

THE PATHOGENESIS OF PERSISTENCE: THE EFFECTS OF
DELIVERY SITE AND BIOFILM FORMATION
BY *MYCOPLASMA OVIPNEUMONIAE*

by

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DEDICATION

To Leif and Adalynn, may you both continue to explore the world, seek out the truth, and challenge your assumptions.

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TABLE OF CONTENTS

1. INTRODUCTION	1
<i>Mycoplasma ovipneumoniae</i>	1
Respiratory Immune Response	2
Physical Barriers	2
Innate Immune Mechanisms	2
Adaptive Immune Mechanisms	3
Immune Evasion	3
Biofilm Formation	3
Management of <i>M. ovipneumoniae</i> Infection in Sheep	4
Research Objectives and Hypotheses	5
References.....	7
2. EXPERIMENTAL INFECTION OF SPECIFIC PATHOGEN FREE DOMESTIC LAMBS WITH <i>MYCOPLASMA OVIPNEUMONIAE</i> CAUSES ASYMPTOMATIC COLONIZATION OF THE UPPER AIRWAYS THAT IS RESISTANT TO ANTIBIOTIC TREATMENT.....	13
Contribution of Authors and Co-Authors	13
Manuscript Information	15
Abstract	16
Introduction.....	17
Methods.....	19
Animals and husbandry.....	19
Health Monitoring.....	20
Experimental infection with <i>Mycoplasma ovipneumoniae</i>	21
Antibiotic Treatment	23
<i>M. ovipneumoniae</i> isolation and culture	23
Antibiotic broth microdilution assay	24
Collection and analysis of bronchoalveolar lavage fluids	24
Detection of <i>M. ovipneumoniae</i> infection.....	25
<i>M. ovipneumoniae</i> serology	25
Statistical analyses	26
Results.....	26
Supervised lambing and motherless rearing successfully prevented colonization of domestic lambs with <i>M. ovipneumoniae</i> and <i>Pasteurellaceae</i>	26
Application of nasal wash fluids from <i>M. ovipneumoniae</i> -infected lambs led to successful colonization of specific-pathogen free lambs and induces <i>M. ovipneumoniae</i> -specific serum antibodies.	27
<i>M. ovipneumoniae</i> infection in domestic SPF lambs did not cause overt respiratory disease.	29

TABLE OF CONTENTS CONTINUED

High dose treatment with the macrolide antibiotic gamithromycin failed to eliminate <i>M. ovipneumoniae</i> colonization.....	31
Discussion	35
Acknowledgements.....	41
Author Contributions	42
References.....	42
3. PATHOGEN DELIVERY ROUTE IMPACTS DISEASE SEVERITY IN EXPERIMENTAL CO-INFECTION OF SHEEP WITH <i>MYCOPLASMA OVIPNEIMONIAE</i> AND <i>MANNHEIMIA HAEMOLYTICA</i>	48
Contribution of Authors and Co-Authors	48
Manuscript Information	50
Abstract.....	51
Keywords	52
Introduction.....	52
Materials and Methods.....	54
Animals and husbandry conditions	54
Preparation of inocula	54
Experimental design and sample collection.....	55
Assessment of clinical signs and other animal characteristics.....	57
Evaluation of pathological and histopathological changes	57
<i>M. ovipneumoniae</i> serology	58
Detection of <i>M. ovipneumoniae</i> and Pasteurellaceae	58
Analysis of cytokine and inflammatory marker gene expression.....	59
Statistical analyses	60
Percent weight gain.....	61
Body temperature.....	62
Results.....	62
Increased pathogen load in the lower respiratory tract following lower-respiratory tract inoculation of lambs with <i>Mycoplasma ovipneumoniae</i>	62
Lower respiratory tract inoculation of lambs with <i>M. ovipneumoniae</i> -positive nasal wash fluid leads to more severe clinical signs and reduced weight loss.	64
Lower respiratory tract inoculation of lambs with <i>M. ovipneumoniae</i> was associated with gross and microscopic lung pathology.....	69
<i>M. ovipneumoniae</i> infection induced significantly increased IFN- γ gene expression in the blood and lungs.	73

TABLE OF CONTENTS CONTINUED

Inoculation of specific pathogen free domestic lambs with ceftiofur-treated nasal wash fluid resulted in co-infection with <i>M.</i> <i>ovipneumoniae</i> and <i>Pasteurellaceae</i> .	76
Discussion	78
Declarations	83
Ethics approval	83
Consent for publication	84
Competing interests	84
Funding	84
Authors' contributions	84
Acknowledgements	84
Availability of data and materials	85
Supplementary Information	85
References	88
4. INNOVATIVE METHODOLOGY FOR ANTIMICROBIAL SUSCEPTIBILITY DETERMINATION IN <i>MYCOPLASMA</i> BIOFILMS	94
Contribution of Authors and Co-Authors	94
Manuscript Information	96
Abstract	97
Keywords	98
1. Introduction	98
2. Materials and Methods	102
2.1 Growth Media	102
2.2 Bacterial isolates	102
2.3 Culture Methods	103
2.4 Biofilm Image Analysis	103
2.5 Acid Production Assay	104
2.6 Quantification of Cells with Flow Cytometry	104
2.7 Biofilm Disruption	105
2.8 Imaging Cytometry Analysis	106
2.9 Small Particle Analysis with Flow Cytometry	106
2.10 Live Dead Percentage Quantification	106
2.11 Disruption Survival Determination	108
2.12 Cell Adhesion Rate for Newly Adhered Biofilms	108
2.13 Broth Microdilutions	109
2.14 LD-AST Assays for Planktonic <i>Mycoplasma</i> spp., Newly Adhered and Mature Biofilm	109
2.15 Statistical Analysis	110
3. Results	113
3.1 Biofilm Maturity Determination	113

TABLE OF CONTENTS CONTINUED

3.2 Acid Production	116
3.3 Particle Size Standardization	117
3.4 Small Particle Analysis	120
3.5 Disaggregation Method Survival	121
3.6 Cell Adhesion Rate for Newly Adhered Biofilms.....	122
3.7 Antimicrobial Susceptibility Testing.....	123
3.8 Statistical Diagnostics Assessment	125
4. Discussion	126
Supplementary Materials	133
Author Contributions	134
Funding	135
Data Availability Statement	135
Acknowledgments.....	135
Conflicts of Interest.....	135
References.....	135
5. MECHANISMS OF BIOFILM FORMATION AND ANTIBIOTIC TOLERANCE IN <i>MYCOPLASMA OVIPNEUMONIAE</i>	147
Contribution of Authors and Co-Authors	147
Manuscript Information	149
Abstract	150
Introduction.....	152
Methods.....	154
Bacterial Strains	154
Quantification of Cells with Flow Cytometry	154
Culture Methods.....	155
Biofilm Staining.....	156
Crystal Violet	156
DNA and EPM Staining.....	156
Confocal Microscopy.....	156
Biofilm Image Analysis	157
Colony Morphology.....	157
Biofilm Disaggregation.....	157
Small Particle Analysis with Flow Cytometry.....	158
Hydrogen Peroxide Production.....	158
Broth Microdilutions.....	159
Live Dead Antibiotic Susceptibility Testing (LD-AST)	159
Biofilm Confluence After Antibiotic Exposure	160
<i>M. ovipneumoniae</i> Whole Genome Sequencing and Genome Assembly.....	160
Hybrid Assembly	160

TABLE OF CONTENTS CONTINUED

Genome analysis	161
Protein sequence comparisons	161
Statistical Analysis	162
Results.....	164
<i>Mycoplasma ovipneumoniae</i> forms robust biofilms <i>in vitro</i>	164
Colony Morphology.....	169
Hydrogen Peroxide Production.....	169
<i>M. ovipneumoniae</i> Biofilm formation is associated with increased antibiotic tolerance.....	170
Antibiotic exposure can lead to increased growth of <i>M.</i> <i>ovipneumoniae</i> biofilms.	173
High quality assembled genomes show strain-level diversity	174
Adhesins and Nucleases show differing levels of conservation	175
Discussion	177
References.....	182
6. CONCLUSIONS AND FUTURE DIRECTIONS.....	191
Future Directions	193
Using <i>in vitro</i> biofilm formation to study immune evasion.....	193
Determining sites of <i>in vivo</i> biofilm formation.....	194
Further investigation into adhesins and nucleases in biofilm formation.....	194
Final Remarks	195
References.....	195
7. TRANSFER AND PERSISTENCE OF BOVINE IMMUNOGLOBULINS IN LAMBS FED A COLOSTRUM REPLACER	198
Contribution of Authors and Co-Authors	198
Manuscript Information	200
8. PATHOPHYSIOLOGY OF INFLUENZA D VIRUS INFECTION IN SPECIFIC-PATHOGEN-FREE LAMBS WITH OR WITHOUT PRIOR <i>MYCOPLASMA OVIPNEIMONIAE</i> EXPOSURE	207
Contribution of Authors and Co-Authors	207
Manuscript Information	209
CUMULATIVE REFERENCES CITED.....	230

LIST OF TABLES

Table	Page
1. Table 1. Parameters for lamb health scoring during challenge experiment.	21
2. Table 2. Characteristics of experimental animals	22
3. Table 3. Characteristics of experimental animals	56
4. Table 4. Scoring criteria for histopathological assessment of lung tissues, based on Passmore 2018, Respiratory Research [119]	58
5. Table 5. Primer sequences.....	59
6. Table 6. Weight gain model	66
7. Table 7. Health score model.....	67
8. Table 8. Body temperature model.....	68
9. Table 9. Histopathological scoring of lungs from sheep infected with PBS (control) or with <i>M. ovipneumoniae</i> containing nasal-wash fluids through inoculation of the upper (URT) or lower respiratory tract (LRT)	73
10. Table 10: Scoring rubric for lamb health, based on Johnson et al. [24]	85
11. Table 11. Characteristics of bovine <i>Mycoplasma</i> isolates used in this study.....	103
12. Table 12. The peak maturity metrics observed over 6 days of growth.	115
13. Table 13. $MBC_{\geq 5\%}$ concentration in $\mu\text{g/mL}$ by organism state (planktonic, newly adhered biofilms, mature biofilm).....	126
14. Table 14 Characteristics of <i>Mycoplasma ovipneumoniae</i> strains used in this study.	154
15. Table 15: The determined maturity metrics for <i>M. ovipneumoniae</i> biofilms.	164
16. Table 16. <i>Mycoplasma ovipneumoniae</i> $MBC_{\geq 5\%}$ values for each strain.....	173
17. Table 17. Genome quality metrics calculated with CheckM	175
18. Table 18. Genome Characteristics, after annotating with PGAP	175

LIST OF FIGURES

Figure	Page
1. Figure 1. <i>Mycoplasma ovipneumoniae</i> commonly infects domestic and bighorn sheep but also affects various other hosts with differing levels of disease severity.....	1
2. Figure 2. The Biofilm Life Cycle.	4
3. Figure 3. <i>Mycoplasma ovipneumoniae</i> colonization and antibody responses in experimentally infected lambs.	28
4. Figure 4. Clinical scoring of lambs following experimental inoculation with <i>M. ovipneumoniae</i>	32
5. Figure 5. Bronchoalveolar lavage fluid from SPF-lambs experimentally infected with <i>M. ovipneumoniae</i> showed no evidence of lung inflammation.	33
6. Figure 6. Antibiotic treatment with gamithromycin did not eliminate asymptomatic <i>M. ovipneumoniae</i> infection.	34
7. Figure 7. Both upper and lower respiratory tract pathogen delivery leads to stable colonization with <i>M. ovipneumoniae</i>	65
8. Figure 8. Lower respiratory tract inoculation with <i>M. ovipneumoniae</i> leads to increased respiratory disease and decreased weight gains compared to upper respiratory tract inoculation.	69
9. Figure 9. Increased gross pathological changes in cranial lung lobes of lambs after lower respiratory tract, but not upper respiratory tract inoculation with <i>M. ovipneumoniae</i>	71
10. Figure 10. Increased histopathological changes in cranial lung lobes of lambs after lower respiratory tract compared to upper respiratory tract inoculation with <i>M. ovipneumoniae</i>	72
11. Figure 11. <i>M. ovipneumoniae</i> infection drives IFN- γ gene expression in blood and lungs.	75
12. Figure 12. Identification of <i>Mannheimia haemolytica</i> in <i>M. ovipneumoniae</i> infected lambs.	77

LIST OF FIGURES CONTINUED

Figure	Page
13. Figure 13: Linear mixed modeling of lamb weight development and health.	86
14. Figure 14: Health scores for behavior, appetite, and administered medications, and body temperatures.	87
15. Figure 15: Identification of <i>Pasteurellacea</i> in <i>M. ovipneumoniae</i> -infected lambs.	87
16. Figure 16. Representative FACS dot plots of live and dead <i>M. bovis</i> samples.	108
17. Figure 17. Brightfield images of <i>Mycoplasma</i> biofilm formation on glass- bottom plates over 6 d.	115
18. Figure 18. Maturation of <i>M. bovis</i> PG45 biofilms over time.	116
19. Figure 19. Standard color change assay is inappropriate for measuring <i>Mycoplasma</i> spp. biofilm growth.	117
20. Figure 20. Impact of biofilm disruption treatment on <i>M. bovis</i> particle size.	119
21. Figure 21. Impact of 10 minutes of sonication on the particle size of <i>M.</i> <i>bovis</i> PG45 biofilms.	121
22. Figure 22. Impact of 10 minutes of sonication on the mean fluorescence of the <i>M. bovis</i> PG45 particles.	121
23. Figure 23. Analysis of cell viability and density for the LD-AST.	122
24. Figure 24. The LD-AST reveals increased MBCs for <i>M. bovis</i> PG45 biofilms compared to planktonic bacteria.	125
25. Figure 25. Graphical Abstract.	151
26. Figure 26. The LD-AST methods used for determining the antibiotic susceptibility of planktonic and biofilm states of <i>M. ovipneumoniae</i>	159
27. Figure 27. Biofilm formation varies between strains.	165

LIST OF FIGURES CONTINUED

Figure	Page
28. Figure 28. <i>M. ovipneumoniae</i> consistently forms biofilms in vitro.....	166
29. Figure 29. Biofilm thickness varies between different strains.....	168
30. Figure 30. Colony morphology shows typical round centerless colonies of <i>M. ovipneumoniae</i> when isolated, and rougher morphology when observing Y98 and LS2.2 as a lawn. Bar for all panels=100 μ m.	169
31. Figure 31. Biofilm formation is linked to increased antibiotic resistance.	172
32. Figure 32. Biofilm confluence significantly increased (Color matched stars to samples) or stayed the same after treatment with antibiotics.	174
33. Figure 33. While there is strain-level variation between all genomes, PdhD protein sequences showed high levels of conservation, and MnuA and Nuc showed high levels of variability.	177

ABSTRACT

Mycoplasma ovipneumoniae is a respiratory pathogen in domestic sheep that is typically associated with polymicrobial pneumonia. *M. ovipneumoniae* infections can lead to decreased weight gains, increased susceptibility to secondary pathogens, and increased lamb mortality. Current understanding of how *M. ovipneumoniae* affects its host in the absence of a secondary pathogen remains limited, as are the site-specific effects of infection. *M. ovipneumoniae* infections have been observed to cause persistent infections that resist both the immune system and antimicrobial treatment. Related organisms in the *Mycoplasmataceae* family evade the immune system and treatment through the formation of a biofilm. Biofilms are formed of aggregated bacteria that adhere to a surface and form a protective extracellular polymeric matrix. However, it is difficult to assess the impacts of *Mycoplasma* biofilm formation on antibiotic susceptibility due to limitations in current methods for antibiotic susceptibility testing (AST). Additionally, whether *M. ovipneumoniae* can form a biofilm remains a matter of debate. This work aims to address these issues by investigating how *M. ovipneumoniae* infection alone can influence pathogenesis, and how infection with a secondary pathogen can vary due to the site of initial administration. It also seeks to address limitations in the current AST methods for *Mycoplasma* biofilms and to better characterize *M. ovipneumoniae* biofilm formation capacity. In four related publications, we first show that *M. ovipneumoniae* alone causes asymptomatic infections that cannot be cleared with antibiotics. We next compare how infection in the lower respiratory tract results in increased pathogenesis with a secondary pathogen compared to the upper respiratory tract. We then developed a method for AST of *Mycoplasma* spp. biofilms to compare the changes in resistance observed between planktonic cells and biofilms. Finally, we show robust *M. ovipneumoniae* biofilm formation *in vitro*, and associated increases in antibiotic tolerance and cellular survival. These observations suggest that infection with *M. ovipneumoniae* alone results in asymptomatic infection, while coinfection with *M. haemolytica* in the lower respiratory tract leads to more severe disease. We conclude that the observed antibiotic treatment failure *in vivo* was caused by *M. ovipneumoniae* biofilm formation that led to increased antibiotic tolerance.

CHAPTER ONE

INTRODUCTION

Mycoplasma ovipneumoniae

Mycoplasma ovipneumoniae is a sheep respiratory pathogen typically associated with respiratory disease in domestic and bighorn sheep [1, 2]. Although these are the common hosts, *M. ovipneumoniae* can also be found in domestic goats, mountain goats, caribou, and musk oxen [3-6] (Figure 1). In domestic sheep operations, *M. ovipneumoniae* infection is widespread, with between 85-88.5% of herds testing positive in the U.S. [7, 8] and 40.9-54.5% of herds in Europe and China [9-11]. While *M. ovipneumoniae* in domestic sheep is often asymptomatic, infection has been shown to lead to reduced weights in infected lambs [1], increases in stillbirths [7], and decreased lamb survival [7, 12]. Infection can also result in specific respiratory complications such as pulmonary lesions [13-15] and autoantibodies to respiratory cilia [16]. Due to these effects of *M. ovipneumoniae* infection, severe disease can occur in domestic sheep when the animals encounter a secondary respiratory pathogen such as *Mannheimia haemolytica* [17].

Alternative Hosts

Common Hosts	
Domestic Sheep	Muskox
Mild Disease	Severe Disease
Bighorn Sheep	Caribou/Reindeer
Severe Disease	Severe Disease
	Mountain Goat
	Mild or Severe Disease
	Domestic Goat
	Mild Disease

Figure 1. *Mycoplasma ovipneumoniae* commonly infects domestic and bighorn sheep but also affects various other hosts with differing levels of disease severity. While typically mild in adult domestic sheep, negative outcomes can occur in lambs. In bighorn sheep, muskoxen, and caribou/reindeer, infections are often more severe and can be fatal. Mountain goats have variable outcomes, while domestic goats have mild or asymptomatic disease.

Respiratory Immune Response

The respiratory tract is the typical point of infection for *M. ovipneumoniae*. To defend against the bacteria, the respiratory immune system consists of physical barriers as well as innate and adaptive immune mechanisms.

Physical Barriers

The first defense that the respiratory system has against pathogens is a physical barrier composed of airway epithelial cells which can block invasion by foreign pathogens and initiate immune responses[18, 19]. Respiratory mucus production can also act as a protective barrier against inhaled particles and pathogens by trapping them, which can then be removed by ciliated cells through the process of mucociliary clearance [20, 21].

Innate Immune Mechanisms

In healthy domestic sheep, between 70-90% of immune cells in the lungs are alveolar macrophages (AMs) [22-24]. These AMs are part of the innate immune system, and they patrol the lung, phagocytosing both pathogens and foreign debris, and can either initiate an immune response or maintain homeostasis [25]. After initiation of an immune response, another immune cell, the neutrophil, is recruited to the site of infection. These neutrophils also phagocytose foreign pathogens in addition to trapping them in neutrophil extracellular traps (NETs) made of DNA, in a process called NETosis [26, 27]. The respiratory tract also contains peptides that have antimicrobial functions that are secreted by both immune cells and airway epithelial cells to initiate the immune response and function as endogenous antibiotics [28].

Adaptive Immune Mechanisms

The adaptive immune response in the respiratory tract is composed of Th1 cells and both IgA and IgG. Th1 cells help to modulate the immune response through the production of IFN- γ and TNF- α , which can activate macrophages and lead to increased clearance of pathogens [29]. IgA is a secretory immunoglobulin that is often found in mucosa and contributes to its defense. IgA does this by preventing interactions between the pathogen and underlying epithelial cells by binding bacterial adhesins used to attach to the cells. This binding by IgA has been shown to limit the capacity of bacteria to colonize nasal passages [30]. IgG in the respiratory tract usually reaches the lower airways through transudation or through translocation via receptor mediated transcytosis by alveolar epithelium [31, 32]. IgG can then function as an opsonin and encourage phagocytosis or initiate the complement cascade [31].

Immune Evasion

Chronic respiratory disease and persistence often occurs due to the evasion of the immune system by pathogens. In respiratory systems, this can occur by several mechanisms such as intracellular survival, suppression of the immune system, antigenic variation, and what I focus on in this dissertation, biofilm formation [33-36].

Biofilm Formation

A common immune evasion and resistance mechanism used by many bacterial pathogens is the formation of a biofilm [36-40]. Biofilms are structures formed by aggregated bacteria encased in an extracellular polymeric matrix (EPM) consisting of extracellular DNA (eDNA), proteins, lipids, and other polysaccharides [41, 42]. These structures of surface-adhered bacteria

often have increased resistance to clearance by the immune system and antimicrobial treatment [36, 40, 43-46]. After adhesion and formation of an EPM, the biofilm can then continue propagation through the dispersal of cells to form new biofilms [47] (Figure 2). *Mycoplasma* spp. are associated with biofilm formation in human and livestock respiratory infections [37, 44, 48, 49]. Biofilm formation by *M. ovipneumoniae* is poorly characterized, and to date, only three papers describing *M. ovipneumoniae* biofilm formation have been published [49-51]. These studies utilized narrow growth parameters, resulting in limited *M. ovipneumoniae* biofilm formation. My dissertation work seeks to address this gap in knowledge and further determine the capacity and effects of biofilm formation by *M. ovipneumoniae*.

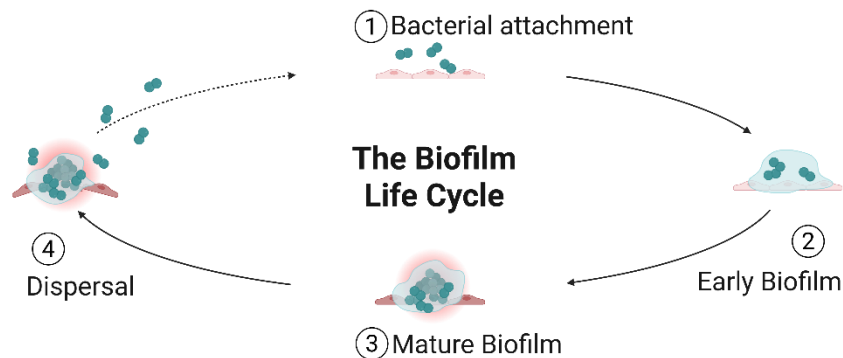


Figure 2. The Biofilm Life Cycle. Biofilm formation in most organisms follows the typical cycle of (1) bacterial attachment, (2) Early biofilm formation with the beginning of an extracellular polymeric matrix (EPM), (3) a mature biofilm, with a thick EPM, and (4) dispersal of bacterial cells to form a new biofilm. Figure created in BioRender.

Management of *M. ovipneumoniae* Infection in Sheep

Management strategies utilizing antibiotic treatments with enrofloxacin, oxytetracycline, lincomycin, or florfenicol have not been successful in the long-term elimination of *M. ovipneumoniae* infections from domestic herds [52, 53]. In bighorn sheep, only one study has been able to clear infection after an aggressive twice daily administration of doxycycline for 8 weeks or longer [54]. Due to these antibiotic treatment failures, *M. ovipneumoniae* infection in

domestic sheep is typically unmanaged, and in bighorn sheep a “test and remove” strategy is currently used [55]. This “test and remove strategy consists of using diagnostic tests to determine *M. ovipneumoniae* infection status of the individuals in a bighorn sheep flock, and then euthanizing positive individuals [55]. Due to the typical observation of treatment failure in domestic sheep, there is a need to determine how *M. ovipneumoniae* infection can be cleared or prevented to ensure the continued success of domestic sheep operations and to reduce the risk of pathogen transmission from domestic to bighorn herds.

Research Objectives and Hypotheses

This dissertation aims to describe the effects of *M. ovipneumoniae* infection alone, as well as in conjunction with a second respiratory infection, in the upper and lower respiratory tract. It also aims to determine whether biofilm formation by *M. ovipneumoniae* leads to the antibiotic resistance observed in both our work and the works of others [52, 53]. My contributions to these works are as follows. In chapter 2, 7 and 8, I served as a co-author of the manuscripts and assisted with the investigation and data analysis of the results. In chapters 3-5, I served as the lead author and contributed to the writing of the manuscript, the experimental design, investigation, and analysis.

Chapter 2 is a manuscript published in *Veterinary Microbiology*, which demonstrates the use of our SPF flock in an experimental infection with *M. ovipneumoniae*. In this we sought to determine if *M. ovipneumoniae* alone can cause respiratory disease. We observed no clinical signs, which are supported by previous observations that infection with *M. ovipneumoniae* alone leads to asymptomatic infection that does not respond to antibiotic treatment.

Chapter 3 is a manuscript published in *Veterinary Research*. This manuscript investigates the changes in the observed pathogenesis between SPF sheep inoculated with *M. ovipneumoniae* and *M. haemolytica* in both the upper and lower respiratory tract. We hypothesized that the infection site would contribute to differing outcomes, and our data suggests that lower respiratory tract infections lead to reduced weight gain, more severe pathological changes, and increased clinical signs.

Chapter 4 is a manuscript published in the journal *Microorganisms* describing the development of an innovative flow cytometry-based antibiotic susceptibility method for *Mycoplasma* spp. biofilms. In this we hypothesized that biofilm formation by *Mycoplasma* species would lead to increased antibiotic tolerance. Our work successfully tested methods for biofilm disruption, quantified adhesion and maturity, and was able to observe increases in antibiotic tolerance by biofilm formation for all the isolates tested.

Chapter 5 is a manuscript prepared for submission to *PLoS Pathogens*. We hypothesized that *M. ovipneumoniae* biofilm formation leads to increased antibiotic tolerance. Our manuscript describes biofilm formation by four *M. ovipneumoniae* strains, the variations observed for maturity and thickness, potential mechanisms of adhesion and disruption, and the increased antibiotic tolerance due to biofilm formation. We observed that the formation of a biofilm by *M. ovipneumoniae* was associated with increased tolerance of antibiotic treatment in support of observations made in other bacterial species.

Chapter 6 concludes this dissertation and discusses the previous chapters in light of one another and other research publications.

Chapters 7 and 8 are additional publications that the author contributed to that are related to the dissertation but are outside the scope of the discussion contained herein.

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CHAPTER TWO

EXPERIMENTAL INFECTION OF SPECIFIC PATHOGEN FREE DOMESTIC LAMBS
WITH *MYCOPLASMA OVIPNEUMONIAE* CAUSES ASYMPTOMATIC COLONIZATION OF
THE UPPER AIRWAYS THAT IS RESISTANT TO ANTIBIOTIC TREATMENT

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Abstract

Mycoplasma ovipneumoniae (*M. ovipneumoniae*) is a respiratory pathogen associated with mild to moderate respiratory disease in domestic lambs and severe pneumonia outbreaks in wild ruminants such as bighorn sheep. However, whether *M. ovipneumoniae* by itself causes clinical respiratory disease in domestic sheep in the absence of secondary bacterial pathogens is still unclear. The goal of our study was to better understand the role of *M. ovipneumoniae* as a respiratory pathogen in domestic sheep and to explore potential antibiotic treatment approaches. Therefore, we inoculated four 4-month-old, specific-pathogen-free lambs with fresh nasal wash fluids from *M. ovipneumoniae*-infected sheep. The lambs were monitored for *M. ovipneumoniae* colonization, *M. ovipneumoniae*-specific antibodies, clinical signs, and cellular and molecular correlates of lung inflammation for eight weeks. All lambs then were treated with gamithromycin and observed for an additional four weeks. *M. ovipneumoniae* inoculation resulted in stable colonization of the upper respiratory tract in all *M. ovipneumoniae*-inoculated, but in none of the four mock-infected control lambs. All *M. ovipneumoniae*-infected lambs developed a robust antibody response to *M. ovipneumoniae* within 2 weeks. However, we did not observe significant signs of respiratory disease, evidence of lung damage or inflammation in any of the infected lambs. Interestingly, treatment with gamithromycin, which blocked growth of the *M. ovipneumoniae* in vitro, failed to reduce *M. ovipneumoniae* colonization. These observations indicate that, in the absence of co-infections, *M. ovipneumoniae* caused asymptomatic colonization of the upper respiratory tract that was resistant to clearance by the host immune response and by gamithromycin treatment.

Introduction

Mycoplasma ovipneumoniae (*M. ovipneumoniae*) is a highly prevalent respiratory pathogen associated with atypical, chronic non-progressive pneumonia in sheep and goats. Colonization of the upper respiratory tract of adult sheep with *M. ovipneumoniae* is increasingly recognized across the world, with infections reported in Europe, North America, Africa, Asia, Australia and New Zealand [56-60]. In the United States, active *M. ovipneumoniae* infection was detected in 88.5% of commercial sheep flocks in a 2011 study [58]. While most cases of *M. ovipneumoniae* infection in adult sheep are thought to be asymptomatic or mild, significant production losses may occur due to reduced weight gains, lower carcass quality and increased mortality in lambs [1, 7, 56]. Moreover, *M. ovipneumoniae*-infected domestic sheep pose a significant threat to other ruminants such as bighorn sheep (*Ovis canadensis*) [2, 61], Dall's sheep (*Ovis dalli dalli*) [62], Argali (*Ovis ammon*) hybrid sheep [63], and Norwegian MuskoX (*Ovibos moschatus*) [5], where *M. ovipneumoniae* infection causes severe pneumonia outbreaks with up to 100% mortality.

However, it is still unclear to what extent *M. ovipneumoniae* causes clinical respiratory disease in sheep in the absence of other pathogens. Several studies have demonstrated that *M. ovipneumoniae* greatly increases the susceptibility for infection with other opportunistic pathogens such as *Mannheimia haemolytica* and *Bibersteinia trehalosi*, which then leads to interstitial pneumonia with severe clinical symptoms and significant mortality [61, 64-66]. However, infection studies with *M. ovipneumoniae* alone have yielded conflicting results. Thus, respiratory symptoms and lung pathology were observed in a subset of sheep following endobronchial application of *M. ovipneumoniae* in two early studies [67, 68], and more recently,

Du et al. described coughing, wheezing and increased body temperatures in Bashbay lambs experimentally inoculated with *M. ovipneumoniae* [69]. In contrast, Buddle et al. [70] did not find evidence of pneumonia in colostrum-deprived lambs after experimental *M. ovipneumoniae* infection. In bighorn sheep, inoculation with cultured strains of *M. ovipneumoniae* did not result in clinical respiratory disease [2], whereas natural transmission of *M. ovipneumoniae* between animals led to severe bronchopneumonia [71]. These conflicting findings point to a need for further experimental investigations.

While the contribution of *M. ovipneumoniae* to sheep respiratory disease is widely acknowledged and elimination of *M. ovipneumoniae* colonization in domestic sheep could prevent severe spillover-associated disease outbreaks in bighorn sheep and other wild ruminants, no vaccines or treatments to combat the infection are currently approved. While experiments have demonstrated the *in vitro* susceptibility of most *M. ovipneumoniae* isolates to a wide range of macrolide, tetracycline and fluoroquinolone antibiotics [59, 72], no studies on antibiotic treatment of *M. ovipneumoniae* infection *in vivo* have been published.

To better understand the role of *M. ovipneumoniae* as a respiratory pathogen in domestic sheep and to explore potential antibiotic treatment approaches, we performed an *M. ovipneumoniae* infection and antibiotic treatment study in four 4-month-old specific-pathogen-free lambs and four age-matched control lambs. Intranasal inoculation of lambs with *M. ovipneumoniae*-positive nasal washes collected from lambs with clinical respiratory disease resulted in consistent colonization of the upper respiratory tract and robust *M. ovipneumoniae*-specific humoral immunity. However, we did not observe clear clinical signs or evidence of lung damage or inflammation in any of the infected lambs. Interestingly, treatment with the macrolide antibiotic

gamithromycin, which has been used successfully to treat clinical *Mycoplasma*-associated pneumonia in cattle, goats and pigs [73-75], failed to reduce *M. ovipneumoniae* colonization in the infected lambs. Overall, these data suggest that, in the absence of other opportunistic pathogens, *M. ovipneumoniae* behaves like an upper respiratory tract commensal that is not affected by the host antibody response or a macrolide antibiotic gamithromycin, which is commonly used to treat respiratory disease in ruminants.

Methods

Animals and husbandry

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Montana State University, protocol #2019-95. To generate specific-pathogen-free lambs, 15 mixed-breed ewes (Rambouillet, Suffolk, Targhee, Columbia, and/or Hampshire) 4-5 years of age were purchased several weeks before the projected lambing date from a local sheep farmer. Pregnancy was confirmed using abdominal ultrasound, and *M. ovipneumoniae* exposure was determined by serology and nasal swab PCR, as described below. Prior to lambing, ewes were fed hay from the Johnson Family Livestock Facility (JFLF) farm, grain, and an appropriate vitamin/mineral supplement.

To derive lambs free from *M. ovipneumoniae* and other facultative respiratory pathogens, we used supervised lambing and motherless rearing, as previously described [76, 77]. Around the projected lambing date, ewes were monitored around the clock for signs of imminent delivery. Ewes developing signs of labor were separated from the flock and moved into a designated lambing pen, and lambs were manually delivered onto sterile towels placed on top of a clean plastic sheet and then were transferred into a separate, heated nursery area within the JFLF

ABSL-2 laboratory. All animal care personnel showered and changed into sterile personal protective equipment prior to entering the nursery, and personnel responsible for the lambs did not have any contact with other non-SPF sheep for the duration of lambing. Lambs were housed in heated animal rooms (15.5-16.8 °C) inside the JFLF in groups of 5-10 animals, with siblings and lambs of similar ages grouped together. During the first 24 h, lambs were bottle-fed a commercial colostrum replacer (Rescue Lamb & Kid Colostrum Replacer, Lifeline Nutrition Solutions/APC, Ankeny, IA), which contains bovine serum as an antibody source. Animals were trained to use bucket feeders and were fed a commercial lamb milk replacer diet (Hubbard Feeds, Mankato, MN) for 5 weeks, with *ad libitum* access to hay and water. Lambs were gradually weaned onto pelleted lamb food starting at 36-48 days of age by decreasing the concentration of milk replacer to 50% for two weeks as previously described [76].

Health Monitoring

Approximately 2 months prior to lambing, ewes were screened for *M. ovipneumoniae*, *Coxiella burnetii* and *Mycobacterium avium paratuberculosis* (MAP) exposure by serum ELISA and for parainfluenza 3 (PI3) exposure by serum-based virus neutralization assay. Nasal swabs from the ewes were also analyzed for *M. ovipneumoniae* by PCR.

Specific-pathogen-free lambs at the JFLF were screened daily for signs of illness or discomfort. In addition, SPF-status of the lambs was confirmed by testing nasal swab samples for *M. ovipneumoniae* infection by quantitative PCR at birth, at the age of 1 month, and seven days prior to the start of the experiment. At 3 months of age, nasal swabs from a subset of lambs were analyzed for *M. ovipneumoniae* by PCR and for the presence of Pasteurellaceae by conventional aerobic culture. All laboratory assays were performed at the Washington Animal

Disease Diagnostic Laboratory (WADDL, accredited by the American Association of Veterinary Laboratory Diagnosticians).

Following experimental *M. ovipneumoniae* infection or mock treatment, lamb health status was assessed twice daily by trained JFLF personnel. The following health parameters were assessed and were used to calculate a clinical disease score: (1) general behavior, (2) appetite, (3) rectal temperature, (4) respiratory symptoms, and (5) any medications administered exclusive of the experimental treatment provided at 8 weeks post infection (p.i.) as detailed in Table 1. Daily scores represent the sum of the two values obtained for each day, and weekly scores represent the sum of all 14 values collected over a 7-day period. In addition, lamb body weights were determined on days 20, 32 and 55 p.i.

Table 1. Parameters for lamb health scoring during challenge experiment.

Parameter	0	1	2	3	4	5
Behavior	bright, alert, responsive	small change in attitude/behavior	less active, visibly appears ill	ill, but still responsive	lethargic	unresponsive
Appetite	ate well	ate most of the ration	ate 3/4 feed	ate 1/2 feed	ate <1/2 feed	not eating
Body temperature	100 °F-102 °F	102.1 °F-103 °F or below 99.9 °F	103.1 °F-104 °F	104.1 °F-104.5 °F	104.6 °F-105 °F	above 105 °F
Respiratory signs	no nasal or ocular discharge, no cough, normal respirations	Panting/increased respiratory rate, or slight clear nasal/ocular discharge	clear nasal or ocular discharge, or coughed 1-5 times	moderate nasal or ocular discharge or frequent coughing	mucous nasal or ocular discharge, coughing, lamb visibly unwell	discharge, coughing, wheezing, labored breathing
Medication	no treatments	electrolytes	NSAID	antibiotic	NSAID and antibiotic	multiple NSAIDs and antibiotics

Experimental infection with *Mycoplasma ovipneumoniae*

Out of thirty live lambs born in our flock, eight SPF lambs aged between 15 and 16 weeks were selected for the experiment and divided into an *M. ovipneumoniae* treatment group (n=4) and a control group (n=4) based on the following criteria: (1) absence of previous clinical disease or injuries that required treatment; (2) equal distribution of available sibling pairs into the two groups; (3) equal numbers of male and female lambs; and (4) group composition matched for age and sex. Animal details are listed in Table 2. The SPF lambs were infected using pooled nasal

Table 2. Characteristics of experimental animals

Animal #	Treatment	Age (days)*	Sex
12	<i>M. ovipneumoniae</i>	109	F (twin of 15)
19	<i>M. ovipneumoniae</i>	108	F
26	<i>M. ovipneumoniae</i>	105	M [#] (twin of 25)
30	<i>M. ovipneumoniae</i>	103	M [#]
15	PBS	109	M [#] (twin of 12)
17	PBS	109	M [#]
25	PBS	105	F (twin of 26)
27	PBS	104	F

* Age at time of infection.

All males were castrated at 11 weeks.

wash fluids from lambs previously determined to be infected with *M. ovipneumoniae*, following published protocols [71, 78, 79]. Animals housed at MSU's Red Bluff Research Ranch, aged 11-13 weeks, were used as donor lambs for nasal washes. We selected eight lambs (4 ewes and 4 wethers) with respiratory disease symptoms including nasal discharge, coughing, labored breathing and low body weights that were confirmed to be infected with *M. ovipneumoniae* based on PCR analysis of nasal swabs. Nasal wash fluids have been successfully used in previous *M. ovipneumoniae* infection studies [71, 78, 79], and this approach prevents potential loss of bacterial virulence due to *in vitro* passaging [80, 81]. To obtain nasal wash fluids, the *M. ovipneumoniae*-infected lambs were restrained using halters, with the heads kept in a slightly lowered position. Nasal washes were performed by squirting 2 x 15 mL of sterile PBS into each nostril and collecting the flush fluid into a clean polyethylene bag. Nasal wash fluids were placed on ice and immediately transferred to the laboratory, where they were pooled, diluted 1 : 1 with tris-buffered saline, and then treated with ceftiofur (100 µg/mL, MWI Animal Health, Boise, ID) for 2 h at 37°C to reduce contamination with *Pasteurellaceae*. Microbiological analysis of the pooled nasal wash fluid confirmed that both *Mannheimia haemolytica* and *Bibersteinia trehalosi* were present before ceftiofur treatment, but were undetectable after the treatment. Within 4 h of nasal wash collection, we then inoculated eight of the SPF lambs (Table

2) with 50 mL of the treated nasal wash fluid (*M. ovipneumoniae* group, n=4) or PBS (control group, n=4) by infusing 15 mL into each nostril, 10 mL into the oral cavity and 5 mL into each conjunctival sac. Following inoculation, the *M. ovipneumoniae* group and the control group were housed in separate rooms of the JFLF to prevent aerosol transmission of *M. ovipneumoniae*. Each room had its own set of equipment so that no equipment was shared between the rooms. For the duration of the infection experiment, staff first performed all necessary husbandry procedures on the control animals before entering the room with the infected animals and showered and changed immediately after leaving the room with the infected lambs.

Antibiotic Treatment

Eight weeks after experimental infection with *M. ovipneumoniae* or mock infection with PBS, all lambs were treated with 6 mg/kg BW gamithromycin (Zactran®, Boehringer Ingelheim, Ridgefield, CT) by subcutaneous injection, with a second dose given 5 days later.

M. ovipneumoniae isolation and culture

Isolation media consisting of *Mycoplasma* Broth Base (Becton Dickinson, Franklin Lakes, NJ) supplemented with dextrose (Thermo Fisher Scientific, Waltham, MA), L-cysteine hydrochloride monohydrate (Thermo Fisher Scientific), beta-NAD (Thermo Fisher Scientific), thallium acetate (Acros Organics, Fair Lawn, NJ), phenol red (Sigma Aldrich, St. Louis, MO), porcine serum (Quad Five, Ryegate, MT), equine serum (Quad Five), and penicillin G potassium salt (Sigma Aldrich) as both broth and agar plates. *Mycoplasma* plates were inoculated with the nasal wash used in the infection experiment. These plates were then incubated under microaerophilic conditions at 37°C for 10 days. Several of the resulting colonies were then determined to be *M. ovipneumoniae* through qPCR targeting the p133 gene [82] with Ct values

<25. These colonies were inoculated into *Mycoplasma* broth and expanded by incubation under microaerophilic conditions at 37°C until acid production caused a color change in the media with the phenol red.

Antibiotic broth microdilution assay

The antibiotic broth microdilution assay was performed according to the methods outlined previously by Hannan [83]. Gamithromycin concentrations from 0.031-128 µg/mL were tested against the nasal wash isolate and the type strain Y98 (ATCC 29419™, NCTC 10151).

Sensitivity of the *Mycoplasma* to gentamicin (0.031-128 µg/mL, Thermo Fisher Scientific) was analyzed for comparison. 96-well plates containing *M. ovipneumoniae* cultures initially were incubated for 48 h under microaerophilic conditions at 37°C and were observed every 24 h for a color change in the media that indicated acid production and growth of the organism. The lowest dilution without a color change after two subsequent 24 h readings without change was recorded as the end-point minimum inhibitory concentration (MIC).

Collection and analysis of bronchoalveolar lavage fluids

To collect bronchoalveolar lavage fluid (BAL), a flexible fiber-optic endoscope measuring 6.6 mm x 100 cm (VFS-2B VetVu, Swiss Precision Products, Inc, Oxford, MA) was introduced into the trachea under local anesthesia with lidocaine. Lavage was performed by instilling 60 mL of sterile saline into the lungs and then aspirating the liquid again; approximately 20 mL of fluid were routinely recovered from the sheep. Bronchoalveolar lavage fluid was stored on ice until transfer to the laboratory, where cells were harvested by centrifugation at 400 g for 10 min. Supernatants were then analyzed for evidence of lung damage using the Lactate Dehydrogenase Assay Kit (Abcam, Cambridge, UK). Cell pellets were processed for absolute cell counts using a

hemocytometer and for differential cell counts using cytopsin preparations stained with a DippKwik stain (ThermoFisher, Waltham, MA). At least 300 cells from each sample were classified by a scientist blinded to the treatment of the animals.

Detection of *M. ovipneumoniae* infection

Detection of *M. ovipneumoniae* infection by PCR was performed at the Washington Animal Disease Diagnostic Laboratory (WADDL, Pullman, WA) using nasal swab samples and standard protocols [84]. Briefly, probe based quantitative PCRs were performed with the following primers and probe: forward: 59-GGG GTG CGC AAC ATT AGT TA-39; reverse: 59-CTT ACT GCT GCC TCC CGT AG-39; and probe: 59-6-FAM-TTA GCG GGG CCA AGA GGC TGT A-BHQ-1-39 derived from GenBank sequences EU290066 and NR_025989 of *M. ovipneumoniae*. Samples with Ct values <40 were considered positive. Data are shown as 40 minus the measured Ct value to enable semi-quantitative comparison between samples.

M. ovipneumoniae serology

Analysis of serum samples for the presence of *M. ovipneumoniae*-reactive antibodies was performed at WADDL using the laboratory's monoclonal antibody-based competitive enzyme-linked immunosorbent assay (cELISA) test, which has a diagnostic sensitivity of 88% and a diagnostic specificity of 99.3%. Validation data for the assays may be obtained directly from the laboratory at http://www.vetmed.wsu.edu/depts_waddl/). Data are shown as % inhibition and represent the reduction in binding of the labelled monoclonal antibody to the *M. ovipneumoniae* test antigen caused by competitive binding of serum antibodies from the diagnostic samples. Inhibition of >50% was considered a positive result, and inhibition between 40% and 50% was considered indeterminate.

Statistical analyses

Data were analyzed by GraphPad Prism, version 9.0 (San Diego, CA) and are shown as mean $\pm$ standard deviation (SD). Differences between groups were analyzed by 2-way ANOVA with Sidak's, Tukey's or Dunnett's multiple comparisons test or by Student's *t* test and were considered significant at $P \leq 0.05$.

Results

Supervised lambing and motherless rearing successfully prevented colonization of domestic lambs with *M. ovipneumoniae* and *Pasteurellaceae*.

Specific-pathogen-free lambs were derived by supervised lambing and artificial rearing from a domestic sheep flock with a history of *M. ovipneumoniae* infection. Pathogen exposure of the ewes was determined by serological analysis prior to lambing. The ewes were free from *C. burnetii* and *M. avium ssp. paratuberculosis*, but had variable serological responses to *M. ovipneumoniae* and parainfluenza virus (PI-3). Notably, nasal swabs collected at the same time as the serum samples tested negative for *M. ovipneumoniae* by PCR, indicating previous exposure of the sheep, but no active pathogen shedding.

All thirty lambs born from our ewe flock including the eight experimental lambs selected for our study were free from *M. ovipneumoniae* on days 0 and 30 after birth and one week prior to the experimental inoculation. We also confirmed the absence of upper respiratory tract colonization by *Pasteurellaceae*, which include the facultative respiratory pathogens *Mannheimia haemolytica* and *Bibersteinia trehalosi*, in all eight experimental lambs at 3 months of age.

Application of nasal wash fluids from *M. ovipneumoniae*-infected lambs led to successful colonization of specific-pathogen free lambs and induces *M. ovipneumoniae*-specific serum antibodies.

To analyze the effects of experimental *M. ovipneumoniae* infection in specific pathogen-free sheep, four lambs each were inoculated with *M. ovipneumoniae*-positive nasal wash fluids or with PBS. Following inoculation, lambs were monitored for clinical signs for twelve weeks, and nasal swabs, serum and BALs for laboratory analyses were collected as shown in Figure 3A. All lambs in the *M. ovipneumoniae* group, but none of the lambs in the control group, showed positive PCR results for *M. ovipneumoniae* at two weeks p.i. (Figure 3B). *M. ovipneumoniae* levels peaked at 2–4 weeks, declined at 6 weeks and then plateaued. Colonization with *M. ovipneumoniae* was confirmed by successful culture of viable *Mycoplasma* in nasal swab samples from two of the experimental lambs at 4 weeks p.i. We did not detect Pasteurellaceae in any nasal swab samples collected on days 0, 56, and 84 of the infection experiment using standard microbiology techniques.

All infected lambs developed a strong *M. ovipneumoniae*-specific antibody response that peaked at 4 weeks p.i. and remained high throughout the experimental period (Figure 3C), whereas no significant *M. ovipneumoniae*-reactive antibodies were detected in the control group. Likewise, no *M. ovipneumoniae*-reactive antibodies were present in any of the animals one day after birth, demonstrating that the colostrum replacer used did not contain any cross-reactive antibodies that might have contributed to *M. ovipneumoniae*-resistance through passive antibody transfer.

