whole blood sample was centrifuged at 500 x RPM for 5 min at room temperature; 1.0 mL of serum was pipetted into a labeled microcentrifuge tube and stored at -20 ℃ for future analysis.

On d 7, 14, 21, and 28 post-treatment, all animals were nasal swabbed using a sterile, polyurethane sponge-tipped swab on a plastic shaft secured to a cap with a plastic transit tube (BD BBL CultureSwab EZ, Becton Dickson Co.). Swabs were labeled, kept in a cooler on ice and out of sunlight during transport, and stored within 1 h of collection at -20 ℃ until further analysis.

Nasal Swab Extraction

Swab tips were aseptically cut from the shaft and placed in a 1.5 mL microcentrifuge tube (Fisher Scientific, Pittsburgh, PA). Three hundred uL phosphate buffered saline (Fisher Scientific) was added and the sample was then pulse vortexed for 15 sec to agitate the nasal mucus from the swab tip into suspension. The resulting suspension was then pipetted into a new microcentrifuge tube, and DNA extracted from the sample using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD). The resulting sample was diluted in 100 uL of Buffer

AE and either proceeded immediately to PCR or stored at -20 ℃ for further analysis. All sample batches included two negative control samples (dry swabs), and two positive control samples (swabs dipped in 15 uL of *M. ovipneumoniae* reference strain Y98; ATCC 29419, ATCC, Manassas, VA) culture broth. The two positive and two negative control swabs were extracted with the study samples and included in the RT-PCR assay of each batch.

Nasal Swab RT-PCR Analysis

Following DNA extraction of the nasal swabs, RT-PCR was conducted using the Applied Biosystems 7500 System using previously described methods (Ziegler et al., 2014) with primers 226Fnew (5′-GGGGTGCGCAACATTAGTTAGTTGGTAG-3′) and LMR1 (59-GACTTCATCCTGCACTCTGT-39), and probe Movip 253P (596-FAM-TTAGCGGGGCCAAGAGGCTGTABHQ-1-3') (Manlove et al., 2019). Bovine serum albumin was added to the PCR mastermix to increase PCR amplification yields in the presence of PCR inhibitors such as dirt and organic material that might be present on the nasal swabs (Lekang et al., 2015). All samples were run in duplicate. A stock sample of *M. ovipneumoniae* genomic DNA from reference strain Y98 was used to create serial dilutions from 10^6 to 10^0 genomic copies. A test assay containing the serial dilutions was run to create a standard curve; the test assay also contained a known positive sample used as an internal control across sample batch assays, and nuclease-free water was used as a negative control. A standard curve, internal control, and negative control were used in all assays ran for samples from Cohort I of the study. An internal control and negative control were included in each assay for samples ran in Cohort II of the study.

Data Analysis

This was an experimental study conducting a controlled trial with a completely randomized block design, where the control group did not receive treatment or a placebo. All statistical analyses were performed with R (R Core Team, 2014) with lme4 (Bates, 2022) to produce a non-linear mixed effects model to evaluate the effect of treatments on Ct values, taking into account the year as a factor, and individual animal and sex as a random effect. The emmeans (Lenauth, 2022) package was used to compare least squares means of Ct

values between treatment and positive control groups overall and at each sample timepoint. The same methods were used to evaluate the effects of treatments on and compare least squares means between treatment and positive control groups of total white blood cell (WBC) count, total neutrophil count, total lymphocyte count, and total monocyte count. Data were visually assessed for normal distribution. Significance was determined at $P < 0.05$.

Results

Standard Curve qPCR

The efficiency of the PCR primers was calculated using the Ct results from the standard curve, repeated six times with serial dilutions from 10^6 to 10^0 genomic copies of *M. ovipneumoniae* reference strain Y98 as well as a known positive internal control and nuclease-free water as a negative control. The PCR primer efficiency for this assay was calculated at 82.0% (Figure 1).

Detection of M.ovi via PCR

Response to treatment was evaluated by comparing mean PCR Ct values of each treatment group to the Ct values of the POS group over time. Mean Ct values for POS were 25.3 overall (Table 2; Figure 2), and trended lower over time (Day 7=29.1, Day 14=26.0, Day 21=25.2, and Day 28=26.8; Tables 4-7; Figure 3).

OXO: Oxytetracycline 20 mg/kg SQ once

There was no difference in mean Ct values between POS and OXO overall ($P = 0.99$; Table 3), nor at any sample time point following treatment Day 7 ($P = 0.33$), Day 14 ($P = 0.98$), Day 21 ($P = 0.95$), Day 28 ($P = 0.83$; Tables 8-11). The mean Ct value for OXO was 25.0 overall (Table 2; Figure 2), and decreased over time indicating an increase in *M. ovipneumoniae* genomic material (Day 7=33.1, Day 14=26.8, Day 21= 23.9, and Day 28= 24.5; Tables 4-7; Figure 3).