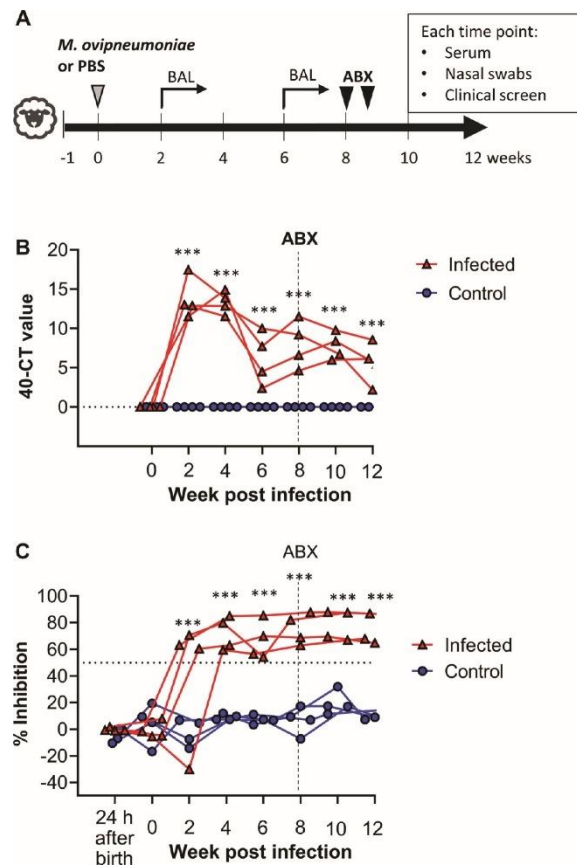


Figure 3. *Mycoplasma ovipneumoniae* colonization and antibody responses in experimentally infected lambs. (A) Experimental schedule. Four SPF lambs aged 3 – 4 months were inoculated with *M. ovipneumoniae*-positive nasal washes, and four control lambs were mock-infected with PBS. All lambs received an antibiotic treatment (gamithromycin) after 8 weeks and were monitored for a total of 12 weeks. (B) *M. ovipneumoniae* infection levels in nasal swab samples were determined by qPCR. Data are shown as 40 minus Ct value. Red triangles represent individual lambs from *M. ovipneumoniae* infection group, blue circles represent lambs from control group. Data were analyzed by 2-way ANOVA with Dunnett's multiple comparisons test for differences between week 0 and other time points. *** $P \leq 0.001$ for the *M. ovipneumoniae* infected lambs. (C) *Mycoplasma ovipneumoniae*-specific antibody levels were determined by competitive ELISA analysis of serum samples. Red triangles represent individual lambs from *M. ovipneumoniae* infection group, blue circles represent lambs from control group. Data were analyzed by 2-way ANOVA with Dunnett's multiple comparisons test for differences between week 0 and other time points. *** $P \leq 0.001$ for the *M. ovipneumoniae*-infected lambs.

To assess whether pathogen loads were similar in experimentally and in naturally infected lambs, we compared *M. ovipneumoniae* colonization levels detected at different time points throughout the experiment in the experimentally infected lambs to those seen in the nasal wash

donor lambs, which were sampled at one time point. No significant difference in *M. ovipneumoniae* colonization was detected between the two groups (Figure 4A). Likewise, antibody levels detected by ELISA in the experimentally infected lambs at multiple time points did not differ significantly from antibody levels detected in the ewes (Figure 4B). These observations suggest that our experimental *M. ovipneumoniae* infection closely replicated natural infection with regards to pathogen load and immune response.

M. ovipneumoniae infection in domestic SPF lambs did not cause overt respiratory disease.

Interestingly, careful monitoring of *M. ovipneumoniae*-inoculated and control lambs for signs of illness including loss of appetite, altered behavior, and respiratory changes did not reveal consistent signs of respiratory disease in response to *M. ovipneumoniae* infection (Figure 3A-F). Our comprehensive and objective scoring system (Table 1) showed increased respiratory signs in the *M. ovipneumoniae*-infected lambs during weeks 1-3 p.i. and a similar increase in the control group during weeks 6-8 p.i. A detailed review of our animal records showed that one of the four lambs in the infected group displayed nasal mucus accumulation in week 1 after inoculation, and coughing was observed on two separate days during week 3, possibly representing a mild, transient respiratory illness in this animal (Figure 3E). However, coughing was also observed in two control lambs on various days during the experiment. Other respiratory scores were assigned for panting respirations, which again were observed in both the *M. ovipneumoniae*-infected and the control group. Appetite and body temperatures also varied widely in both groups, and decreased appetite was seen in the control group at several time points (Figure 3C,D). As described in more detail below (Figure 6), lambs in both groups had increased clinical scores and received anti-inflammatory treatment following administration of the antibiotic

gamithromycin (Figure 3A,F). Lamb body weights and daily gains measured between days 20 and 55 of the experiment, prior to administration of antibiotics, also did not differ significantly between *M. ovipneumoniae*-infected and control lambs (Figure 4A,B). Overall, clinical signs and scores in the *M. ovipneumoniae*-infected lambs did not differ markedly from the scores in the non-infected control lambs, indicating that *M. ovipneumoniae* alone did not consistently cause clinical disease in domestic SPF-lambs under controlled laboratory conditions.

Differential cell counts performed on BAL samples in order to determine a potential influx inflammatory cells into the lungs showed that >90% of cells present in BAL from both *M. ovipneumoniae*-infected and uninfected lambs had a typical alveolar macrophage morphology, with oval or round nuclei and cytoplasmic vacuoles (Figure 5A-C). A small number of lymphocytes, neutrophils and eosinophils were also detected (Figure 5C). Neither cell composition nor total cell counts differed significantly between the *M. ovipneumoniae*-infected and non-infected lambs (Figure 5C,D). We also analyzed the cell-free supernatants for the presence of lactate dehydrogenase (LDH) as a marker of lung injury [85]. Although there was a trend for increased LDH release upon *M. ovipneumoniae* infection 14 days after experimental inoculation, no statistically significant differences between the two groups were found at either of the time points analyzed (Figure 5E), although this may have been due to the small group size in our study. These data suggest that the experimental infection did not cause overt lung inflammation in the SPF lambs, consistent with the clinical findings presented above.

High dose treatment with the macrolide antibiotic gamithromycin failed to eliminate *M. ovipneumoniae* colonization.

We next sought to determine whether *M. ovipneumoniae* infection could be eliminated by antibiotic treatment with gamithromycin, which is currently used therapeutically to treat *M. bovis* infection in cattle [74]. *M. ovipneumoniae* that we cultured from the pooled nasal wash fluids used to inoculate the lambs were analyzed for susceptibility to antibiotics using a broth microdilution assay [83]. As shown in Figure 6 A,B, both gentamicin and gamithromycin inhibited growth of the nasal wash isolate of *M. ovipneumoniae* with MICs of 8 µg/mL and 16 µL, respectively. Similarly, minimum inhibitory concentrations of 4 µg/mL (gentamicin) and 32 µg/mL (gamithromycin) were effective against the *M. ovipneumoniae* type strain Y98.

To evaluate the efficacy of gamithromycin *in vivo*, all lambs were treated with two doses of gamithromycin on days 56 and 61 after inoculation with *M. ovipneumoniae* or mock-treatment. (Figure 6C). However, the antibiotic treatment neither eliminated nor significantly decreased the level of *M. ovipneumoniae* infection as determined by qPCR in any of the lambs either at 2 or at 4 weeks after the treatment (Figure 6D). Notably, statistically significant side effects of the gamithromycin treatment including changes in behavior, decreased appetite, and increased body temperatures, were observed after treatment in both the *M. ovipneumoniae*-infected lambs and the control group (Figure 6E). To counteract these side effects, anti-inflammatory treatment was administered to some of the animals (Figure 3F). These data strongly indicate that gamithromycin is unsuitable for treatment of *M. ovipneumoniae* infection in domestic lambs, since it does not eliminate infection, but has considerable adverse effects.

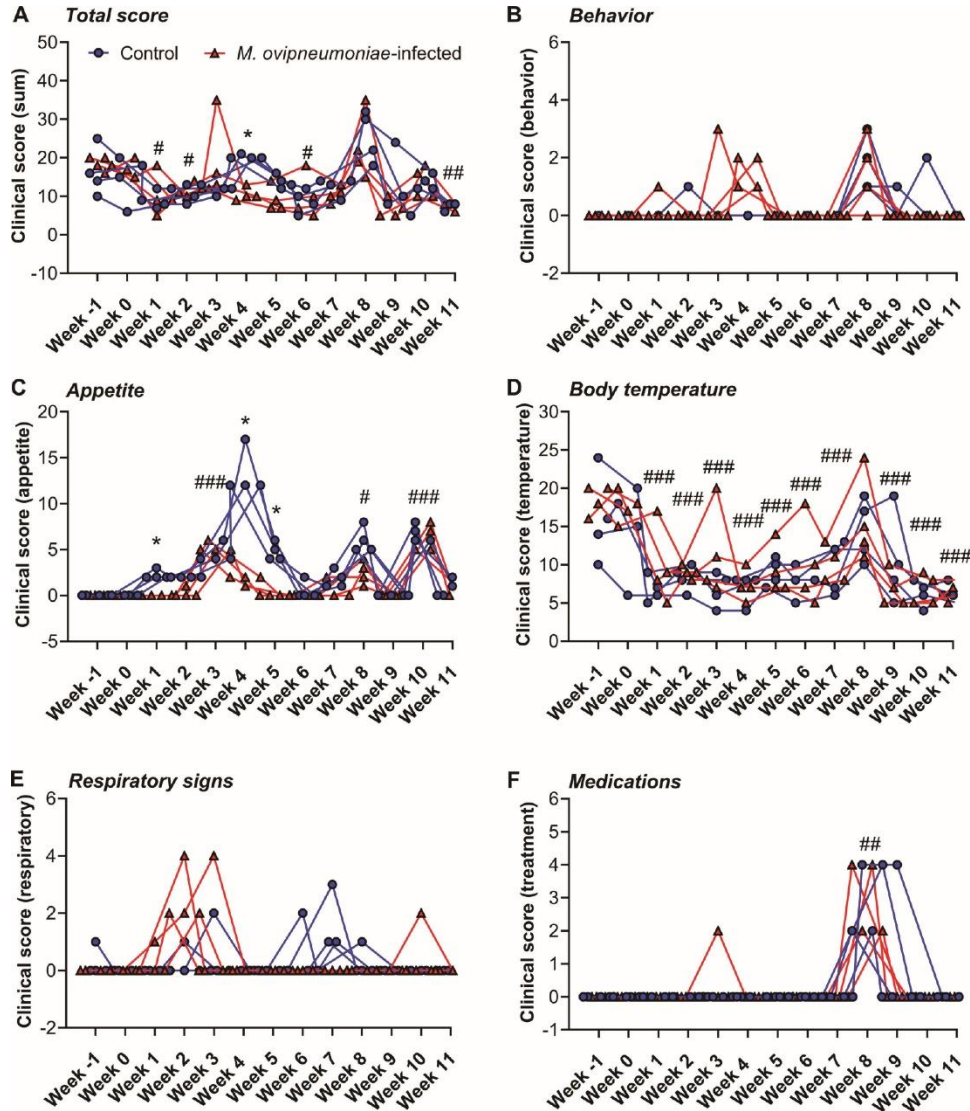


Figure 4. Clinical scoring of lambs following experimental inoculation with *M. ovipneumoniae*. All lambs were screened twice daily for changes in behavior and appetite and for signs of respiratory disease; body temperatures and administered medications were also recorded. Scores were determined using the criteria listed in Table 1. Weekly scores are sums of all 14 scores obtained for each lamb per week and per category. Red triangles represent individual lambs from *M. ovipneumoniae* infection group, blue circles represent lambs from control group. (A) Total clinical scores, sum of B–F; (B) Behavior scores, (C) Appetite scores, (D) Body temperature scores; (E) Respiratory score; (F) Medication score. Data were analyzed by 2-way ANOVA with Sidak's multiple comparisons test for differences between the *M. ovipneumoniae*-infected and control groups. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Differences between individual time points and baseline values (week -1 p.i.) for all lambs are indicated by # $P \leq 0.05$; ## $P \leq 0.01$; ### $P \leq 0.001$.

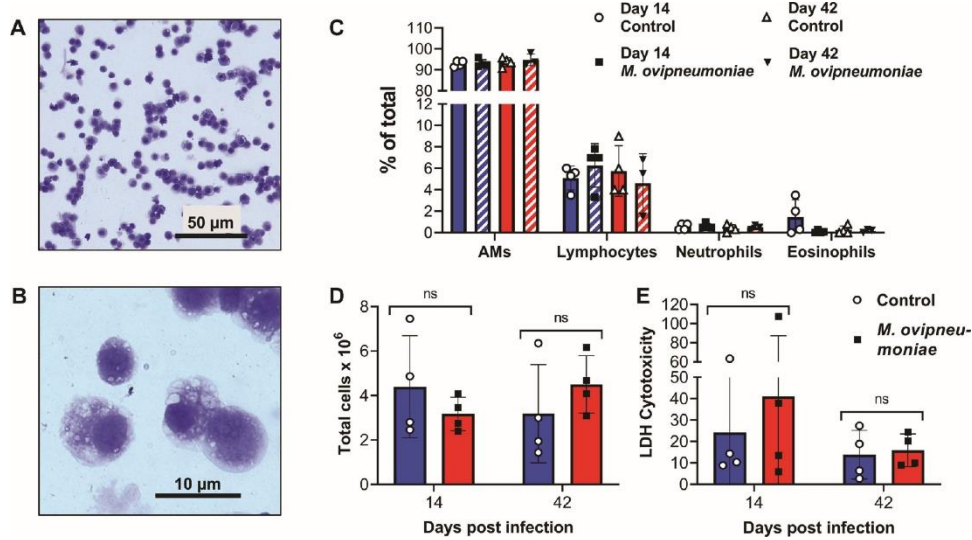


Figure 5. Bronchoalveolar lavage fluid from SPF-lambs experimentally infected with *M. ovipneumoniae* showed no evidence of lung inflammation. (A, B) Cellular composition of BAL from an *M. ovipneumoniae*-infected lamb 14 days after experimental inoculation. Representative image from one of four infected sheep shows typical macrophage morphology of the majority of the cells at (A) low and (B) high magnification. (C) Differential cell counts of BAL collected on days 14 and 42 after experimental inoculation from *M. ovipneumoniae* infected and non-infected lambs ($n = 4$). (D) Total cell counts of BAL collected on days 14 and 42 after experimental inoculation from *M. ovipneumoniae*-infected and mock-infected lambs ($n = 4$). (E) Analysis of lactate dehydrogenase in cell-free supernatants of BAL collected on days 14 and 42 p.i. Individual data points and mean $\pm$ SD are shown. Differences between groups were analyzed by Student's t test.

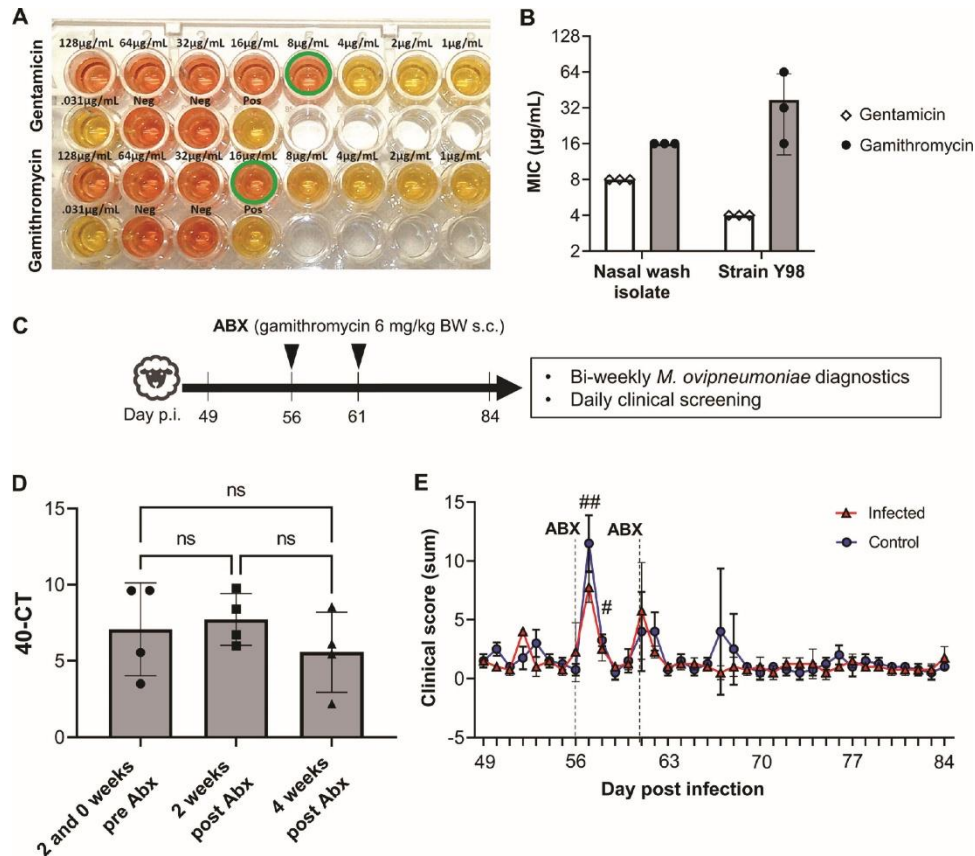


Figure 6. Antibiotic treatment with gamithromycin did not eliminate asymptomatic *M. ovipneumoniae* infection. (A) Gamithromycin and gentamicin inhibit growth of *M. ovipneumoniae* in vitro in a broth microdilution assay. Representative image of a culture plate showing color change from red to yellow reflecting growth of *M. ovipneumoniae* (nasal wash isolate). Wells with minimum inhibitory concentrations are highlighted by a green circle. (B) Minimum inhibitory concentration (MIC) for gentamicin and gamithromycin detected in three independent broth microdilution experiments performed with the nasal wash isolate and strain Y98. Individual values, mean $\pm$ SD are shown. (C) Detailed timeline for antibiotic administration. (D) *M. ovipneumoniae* infection levels in nasal swab samples of lambs inoculated with *M. ovipneumoniae*-positive nasal washes were determined by qPCR at 14 and 0 days before administration of gamithromycin (average of two time points) and at 14 and 28 days post administration of gamithromycin. Data are shown as 40 minus Ct value. Differences between time points were analyzed by 1-way ANOVA with Tukey's multiple comparisons test; ns: not significant. (E) Clinical scores of *M. ovipneumoniae*-positive and negative lambs after gamithromycin treatment show side effects of antibiotic administration. All lambs were screened twice daily for changes in behavior and appetite and for signs of respiratory disease; body temperatures and administered medications were also recorded. Scores were determined using the criteria listed in Table 1. Daily scores reflect the sum of two scores per day for all five criteria. Red triangles represent individual lambs from *M. ovipneumoniae* infection group, blue circles represent lambs from control group. Significant differences ($P \leq 0.05$) between individual time points and baseline values (day 49 p.i.) for all lambs are indicated by #.

Discussion

Mycoplasma ovipneumoniae is a key co-factor in the pathogenesis of chronic, atypical pneumonia in sheep, which has been associated with production losses in domestic lambs and significant deaths in wild sheep populations. In our study, we sought to define whether *M. ovipneumoniae* infection causes clinical respiratory disease in immunologically naïve, SPF domestic lambs. We also tested whether established *M. ovipneumoniae* infection could be eliminated by antibiotic treatment. We found that *M. ovipneumoniae* mostly caused stable, asymptomatic colonization of the upper respiratory tract that induced a significant antibody response, but that could not be cleared by gamithromycin application.

Our SPF protocol resulted in lambs that were initially free from *M. ovipneumoniae*-infection and *M. ovipneumoniae*-specific antibodies, with no indication of transplacental transfer of *M. ovipneumoniae* or antibodies to the lambs. In humans, vertical transmission of *Mycoplasmas* that colonize the genitourinal tract across the placenta has been described [86], and in cattle, blood-borne *Mycoplasma* species such as *Candidatus M. haemobos* and *M. wenyonii* can be transmitted *in utero* [87-89], in spite of the multi-layered synepitheliochorial placenta found in ruminants. Since *M. ovipneumoniae* is a respiratory tract bacterium that is not known to spread systemically in sheep, vertical transmission is unlikely, but cannot be excluded. However, we did not find any evidence that such transmission occurred.

Whether *M. ovipneumoniae* causes respiratory disease in healthy domestic lambs has been a matter of debate [67-70, 90]. Our experimental conditions that included supervised lambing followed by artificial rearing inside an ABSL-2 facility were specifically designed to prevent exposure of the experimental lambs to respiratory pathogens other than the *M. ovipneumoniae*

used in the infections. Repeated testing confirmed that Pasteurellaceae, which are major causative agents of pneumonia in lambs, were not present in any of the lambs before or at any time during the experimental period. While it was not possible to screen for all relevant respiratory pathogens, our protocol likely eliminated additional viruses, parasites and bacteria that may have been present in the ewes, strongly suggesting that *M. ovipneumoniae* was the only relevant respiratory pathogen present in our experiment. The twice-daily clinical screens and cellular and molecular analyses of the bronchoalveolar lavage fluid demonstrated that active *M. ovipneumoniae* colonization of the upper airways did not consistently lead to overt clinical disease or lung pathology in our model. Although we detected mild respiratory signs, i.e. very occasional coughing, in one out of four *M. ovipneumoniae*-infected lambs, identical signs were also observed in the uninfected control lambs at a similar frequency, albeit at different times. Therefore, it is unclear whether these mild signs were associated with the ongoing *M. ovipneumoniae* infection. In contrast to recent studies, we also did not observe any differences in lamb body weights or daily gains associated with *M. ovipneumoniae* infection [1, 7].

Multiple different hypotheses have been proposed to explain why *M. ovipneumoniae* causes asymptomatic airway colonization in some instances, but severe pneumonia in other cases: (1) differences in *M. ovipneumoniae* strain virulence; (2) age-related differences in disease susceptibility; (3) differences in the immune response to *M. ovipneumoniae*; and (4) presence of additional facultative pathogens such as *Mannheimia haemolytica*. Asymptomatic or mild *M. ovipneumoniae* infections as seen in our study may be caused by experimental inoculation with non-pathogenic *M. ovipneumoniae* variants, since *M. ovipneumoniae* strains are highly diverse and can differ in their virulence [80]. Moreover, *Mycoplasma* may lose their virulence upon

prolonged culture [80]. Such unintended, culture-dependent attenuation of *M. ovipneumoniae* isolates has been discussed as the cause for asymptomatic *M. ovipneumoniae* infection in several previous studies [70, 71]. Therefore, we used fresh nasal washes obtained from *M. ovipneumoniae*-positive lambs that showed signs of respiratory disease at the time of nasal wash collection as the infectious inoculum in our study. Because of this approach, that the lack of consistent clinical signs upon experimental *M. ovipneumoniae* infection likely was not due to the use of an avirulent or attenuated *M. ovipneumoniae* strain. A limitation of using nasal washes as a source of *M. ovipneumoniae* is that the exact bacterial strain and its characteristics remain unknown, and infections with multiple strains of *M. ovipneumoniae* or other pathogenic contaminants cannot be excluded.

With regard to the hypothesis that age-related differences are crucial, it is generally accepted that clinical infections with *M. ovipneumoniae* most commonly occur in lambs under one year of age, while adult sheep serve as asymptomatic *M. ovipneumoniae* carriers [58, 91]. Plowright et al. showed that, in bighorn sheep, prevalence of *M. ovipneumoniae* was highest in lambs and aged animals, but low in adults, pointing to age-dependent infection dynamics [92]. However, it is unclear whether the increased susceptibility of lambs was due to the lack of established adaptive immune responses to *M. ovipneumoniae* or other age-related differences in respiratory anatomy, physiology, or immunity. Interestingly, Gilmour et al. found increased lung pathology in seven-month-old lambs compared to five-week-old lambs upon experimental *M. ovipneumoniae* infection [90]. In our study, *M. ovipneumoniae* infection in four-month old lambs led to colonization in the absence of overt respiratory disease. In bighorn sheep, yearlings and older adults with no previous *M. ovipneumoniae* infection were equally susceptible to *M.*

ovipneumoniae-induced pneumonia [71]. Thus, age-dependent mechanisms alone do not explain the variable susceptibility of sheep to *M. ovipneumoniae* disease.

In bighorn sheep, introduction of *M. ovipneumoniae* into herds with no prior exposure to the pathogen leads to particularly devastating disease outbreaks, suggesting that immunologically naïve populations are especially prone to symptomatic infections. *In vitro* studies have shown that *M. ovipneumoniae* specific antibodies can have protective functions by mediating opsonization and phagocytosis of the *Mycoplasma* [93]. Likewise, Niang et al. [94] found increased levels of *M. ovipneumoniae*-reactive antibodies in lambs that had recovered from the clinical *M. ovipneumoniae* infection. In our study, lambs had no detectable *M. ovipneumoniae* specific antibodies at any point before experimental inoculation, consistent with the absence of *M. ovipneumoniae*-specific antibodies in the colostrum replacer and the lack of placental antibody transfer in sheep [95]. However, all inoculated lambs developed a significant, stable antibody response within two weeks after infection, confirming previous studies that have tracked humoral responses to *M. ovipneumoniae* [96]. These observations indicate that lack of existing immunity does not lead to more severe disease in *M. ovipneumoniae* infected sheep. Interestingly, the development of an antibody response was not associated with decreased *M. ovipneumoniae* colonization in our study. A possible explanation for this phenomenon is that antibody levels measured in serum consist mainly of IgG, which may have protective functions within the lungs, whereas a strong mucosal IgA response may be necessary to eliminate asymptomatic *M. ovipneumoniae* colonization from the upper respiratory tract. Antibody isotypes and mucosal antibodies were not analyzed in this study but may be a subject of future investigations.

Overall, our findings that *M. ovipneumoniae* application in domestic lambs did not cause significant respiratory disease were in line with earlier studies that showed that pneumonia develops as a result of *M. ovipneumoniae* co-infections with other facultative bacterial pathogens [61, 66, 70, 97]. In bighorn sheep, where a large number of studies have been performed, *M. ovipneumoniae* is likely essential for causing severe respiratory disease [98], but consistent clinical disease and lung pathology only developed when additional agents such as *Mannheimia haemolytica*, *Bibersteinia trehalosi*, *Pasteurella multocida* or *Fusobacterium necrophorum* [61, 64, 97] also were present. In further support of this hypothesis, the lambs that served as donors for the nasal wash fluids that we used to inoculate the experimental animals in our study showed clinical signs of respiratory disease and tested positive for *Mannheimia haemolytica* and *Bibersteinia trehalosi* in addition to the *M. ovipneumoniae*.

Even if *M. ovipneumoniae* alone does not cause clinical respiratory disease in domestic sheep, an effective treatment strategy that eliminates *M. ovipneumoniae* infection from domestic sheep flocks could reduce production losses due to complex respiratory disease and protect bighorn sheep from devastating pneumonia epizootics. To address this issue, we analyzed whether antibiotic therapy could eliminate *M. ovipneumoniae* colonization in our asymptotically infected lambs. We chose the macrolide antibiotic gamithromycin, because gamithromycin application in goats infected with *Mycoplasma* and *Mannheimia* was successful in treatment of clinical pneumonia [2], and preventive treatment with gamithromycin in cattle was successful for reducing bovine respiratory disease, which commonly involves *Mycoplasma* spp. [61]. A recent study by Jay *et al.* on the antibiotic susceptibility of *M. ovipneumoniae* showed homogenously low minimum inhibitory concentrations for a wide range of

antimicrobials including macrolides, with no indication of significant antimicrobial resistance [59]. Since gamithromycin is currently not approved for use in sheep in the US, the presence of resistant *M. ovipneumoniae* strains in the infected donor sheep was unlikely, and our *in vitro* analysis demonstrated that the strain used to infect the lambs in our experiment was indeed susceptible to gamithromycin. However, our *in vivo* data showed no significant reduction in *M. ovipneumoniae* colonization levels in our experimental lambs following two injections of the recommended gamithromycin dose. It remains to be investigated whether failure of the antibiotic treatment was due to bacterial biofilm formation [49], intracellular location of the *Mycoplasma* [99] or an alternative antimicrobial resistance mechanism. Notably, all lambs developed significant side effects following gamithromycin administration, including increased body temperatures, decreased appetites and altered behavior as well as local inflammation at the injection sites that warranted the application of analgesics. These side effects were greatly more severe than the transient mild to moderate swellings at the site of injection described for this drug by the European Medicines Agency [100].

Why domestic lambs tolerate *M. ovipneumoniae* infection, while bighorn lambs suffer severe clinical disease may thus be due to host genetics and will require further investigations. The inoculation approach that involved nasal, oral and ocular application of four-month-old domestic SPF lambs with ceftiofur-treated nasal washes from naturally infected, symptomatic lambs led to 100% successful upper airway colonization with *M. ovipneumoniae*, but not with *Pasteurellaceae*, confirming earlier studies [71, 78, 79]. Colonization levels as determined based on the Ct values in the qPCR reactions was similar in experimentally and naturally infected lambs, and antibody levels measured by *M. ovipneumoniae*-specific ELISA also closely

replicated antibody levels seen upon natural infection. Thus, our infection model represents a valid tool to investigate the role of *M. ovipneumoniae* in sheep respiratory disease. In future studies, we plan to utilize this *M. ovipneumoniae* infection model to explore novel preventative or treatment approaches and to define how interactions between *M. ovipneumoniae* and other facultative respiratory pathogens lead to clinical respiratory disease and pulmonary pathology in domestic sheep.

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Author Contributions

DB, MJ, ARA, TJ, KJ and TB designed the study; TJ, KJ, BTJ, JS, NA and IT performed the experiments; TJ, BTJ and DB analyzed the data; DB, TJ, ARA and MJ interpreted the data; DB obtained funding for the study; DB and TJ wrote the manuscript; all authors have read and approved the final manuscript.

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CHAPTER THREE

PATHOGEN DELIVERY ROUTE IMPACTS DISEASE
SEVERITY IN EXPERIMENTAL CO-INFECTION OF SHEEP
WITH *MYCOPLASMA OVIPNEUMONIAE* AND *MANNHEIMIA*
HAEMOLYTICA

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Abstract

M. ovipneumoniae is a respiratory pathogen that can cause mild to moderate pneumonia and reduced productivity in domestic lambs. However, studies on both natural and experimental *M. ovipneumoniae* infection have reported highly variable clinical signs and pathology. Here, we assessed the impact of administering *M. ovipneumoniae* to the upper respiratory tract (URT) or to the lower respiratory tract (LRT) of two-months-old specific pathogen-free lambs. Lambs were inoculated with PBS (control) or with ceftiofur-treated nasal wash fluid obtained from sheep with natural *M. ovipneumoniae* infection, monitored for eight weeks, and subsequently euthanized. All lambs in the URT and LRT groups developed a stable infection with *M. ovipneumoniae*. *M. ovipneumoniae* infection led to lower weight gains and mild respiratory disease, with significantly greater effects following LRT inoculation compared to URT inoculation. At necropsy, lambs inoculated via the LRT showed consolidation of the cranial and alveolar, bronchiolar, and interstitial inflammation that was significantly more severe in the LRT compared to the URT group. *M. ovipneumoniae* loads in the trachea and bronchi also were significantly higher after LRT than URT inoculation. Interestingly, 9/10 inoculated lambs also tested positive for *M. haemolytica* in nasal swab but not in bronchial swab samples. In summary, our study suggests that bypassing protective mechanisms of the URT by delivering respiratory pathogens to the LRT leads to more severe respiratory disease and lung damage than delivery to the URT. We also confirmed that *M. ovipneumoniae* significantly enhances the susceptibility of lambs to secondary respiratory pathogens such as *M. haemolytica*.

Keywords

Mycoplasma ovipneumoniae, *Mannheimia haemolytica*, sheep, pneumoniae, delivery route

Introduction

Mycoplasma ovipneumoniae is a respiratory pathogen of domestic and wild sheep that is associated with atypical pneumonia and decreased productivity in lambs [1, 7, 34] and that is highly common across the world [14, 58, 101-103]. However, results from experimental *M. ovipneumoniae* infections have been inconsistent, ranging from severe respiratory illnesses with weight loss and behavioral changes to asymptomatic infections [17, 34, 63, 69, 71, 104, 105]. These variable results have been partially attributed to the presence or absence of bacterial and viral co-infections [17, 106-108], differences in the virulence of *M. ovipneumoniae* strains [24, 34], and genetic differences in host susceptibility [109]. Current evidence from infection experiments and observational studies point to two potential outcomes of *M. ovipneumoniae* infection: Asymptomatic carrier status, with colonization of the upper airways and no clinical disease [24, 92, 105, 110], or clinical infection with atypical pneumonia, where *M. ovipneumoniae* is found in diseased lung tissue [2, 71, 105, 110]. While clinical pneumonia is generally an immediate concern, sheep that are asymptomatic carriers for *M. ovipneumoniae* commonly remain undetected. However, these animals play an important epidemiological role, since they can spread infection to susceptible lambs and cause pneumonia outbreaks [111, 112]. Despite these two disparate outcomes, how *M. ovipneumoniae* distribution in the respiratory tract impacts *M. ovipneumoniae* pathogenesis has not yet been systematically analyzed.

The upper respiratory tract (URT), which is comprised of the nasal cavity, pharynx, and cranial portions of the larynx, is the primary site of infection for most respiratory pathogens [113]. Under natural circumstances, respiratory pathogens can reach the lower respiratory tract (LRT) if respiratory defenses such as the mucociliary elevator are damaged due to previous infections or environmental stressors, or in association with physical exercise and exertion [114]. Natural *M. ovipneumoniae* infection is thought to occur via respiratory droplets or secretions following repeated close contact [34], but airborne transmission over a distance of >10 m also has been reported [71]. Upon initial infection, *M. ovipneumoniae* can damage the cilia of the respiratory epithelium [115], which then enables the *M. ovipneumoniae* as well as other pathogens to invade the lower respiratory tract. Ionas et al. proposed that different *M. ovipneumoniae* strains might preferentially colonize the lungs versus the nasal passages [116].

We hypothesized that infection of the lower respiratory tract, achieved experimentally through intratracheal inoculation, would result in clinical disease, whereas pathogen delivery to the upper respiratory tract would result in asymptomatic colonization. We present results from an experimental infection study performed in specific pathogen free (SPF) domestic lambs that were inoculated via either the URT or the LRT with antibiotic-treated nasal wash fluid from lambs naturally infected with *M. ovipneumoniae*. Our results indicate that delivering the infectious inoculum directly to the LRT resulted in increased pathogen load in the trachea and bronchi, decreased weight gains, increased clinical signs, and increased gross and microscopic respiratory tract pathology compared to pathogen delivery to the nasal and oral mucosae. Interestingly, both URT and LRT delivery of *M. ovipneumoniae* enabled expansion of previously undetectable

Mannheimia haemolytica in infected lambs from both groups, which likely contributed to the development of clinical disease in this study.

Materials and Methods

Animals and husbandry conditions

Fifteen two- to three-months-old mixed-breed lambs (nine males, six females) from Montana State University's (MSU's) SPF flock [24, 117] were used in this study. The lambs were born naturally to SPF ewes, weaned at approximately 6 weeks of age, and kept in outdoor paddocks with *ad libitum* access to water, hay, mineral and selenium supplements, and lamb starter pellets. All lambs were vaccinated with a multivalent clostridial vaccine (Covexin® 8, Merck). For experimental infections, the lambs were moved to the Johnson Family Livestock Facility (JFLF) ABSL-2 laboratory, where they were housed in heated animal rooms (15.5-16.8 °C) in pens with five (control group) or ten (*Mycoplasma*-infected group) animals each. The animals were fed a standard diet consisting of hay, lamb concentrate, and starter pellets. Clean water, mineral, salt, and selenium supplements were provided *ad libitum*. All animal care personnel changed into sterile personal protective equipment prior to entering the animal rooms, and personnel responsible for the lambs did not have any contact with other non-SPF sheep for the duration of the experiment. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of MSU, protocol #2022-158-95.

Preparation of inocula

Bacterial inocula containing *M. ovipneumoniae* were prepared from fresh nasal wash fluids of fifteen lambs naturally infected with respiratory pathogens, as described previously [24].

Briefly, *M. ovipneumoniae*-infected lambs at MSU's Red Bluff Research Ranch were selected based on prior positive tests results from nasal swab samples. Nasal washes were performed by squirting a total of 60 mL of sterile PBS into the lambs' nostrils and collecting the flush fluid into clean, single use polyethylene bags (ZipLock®). The nasal wash fluids were pooled, diluted with an equal amount of tryptic soy broth, incubated for 2 h at 37°C, concentrated by centrifugation at 10,000 x g for 30 min. Inocula were prepared by resuspending pellets from equal amounts of starting material in 20 mL of sterile PBS per dose for LRT inoculation and 50 mL per dose for URT inoculation. Aliquots of the untreated and the ceftiofur-treated nasal wash were sent to the Washington Animal Diseases Diagnostic Laboratory for bacteriological analysis and *M. ovipneumoniae* PCR.

Experimental design and sample collection

Fifteen experimental animals were randomly selected from a total of 22 SPF lambs born in our flock in 2022 and randomly assigned to three treatment groups consisting of two female and three male animals: (1) uninfected control; (2) upper respiratory tract (URT) infection with nasal wash fluid; and (3) lower respiratory tract (LRT) infection with nasal wash fluid. "Dam" was used as a blocking variable to ensure that siblings were placed in different groups. Random selection and random assignment was performed using the R package randomizr (v 0.22.0) [118]. Animal characteristics are provided in Table 3. There were no statistically significant differences in sex, litter size, starting weight or age. One week before the experimental start date (day -7), lambs were moved into two separate animal rooms (one for the control group and one for the URT and LRT groups) inside the JFLF. Husbandry staff wore sterile scrubs, head and face coverings, and latex gloves when entering rooms and always handled the control group before

entering the room with the URT and LRT groups. Lambs in the URT and LRT groups were inoculated with the infected nasal wash fluid, and control lambs received PBS. For URT infection, 30 mL of inoculum was administered into the lamb's nostrils, 10 mL into the conjunctival sacs, and 10 mL into oral cavity. For LRT infection, 20 mL of inoculum was administered directly into the distal trachea using a flexible fiber-optic endoscope measuring 6.6 mm x 100 cm (VFS-2B VetVu, Swiss Precision Products, Inc, Oxford, MA) under local anesthesia with lidocaine. Serum samples, heparinized blood samples, and PAXgene® tube (BD Biosciences) samples for RNA isolation were collected every two weeks by jugular venipuncture. Bronchoalveolar lavage fluid was collected at -1, 2, and 5 weeks *p.i.* (post inoculation) using a fiberoptic endoscope, and cell concentration was determined with a Neubauer counting chamber. At the experimental endpoint, all lambs were euthanized and transferred to the Montana Veterinary Diagnostic Laboratory (MVDL) for necropsy and tissue collection. One lamb in the URT group developed a severe urinary tract disease that did not respond to treatment and was therefore euthanized during week five of the study, and no endpoint samples were collected.

Table 3. Characteristics of experimental animals

Experimental group	N	Treatment	Route	Males	Litter size	Weight at start (kg)	Age at start (d)
Control	2	PBS	URT	3	2.2±0.4	27.7±9.1	64.0±11.1
	3	PBS	LRT				
URT ¹	5 (4) ³	Nasal wash	URT	3	1.6±0.5	28.5±4.5	59.8±7.2
LRT ²	5	Nasal wash	LRT	3	1.6±0.9	31.2±6.5	67.2±8.6
<i>P</i> value				> 0.99	0.288	0.709	0.460

¹ Inoculation of upper respiratory tract via nasal, conjunctival, and oral mucosae.

² Inoculation of lower respiratory tract via endoscope.

³ One lamb was euthanized prior to the experimental endpoint due to urinary tract disease. Groups were compared using one-way ANOVA with Tukey's multiple comparisons test.

Assessment of clinical signs and other animal characteristics

Following experimental *M. ovipneumoniae* infection or mock treatment, lamb health status was assessed twice daily by trained JFLF personnel, as previously described [24, 117]. Rectal temperatures were measured twice a week and whenever an animal showed any signs of disease. The following health parameters were assessed and were used to calculate a clinical disease score: (1) respiratory signs; (2) general behavior; (3) appetite, and (4) any medications administered exclusive of the experimental antibiotic treatment as detailed in Supplemental Figure 14.1. Daily scores represent the sum of the two values obtained for each day. Body temperatures were measured twice per week. Lamb body weights were determined weekly using a digital scale.

Evaluation of pathological and histopathological changes

Sheep were necropsied at the MVDL within 30 min of euthanasia, and the respiratory tract including lungs and the pleural cavity were evaluated for pathological changes by trained veterinary pathologists (S.S. and J.S.). Representative hematoxylin/eosin-stained formalin-fixed paraffin-embedded sections of the cranial and caudal right and left lung lobes were scored for the presence of histopathological alterations. The scoring criteria were based on a publication by Passmore et al. [119], with slight modifications (see Table 4), and the pathologist was blinded to the experimental groups.

M. ovipneumoniae serology

Table 4. Scoring criteria for histopathological assessment of lung tissues, based on Passmore 2018, Respiratory Research [119]

Score	Pulmonary edema and congestion	Alveolar and interstitial inflammation	Bronchiolar inflammation	BALT hyperplasia
0	Minimal/Normal	Minimal/Absent	Minimal/Normal	None
1	Mild edema and/or congestion	Mild inflammatory exudate	Mild infiltration of inflammatory cells	Mild
2	Moderate edema and/or congestion	Moderate inflammatory exudate	Moderate infiltration of inflammatory cells	Moderate
3	Severe edema and congestion ± intra-alveolar hemorrhage	Marked-severe inflammatory exudate	Marked/severe infiltration by inflammatory cells, ± epithelial damage	Marked

Analysis of serum samples for the presence of *M. ovipneumoniae*-reactive antibodies was performed at the Washington Animal Disease Diagnostic Laboratory (WADDL) using the laboratory's competitive enzyme-linked immunosorbent assay (cELISA) test, which has a diagnostic sensitivity of 88% and a diagnostic specificity of 99.3%. Data are shown as % inhibition and represent the reduction in binding of the labelled monoclonal antibody to the *M. ovipneumoniae* test antigen caused by competitive binding of serum antibodies from the diagnostic samples.

Detection of *M. ovipneumoniae* and Pasteurellaceae

Flocked polyester swabs (Puritan) were stored at -20°C in transport medium composed of DMEM with 15% glycerol, and aliquots were shipped on dry ice to WADDL. Standard microbiological assays, performed at WADDL, were used to detect and identify *Pasteurellaceae*. A subset of samples that tested positive for *M. haemolytica* also was tested for the presence of leukotoxin by PCR [120]. *M. ovipneumoniae* was detected by quantitative PCR (qPCR) using WADDL's standard diagnostic assay. In addition, qPCR detection of *M. haemolytica* was performed in our laboratory using a protocol previously described by Shanthalingam et al. 2014

[120]. Primer sequences are provided in Table 5. All PCR data are expressed as the 40 -cT value of the sample.

Table 5. Primer sequences.

Target	Sequence (5'-3')	Product size	References
<i>M. ovipneumoniae</i> p113		295 bp	[24, 82]
Forward	GCG GTG CGC AAC ATT AGT TA		
Reverse	CTT ACT GCT GCC TCC CGT AG		
<i>M. haemolytica</i> o-sialoglycoprotein endopeptidase		227 bp	[120]
Forward	TGG GCA ATA CGA ACT ACT CGG G		
Reverse	CTT TAA TCG TAT TCG CAG		
<i>M. haemolytica</i> Leukotoxin (<i>lktA</i>)		497 bp	[120]
Forward	CTT ACA TTT TAG CCC AAC GTG		
Reverse	TAA ATT CGC AAG ATA ACG GG		

Analysis of cytokine and inflammatory marker gene expression

Whole blood collected into PAXgene® tubes was processed for RNA isolation and qRT-PCR analysis as previously described [121]. Lung tissue collected at the experimental endpoint from two regions of the cranial lung and two regions of the caudal lungs was frozen at -80 °C and stored until analysis. Total RNA was extracted from homogenized tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen; Germantown, MD, USA). To remove contaminating genomic (g)DNA, the spin columns were treated with RNase-free DNase I (DNase, Amersham Pharmacia Biotech Inc.; PA, USA) at 20 °C for 15 min. cDNA synthesis was performed on 2 µg of RNA template from each tissue. Reaction conditions included 4 U reverse transcriptase (Omniscript, Qiagen, Valencia, CA, USA), 1 µM random hexamers, 0.5 mM of each dNTP, and 10 U RNase inhibitor, in RT buffer (Qiagen, Valencia, CA, USA). Reactions were incubated for 60 min at 37 °C, followed by an enzyme inactivation step of 5 min at 93 °C, and then stored at -20 °C until further analysis. Expression of the following genes was measured: aryl hydrocarbon receptor (*AHR*), heat shock protein 70 (*HSP70*), myeloid differentiation primary response 88 (*MYD88*),

myxovirus resistance-1 (*MX1*), interferon- γ (*IFNG*), interleukin-1 β (*IL1B*), *IL10*, *CD69*, Fas-associated death domain (*FADD*), AMP-activated protein kinase (*AMPK*), tumor necrosis factor- α (*TNFA*), GATA binding protein 3 (*GATA3*), T-box expressed in T cells (*TBX21*), and transforming growth factor- β (*TGFB*). Primer sequences are provided in [121]. Data were analyzed using the delta-delta Ct method ($2^{-\Delta\Delta C_t}$), with S9 used as housekeeping gene. Gene expression for each animal was normalized to values obtained immediately prior to inoculation (week 0) for blood samples, or to the average gene expression of the control group for tissue samples.

Statistical analyses

Data were analyzed using R Statistical Software (v4.3.3; R Core Team 2024) [122] and GraphPad Prism 10.1.2 (Boston, MA) and are shown as mean $\pm$ SD unless stated otherwise. Data for the total health score, percent weight gain, and temperature models was processed into data structures with the tidyverse package (v2.0.0) [123], the linear mixed-models were run with the nlme package (v3.1.164) [124], and the negative binomial mixed-model was run with the MASS package (v7.3.60.1) [125]. Mixed model tables were generated with the sjPlot package (v2.8.1.5) [126]. Plots related to these models were generated with the ggplot2 package (v3.5.0) [127] and ggthemes package (v5.1.0) [128]. Other data types were analyzed for statistically significant differences using one-or two-way ANOVA or mixed effects analysis with Tukey's or Dunnett's multiple comparisons test, or with the non-parametric Wilcoxon's signed ranks test.

In the total health score statistical modeling analysis, we used a generalized linear model with a negative binomial distribution with fixed effects for sex, age at infection, and the interaction terms between week post inoculation (*p.i.*) and treatment.

The model for total health score can be written as follows:

$$\begin{aligned} \log(\mu_i) &\sim \beta_1 X_{Sex_i} + \beta_2 X_{age\ at\ infection_i} + \beta_3 X_{Week_i} + \beta_4 X_{LRT_i} + \beta_5 X_{URT_i} + \beta_6 X_{Week} X_{LRT_i} \\ &\quad + \beta_7 X_{Week_i} X_{URT_i} \\ TotalScore_i &\sim NB(\mu_i, \phi) \end{aligned}$$

Where i represents the observation. The overdispersion parameter with respect to the Poisson distribution is estimated to be 0.76.