OXD: Oxytetracycline 10 mg/kg IM once daily for five days

Mean Ct values were different between POS and OXD overall (*P* = 0.0027; Table 3, Figure 4). Mean Ct of OXD compared to POS was different on Day 7 (*P* = 0.0039), Day 14 (*P* = 0.0005), and Day 21 (*P* = 0.0077; Tables 8-10), but not at Day 28 (*P* = 0.5; Table 11). Mean Ct values for OXD were 31.4 overall (Table 2; Figure 2) and decreased over time indicating an increase in *M. ovipneumoniae* genomic material (Day 7=35.7, Day 14=32.6, Day 21= 30.6, and Day 28= 29.6; Tables 4-7; Figure 3).

OXB: Oxytetracycline 10mg/kg intramuscularly once daily for five days with a dilute betadine nasal flush once daily for five days

There was no difference in mean Ct values between POS and OXB overall (*P* = 0.97; Table 3), but there was a difference only on Day 7 (*P* = 0.0339). There was no significant difference at any other time points following treatment Day 14 (*P* = 0.73), Day 21 (*P* = 0.99), or Day 28 (*P* = 1; Tables 8-11). The mean Ct value for OXB was 26.4 overall (Table 2; Figure 2), and trended lower over time indicating an increase in *M. ovipneumoniae* genomic material (Day 7=35.1, Day 14=28.3, Day 21= 25.5, and Day 28= 26.8; Tables 4-7; Figure 3).

OXC: Oxytetracycline 10mg/kg intramuscularly once daily for five days with a dilute chlorhexidine nasal flush once daily for five days

There was no significant difference in mean Ct values between POS and OXC overall (*P* = 0.67; Table 3), nor at any sample time point following treatment Day 7 (*P* = 0.99), Day 14 (*P* = 0.75), Day 21 (*P* = 0.65), Day 28 (*P* = 0.96; Tables 8-11). The mean Ct value for OXC was 22.7 overall (Table 2; Figure 2), and trended lower over time indicating an increase in *M. ovipneumoniae* genomic material (Day 7=28.6, Day 14=23.7, Day 21= 22.6, and Day 28= 25.3; Tables 4-7; Figure 3)

LIN: Lincomycin 5mg/kg intramuscularly every 48 hours for three doses

There was no significant difference in mean Ct values between POS and LIN overall (*P* = 0.94; Table 3), nor at any sample time point following treatment Day 7 (*P* = 0.95), Day 14 (*P* = 0.97), Day 21 (*P* = 0.96), Day 28 (*P* = 0.98; Tables 8-11). The mean Ct value for

LIN was 26.8 overall (Table 2; Figure 2), and decreased over time indicating an increase in *M. ovipneumoniae* genomic material (Day 7=27.5, Day 14=27.0, Day 21= 26.5, and Day 28= 25.7; Tables 4-7; Figure 3).

LIF: Lincomycin 5mg/kg intramuscularly every 48 hours for three doses with dilute lincomycin nasal flush once daily for five days

There was no significant difference in mean Ct values between POS and LIF overall ($P = 0.80$; Table 3), nor at any sample time point following treatment Day 7 ($P = 0.86$), Day 14 ($P = 0.76$), Day 21 ($P = 0.84$), Day 28 ($P = 0.98$; Tables 8-11). The mean Ct value for LIF was 23.2 overall (Table 2; Figure 2), and trended lower over time indicating an increase in *M. ovipneumoniae* genomic material (Day 7=26.8, Day 14=23.7, Day 21= 23.3, and Day 28= 25.6; Tables 4-7; Figure 3).

FLO: Florfenicol 20mg/kg intramuscularly every 48 hours for three doses

There was no significant difference in mean Ct values between POS and FLO overall ($P = 0.91$; Table 3; Figure 2), nor at any sample time point following treatment Day 7 ($P = 0.22$), Day 14 ($P = 0.75$), Day 21 ($P = 0.95$), Day 28 ($P = 0.93$; Tables 8-11). The mean Ct value for FLO was 23.7 overall (Table 2), and trended lower over time indicating an increase in *M. ovipneumoniae* genomic material (Day 7=24.2, Day 14=23.7, Day 21= 24.0, and Day 28= 25.0; Tables 4-7; Figure 3).

FLF: Florfenicol 20mg/kg intramuscularly every 48 hours for three doses with dilute florfenicol nasal flush once daily for five days

There was no significant difference in mean Ct values between POS and FLF overall ($P = 1$; Table 3), nor at any sample time point following treatment Day 7 ($P = 0.14$), Day 14 ($P = 0.98$), Day 21 ($P = 0.99$), Day 28 ($P = 0.98$; Tables 8-11). The mean Ct value for FLF was 25.4 overall (Table 2; Figure 2), and trended higher over time indicating a decrease in *M. ovipneumoniae* genomic material (Day 7=23.7, Day 14=25.1, Day 21= 25.7, and Day 28= 25.6; Tables 4-7; Figure 3).

Leukogram

There was no significant difference in mean WBC count, mean neutrophil count, mean lymphocyte count, or mean monocyte count when comparing *P*-values between POS or any of the treatment groups (Tables 13, 15, 17, and 19). Multiple groups had mean cell counts that were higher than reported reference ranges for healthy adult sheep (Frye et al., 2022). In Table 12, overall mean total WBC count was increased in OXO at 12.63×10^9/L (reference interval 4.5-11.8 $\times 10^9$/L). In table 14, overall mean total lymphocyte count was increased in OXO at 10.61×10^9/L, OXB at 9.46×10^9/L, and FLF at 8.37×10^9/L (reference interval 1.5-8.3 $\times 10^9$/L).