Percent weight gain

For weight gain, we selected a mixed-effect model with fixed effects for sex, age at infection, and the interaction terms between week and treatment. Weight gain was calculated as the percent change compared to baseline weights at 1 week before inoculation. The model for weight percentage can be written as follows:

$$\begin{aligned} PercentWeight_i &\sim \alpha_{m[i]} + \delta_{j[i]} + \beta_1 X_{Sex_i} + \beta_2 X_{age\sim at\sim infection_i} + \beta_3 X_{Week_i} \\ &\quad + \beta_4 X_{Lower_i} + \beta_5 X_{Upper_i} + \beta_7 X_{Week} X_{Lower_i} + \beta_8 X_{Week_i} X_{Upper_i} + \epsilon_i \end{aligned}$$

$$\alpha_m \sim N(0, \sigma_{ewe}^2)$$

$$\delta_j \sim N(0, \sigma_{lambID}^2)$$

$$\epsilon_i \sim N(0, \sigma^2 e^{2\theta * X_{Age\ at\ Infection_i}})$$

$$cor(\epsilon_s, \epsilon_t) = \begin{cases} 1 & \text{if } s = t \\ \rho^{|t-s|} & \text{otherwise} \end{cases}$$

Where i represents the individual observation (% weight change), m represents the ewe, j represents the lamb ID, and t represents the time in weeks.

Body temperature

For body temperature post inoculation, we chose a mixed-effect model with fixed effects for sex, age at infection and the interaction terms between week and treatment. The model for body temperature can be written as follows:

$$\begin{aligned} Temperature_i \sim & \alpha_{m[i]} + \delta_{j[i]} + \beta_1 X_{Sex_i} + \beta_2 X_{age\ at\ infection_i} + \beta_3 X_{Week_i} \\ & + \beta_4 X_{Lower_i} + \beta_5 X_{Upper_i} + \beta_7 X_{Week_i} X_{Lower_i} + \beta_8 X_{Week_i} X_{Upper_i} + \epsilon_i \end{aligned}$$

$$\alpha_m \sim N(0, \sigma_{ewe}^2)$$

$$\delta_j \sim N(0, \sigma_{lambID}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation (body temperature), m represents the ewe, and j represents the lamb ID.

Results

Increased pathogen load in the lower respiratory tract following lower-respiratory tract inoculation of lambs with *Mycoplasma ovipneumoniae*.

In a previous study, we showed that experimental *M. ovipneumoniae* infection, achieved by inoculating the nasal, oral and conjunctival mucosae of specific pathogen-free lambs with ceftiofur-treated nasal wash fluid from conventional lambs with natural *M. ovipneumoniae* infection, caused chronic colonization of the nasal cavity in the absence of clinical signs [24]. Here, we compared inoculation of the nasal, oral, and conjunctival mucosae, i.e., the upper respiratory tract (URT), to endoscopic intratracheal inoculation of the lower respiratory tract

(LRT; Figure 7A). Analysis of nasal swab samples for *M. ovipneumoniae* showed that both URT and LRT delivery of *M. ovipneumoniae*-positive, antibiotic-treated nasal wash fluid resulted in consistent colonization of the nasal mucosae with *M. ovipneumoniae* over eight weeks (Figure 7B). Interestingly, swab samples collected at necropsy from both URT (nose and nasopharynx) and LRT (bronchi, and trachea) at eight weeks *p.i.* were positive for *M. ovipneumoniae* in lambs from both infection groups, suggesting that inoculation of the URT also leads to infection of the LRT, and vice versa (Figure 7C). However, *M. ovipneumoniae* pathogen load in the bronchi and trachea, i.e., the LRT, was significantly higher in the LRT than the URT group, confirming that delivery route influenced pathogen distribution. None of the samples from control lambs that had received PBS tested *M. ovipneumoniae*-positive at any time during the study. We also analyzed sera collected throughout the study for antibodies to *M. ovipneumoniae* to elucidate whether pathogen delivery route affected the induction of humoral immunity. Both URT and the LRT inoculation with *M. ovipneumoniae* led to significant induction of anti-*M. ovipneumoniae* antibodies within two weeks that plateaued by six weeks *p.i.* and remained high at eight weeks *p.i.* (Figure 7D). There was a non-significant trend for higher antibody responses in the LRT compared to the URT groups. Bronchoalveolar lavage fluids (BALF) were collected at one week before and at two and five weeks *p.i.*, and total cell counts were determined. No significant differences in total cell counts were found between experimental groups at -1 and +2 weeks. However, at five weeks post infection, BALF cell counts were significantly higher in the LRT group ($2.47 \pm 0.91 \times 10^6$) compared to the control group ($0.88 \pm 0.55 \times 10^6$, $P=0.0073$), with intermediate cell numbers recovered in the URT group ($2.04 \pm 0.85 \times 10^6$; Figure 7E). These data

indicate that pathogen delivery route alters pathogen distribution across the respiratory tract and that LRT delivery of *M. ovipneumoniae* induces an increased local response.

Lower respiratory tract inoculation of lambs with *M. ovipneumoniae*-positive nasal wash fluid leads to more severe clinical signs and reduced weight loss.

To assess the impact of *M. ovipneumoniae* infection on growth and productivity, we measured body weights of the experimental animals weekly over the course of the study. Interestingly, lambs in the LRT group gained less weight by week than lambs in control group, while the URT group had intermediate weight gains (Figure 8A). Statistical analysis of these data was performed using a mixed linear model (Table 4, Supplemental Figure 13). The model showed strong evidence for an interaction between week and LRT treatment that corresponded to a 3% decrease in weight gain compared to the control animals (Wald's test, $P < 0.001$) after accounting for sex and age at infection. Interestingly we also observed strong evidence to suggest that the age at infection was associated with a 1% decrease in percent weight gain (Wald's test, $P < 0.01$) after accounting for week, sex, and age at infection.

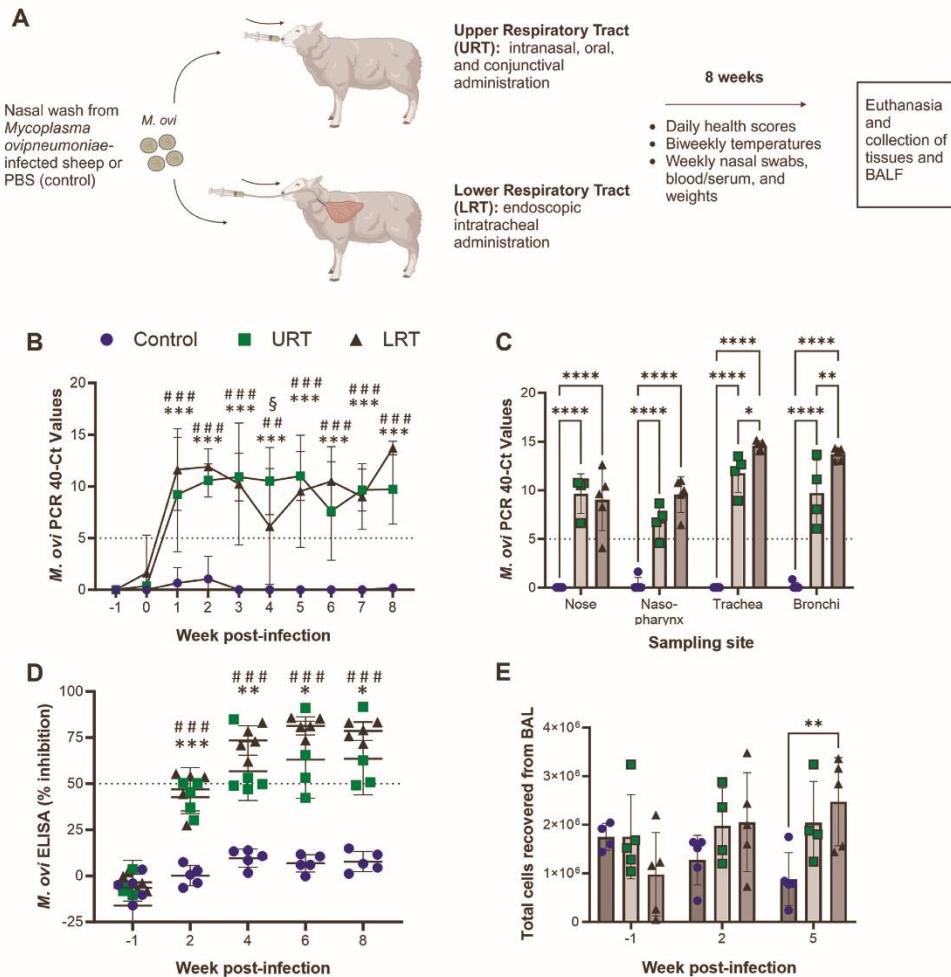


Figure 7. Both upper and lower respiratory tract pathogen delivery leads to stable colonization with *M. ovipneumoniae*. A Experimental design. Five three-month-old lambs per group were inoculated with PBS or with Ceftiofur-treated nasal wash fluid collected from sheep with natural *M. ovipneumoniae* infection via the upper (URT) or the lower respiratory tract (LRT) and then monitored over eight weeks. Created with BioRender.com. B *M. ovipneumoniae* infection levels in nasal swab samples were determined by qPCR. Data are shown as 40 minus Ct value. Mean $\pm$ SD. Statistically significant differences between control and URT (***) $P \leq 0.001$, control and LRT (###) $P \leq 0.001$, and LRT and URT (§) $P \leq 0.05$ were determined by mixed-effects analysis with Tukey's multiple comparisons test. C *M. ovipneumoniae* infection levels in swab samples collected at necropsy from the nose, nasopharynx, trachea, and bronchi were determined by qPCR. Individual data points, mean $\pm$ SD. Statistically significant differences (**** $P \leq 0.0001$, ** $P \leq 0.01$, * $P \leq 0.05$) were determined by 2-way ANOVA with Tukey's multiple comparisons test. D *M. ovipneumoniae*-specific antibody levels were determined by competitive ELISA analysis of serum samples before (week-1) and after URT or LRT inoculation with *M. ovipneumoniae* or PBS (control). Statistically significant differences between control and URT (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$), or control and LRT (### $P \leq 0.001$) were determined by mixed-effects analysis with Tukey's multiple comparisons test. Individual data points, mean $\pm$ SD. E Total cell counts in bronchoalveolar lavage fluid (BAL) collected via endoscope one week

before and two or five weeks after experimental inoculation of lambs. Individual data points, mean $\pm$ SD. Statistically significant differences between control and LRT (## $P \leq 0.01$) were determined by 2-way ANOVA with Tukey's multiple comparisons test.

Table 6. Weight gain model

Predictors	Estimates	CI	<i>P</i>
(Intercept)	1.42	1.17 to 1.67	< 0.001
Sex [m]	0.03	−0.38 to 0.44	0.538
Age at infection	−0.01	−0.01 to −0.00	0.009
Week	0.07	0.06 to 0.08	< 0.001
Treatment [URT]	−0.00	−0.58 to 0.58	0.998
Treatment [LRT]	−0.07	−0.72 to 0.58	0.398
Treatment [URT] x Week	0.00	−0.01 to 0.02	0.702
Treatment [LRT] x Week	−0.03	−0.04 to −0.01	< 0.001
N _{Ewe}	11		
N _{lambID}	15		
Observations	130		

Mixed linear model coefficients for weight development based on percent weight change. *P* values considered statistically significant are bolded.

We also monitored lamb health, behavior and food intake two times per day, using our previously published scoring system [24] with slight modifications (Supplementary Table 10). In contrast to our earlier study [24], all lambs that had received the *M. ovipneumoniae*-containing nasal wash fluid developed significant respiratory disease (Figure 8B,C). Total clinical scores in the LRT group peaked at two weeks post infection, with a second peak seen at about 7 weeks p.i. (Figure 8B). In the URT group, clinical scores peaked at 4 and 7 weeks p.i. (Figure 8B). In particular, respiratory scores, representing incidences of coughing, nasal discharge and/or labored breathing, were significantly higher for infected lambs compared to uninfected controls, again with highest scores seen following LRT inoculation, but with a peak at six weeks post infection (Figure 8C). Conversely, no apparent changes in behavior, appetite, or administered

medications were detected, although the control lambs consistently scored lower than the infected lambs (Figure 8B-D, Supplemental Figure 14A-C). Using a mixed linear model that accounts for sex, week *p.i.*, and age at infection (Table 5, Supplemental Figure 13), we found a 31.5-times increase in the total score between the control group and the LRT group (Wald's test, $P < 0.001$) and a 15.5-times increase in total score between the control group and the URT group (Wald's test, $P < 0.001$). We also noted that males had a 34% increase in total score (Wald's test, $P < 0.01$) after accounting for week, treatment, and age at infection (Supplemental Figure 13, Table 5). There was also moderate evidence for an interaction between week *p.i.* and treatment

Table 7. Health score model

Predictors	Incidence rate ratios	CI	<i>P</i>
(Intercept)	0.02	0.00–0.23	0.001
Sex [m]	1.41	0.88–2.27	0.157
Age at infection	1.00	0.97–1.03	0.828
Treatment [URT]	15.16	5.12–44.86	< 0.001
Treatment [LRT]	29.18	10.00–85.16	< 0.001
Week	0.81	0.61–1.08	0.148
Treatment [URT] x Week	1.38	1.03–1.85	0.029
Treatment [LRT] x Week	1.38	1.04–1.85	0.028
Observations	1840		

Mixed linear model coefficients for health scores. *P* values considered statistically significant are bolded.

that led to a 37% increase in total health scores for the URT group (Wald's test, $P = 0.030$) and a 35% increase in total health scores for the LRT group (Wald's test, $P = 0.039$; Supplemental Figure 13, Table 5).

Lambs in the LRT group also had increased incidences of elevated body temperatures that exceeded 104 °F (40 °C) at multiple time points, whereas body temperatures remained in the

normal range in the URT and control groups (Figure 8D). In our linear mixed model for temperature (Table 8), there was no significant evidence for a difference in temperature between the control animals and the URT or LRT treatments (Wald's test, $P > 0.05$), but moderate evidence for a 0.07 degree decrease in temperature over time (Wald's test, $P = 0.038$) after accounting for treatment, age at infection, and sex. In summary, data analysis using a mixed linear model confirmed that the site of pathogen introduction to the respiratory tract can significantly impact disease severity in *M. ovipneumoniae*-infected lambs.

Table 8. Body temperature model

Predictors	Estimates	CI	<i>P</i>
(Intercept)	102.78	101.43–104.12	< 0.001
Sex [m]	−0.13	−2.04–1.77	0.540
Age at infection	0.01	−0.02–0.03	0.457
Treatment [URT]	−0.05	−3.08–2.98	0.865
Treatment [LRT]	0.69	−2.29–3.68	0.208
Week	−0.07	−0.13 to −0.00	0.038
Treatment [URT] x Week	0.03	−0.06–0.12	0.529
Treatment [LRT] x Week	−0.07	−0.16–0.02	0.148
N _{Ewe}	11		
N _{lamblD}	15		
Observations	241		

Mixed linear model coefficients for body temperatures. *P* values considered statistically significant are bolded.

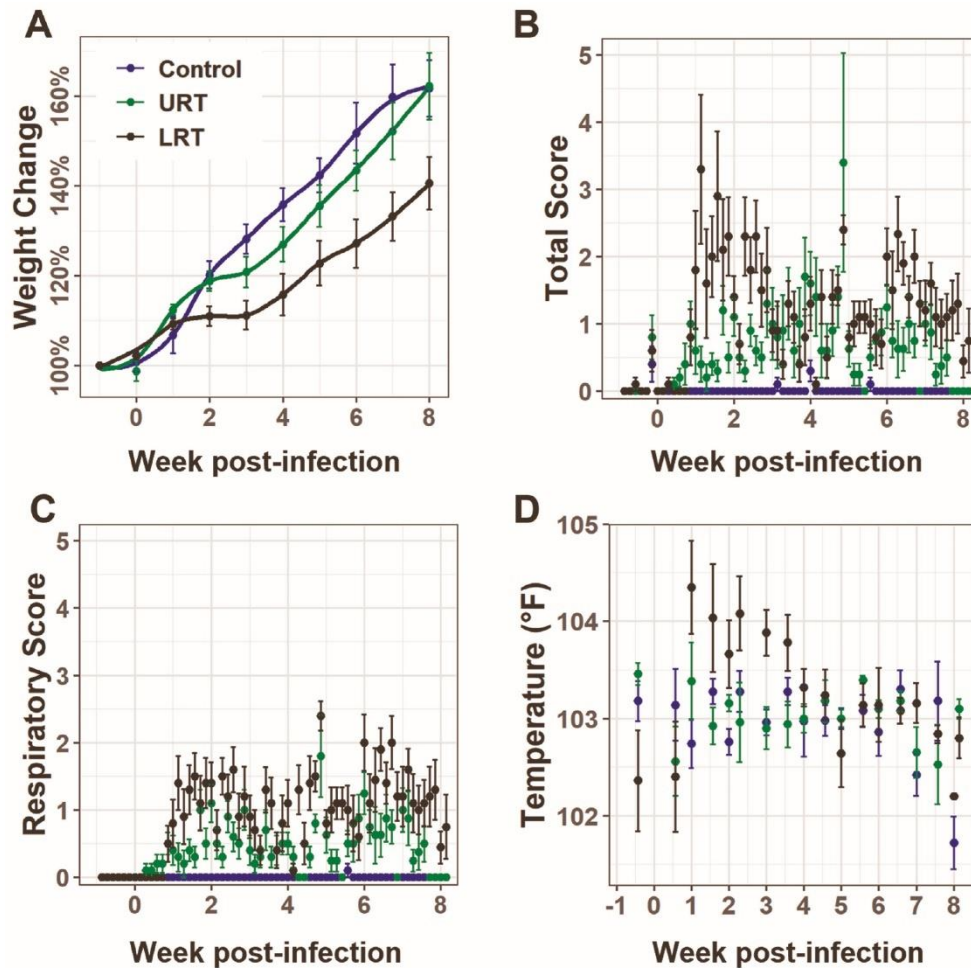


Figure 8. Lower respiratory tract inoculation with *M. ovipneumoniae* leads to increased respiratory disease and decreased weight gains compared to upper respiratory tract inoculation. A Development of lamb body weights over time, shown as percent change from baseline weight measured one week before inoculation with *M. ovipneumoniae* or PBS. Graph shows predicted mean (line) and SD. B, C All lambs were screened twice daily for signs of respiratory disease, for changes in behavior and appetite, and administered medications were also recorded (see Supplementary Table 10 for scoring rubric). Daily scores were the sum of the two individual measurements. B Total health scores, calculated as the sums of the daily scores in the four categories. C Respiratory scores. D Biweekly rectal temperatures measured in the three different treatment groups. Graphs show mean $\pm$ SD of five lambs per group.

Lower respiratory tract inoculation of lambs with *M. ovipneumoniae* was associated with gross and microscopic lung pathology.

Lungs collected after euthanasia at 8 weeks *p.i.* were analyzed for gross and microscopic abnormalities. The respiratory tract of the control lambs had no visible abnormalities. In contrast,

enlarged tracheobronchial lymph nodes were found in three out of four lambs in the URT inoculation group, and patchy areas of consolidated lung tissue were present in one lamb (Figure 9A,B). More strikingly, all lambs in the LRT inoculation group (5/5) had consolidated cranial lung lobes, three had enlarged tracheobronchial nodes, and two had fibrotic lung adhesions, all indicative of significant pneumonia (Figure 9A,B).

These observations were confirmed by histopathological analysis of lung tissue and bronchial regions (Figure 10 and Table 9). A significant increase in interstitial, alveolar, and bronchiolar inflammation was detected in the cranial lungs of the LRT group compared to the control group. Cranial lungs of the URT group also had significantly increased alveolar and interstitial inflammation compared to the control group, with a trend for increased bronchiolar inflammation (Figure 10A,B). Some bronchioles in lambs from the LRT infection group were filled with large numbers of neutrophilic granulocytes, indicative of significant acute inflammation (Figure 10B, Table 9). We also observed inflammatory infiltrates around the bronchi of *M. ovipneumoniae*-infected lambs that were mild in the URT group, moderate to severe in the LRT group, but absent from healthy or control lambs (Figure 10C). Interestingly, bronchus-associated lymphoid tissue (BALT) developed to a similar extent in both the URT and LRT groups but was generally absent from lungs of control animals (Figure 10D, Table 9). No significant differences were found for any group or parameter for the caudal lung lobes, indicating that the disease was limited to the cranial lung (data not shown). Overall, these data indicate that the increased pathogen load in the lower respiratory tract of lambs in the LRT inoculation group was associated with more severe respiratory pathology, but that BALT was induced independent of inoculation route or pathogen load.

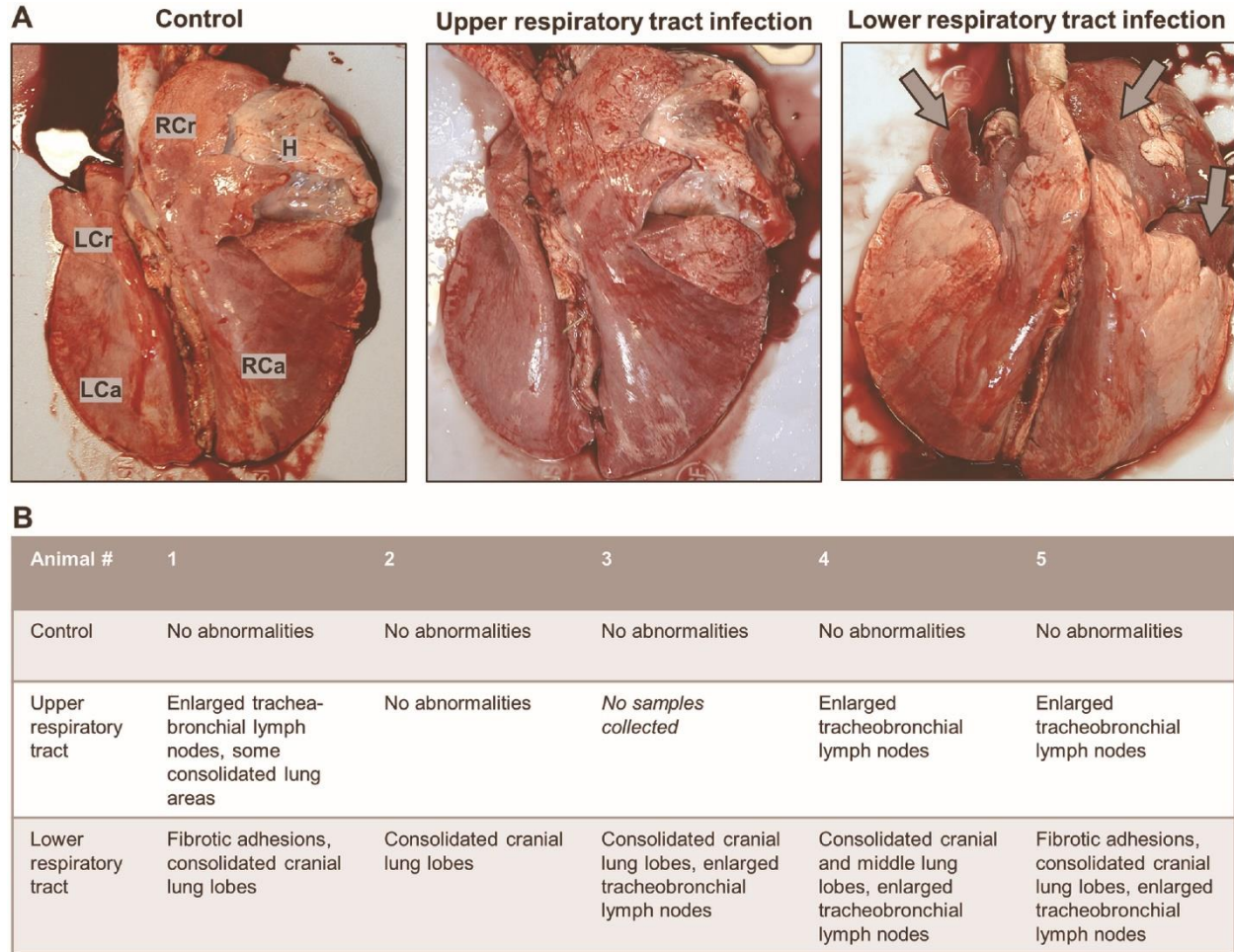


Figure 9. Increased gross pathological changes in cranial lung lobes of lambs after lower respiratory tract, but not upper respiratory tract inoculation with *M. ovipneumoniae*. (A) Representative lungs from lambs with upper and lower respiratory tract inoculation at eight weeks post inoculation with PBS or with *M. ovipneumoniae*. LCr—left cranial lung, RCr—right cranial lung, LCa—left caudal lung, RCa—right caudal lung, H heart. Arrows in the right panel point to consolidated lung tissue. (B) Gross pathological findings for lungs from individual animals at necropsy. One animal in the URT group developed a urinary tract disease and was euthanized prior to the experimental endpoint, so that no samples could be collected.

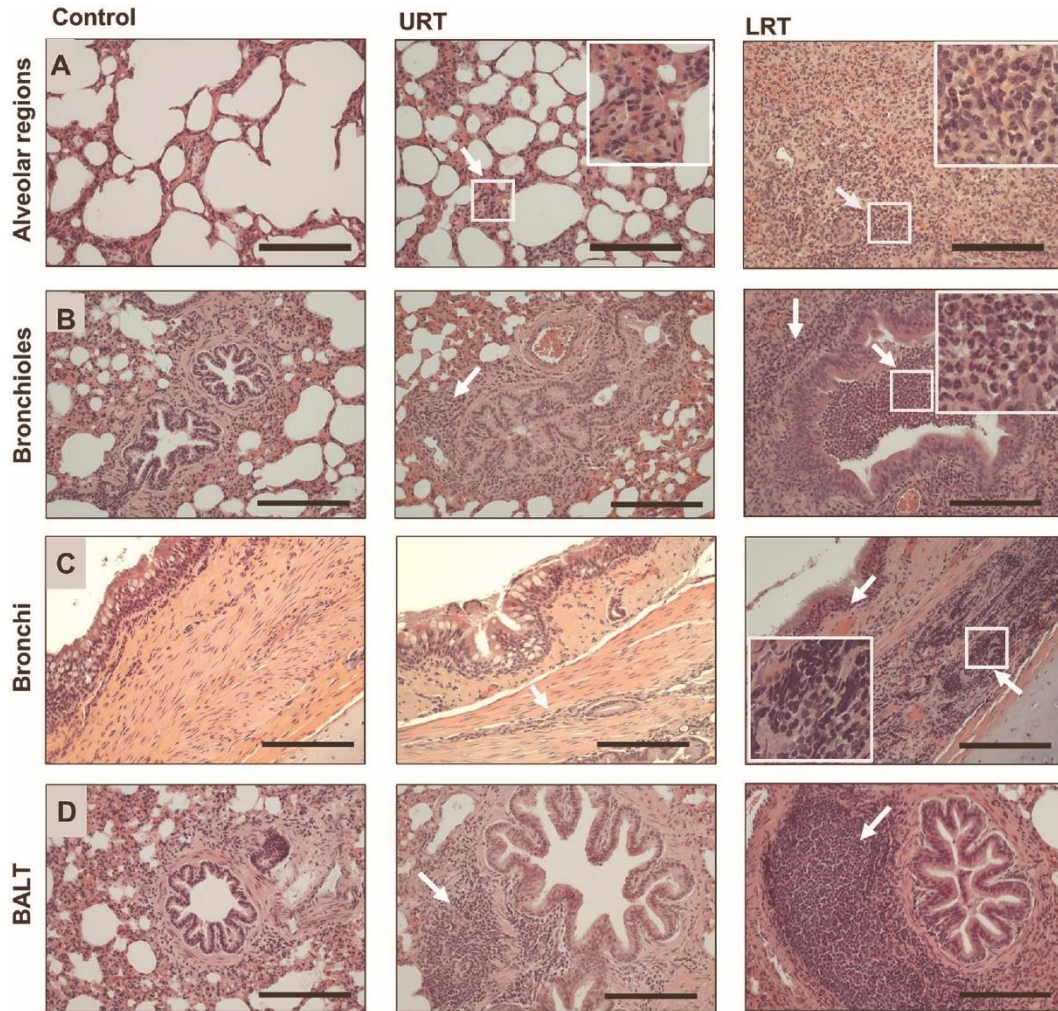


Figure 10. Increased histopathological changes in cranial lung lobes of lambs after lower respiratory tract compared to upper respiratory tract inoculation with *M. ovipneumoniae*. Histopathological analysis of paraffin-embedded, H&E-stained sections of respiratory tract tissues collected upon necropsy at eight weeks post inoculation. (A) Representative images of the alveolar regions with interstitium show healthy tissue from a representative control lamb. Samples from *M. ovipneumoniae*-infected animals show thickening of the alveolar septa and infiltration of alveolar spaces with neutrophils and mononuclear cells that were mild to moderate in the URT group and severe in the LRT group. (B) Bronchioles show peribronchiolar lymphoid cuffs in the URT and LRT groups and severe luminal infiltration with neutrophils and necrotic material in the LRT group. (C) Severe submucosal inflammatory infiltrates in the bronchial submucosa of a lamb from the LRT group. Some infiltrating immune cells are also present in tissue from the URT group. (D) Formation of bronchus-associated lymphoid tissue (BALT) is observed in the URT and LRT groups, but not in the control group. All bars are 100 μ m.

Table 9. Histopathological scoring of lungs from sheep infected with PBS (control) or with *M. ovipneumoniae* containing nasal-wash fluids through inoculation of the upper (URT) or lower respiratory tract (LRT)

	Control (n = 5)	URT (n = 4)	LRT (n = 5)	P-value*
Pulmonary edema and congestion				
Cranial	3.4 ± 1.1	3.8 ± 0.5	3.2 ± 0.8	0.6631
Caudal	3.2 ± 0.8	3.5 ± 0.6	3.6 ± 0.9	0.7212
Alveolar and interstitial inflammation				
Cranial	0.8 ± 1.1 ^a	2.8 ± 0.5 ^b	5.0 ± 1.2 ^c	0.0002
Caudal	0.2 ± 0.4	0.8 ± 1.0	1.0 ± 0.0	0.1207
Bronchiolar inflammation				
Cranial	0.2 ± 0.4 ^a	1.5 ± 0.6 ^a	4.4 ± 1.5 ^b	0.0001
Caudal	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4	0.4406
BALT hyperplasia				
Cranial	0.6 ± 0.5 ^a	3.3 ± 1.0 ^b	4.2 ± 0.8 ^b	<0.0001
Caudal	0.4 ± 0.5	1.0 ± 0.0	1.2 ± 0.8	0.1406
Total score	8.8 ± 3.1 ^a	11.3 ± 1.3 ^a	22.8 ± 4.1 ^b	<0.0001

* ANOVA analysis. P values considered statistically significant are bolded.

^{a,b,c} Different letters denote statistically significant differences ($P < 0.05$) in Tukey's pairwise comparisons test.

M. ovipneumoniae infection induced significantly increased IFN- γ gene expression in the blood and lungs.

A transcriptomics panel for fourteen immune related genes was applied to tissue samples from cranial and caudal lungs at necropsy (8 weeks p.i.) and to blood samples collected at 0, 2, 4, 6, and 8 weeks p.i. [121]. Interestingly, *M. ovipneumoniae* infection was associated with increased expression of the Th1 polarization marker IFN- γ in all three types of samples (Figure 11). For blood samples, no clear trends over time were apparent, so that gene expression from the 2, 4, 6, and 8 week time points relative to baseline expression at week 0 was averaged for each animal (Figure 11A). Blood samples from LRT lambs showed a significant increase in IFN- γ

gene expression compared to the URT group ($P \leq 0.05$) and compared to the control group ($P \leq 0.001$). In addition, there was a trend for increased expression of the T cell activation marker *CD69* in blood from both URT and LRT lambs compared to the control group, and a trend for increased expression of the anti-inflammatory cytokine *IL10* in lambs from the URT group alone that was also seen in cranial lung tissue. Interestingly, both cranial and caudal lung tissue from lambs inoculated via the URT showed significantly increased expression of *IFNG* compared to the control group (Figure 11B, C). *IFNG* expression also was increased in the LRT group, but this was not significant. No other significant changes were detected. Both cranial and caudal lungs from URT and LRT lambs showed a trend for increased expression of *CD69*. Cranial lung tissue from URT and LRT lambs also showed a trend for increased expression of the Th1 transcription factor *TBET* and the pro-inflammatory cytokines *TNFA* and *IL1B*. Overall, these data suggest that *M. ovipneumoniae* infection is associated with Th1 polarization both locally and systemically.

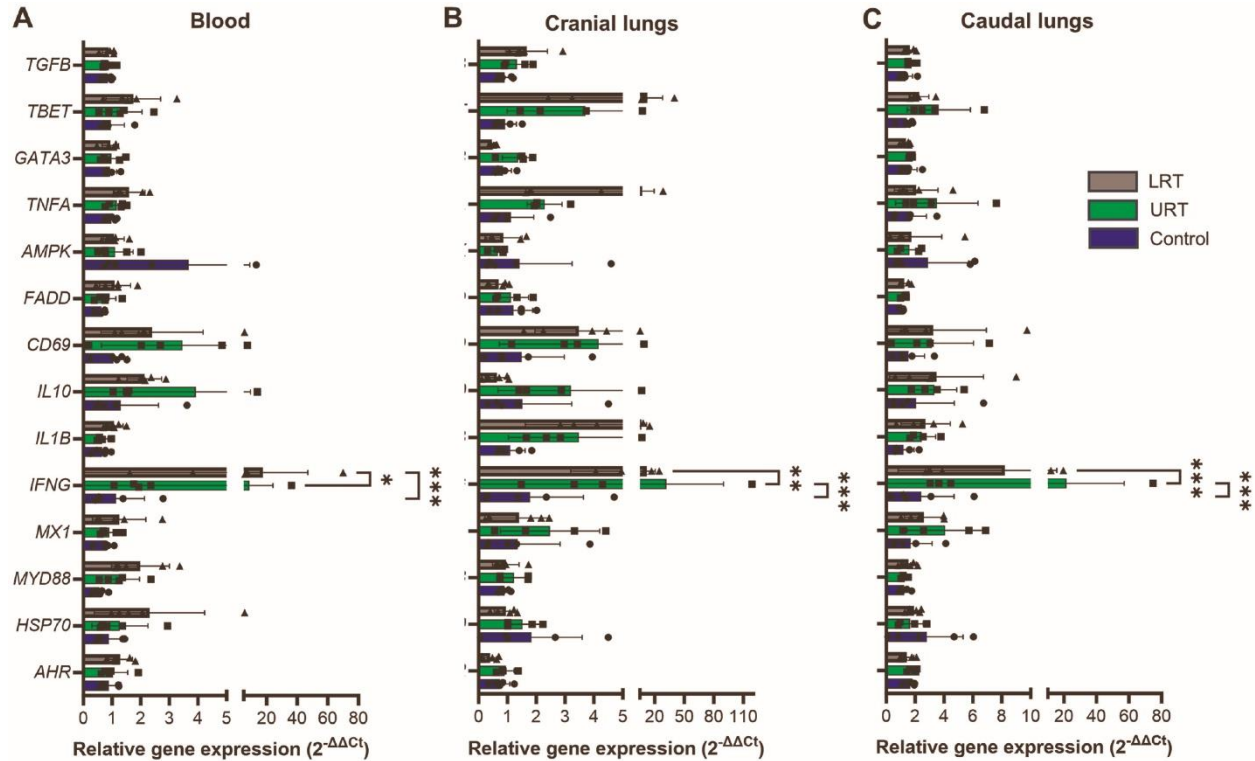


Figure 11. *M. ovipneumoniae* infection drives IFN- γ gene expression in blood and lungs. Gene expression analysis for a panel of immune related genes [121] was performed using quantitative RT-PCR, with the delta-delta Ct method for data analysis. (A) Whole blood samples were collected at weeks 0, 2, 4, 6 and 8 pi. Datapoints for each lamb represent average expression for weeks 2, 4, 6 and 8 pi normalized to the baseline samples collected at week zero before inoculation. (B, C) RNA was isolated from cranial and caudal lung tissues collected at necropsy (week 8 pi). Data were normalized to the average gene expression of the control group. For all three panels, individual data points, mean and SD are shown. Data were analyzed by 2-way ANOVA with Tukey's multiple comparisons test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

Inoculation of specific pathogen free domestic lambs with ceftiofur-treated nasal wash fluid resulted in co-infection with *M. ovipneumoniae* and *Pasteurellaceae*.

Given that our previous studies investigating monoinfection with *M. ovipneumoniae* resulted in subclinical colonization [24, 117], whereas the lambs exhibited significant clinical signs in the current experiment, we asked whether additional respiratory pathogens were present that might have contributed to disease pathogenesis. The SPF flock used in this study consistently tested negative for nasal *Pasteurellaceae* and *M. ovipneumoniae* colonization. Nasal wash fluids used for inoculation had been treated with ceftiofur, which was expected to eliminate respiratory bacterial pathogens other than *Mycoplasma* spp. [71, 78]. However, standard aerobic culture revealed that low levels of viable *Pasteurellaceae* (i.e., *Bordetella bronchiseptica*) were present in the inoculum even after the ceftiofur treatment, although the major respiratory pathogen *M. haemolytica* was absent (data not shown). Having detected *Pasteurellaceae* in the nasal wash inoculum, we subsequently tested all nasal swab samples collected throughout the study for aerobic bacteria using standard diagnostic microbiology methods. We found that nasal swabs from all lambs from both the URT and LRT groups, but not the control group, contained culturable *Bordetella* sp. (*bronchiseptica* and/or *parapertussis*) at three weeks p.i., with inconsistent detection thereafter (Figure 12A-C). In addition, *M. haemolytica* was isolated from one lamb in the URT group and three lambs in the LRT group at various time points p.i. but was never detected in any of the control lambs. Three isolates of *M. haemolytica* were cultured, and the presence of the leukotoxin gene *lktA* was confirmed by PCR [120] in all isolates (data not shown).

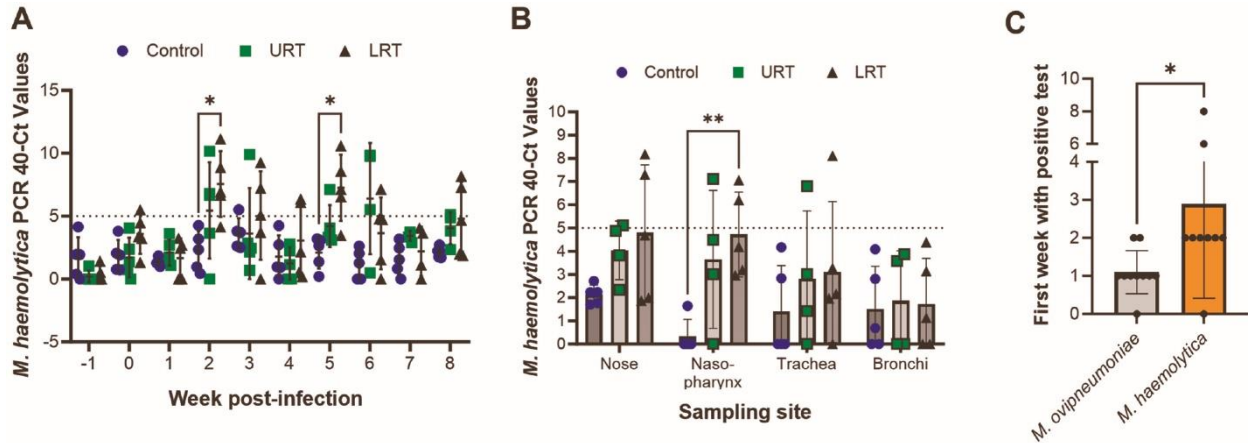


Figure 12. Identification of *Mannheimia haemolytica* in *M. ovipneumoniae* infected lambs. (A) *Mannheimia haemolytica* infection levels in nasal swab samples collected throughout the experiment were determined by qPCR. Data are shown as 40 minus Ct; individual data points, mean $\pm$ SD. Statistically significant differences between groups (* $P \leq 0.05$) were determined by mixed-effects analysis with Tukey's multiple comparisons test. Dotted line indicates limit of detection. (B) *M. haemolytica* infection levels in swab samples collected at necropsy from the nose, nasopharynx, trachea, and bronchi were determined by qPCR. Individual data points, mean $\pm$ SD. Statistically significant differences (** $P \leq 0.01$) were determined by 2-way ANOVA with Tukey's multiple comparisons test. (C) Timepoint for first detection of a PCR-positive sample (Ct ≤ 35) for *M. ovipneumoniae* or *M. haemolytica* in each animal. Only lambs from the URT and LRT groups were included in the analysis. Individual datapoints, mean $\pm$ SD; * $P \leq 0.05$ Wilcoxon matched-pairs signed rank test.

To further investigate the apparent *M. haemolytica* co-infection in the lambs, we also performed quantitative PCR screening of the nasal swab samples collected throughout the experiment for *M. haemolytica* (Figure 12D and Supplemental Figure 14). A subset of samples from both groups of inoculated lambs, but not the control group, tested positive for *M. haemolytica* throughout the study, with a significant increase in positive samples detected for the LRT group at weeks two and five p.i. (Figure 12D). In general, more *M. haemolytica*-positive samples were detected in the LRT compared to the URT groups (Figure 12E). Importantly, all inoculated lambs except one animal in the LRT group tested PCR positive for *M. haemolytica* at some point during the study. When looking at associations between *M. ovipneumoniae* and *M. haemolytica* infection, we found that nasal swab samples tested positive for *M. ovipneumoniae*

significantly earlier (at 1.1 ± 2.6 weeks p.i.) than for *M. haemolytica* (at 2.9 ± 2.5 weeks p.i.; Figure 12F, Supplemental Figure 14). Across all time points, there was a weak but significant correlation between *M. ovipneumoniae* and *M. haemolytica* pathogen load based on cT values obtained by qPCR analysis (Figure 12G).

To assess distribution of *M. haemolytica* throughout the ovine respiratory tract, we analyzed swab samples collected from different regions of the respiratory tract at the experimental endpoint. Interestingly, only a small proportion of swabs from lambs in the URT and LRT groups collected from the nose, deep nasopharynx, and trachea tested positive for *M. haemolytica* (Figure 12H), and *M. haemolytica* detection was statistically significant only in nasopharyngeal swabs collected from LRT group lambs. Surprisingly, we were unable to detect *M. haemolytica* in swab samples collected from bronchi, indicating that *M. haemolytica* did not cause significant lung infection in this study.

Discussion

Mycoplasma ovipneumoniae is a facultative pathogen associated with ovine respiratory disease that ranges from asymptomatic colonization to lethal pneumonia [24, 34, 78]. The factors that determine the outcome of infection are still incompletely understood but include bacterial strain characteristics [3], genetic susceptibility of the host [109, 129], and presence of co-infections [106]. In two recent challenge studies that we performed with specific pathogen-free lambs with *M. ovipneumoniae*, the infection had no measurable impact on weights, lamb health, or lung inflammation [24, 117]. In contrast, studies from China described coughing, wheezing, and lethargy in response to infection with the *M. ovipneumoniae* type strain Y-98 [63, 69].

Notably, *M. ovipneumoniae* was directly administered into the bronchi or trachea in these experiments [63, 69], whereas we had used an upper respiratory tract inoculation protocol based on a publication by Besser et al. [24, 71, 117]. Here, we performed a side-by-side comparison of endoscopic LRT inoculation with *M. ovipneumoniae* and inoculation of the URT i.e., the nasal cavity, conjunctiva, and oral cavity. Under normal circumstances, *M. ovipneumoniae* transmission between sheep is thought to occur via the URT through droplets [34]. However, it is conceivable that *M. ovipneumoniae* may reach the lower airways if the animal is panting due to stress or exertion, if pathogen loads are extremely high, or if mucociliary defense mechanisms are compromised due to ongoing disease processes or infections. PCR analysis of swab samples collected at necropsy confirmed that, compared to URT inoculation, pathogen administration to the LRT via endoscopic intratracheal inoculation indeed led to significantly higher *M. ovipneumoniae* loads in the trachea and bronchi, but similar loads in the nasal cavity and nasopharynx. These results corroborated that different distribution patterns of the pathogen could be effectively modeled using different experimental inoculation methods. Importantly, LRT *M. ovipneumoniae* administration led to significantly more severe clinical disease in our study than URT administration. Our results also are consistent with findings from other experimental models that showed that outcomes of respiratory infections can vary depending on where *Mycoplasma* or other pathogens are delivered. In a hamster model of *M. pneumoniae* infection small particle aerosols and large volume inocula reached the lungs and subsequently caused lung pathology, whereas *Mycoplasma* from small inocula or large particle aerosols remained in the URT and did not cause lung lesions [130]. Likewise, pneumonia in mice inoculated with *Bordetella pertussis* was more severe and less variable following aerosol inoculation compared

to intranasal delivery [131]. A study that compared intranasal and intratracheal inoculation of Cebus monkeys with influenza A virus found that only intratracheal pathogen delivery resulted in clinical disease [132]. Together, these findings indicate that circumventing critical protective mechanisms of the upper respiratory tract, such as the tonsils and mucociliary clearance, consistently results in pathogen growth in the lower airways.

Importantly, our results provide further support for the hypothesis that a secondary pathogen may be required to induce respiratory disease and airway lesions upon *M. ovipneumoniae* infection [106]. Despite treatment of the nasal wash inocula with ceftiofur, we found that all lambs in the *M. ovipneumoniae*-infected groups except one also got infected with leukotoxin-positive *M. haemolytica* and *Bordetella* spp. Our observations are consistent with previous reports that showed that clinical *M. ovipneumoniae* infection in both domestic and wild sheep predominantly occurs when the animals are co-infected with *Pasteurellaceae* such as *Mannheimia haemolytica* and *Bibersteinia trehalosi*, or with *Fusobacterium necrophorum* [66, 133]. The ability of *M. ovipneumoniae* to support expansion of *M. haemolytica* in our study was extremely impressive, since no detectable *M. haemolytica* were present in the original inocula or in any of our SPF sheep prior to the study, and none of the uninfected control lambs ever tested positive for this organism. Notably, all three representative *M. haemolytica* strains that were cultured from the experimental animals were positive for the virulence factor leukotoxin (*lktA*) by PCR, [120, 134]. *M. haemolytica* leukotoxin is a cytolytic factor that targets the integrin CD18 (integrin $\beta 2$), which is expressed on all ovine leukocyte subsets, and causes leukocyte death and local immunosuppression, which can facilitate the expansion of secondary pathogens [135]. The role of the *Bordetella bronchiseptica* and *parapertussis* that we also detected in the

inoculated sheep remains less clear. These organisms likewise are considered facultative pathogens, and they are commonly found in both healthy and pneumonic lungs [107, 136]. We focused our investigations on *M. haemolytica* but acknowledge that more research needs to be performed to better understand the potential contributions of *Bordetella sp.* to ovine polymicrobial pneumonia.

Intriguingly, *M. ovipneumoniae* was generally detected in the nasal swabs at earlier timepoints than the *M. haemolytica*, suggesting that *M. ovipneumoniae* was the driving force for respiratory disease development in our study. Moreover, *M. haemolytica* colonization of the nasal cavity was variable, with positive PCR test results only in 20% of samples collected p.i. from the URT group and in 35% of samples from the LRT group. Also, while Shanthalingam et al. [120] report PCR detection of *M. haemolytica* in lung tissue, none of the postmortem swabs obtained from the lower respiratory tract tested positive for this organism, indicating that pulmonary lesions found in our study were caused by the *Mycoplasma*. Our observations are consistent with earlier reports that did not find *M. haemolytica* in the lungs of sheep co-infected with *M. ovipneumoniae* [17, 137]. Together, these data suggest that, while *M. haemolytica* was likely a crucial contributor, the driving force for lung damage and clinical signs in our challenge study was *M. ovipneumoniae*.