Discussion

All antibiotics evaluated (oxytetracycline, florfenicol, and lincomycin) are bacteriostatic. Efficacy of bacteriostatic agents depends on the ability of the antimicrobial agent to suppress the bacteria long enough for the host immune system to respond to the pathogen, and for the host to recognize and respond to the presence of a pathogen (Murray et al., 2020). Daily administration of oxytetracycline injections (OXD) resulted in statistically significant changes in Ct values when compared to that for the positive control group. A single-dose therapy of antibiotic (OXO) likely did not achieve therapeutic levels of the antibiotic for a length of time to allow the host's immune system to mount a response to the presence of the bacteria. An *in vitro* study evaluating susceptibility of *M. ovipneumoniae* to various antibiotics found that while *M. ovipneumoniae* was susceptible to florfenicol and oxytetracycline, florfenicol had the highest MIC_{50} value and oxytetracycline the highest MIC_{90} value (Maksimović et al., 2020), indicating that high concentrations of each of these antibiotics was needed for an inhibitory effect on *M. ovipneumoniae*.

Daily flush with either betadine or chlorhexidine paired with daily oxytetracycline injections should work synergistically and be the most efficacious in disrupting any *M. ovipneumoniae* biofilm by physically removing bacteria from the animal's nasal passages. Betadine and chlorhexidine are bacteriocidal and bacteriostatic, respectively, with chlorhexidine being bacteriocidal at high concentrations but diminishing in efficacy in the

face of organic matter (Mcdonnell & Russell, 1999) such as nasal mucus. Increased serous nasal discharge was observed in the study animals receiving nasal flush treatments, regardless of flush material used.

Although topical application of lincomycin or florfenicol as a nasal flush was anticipated to improve clearance of *M. ovipneumoniae* when in combination with systemic antibiotic use, both of these antibiotics are bacteriostatic and were unable to decrease bacterial presence through a local application. In fact, LIF and FLF were among the least efficacious treatments, performing close to the positive control groups receiving no treatments. Topical application of these antibiotics likely caused local irritation or dysfunction to the cilia, resulting in little to no disruption of the *M. ovipneumoniae* biofilm. Substances which cause irritation of nasal mucosa cause increased production of nasal mucous, which dilutes the substance and facilitates clearance of the irritant but causes the material to be removed faster than it has time to act locally in the nasal passages (Gizurarson, 2015). Both lincomycin and florfenicol have basic pH of 4 and 5, respectively (Chen et al., 2019; Czarniak et al., 2016), which would cause dehydration of nasal cilia, disrupt the ciliary beat, and reduce mucociliary clearance (Bustamante-Marin & Ostrowski, 2017).

Animals receiving oxytetracycline, florfenicol, or lincomycin in our study were receiving label doses for the given route and dosing interval; proportionately increasing dosages *in vivo* may result in side effects or be unfeasible because of extended meat withdrawal times. An alternative to increasing the dosage would be to change the route of administration, such as in the case of OXD where the oxytetracycline could instead be given intravenously daily to change pharmacokinetics with regards to distribution rate and tissue compartments.

PCR Ct values are specific for genomic material; in this case, it is possible that animals with detected *M. ovipneumoniae* genomic material could have nonviable genomic material present in their nasal passages instead of live *M. ovipneumoniae* organisms (Ackerman et al., 2019). Variation in Ct values can occur with variations in nasal swab sampling technique; though all individuals conducting sampling were trained to use a similar technique and depth of swabbing, four individuals collected the samples during the study. There is also potential for variation between *M. ovipneumoniae* organism load between

swabs when multiple swabs are taken from an individual at the same time, and presence or absence of *M. ovipneumoniae* organisms in the upper respiratory tract (nasal passages and nasopharynx) compared to the lower airway (trachea, bronchi, and lungs) (Yang et al., 2014).

In mammals, *Mycoplasma spp.* have been demonstrated to modulate the host immune system by suppressing the inflammatory response (Borchsenius et al., 2020). Host adaptation, virulence, and response to antimicrobial therapy is likely influenced by *M. ovipneumoniae* strain type. In a study by Kamath et al, 187 genetically different strains of *M. ovipneumoniae* were identified in 207 domestic sheep, with 77% of individual sheep having unique strains (Kamath et al., 2019). There does not appear to be a maximum number of strains with which an individual domestic sheep can be simultaneously infected (Harvey et al., 2007). In bighorn sheep and domestic sheep, immunity to *M. ovipneumoniae* appears to be strain specific and not long lived (Cassirer et al., 2017; Ziegler et al., 2014). The sheep in our study did not have *M. ovipneumoniae* strain typed, though previous studies suggest the strong likelihood of multiple stains within individual study animals and variations related to strain type influencing short- and long-term treatment outcomes (Harvey et al., 2007; Lonas et al., 1991; Maksimović et al., 2020). Over the course of the study, all treatment cohorts were observed to have Ct values trending lower over time, indicating an increase in *M. ovipneumoniae* genomic material, presumably due to increased growth of *M. ovipneumoniae* in these animals over time, with more bacteria present as time since treatment increased. The presence, and increase, of bacteria could be due to incomplete response to treatment due to antibiotic susceptibility differences in strain type; re-colonization of an individual's nasal passages with *M. ovipneumoniae* which remained present in the cornual sinus or lower airway (Besser et al., 2017); or re-infection. Oxytetracycline, a bacteriostatic antibiotic, administered daily allowed animals to mount an immune response to *M. ovipneumoniae* but failed to completely eliminate the bacteria, and animals receiving this treatment showed an increase in bacterial presence over time once the bacteriostatic agent was no longer present systemically (Aktas & Yarsan, 2017; Craigmill & Craigmill, n.d.; Sun et al., 2002). Prolonged exposure to pen-mates that continued to be positive for *M. ovipneumoniae* could have resulted in animals that initially responded to therapy being re-infected with the same or different strains.

Overall mean lymphocyte counts greater than the reference range for healthy sheep were observed in multiple groups. Although the sheep in our study underwent an acclimation period prior to initiating treatment and sampling, they were not habituated to being handled or having humans in close proximity on a frequent basis. These animals were likely experiencing a physiologic or 'shift' lymphocytosis, which occurs transiently in multiple animal species when the 'fight or flight' response is initiated; endogenous catecholamine release causes release of lymphocytes to the circulating pool and decreased adherence of lymphocytes to the endothelium (Stockham & Scott, 2008). No significant differences were anticipated in total WBC count or other parameters when comparing treated and positive control groups, in part because they were enrolled in the study based on PCR results and not because they exhibited clinical signs of respiratory disease.

Conclusion

The goal of this study was to find an antibiotic therapy that was effective in clearing *M. ovipneumoniae* from lambs, which could then be recommended to producers to use to treat *M. ovipneumoniae* positive animals in their flock. Of proposed treatment options, antibiotics and topical nasal flushes were selected based on ease of administration, cost, and ability to be used in food-producing animals in the United States. To be considered effective, treatment needed to clear *M. ovipneumoniae* from the animal, and provide reasonable duration of effect.

In our study, sheep were enrolled once they were identified as positive for natural infection with *M. ovipneumoniae* via PCR. These sheep could be considered 'asymptomatic' as they did not exhibit clinical signs of respiratory disease including coughing, nasal discharge, depression, elevated rectal temperature, or being off feed. Response to treatment and efficacy of the antibiotic therapy was based on evaluating mean PCR Ct values for *M. ovipneumoniae* on nasal swabs of each group, with the desired outcome specifically a negative PCR test for *M. ovipneumoniae* (Ct >40) in most of the animals.

Although daily oxytetracycline injections increased Ct values when compared to that for the positive control, it did not induce complete response to treatment by achieving Ct

values ≥40 in the majority of animals, nor were the Ct values maintained over time. Decreasing Ct values over the course of the study (i.e. increasing presence of *M. ovipneumoniae* genomic material in nasal samples) were possibly related to the animals being re-infected with *M. ovipneumoniae* because of prolonged exposure to positive individuals that did not respond to treatment. Failure to respond to treatment can likely be attributed to variations in *M. ovipneumoniae* strain type susceptibility to each antibiotic and antibiotic dosage. Complete response to treatment in the majority of the study animals is needed in order to recommend the antibiotic be used to treat animals with clinical disease. Further studies identifying the strain types present in a study cohort, and testing and segregating animals after treatment to prevent re-infection from a positive cohort is warranted.

Tables

Table 1. Food Animal Residue Avoidance Databank recommended meat withdrawal intervals for antibiotics administered to sheep in this study

Drug	Dose	# doses	Route	Frequency	Withdrawal
Oxytetracycline 200mg/mL	20 mg/kg	1	SQ	Once	35 day meat
Oxytetracycline 200mg/mL	10 mg/kg	5	IM	Q 24 hrs	56 day meat
Lincomycin 100mg/mL	5 mg/kg	3	IM	Q 48 hrs	19 day meat
Lincomycin 100mg/mL	0.5 mg/kg	5	Intranasal	Q 24 hrs	26 day meat (paired w/Lincomycin injectable treatment)
Florfenicol 300mg/mL	20 mg/kg	3	IM	Q 48 hrs	82 day meat
Florfenicol 300mg/mL	2 mg/kg	5	Intranasal	Q 24 hrs	110 day meat (paired w/Florfenicol injectable treatment)

Table 2. Least square means of overall Ct values

Treatment	Least square mean Ct	SE
POS	25.3	1.20
FLO	23.7	1.76
FLF	25.4	1.76
LIN	26.8	1.76
LIF	23.2	1.76
OXB	26.4	1.68
OXC	22.7	1.68
OXD	31.4	1.20
OXO	25.0	1.68

Confidence level used: 0.95

Table 3. Contrast of overall positive control vs treatment group Ct values via least square means

Contrast	estimate	SE	*P*-value
FLO – POS	-1.620	2.08	0.9193
FLF – POS	0.115	2.08	1.0000
LIN – POS	1.434	2.08	0.9449
LIF – POS	-2.178	2.08	0.8070
OXB – POS	1.076	2.08	0.9783
OXC – POS	-2.658	2.08	0.6749
OXD – POS	6.097	1.67	0.0027
OXO – POS	-0.374	2.08	0.9994