One other key finding from the present study was the observation that *M. ovipneumoniae* infection was associated with increased gene expression of *IFNG* in the blood, the cranial and the caudal lungs. This was significant for the LRT group for blood samples and the URT for lung samples, but similar trends were seen for both infection groups. So far, very little information is available regarding the immune response to *M. ovipneumoniae*. The increase in *IFNG* together

with a trend for increased expression of *TBET* both locally and systemically are indicative of a Th1 polarized response, which is typically induced in response to intracellular pathogens and viruses. Whether *M. ovipneumoniae* can survive intracellularly is still a matter of ongoing investigations. Importantly, in a murine model of *Mycoplasma pulmonis* infection, IFN- γ had a protective function and was associated with a reduced pathogen load and disease severity, particularly in the LRT [138, 139]. A few previous studies have analyzed cytokine responses in *M. ovipneumoniae*-infected sheep, with highly variable results. Bowen et al. [121] analyzed blood samples from adult Bighorn sheep with acute or chronic *M. ovipneumoniae* found that *MX1*, *TGFB*, and *IL1B* gene expression increased as infection progressed, while *IFNG* was only increased in early infection. Conversely, bulk RNASeq and pathway analysis of lung tissue analyzed at 4 and 14 days p.i. revealed that *M. ovipneumoniae* infection predominantly led to an increased expression of toll-like receptor pathway genes in Bashbay sheep and of primary immunodeficiency genes in Argali hybrid sheep [63, 69]. Li et al. [15] analyzed the respiratory tracts of domestic sheep naturally infected with *M. ovipneumoniae* and found increased gene expression of *IFNG* in the trachea, but not the lungs, whereas the expression of *IL1B* and *TNFA* was increased across the entire respiratory tract. These different findings may be related to differences in sheep species and experimental setup between the studies.

Our study did have several limitations. First, although we did find significant differences in pathogen distribution between lambs inoculated via LRT vs. URT, *M. ovipneumoniae* was still detected in samples from all anatomical sites in both groups. Moreover, although we and others have previously performed successful monoinfection of lambs with *M. ovipneumoniae* using ceftiofur-treated nasal washes from naturally infected animals [24, 71, 78, 79], we achieved an

unintentional polymicrobial infection in the present study. Ceftiofur is a beta lactam antibiotic in the cephalosporin group that exerts antibacterial effects by interfering with synthesis of the key bacterial cell wall component peptidoglycan [140]. It is therefore ineffective against *Mycoplasma*, which do not have a cell wall, but is highly effective against Pasteurellaceae [141]. Although no growth of *M. haemolytica* was detected in the ceftiofur-treated nasal washes used as the inoculum, we cannot exclude that small numbers of viable bacterial were still present that then were able to proliferate within the respiratory tract of the sheep. Of note, a recent study has described rare ovine isolates of *M. haemolytica* with ceftiofur resistance [142].

In summary, our study showed that pathogen delivery route impacts disease severity in experimental infection of sheep with *M. ovipneumoniae*. Specifically, deep intratracheal inoculation with the organisms resulted in more severe clinical disease, reduced weight gains, increased macroscopic and microscopic lung pathology, and increased pathogen load in the LRT compared to inoculation of URT mucosal surfaces. In addition, our study provided further support for the premise that secondary bacterial pathogens exacerbate clinical disease upon *M. ovipneumoniae* infection. Clinical respiratory disease involved lower weight gains in the lambs, confirming the negative impact of *M. ovipneumoniae* infection on productivity [1, 7]. We also show, for the first time, that *M. ovipneumoniae* infection in domestic sheep leads to a Th1-polarized immune response.

Declarations

Ethics approval

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of MSU, protocol #2022-158-95.

Consent for publication

All co-authors concur with the publication of this manuscript.

Competing interests

No potential competing interest was reported by the authors.

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Authors' contributions

Conceptualization: BTJ, KJ, DB; methodology: BTJ, KJ, LB, StS, DB; software: BTJ, CH, MT; formal analysis: BTJ, CH, MT, LZ, LB, DB; investigation: BTJ, LZ, JD, SoS, PB, KA, JS, StS, LB, DB; resources: KJ, DB; writing - original draft: BTJ, DB; writing - review & editing: all authors; visualization: DB, BTJ, JD, SoS; supervision: KJ, DB; project administration: DB; funding acquisition: DB.

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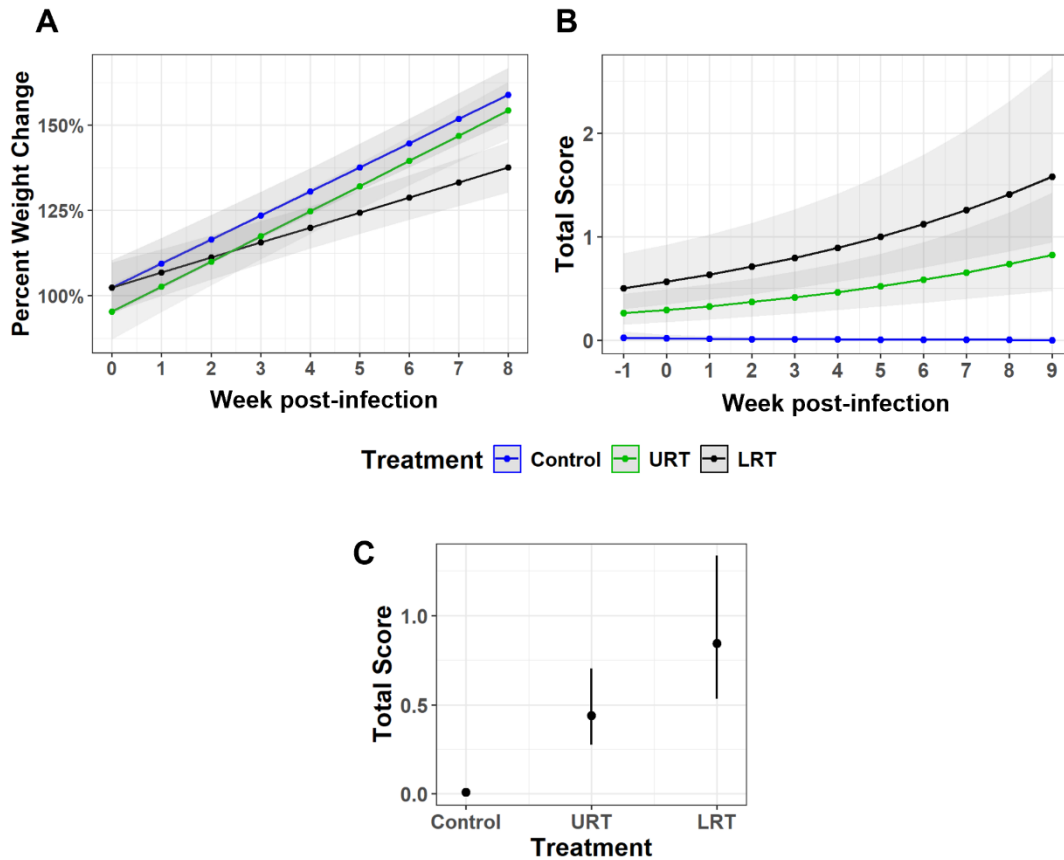
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

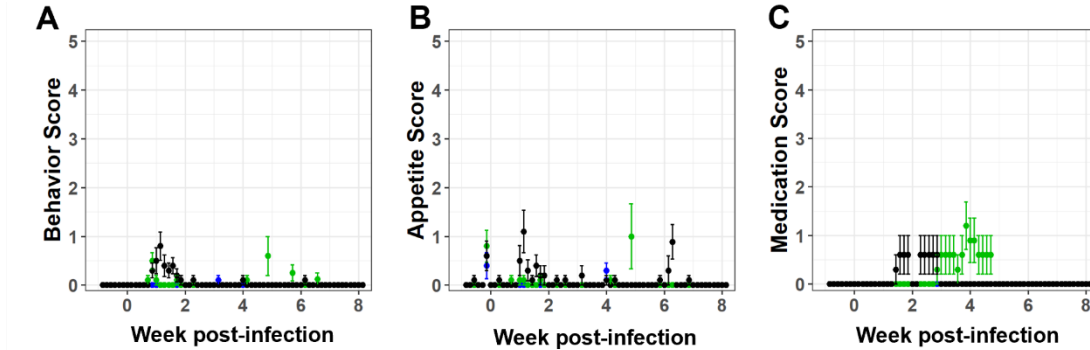
Supplementary Information

Supplemental Table 10: Scoring rubric for lamb health, based on Johnson et al. [24]

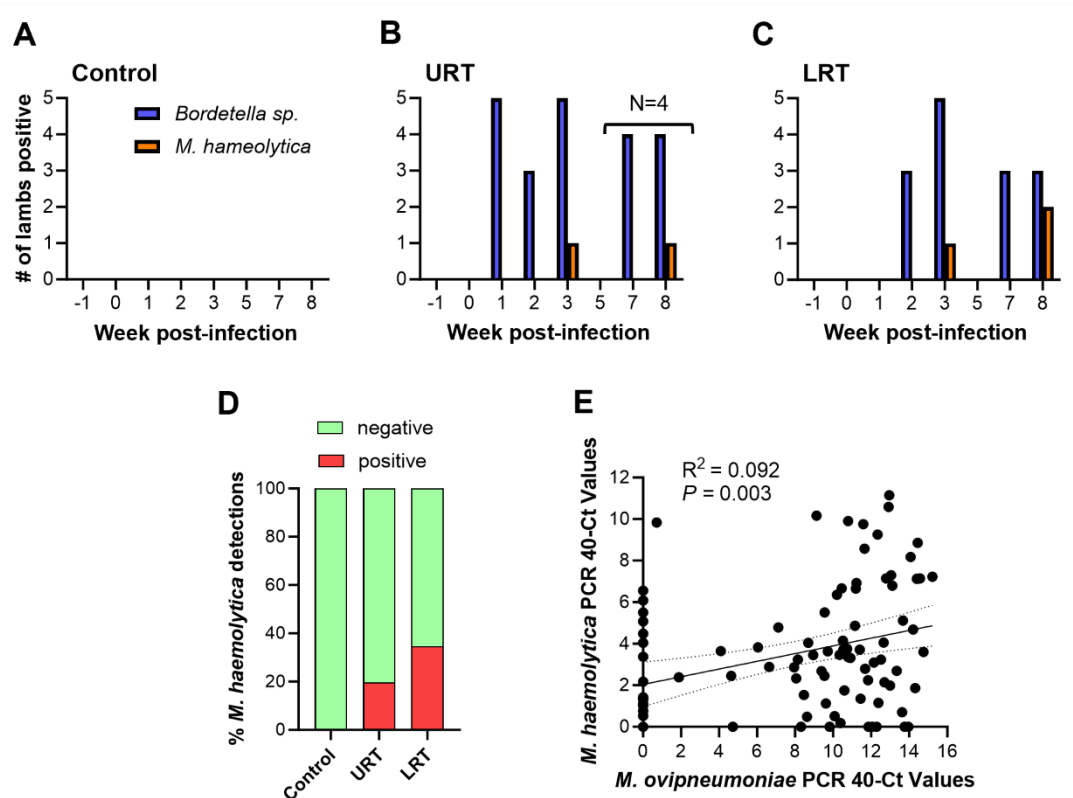
Parameters	0	1	2	3	4	5
Behavior	Bright, alert, responsive	Small change in attitude/behavior	Less active, visibly appears ill	Ill but still responsive	Lethargic	Unresponsive
Appetite	Ate well	Ate most of the ration	Ate $\frac{3}{4}$ feed	Ate $\frac{1}{2}$ feed	Ate $< \frac{1}{2}$ feed	Not eating
Respiratory Signs	No nasal or ocular discharge, no cough, normal respirations	Panting/increased respiratory rate, or slight clear nasal/ocular discharge	Clear nasal or ocular discharge, or coughed 1-5 times	Moderate ocular or nasal discharge or frequent coughing	Mucous, nasal, or ocular discharge, coughing, lamb visibly unwell	Discharge, coughing, wheezing, labored breathing
Medication	No treatments	Electrolytes	NSAID	Antibiotic	NSAID and antibiotics	Multiple NSAIDs and antibiotics



Supplemental Figure 13: Linear mixed modeling of lamb weight development and health. (A) The predicted percent weight change effect is shown for the treatments across the weeks while holding sex and age at infection constant. The grey shaded area corresponds to the 95% confidence interval. (B) The predicted total score effect is shown for each treatment group across the weeks post-infection holding sex and age at infection constant. The grey shaded area corresponds to the 95% confidence interval. (C) The predicted total score effect is shown for just the treatment groups while holding week, sex, and age at infection constant, the lines correspond to the 95% confidence interval.



Supplemental Figure 14: Health scores for behavior, appetite, and administered medications, and body temperatures. All lambs were screened twice daily for changes in (A) behavior, and (B) appetite. (C) Administered medications were also recorded (see Supplementary Table 10 for scoring rubric). Daily scores were the sum of the two individual measurements. Graphs show mean $\pm$ SD of five lambs per group.



Supplemental Figure 15: Identification of *Pasteurellaceae* in *M. ovipneumoniae*-infected lambs. Routine bacteriological analysis of nasal swab samples collected throughout the study from lambs in (A) the control group, (B) the upper respiratory tract (URT) infection group, and (C) the lower respiratory tract (LRT) infection group for the presence of *Pasteurellaceae* was performed at the Washington Animal Diseases Diagnostic Laboratory. (D) Percentage of *Mannheimia haemolytica* positive nasal swab samples out of all samples in lambs from the three experimental groups. (E) Weak correlation between Ct values from *M. ovipneumoniae* and *M. haemolytica*

PCRs across all animals from the URT and LRT groups and all time points post inoculation. Data were analyzed by simple linear regression analysis.

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CHAPTER FOUR

INNOVATIVE METHODOLOGY FOR ANTIMICROBIAL SUSCEPTIBILITY DETERMINATION IN *MYCOPLASMA* BIOFILMS

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Abstract

Mycoplasma spp. are facultative pathogens that contribute to the pathogenesis of multiple bovine diseases, including the bovine respiratory disease complex, and have been shown to form biofilms. Biofilm formation is associated with increased antibiotic resistance in many organisms, but accurate determination of antimicrobial susceptibility in biofilms is challenging. In *Mycoplasma* spp., antimicrobial susceptibility is routinely determined using metabolic pH-dependent color change. However, biofilm formation can lead to reduced metabolism, making interpretation of metabolic readouts difficult. Therefore, we developed and optimized a new flow cytometry-based method for antimicrobial susceptibility testing in biofilm-forming *Mycoplasma*, termed the live/dead antimicrobial susceptibility test (LD-AST). The LD-AST measures the proportion of live bacteria upon exposure to antibiotics, works robustly with both planktonic and biofilm cultures, and enables the determination of the minimum bactericidal concentration (MBC) for a given antibiotic. We used two strains of *Mycoplasma bovis* (Donetta PG45 and Madison) and two clinical *Mycoplasma bovoculi* isolates (MVDL1 and MVDL2) to determine the impact of biofilm growth on antimicrobial susceptibility for gentamicin, enrofloxacin, or tetracycline. All *Mycoplasma* strains were susceptible to all antibiotics when cultured as planktonic cells, with MBCs in the expected range. However, three out of four strains (Donetta PG45, MVDL1, and MVDL2) were completely resistant to all three antibiotics when newly adhered biofilms were analyzed, whereas *M. bovis* Madison gave variable results. For mature biofilms that were cultured for 4–5 days before antibiotic exposure, results also were variable, with some strains showing an increased resistance with certain antibiotics and a decreased

resistance with others. Overall, these results are consistent with earlier reports that biofilms can exhibit increased antimicrobial resistance.

Keywords

antimicrobial resistance; *Mycoplasma bovis*; *Mycoplasma sp.*; biofilm formation; flow cytometry; live/dead staining; assay development

1. Introduction

Mycoplasma spp. are common facultative pathogens in cattle and other livestock species, where they contribute to significant diseases of the respiratory tract and of other organ systems. In domestic cattle, *Mycoplasma bovis* is considered a key pathogen that significantly contributes to the bovine respiratory disease complex, which causes significant economic losses to the cattle industry globally [143, 144]. *M. bovis* is well established as an important cause of pneumonia, mastitis and arthritis, but has also been associated with ocular infections, otitis media, meningitis, endocarditis and reproductive disorders [145-150]. Clinical signs among cattle infected with *M. bovis* are often inconsistent and unspecific, making infections difficult to identify and control [145, 146]. Importantly, studies have shown that *M. bovis* has developed increased levels of resistance to a wide range of antimicrobial classes, including aminocyclitols (spectinomycin), fluoroquinolones (enrofloxacin), lincosamides (clindamycin and lincomycin), macrolides (erythromycin, gamithromycin, tilmicosin, tulathromycin, and tylosin), pleuromutilins (tiamulin), and tetracyclines (oxytetracycline). [151-158]. A second species, *Mycoplasma bovovuli* is a frequent isolate of the bovine eye [159] and is associated with both bovine conjunctivitis and keratoconjunctivitis [160-163]. While conjunctivitis has been observed

in herds infected with *M. bovoculi* alone [164], *M. bovoculi* has been found to enhance keratoconjunctivitis caused by *Moraxella bovis* [165, 166]. Notably, related *Mycoplasma* species have been shown to contribute to similar respiratory diseases in pigs (*Mycoplasma hyopneumoniae*), sheep (*Mycoplasma ovipneumoniae*), and goats (*Mycoplasma ovipneumoniae* and *Mycoplasma arginini*) [1, 6, 167] and to conjunctivitis in pigs (*Mycoplasma hyorhinitis*), birds (*Mycoplasma gallisepticum*), and cats (*Mycoplasma felis*) [168-170].

Antibiotic susceptibility testing (AST) of bacterial isolates is commonly quantified *in vitro* with the metrics of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) [171]. The first metric, MIC, is the antibiotic concentration where the growth of the organism is inhibited. The inhibition of an organism's growth can have clinical relevance, as slowing the spread of the infection can allow for the host immune response to clear the infection. The second metric, MBC, is commonly defined as the minimum concentration of an antibiotic that can kill 99.9% of the viable organisms [171]. The bactericidal effect of the antibiotic can also be interesting clinically, because rather than just inhibiting growth, this can give insight into whether the antibiotic alone can kill the infecting organism. These two metrics can also allow for the comparison between organisms and isolates *in vitro*, to determine the relative differences in susceptibility to treatment.

There is a need for improved diagnostic methods for *Mycoplasma* spp., specifically in regard to *Mycoplasma* biofilms. To date, there has only been limited research into the effect that biofilm formation has on both the pathogenicity and the antimicrobial resistance of *Mycoplasma* spp. . The current method for MIC determination recommended by the International Research Programme on Comparative Mycoplasmaology (IRPCM) for antimicrobial susceptibility testing

(AST) of veterinary *Mycoplasma* spp. utilizes a metabolic assay, termed color change assay, that is based on pH-dependent color change of culture media in a microtiter plate over the course of 7 – 14 d [83]. Some *Mycoplasma* spp. can produce acid as they metabolize nutrients and grow, creating a correlated change in acidity. When an antibiotic inhibits growth, no acid production and hence no color change occurs in the culture. However, the color change assay can be difficult to interpret, due to the variability of acid production between different *Mycoplasma* strains and under different culture conditions. In particular, metabolic differences between laboratory-adapted *Mycoplasma* strains and clinical isolates can result in variations in acid production [172]. While all planktonic bovine *Mycoplasma* spp. analyzed in our laboratory caused a color change that correlated with growth, others have reported that some *Mycoplasma* spp. do not acidify their media [173]. Previously developed assays to determine the MBC for *Mycoplasma* spp. have been based on the growth rate of the organism or time-kill assays. These methods also rely on a color change by the pH indicator in broth media [174-176] or on colony counts on plates [177], which is not always feasible depending on the *Mycoplasma* spp. . These issues with the current methods for both MIC and MBC determination point to a need for alternative AST methods.

Biofilm formation is a defense strategy employed by a variety of microorganisms, where the adherence of the cells to a surface and the formation of an extracellular polymeric matrix (EPM) commonly results in both immune evasion and antibiotic resistance [43, 46, 178]. The increased resistance of biofilms to normal defense mechanisms employed by the host may enable chronic colonization, leading to the presence of persistent carrier animals that pose a significant transmission risk to other animals in the population [49, 179, 180]. In other bacterial species,

biofilm formation has been associated with increased antibiotic resistance and negative outcomes in livestock health [181, 182]. Biofilm formation by *M. bovis* [49, 183] and by other *Mycoplasma* spp. [49, 184, 185] has been documented in previous studies. A recent review by Andrés-Lasheras et al. identified significant knowledge gaps in livestock biofilm research [37]. Specifically, effects of *Mycoplasma* biofilm formation on antimicrobial resistance needs to be addressed due to their potential implications on treatment decisions.

One common feature of biofilms, including those formed by other *Mycoplasma* spp. , is a significantly reduced bacterial metabolism, as some bacteria in the biofilm structure enter a dormant metabolic state [186-188]. This metabolic dormancy could also lead to a decrease in acid production by *M. bovis*, which could skew the results of the metabolic-based AST method. Due to potential differences in metabolic state and antibiotic susceptibility between planktonic bacteria and biofilm-forming bacteria, antibiotic concentrations may need to be adapted to effectively treat bacterial biofilms. The potential inaccuracy of using the color change assay [83] as well as the rising need for susceptibility information necessitates new approaches to the development of sensitive and rapid AST methods to compare both planktonic *Mycoplasma* spp. as well as *Mycoplasma* biofilms. Therefore, as there are no currently published biofilm-specific methods to address antibiotic susceptibilities for *Mycoplasma* biofilms, we have developed a new method of antimicrobial susceptibility testing to address this. This method, termed the live-dead AST (LD-AST), is based on the quantification of live and dead bacteria in disaggregated biofilms by flow cytometry.

Due to the lack of a cell wall, *Mycoplasma* spp. have an inherent resistance to common antibiotics such as beta-lactams and glycopeptides [189]. Antibiotics utilizing different

mechanisms such as inhibiting protein synthesis (tetracyclines and aminoglycosides), or DNA synthesis (fluoroquinolones) have been used to successfully treat *Mycoplasma* infections [189, 190]. Therefore, we have selected the antibiotics tetracycline, gentamicin (aminoglycoside), and enrofloxacin (fluoroquinolone) which have been approved for use in clinical practice to treat both bovine respiratory infections (tetracycline and enrofloxacin) and bovine conjunctivitis (gentamicin) [191-194].

2. Materials and Methods

2.1 Growth Media

Broth media consisted of *Mycoplasma* PPLO Broth Base (19.5 mg/mL) (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with L-cysteine hydrochloride monohydrate (8.7×10^{-2} mg/mL) (Thermo Fisher Scientific, Waltham, MA, USA), beta-nicotinamide adenine dinucleotide (NAD) (8.7×10^{-2} mg/mL) (Thermo Fisher Scientific), dextrose (2.6 mg/mL) (Thermo Fisher Scientific), thallium acetate (0.43 mg/mL) (Acros Organics, Fair Lawn, NJ, USA), porcine serum (6.5%) (Quad Five, Rygate, MT, USA), horse serum (6.5%) (Quad Five), and phenol red (2.1×10^{-2} mg/mL) (Sigma Aldrich, St. Louis, MO, USA) as a pH indicator.

2.2 Bacterial isolates

Mycoplasma isolates (Table 11) were obtained from either the American Type Culture Collection (ATCC, Manassas, VA, USA) or the Montana Veterinary Diagnostics Laboratory (MVDL, Bozeman, MT, USA). The *M. bovoculi* isolates were identified by whole genome sequencing with Illumina MiSeq (Wyoming State Veterinary Laboratory, Laramie, WY). The *M. bovis* PG45 isolate (ATCC, 25523) was used as the reference strain and as a baseline for method

development. The resulting optimized methods were then applied to the additional isolates with minimal adjustments.

Table 11. Characteristics of bovine *Mycoplasma* isolates used in this study.

Mycoplasma Isolate	Source	Isolation Source	Year Isolated
<i>M. bovis</i> strain Donetta PG45	American Type Culture Collection (ATCC) 25523	Bovine mastitis	1962
<i>M. bovis</i> strain Madison	ATCC 27368	Dairy products	1971
<i>M. bovoculi</i> Clinical Isolate MVDL1	Montana Veterinary Diagnostic Lab (MVDL)	Bovine conjunctivitis	2023
<i>M. bovoculi</i> Clinical Isolate MVDL2	MVDL	Bovine conjunctivitis	2023

2.3 Culture Methods

Each strain was grown for 2 days (d) in the growth media described above at 37 °C and 5% CO₂, which were considered the standard conditions for incubation. The culture was incubated in either a polystyrene 96-well or 6-well plate (Table S1). *Mycoplasma* spp. typically do not adhere well to polystyrene, so these were considered planktonic cultures. To stimulate formation of biofilms, the *Mycoplasma* spp. were incubated in a 96-well glass-bottom plate (Cellvis, Product Number: P96-1.5H-N) under the same conditions as above.

2.4 Biofilm Image Analysis

Mycoplasma biofilm growth over time was determined by inoculating 200 µL of culture at a concentration of 1×10^4 cells/mL into a 96-well glass-bottom plate. The plate was incubated for 6 d under standard conditions. Brightfield images of each well were taken daily using a Keyence BZ-X 810 microscope. Images were then analyzed using FIJI software (v2.14.0) [195] to assess structure size and confluency of adhered cells. To measure structure size, the images were set to scale in the FIJI software and manually measured using the measurement tool. For confluency

analysis of the biofilm, images were analyzed with FIJI software (v2.14.0) [195] using methods adapted from Busschots et al. with some slight modifications. Briefly, the rolling ball radius was reduced until only the background was removed, the threshold was reduced so that only the biofilm was selected, and instead of counting cells, only the area fraction was used [196].

2.5 Acid Production Assay

To assess acid production by *Mycoplasma* biofilms, new broth media was placed on the mature biofilms and incubated at standard conditions for 2 d. This was compared to planktonic cultures that were incubated at standard conditions for 2 d. These wells were then imaged to assess visible color change. *M. bovis* PG45 was further investigated over a total of 6 d by performing the following. A standard curve of pH values was created to estimate the pH in each well using a SpectraMax ABS Plus plate reader (Molecular Devices, San Jose, CA). The samples and the standard curve were read at 620 nm and 700 nm. Each well was then examined for visual signs of contamination or debris, and any wells that were obviously contaminated or contained debris were excluded from the data set.

2.6 Quantification of Cells with Flow Cytometry

The ZE5™ Cytometer (Bio-Rad, Hercules, CA, USA) was used for cell quantification of cells, so that standardized concentrations of inoculum could be used. Briefly, 10 µL of ~4 µm Countbright Plus Absolute Counting Beads (Reference Number: C36995, Invitrogen, Waltham, MA, USA) were added to 200 µL of FACS buffer, and 100 µL of *Myoplasma sp.* sample. FACS buffer was made using phosphate buffered saline (PBS) (Cytiva, Logan, UT), FBS (2%) (SeraPrime, Fort Collins, CO), and sodium azide (0.1%) (Aqua Solutions Inc., Deer Park, TX). One thousand bead events were collected and then analyzed with the Everest analysis software

(v3.2.12.0, Bio-Rad) [197]. The overall concentration for the sample was then calculated with the equation below.

$$\text{Absolute count} \left(\frac{\text{cells}}{\mu\text{L}} \right) = \frac{(\text{Cell count} \times \text{Counting beads volume} [\mu\text{L}])}{(\text{Counting beads count} \times \text{Cell volume} [\mu\text{L}])} \times \text{Counting beads concentration} \left(\frac{\text{beads}}{\mu\text{L}} \right)$$

2.7 Biofilm Disruption

To analyze *M. bovis* PG45 biofilms using imaging cytometry, the media was removed from the mature biofilms and replaced with 0.2 μm filtered, degassed PBS (Cytiva). The biofilms were then scraped from the bottom of a glass-bottom well with a mini cell scraper (United Biosystems, Herndon, VA) and exposed to either 6 min sonication, 10 min sonication, or Tween-20 (Fisher Scientific) treatment. After scraping, the biofilm suspension was transferred to 1.5 mL microcentrifuge tube (Fisherbrand, Pittsburgh, PA). Using a protocol modified from Totten et al. [198], sonication samples were treated for either 6 min or 10 min at 45 kHz (VWR Scientific, Aquasonic Model 550T). Briefly, *Mycoplasma* cultures were sonicated for a longer period using a higher frequency sonicating water bath than was performed by the previous authors. Biofilm disruption with 0.01% Tween-20 was performed for 15 min at room temperature, following a protocol modified from Alsharif and Godfrey [199]. Briefly, Tween-20 was substituted as an alternative for Pluronic F-68 as suggested by the authors. Samples were then stained with SYTO9 (Invitrogen) at a final concentration of 1.36 μM for 15 min at 4 $^{\circ}\text{C}$ and analyzed by imaging cytometry.

2.8 Imaging Cytometry Analysis

Imaging cytometry was performed with the Cytex Amnis Imagestream^X MkII Imaging flow cytometer. The particle images were obtained with the 20 x objective so that the field would capture the widest range of particle sizes. Due to the lower limit of size measurement capabilities of the device, the particles were compared by utilizing relative fluorescent units (RFU) following manufacturer recommendations for small particle analysis. Each sample was run on the instrument for 2 min and subsequently analyzed with Amnis IDEAS® 6.2 software. For the analysis, first the samples were gated for a 2 min runtime to ensure consistency, next the samples were gated for focus in the fluorescent channel. After these first steps were completed, a built-in machine learning module for counting the spots on each image was trained to identify images with multiple particles for removal from the dataset. At this point, the in-focus single particle image fluorescent intensity feature for each particle was exported to a *.csv file for analysis with the R statistical software (v4.3.3, R core Team) [122].

2.9 Small Particle Analysis with Flow Cytometry

The disruption method that was determined to be successful by imaging cytometry analysis in section 2.7 was then analyzed via flow cytometry with the ZE5™ Cytometer (Bio-Rad) utilizing the 405 nm small particle laser. Briefly, the *M. bovis* PG45 culture biofilms were sonicated for 10 minutes, stained with a 1:6,000 dilution of SYBR Safe DNA gel stain (Thermo Fisher) and compared to untreated biofilm and planktonic samples. To determine the specific size of the disrupted particles, a Flow Cytometry Sub-micron Particle Size Reference Kit (Invitrogen) was used. The resulting data was then analyzed with FlowJo software (BD, v. 10.10.0).

2.10 Live Dead Percentage Quantification

To quantify cell death using flow cytometry, 2 d *Mycoplasma* spp. planktonic or biofilms grown in 96-well plates as described above were collected for analysis. The biofilm was collected by removing the media on top of the biofilm and replacing it with an equal volume of FACS buffer. A mini cell scraper was then used to scrape the bottom of each well and remove the adherent biofilm from the glass-bottom plate. For the planktonic cells 100 μ L of the culture was added to 100 μ L of FACS buffer and transferred to a capless 1.5 mL microcentrifuge tube (Research Products International, Mt Prospect, IL) and covered with foil (Kirkland Signature, Seattle, WA). Additional disruption treatments were applied after this step as described below. The suspensions of cells and FACS buffer from each tube were individually transferred to the wells of a polystyrene 96-well plate (Greiner, Kremsmünster, Austria) and stained with SYTO9 at a final concentration of 1.36 μ M and propidium iodide (PI) at a final concentration of 8 μ M according to methods described by Assunção et al. [200]. A ZE5™ Cytometer (Bio-Rad) was used to analyze 50 μ L of each sample. The cell concentration for each sample was then calculated based upon this known volume instead of using the counting beads mentioned above. Data were analyzed using Bio-Rad Everest software (v3.2.12.0) [197] and FlowJo software (v10.10.0). Gates for live and dead cells were applied using the fluorescent intensity of SYTO9 versus PI (Figure 16), as described by Assunção et al. [200]. Separate populations were then visualized for live (Figure 16A) and dead (Figure 16B) bacteria after treating the various *Mycoplasma* spp. with 0.01% Triton X-100 [201] to create a positive control for dead cells (Figure 16B).

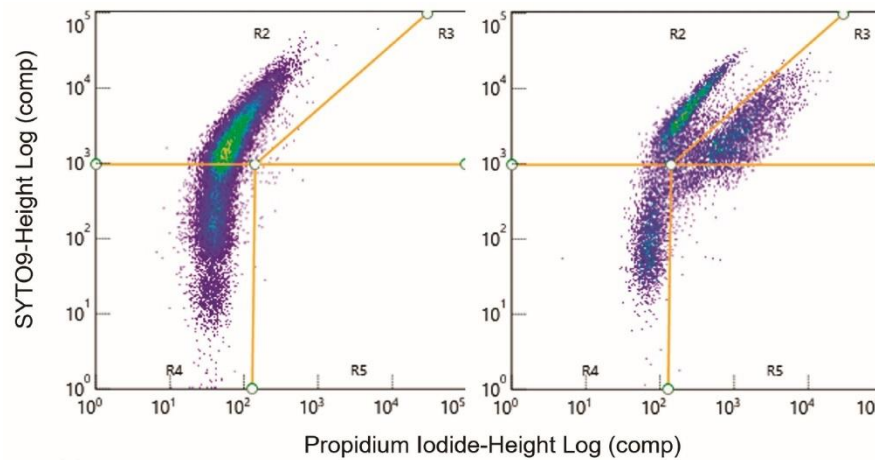


Figure 16. Representative FACS dot plots of live and dead *M. bovis* samples. The live/dead stain was visualized as the log intensity of SYTO9 versus the log intensity of the PI (A) Live cells were gated as SYTO9-positive, PI-negative cells in gate R2. (B) TritonX-100 treatment was used to kill a proportion of the *M. bovis* to create a positive control. Dead cells are found as SYTO9-positive, PI positive cells in gate R3. Representative data from one experiment.

2.11 Disruption Survival Determination

To ensure that the biofilm disruption treatment did not result in a decrease in cell viability, 2 d planktonic *M. bovis* PG45 cultures were filtered with a 0.45 μm low protein-binding syringe filter to remove any aggregates. These filtered cells were either left untreated or sonicated for 10 min as previously described. The cells were then live-dead stained and quantified with both flow cytometry and imaging cytometry.

2.12 Cell Adhesion Rate for Newly Adhered Biofilms

To determine the rate at which *M. bovis* PG45 cells adhere to glass, 200 μL of culture were inoculated at a concentration of 2×10^4 cells/mL into the wells of a 96-well glass-bottom plate. After 1, 2.5, or 4 h, the media was removed and replaced with 200 μL of FACS buffer. The cells were then disrupted with a mini cell scraper, transferred to a 1.5 mL microcentrifuge tube (Fisherbrand) and sonicated for 10 min at 45 kHz. These removed cells were then stained with SYTO9 and the cell concentration was determined via flow cytometry.

2.13 Broth Microdilutions

Broth microdilutions were made with the growth media mentioned above, but without penicillin and were instead made with either gentamicin (IBISCI, Dubuque, IA), enrofloxacin (Sigma Aldrich, St. Louis, MO), or tetracycline (Sigma Aldrich, St. Louis, MO). Each antibiotic was used with two technical replicates per plate and was diluted with 2-fold serial dilutions into 9 wells. Broth media without antibiotics was used as a growth control. Gentamicin concentrations were 2-fold dilutions from 128 $\mu\text{g/mL}$ – 0.5 $\mu\text{g/mL}$, enrofloxacin from 10 $\mu\text{g/mL}$ – 0.039 $\mu\text{g/mL}$, and tetracycline from 16 $\mu\text{g/mL}$ – 0.0625 $\mu\text{g/mL}$. While both gentamicin and enrofloxacin are considered bactericidal, tetracycline is typically considered bacteriostatic, but bactericidal activity also has been described for certain bacteria [202].

2.14 LD-AST Assays for Planktonic *Mycoplasma* spp., Newly Adhered and Mature Biofilm

For planktonic growth, the microdilutions described above were prepared, and planktonic culture quantified with flow cytometry was inoculated at 200 μL of 1×10^4 cells/mL per well of a polystyrene 96-well plate as recommended by Hannan et al. [83]. The interior wells were inoculated, with the outer edge filled with 1X PBS (Cytiva) to reduce evaporation of the sample wells. Plates were incubated using standard culture conditions for 2 d and then quantified using the LD-AST.

For newly adhered biofilm growth, 200 μL of 2×10^4 cells/mL of planktonic culture was inoculated into the interior wells of a 96-well glass-bottom plate, with the outer edge filled with 1X PBS to reduce evaporation of the sample wells. This plate was then incubated for 1 h at standard conditions so that the adhered cells would fall into the range 10^3 – 10^5 cells/mL suggested by Hannan et al [83]. To ensure that the cells were in range, three wells grown for

concentration verification were scraped with a mini cell scraper, sonicated for 10 min, and quantified with flow cytometry as described above. The media on the rest of the newly adhered cells was removed and the prepared antibiotic microdilutions described above were added. Plates were incubated using standard culture conditions for 2 d and then quantified using the LD-AST.

For mature biofilms, 200 μL of 1×10^4 cells/mL of planktonic culture was inoculated into the interior wells of a 96-well glass-bottom plate, with the outer edge filled with 1X PBS to reduce evaporation of the sample wells. This plate was then incubated for 4 d at standard conditions to produce a mature biofilm. The wells were then observed microscopically to ensure consistency of biofilm formation. The media was removed from all sample wells, and newly prepared antibiotic microdilutions as described above were added to the mature biofilms.

2.15 Statistical Analysis

All analyses were performed using R Statistical Software (v4.3.3; R Core Team 2024) [122]. Data was processed into data structures with the tidyverse package (v2.0.0) [123]. Linear mixed-models were performed with the nlme package (v3.1.164) [124], generalized linear mixed-models were performed with the lme4 package (v1.1.35.1) [203], and the diagnostic fit was assessed using the ggResidPanel package (v0.3.0) [204]. Mixed model tables were generated with the sjPlot package (v2.8.1.5) [126]. Imaging cytometry particle size data was processed and combined with the purr package (v1.0.2) [205] and stringr package (v1.5.1) [206]. The disruption method survival statistics were performed with the rstatix package (v0.7.2) [207]. All figure plots were made with the ggplot2 package (v3.5.0) [127], ggthemes package (v5.1.0) [128] and ggpubr package (v0.6.0) [208]. For all tests, significance was considered at an $\alpha < 0.05$.

When determining the maturity of the biofilm described in section 2.4, we quantified both the largest structure size and the confluence. The effect of incubation times on largest structure size was tested with a separate means mixed model with the day post-inoculation as a fixed effect and the technical replicate as a random effect according to the following:

$$Measurement_i \sim \delta_{m[i]} + \beta_1 X_{DayPostInoculation_i} + \epsilon_i$$

$$\delta_m \sim N(0, \sigma_{TechnicalReplicate}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation level and where m represents the technical replicate.

The effect of incubation time on biofilm confluence was tested with a separate means mixed model with the day post-inoculation as a fixed effect and the technical replicate as a random effect according to the following:

$$PercentConfluence_i \sim \delta_{m[i]} + \beta_1 X_{DayPostInoculation_i} + \epsilon_i$$

$$\delta_m \sim N(0, \sigma_{TechnicalReplicate}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation level and where m represents the technical replicate.

The timepoint post-inoculation that was observed to have the largest mean for model was set as the baseline and compared to all other timepoints post-inoculation.

For the acid production assay described in section 2.5, a mixed model was applied with an interaction between the inoculation state (uninoculated controls or *M. bovis* PG45) and the antibiotic concentration as the fixed effects and the individual plate variance as a random effect according to the following:

$$pH_i \sim \delta_{m[i]} + \beta_1 X_{InoculationState_i} + \epsilon_i$$

$$\delta_m \sim N(0, \sigma_{Plate}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation level, where m represents the different 96-well plate blocks, and where j represents the biofilm percentage.

For the disrupted biofilm data obtained by imaging cytometry described in sections 2.7 and 2.8, the percentage of the population $\leq 1.75 \times 10^3$ RFU for each replicate was calculated and a separate means mixed model was performed with the treatment as a fixed effect and the replicate culture as a random effect according to the following:

$$Percent_i \sim \delta_{m[i]} + \beta_1 X_{Treatment_i} + \epsilon_i$$

$$\delta_m \sim N(0, \sigma_{ReplicateCulture}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation level and where m represents the biological replicate.

For the small particle 10 minute sonication data obtained with the 405 nm small particle laser described in section 2.9, a generalized linear model was performed with the treatment as a fixed effect according to the following:

$$\log(\mu_i) = \beta_0 + \beta_1 X_{Treatment_i}$$

$$FSC_i \sim IG(\mu, \lambda)$$

Where i represents the individual observation, and IG refers to the Inverse Gaussian distribution.

For the experiments performed to assess the impact of biofilm disruption on cell viability described in section 2.11, we wanted to determine whether the treatment resulted in a decrease in live cells. A one-sided Welch's T-test was performed to compare the untreated samples to their

paired samples that were sonicated for 10 min. To determine whether there were significant differences between imaging cytometry and flow cytometry, a two-sided Welch's T-test was performed to compare the flow cytometry samples at each treatment to their paired samples analyzed with the imaging cytometer.

For the AST assay described in section 2.14, we defined the MBC as the lowest dilution that resulted in a significant decrease in live cell percentage that was $\geq 5\%$, which we notated as $MBC_{\geq 5\%}$. To determine the $MBC_{\geq 5\%}$, a separate means mixed model was performed for each organism state and antibiotic combination with concentration as a fixed effect and the biological replicate as a random effect according to the following:

$$PercentLiveCells_i \sim \delta_{m[i]} + \beta_1 X_{concentration_i} + \epsilon_i$$

$$\delta_m \sim N(0, \sigma_{ReplicateCulture}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation level and where m represents the biological replicate.

3. Results

3.1 Biofilm Maturity Determination

First, we confirmed that all four *Mycoplasma* strains produced biofilms following adherence to glass-bottom plates. As shown in Figure 17, adhered bacterial aggregates were visible after 6 days of incubation for all strains, although aggregate sizes varied. Moreover, the biofilms could be detected using image analysis software. Next, we sought to define the point at which the *Mycoplasma* biofilms reached their maximum size and thus could be considered mature based on both the area of the plate covered by the biofilm (confluence) and the size of individual aggregates (structure size). We used digital image analysis to quantify the percent confluence and

the diameter of the largest structure after 0-6 d of culture (Figure 18). For *M. bovis* PG45, biofilm confluence peaked after 4 d with a mean of $10.42 \pm 1.89\%$ coverage of the culture plate (Figure 18A, Table 12). Biofilm confluence at 4 d showed moderate to strong evidence for a difference (Wald's test, $p < 0.05$) compared to all other days post-inoculation. Structure size peaked at 6 d with a mean size of $29.50 \pm 8.10 \mu\text{m}$ (Figure 18B, Table 12), with no evidence of differences between any of the time points analyzed (Wald's test, $p > 0.05$). Based on these results, we considered *M. bovis* PG45 biofilms cultured for 4 d to be mature, since a longer culture did not lead to an increase in biofilm mass. When determining maturity for the additional isolates (Table 12), we found that the *M. bovis* Madison strain had significantly higher confluence (Wald's Test, $p < 0.001$) at 5 d, with no significant difference in the largest structure size between the rest of the days (Wald's Test, $p > 0.05$). Therefore, the Madison strain was considered mature after 5 d of incubation. For the *M. bovoculi* MVDL1 isolate, the highest level of confluence was found at 5 d, when it was significantly greater than all other days except 4 d (Wald's Test, $p < 0.001$), and the largest structure size were present at 3 d, when they were significantly larger than all the other days (Wald's Test, $p < 0.001$). Therefore, MVDL1 biofilms were considered mature after 4 d. For the *M. bovoculi* MVDL2 isolate, we observed the highest level of confluence at 5 d that was significantly higher than all the other days (Wald's Test, $p < 0.001$), and we observed the largest structure size at 2 d that was only significantly different than the 3 d and 6 d observations (Wald's Test, $p < 0.05$). Therefore, MVDL2 biofilms were considered mature after 5 d.

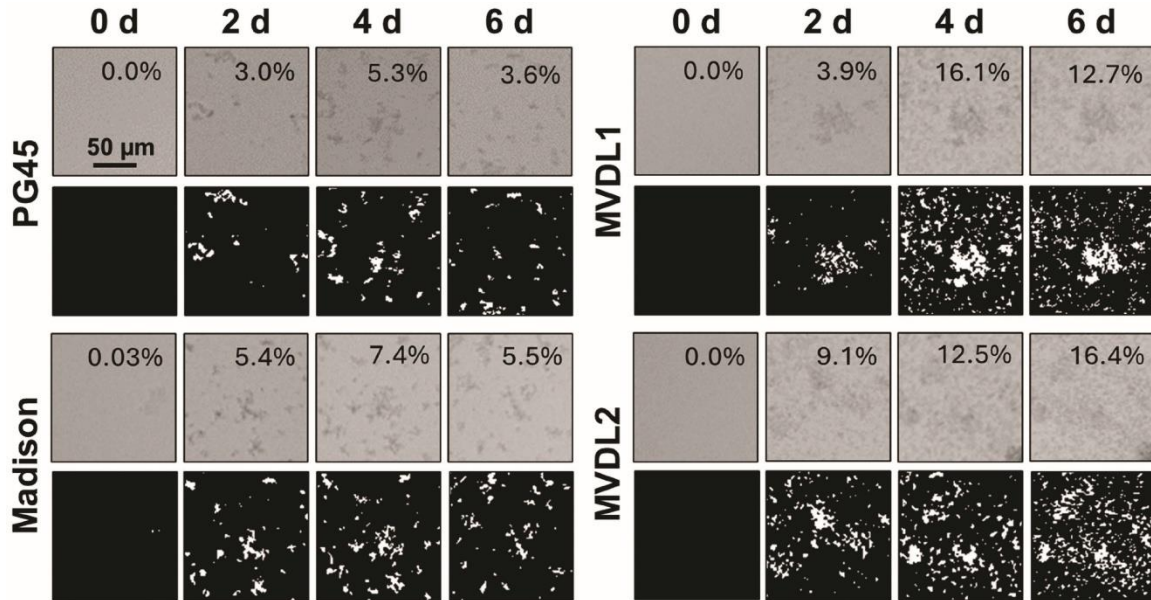


Figure 17. Brightfield images of *Mycoplasma* biofilm formation on glass-bottom plates over 6 d. Top rows: phase contrast images; bottom rows: thresholded images used to estimate the percent confluence of the biofilm in the field of view. Data are representative of one experiment with 18 technical replicates.

Table 12. The peak maturity metrics observed over 6 days of growth.