Table 4. Least square means of Ct values on Sample Day 7

Sample Day 7		
Treatment	**Least square mean Ct**	**SE**
POS	29.1	1.33
FLO	24.2	1.95
FLF	23.7	1.95
LIN	27.5	1.95
LIF	26.8	1.95
OXB	35.1	1.88
OXC	28.6	1.88
OXD	35.7	1.33
OXO	33.1	1.88

Confidence level used: 0.95

Table 5. Least square means of Ct values on Sample Day 14

Sample Day 14		
Treatment	**Least square mean Ct**	**SE**
POS	26.0	1.15
FLO	23.7	1.70
FLF	25.1	1.70
LIN	27.0	1.70
LIF	23.7	1.70
OXB	28.3	1.62
OXC	23.7	1.62
OXD	32.6	1.15
OXO	26.8	1.62

Confidence level used: 0.95

Table 6. Least square means of Ct values on Sample Day 21

Sample Day 21		
Treatment	**Least square mean Ct**	**SE**
POS	25.2	1.15
FLO	24.0	1.70
FLF	25.7	1.70
LIN	26.5	1.70
LIF	23.3	1.70
OXB	25.5	1.62
OXC	22.6	1.62
OXD	30.6	1.15
OXO	23.9	1.62

Confidence level used: 0.95

Table 7. Least square means of Ct values on Sample Day 28

Sample Day 28		
Treatment	**Least square mean Ct**	**SE**
POS	26.8	1.33
FLO	25.0	1.95
FLF	25.6	1.95
LIN	25.7	1.95
LIF	25.6	1.95
OXB	26.8	1.88
OXC	25.3	1.88
OXD	29.6	1.33
OXO	24.5	1.88

Confidence level used: 0.95

Table 8. Contrast of positive control vs treatment group Ct values via least square means on Sample Day 7

Sample Day 7			
Contrast	**estimate**	**SE**	***P*-value**
FLO – POS	-4.7698	2.33	0.2213
FLF - POS	-5.2261	2.33	0.1477
LIN - POS	-1.4710	2.33	0.9581
LIF - POS	-2.1259	2.33	0.8689
OXB - POS	6.5939	2.33	0.0339
OXC - POS	-0.4127	2.33	0.9995
OXD - POS	6.5993	1.87	0.0039
OXO - POS	4.1426	2.33	0.3569

Table 9. Contrast of positive control vs treatment group Ct values via least square means on Sample Day 14

Sample Day 14			
Contrast	**estimate**	**SE**	***P*-value**
FLO - POS	-2.3094	2.02	0.7534
FLF - POS	-0.9314	2.02	0.9851
LIN - POS	1.0621	2.02	0.9769
LIF - POS	-2.2831	2.02	0.7607
OXB - POS	2.3655	2.02	0.7374
OXC - POS	-2.3173	2.02	0.7512
OXD – POS	6.6642	1.62	0.0005
OXO - POS	0.8227	2.02	0.9902

Table 10. Contrast of positive control vs treatment group Ct values via least square means on Sample Day 21

Sample Day 21			
Contrast	**estimate**	**SE**	***P*-value**
FLO - POS	-1.2833	2.02	0.9572
FLF - POS	0.4340	2.02	0.9989
LIN - POS	1.2181	2.02	0.9638
LIF - POS	-1.9435	2.02	0.8469
OXB - POS	0.1883	2.02	0.9999
OXC - POS	-2.6509	2.02	0.6511
OXD - POS	5.4087	1.62	0.0077
OXO - POS	-1.3236	2.02	0.9527

Table 11. Contrast of positive control vs treatment group Ct values via least square means on Sample Day 28

Sample Day 28			
Contrast	**estimate**	**SE**	***P*-value**
FLO - POS	-1.6915	2.33	0.9347
FLF - POS	-1.1301	2.33	0.9824
LIN - POS	-1.0030	2.33	0.9882
LIF - POS	-1.1072	2.33	0.9836
OXB - POS	0.0624	2.33	1.0000
OXC - POS	-1.4135	2.33	0.9632
OXD - POS	2.8329	1.87	0.5234
OXO - POS	-2.2964	2.33	0.8361

Table 12. Least square means of overall WBC count

Treatment	**Mean WBC (x 10^9/L)**	**SE**
POS	10.59	0.894
FLO	10.62	1.315
FLF	10.04	1.315
LIN	8.21	1.315
LIF	7.69	1.315
OXB	11.08	1.256
OXC	9.57	1.256
OXD	10.54	0.905
OXO	12.63	1.256

Confidence level used: 0.95

Table 13. Contrast of overall positive control vs treatment group WBC count

Contrast	**estimate**	**SE**	***P*-value**
FLO – POS	0.0301	1.55	1.0000
FLF – POS	-0.5500	1.55	0.9940
LIN – POS	-2.3744	1.55	0.5133
LIF – POS	-2.8996	1.55	0.3113
OXB – POS	0.4880	1.55	0.9960
OXC – POS	-1.0229	1.55	0.9523
OXD – POS	-0.0525	1.26	1.0000
OXO – POS	2.0443	1.55	0.6504

Table 14. Least square means of overall lymphocyte count

Treatment	Mean Lymphocytes (x 10^9/L)	SE
POS	8.28	0.737
FLO	8.07	1.088
FLF	8.37	1.088
LIN	6.47	1.088
LIF	6.78	1.088
OXB	9.46	1.036
OXC	8.02	1.036
OXD	8.07	0.745
OXO	10.61	1.036

Confidence level used: 0.95

Table 15. Contrast of overall positive control vs treatment group lymphocyte count

Contrast	estimate	SE	*P*-value
FLO – POS	-0.2085	1.28	0.9996
FLF – POS	0.0971	1.28	1.0000
LIN – POS	-1.8095	1.28	0.5889
LIF – POS	-1.5000	1.28	0.7391
OXB – POS	1.1840	1.28	0.8647
OXC – POS	-0.2555	1.28	0.9992
OXD – POS	-0.2111	1.03	0.9991
OXO – POS	2.3282	1.28	0.3388

Table 16. Least square means of overall neutrophil count

Treatment	Mean Neutrophils (x 10^9/L)	SE
POS	2.260	0.327
FLO	2.498	0.478
FLF	1.616	0.478
LIN	1.703	0.478
LIF	0.876	0.478
OXB	1.562	0.460
OXC	1.497	0.460
OXD	2.425	0.332
OXO	1.967	0.460

Confidence level used: 0.95

Table 17. Contrast of overall positive control vs treatment group neutrophil count

Contrast	estimate	SE	*P*-value
FLO – POS	0.238	0.568	0.9893
FLF – POS	-0.643	0.568	0.7600
LIN – POS	-0.556	0.568	0.8393
LIF – POS	-1.384	0.568	0.0955
OXB – POS	-0.698	0.568	0.7035
OXC – POS	-0.763	0.568	0.6317
OXD – POS	0.165	0.461	0.9938
OXO – POS	-0.293	0.568	0.9785

Table 18. Least square means of overall monocyte count

Treatment	Mean Monocytes (x 10^9/L)	SE
POS	0.0530	0.00447
FLO	0.0525	0.00658
FLF	0.0497	0.00658
LIN	0.0394	0.00658
LIF	0.0377	0.00658
OXB	0.0557	0.00629
OXC	0.0466	0.00629
OXD	0.0520	0.00453
OXO	0.0614	0.00629

Confidence level used: 0.95

Table 19. Contrast of overall positive control vs treatment group monocyte count

Contrast	estimate	SE	*P*-value
FLO - POS	-0.000481	0.00777	1.0000
FLF - POS	-0.003304	0.00777	0.9888
LIN - POS	-0.013616	0.00777	0.3748
LIF - POS	-0.015285	0.00777	0.2607
OXB - POS	0.002685	0.00777	0.9945
OXC - POS	-0.006384	0.00777	0.9048
OXD - POS	-0.001040	0.00629	0.9996
OXO - POS	0.008407	0.00777	0.7885

Figures

Figure 1. Correlation between Ct values and genome copy numbers for *M. ovipneumoniae* reference strain Y98