Mycoplasma Isolate	Mature Biofilm Confluence	Largest Structure Size
PG45	$10.42 \pm 1.89\%$ (4 days)	$29.50 \pm 8.10 \mu\text{m}$ (4 days)
Madison	$8.99 \pm 0.81\%$ (5 days)	$30.40 \pm 9.38 \mu\text{m}$ (2 days)
MVDL1	$26.38 \pm 4.12\%$ (5 days)	$60.48 \pm 25.18 \mu\text{m}$ (3 days)
MVDL2	$22.84 \pm 2.94\%$ (5 days)	$50.09 \pm 13.82 \mu\text{m}$ (2 days)

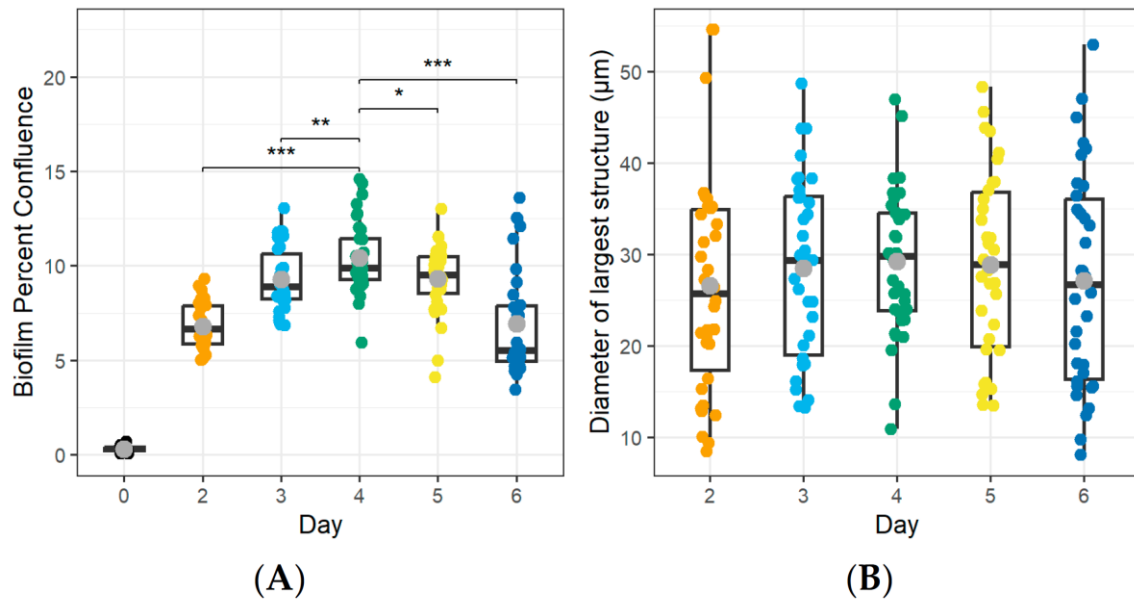


Figure 18. Maturation of *M. bovis* PG45 biofilms over time. Biofilm maturity was assessed based on the percent confluence of the cells and the largest structure diameter observed. (A) Biofilm confluence peaked at 4 d, which showed strong evidence of a difference (Wald's Test, $p < 0.01$) compared to all other days post-inoculation. (B) The diameter of the largest biofilm structure did not vary across the different days (Wald's test, $p > 0.05$). The boxplot displays the median and quartiles of the population, the grey point indicates the mean. Representative of two experiments, each with 16–18 technical replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2 Acid Production

We first tested the hypothesis that the standard color change assay is not suitable to assess growth and antimicrobial susceptibility of our selected *Mycoplasma* spp. biofilms (Figure 19A). Indeed, while a color change of the media is clearly visible for cultured planktonic *Mycoplasma* spp. compared to the uninoculated control media, no color change was seen in the media of mature *Mycoplasma* spp. biofilms after 2 days. It is interesting to note that variation in acidification was observed between the different organisms. Moreover, additional pH measurements performed with mature *M. bovis* PG45 biofilms over 6 d of incubation showed no evidence (Wald's test, $p > 0.05$) for a difference in the pH after accounting for the variation by

plate (Figure 19B). These data confirm the need for alternative assays to measure antimicrobial effects in *M. bovis* biofilm cultures.

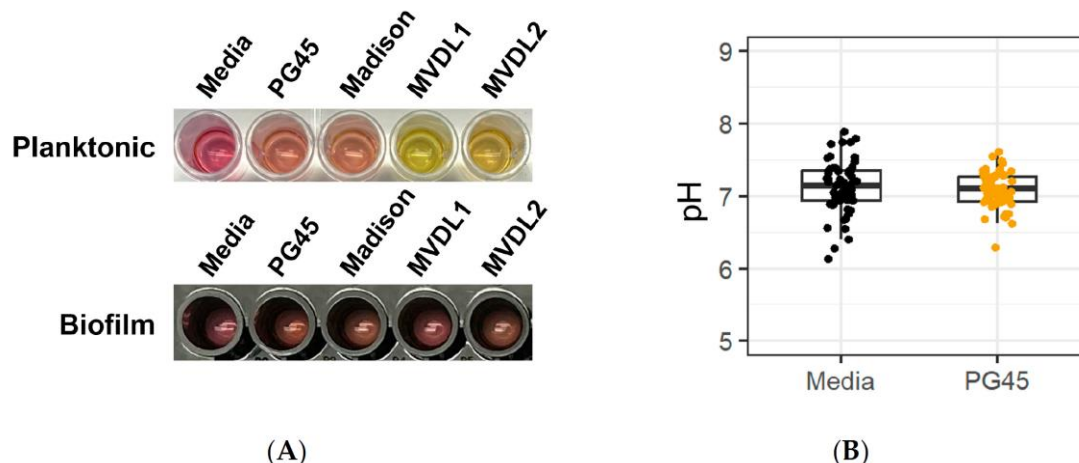


Figure 19. Standard color change assay is inappropriate for measuring *Mycoplasma* spp. biofilm growth. (A) A color change due to acid production is visible for planktonic cells (top, grown for 2 d on a clear polystyrene plate), but not for mature biofilms (bottom, incubated with new media for 2 d after biofilm formation on a glass-bottom plate with black polystyrene wells). (B) Quantification of medium pH for *M. bovis* PG45 grown as a biofilm for 6 d. No significant difference in pH between the media control and PG45 (Student's t-test, $p > 0.05$). Representative of one experiment with 60 technical replicates.

3.3 Particle Size Standardization

Having observed that the pH did not change after incubation of a mature biofilm, we developed an innovative flow cytometry-based assay to assess biofilm growth and survival. This assay, termed the live-dead antimicrobial susceptibility test (LD-AST), involves the analysis of disrupted biofilms on a flow cytometer or imaging cytometer. First, we sought to standardize the particle size of the disrupted biofilms as the staining of large cellular aggregates can cause intra-assay variability. *M. bovis* PG45 biofilms were scraped from the plate (untreated control) and then underwent either sonication or detergent treatment with Tween-20 to break up aggregates.

The fluorescence intensity distribution of the planktonic and differentially treated biofilm samples, which correlates with particle size, was measured using imaging cytometry (Figure 20).

We observed a bimodal size distribution for planktonic *M. bovis* PG45 (Figure 20A) that we used to define our threshold for large and small particles. The separation occurred at 1.75×10^3 RFU which corresponded to particles with a diameter of $5.05 \pm 0.74 \mu\text{m}$ (Figure 20A). Based on this definition, planktonic *M. bovis* samples contained $55.01 \pm 12.32\%$ of small particles. In contrast, the percentage of small particles in untreated biofilms was $22.32 \pm 6.52\%$. Sonication treatment resulted in a moderate increase in small particles to $25.69 \pm 6.33\%$ after 6 min, and a significant increase to $31.20 \pm 4.52\%$ (Wald's test, $p = 0.015$) after 10 min of sonication, whereas Tween-20 treatment resulted a decrease in small particles to $13.45 \pm 1.69\%$ (Figure 20B).

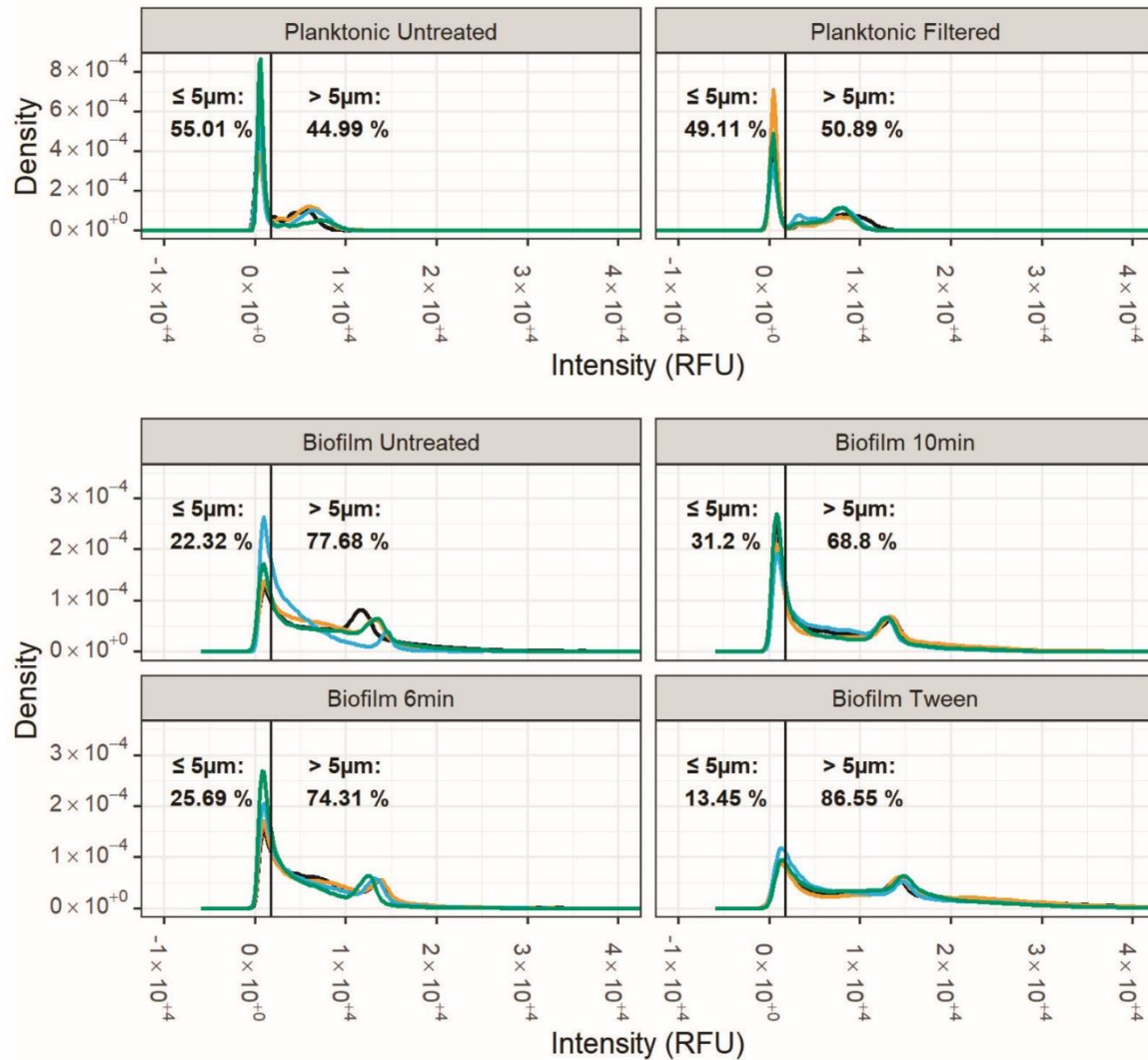


Figure 20. Impact of biofilm disruption treatment on *M. bovis* particle size. Particle size of disrupted *M. bovis* biofilms was determined using SYTO9 stained cells with an imaging cytometer. (A) Two populations for the planktonic cells were noted, with a natural division observed at 1.75×10^5 RFU, which corresponds with a particle diameter of approximately 5 μm . (B) For the disrupted biofilms, the 10 min sonication had strong evidence (Wald's test, $p = 0.015$) for an increase in small particles compared to the untreated biofilms. The percentage of particles above and below 5 μm was calculated for each treatment and compared. Representative of one experiment with two replicate cultures, each with two technical replicates (represented by different colored lines). Each technical replicate had $\sim 5 \times 10^4$ – 8×10^4 particles/biofilm replicate and $\sim 8 \times 10^3$ – 16×10^3 particles/planktonic replicate.

3.4 Small Particle Analysis

Having observed that the 10 min sonication was the most effective disruption technique according to the imaging cytometry data, we confirmed these results using a flow cytometer equipped with a 405 nm small particle laser and size calibration beads. Planktonic *M. bovis* samples had a right skewed distribution with a peak corresponding to $< 0.5 \mu\text{m}$ particles (Figure 21A). The untreated biofilm had a bimodal distribution with the first peak corresponding to $\sim 0.5 \mu\text{m}$ and the second peak corresponding to $1.0 - 2.0 \mu\text{m}$ particles (Figure 21B). The 10 min sonicated biofilms had a single peak corresponding to $0.5 - 1.0 \mu\text{m}$ particles (Figure 21C). Consistent with these observations, mean fluorescence values corresponding to size observed were lowest for the planktonic samples, higher for the sonicated biofilms, and highest for the untreated biofilms (Figure 22), with a significant $9.37 \pm 0.57\%$ decrease (Wald's Test, $p < 0.001$) in mean fluorescence after 10 min of sonication compared to the untreated biofilm (Figure 22). Similar findings were obtained with the other *Mycoplasma* isolates (data not shown).

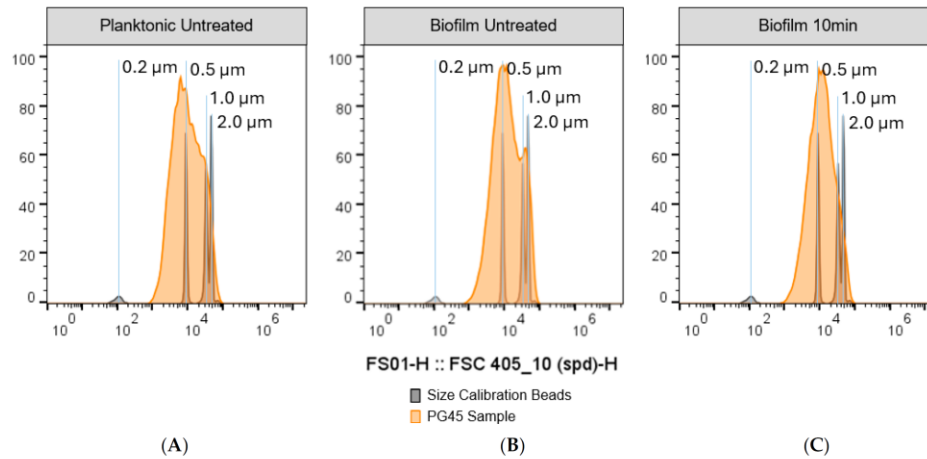


Figure 21. Impact of 10 minutes of sonication on the particle size of *M. bovis* PG45 biofilms. The particle size of disrupted biofilms was further analyzed by observing the forward scatter from a 405 nm small particle laser and comparing it to size calibration beads. (A) Planktonic *M. bovis* PG45 culture. (B) Untreated *M. bovis* PG45 biofilm. (C) *M. bovis* PG45 biofilm after 10 min sonication. Representative graphs were generated by randomly selecting and concatenating 1×10^6 particles from each technical replicate. Representative of one experiment with 4 technical replicates.

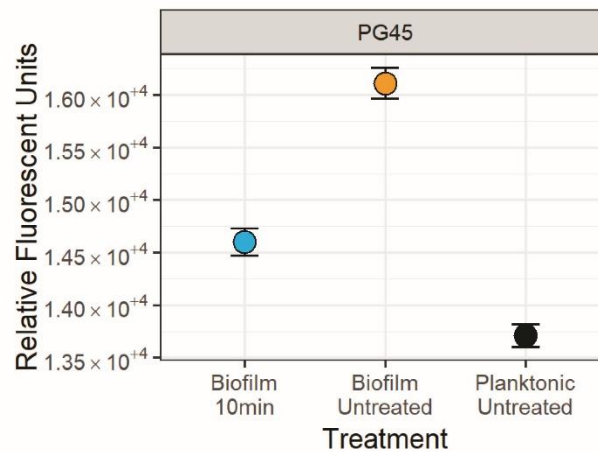


Figure 22. Impact of 10 minutes of sonication on the mean fluorescence of the *M. bovis* PG45 particles. There was a $9.37 \pm 0.57\%$ decrease (Wald's Test, $p < 0.001$) in mean particle size between the untreated biofilm and the 10 minutes sonicated biofilm. The point is the estimated mean and the 95% confidence interval from the separate means model. Representative of one experiment with four technical replicates.

3.5 Disaggregation Method Survival

Having demonstrated that a 10 min sonication increased the proportion of small particles in the *M. bovis* PG45 biofilms, we next assessed whether the cells survived the sonication

treatment. We also sought to ensure that there were no significant differences between measurements obtained using flow cytometry and imaging cytometry. The percentage of live cells (Figure 23A) was generally high, with > 90% live bacteria detected with both methods. There was no difference between the untreated and the sonication treated biofilms (one-sided Welch's t-Test, $p > 0.05$), and between measurements obtained using the imaging cytometry and flow (two-sided Welch's t-Test, $p > 0.05$).

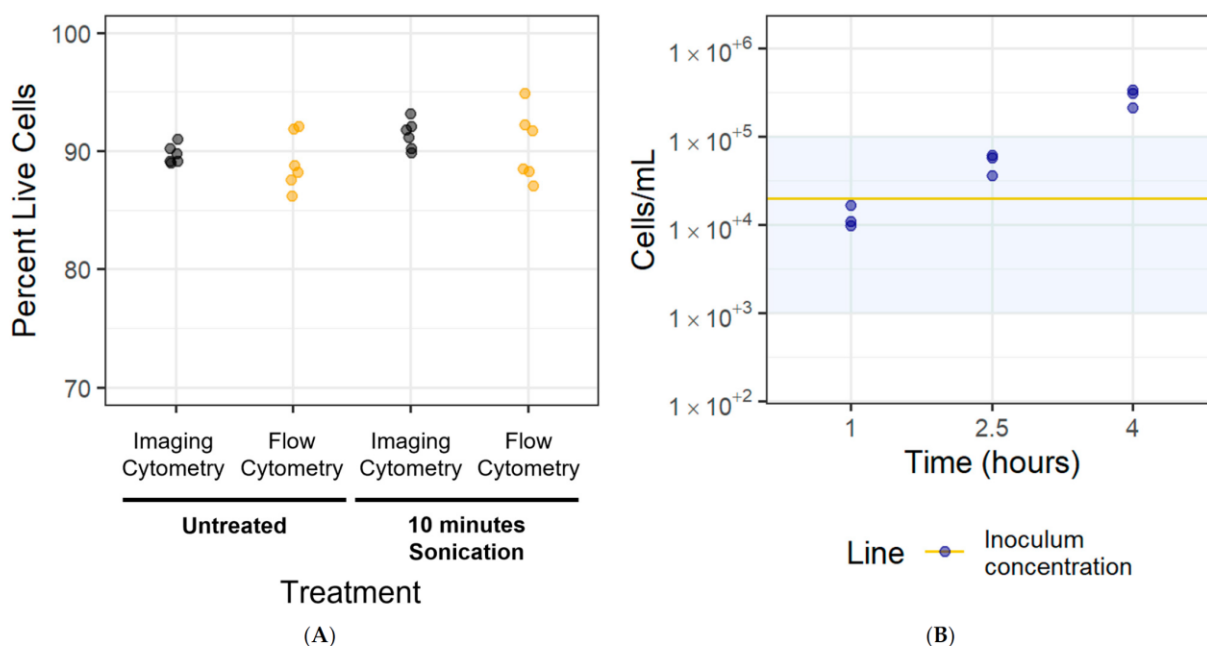


Figure 23. Analysis of cell viability and density for the LD-AST. (A) The survival of filtered cells exposed to 10 min of sonication and the rate of cell adherence was assessed. Filtered planktonic cells were sonicated for 10 min to determine if the treatment would lead to a decrease in live cells. The imaging and flow cytometry results were compared. Representative of one experiment with six technical replicates. (B) Cells inoculated at 2×10^4 cells/mL were imaged at 1, 2.5, and 4 h intervals to determine the rate of adhesion to the glass-bottom plate. The line represents the inoculum concentration while the shaded area represents the 103–105 cells/mL target range. Representative of one experiment with three technical replicates.

3.6 Cell Adhesion Rate for Newly Adhered Biofilms

To standardize cell counts for the newly adhered biofilm-based assay, the rate at which the cells adhered to the glass-bottom plate was investigated over 1-4 h. Our goal was to achieve a

cell concentration of 10^3 - 10^5 cells/mL, which is used in color change MIC assays for *Mycoplasma* [83]. We considered these newly adherent cells as our newly adhered biofilm. *M. bovis* PG45 cells were inoculated at 2×10^4 cells/mL into the wells, incubated for 1-4 h, and then were recovered and analyzed. The mean concentration of adhered cells was $1.26 \times 10^4 \pm 3.76 \times 10^3$ cells/mL after 1 h of incubation, $5.19 \times 10^4 \pm 1.39 \times 10^4$ cells/mL after 2.5 h of incubation, and $2.87 \times 10^5 \pm 6.89 \times 10^4$ cells/mL after 4 h (Figure 23B). Based on these results, we selected an incubation time of 1 h for the newly adhered *M. bovis* PG45 biofilms to achieve a cell concentration in the required 10^3 - 10^5 cells/mL range. When repeating this for the *M. bovis* Madison strain and the *M. bovoculi* MVDL1 and MVDL2 clinical isolates, an incubation time of 30 minutes was sufficient for reaching the desired cell concentration (data not shown).

3.7 Antimicrobial Susceptibility Testing

We next integrated the optimized culture and disruption methods for *Mycoplasma* biofilms into our novel flow cytometry-based LD-AST protocol to measure antibiotic susceptibility of *Mycoplasma* biofilms. The metric defined for $\text{MBC}_{\geq 5\%}$ is the lowest concentration that led to a $\geq 5\%$ decrease in the proportion of live cells. Based on this definition, we observed an $\text{MBC}_{\geq 5\%}$ for *M. bovis* PG45 exposed to enrofloxacin of 0.156 $\mu\text{g/mL}$ for the planktonic cells, $> 10 \mu\text{g/mL}$ for newly adhered biofilms, and 0.078 $\mu\text{g/mL}$ for mature biofilms (Figure 24A and Table 13). This represents a > 64 -fold increase in resistance with the newly adhered biofilms compared to the planktonic cells, but a 2-fold decrease in the resistance for the mature biofilm. The $\text{MBC}_{\geq 5\%}$ for gentamicin with *M. bovis* PG45 was 4 $\mu\text{g/mL}$ for planktonic cells, $> 128 \mu\text{g/mL}$ for newly adhered biofilms, and 8 $\mu\text{g/mL}$ for mature biofilms (Figure 24B and Table 13). This represents a > 32 -fold and a 2-fold increase in antibiotic resistance for both the newly adhered biofilm and

mature biofilms, respectively. The $MBC_{\geq 5\%}$ for *M. bovis* PG45 exposed to tetracycline was 0.125 $\mu\text{g/mL}$ for planktonic cells and increased by >128-fold to >16 $\mu\text{g/mL}$ for newly adhered biofilms, and by 2-fold to 0.25 $\mu\text{g/mL}$ for the mature biofilm (Figure 24C and Table 13). Paradoxically, at the highest concentrations of enrofloxacin (10 $\mu\text{g/mL}$) and tetracycline (16 $\mu\text{g/mL}$), the mature biofilm showed an increase in live cells compared to baseline levels, representing resistance to treatment.

When we performed the LD-AST with the additional *Mycoplasma* isolates we observed that the *M. bovis* Madison strain exposed had no change in susceptibility to enrofloxacin between the planktonic, newly adhered biofilm, and mature biofilm (Table 13). For gentamicin we observed a 2-fold increase in antibiotic resistance between the planktonic cells and newly adhered biofilm, but a 4-fold decrease in resistance with the mature biofilm (Table 13). We also observed that for tetracycline *M. bovis* Madison had a >2-fold decrease in resistance for both the newly adhered and mature biofilms compared to planktonic cells (Table 13).

For the *M. bovoculi* MVDL1 isolate exposed to enrofloxacin, we observed a >256-fold increase in resistance for the newly adhered biofilms and >8-fold resistance for the mature biofilm compared to the planktonic cells. For gentamicin, we observed that the MVDL1 isolate had >64-fold increase in resistance for the newly adhered biofilm and a >8-fold increase in resistance for the mature biofilm. With tetracycline exposure the MVDL1 isolate had a >256-fold increase in resistance for the newly adhered biofilm and a >8-fold increase in resistance for the mature biofilm (Table 13). These results show a general trend of large increases in resistance for both the newly adhered biofilms and the mature biofilms for the MVDL1 isolate.

For the *M. bovoculi* MVDL2 isolate exposed to enrofloxacin we observed a >256- fold increase in resistance for the newly adhered biofilm and no change for the mature biofilm compared to the planktonic cells. For gentamicin we observed >8-fold increase in resistance with the newly adhered biofilms, and a >4-fold decrease in resistance for the mature biofilms. For tetracycline we observed a >128-fold increase in resistance for the newly adhered biofilm and >2-fold decrease in resistance for the mature biofilm (Table 13).

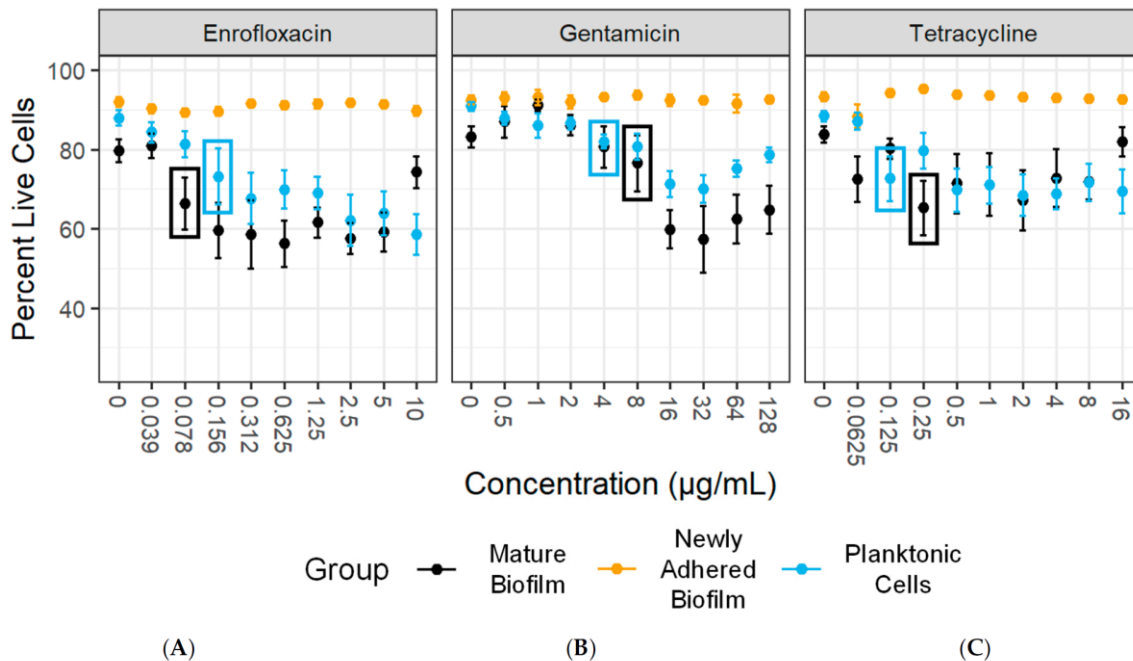


Figure 24. The LD-AST reveals increased MBCs for *M. bovis* PG45 biofilms compared to planktonic bacteria. Minimum bactericidal concentration of a decrease in live cells $\geq 5\%$ (MBC $\geq 5\%$) data showing the percentage of live cells compared to (A) enrofloxacin, (B) gentamicin, and (C) tetracycline concentrations for each of the organism states. The colored boxes correspond to the lowest antibiotic concentration that resulted in a significant drop in the percentage of live cells. The mean and SEM are shown for each group. Representative of three independent experiments with 2 technical replicates.

3.8 Statistical Diagnostics Assessment

The model fit for the separate means mixed models was assessed for both linearity and normality of the residuals and no severe violations were noted for the linearity, but some of the

live percentage separate means mixed models had severe violations of normality. Mixed-effect models however are robust to violations of normality with the resulting effect of these violations being a larger uncertainty estimate [209]. The Welch's t-Tests that were performed on the disruption survival data were determined to be appropriate as there were no severe violations of the normality assumption.

Table 13. $MBC_{\geq 5\%}$ concentration in $\mu\text{g/mL}$ by organism state (planktonic, newly adhered biofilms, mature biofilm).

<i>Mycoplasma</i> Isolate	Antimicrobial Agent	<i>Mycoplasma bovis</i> State		
		Planktonic	Newly Adhered Biofilm	Mature Biofilm
PG45	Enrofloxacin	0.156	$>10^1$	0.078
	Gentamicin	4	$>128^1$	8
	Tetracycline	0.125	$>16^1$	0.25
Madison	Enrofloxacin	0.078	0.078	0.078
	Gentamicin	8	16	2
	Tetracycline	0.125	$<0.0625^2$	$<0.0625^2$
MVDL1	Enrofloxacin	$<0.039^2$	$>10^1$	0.312
	Gentamicin	2	$>128^1$	16
	Tetracycline	$<0.0625^2$	$>16^1$	0.5
MVDL2	Enrofloxacin	0.078	$>10^1$	0.078
	Gentamicin	16	$>128^1$	4
	Tetracycline	0.125	$>16^1$	$<0.0625^2$

¹ $MBC \geq 5\%$ higher than the concentrations of the tested range, ² $MBC > 5\%$ lower than the concentrations of the tested range.

4. Discussion

In this study, we developed an innovative method for antimicrobial susceptibility testing in *Mycoplasma* biofilms. The standard color change assay was suitable to monitor growth of planktonic cells (Figure 19A), albeit with variable acidification observed with different *Mycoplasma* strains and isolates. This color change assay however was unsuitable for biofilms, since biofilm cultures of bovine *Mycoplasma* spp. did not cause a color change of the culture media upon growth, as we show. The lack of color change in the media found in our experiment was likely related to either the changes in biofilm metabolism and the metabolic dormancy in the

biofilm formation reported for various bacterial species including *Mycoplasma* or the physical barrier of the EPM altering the diffusion of metabolites [179, 186-188, 210-212].

We here demonstrate an innovative AST for *Mycoplasma* biofilms using flow cytometry with live dead staining with disaggregated *Mycoplasma* biofilms. The application of flow cytometry for the analysis of antimicrobial effects on *Mycoplasma* spp. has been described previously, but only with planktonic organisms [213, 214]. Our study sought to standardize methods for utilizing this technique for biofilm forming *Mycoplasma* spp. Importantly, results for planktonic *M. bovis* PG45 analyzed with our LD-AST ($\text{MBC}_{\geq 5\%}$ values) matched previously published values for MIC at 4 $\mu\text{g/mL}$ for gentamicin [172, 215] 0.16 $\mu\text{g/mL}$ for enrofloxacin [215], and 0.125 $\mu\text{g/mL}$ for tetracycline [172]. Interestingly however, no antibiotic concentration was able to reduce the mean percentage of live cells to <45% for any of the tested organisms. This might be due to autoaggregation of the cells, as evidenced from our imaging cytometry data where the population of planktonic cells was $44.99 \pm 12.32\%$ larger than 5 μm . Autoaggregation has also been shown to be part of the biofilm formation cycle and has been previously associated with increases in antibiotic resistance with other organisms even when not attached to a surface [216, 217].

What is interesting about these results generally is that the newly adhered biofilm led to increased resistance in all the tested isolates and antibiotics except for *M. bovis* Madison exposed to enrofloxacin or gentamicin. This phenomenon of biofilms to withstand increased concentrations of antibiotics has also been termed “tolerance” in several studies [218, 219], since decreased susceptibility to antibiotics in biofilms is thought to be caused by the expression of wild-type genes rather than due to genetic mutation or acquisition [220]. However, we use the term “resistance” based on the general definition that resistance is the “development of the

ability to withstand the previously destructive effect of a drug by microorganisms” [221] and previous usage of the term “resistance” by other studies to describe this innate resistance by biofilm formation [37, 184, 222, 223]. For newly adhered biofilms, we observed increases in resistance for all three antibiotics in the *M. bovis* PG45, *M. bovoculi* MVDL1 and MVDL2 isolates. For the remaining isolate *M. bovis* Madison, we observed an increase in resistance to gentamicin. These differences in observed susceptibility between the planktonic cells and the biofilm states match previous studies on increased antimicrobial resistance in biofilms formed by other biofilm forming bacteria [185, 224].

Data were more variable for mature biofilms. We observed a 2-fold decrease in enrofloxacin resistance for mature biofilms of *M. bovis* PG45 and no change in susceptibility for mature biofilms of *M. bovis* Madison and *M. bovoculi* MVDL2. This might be due to the inhibition of nuclease synthesis by enrofloxacin, as extracellular DNA (eDNA) is important in adherence of other *Mycoplasma* biofilms [225]. The *M. bovis* Madison strain and *M. bovoculi* MVDL2 isolate are also interesting as both organisms saw a ≥ 2 -fold decrease in susceptibility for both gentamicin and tetracycline. As both organisms took longer to reach peak biofilm maturity (5 d) compared to the other isolates (4 d), and as peak biofilm confluence for *M. bovis* Madison was low ($8.99 \pm 0.81\%$), the differential results obtained with the different strains might reflect differences in their ability to form biofilms. For *M. bovis* PG45 and *M. bovoculi* MVDL1, 2-fold and 8-fold increases in resistance respectively were observed for both gentamicin and tetracycline $\text{MBC}_{\geq 5\%}$ compared to the planktonic cells. The EPM and eDNA of the mature biofilm in these organisms likely provided resistance from gentamicin [224-226].

Interestingly, we observed that mature biofilms of *M. bovis* PG45 were completely resistant to the bactericidal effects of the highest concentration of enrofloxacin (10 µg/mL). Similar observations were made for mature biofilms of *M. bovis* PG45, *M. bovis* Madison, and *M. bovoculi* MVDL2 treated with tetracycline, where the highest concentration (16 µg/mL) failed to kill the bacteria, but lower concentrations led to significantly decreased viability. This decrease in efficacy of enrofloxacin in the mature biofilms at those highest concentrations may represent the “paradoxical” or “Eagle effect” [171, 227]. Eagle effect is a phenomenon where higher drug concentrations can result in increased resistance by the organism, while it shows susceptibility to lower drug concentrations [171]. This effect has been seen in both bacterial and fungal biofilms, where increases in antimicrobial concentrations resulted in increased cellular survival in the biofilm [43, 228].

Notably, a previous study by McAuliffe et al. [49] investigated *M. bovis* biofilms and found no difference in antimicrobial susceptibility upon biofilm formation. However, the color change assay was used in their study, which cannot accurately determine biofilm growth. Moreover, we showed that the effect of biofilm formation on antibiotic resistance is highly variable and depended on the *Mycoplasma* isolate and the time after biofilm formation, with a strong reduction in antimicrobial susceptibility seen with newly adhered biofilms only. In McAuliffe’s study, biofilms were cultured for 24 h before antibiotics were added [49], whereas we exposed biofilms to antibiotics after 1 h or 4-5 d of growth.

Our results support the hypothesis that *Mycoplasma* biofilms have increased resistance to antibiotics, which may be due to several biofilm-associated mechanisms. First, the EPM can act as a physical barrier to antibiotic diffusion, slowing or preventing interaction of the antibiotics

with their cellular targets [229]. Enrofloxacin is a fluoroquinolone antibiotic that inhibits nucleic acid synthesis by disrupting the DNA gyrase and topoisomerase IV enzymes [230]. Resistance to fluoroquinolones has been positively correlated with the bacterial SOS response, which is a series of proteins produced during cellular distress to maintain DNA integrity [231]. This SOS response has been observed as a likely cause of fluoroquinolone resistance in *Mycoplasma gallisepticum* biofilms [48], and genes for the SOS system have been described in *M. bovis* [232]. The second antibiotic we tested, gentamicin, is an aminoglycoside antibiotic that inhibits protein synthesis by causing conformational changes to the 16S ribosomal RNA component of the 30S ribosome subunit which results in the mistranslation of proteins [226]. Biofilm resistance to aminoglycosides may be partially due to the negative charge of the EPM and the positive charge of the antibiotic, which limits penetration [225]. The eDNA component of the EPM also can contribute to resistance as even low concentrations in biofilms have been shown to chelate cations and lead to fluoroquinolone resistance [233]. *Mycoplasma* biofilms in other species have both negatively charged EPM as well as eDNA [184, 234], which supports a role for both the EPM and the eDNA in the resistance of *M. bovis* biofilms to gentamicin. The third antibiotic that we tested, tetracycline, inhibits protein synthesis by preventing attachment of aminoacyl-tRNA to the ribosomal A acceptor site [235]. In many organisms expression of efflux pumps that remove antibiotics such as tetracycline from the organism is associated with resistance [225]. Efflux pumps have also been associated with general biofilm formation as they can contribute to biofilm formation by the secretion of EPM components [45]. Efflux pump associated genes have also been observed in *M. bovis*, which may help explain our observed resistance to tetracycline by *M. bovis* PG45 [236].

The CLSI states that while the minimal concentration needed to kill 99.9% of the cells is the most common estimation of MBC, this definition is arbitrary and may lack biological relevance [171]. Notably, the $MBC_{\geq 5\%}$ determined with our assay matched the published MIC values obtained by the standard microdilution assay for planktonic *M. bovis* PG45 for all the antibiotics tested, supporting the validity of our approach [172, 215].

Glass often allows for the formation of biofilms and *M. bovis* biofilm formation on glass has been previously described [49] as well as for other *Mycoplasma* spp. [234, 237]. This informed our choice to use glass-bottom plates in both the newly adhered and mature biofilms. For the newly adhered biofilms, the determination of the cell adherence rate of *M. bovis* was crucial for allowing us to obtain the same starting concentration as our planktonic cells. This approach allowed us to directly compare the new LD-AST to the standard colorimetric assay, as the cell concentration was in the same range when the antibiotics were added.

For the mature biofilms we wanted to define experimental conditions that were in line with the current understanding of the biofilm life cycle, which involves cell attachment, maturation, and dispersion [37]. Unfortunately, the biofilm formation cycle for *Mycoplasma* spp. is not well described. A few studies have worked to address this in various *Mycoplasma* spp. by characterizing the adhesion, aggregation, and percentage of area covered [238, 239], the extracellular matrix composition [234], and gene expression [239]. Even with these studies, a complete understanding of the specific cycle of formation and dispersion is not well understood, and so the application of knowledge from other bacterial species has been applied in this study. In the classical understanding of biofilm formation cycles by other bacteria, the development of a 3-dimensional structure is associated with maturity, after which the bacteria disperse, which

leads to both the spread of the organism and a decrease in the biofilm biomass [41]. This model matches our observation that the biofilm biomass peaked at 4-5 d, likely representing maturation, and decreased significantly afterwards, possibly reflecting dispersion.

When we investigated the particle size of the planktonic cells, an interesting trend was noted where the filtered cells still had a diameter larger than the 0.45 μm pore size for the syringe filter. The diameter for single *M. bovis* cells is between 0.2-1.0 μm [240, 241], and our flow cytometry experiments confirmed that planktonic *M. bovis* was within the 0.5-1.0 μm range. In a study investigating filtration, soft particles were able to deform and translocate across filter pores that were smaller than the particles [242]. As *Mycoplasma* spp. lack a cell wall, the bacteria likely passed through the filter pores by deformation.

We are aware that this study still had some limitations as we only utilized four *Mycoplasma* isolates, three antibiotics, and a limited number of disruption techniques. Our study also only performed a limited investigation into *Mycoplasma* biofilm maturity and did not investigate any mechanisms of resistance conferred by the formation of the biofilms. These are important factors for the understanding and characterization of both biofilms and resistance by *M. bovis* and should be explored further. Also, we did not validate our AST results with the gold standard color change assay, but instead relied on published MIC values. While these limitations do exist, the overall methodology should remain viable with minimal adaptation for other antibiotics, *M. bovis* and *M. bovoculi* strains, and *Mycoplasma* spp.

In summary, we utilized the LD-AST to directly compare the antibiotic susceptibilities of *M. bovis* biofilms and planktonic cells, with reproducible results. The $\text{MBC}_{\geq 5\%}$ we observed for planktonic cells corresponded with the MIC values obtained with the standard microdilution

method. We anticipate that the new LD-AST method will allow for further comparison of the resistive effects of biofilm formation by other *Mycoplasma* spp. and will better inform whether antibiotic treatments might be successful in treating infection.

Supplementary Materials

The following supporting information can be downloaded at:

<https://www.mdpi.com/article/10.3390/microorganisms12122650/s1>, Table S1: Catalog numbers and manufacturers for the materials used, Table S2: Estimated pH model output, Table S3: Particle disruption model output, Table S4: Small particle (405 nm) model output, Table S5: PG45 maturity structure measurement model, Table S6: PG45 maturity confluence model, Table S7: Madison maturity structure measurement model, Table S8: Madison maturity confluence model, Table S9: MVDL1 maturity structure measurement model, Table S10: MVDL1 maturity confluence model, Table S11: MVDL2 maturity structure measurement model, Table S12: MVDL2 maturity confluence model, Table S13: Mature PG45 percent live model (PLM) for enrofloxacin, Table S14: Mature PG45 PLM for gentamicin, Table S15: Mature PG45 PLM for tetracycline, Table S16: NAB PG45 PLM for enrofloxacin, Table S17: NAB PG45 PLM for gentamicin, Table S18: NAB PG45 PLM for tetracycline, Table S19: Planktonic PG45 PLM for enrofloxacin, Table S20: Planktonic PG45 PLM for gentamicin, Table S21: Planktonic PG45 PLM for tetracycline, Table S22: Mature Madison PLM for enrofloxacin, Table S23: Mature Madison PLM for gentamicin, Table S24: Mature Madison PLM for tetracycline, Table S25: NAB Madison PLM for enrofloxacin, Table S26: NAB Madison PLM for gentamicin, Table S27: NAB Madison PLM for tetracycline, Table S28: Planktonic Madison PLM for enrofloxacin, Table S29: Planktonic Madison PLM for gentamicin, Table S30: Planktonic Madison PLM for

tetracycline, Table S31: Mature MVDL1 PLM for enrofloxacin, Table S32: Mature MVDL1 PLM for gentamicin, Table S33: Mature MVDL1 PLM for tetracycline, Table S34: NAB MVDL1 PLM for enrofloxacin, Table S35: NAB MVDL1 PLM for gentamicin, Table S36: NAB MVDL1 PLM for tetracycline, Table S37: Planktonic MVDL1 PLM for enrofloxacin, Table S38: Planktonic MVDL1 PLM for gentamicin, Table S39: Planktonic MVDL1 PLM for tetracycline, Table S40: Mature MVDL2 PLM for enrofloxacin, Table S41: Mature MVDL2 PLM for gentamicin, Table S42: Mature MVDL2 PLM for tetracycline, Table S43: NAB MVDL2 PLM for enrofloxacin, Table S44: NAB MVDL2 PLM for gentamicin, Table S45: NAB MVDL2 PLM for tetracycline, Table S46: Planktonic MVDL2 PLM for enrofloxacin, Table S47: Planktonic MVDL2 PLM for gentamicin, Table S48: Planktonic MVDL2 PLM for tetracycline.

Author Contributions

The contributor roles for this publication are defined as follows: Conceptualization, B.T.J., E.R.S., D.B., and J.D.; methodology, B.T.J.; software, B.T.J., M.T., and C.C.; validation, J.D., B.T.J., and E.T.S.; formal analysis, B.T.J., M.T., and C.C.; investigation, J.D., B.T.J., E.T.S., M.Q., L.Z., and S.S.; resources, D.B. and B.T.J.; data curation, B.T.J., J.D., and E.T.S.; writing—original draft preparation, B.T.J. and J.D.; writing—review and editing, B.T.J., J.D., D.B., E.R.S., E.T.S.; visualization, B.T.J. and J.D.; supervision, B.T.J. and D.B.; project administration, B.T.J. and D.B.; funding acquisition, D.B., B.T.J., and J.D. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data for the experiments described above can be found at:

<https://doi.org/10.5061/dryad.m905qfv8m>

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Conflicts of Interest

The authors declare no conflicts of interest.

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CHAPTER FIVE

MECHANISMS OF BIOFILM FORMATION AND ANTIBIOTIC
TOLERANCE IN *MYCOPLASMA OVIPNEUMONIAE*

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Abstract

Mycoplasma ovipneumoniae is a respiratory pathogen in both domestic and wild sheep that causes mild to severe respiratory illnesses and persistent infections of the upper respiratory tract that antibiotics typically fail to clear. Related organisms in the *Mycoplasmataceae* family form biofilms that can tolerate high concentrations of various antibiotics. However, whether *M. ovipneumoniae* forms biofilms has not been systematically analyzed and remains a matter of debate, with some previous work observing weak or no biofilm formation. To determine the ability and impact of biofilm formation by *M. ovipneumoniae*, we investigated a well-characterized strain (Y98) and three clinical strains (MSU-NW4-2021, MSU-LS2.2-2024, and MSU-LS3.1-2024). Using crystal violet staining and fluorescent confocal microscopy, we show that all strains analyzed formed biofilms, but that size and morphology of the biofilms was variable. Additionally, when grown as biofilms, all strains showed increased antibiotic tolerance to enrofloxacin, gentamicin, and tetracycline in a flow cytometry based live-dead antibiotic susceptibility test (LD-AST). Whole genome sequencing of the clinical strains for further *in silico* analysis detected several potential genes associated with adhesion (*pdhD*) and dispersal (*mnuA* and *nuc*) of biofilms. Further *in silico* investigation into the protein sequences showed high levels of conservation for PdhD, and increased variation for MnuA and Nuc. Interestingly the Y98 and MSU-LS2.2-2024 strains showed the highest similarity with PdhD, while the MSU-NW4-2021 and MSU-LS3.1-2024 strains were most similar for MnuA. The Y98 and MSU-LS2.2-2024 strains formed the most robust biofilms and had similar dispersal characteristics, potentially pointing towards PdhD as a contributor to biofilm adhesion. Alternatively, as MSU-NW4-2021 and MSU-LS3.1-2024 had lower levels of biofilm formation and more similar MnuA

sequences, this might suggest that MnuA contributes to biofilm dispersion. Overall, this research suggests that biofilm formation in *M. ovipneumoniae* leads to antibiotic tolerance and potentially contributes to chronic persistence and disease.

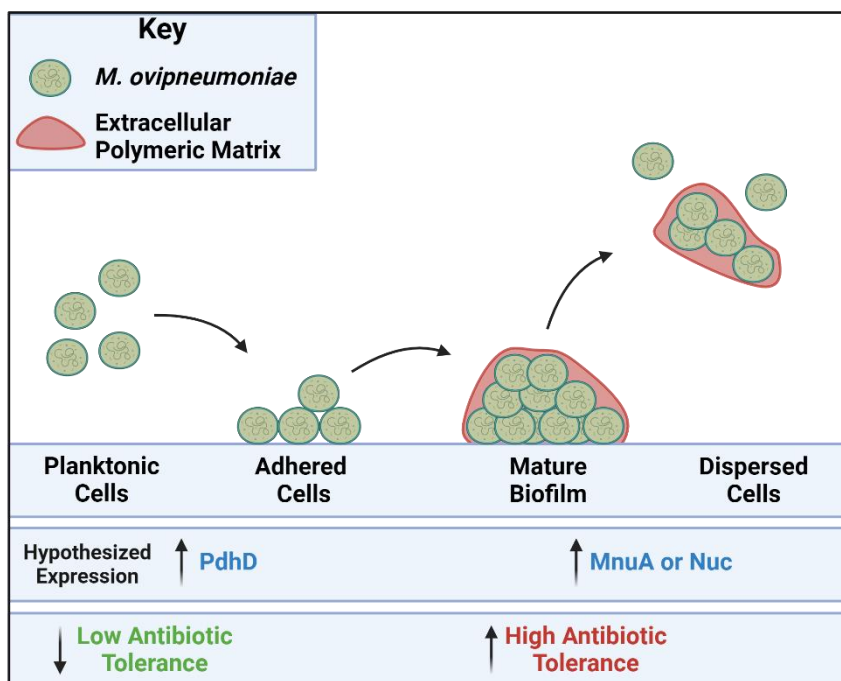


Figure 25. Graphical Abstract. *M. ovipneumoniae* formation is hypothesized to be instigated by the putative adhesin PdhD and dispersed with the nucleases MnuA or Nuc. We have also observed low antibiotic tolerance by planktonic cells and high antibiotic tolerance after biofilm formation.

Introduction

The bacterial pathogen *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) is associated with severe respiratory disease in bighorn sheep (*Ovis canadensis*) [106, 243] and atypical pneumonia in domestic sheep (*Ovis aries*) [244]. *M. ovipneumoniae* infection commonly occurs in domestic sheep, with infection detected in 40.9-88.5% of domestic sheep operations worldwide [7-11]. Infection with *M. ovipneumoniae* causes persistent colonization of the upper respiratory mucosae and is resistant to various antibiotic treatments [24, 53, 245].

In addition to genetic mechanisms of antibiotic resistance, antibiotic treatments can fail due to antibiotic tolerance associated with biofilm formation, which involves metabolic changes that result in the self-aggregation or adhesion of an organism to a surface and the formation of an extracellular polymeric matrix (EPM). This EPM is composed of extracellular DNA (eDNA) and polysaccharides which have been shown to reduce penetration and activity of antimicrobial drugs [37, 181]. As we and others have shown, this reduced penetration can lead to increases in the minimum inhibitory concentration and minimum bactericidal concentration (MIC and MBC respectively) [233, 246, 247]. Antibiotic tolerance correlated with biofilm formation has been described for other members of the *Mycoplasmataceae* family [185, 248], but has not been studied in *M. ovipneumoniae*.

Prior research on *M. ovipneumoniae* biofilm formation has been limited, with previous reports observing no formation, weak formation, or limited staining [49, 50]. However, formation of an exopolysaccharide capsule by *M. ovipneumoniae* has been reported [249], which can serve to foster both bacterial adhesion and biofilm formation [250].

Due to the effects that biofilm formation has on antimicrobial therapy, further understanding of the mechanisms of biofilm formation and dispersal could reveal potential new therapeutic targets [251]. It is widely understood that biofilm formation in most species proceeds through the steps of adhesion, growth, and detachment [252]. Each phase of this cycle corresponds with the production and expression of specific proteins and genes [253, 254]. These phases of biofilm adhesion and dispersal are of specific interest due to their potential as a target for inhibiting or disrupting biofilms [251]. The adhesion step of many species involves the expression of adhesins that enable the self-aggregation of cells or the attachment of the cells to a surface [255]. The dispersal step of the cycle typically involves the regulation of one or more nucleases that can disrupt the EPM of a formed biofilm by degrading the eDNA [254, 256]. In *M. ovipneumoniae*, other *Mycoplasmataceae*, and other bacterial species, the adhesin pyruvate dehydrogenase complex component E3 (PdhD) has been identified as a potential contributing factor to biofilm adhesion and survival of stationary cells [48, 51, 257]. In other members of *Mycoplasmataceae*, the virulence factor membrane nuclease A (MnuA) has been identified as a primary contributor to nuclease activity [258, 259].

Previous work by McAuliffe et al. mainly focused on *Mycoplasma bovis* biofilm formation, and noted that *M. ovipneumoniae* was unable to form a biofilm in their study [49]. Similarly, Osman et al. reported that the strains tested in their work showed weak tendency for biofilm formation [50]. A recent study by Ge et al. did observe biofilm formation by *M. ovipneumoniae*, but only utilized limited methods for quantification [250]. Our work is the first to describe both the consistent and robust biofilm formation by *M. ovipneumoniae* and the effects

that formation has on antibiotic tolerance. This work also is the first to investigate the role that nuclease activity has on the formation cycle of *M. ovipneumoniae* biofilms.

Methods

Bacterial Strains

Three *M. ovipneumoniae* strains (Table 14) were isolated from domestic sheep (Montana State University, MT, USA), and the reference strain Y98 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All strains were verified as *M. ovipneumoniae* via qPCR for the *M. ovipneumoniae* P113 fragment as previously described [24].

Table 14 Characteristics of *Mycoplasma ovipneumoniae* strains used in this study.

<i>M. ovipneumoniae</i> Strain	Source	Collection Source	Year Collected
<i>M. ovipneumoniae</i> Strain Y98 (29419)	American Type Culture Collection (ATCC)	Ovine Respiratory Tract	1972
<i>M. ovipneumoniae</i> Clinical Strain MSU-NW4 -2020 (NW4)	Montana State University (MSU)	Ovine Nasal Wash	2020
<i>M. ovipneumoniae</i> Clinical Strain MSU-LS2.2-2024 (LS2.2)	MSU	Ovine Lung Swab	2024
<i>M. ovipneumoniae</i> Clinical Strain MSU-LS3.1-2024 (LS3.1)	MSU	Ovine Lung Swab	2024

Quantification of Cells with Flow Cytometry

The *M. ovipneumoniae* cells were quantified with flow cytometry as previously described [200, 260]. Briefly, cells were stained with a 1:6,000 dilution of SYBR Safe DNA gel dye (Invitrogen, Waltham, MA, USA) for 15 minutes at 4 °C and then counted on a ZE5™ flow

cytometer (Bio-Rad, Hercules, CA, USA) with CountBright Plus Absolute Counting Beads (Invitrogen).

Culture Methods

M. ovipneumoniae was grown in a modified PPLO broth media prepared as previously described [24, 247]. Briefly, the media was composed of *Mycoplasma* PPLO Broth Base (19.5 mg/mL) (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with L-cysteine hydrochloride monohydrate (8.7×10^{-2} mg/mL) (Thermo Fisher Scientific, Waltham, MA, USA), beta-nicotinamide adenine dinucleotide (NAD) (8.7×10^{-2} mg/mL) (Thermo Fisher Scientific), dextrose (2.6 mg/mL) (Thermo Fisher Scientific), thallium acetate (0.43 mg/mL) (Acros Organics, Fair Lawn, NJ, USA), porcine serum (6.5%) (Quad Five, Rygate, MT, USA), horse serum (6.5%) (Quad Five), and phenol red (2.1×10^{-2} mg/mL) (Sigma Aldrich, St. Louis, MO, USA) as a pH indicator. Agar plates were made using the components listed above in addition to agarose (12 mg/mL) (Fisher Scientific) The cultures were grown in broth media at atmospheric conditions at 37 °C for 1-3 days (d) until a color change was observed due to acid production. Cultures were incubated in 6-well or 96-well polystyrene plates (Celltreat, Ayer, MA, USA) for use in additional assays. To stimulate *in vitro* biofilm formation, the *M. ovipneumoniae* isolates were incubated in 96-well glass-bottom plates (Greiner Bio-one, Monroe, NC, USA) under the same conditions. When cultures were grown on agar instead of the broth media, they were incubated with microaerophilic pouches (Mitsubishi Chemical America, New York, NY, USA), at 37 °C as previously described [24].

Biofilm Staining

Crystal Violet: Crystal violet staining of *M. ovipneumoniae* biofilms on glass-bottom plates was performed according to previously published methods [261] with some slight alterations. Briefly, the media and non-adhered cells were removed via pipetting, and then each well was washed with phosphate-buffered saline (PBS) three times and allowed to dry. Next, 100% ethanol was added to each well to fix the biofilms and then allowed to dry. Once the samples dried, 0.1% crystal violet dye was added to each well and incubated for 15 min at room temperature. The dye was then removed via pipetting, and the plate was washed three times before imaging with a Keyence BZX-810 microscope (Keyence, Itasca, IL, USA).

DNA and EPM Staining: Planktonic *M. ovipneumoniae* Y98 cultures were grown in PPLO broth for 2 d and then diluted to a concentration of 5×10^5 cells/mL. 200 μ L of the diluted culture was added to each well of a 96 well glass bottom plate, except for the outermost border wells, which were filled with PBS to prevent evaporation. The cultures were then incubated for 4-6 d depending on the peak confluence determined for each isolate. The biofilms were then stained with 50 μ L of 1 μ M TOTO-1 DNA dye and 10 μ g/mL wheat germ agglutinin (WGA) with an Alexa Fluor 488 conjugate and incubated for 1 h at ambient temperature. After 1 h, the stain was removed, and wells were washed gently 2x with 50 μ L of PBS. Cells were subsequently imaged on a Keyence BZ-X 810 microscope.

Confocal Microscopy

Biofilms were imaged using a Leica Stellaris 8 Confocal Scanning Laser Microscope and image analysis was performed using Imaris® software (v10.2.0; Oxford Instruments) [262]. To identify biofilm structures, surface analysis was performed using TOTO-1 positive signal using

the following criteria: threshold between 3-5, set manually based on image quality, a surface grain size of 0.361 μm , maximum object diameter of 1.35 μm , with object splitting performed after 1.8 μm diameter. Biofilm height was determined by the Z position of objects compared to a reference frame set to $Z = 0$, with the greatest Z position equivalent to maximum height.

Biofilm Image Analysis

M. ovipneumoniae biofilm growth was observed by inoculating a culture of 5×10^4 cells/mL into a 96-well glass-bottom plate. The cells were then incubated for 6 d under standard conditions. The adhered cells were quantified with brightfield images taken daily using a Keyence BZ-X 810 microscope. The images were then analyzed with FIJI software (v2.14.0) [195] to assess the confluence of adhered cells. Confluence was assessed using methods described previously [260]. Briefly, image thresholding was used to better visualize the adhered cells, and a percent coverage of the field of view was then calculated.

Colony Morphology

Colony morphologies were imaged using a Fisher Science Education 430TBL dissection microscope (Fisher Scientific) and a cellphone camera with an ocular mount.

Biofilm Disaggregation

To homogenize samples for flow cytometry analysis, we optimized methods for disaggregation of mature *M. ovipneumoniae* biofilms. Mature *M. ovipneumoniae* Y98 biofilms were grown and disrupted as follows. First, the broth media was removed from the top of the biofilm and replaced with FACS buffer (phosphate buffered saline (PBS) (Cytiva, Logan, UT), FBS (2%) (SeraPrime, Fort Collins, CO), and sodium azide (0.1%) (Aqua Solutions Inc., Deer

Park, TX)). The biofilms were then scraped with a mini cell scraper (United Biosystems, Herndon, VA) and treated with Tween-20 (Fisher Scientific) or with sonication for 6, 10, or 15 min. The resulting suspensions were transferred to 1.5 mL microcentrifuge tubes (Fisherbrand, Pittsburg, PA). Sonication was performed at 45 kHz in a bath sonicator (VWR Scientific, Aquasonic Model 550T) based on a protocol modified from Totten et al. [198]. Disruption with Tween-20 was performed by incubation for 15 min at room temperature as previously described [260].

Small Particle Analysis with Flow Cytometry

The disrupted biofilms described above were compared to biofilms that had not been sonicated or treated with Tween and to planktonic cells also stained with 1:6,000 SYBR safe DNA gel stain (Invitrogen). These samples were then analyzed with the ZE5™ Flow cytometer (Bio-Rad) with a 405 nm small particle laser. The samples were then compared to a Flow Cytometry Sub-micron Particle Size Reference Kit (Invitrogen) to determine the specific range in particle size.

Hydrogen Peroxide Production

Hydrogen peroxide production by the four *M. ovipneumoniae* strains was stimulated using methods modified from a recent study [263]. Briefly, broth media described above was supplemented with 0.8 M glycerol and added to a polystyrene plate with 5×10^4 cells/mL of the planktonic *M. ovipneumoniae* cultures and compared to 0.8 M glycerol added to the top of mature *M. ovipneumoniae* biofilms grown on 96-well glass-bottom plate. Over the course of 6 d, the H₂O₂ concentration was quantified with MQuant peroxide test strips (Supelco, Bellefonte, PA, USA).

Broth Microdilutions

Broth microdilutions in penicillin-free modified PPLO were made with enrofloxacin (Sigma Aldrich, St. Louis, MO), gentamicin (Gibco, Waltham, MA), or tetracycline (Sigma Aldrich). 2-fold dilutions of enrofloxacin (10 µg/mL – 0.039 µg/mL), gentamicin (128 µg/mL – 0.5 µg/mL), and tetracycline (16 µg/mL – 0.0625 µg/mL) were used for testing.

Live Dead Antibiotic Susceptibility Testing (LD-AST)

The LD-AST methods for determining antibiotic susceptibility in mature *M. ovipneumoniae* biofilms and planktonic bacteria were performed as previously described with few modifications [260]. Briefly, cells were inoculated onto the plates at 5×10^4 cells/mL, 15 min of sonication was used to disaggregate the biofilms (Figure 26).

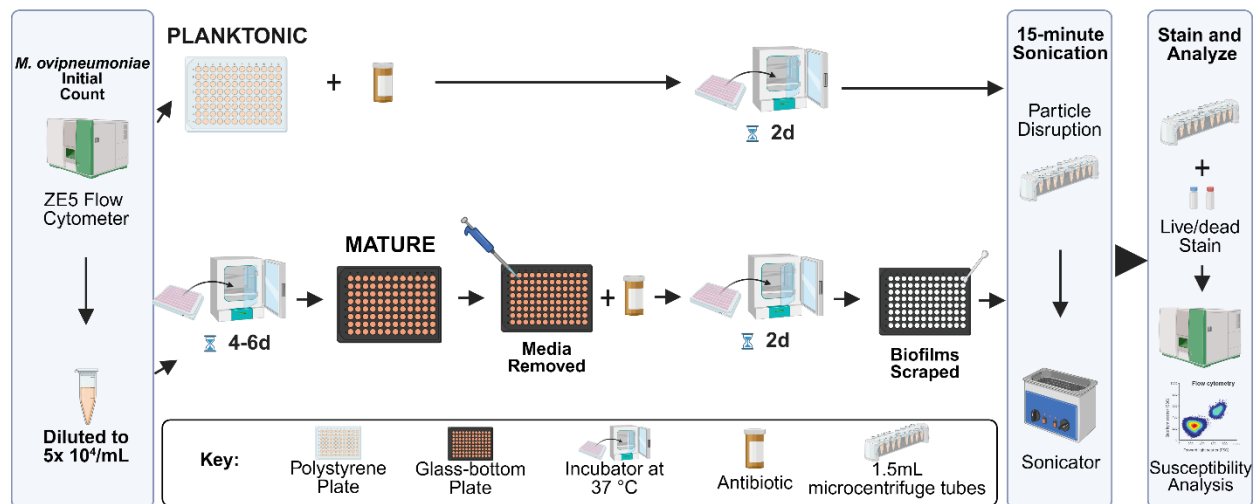


Figure 26. The LD-AST methods used for determining the antibiotic susceptibility of planktonic and biofilm states of *M. ovipneumoniae*.

Biofilm Confluence After Antibiotic Exposure

The mature biofilms were exposed to the antibiotic concentrations described above for 4 d at 37 °C. After incubation the plates were imaged with the Keyence BZ-X 810 microscope to determine biofilm confluence at each of the antibiotic concentrations.

M. ovipneumoniae Whole Genome Sequencing and Genome Assembly

30 mL of *M. ovipneumoniae* broth culture for each strain was centrifuged at $10,000 \times g$ for 30 min and resuspended in 1 mL of sterile PBS. 200 μ L of the resuspended cells were then treated with 20 μ L of 10mg/mL RNase A (Thermo Scientific, Waltham, MA, USA) for 15 min. Next, the samples were extracted according to manufacturer instructions using a Mag-Bind Viral DNA/RNA Kit (Omega Bio-Tek, Norcross, GA, USA) with an EpMotion 5070 Robotic Liquid Handler (Eppendorf, Framingham, MA, USA). Next, Illumina iSeq100 short-read and Oxford Nanopore Technologies (ONT) long-read sequencing was performed by the Wyoming State Veterinary Laboratory (Laramie, WY, USA).

Hybrid Assembly

Raw Illumina sequence reads were cleaned with BB DuK (v38.95) [264] and ONT sequence reads were filtered with Chopper (v0.9.0) [265]. Assembly was performed using the ONT sequences reads with three independent methods (Flye v2.9.5-b1801, Minipolish v0.1.3, and Raven v1.8.3) [266-268]. These three methods were run separately due to the robustness of using separate algorithms and then combining them into a consensus. The contigs created by these assemblers (Flye, Minipolish, and Raven) with the expected read length for *M.*

ovipneumoniae (~ 1.0 Mbp) were manually selected and then combined as a consensus sequence using Tricycler (v0.5.5) [269]. The Illumina sequence reads were then aligned to the consensus

sequences with the Burrows-Wheeler Alignment Tool (BWA, v0.7.17-r1188), and errors on the consensus sequences were corrected with the aligned Illumina sequences using polypolish (v0.6.0) [270]. The quality of these polished hybrid assemblies was then assessed using CheckM (v1.2.3) [271]. Next, the polished hybrid assemblies were then annotated with the NCBI Prokaryote Genome Annotation Pipeline (PGAP, v2024-07-18.build7555) [272].

Genome analysis

To compare the difference between the *M. ovi* strains, we used FastANI (v1.33) [273] to calculate the Average Nucleotide Identity between the strains in a pairwise fashion.

To identify additional nucleases that could contribute to biofilm formation by the *M. ovipneumoniae* strains, we used VFAnalyzer (v2022) to identify potential virulence genes in our assemblies by comparing them to a virulence factor database [274].

Protein sequence comparisons

Clustal Omega (v1.2.4) [275] was used to perform a multiple sequence alignment of the transcribed proteins for PdhD, MnuA, and Nuc for all of the *M. ovipneumoniae* strains. The transcribed amino acid sequences for the PdhD, MnuA, and Nuc proteins from each of the strains were then submitted to the AlphaFold 3 Server [276] for structural prediction. Next, we used the protein structure for each strain that was identified as having the largest sequence for PdhD, MnuA, or Nuc as a template. To this template we visually annotated the conservation information derived from our Clustal Omega multiple sequence alignment. Blue coloration indicates complete conservation at that location across all strains, yellow indicates strong conservation (residues of similar properties), orange represents weak conservation (residues with weakly similar properties), and red represents no conservation.

Statistical Analysis

The analyses were performed with R Statistical software (v4.3.3; R Core Team 2024) [122]. Data processing was performed with the tidyverse package (v2.0.0) [123]. The nlme package (v3.1.164) [124] was used for the linear mixed-model analyses and mixed-model tables were created with the sjPlot package (v2.8.1.5) [126]. All figure plots were made with the ggplot2 package (v3.5.0) [127], ggthemes package (v5.1.0)[128] and ggpubr package (v0.6.0) [208]. The trees for both the ANI and multiple sequence alignment were made with Ape (v5.8-1) [277]. The heatmap for the ANI was generated with ComplexHeatmap (v2.22.0) [278, 279]. For all tests, significance was considered at an $\alpha < 0.05$.

The model used for biofilm maturity was performed utilizing confluence calculated from the brightfield imaging methods described above. To determine the effect of incubation time on biofilm confluence, a separate means mixed model analysis was performed. The baseline was set to the day with the highest observed confluence, and then compared to the previous 2 d. The day post-inoculation was a fixed effect, and the experimental replicate was a random effect according to the following:

$$PercentConfluence_i \sim \delta_{m[i]} + \beta_1 X_{DayPostInoculation_i} + \epsilon_i$$

$$\delta_m \sim N(0, \sigma_{ReplicateCulture}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation level and where m represents the experimental replicate.

For the small particle analysis of the disaggregation methods, a generalized linear model was performed with the treatment as a fixed effect according to the following:

$$\log(\mu_i) = \beta_0 + \beta_1 X_{Treatment_i}$$

$$FSC_i \sim IG(\mu_i, \lambda)$$

Where i represents the individual observation, FSC represents the forward scatter intensity from the flow cytometry data, and IG refers to the Inverse Gaussian distribution.

For the LD-AST assay, the MBC was defined as the lowest dilution that resulted in a significant decrease in live cell percentage that was $\geq 5\%$ ($MBC_{\geq 5\%}$). To determine this, a separate means mixed model was performed for each organism state and antibiotic combination with concentration as a fixed effect and the biological replicate as a random effect according to the following:

$$PercentLiveCells_i \sim \delta_{m[i]} + \beta_1 X_{concentration_i} + \epsilon_i$$

$$\delta_m \sim N(0, \sigma_{ReplicateCulture}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation level and where m represents the biological replicate.

In a similar manner, biofilm confluence after 4 d of exposure to antibiotics was determined with a separate means mixed model for each isolate and antibiotic combination with concentration as a fixed effect and the biological replicate as a random effect according to the following:

$$PercentConfluence_i \sim \delta_{m[i]} + \beta_1 X_{concentration_i} + \epsilon_i$$

$$\delta_m \sim N(0, \sigma_{ReplicateCulture}^2)$$

$$\epsilon_i \sim N(0, \sigma^2)$$

Where i represents the observation level and where m represents the biological replicate.

Results

Mycoplasma ovipneumoniae forms robust biofilms *in vitro*.

To determine whether *M. ovipneumoniae* can form biofilms, we cultured different *M. ovipneumoniae* strains of the bacteria on glass-bottom plates, which promote biofilm formation in other species [47]. Using brightfield imaging, we observed the development of adherent cell clusters, consistent with a typical biofilm morphology (Figure 27). We next performed a time course analysis of biofilm formation, using microscopic analysis of biofilm confluence as the readout (Figure 27). We defined the point of maturity as the highest level of confluence that was significantly increased compared to the previous 2 days (Student's T-test, $p < 0.05$). *M. ovipneumoniae* Y98 biofilms had the highest levels of confluence with maturity determined to be a confluence of $76.28 \pm 4.98\%$ at 5 d of incubation (Figure 27B, Table 15). Alternatively, *M. ovipneumoniae* NW4 biofilms achieved the lowest levels of confluence with maturity reached at

Table 15: The determined maturity metrics for *M. ovipneumoniae* biofilms.

<i>M. ovipneumoniae</i> Strain	Mature Biofilm Confluence	Peak Thickness
Y98	$76.28 \pm 4.98\%$ (5 days)	42.1 μm
NW4	$22.48 \pm 13.09\%$ (5 days)	6.3 μm
LS2.2	$48.42 \pm 8.25\%$ (4 days)	48.8 μm
LS3.1	$57.69 \pm 7.10\%$ (6 days)	32.9 μm

$22.48 \pm 13.09\%$ confluence at 5 d, (Wald's Test, $p < 0.001$) (Figure 27B, Table 15). The *M.*

ovipneumoniae LS2.2 isolate reached biofilm maturity at $48.42 \pm 8.25\%$ confluence at 4 d (Wald's Test, $p < 0.05$) (Table 15). Biofilms of the *M. ovipneumoniae* LS3.1 isolate matured the latest, with $57.69 \pm 7.10\%$ confluence at 6 d (Wald's Test, $p < 0.001$) (Figure 27B, Table 15).

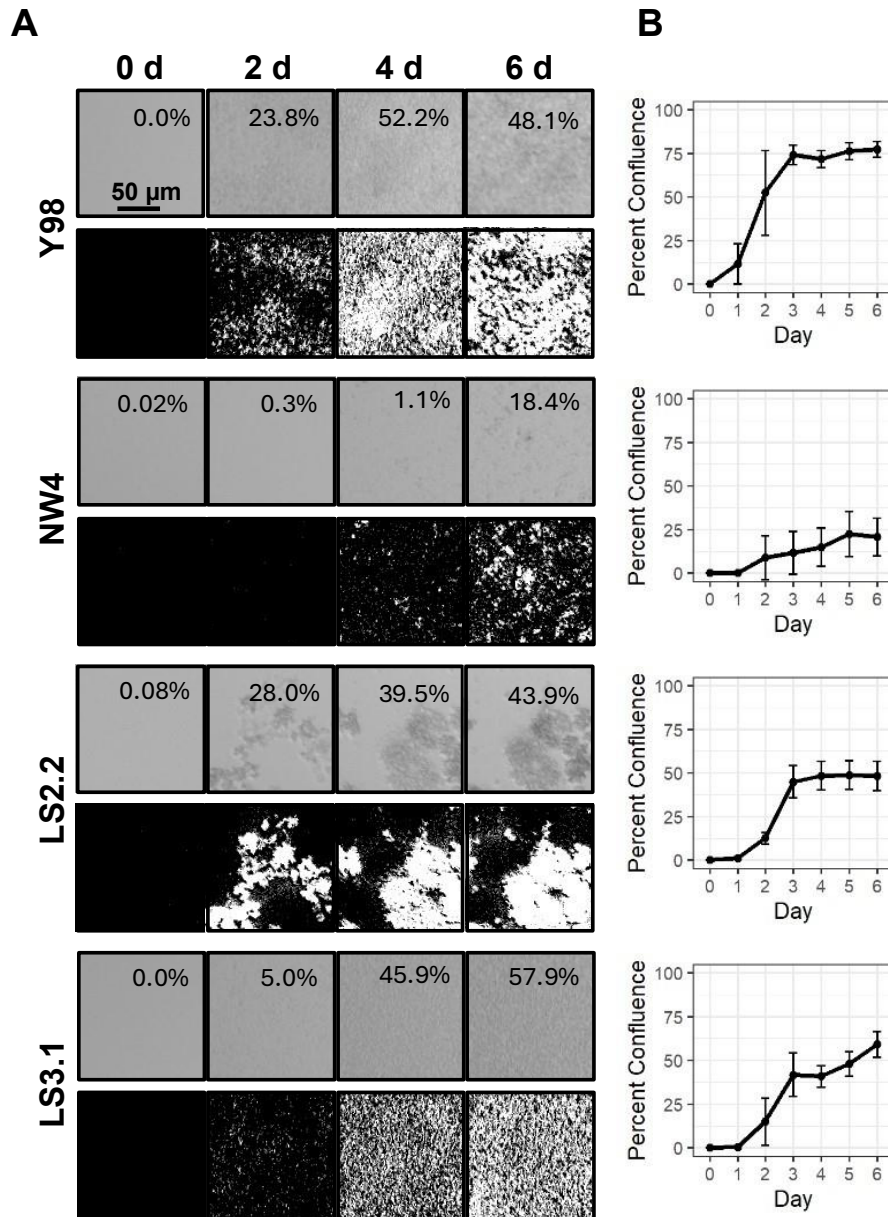


Figure 27. Biofilm formation varies between strains. (A) Image thresholding was used to estimate the percent confluence of the biofilms formed by the different strains. (B) The biofilm confluence increased for all strains over time, and the highest observed confluence that was significantly increased compared to the previous 2 days was considered to be the point of maturity. Data represents pooled data from two experiments with 18 technical replicates each (mean $\pm$ SD).

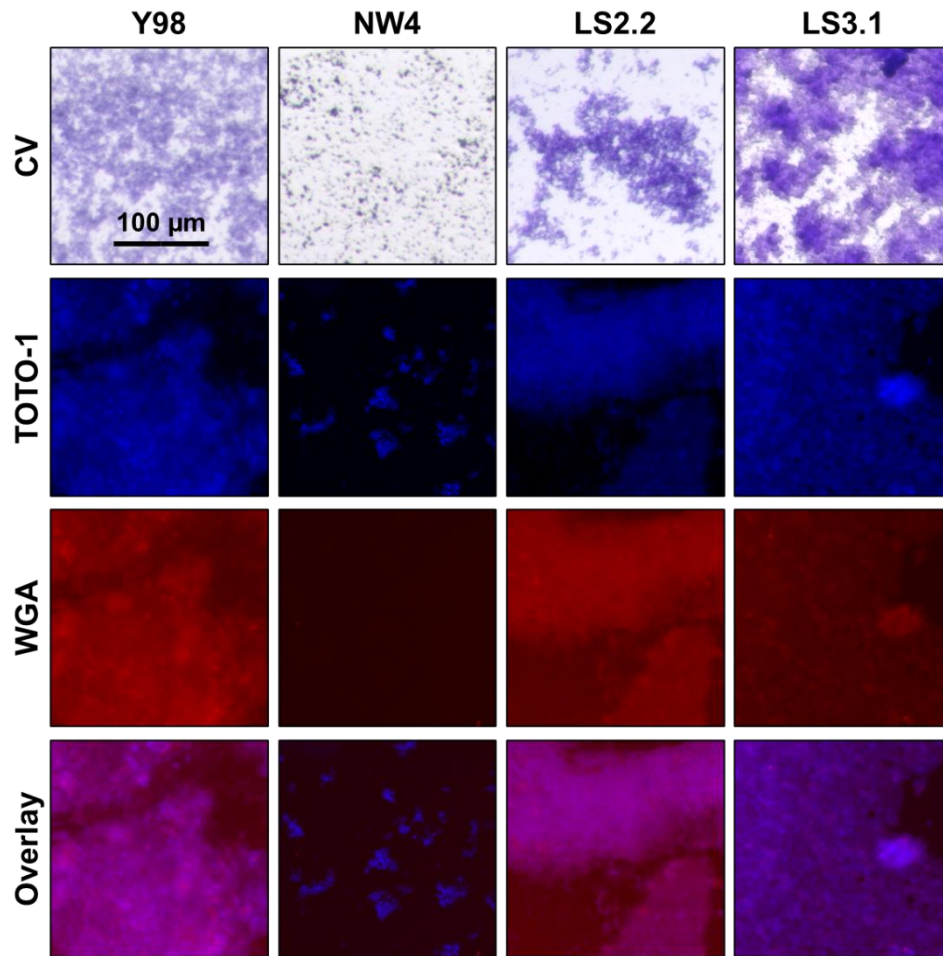


Figure 28. *M. ovipneumoniae* consistently forms biofilms in vitro. (CV) Crystal violet staining shows typical biofilm morphology with high confluence for the Y98, LS2.2, and LS3.1 strains, and weak formation for the strain NW4. The extracellular polymeric matrix is visualized by staining extracellular DNA (eDNA) with TOTO-1 fluorescent dye in blue, and polysaccharides with wheat germ agglutinin (WGA) in red. Data are representative of two experiments with 12 technical replicates. The 100 μm size bar applies to all panels.

Crystal violet staining was performed at the point of maturity determined above for each *M. ovipneumoniae* strain. Adherent *M. ovipneumoniae* clusters with a typical biofilm morphology were found for all four strains that we analyzed (Figure 28 – CV). These observations were confirmed using TOTO-1 DNA dye and wheat germ agglutinin (WGA), which demonstrated the presence of an eDNA- and carbohydrate-rich EPM that is typical of bacterial biofilms (Figure 28 – TOTO-1, WGA, and Overlay). We again observed the most robust WGA staining with the Y98 and LS2.2 isolates, decreased levels of fluorescence with the LS3.1 isolate and almost no reactivity for the NW4 isolate, reflecting strain-specific variation in *M. ovipneumoniae* biofilm formation.

Confocal imaging of the mature biofilms was used to determine the 3-dimensional structure of TOTO-1 stained biofilms for each isolate. The *M. ovipneumoniae* Y98 isolate had a peak biofilm thickness of 42.1 μm . *M. ovipneumoniae* strain LS2.2 had a similar peak thickness of 48.8 μm . *M. ovipneumoniae* strains NW4 and LS3.1 had lower peak thicknesses of 6.33 μm and 32.9 μm respectively (Figure 29, Table 15).

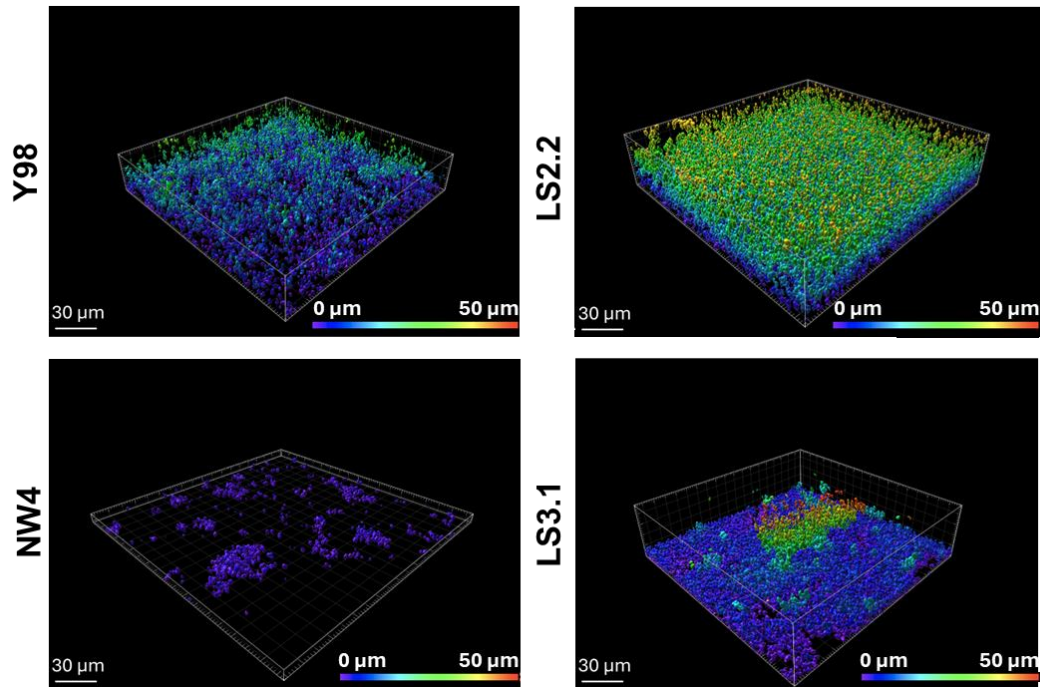


Figure 29. Biofilm thickness varies between different strains Biofilm thickness varied between TOTO-1 stained strains, with Y98 and LS2.2 having the thickest observed structures. Data are representative of 1 experiment with 6 technical replicates.

Colony Morphology

Rough colony morphology has been previously described as potentially being linked to potential biofilm formation in the related organism *Mycoplasma mycoides* [49, 280]. In general, isolated colonies for all four strains were centerless colonies typical of *M. ovipneumoniae* grown on agarose [281] (Figure 30). The lawn cultures for the *M. ovipneumoniae* Y98 and LS2.2 strains had a rougher appearance with a highly confluent lawn, while the *M. ovipneumoniae* NW4 and LS3.1 strains had a smoother appearance and a lower level of lawn confluence.

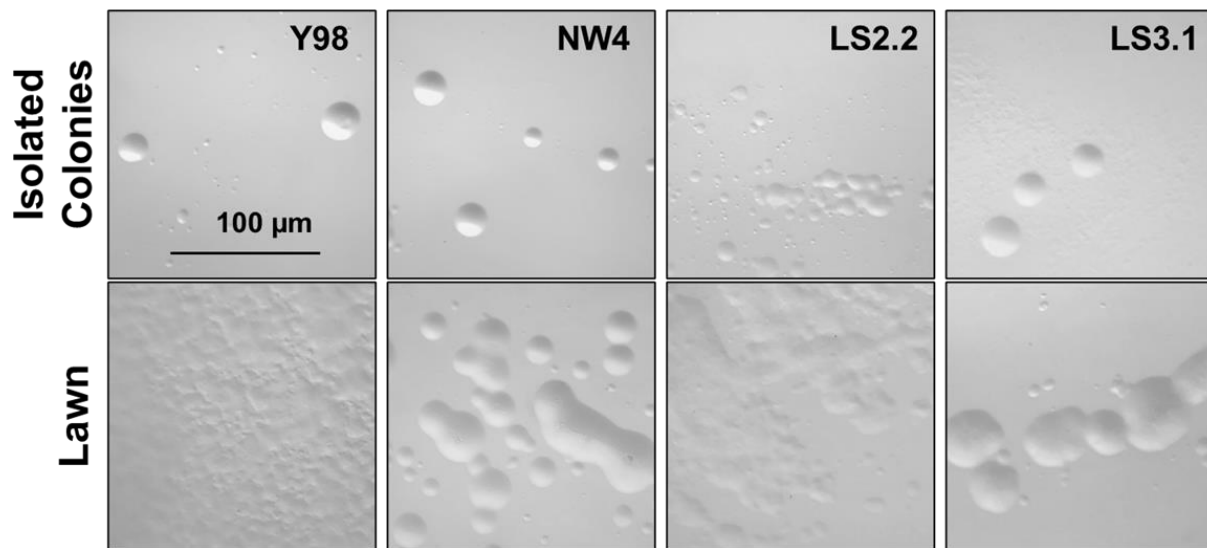


Figure 30. Colony morphology shows typical round centerless colonies of *M. ovipneumoniae* when isolated, and rougher morphology when observing Y98 and LS2.2 as a lawn. Bar for all panels=100 μ m.

Hydrogen Peroxide Production

To test whether production of the virulence factor hydrogen peroxide changed between the planktonic cells and biofilm, we measured production between the two states over time. Hydrogen peroxide levels did not differ between the planktonic state and the biofilms at any point across the 6 d of growth and remained below the limit of detection for the assay (<1 mg/mL) for all isolates tested.

M. ovipneumoniae Biofilm formation is associated with increased antibiotic tolerance

For biofilm analysis in our LD-AST and flow cytometry quantification, we disrupted the scraped biofilms with either sonication or Tween treatment. All treatments were able to decrease the mean size of the particles (data not shown). The untreated biofilm had a size of 1.81×10^4 RFU, while 15 min of sonication resulted in a 22.5% (22.3-22.7%, 95% CI) reduction in mean particle size (Wald's Test, $p < 0.001$) to 1.40×10^4 RFU, corresponding to a diameter of $\sim 0.5 \mu\text{m}$. As the 15 min of sonication led to the largest reduction in particle size, it was utilized as the disruption technique for the LD-AST.

Previous studies with other bacterial species have shown that biofilms are commonly associated with increased levels of antibiotic tolerance [43, 223]. Therefore, we explored whether minimum bactericidal concentrations (MBCs) for three different antibiotic classes differed between *M. ovipneumoniae* grown as a biofilm and those grown in planktonic cultures, using a recently developed flow cytometry-based live/dead AST assay [247]. As previously, the $\text{MBC}_{\geq 5\%}$ was defined as the lowest concentration that led to a $\geq 5\%$ decrease in the proportion of live cells. Planktonic *M. ovipneumoniae* Y98 was highly susceptible to enrofloxacin, gentamicin, and tetracycline, with an $\text{MBC}_{\geq 5\%}$ of $<0.039 \mu\text{g/mL}$, $2 \mu\text{g/mL}$, and $<0.0625 \mu\text{g/mL}$ respectively (Figure 31A, Table 16). In contrast, antibiotics had no effect on viability of Y98 biofilms, with $\text{MBC}_{\geq 5\%}$ values outside of the range of the concentrations tested for enrofloxacin ($\text{MBC}_{\geq 5\%} > 20 \mu\text{g/mL}$), gentamicin ($\text{MBC}_{\geq 5\%} > 128 \mu\text{g/mL}$) and tetracycline ($\text{MBC}_{\geq 5\%} > 16 \mu\text{g/mL}$ respectively) (Figure 31A, Table 16). This represents a >512 -fold increase in resistance to enrofloxacin and tetracycline, and a >64 -fold increase in resistance to gentamicin for the mature biofilms. Similarly, the $\text{MBC}_{\geq 5\%}$ for NW4 planktonic cells was low for enrofloxacin, gentamicin, and tetracycline ($0.039 \mu\text{g/mL}$, $2 \mu\text{g/mL}$, and $0.5 \mu\text{g/mL}$ respectively). Again, the mature biofilms for

NW4 had increased resistance, albeit at lower rates than were observed for Y98 with 0.156 µg/mL, 32 µg/mL, and 1 µg/mL for enrofloxacin, gentamicin, and tetracycline respectively (Table 16). These changes represent a 4-fold increase for enrofloxacin, a 16-fold increase for gentamicin, and a 2-fold increase for tetracycline for mature NW4 biofilms. The $MBC_{\geq 5\%}$ for LS2.2 planktonic cells was low for enrofloxacin, gentamicin, and tetracycline (0.078 µg/mL, 4 µg/mL, and 0.125 µg/mL respectively). The $MBC_{\geq 5\%}$ for mature biofilms of LS2.2 were identical to those of Y98 as they were outside of the tested ranges for all three antibiotics. These changes represents a >256-fold increase for enrofloxacin, >32-fold increase for gentamicin, and >128-fold increase for tetracycline for mature LS2.2 biofilms. The $MBC_{\geq 5\%}$ values for the LS3.1 planktonic cells were also similarly low for enrofloxacin, gentamicin, and tetracycline (0.312 µg/mL, 1 µg/mL, and 0.125 µg/mL respectively). However, in contrast to the significantly increased antibiotic tolerance for biofilms of the other *M. ovipneumoniae* strains, the $MBC_{\geq 5\%}$ for mature biofilms of strain LS3.1 remained low with 0.039 µg/mL for enrofloxacin, 1 µg/mL for gentamicin, and < 0.0625 for tetracycline. This corresponds to an 8-fold reduction for enrofloxacin, no change for gentamicin, and a 2-fold reduction for tetracycline. Interestingly however, at all concentrations above the $MBC_{\geq 5\%}$ for LS3.1 biofilms exposed to enrofloxacin and gentamicin there were no differences in live cells compared to the controls (Wald's Test, $p > 0.05$) or increases in live cells (Wald's Test, $p < 0.01$) (Figure 31B, Table 16). Similarly, the concentrations above 0.125 µg/mL for tetracycline for the mature LS3.1 biofilm showed no difference in live cells compared to the controls (Wald's Test, $p > 0.05$) (Figure 31B, Table 16).

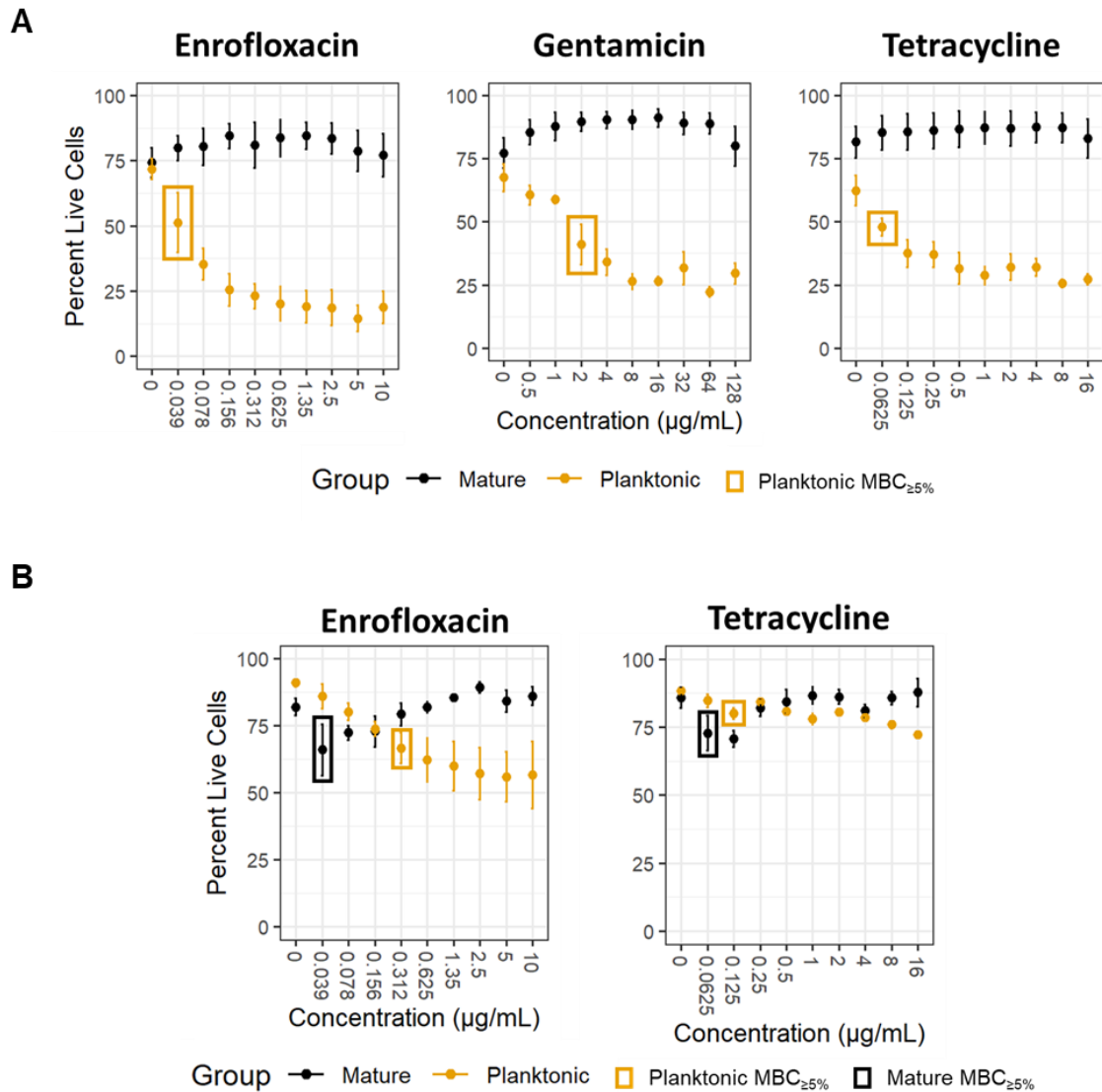


Figure 31. Biofilm formation is linked to increased antibiotic resistance. (A) Antibiotic exposure of *M. ovipneumoniae* Y98 led to a significant decrease in live planktonic cells, but no significant decrease in the mature biofilm. (B) Biofilm formation showed a decreased MBC_{50%} for LS3.1 exposed to enrofloxacin and tetracycline, but increased antibiotic concentrations showed evidence of the eagle effect. Data are representative of two experiments with two technical replicates.

Table 16. *Mycoplasma ovipneumoniae* $MBC_{\geq 5\%}$ values for each strain

<i>M. ovipneumoniae</i> strain	Antimicrobial Agent	<i>Mycoplasma ovipneumoniae</i> state	
		Planktonic ($\mu\text{g/mL}$)	Mature Biofilm ($\mu\text{g/mL}$)
Y98	Enrofloxacin	<0.039*	>20 [†]
	Gentamicin	2	>128 [†]
	Tetracycline	<0.0625*	>16 [†]
NW4	Enrofloxacin	<0.039*	0.156
	Gentamicin	2	32
	Tetracycline	0.5	1
LS2.2	Enrofloxacin	0.078	>20 [†]
	Gentamicin	4	>128 [†]
	Tetracycline	0.125	>16 [†]
LS3.1	Enrofloxacin	0.312	<0.039* ^{††}
	Gentamicin	1	>128
	Tetracycline	0.125	<0.0625* ^{††}

*Value below the tested range, Value above the tested range, ^{††}Higher concentrations were observed to have the same or more live cells than the controls.

Antibiotic exposure can lead to increased growth of *M. ovipneumoniae* biofilms.

We analyzed biofilm confluence after exposure to antibiotics to determine whether antibiotic treatment would reduce the overall biomass. However, for all the isolates and antibiotics tested, there was only one significant decrease in biofilm confluence compared to untreated controls. This decrease was observed only at the lowest concentration of gentamicin for the LS2.2 isolate with an estimated decrease of $7.2 \pm 6.82\%$ (Wald's Test, $p < 0.05$) (Figure 32). In contrast, there were significant increases in biofilm confluence for Y98 compared to untreated controls, with an estimated increase of $11.23 \pm 9.65\%$ at 5 $\mu\text{g/mL}$ of enrofloxacin (Wald's Test, $p < 0.05$) (Figure 32) and an estimated increase of $10.30 \pm 7.74\%$ at 128 $\mu\text{g/mL}$ of gentamicin (Wald's Test, $p < 0.05$) (Figure 32). Likewise, biofilm confluence for the NW4 isolate was increased at 5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 20 $\mu\text{g/mL}$ of enrofloxacin that were >13% (Wald's Test, $p < 0.05$) (Figure 32) compared to untreated controls. NW4 biofilms exposed to gentamicin

grew to an increased confluence at all tested concentrations except 128 $\mu\text{g/mL}$ that were $>8\%$ (Wald's Test, $p < 0.01$) (Figure 32) compared to untreated controls. Similar trends were observed for the LS3.1 isolate where the confluence did not change when exposed to enrofloxacin and tetracycline, but increased $>11\%$ (Wald's Test, $p < 0.05$) (Figure 32) with all concentrations gentamicin compared to untreated controls except 0.5, 8, and 128 $\mu\text{g/mL}$ where the confluence did not differ compared to the controls (Wald's Test, $p > 0.05$) (Figure 32).