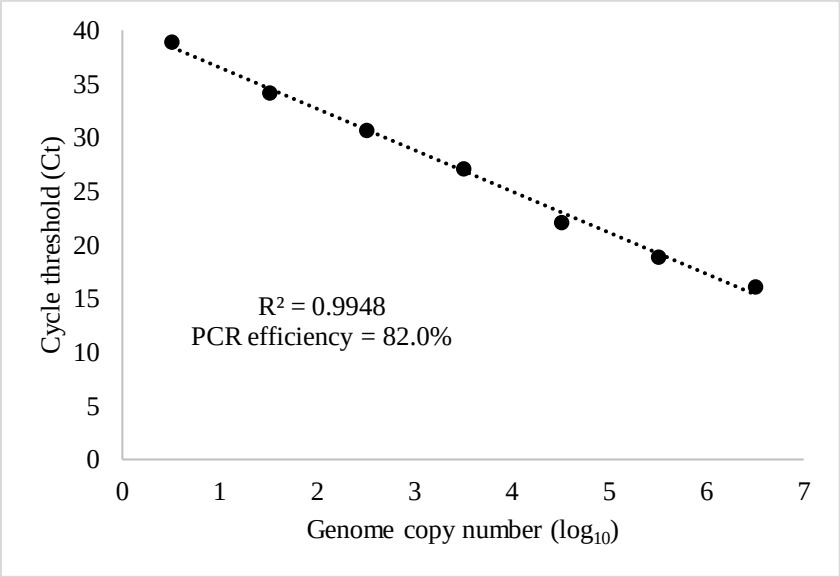


Figure 2. Mean Ct values of treatment groups overall

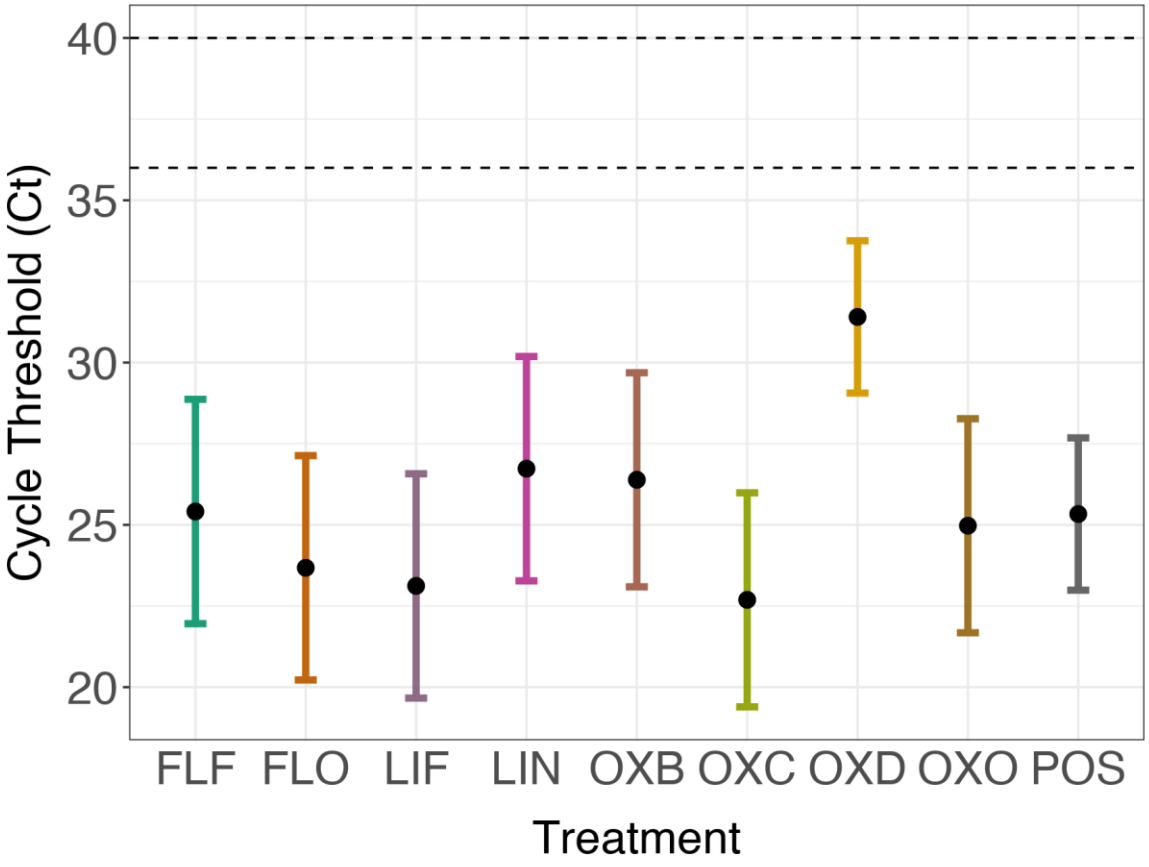


Figure 3. Mean Ct values of treatment groups on each sample date

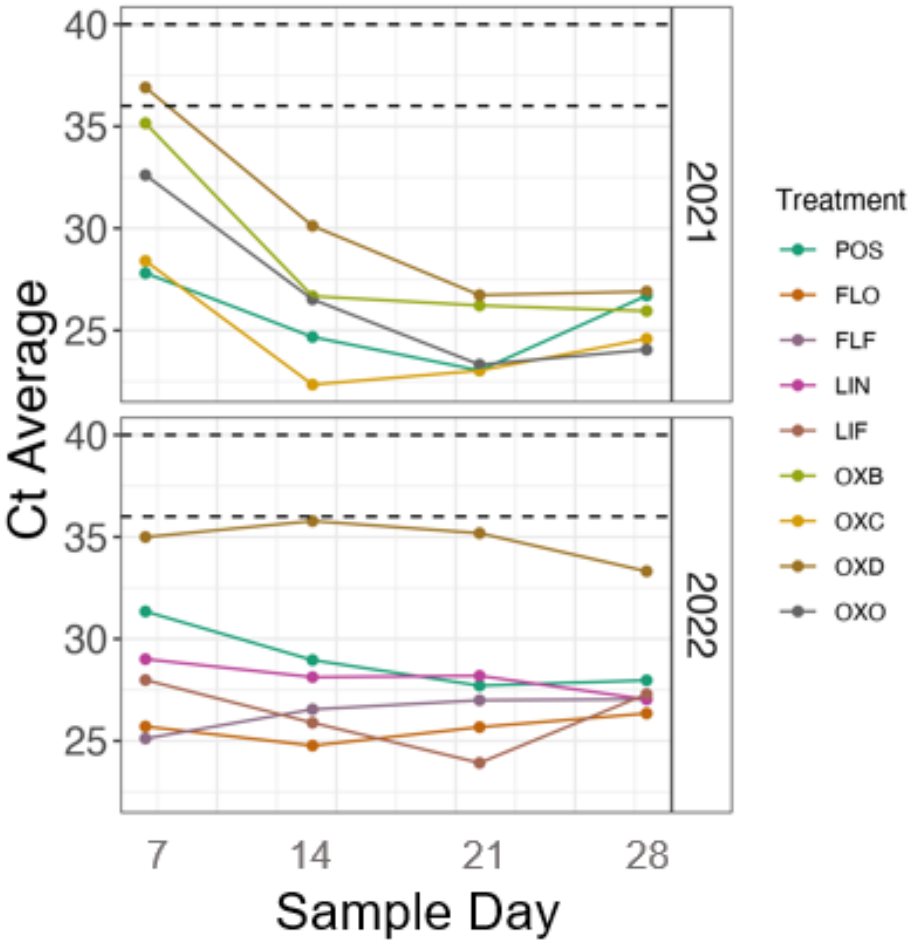
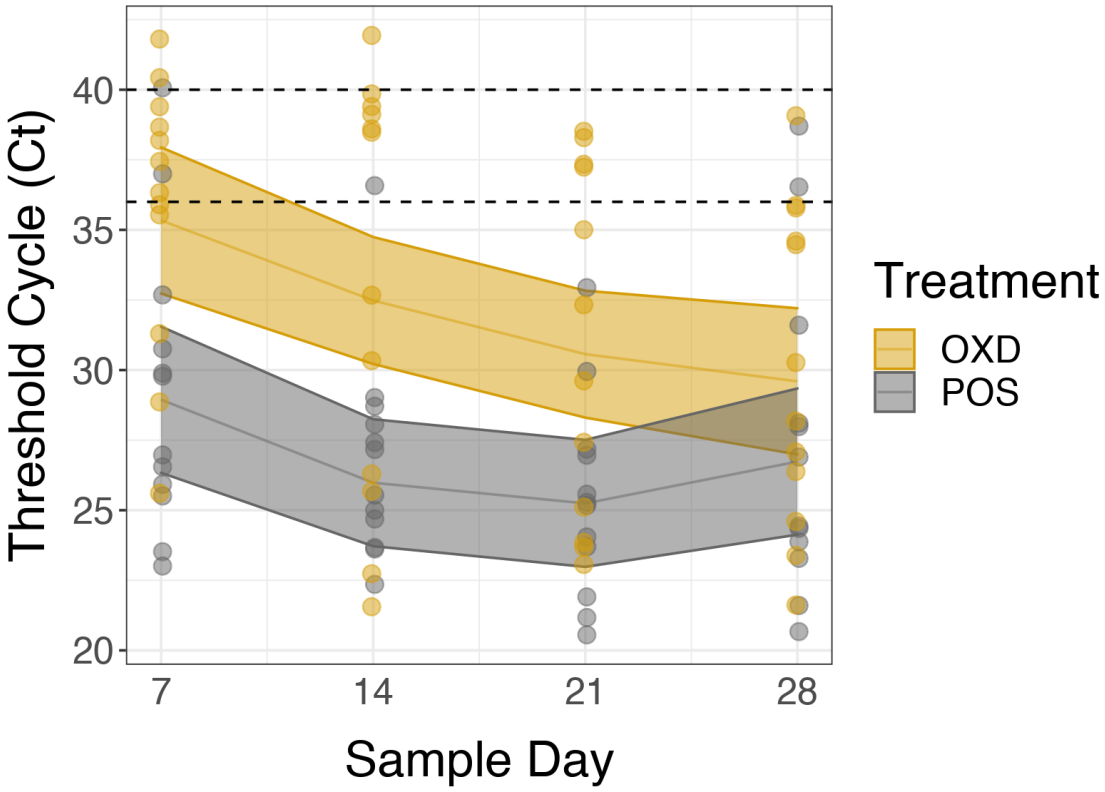


Figure 4. Predicted mean Ct values for POS and OXD groups over time. Dots represent actual data points for each group (POS n=12 and OXD n=12)



Appendix: Animal Care and Use Committee Approval Letter

University of Idaho
Office of Research Assurances
Institutional Animal Care and Use Committee
875 Perimeter Drive, MS 3010
Moscow, ID 83844-3010
Phone: 208-885-6162
Fax: 208-885-6014
Email: iacuc@uidaho.edu

Date: December 21, 2021

To: Denise Konetchy

From: University of Idaho Institutional Animal Care and Use Committee

Re: Protocol IACUC-2020-63 *Use of systemic Oxytetracycline with or without topical nasal treatments to clear Mycoplasma ovipneumoniae (Movi) from domestic lambs*

Your personnel amendment request, 015764, submitted on 12/10/2021 02:43:16 PM PST to the animal care and use protocol listed above was administratively reviewed and approved by the Institutional Animal Care and Use Committee on 12/21/2021.

The original approval date for this protocol was: 11/05/2020
This protocol approval will remain in effect until: 11/15/2022
The protocol may be continued by annual updates until: 11/04/2023

Currently approved internal personnel on this protocol are: Arnett, Sarah; Bennett, Joyce; Chapleski, Anna, Bachelor of Science Animal & Vet Sci; Christensen, Lauren; Elmore, Kylee; Goertzen, Tara, Animal Science; Heimbuch, Mikayla; Konetchy, Denise; Larson, Mallery; McGuire, Mark A.; Myers, Cheyanne; Peterson, Sarah; Vinci, Dino, B.S.; Ware, Maria; Weinstein, Kyleigh; Woods, Julia

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Janet Rachlow, IACUC Chair

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