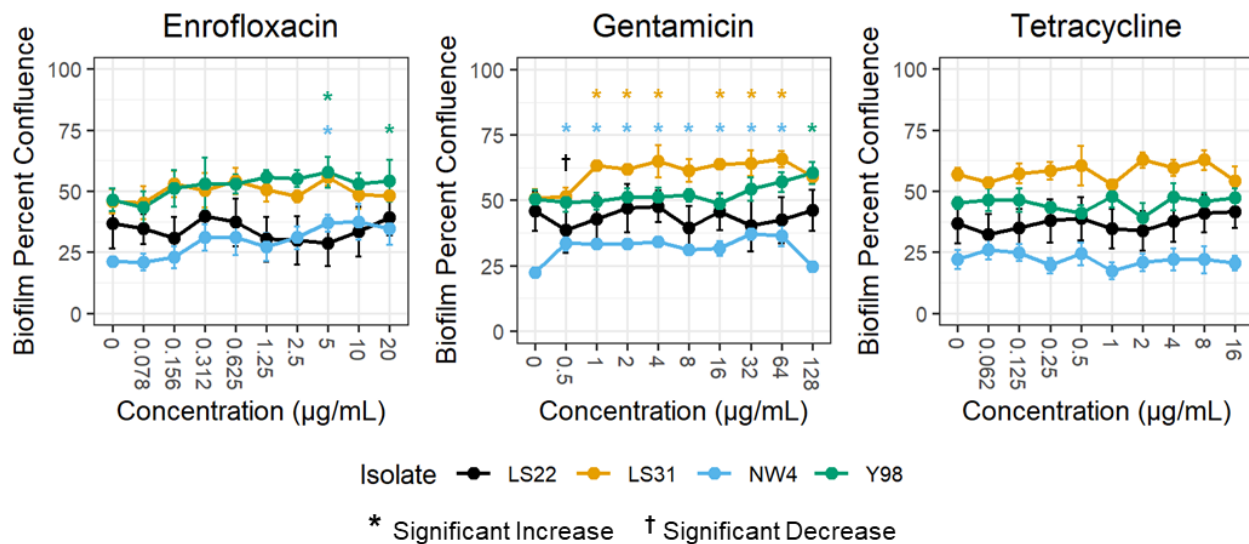


Figure 32. Biofilm confluence significantly increased (Color matched stars to samples) or stayed the same after treatment with antibiotics. Antibiotic treatment led to either significant increases (Wald's Test, $p < 0.05$) in biofilm confluence or no change (Wald's Test, $p > 0.05$) compared to untreated controls. Only one treatment with one strain (LS2.2 at 0.5 $\mu\text{g/mL}$ of gentamicin) resulted in a significant decrease (Wald's Test $p < 0.05$) in biofilm confluence of 7.2%. Data are representative of two experiments with two technical replicates.

High quality assembled genomes show strain-level diversity

To determine why biofilm formation differs between strains we performed a whole genome analysis. Once we had our sequences for the clinical *M. ovipneumoniae* strains, we compared them to each other and the Y98 reference strain [282]. The assembled genomes for the clinical strains were high quality, with all the isolates having high completeness ($> 99\%$), low

contamination (< 1%), and 0% heterogeneity (Table 17). All assembled genomes had lengths between ~1.06-1.07 Mbp, with 778-803 genes identified by PGAP (Table 18). The genomes were all identified as the same species with > 95% average nucleotide identity (ANI), but were identified as separate strains (> 99% ANI) [273, 283]. The strains showed a moderate level of strain-level diversity with between ~96-97.5% ANI (Figure 33A and B).

Table 17. Genome quality metrics calculated with CheckM

<i>M. ovipneumoniae</i> Strain	Completeness	Contamination	Heterogeneity
NW4	99.28%	0.18%	0%
LS2.2	99.28%	0.72%	0%
LS3.1	98.91%	0.72%	0%

Table 18. Genome Characteristics, after annotating with PGAP

<i>M. ovipneumoniae</i> Strain	Genome Length	Genes (Total)	Genes (Coding)
Y98 ^[282]	1081520 bp	805	762
NW4	1077622 bp	803	760
LS2.2	1063126 bp	784	738
LS3.1	1056336 bp	778	731

Adhesins and Nucleases show differing levels of conservation

Due to previous publications suggesting that the putative adhesin PdhD and nucleases might impact biofilm formation [51, 254], we performed *in silico* analyses of the protein sequences for PdhD [51], and the MnuA [258] and Nuc (Identified by VFAnalyzer) nucleases.

Adhesins and eDNA accumulation may influence the adhesion of planktonic cells to a surface and lead to the formation of a biofilm, while nucleases can degrade the eDNA component of the EPM and lead to biofilm dispersal. The putative adhesin PdhD showed high levels of conservation between strains (Figure 33C). Alternatively, the nuclease MnuA showed large areas of the protein where amino acid residues were not conserved between strains (Figure 33D). The nuclease Nuc also showed increased levels of amino acids residues with weak or no conservation, but at a lower level than MnuA (Figure 33E). The distances calculated with the multiple sequence alignment (MSA) displayed a similar trend, where there are increased distances between the strains for MnuA and Nuc compared to the more conserved PdhD (Figure 33F-H).

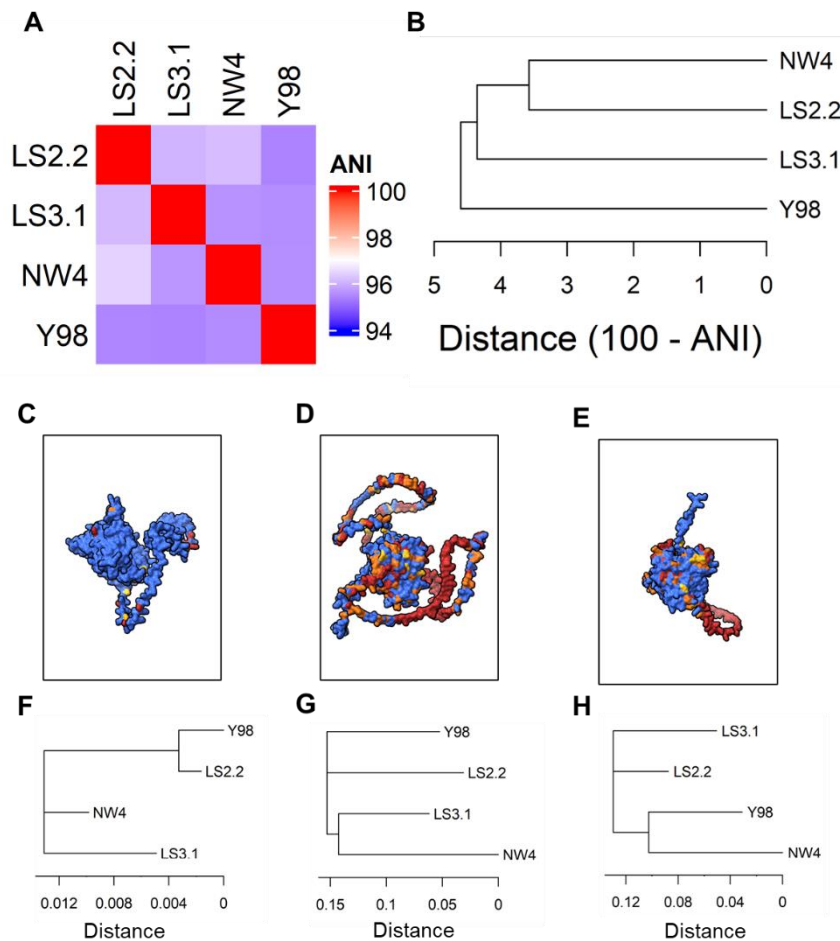


Figure 33. While there is strain-level variation between all genomes, PdhD protein sequences showed high levels of conservation, and MnuA and Nuc showed high levels of variability. The Average Nucleotide Identity (ANI) for each of the strains is displayed as a (A) heatmap and a (B) distance tree. Predicted proteins are shown for (C) PdhD, (D) MnuA, and (E) Nuc, where blue indicates fully conserved amino acids, yellow indicates strongly conserved amino acids, orange indicates weakly conserved amino acids, and red indicates non-conserved amino acids. Trees for the strains are based on the protein sequences for (F) PdhD, (G) MnuA, and (H) Nuc.

Discussion

This study is the first in-depth investigation into biofilm formation by *M. ovipneumoniae*. Previous work has found only limited biofilm formation by *M. ovipneumoniae* isolates, but only utilized a single analysis method (crystal violet microplate assay) [50] or a single isolate [49]. Our results have shown robust formation of biofilms for three out of four strains. This variability

in biofilm formation between strains may contribute to both previous observations of variable pathogenicity by *M. ovipneumoniae* and help explain why antibiotic treatment failure has occurred in previous studies with *M. ovipneumoniae* [2, 24, 52, 53, 78, 110].

Our data has shown that given the right growth conditions and media, *M. ovipneumoniae* can form robust biofilms. We observed biofilm peak thicknesses of $> 30 \mu\text{m}$ for three strains, and as *M. ovipneumoniae* cells are between $0.3\text{-}0.8 \mu\text{m}$ in diameter [284], this thickness corresponds with a large aggregation of cells. This variation between our observations of robust biofilm formation and previous studies showing limited formation is unsurprising as different media and growth conditions were used [36, 37]. Additionally, another study into *M. ovipneumoniae* capsular formation by Niang et al. also observed variations in capsule thickness and presence depending on media and growth conditions [249]. This study investigating capsular formation also supports our observations of biofilm formation, as the capsule is formed of exopolysaccharides, which can facilitate bacterial adhesion and biofilm formation [249, 250]. The presence of an exopolysaccharide capsule on individual cells, and our observations of an EPM composed of exopolysaccharides and eDNA surrounding adhered cells, is a natural progression in understanding of the mechanisms underlying biofilm formation in *M. ovipneumoniae* [249, 250, 285, 286]

No previous studies with *M. ovipneumoniae* have investigated the effect of biofilm formation on antibiotic tolerance. Our study demonstrated increased levels of antibiotic tolerance after biofilm formation for all the strains and antibiotics tested. There were increases in the $\text{MBC}_{\geq 5\%}$ for all tested strains and antibiotics except for LS3.1 exposed to gentamicin and tetracycline. While a decrease in $\text{MBC}_{\geq 5\%}$ was observed for LS3.1 exposed to these two

antibiotics, higher concentrations of both antibiotics ($> 0.312 \mu\text{g/mL}$ of enrofloxacin, and $> 0.25 \mu\text{g/mL}$ of tetracycline) showed an increase in cellular survival for the biofilms. We and others have demonstrated this increased survival by biofilms at the highest tested concentrations of antibiotics in what is termed the “paradoxical” or “eagle effect” [43, 171, 227, 228, 247]. The eagle effect occurs when higher drug concentrations result in increased tolerance or resistance by the organism, when initially susceptibility is seen at lower concentrations [171]. We also have shown that biofilm biomass did not decrease after antibiotic treatment for all tested strains and antibiotics except one. Rather, the biofilms had no change in confluence or increased levels of confluence after exposure, pointing towards antibiotic treatment as potentially leading to increased biofilm formation in certain conditions. This increase in biofilm formation is supported by other works, where exposure to antibiotics triggered or enhanced biofilm formation [287, 288].

These results support our hypothesis that *M. ovipneumoniae* biofilm formation results in antibiotic tolerance. The three antibiotics used here have different mechanisms of action, with enrofloxacin inhibiting nucleic acid synthesis, and both gentamicin and tetracycline inhibiting protein synthesis through two separate pathways [226, 230, 235]. The EPM that is formed in a mature biofilm can act as a physical barrier that prevents penetration of these antibiotics [229]. The EPM also reduces penetration of aminoglycosides, such as the gentamicin we tested here, due to the positive charge of the antibiotic and the negative charge of the EPM. These opposite charges can keep the antibiotic from reaching the cellular target by trapping it in the EPM [225]. The eDNA component of the EPM can also function to increase tolerance of fluoroquinolones such as the enrofloxacin we tested here. This occurs because the eDNA can chelate cations which

can then further bind to the fluoroquinolone and decrease its bioavailability [233, 289]. The observed increase in tolerance of tetracycline may be due to the presence of efflux pumps in the biofilm which secrete EPM components. These efflux pumps in biofilms can also function to remove tetracycline from the cells and increase antibiotic tolerance [45, 225].

When we investigated the potential proteins involved in biofilm formation by *M. ovipneumoniae* we observed high conservation with the PdhD protein, which has also been reported in a previous study [51], and moderate conservation for the putative nuclease Nuc. The high levels of variation observed between strains in the MnuA protein suggest that MnuA might be a contributing factor in the biofilm variation between the strains. Our MSA distance tree shows that the Y98 and LS2.2 strains have the closest protein sequences for PdhD in the 4 strains, potentially contributing to the similar adhesion dynamics between them (Figure 3F). Alternatively, the MnuA protein sequence for NW4 and LS3.1 showed the smallest distance, potentially contributing to the lower levels of biofilm thickness and delayed maturity observed between these two strains. Previous research on the related organisms *Mycoplasma bovis* and *Mycoplasma hyopneumoniae* have observed MnuA as a primary contributor to the degradation of eDNA [258, 259]. The observed variation in the MnuA sequence between our strains underscores its potential role in biofilm dispersion and provides a valuable target for future investigation.

We are aware that our study has several limitations as we utilized only four *M. ovipneumoniae* strains, three antibiotics, and limited growth conditions. The limited number of *M. ovipneumoniae* strains from two geographical locations and one host species could be improved to include a wider range of geographies and hosts to determine if there are regionally or host-specific patterns. Our study was done in a controlled but limited laboratory environment,

and fluctuations in temperature, humidity, and nutrient availability could induce more robust biofilm formation as a survival response. Our study also only looked at *in silico* protein structures which have reduced levels of accuracy compared to protein purification and crystallography. We also did not explore the relative expression of adhesins and nuclease genes in the different planktonic and biofilm states, which would further elucidate the contributions that these genes have in the formation of biofilms. While these limitations do exist, our observations of robust biofilm formation and antibiotic tolerance have important implications for future work in *M. ovipneumoniae* pathogenesis and treatment.

In summary, we are the first to demonstrate that *M. ovipneumoniae* generates robust biofilms and to quantify both the thickness and speed of biofilm formation. We have also shown the effect that biofilm formation has on antibiotic tolerance. The protein targets, i.e. (PdhD, MnuA, and Nuc) that we have identified may also be valuable for future studies into the mechanisms of *M. ovipneumoniae* biofilm formation. We anticipate that this new information into a mechanism of pathogenesis and survival for *M. ovipneumoniae* will lead to further avenues of investigation into the treatment and prevention of this disease.

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CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

As *M. ovipneumoniae* is an important and prevalent contributor to respiratory disease in both domestic and wild sheep [7, 12, 103, 244], this dissertation sought to address the following questions. 1) What are the effects of *M. ovipneumoniae* infection of domestic sheep in the absence of a secondary infection? 2) What are the effects of upper vs lower respiratory infection in domestic sheep by *M. ovipneumoniae*? 3) Can we develop a test for antibiotic susceptibility of *Mycoplasma* biofilms? 4) Does *M. ovipneumoniae* form biofilms and does this lead to increased antibiotic tolerance?

This dissertation addressed these questions by first analyzing the effects of *M. ovipneumoniae* mono-infection in a specific pathogen free (SPF) flock of domestic sheep, where asymptomatic infection was observed. It then was able to take this a step further by investigating the effects of *in vivo* *M. ovipneumoniae* infection in both the upper and lower respiratory tracts in combination with a secondary pathogen. Due to the observed failure to treat *M. ovipneumoniae* with antibiotics in our first experiment, we then introduced a novel method for antibiotic susceptibility testing, termed the live-dead antibiotic susceptibility test (LD-AST). This test was successfully applied to both *Mycoplasma bovis* and *Mycoplasma bovoculi* biofilms to validate its efficacy for *Mycoplasma* biofilms. Last we were able to observe biofilm formation by *M. ovipneumoniae* in four strains and determine its impact on antibiotic tolerance.

This work has demonstrated the efficacy of our domestic sheep infection model and highlighted differences in pathogenesis based on the infection site. Furthermore, it has provided an innovative approach for investigating antibiotic susceptibility in *Mycoplasma* spp. biofilms

and their formation dynamics. This work is the first to characterize biofilm formation by *M. ovipneumoniae* and conduct an *in silico* analysis of potential proteins involved in adhesion and dispersion. It also identified increased antibiotic tolerance in *M. ovipneumoniae* biofilms, which may explain previous observations of antibiotic treatment failure.

In chapter 2 we were able to establish infection in our specific pathogen free (SPF) flock of sheep and observed an asymptomatic infection. When we attempted to clear the infection with gamithromycin treatment, we found that it was not effective, even though *in vitro* antibiotic susceptibility testing showed efficacy.

In chapter 3 we established an infection with both *M. ovipneumoniae* and a secondary pathogen *Mannheimia haemolytica* in both the upper and lower respiratory tract. We observed increased clinical signs, lung pathology, bacterial load, and decreased weight gain in animals that were inoculated in the lower respiratory tract.

In chapter 4 we developed an innovative test for antibiotic susceptibility in *Mycoplasma* biofilms and were able to define maturity, optimize disruption for analysis, and implement live-dead staining. This allowed us to compare planktonic cells, newly adhered biofilms, and mature biofilms in order to determine the effects that biofilm formation has on the survival of cells after antibiotic exposure.

In chapter 5 we were able to demonstrate biofilm formation by four *M. ovipneumoniae* strains. We characterized both the speed of biofilm formation and the peak thickness for all strains. We also analyzed the genomes of our selected strains and performed an *in silico* analysis of selected protein sequences looking for patterns that might explain the observed variability in formation dynamics. Last we showed increases in antibiotic tolerance for all tested strains, both

as an $\text{MBC}_{\geq 5\%}$ and in both a failure to reduce biomass and even observations of increased biofilm biomass after antibiotic exposure.

Overall we have been able to generate significant insight into the behavior and impact of *M. ovipneumoniae* persistence and pathogenesis. We have demonstrated that 1) *M. ovipneumoniae* can persist as an asymptomatic chronic infection *in vivo* despite antibiotic treatment, highlighting the challenge of eradication in the host. 2) Our findings have also revealed distinct differences in pathogenesis in the upper and lower respiratory tract when co-infected with a secondary pathogen. The lower respiratory tract infection led to more severe clinical disease and lung pathology. 3) We have also developed an innovative antibiotic susceptibility test that revealed increased antibiotic tolerance in *Mycoplasma* biofilms, adding support to the need for alternative treatment strategies. 4) Finally, we characterized *M. ovipneumoniae* biofilm formation, identified potential mechanisms of formation and dispersal, and observed increased levels of antibiotic tolerance. This important survival mechanism that resists clearance with antibiotics points towards the need for new approaches to disease treatment in *M. ovipneumoniae*. These findings reflect the multifaceted issue of *M. ovipneumoniae* infection and have identified important factors that contribute to severe clinical disease, persistence, and antibiotic treatment failure.

Future Directions

Using *in vitro* biofilm formation to study immune evasion

Another feature of persistence by biofilms is their ability to evade the immune system and resist phagocytosis [36, 290, 291]. *M. ovipneumoniae* infection has often been observed in chronic respiratory infections [34, 292], and other biofilm-forming members of the

Mycoplasmataceae family have also been associated with persistence [33, 184]. As persistent infection that can evade the immune system has been observed, more work needs to be done to fully understand the implications of biofilm formation as it relates to the immune response. Our reproducible method for initiating biofilm formation by *M. ovipneumoniae* can be used in conjunction with phagocytosis assays to determine if this also plays a role in immune evasion [290, 291].

Determining sites of *in vivo* biofilm formation

We have investigated the localization of *M. ovipneumoniae* along the respiratory tract [293], and have observed the *in vitro* formation of biofilms by *M. ovipneumoniae*. To fully understand where *M. ovipneumoniae* localization can occur, and whether it does form biofilms *in vivo*, work needs to be done to observe these structures during infection. Many bacterial biofilms are formed in a multi-species complex, and often result in synergistic effects [294, 295]. *M. ovipneumoniae* is often associated with polymicrobial infection [3, 61, 107, 108, 296], and if this can be associated with a multi-species biofilm, this might allow for better understanding of the role that *M. ovipneumoniae* has in respiratory disease.

Further investigation into adhesins and nucleases in biofilm formation

As our work has only performed *in silico* investigation into the role of both adhesins and nucleases on the formation and dispersion of *M. ovipneumoniae* biofilms, more work should be done to fully characterize the proteins involved in the biofilm formation cycle. As we and others have identified genes that may contribute to biofilm formation, there is a need to elucidate their full contributions at different stages [51]. Gene expression and functional analyses of the putative

adhesin and nucleases would further elucidate the contributions that these have on biofilm formation. Other bacterial organisms observe changing dynamics in biofilm adhesion depending on nuclease expression, with increased expression resulting in decreased biofilm formation and dispersal [254, 256].

Final Remarks

This research has contributed to the understanding of both *M. ovipneumoniae* infection in the absence of a secondary pathogen and to the effects of *M. ovipneumoniae* administration to either the upper or the lower respiratory tract with a secondary pathogen. This work has also provided a diagnostic tool for future studies into the effects of *Mycoplasma* biofilm formation on antibiotic tolerance, and is the first to robustly characterize *M. ovipneumoniae* biofilm formation. Future work can use these findings as a foundation for further advancement into understanding and treatment of *M. ovipneumoniae* infection.

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CHAPTER SEVEN

TRANSFER AND PERSISTENCE OF BOVINE
IMMUNOGLOBULINS IN LAMBS FED A COLOSTRUM
REPLACER

Contribution of Authors and Co-Authors

Manuscript in Chapter 7

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Contributions: Conceptualization, methodology, investigation and writing - original draft

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Contributions: Software, formal analysis, investigation and writing - review and editing

Co-Author: Kerri Jones

Contributions: Conceptualization, investigation, resources, supervision and project
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Co-Author: Cassie Mosdal

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Contributions: Investigation and formal analysis

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Contributions: Conceptualization, formal analysis, writing—original draft, review and editing, visualization, supervision, project administration and funding acquisition

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SHORT COMMUNICATION

Transfer and persistence of bovine immunoglobulins in lambs fed a colostrum replacer

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Abstract

Background: Colostrum-derived antibodies are crucial for the protection of newborn lambs from infectious diseases. Several colostrum replacer products that contain bovine antibodies are on the market. We investigated the absorption and persistence of bovine antibodies from a powdered colostrum replacer in newborn lambs.

Methods: We tested a lamb colostrum replacer containing bovine serum in lambs that were separated from their dams at birth. Immunoglobulin G (IgG) uptake was analysed by ELISA, and the persistence of antigen-specific antibodies was analysed by parainfluenza 3 virus (PI-3) neutralisation assay.

Results: Serum antibody ELISA performed on days 1 and 14 revealed IgG levels of 17.9 ± 2.8 and 27.5 ± 2.5 mg/ml, respectively. PI-3 antibodies derived from the colostrum replacer were present for 86.3 ± 10.6 days.

Conclusions: Antibodies derived from bovine serum protein delivered to lambs via a commercial colostrum replacer are readily absorbed and persist for months, suggesting that these products may offer adequate protection.

KEYWORDS

artificial rearing, colostrum replacer, immunoglobulin, lamb

INTRODUCTION

Colostrum is the only source of antibodies in newborn lambs, since the ruminant placenta is impermeable to immunoglobulins (Igs).¹ Consumption of sufficient high-quality colostrum is linked to improved lamb survival, growth, gut maturation and immune status.^{2–5} Thus, colostrum replacers and supplements play a critical role in ovine production in situations where sufficient high-quality ewe colostrum is not available, such as periparturient death of the dam, multiple births or artificial rearing systems.^{6–8} Bovine colostrum has traditionally been used to substitute ewe colostrum.^{9–10} Ig genes are highly conserved between cattle and sheep,¹¹ and a significant number of pathogens target both sheep and cattle, so bovine antibodies are likely to provide relevant protection in lambs. However, bovine colostrum may be contaminated with pathogens, including rotavirus, *Cryptosporidium*, bovine leukosis virus or *Mycobacterium avium* subsp. *paratuberculosis*, thus posing an infection risk.^{12–15}

Colostrum replacers are synthetic feed products designed to completely replace maternal colostrum.¹⁶ However, few studies have analysed the impact of feeding colostrum replacers to lambs.^{6,17} We analysed Ig uptake and persistence in lambs that were fed a commercial colostrum replacer that contained bovine serum.

METHODS

Animals, housing and husbandry

Animal experiments were approved by the Institutional Animal Care and Use Committee of Montana State University, protocol #2019-95. Our investigations were part of a larger study performed between March and October 2020 that involved the generation of a specific-pathogen-free (SPF) research flock.¹⁸ Therefore, a matched control group that did not receive colostrum replacer was not available. Fifteen pregnant Rambouillet/Targhee ewes were purchased and closely monitored prior to lambing and were then

isolated as soon as they displayed signs of labour. Lambs were manually delivered onto clean sheets and immediately transferred to the large animal biosafety level 2 (LABSL-2) facility, where they were raised isolated from other livestock. Lamb rooms had a combination of concrete and slatted flooring and were maintained at a temperature of 16°C with a natural light cycle. Lambs were weighed weekly and monitored by trained animal care staff twice per day to assess food intake, behaviour or any signs of illness. Animals that showed signs of ill health were examined and treated by a veterinarian if indicated.

Administration of colostrum replacer

Lambs were bottle-fed Rescue Lamb & Kid Colostrum Replacer (Lifeline Nutrition Solutions/APC, Ankeny, IA, USA) at 40 g of powdered colostrum replacer diluted in 189 ml water per kg bodyweight over 24 hours and divided into six meals. Colostrum replacer consumption was recorded after every meal. Per manufacturer's analyses, the colostrum replacer contained 44% crude protein, 24% globulin protein (Ig derived from bovine serum), 24% crude fat and 0.5% crude fiber. Based on this information, the concentration of bovine Ig in the prepared colostrum replacer was 50.8 mg/ml.

Lamb diet and weaning

After the first 24 hours, lambs received SuperLamb Milk Replacer (Milk Specialties Global, New Holstein, WI, USA), which contained 24% crude protein, 30% crude fat and 0.1% crude fibre. The protein in the milk replacer contained dried whey and whey protein concentrate. Lambs were bottle fed for the first week, followed by bucket feeding. Gradual weaning onto a mixture of corn, oats and barley with molasses as well as Lamb Starter Pellets (Payback, Sioux Falls, SD, USA) was initiated at around 45 days. Hay, water and a salt/mineral supplement were freely available throughout the study from birth.

Measurement of serum IgG, total serum protein and parainfluenza 3 virus neutralisation titres

Blood samples (3–7 ml) were collected from the external jugular vein using a Vacutainer system and centrifuged to obtain serum. Day 1 blood samples were collected between 23 and 25 hours after birth. Serum IgG concentrations were measured using a bovine IgG ELISA kit (MyBioSource, San Diego, CA, USA). Total serum protein concentrations were measured using the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Parainfluenza 3 (PI-3) serum virus neutralisation titres were measured by the Washington Animal

Disease Diagnostic Lab (Pullman, WA, USA) using bovine turbinate cells and the Reisinger SF-4 strain of PI-3 virus. The titre was determined as the highest dilution showing complete neutralisation with no evidence of a cytopathic effect.

Statistical analysis

Data were analysed using GraphPad Prism 9.3.0 and are shown as the mean $\pm$ standard deviation. Normality of distribution was determined using a Shapiro–Wilk test ($\alpha = 0.05$). Differences between time points were analysed using a Wilcoxon matched-pairs signed rank test or ANOVA with Tukey's multiple comparisons test and were considered significant at $p \leq 0.05$. A simple linear regression analysis was performed to assess relationships between different parameters.

RESULTS

Characteristics and survival rates of artificially reared lambs

Out of 32 live births, 30 lambs (93.8%) survived and were included in the data analysis. One lamb was euthanised 2 days after birth due to lethargy and inappetence, and a second lamb born with neurological issues was euthanised 6 days after birth. The study group was composed of 12 males and 18 females, with two single lambs, 12 twins, 10 triplets and six quadruplets. The average daily gains were 0.36 ± 0.04 kg/day during the first 8 weeks of life. Detailed health records showed that six out of 30 lambs developed mild diarrhoea during the study that generally resolved within 1 day, but no other signs of infectious disease were observed.

IgG and total serum protein concentrations in colostrum-replacer-fed lambs

Lambs weighed 4.9 ± 1.1 kg at birth and thus received colostrum replacer with a total of 47.0 ± 10.6 g of IgG over 24 hours. Serum IgG levels of 17.9 ± 15.7 mg/ml were detected 1 day after birth. On day 14, IgG levels were significantly higher at 27.5 ± 13.9 mg/ml ($p \leq 0.01$; Figure 1a). Conversely, total serum protein levels were 97.6 ± 17.6 mg/ml 1 day after birth but decreased significantly by day 14 (68.1 ± 20.0 mg/ml; $p \leq 0.001$; Figure 1b).

Antibodies derived from bovine colostrum persist for several months

Our unique study design, where lambs were raised artificially in an ABL-2 facility, enabled us to analyse the persistence of antigen-specific antibodies derived from the colostrum replacer. To that end,

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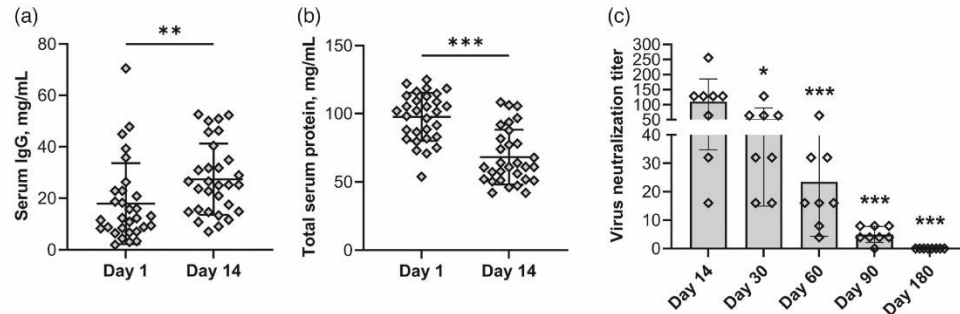


FIGURE 1 Serum concentrations of immunoglobulin G (IgG) and total protein in artificially raised lambs. Serum samples from 30 lambs that received colostrum replacer were collected 1 and 14 days after birth. (a) The concentration of total bovine IgG in lamb serum was determined by bovine IgG ELISA. (b) Total protein concentrations in lamb serum on days 1 and 14 after birth were determined by bicinchoninic acid assay. (a and b) Individual data points, mean $\pm$ standard deviation are shown, Wilcoxon matched-pairs signed rank test, ** $p \leq 0.01$, *** $p \leq 0.001$. (c) Virus neutralisation titres for parainfluenza 3 virus on days 14, 30, 60, 90 and 180 after birth. Days 30, 60, 90 and 180 were compared to day 14 using ordinary one-way ANOVA with Tukey's post hoc test, * $p \leq 0.05$, *** $p \leq 0.001$

we measured PI-3 neutralising antibodies in nine randomly selected lambs. Since PI-3 infections or vaccinations are extremely common in bovine herds,¹⁹ reactive antibodies were expected to be present in the colostrum replacer, which contains bovine serum, and our management practices prevented natural PI-3 infection with resulting antibody production in the lambs. The PI-3 neutralising activity of lamb sera was measured on days 14, 30, 60, 90 and 180. A 24-hour sample was not analysed due to insufficient sample volumes. The sera of all lambs contained PI-3 neutralising antibodies, with average titres of 110.0 ± 75.3 at 14 days after birth. Titres were highest on day 14 and decreased steadily until they dropped below the limit of detection at day 180 (Figure 1c). In seven out of eight lambs (87.5%), PI-3 neutralising activity was still observed at 90 days. The average persistence of PI-3 neutralising activity was 86.3 ± 10.6 days.

Colostrum consumption did not correlate with IgG or total serum protein concentrations

The actual uptake of colostrum replacer by the lambs varied between 63% and 155% of the volume recommended by the manufacturer (mean: 94.7%). However, there was no significant correlation between colostrum replacer consumption and either IgG or serum protein concentrations measured at 24 hours after birth (Figure 2a,b). Unexpectedly, there was also no correlation between serum IgG and protein concentrations (Figure 2c).

DISCUSSION

Our data indicate that bovine serum antibodies in a commercial lamb colostrum replacer are readily absorbed into the bloodstream of lambs and persist

for up to 3 months. Moreover, we did not observe any negative effects of colostrum replacer administration on lamb health and performance, indicating that colostrum replacers with bovine serum are a viable replacement for ewe colostrum.

We showed that the commercial lamb colostrum replacer used resulted in significant and sufficient IgG uptake into the lambs' circulation. The average concentration of serum IgG 24 hours after birth was 17.9 ± 15.7 mg/ml, which is above the threshold of 15 mg/ml that is used to indicate failure of passive transfer⁵ and comparable to the IgG concentrations reported for 24-hours-old lambs fed ewe colostrum (19.6 mg/ml, Barta²⁰ and 24.6 mg/ml, Masimini et al.²¹). We thus confirm previous reports of bovine IgG absorption in lambs.^{6,17,22} The pre-colostrum IgG concentration in lambs was reported to be only 0.22 ± 0.01 μ g/ml,²³ strongly suggesting that the IgG we measured was derived from the colostrum replacer. Although the milk replacer product used contained some whey-derived Ig, absorption of IgG from the gut ceases within 24 hours in lambs.²⁴ Therefore, the increased IgG concentrations detected on day 14 compared to 1 day after birth likely reflect the initiation of antibody production by the lambs, consistent with previous studies.^{25,26}

Interestingly, there was no significant correlation between the volume of colostrum replacer consumed and IgG or serum protein concentrations. While the serum protein concentrations measured here were similar to those reported elsewhere,^{27,28} they did not correlate with IgG concentrations. This was unexpected, since serum protein is frequently used to assess colostrum intake.^{5,29} However, serum protein concentrations vary widely depending on hydration status, liver function, genetics and other factors. Moreover, since all lambs in our study were supplied with an adequate amount of colostrum with sufficient IgG, it is possible that IgG transport across the gut wall was saturated and that a correlation between serum protein

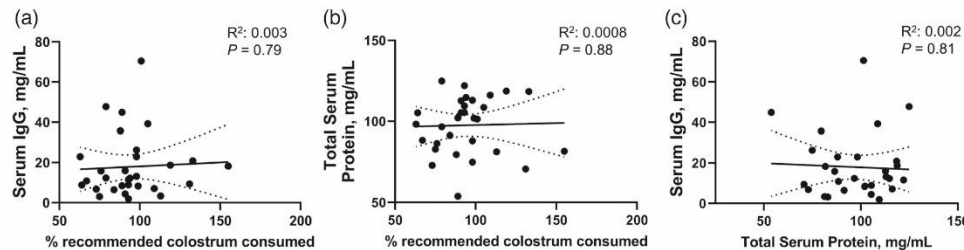


FIGURE 2 Serum immunoglobulin G (IgG) and total protein in artificially raised lambs do not correlate with colostrum replacer intake. (a) Relationship between the concentration of total bovine IgG in lamb serum 24 hours after birth and the percentage of recommended colostrum consumed. (b) Relationship between total serum protein and percentage of recommended colostrum consumed at 24 hours after birth. (c) Relationship between total bovine IgG and protein concentration in lamb serum 24 hours after birth. (a–c) Data were analysed using a simple linear regression model, and dotted lines indicate the 95% confidence interval. R^2 denotes the square of the correlation coefficient

and IgG would only be detectable with suboptimal colostrum intake.

Our unique experimental setup, where lambs were raised in isolation under SPF conditions, enabled us to monitor the degradation of antigen-specific antibodies over time without interference from developing natural immune responses in the lambs. We showed that PI-3 neutralising antibodies from bovine serum incorporated into a powdered colostrum replacer maintained a physiological half-life in the lambs' sera and remained functional for up to 3 months. The observed kinetics are consistent with the expected rate of Ig degradation in lambs.³⁰

Notably, lamb colostrum replacers have been associated with variable effects on lamb survival and performance.^{6,22,31,32} This may be due to differences in the IgG content of different lamb colostrum supplements and lots¹⁶ and other study parameters. In our study, the SPF conditions and intensive management likely improved animal health, survival and growth, so our observations need to be interpreted with caution. Additional limitations of our study were the lack of control groups that received ewe colostrum or no colostrum, the lack of pre-colostrum antibody and protein data, and the lack of PI-3 neutralisation data at 24 hours. Overall, our findings of sufficient antibody uptake, long-term antibody persistence and excellent lamb health corroborate other publications^{17,22} that support the use of high-quality colostrum replacers with a bovine Ig source in lambs.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS STATEMENT

Animal experiments were approved by the Institutional Animal Care and Use Committee of Montana State University, protocol #2019-95.

AUTHOR CONTRIBUTIONS

Conceptualisation, methodology, investigation and writing—original draft: Thea Johnson. *Software, formal analysis, investigation and writing—review and editing:* Bryan Tegner Jacobson. *Conceptualisation, investigation, resources, supervision and project administration:* Kerri Jones. *Investigation:* Cassie Mosdal, Steve Jones, Maia Vitkovic and Sam Kruppenbacher. *Investigation and formal analysis:* Andy Sebrell. *Conceptualisation, formal analysis, writing—original draft, review and editing, visualisation, supervision, project administration and funding acquisition:* Diane Bimczok.

DATA AVAILABILITY STATEMENT

The datasets generated during the current study are available from the corresponding author on reasonable request.

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CHAPTER EIGHT

PATHOPHYSIOLOGY OF INFLUENZA D VIRUS INFECTION
IN SPECIFIC-PATHOGEN-FREE LAMBS WITH OR WITHOUT
PRIOR *MYCOPLASMA OVIPNEUMONIAE* EXPOSURE

Contribution of Authors and Co-Authors

Manuscript in Chapter 8

Author: Ema Robinson

Contributions: Conceptualization, methodology, validation, formal analysis,
investigation, data curation, writing – original draft, visualization,

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Pathophysiology of Influenza D Virus Infection in Specific-Pathogen-Free Lambs with or without Prior *Mycoplasma ovipneumoniae* Exposure

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Abstract: Polymicrobial pneumonias occur frequently in cattle, swine, and sheep, resulting in major economic losses. Individual pathogens comprising these complex infections may be mild on their own but can instead exhibit synergism or increase host susceptibility. Two examples of such pathogens, *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) and influenza D viruses (IDVs), naturally infect domestic sheep. In sheep, the role of *M. ovipneumoniae* in chronic nonprogressive pneumonia is well-established, but the pathogenesis of IDV infection has not previously been studied. We utilized a specific-pathogen-free sheep flock to study the clinical response to IDV infection in naïve vs. *M. ovipneumoniae*-exposed lambs. Lambs were inoculated intranasally with *M. ovipneumoniae* or mock infection, followed after four weeks by infection with IDV. Pathogen shedding was tracked, and immunological responses were evaluated by measuring acute phase response and IDV-neutralizing antibody titers. While lamb health statuses remained subclinical, *M. ovipneumoniae*-exposed lambs had significantly elevated body temperatures during IDV infection compared to *M. ovipneumoniae*-naïve, IDV-infected lambs. Moreover, we found a positive correlation between prior *M. ovipneumoniae* burden, early-infection IDV shedding, and IDV-neutralizing antibody response. Our findings suggest that IDV infection may not induce clinical symptoms in domestic sheep, but previous *M. ovipneumoniae* exposure may promote mild IDV-associated inflammation.

Keywords: influenza D virus; *Mycoplasma ovipneumoniae*; polymicrobial infection; specific-pathogen-free sheep; respiratory coinfection; chronic nonprogressive pneumonia

1. Introduction

Polymicrobial pneumonias in cattle, swine, and sheep result in major annual economic losses in the livestock industry. It is hypothesized that during these complex infections, the primary pathogen may drive disease severity by remodeling or suppressing the host immune response, thereby facilitating infection by additional pathogens and/or limiting the host's ability to combat the new infections [1–6]. Understanding the immunological processes driving these events is critical for effective prevention and management. Our group has investigated influenza-associated polymicrobial infections and identified several processes by which influenza A virus infection modulates host susceptibility to subsequent bacterial secondary infection [7–9]. We subsequently extended these investigations to include influenza D virus (IDV)-*Staphylococcus aureus* sequential infection

using a mouse model [10]. In the current study we utilized a recently developed specific-pathogen-free (SPF) sheep flock to investigate the impact of prior *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) infection on IDV pathogenesis in a natural host [11].

M. ovipneumoniae is a respiratory bacterium commonly detected in healthy and diseased lambs [12–14]. *M. ovipneumoniae* infection can induce chronic nonprogressive pneumonia, particularly in 2- to 12-month-old lambs [15–18]. While its prevalence in healthy domestic lambs demonstrates that *M. ovipneumoniae* infection can be asymptomatic, evidence suggests that asymptomatic carriage may reduce lamb growth rates [19–22]. Importantly, while domestic sheep tolerate *M. ovipneumoniae* infection, wild sheep species such as Rocky Mountain Bighorn sheep (*Ovis canadensis*) and argali (*Ovis ammon*) are highly susceptible to *M. ovipneumoniae* infection, which predisposes them to severe-to-lethal polymicrobial interstitial pneumonia [19–21,23–25]. The mechanisms through which *M. ovipneumoniae* promotes secondary infection are not fully understood. Ex vivo studies have demonstrated that *M. ovipneumoniae* damages tracheal epithelium through the generation of reactive oxygen species, which may increase susceptibility to a secondary pathogen [4,26]. *M. ovipneumoniae* also induces ciliostasis [27], promotes anti-ciliary autoantibody production [28], and may compromise the mucociliary escalator and enable the migration of inert upper respiratory tract resident microbes such as *Mannheimia haemolytica* into the lower respiratory tract, which can result in severe interstitial pneumonia [23]. In the lower respiratory tract, *M. ovipneumoniae* reduces the phagocytic capacity of alveolar macrophages and impairs pathogen clearance [5,29], while stimulating proinflammatory cytokine secretion, which damages host tissue [4,30]. *M. ovipneumoniae* may also impair the adaptive immune response as it bears a surface protein that directly inhibits mitogen-stimulated expansion of T-cells and B-cells [31,32]. Each of these mechanisms may confer an immunosuppressed state and contribute to the increased disease severity associated with *M. ovipneumoniae* colonization.

IDVs comprise a recently identified genus of *Orthomyxoviridae* about which little is known [33,34]. The natural host range is broad, including (in order of known seroprevalence) livestock like cattle (40–95%) [35–38], swine (6–12%) [33,39,40], horses (12%) [41], sheep (2–6%) [38,39,42–44], goats (1–4%) [38,42,44,45], dromedary camels (6–99%, possible cross-reactivity with influenza C virus) [44,46], as well as wild ungulates including feral swine [40,47] and water buffalo [45]. There is also serological evidence of human infection [33,48–50], and experimental infections have been carried out in model organisms of the human respiratory tract (mice [10,51], guinea pigs [52], and ferrets [33,53]). In most species, IDV primarily infects ciliated cells of the upper respiratory tract and soft palate [4,33,53,54]. Cellular tropism varies between species due in part to differences in respiratory system glycosylation patterns [54,55]. IDV infections depend on docking of the major surface glycoprotein (hemagglutinin-esterase fusion protein) to extracellular 9-O-acetylated sialic acid residues, and as such, cellular permissivity is dependent on the presence of these modified glycans [56,57]. IDV infections typically remain subclinical [35,53,58–61], but metagenomics studies suggest that IDVs may play an etiologic role in a bovine respiratory disease (BRD), a severe-to-lethal polymicrobial infection common in pre- and post-weaned calves [35,62,63]. In cattle, IDVs infect both the upper and lower respiratory tract and can cause mild-to-moderate respiratory symptoms [53,58,61,64]; however, respiratory tissue tropism has not been established for most hosts, including sheep. Until now, active IDV infection has never been documented in sheep, but serostudies in the United States [42], Ireland [43], France [38], and West Africa [39,44] report IDV-neutralizing antibodies in domestic sheep, suggesting that IDV infections in sheep occur commonly across multiple continents.

Despite evidence of circulation of both *M. ovipneumoniae* and IDV in sheep, no studies have characterized infections involving both pathogens. Our previous work and the work of others suggests that *M. ovipneumoniae* monoinfection does not consistently induce clinical illness in SPF lambs [11,19,65,66]. To date, experimental coinfection of an IDV with a bacterial pathogen has been limited to two studies in calves and one in mice [10,58,64].

In the current study, we infected SPF lambs with *M. ovipneumoniae* or mock treatment. After four weeks, we infected all animals with IDV to characterize IDV pathogenesis with and without a recent or ongoing *M. ovipneumoniae* infection. We found that on their own, both *M. ovipneumoniae* and IDV infections were asymptomatic. While we observed no significant difference in overt respiratory disease or shedding when *M. ovipneumoniae*-infected versus naïve lambs were inoculated with IDV, we found subclinical ($<40^{\circ}\text{C}$) elevation in rectal temperature, as well as elevated serum protein concentrations in lambs exposed to both pathogens. Our data also allowed for correlative analysis which suggests that the amount of recent *M. ovipneumoniae* burden may correspond to the degree of early-phase IDV replication and the endpoint IDV-neutralizing antibody titer. Because no causal relationship has been established, these data must be confirmed by additional experiments.

2. Materials and Methods

2.1. Animals and Husbandry

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Montana State University, protocol# 2019-109. We previously reported the derivation of a SPF sheep flock through established motherless rearing methods using mixed-breed (Rambouillet/Targhee) founders (F0) [11]. The SPF flock was maintained under SPF conditions at the Johnson Family Livestock Facility (JFLF) in Bozeman, MT. Of the F1 SPF sheep, 2 rams and 11 ewes were bred, and of the resulting progeny (F2), 3 ewes and 4 wethers were selected for each experimental group in this study ($n = 7$ lambs/group).

Four days prior to the study start date, 12–15-week-old lambs were weaned and moved into a temperature-controlled room ($15.5\text{--}16.8^{\circ}\text{C}$) in the JFLF ABSL-2 facility for acclimation. Experimental groups were housed in different rooms, separated by a procedure area. To minimize the possibility of cross-contact or accidental exposures, all personnel showered and donned sterile personal protective equipment before and after entering either room. All protocols were performed on the *M. ovipneumoniae*-naïve group prior to the *M. ovipneumoniae*-inoculated group. Equipment was sterilized between experimental groups and, when possible, separate sets of procedural equipment were used for each group.

2.2. Health Monitoring

Lamb health status was monitored daily, and combined clinical scores were assigned daily using previously established metrics [11]. To summarize, lambs were scored in five categories: (1) behavior, (2) appetite, (3) respiratory symptoms, (4) clinical interventions, and (5) body temperature (measured rectally). Each category uses a scale from 0 to 5 with higher scores indicating a worsening state, giving a maximum possible combined clinical score of 25. Lambs were also weighed every 14 days. Trained animal husbandry staff examined lambs twice daily and monitored them by video feed throughout each day.

2.3. *M. ovipneumoniae* Preparation

M. ovipneumoniae inoculum was grown from a previously described nasal wash isolate (MSU NW-4) and expanded exactly as described in the original publication [11]. Briefly, inoculum was grown at 37°C in Mycoplasma broth under microaerophilic conditions. On the day of inoculation, media was removed ($10,000\times g$, 10 min , 4°C) and culture was resuspended in sterile FACS buffer (2% fetal bovine serum and 0.1% sodium azide in DPBS) for counting by flow cytometry [67]. Cells were subsequently stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) at 4°C for 30 min and Absolute Counting Beads (Invitrogen, Carlsbad, CA, USA) were added to solution immediately prior to analysis using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.4. Virus Preparation

Influenza D virus (D/swine/Oklahoma/1334/2011) was propagated in embryonated chicken eggs, and inoculum titer was determined by plaque assay as previously described with modifications [68,69]. On the day of inoculation, an aliquot of inoculum was serially diluted in 1X inoculation media (1X MEM, 0.3% bovine serum albumin, 2 mM HEPES, with penicillin/streptomycin) containing 1 µg/mL TPCK-trypsin (Thermo Fisher Scientific, Waltham, MA, USA). Dilutions were plated in duplicate onto 90–100% confluent monolayers of Madin-Darby Canine Kidney cells (MDCK, ATCC# NBL-2), a kind gift from Dr. Benfeng Lei. After a 1 h infection period at 33 °C, cells were washed, and a semisolid overlay was added (1X inoculation media, 1 µg/mL TPCK-trypsin (Thermo Fisher Scientific, Waltham, MA, USA), 0.6% agarose, 100 µg/mL DEAE-dextran (Sigma Aldrich, St. Louis, MO, USA)). Plates incubated for 5 days at 33 °C. Plaques were visualized by tetrazolium dye vital staining by dispensing 0.5 mL dye solution (3 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT, Thermo Fisher Scientific, Waltham, MA, USA) in 150 mM NaCl) on top of each overlay as previously described [70]. Cells were incubated with the dye for 60 min at 37 °C, 5% CO₂, or until blue puncta developed and plaques were enumerated immediately.

IDV for hemagglutinin inhibition assays was propagated in swine testicular cells (ST, ATCC# CRL-1746, a kind gift from Dr. Feng Li). Upon reaching 60% confluence, cells were inoculated (MOI = 0.1) in a minimal volume of inoculation media at 33 °C, 5% CO₂ for 1 h. Cells were then cultured for 5 days at 37 °C, 5% CO₂ in inoculation media with TPCK-trypsin reduced to 0.1 µg/mL. After outgrowth period, media was collected and virus was concentrated by ultracentrifugation over a 30% sucrose cushion at 100,000 × g, 90 min, 4 °C. Pellets were resuspended in sterile DPBS and stored at −80 °C until use.

2.5. Intranasal Inoculations

Each experimental group consisted of 3 ewes and 4 wethers aged 12–15 weeks old. Among the entire cohort were 3 sets of twins, which were distributed evenly across groups. On the first day of the study, lambs were inoculated with either sterile DPBS ($n = 7$) or 3.5×10^8 CFU *M. ovipneumoniae* ($n = 7$) by instilling 15 mL into each naris and 10 mL orally similar to what has previously been described [11,25,71–73]. On day 28 of the study, when in our previous study the peak *M. ovipneumoniae* infection occurred in SPF sheep [11], all lambs were inoculated intranasally with 1.86×10^5 PFU egg grown IDV in DPBS as previously described, with modifications [33,74].

2.6. Sampling

2.6.1. Nasal and Rectal Swabs

At indicated timepoints, either both nares or rectum were swabbed, and swabs were transported in DPBS (2 mL). Swabs were vortexed briefly and transport media was aliquoted and stored at −20 °C until use.

2.6.2. Serum Collection

At the stated timepoints, whole blood was collected by jugular venipuncture using BD Vacutainer serum tubes (Becton, Dickson and Company, Franklin Lakes, NJ, USA) and serum was separated by centrifugation at 1000 × g, 10 min, 4 °C. Aliquots were stored at −20 °C until use.

2.6.3. Fecal Samples

Fecal samples were obtained directly from the rectum and were transported on ice. Samples (80–120 mg) were subsequently homogenized in 500 µL TRIzolTM Reagent (Invitrogen, Carlsbad, CA, USA) using a bead beater, then stored at −80 °C until time of RNA extraction.

2.7. Determining Pathogen Burden

2.7.1. *M. ovipneumoniae* Quantification

DNA was extracted from nasal swab samples (500 µL) using the DNeasy Ultraclean Microbial Kit (Qiagen, Redwood City, CA, USA), with modifications. Briefly, cells were pelleted at $10,000\times g$ for 10 min, resuspended in lysis buffer, and incubated at 95 °C for 10 min prior to proceeding with the remainder of the manufacturer's protocol. Each sample was eluted in 50 µL of provided elution buffer. *M. ovipneumoniae* burden was determined by qPCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and the following previously validated primers [75]: Fwd: 5'-TCTCCCAGATGATGCTAACC-3'; Rev: 5'-TGAAAATCAACTGGTCTAA-3'. Samples were volume-normalized using 0.5 µL DNA per reaction. Genome copy number was interpolated from a standard curve using a fragment of the *p113* gene (Twist Bioscience, San Francisco, CA, USA; sequence listed below). A standard curve was analyzed in each sample plate. Thermal cycling reactions proceeded in 10 µL volumes using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the following cycling conditions: 3-min polymerase activation at 98 °C followed by 40 cycles of denaturation for 15 s at 98 °C and annealing/extension for 30 s at 60 °C.

p113 fragment sequence (5'→3'): AGCTACCAAGTCGACATTCTAGAAAATTCTC-CCAGATGATGCTAACCAAAATTTT AAAGTCAAATTTCAAGCTAGCCAAAAATTAG-CAAACGGTGACATCGCCAAATCT GACATTATGAACAAGTTGTTCTTTTGTCAAA-GAATCAACTATTTTAATTGCCGA ATTTAATTTTTCTTACAAAAAATTACAAGCAGA CTTAATCAACAAGTCCAAAAAT TTAATTTCTGCTCGAACC GCCAATTTTGCTGATCAA AATTCAGCTACTTCAAATC CAACAGATCCTAGCACAAATTAGACCAGTTGATTTTCAA-CATGACTTAAGAATTC ATAAAGCAAA

2.7.2. IDV Quantification

Viral RNA was extracted from nasal and rectal swabs (140 µL) with the QIAamp Viral RNA Mini Kit (Qiagen, Redwood City, CA, USA) as directed, using the provided carrier RNA, and eluted in 60 µL nuclease-free water. Fecal samples were processed exactly as previously described [76]. Blood samples (100 µL) were processed using the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosciences™, Cheshire, UK) using 10 µL RNA extract per reaction. Prior to IDV quantification, successful RNA extraction was confirmed using qRT-PCR to probe for pan-eukarya 18S rRNA (Hs99999901_s1; Applied Bioscience, Cheshire, UK). Extractions with Cq > 25 were excluded from analysis and extraction was repeated. IDV burden was determined by qRT-PCR using SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and the following previously validated FAM-labeled primers targeting the segment encoding PB1 [76]: Fwd: 5'-CAGCTGCGATGTCTGTCATAAG-3'; Rev: 5'-ACAAATTCGAGGGCCATTA-3'; Probe: 5'-FAM-AATGGACTTTCTCCTGGGACTGCT-TAMRA-3'. To generate a standard curve for interpolation of PFU equivalents (PFU Equiv.), IDV inoculum was serially diluted in sterile DPBS and RNA was extracted from each dilution as described for nasal and rectal swabs. A plaque assay was performed in parallel as described above to confirm the inoculum concentration. cDNA libraries were generated as described above and the resulting standard curve was analyzed in each sample plate. Thermal cycling reactions proceeded as for *M. ovipneumoniae* except with the following cycling conditions: 30 s polymerase activation at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 30 s at 60 °C.

2.8. Hemagglutinin Inhibition (HI) Assay

IDV neutralizing antibody titers were determined by HI as previously described, with minor modifications [77]. Briefly, serum was treated with receptor-destroying enzyme II (Denka Seiken, Tokyo, Japan) at a 3:1 ratio for 18 h at 37 °C, followed by enzyme

inactivation at 56 °C for 30 min. Sera were diluted to 0.1X in PBS and stored at −20 °C until use. HI was performed on sera diluted in series from 1:10 to 1:1280 using a 1% suspension of washed chicken red blood cells (Lampire Biological Laboratories, Pipersville, PA, USA) and in the presence of 4 HAU of ST-grown IDV. The agglutination reaction proceeded for 45 min at room temperature in V-bottom plates and titers were read by tilting the plate 90° for 30 s. Viral HA activity was determined just prior to test and confirmed with an in-plate dilution series. Tests were performed on two independent days, twice each day. Partially agglutinated wells were considered agglutinated. Titers are reported as inverse dilution, and sera with titers < 10 were assigned a titer of 5 for statistical analysis and graphical representation.

2.9. Acute Phase Protein and L-Lactate Quantification

Total protein was measured by BCA assay as directed (Thermo Fisher Scientific, Waltham, MA, USA). Albumin was determined using a modified bromocresol green (BCG) protocol. Briefly, 50 µL BCG reagent (Eagle Diagnostics, De Soto, TX, USA) was combined with 50 µL serum diluted appropriately in 0.9% saline. Reaction incubated 15 min at room temperature before measuring absorbance at 630 nm. Each test plate included a 125–1500 µg/mL standard curve using a commercially-available 2 mg/mL bovine serum albumin standard (Thermo Fisher Scientific, Waltham, MA, USA). Serum amyloid A was measured using a commercially available ELISA (PHASE™ RANGE Multispecies SAA ELISA kit, Tridelta Development, Ltd., Maynooth, Co., Kildare, Ireland) using the bovine standard. L-lactate was measured using the EnzyChrom™ L-lactate Assay Kit (BioAssay Systems, Hayward, CA, USA) as directed, scaled to a final volume of 100 µL/reaction. Sera exhibiting gross hemolysis (background-corrected absorbance at 520 nm > 0.5) were omitted from statistical analyses.

2.10. Statistical Analyses

Data are presented as means of at least two technical replicates per sample. Unless otherwise stated in figure legend, differences between treatment groups were analyzed using unpaired two-tailed Student's *t*-test and all data are reported as means ± standard deviation (SD). When multiple comparisons were made, *p*-values were adjusted by the Bonferroni–Dunn method. Confidence in Spearman's rank-order correlation analyses was expressed using a two-tailed *p*-value. All analyses were performed using GraphPad Prism 9 software.

3. Results

3.1. Prior *M. ovipneumoniae* Infection Elicits a Subclinical Response in IDV-Infected Lambs

To assess IDV pathogenesis with and without prior *M. ovipneumoniae* infection, fourteen SPF lambs were divided into two groups housed in separate rooms (*n* = 7 lambs/group). On day 0 (initiation of Phase 1), *M. ovipneumoniae* (3.5×10^8 CFU) or phosphate-buffered saline sham were introduced intranasally. After 4 weeks, all lambs were intranasally inoculated with IDV (1.86×10^5 PFU), resulting in a double-infected group (*Mo-IDV*) and an *M. ovipneumoniae*-naïve group infected only with IDV (*sal-IDV*) (Figure 1A). Lambs were monitored for an additional 22 (*sal-IDV*) or 23 (*Mo-IDV*) days before necropsy. For clarity, the first four weeks of the study during which time we evaluated the difference between *M. ovipneumoniae*-inoculated lambs and immunologically naïve saline controls is denoted as phase 1, and the time period after IDV infection, comparing IDV pathogenesis with versus without prior *M. ovipneumoniae* exposure, is denoted as phase 2 (Figure 1A).

Overall health, respiratory symptoms, demeanor, weight, and rectal temperature were documented throughout the study to identify signs of illness (Table 1 and Figure 1B,C). For reasons deemed unrelated, the experimental conditions, one *sal-IDV* wether (4104) was euthanized 13 days post-IDV after exhibiting signs of abdominal discomfort and anorexia starting 8 days post-IDV. Data collected from this lamb after 7 days post-IDV were omitted from analysis.

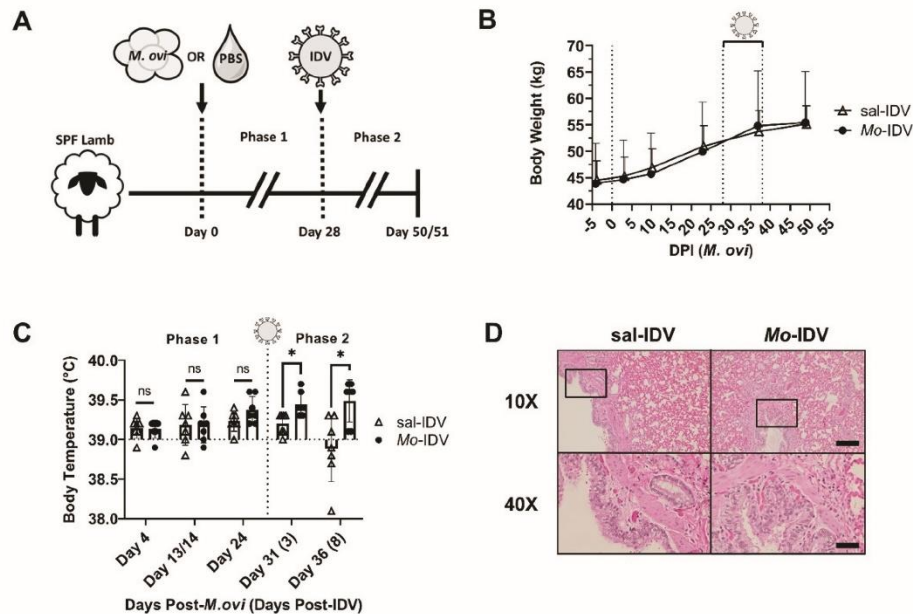


Figure 1. IDV with or without recent *M. ovipneumoniae* infection does not induce clinical symptoms in SPF lambs. (A) Schematic representation of experimental design. On day 0, seven lambs were administered *M. ovipneumoniae* (3.5×10^8 CFU) or PBS control ($n = 7$ lambs/group); at day 28, all lambs were inoculated with IDV (D/Oklahoma/1337/2011; 1.86×10^5 PFU). (B) Weights and (C) rectal temperatures were monitored throughout the study. (D) Representative histological analysis of distal lungs from each treatment group. Boxes on 10 $\times$ images denote 40 $\times$ field. 10 $\times$ scale bar: 500 μ m; 40 $\times$ scale bar: 100 μ m; *M. ovi*: *M. ovipneumoniae*; comparisons evaluated by Student's *t*-test; * Bonferroni–Dunn-adjusted $p < 0.05$, ns: $p > 0.05$. Vertical dashed lines denote IDV shedding period (B) or inoculation (C), horizontal line indicates average clinically normal lamb rectal temperature (39 °C). Data are represented as mean $\pm$ standard deviation (SD).

As expected, based on our previous work, during phase 1 of the study, *M. ovipneumoniae*-infected and sham-treated lambs remained asymptomatic, gained weight at the same rate, and showed no significant difference in rectal temperatures (Table 1 and Figure 1B,C) [11]. Following IDV infection (phase 2), we observed no clinical manifestations in any lamb, and no difference in growth between groups (Table 1 and Figure 1B). We did find that Mo-IDV lambs had significantly higher rectal temperatures versus sal-IDV lambs at both 3 and 8 days post-IDV (Table 1 and Figure 1C). While all temperatures remained in the normal range (<40 °C) [78], this finding could indicate that recent *M. ovipneumoniae* infection promotes a mild inflammatory response during subsequent IDV infection.

Necropsy was performed on 5–6 lambs per group at 22 (sal-IDV) or 23 (Mo-IDV) days post-IDV. All lambs in both groups were apparently healthy at necropsy, with no abnormalities or signs of pathogen-associated inflammation. Lung tissue featured mild-to-moderate congestion, petechiae, hemorrhage, edema, and/or atelectasis, all of which were deemed agonal by a board-certified veterinary pathologist. Accordingly, histological evaluation showed no evidence of microscopic lesions, and no evidence of inflammation-associated immune cell recruitment (Figure 1D). Similarly, concurrent evaluation of bronchoalveolar lavages collected at necropsy did not reveal any differences in either numbers or composition of cellular infiltrates between sal-IDV and Mo-IDV infected lambs (data not shown).

Table 1. Clinical parameters from each group at indicated timepoints.

	DPI (<i>M. ovi</i>)	DPI (IDV)	sal-IDV (SD)	Mo-IDV (SD)
Attitude ^a	2	-	0 (0)	0.14 (0.38)
	3	-	0.14 (0.38)	0 (0)
	14	-	0 (0)	0.17 (0.41)
	21	-	0 (0)	0.14 (0.38)
	23	-	0 (0)	0.14 (0.38)
	42	14	0 (0)	0.14 (0.38)
Body Condition ^b	2	-	3.29 (0.27)	3.36 (0.24)
	9	-	3.29 (0.39)	3.29 (0.39)
	16	-	3.21 (0.27)	3.07 (0.35)
	23	-	3.36 (0.38)	3.57 (0.45)
	30	2	3.64 (0.38)	3.5 (0.29)
	37	9	3.86 (0.24)	3.79 (0.39)
	44	16	3.42 (0.2)	3.57 (0.35)
Clinical Signs ^c	2	-	0 (0)	0.14 (0.38)
	3	-	0.14 (0.38)	0 (0)
	23	-	0 (0)	0.14 (0.38)
	25	-	0.14 (0.38)	0 (0)
	27	-	0 (0)	0.14 (0.38)
	28	0	0 (0)	0.14 (0.38)
	28	0	0 (0)	0.14 (0.38)

^a **Attitude.** 0: bright, alert, responsive (BAR, ideal state); 1: less BAR; 2: beginning to appear visibly ill; 3: obviously ill but alert and responsive; 4: depressed, unresponsive; 5: appears critically ill. ^b **Body condition.** 1: very lean/bony; 2: lean; 3: ideal; 4: fat; 5: obese. ^c **Clinical signs.** 0: healthy; 1: mild discomfort and/or minimal nasal/ocular discharge, panting; 2: clear nasal discharge, attitude change, with or without coughing 1–5 times; 3: major attitude change, constant coughing with decreased appetite and/or moderate nasal or ocular discharge; 4: appears very ill, prominent nasal or ocular discharge, still eating; 5: anorexic, lethargic, unresponsive. DPI: days post-infection; ND: not done.

3.2. Recent *M. ovipneumoniae* Infection Has a Minimal Impact on IDV Shedding

Nasal swabs were collected throughout the study to determine bacterial and/or viral burden. Pathogen burden (*M. ovipneumoniae*: Genome Copies, G.C.; IDV: plaque-forming unit equivalents, PFU Equiv., see methods for details) was quantified by qPCR or RT-qPCR using the standard curve method (Figure 2A,B and Supplementary Figure S1A,B). We found that after intranasal inoculation, nasal *M. ovipneumoniae* shedding varied widely between lambs and over time (Figure 2A). Shedding was never detected for two wethers (3625 and 2811). One ewe and one wether (4527 and 3713) were *M. ovipneumoniae*-positive at only one timepoint (day 14 or 20, respectively), and one wether (4305) shed only at the day 14 and day 20 timepoints. Two ewes (3318 and 4205) shed *M. ovipneumoniae* throughout phase 1 (until 1 day prior to IDV inoculation) and were presumably shedding at the time of IDV infection. Of those two, *M. ovipneumoniae* titers dropped sharply at the next collection timepoint (3 days post-IDV) and remained undetectable for the duration of the study (4205) or remained relatively low (3318) until IDV shedding resolved (Figure 2B and Supplementary Figure S1A). Presence of IDV in blood (at day 3 post-IDV) and rectal swabs (at day 7 post-IDV) was determined based on the pattern of nasal IDV shedding in Figure 2B. At these selected time points, we found no signs of viremia or rectal shedding (data not shown).

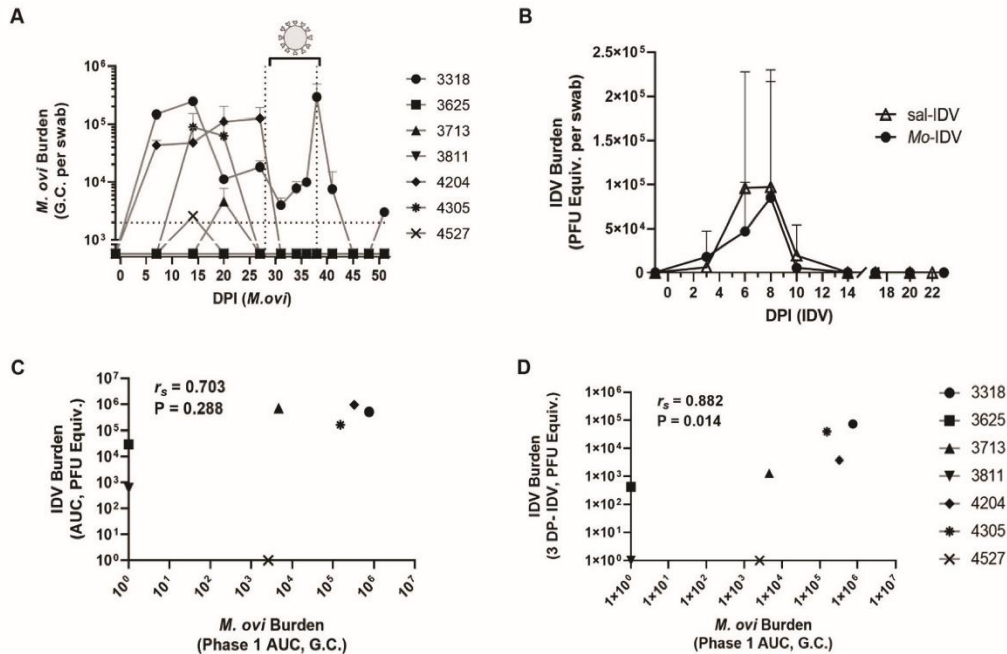


Figure 2. Recent *M. ovipneumoniae* infection correlates to early IDV shedding. Nasal shedding was monitored over time for (A) *M. ovipneumoniae*, individual lambs and (B) IDV, mean for each group. (C,D) Scatterplot showing correlation between total *M. ovipneumoniae* burden and (C) total IDV shedding, and (D) IDV shedding at 3 days post-IDV. Dotted horizontal line represents assay detection limit. Phase 1 AUC: area under the curve for phase 1 section of traces in (A); G.C.: enumeration of genome copies by qPCR; PFU Equiv.: enumeration of plaque-forming units by qRT-PCR; r_s : Spearman correlation coefficient; P: p-value. Data presented as mean $\pm$ SD.

All lambs were inoculated intranasally with IDV at the start of phase 2 (28 days post-*M. ovipneumoniae*). We found no significant difference in IDV shedding between groups at any discrete collection timepoint and no difference in total shedding as reflected by area under the curve (AUC) (Figure 2B and Supplementary Figure S1A,B). IDV remained undetectable for one ewe from each group (sal-IDV: 4016, Mo-IDV: 4527) (Supplementary Figure S1A,B). The remaining 6 sal-IDV lambs and 5 of the remaining 6 Mo-IDV lambs shed IDV at 3, 6, 8, and 10 days post-IDV. One Mo-IDV ewe (3811) shed only at 6, 8, and 10 days post-IDV. While not significant, Mo-IDV lambs showed a trend towards decreased IDV burden compared to sal-IDV animals at 6 days post-IDV (sal-IDV = $9.60 \times 10^4 \pm 1.32 \times 10^5$ PFU Equiv.; Mo-IDV = $4.71 \times 10^4 \pm 5.55 \times 10^4$ PFU Equiv.; ratio-paired *t*-test, adjusted *p*-value = 0.08, Figure 2B).

The wide distribution in degree and timing of shedding of both pathogens allowed us to assess whether the amount of *M. ovipneumoniae* shedding correlated to the amount of IDV shedding within the Mo-IDV lambs. To test this, we utilized a Spearman's rank-order correlation analysis, which separately ranked lambs by order of their degree of IDV shedding and by order of their *M. ovipneumoniae* shedding, then determined how similar those rankings are between the two rank orders. This test describes both the strength and directionality of the correlation (Spearman's coefficient, r_s ; positive correlation: 0 to 1, negative correlation: 0 to -1, with 1 or -1 being perfect correlations) as well as the certainty of that strength (a standard *p*-value, P). We found a strong positive correlation between total

M. ovipneumoniae shedding during phase 1 of the study (days 0–28 post-*M. ovipneumoniae*) and total IDV shedding, but this correlation had weak certainty ($r_s = 0.703$, $p = 0.228$) (Figure 2C). Interestingly, we found a very strong positive correlation with high certainty between phase 1 *M. ovipneumoniae* shedding and subsequent IDV shedding at 3 days post-IDV ($r_s = 0.882$, $p = 0.014$) (Figure 2D). While causation cannot be determined, these data suggest that *M. ovipneumoniae* infection is associated with subsequent accelerated early-infection IDV replication.

3.3. Total Serum Protein and Acute Phase Response

Increased total protein in serum can occur during prolonged infection due to the generation of acute-phase proteins during an inflammatory response. We collected serum every 7 or 14 days throughout the study and found no difference in total serum protein levels between treatment groups at any serum collection timepoint (Table 2 and Figure 3A). Because the total serum protein response occurs over time and may not be reflected by single-timepoint or even sequential-timepoint comparisons, we also compared total serum protein concentrations from serum collected before versus after the IDV shedding period (1 day prior to IDV and 14 days post-IDV) (Figure 1B). Consistent with our earlier findings that recent *M. ovipneumoniae* infection may increase IDV-associated body temperature and early viral replication, we found that Mo-IDV lambs also showed an increase in total serum protein concentration after IDV infection, while sal-IDV lambs showed a small decrease in total serum protein during this time period (Mo-IDV: 2.00 ± 1.43 g/dL versus sal-IDV: -0.413 ± 2.27 g/dL; adjusted p -value = 0.035) (Figure 3B).

Table 2. Total serum protein and acute-phase response. Start denotes 1 day prior to *M. ovipneumoniae* infection. All other days relative to IDV infection. Endpoint denotes 22 days post-IDV (sal-IDV) or 23 days post-IDV (Mo-IDV). SAA: serum amyloid A; P: Bonferroni–Dunn adjusted p -value. Comparisons evaluated by Student’s t -test.

	Timepoint	sal-IDV			Mo-IDV			p
		Mean	Median	Range	Mean	Median	Range	
Total Serum Protein (g/dL)	Start	7.66	7.3	6.2–8.8	7.6	7.6	6.5–8.7	>0.999
	Day-1	10.8	11	9.1–11.7	9.6	9.7	9.1–10.1	0.244
	Day 6	10.4	9.9	7.8–13.1	9	9.5	6.8–10	0.236
	Day 14	10.4	10.3	8–13.5	11.6	11.4	10.5–14.2	0.581
	Endpoint	10.5	10.7	8.6–12.2	10.7	10.5	8.8–13	>0.999
Albumin (g/dL)	Start	4.2	4.2	3.9–4.4	4.2	4.3	3.3–4.7	>0.999
	Day-1	4.9	4.9	4.6–5.5	4.8	4.8	3.3–5.9	>0.999
	Day 6	4.9	4.9	4.7–5.3	4.6	4.8	3.3–5.5	>0.999
	Day 14	4.4	4.4	3.9–4.6	4.4	4.4	3.6–5.2	>0.999
	Endpoint	4.6	4.6	4.4–4.7	4.2	4.3	3.1–5	0.546
SAA (ng/mL)	Day-1	618.5	58.5	3.4–2902.2	499.7	42.4	0–2936.8	>0.999
	Day 6	158.4	30.8	0–838	667.3	231.2	36.2–2793.5	0.587
L-lactate (mM)	Day-1	1.9	2	1.4–2.2	1.7	1.8	1.1–2.3	0.495
	Day 6	1.8	1.9	0.3–3	1.9	1.8	0.8–2.8	0.838

We also measured acute-phase response via serum albumin (a negative acute-phase protein), serum amyloid A (a positive acute-phase protein), and L-lactate (a metabolic marker of critical illness). As expected, each group displayed decreased serum albumin levels following IDV infection (14 days post-IDV), although this change was not statistically significant (Table 2 and Figure 3C). Albumin fraction (albumin/total protein) remained largely stable, and no significant differences were observed between groups at any timepoint, indicating overall relative health (Figure 3D). Serum amyloid A levels varied over time and between lambs but were not affected by treatment (Table 2).

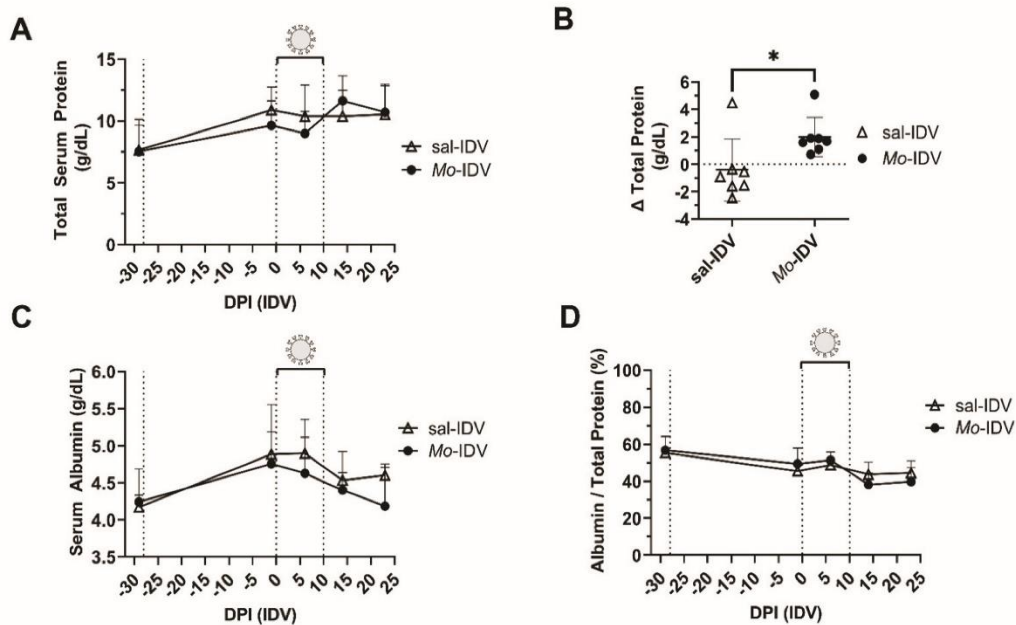


Figure 3. Prior infection with *M. ovipneumoniae* has a minimal effect on IDV-associated acute-phase response. (A) Total serum protein was measured throughout duration of study. (B) Change in total serum protein between 1 day prior to IDV infection and 14 days post-infection. (C) Serum albumin concentration were measured throughout duration of study. (D) Ratio of serum albumin:total protein. Vertical dotted lines signify inoculation with *M. ovipneumoniae* or IDV, or the last observed timepoint of IDV shedding, respectively. DPI: days post-infection. Data are presented as mean ± SD. Comparisons were evaluated by Student's *t*-test. * Bonferroni–Dunn adjusted *p* < 0.05.

While it has been used as a prognostic indicator in some species, the predictive power of L-lactate in sheep infection outcomes remains unexplored in a controlled infection setting. To investigate whether L-lactate was a predictor of IDV-associated pathology, we compared serum L-lactate at 1 day prior to and 6 days post-IDV and found no significant difference between experimental groups at either timepoint (Table 2). L-lactate also failed to predict viral load or reflect past (phase 1) or ongoing bacterial burden (correlative data not shown).

3.4. IDV Infection Elicits a Neutralizing Antibody Response in SPF Lambs

To date, studies have reported domestic sheep seroconversion against D/OK, D/660, and D/IM lineage IDVs [42]. To investigate the induction of a neutralizing antibody response in sal-IDV and Mo-IDV lambs, we evaluated titers by HI assay (Figure 4A). Lambs showed seropositivity beginning 14 days post-IDV, with all but 2 sal-IDV (3525 and 4016) and all but 2 Mo-IDV (3318 and 3811) lambs reaching the defined seropositivity threshold (geometric mean titer (GMT) ≥ 10; sal-IDV GMT = 21.5; Mo-IDV GMT = 17.2). Interestingly, overall titers were diminished at the experimental endpoint (22–23 days post-IDV; sal-IDV GMT = 14.1; Mo-IDV GMT = 11.2), when all but 2 sal-IDV (3525 and 3418) and all but 3 Mo-IDV (3713, 3811, and 4527) lambs remained above the seropositivity threshold. No IDV-neutralizing antibody response was detected in one IDV-positive wether from the Mo-IDV group (3811; HI ≤ 5). This lamb shed only low levels of IDV at 6, 8, and 10 DP-IDV (6 DP-IDV: 9.0 ± 17.0 PFU/mL; 8 DP-IDV: 99.0 ± 64.1 PFU/mL; 10 DP-IDV: 86.8 ± 65.2 PFU/mL) and never shed *M. ovipneumoniae*, despite having been inoculated.

Conversely, both lambs with subdetectable IDV shedding (sal-IDV: 4016; Mo-IDV: 4527) showed a measurable neutralizing antibody response at either 14 days post-IDV (4527; GMT = 16.8) or 22 days post-IDV (4016; GMT = 7.1). Interestingly, Mo-IDV lamb 4527 exhibited the lowest shedding of any lamb with detectable *M. ovipneumoniae* and no detectable IDV, but still produced IDV-neutralizing antibodies. Notably, these data support serosurveillance data which suggests that in natural IDV infections, sheep mount a neutralizing antibody response, but that HI titers remain lower than those in some other species such as cattle and swine [33,34,42,47,79].

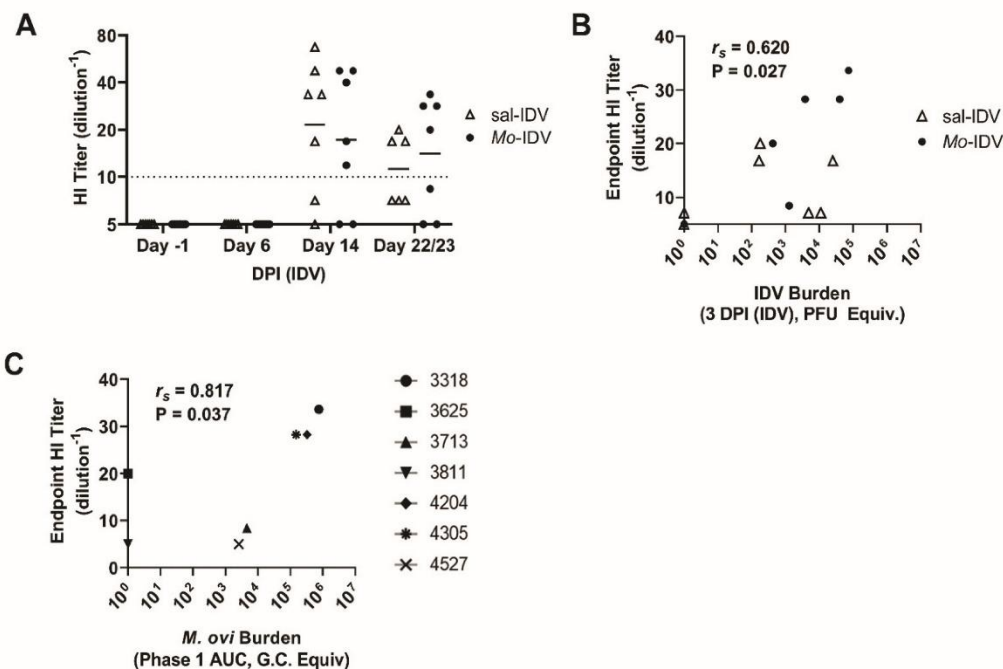


Figure 4. IDV antibody response correlates to prior *M. ovipneumoniae* burden and early-IDV-infection viral load. (A) Neutralizing antibody titer by HI assay. (B,C) Correlations between HI titer and (B) viral burden at 3 days post-IDV or (C) total *M. ovipneumoniae* burden during phase 1 (Phase 1 AUC). AUC: area under the curve; r_s : Spearman correlation coefficient; P : p -value. Horizontal line denotes HI cutoff value (10).

As with the viral and bacterial shedding data, the HI results were heterogeneous, providing an opportunity for correlative analysis. We found a moderate positive correlation between IDV shedding at 3 days post-IDV and neutralizing antibody response at the endpoint of the study (22 or 23 days post-IDV) ($r_s = 0.671$, $p = 0.012$). Interestingly, this effect was strongly driven by the Mo-IDV lambs ($r_s = 0.954$, $p = 0.031$) and was not observed within the sal-IDV group ($r_s = 0.031$, $p = 0.967$) (Figure 4B). Moreover, we also found a strong positive correlation between phase 1 *M. ovipneumoniae* burden and endpoint IDV neutralizing antibody titers ($r_s = 0.817$, $p = 0.037$) (Figure 4C). Taken together, these correlative data imply a connection between recent *M. ovipneumoniae* burden, early IDV infection, and neutralizing antibody response. Moreover, differential responses between Mo-IDV versus sal-IDV lambs suggest that while subtle, recent *M. ovipneumoniae* infection does in some way modify the immunological response to IDV in SPF lambs.

4. Discussion

IDV is a recently discovered pathogen, and so far, its pathogenesis has only been studied in a small subset of its natural hosts [33,47,53,58,64,74,80]. The primary goal of this study was to characterize IDV infection in immunologically naïve domestic lambs, which to our knowledge, has not been studied previously. Because lambs are commonly transiently colonized by *M. ovipneumoniae* early in life (2–12 months), we also sought to establish whether IDV progression is altered in the context of a recent or ongoing *M. ovipneumoniae* infection [81]. Our experimental design also allowed us to compare our current findings regarding *M. ovipneumoniae* infection to those we obtained with the previous year's flock [11]. Finally, we found variable shedding, acute phase responses, and neutralizing antibody titers from lamb to lamb, which we leveraged to identify correlations between recent *M. ovipneumoniae* burden, early-infection IDV replication, and neutralizing antibody response (Figure 2C,D and Figure 4B,C).

In phase 1 of this study, lambs were infected intranasally with a cultured *M. ovipneumoniae* isolate (NW-4) or mock-infected with phosphate-buffered saline, whereas in our previous study, lambs were infected using fresh nasal washes from confirmed *M. ovipneumoniae*-positive sheep [11]. While all nasal wash-inoculated lambs in our previous study sustained a detectable *M. ovipneumoniae* infection for 12 weeks, only 5 of 7 isolate-inoculated lambs in the current study ever detectably shed *M. ovipneumoniae*. Moreover, 6 of 7 lambs in the current study apparently cleared the infection by week 5 (Figure 1A). Importantly, lambs in neither study showed overt clinical signs in response to *M. ovipneumoniae* infection [11]. While our inoculum was a higher dose than that which was previously used by others [25,65,71,72,82,83], it is well established that individual *M. ovipneumoniae* strains vary widely in virulence and that *M. ovipneumoniae* is readily culture-attenuated, both of which could have contributed to our comparatively early *M. ovipneumoniae* clearance and complete lack of respiratory symptoms [28,66,81,84].

In phase 2, all lambs were inoculated with IDV. Similar to *M. ovipneumoniae*, IDV shedding was variable, and 2 lambs failed to detectably shed virus at any point. Our shedding data show that in sheep, IDV maintains a replication pattern similar to IDVs in other species, inducing an acute infection which persists for less than 2 weeks (Figure 2B and Supplemental Figure S1A,B) [33,47,51–53,61]. Moreover, this experiment demonstrated that IDV infection can remain subclinical in lambs, even after recent or ongoing *M. ovipneumoniae* infection, and that virus readily sheds during asymptomatic infection (Figure 2B and Supplemental Figure S1A,B). Although others have observed rectal shedding and viremia in cattle and swine, we found no evidence of either at the chosen timepoints (rectal sample: day 7 post-IDV; blood samples: day 3 post-IDV) [45,47,58]. To date, there are no reported IDV isolates from small ruminant respiratory samples, although there is one report of IDV recovered from a rectal swab from a goat that was critically ill with severe diarrhea [45]. This goat isolate belongs to the same phylogenetic lineage as the IDV used in this study (D/OK) and is closely related to both bovine and swine IDVs, suggesting that some IDVs may contribute to illness (albeit gastric) in sheep under certain circumstances [45]. A more thorough investigation of possible gastrointestinal transmission of IDVs should be pursued in the future to gain insight into this possible mechanism of transmission.

IDV is often asymptomatic in cattle but may predispose its hosts to secondary infections by facultative pathogens, including *Mycoplasma* spp. This effect was demonstrated in a study by Lion and colleagues which found that in immunologically naïve calves, simultaneous IDV-*Mycoplasma bovis* (*M. bovis*) infection increased early-infection *M. bovis* nasal shedding and promoted *M. bovis* colonization in the lower respiratory tract compared to calves that were not coinfecting with IDV [64]. Moreover, Nissly and colleagues found that among IDV-positive cattle, those coinfecting by additional respiratory pathogens had a higher IDV burden than those solely infected with IDV [63]. While etiology remains unclear, these data suggest that IDV may act synergistically with other respiratory pathogens. In this experiment, *M. ovipneumoniae* was introduced first and had cleared for most lambs by the time IDV was introduced, so for most lambs, any impact of *M. ovipneumoniae* would

be due to recent rather than ongoing infection, and therefore, true synergism would not be possible.

Accordingly, in phase 2, we found no difference in clinical symptoms or overall viral shedding between groups. Despite this, we found several indicators that recent *M. ovipneumoniae* infection impacted IDV pathogenesis. For instance, during IDV infection *Mo*-IDV lambs had significantly elevated rectal temperatures compared to the *sal*-IDV (Figure 1C). While these temperatures always remained subclinical, elevated body temperature is a hallmark response to influenza infection in many species. Interestingly, in our study, *Mo*-IDV group showed a strong positive correlation between total phase 1 *M. ovipneumoniae* burden and early-infection IDV shedding (3 days post-IDV) (Figure 2D). While these data are correlative, one possible explanation is that recent *M. ovipneumoniae* infection expedites the establishment of a subsequent IDV infection. In an immunocompromised host, this might result in a more severe infection, but if the immune system is functioning optimally, as viral burden rises, there is a proinflammatory response which in turn contains the infection to result in asymptomatic carriage, as we observed in these lambs. During an inflammatory response, serum protein levels rise as acute phase proteins are produced. Consistent with our observation of elevated body temperature in *Mo*-IDV lambs during viral infection, we found that *Mo*-IDV total serum protein level was elevated at 14 days post-IDV as compared to 1 day before IDV (Figure 3B). Conversely, serum protein levels in *sal*-IDV lambs fell for all but one animal, suggesting that recent *M. ovipneumoniae* infection promoted inflammation during IDV infection, whereas IDV alone did not. Collectively, these data suggest the presence of a subclinical immune response to IDV which is enhanced by recent or ongoing *M. ovipneumoniae* infection.

While an innate immunity-driven inflammatory response may partially mitigate an infection, neutralizing antibodies are typically required for complete clearance of a pathogen. Interestingly, hemagglutinin inhibition assays on serum from naturally infected sheep tend to show lower neutralizing antibody titers than those from naturally infected cattle. In a U.S. retrospective study, Quast et al. reported that more than half of seropositive sheep (defined as HI $\geq$ 1:40) had titers of 1:40, with a maximum documented titer of 1:320 [42]. This is not specific to the United States; in France, no reported titers have exceeded 1:160, and in West Africa, the maximum reported titer to date is 1:80 [38,39,44]. By comparison, HI results in 2400 randomly sampled Irish cattle reported only 7% of seropositive cattle to have titers as low as 1:40, with over 15% of titers $\geq$ 1:640–1:10,240 [43]. A recent study established that a 1:10 HI cutoff had equivalent specificity and sensitivity to viral microneutralization-based IDV diagnostics, and it may therefore be appropriate to consider whether a lower seropositivity cutoff value should be employed for reporting purposes [85]. Herein we used a cutoff value of GMT $\geq$ 1:10, i.e., excluding samples for which any replicate is well agglutinated. Mirroring surveillance data, we observed a maximum GMT of 1:67, with a minority of responses exceeding the traditional influenza seropositivity threshold of $\geq$ 1:40 (*sal*-IDV = 3/7, *Mo*-IDV = 2/7). Lambs failed to maintain a GMT $\geq$ 1:40 to the endpoint of the study (22 or 23 days post-IDV), and only 3/6 *sal*-IDV and 4/7 *Mo*-IDV maintained GMT $\geq$ 1:10 through the endpoint. These findings are in contrast to three separate calf studies, in which animals sustained or increased their titers between the same timepoints [53,61,64]. Notably, no lambs in this study seroconverted at 6 days post-IDV, which diverges from the response reported in intranasally-inoculated feral swine and calves [47,53], but was consistent with colostrum-deprived calves as well as calves infected by contact transmission [61].

In the abovementioned recent study by Lion and colleagues, increased disease severity in calves coinfecting with *M. bovis* and IDV was associated with overexpression of interferon- γ during coinfection as compared to single infections, which the authors attributed to differences in lymphocytic infiltrate between treatment groups [64]. In our study, neither the quality nor the quantity of cellular lung infiltrates at 23 days post-IDV were affected by prior *M. ovipneumoniae* infection. Interestingly, we also found that *M. ovipneumoniae* infection alone does not induce transcription of inflammatory cytokines in PBMCs (data not

shown). Nonetheless, our observation of a positive correlation between *M. ovipneumoniae* burden, early-infection IDV shedding, and IDV-neutralizing antibody response suggest that prior *M. ovipneumoniae* infection could promote anti-IDV inflammation. Thus, future studies will examine the quality of anti-viral immune response to IDV infection in lambs and whether and how this response could be modified by persistence of *M. ovipneumoniae*.

Overall, we observed low and unstable neutralizing antibody response to IDV, which may not be uncommon to sheep. Despite this, we found a correlation between IDV shedding at 3 days post-IDV with endpoint HI titer (Figure 4B), suggesting that those lambs with higher early-infection viral loads may mount a more robust secondary immune response and the strength of this correlation was almost exclusively driven by the Mo-IDV group ($r_s = 0.954$, $p = 0.031$). This finding is somewhat surprising because *M. ovipneumoniae* has been shown to directly suppress mitogenic expansion of T-cells and B-cells, which could plausibly result in a delayed or impaired antibody response [31,32]. Interestingly, the lamb with the highest overall *M. ovipneumoniae* burden also had the highest endpoint HI titer (lamb 3318; GMT = 33.6), and *M. ovipneumoniae* burden had a strong positive correlation with endpoint titer, suggesting that recent or ongoing *M. ovipneumoniae* infection may not suppress a heterologous antibody response in lambs (Figure 4C). Taken together, our serological data support prior evidence that sheep fail to either mount or maintain a robust neutralizing antibody response against IDV, and the data indicate that this response may also wane more rapidly than with other hosts, but that recent or ongoing *M. ovipneumoniae* infection does not inhibit antibody production or efficacy.

In conclusion, this study confirms that IDV replicates in sheep and elicits a neutralizing antibody response, but IDV does not confer pathology or strong acute-phase responses even in sheep infected with *M. ovipneumoniae*. Our findings corroborate serosurveillance data that indicate that IDV neutralizing antibody titers appear relatively weak in comparison to other species' responses, suggesting that they were sufficient to clear the pathogen within two weeks of infection [38,39,42–44]. We also noted rapid waning of the antibody response, which could impact serosurveillance study outcomes. Although subclinical, we found multiple indicators that recent *M. ovipneumoniae* infection stimulated an immunological response, featuring elevated body temperature and an increase in total serum protein in response to IDV infection only in lambs previously infected with *M. ovipneumoniae*. Moreover, we found that at day 3, post-IDV viral titers and endpoint HI titers correlated to prior *M. ovipneumoniae* burden. We posit that on their own, both *M. ovipneumoniae* and IDV are minimally immunostimulatory in sheep, but that *M. ovipneumoniae* infection may prime the lamb immune system to respond more robustly to secondary infection by IDV. In the context of two subclinical infections, this may not lead to overt respiratory symptoms, but virulence varies widely between *M. ovipneumoniae* strains [66,81,86], and IDV is not genetically stable [74,87], it readily reassorts [88], and its tissue tropism and host range can vary by strain [74,89]. Changes in any of these parameters could dramatically alter outcomes to coinfection of both of these pathogens in sheep. Finally, recent work has demonstrated that interspecies transmission is possible and has suggested a model wherein cattle are the primary host of IDV, but periodic spillover events result in contained outbreaks in other species [74]. Still, it is unclear whether sheep are an intermediate host and whether sheep adapted IDVs can infect other species. Therefore, it is important not only that IDV surveillance continues, but also that a standardized HI titer cutoff is implemented, and a sheep IDV isolate(s) is obtained and sequenced for reference.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/v14071422/s1>, Figure S1: IDV nasal shedding patterns in SPF lambs varies within both experimental groups.

Author Contributions: Conceptualization and methodology: all authors; validation, E.R. and C.S.; formal analysis, E.R. and C.S.; investigation, K.J., E.R., C.S., B.T.J. and J.S.; resources, A.R.-A., D.B. and K.J.; data curation, E.R.; writing—original draft preparation, E.R. and A.R.-A.; writing—review and editing, A.R.-A., D.B., M.J. and K.J.; visualization, E.R.; supervision, A.R.-A., D.B., M.J. and V.H.; funding acquisition, A.R.-A. All authors have read and agreed to the published version of the manuscript.

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