Navigating the Complexities of Disease Prevention, Alternative Treatments, and Multi-Drug Resistant *Rhodococcus equi* Mitigation Strategies

by

Courtney Higgins

(Under The Direction Of Dr. Laura Huber)

A thesis submitted to the Graduate Faculty Of Auburn University in partial fulfillment of the requirements for the Degree of
Master of Science

Auburn, Alabama
August 5, 2023

Keywords**:** *Rhodococcus equi*, multidrug resistance, environmental impact, mitigation strategies

Approved by

Laura Huber, Chair, Assistant Professor of Epidemiology, Auburn University CVM
Noah D. Cohen, Distinguished Professor of Large Animal Internal Medicine and Clinical Sciences, Texas A&M School of Veterinary Medicine and Biomedical Sciences
Mark R. Liles, Acting Associate Dean of Research and Graduate Studies, Professor of Biological Sciences, Auburn University
Stuart B. Price, Associate Professor of Pathobiology, Auburn University CVM
Chengming Wang, Professor of Pathobiology, Auburn University CVM

Abstract

The opportunistic pathogen, *Rhodococcus equi*, is a robust soil saprophyte present in the environment of horse-breeding farms and an important cause of pulmonary disease in foals at endemic farms. As an effort to prevent severe disease, endemic farms rely on early identification of lung lesions using thoracic ultrasonographic screening (TUS) of foals and administration of antimicrobials in foals with sonographic evidence of pulmonary abscesses or consolidations irrespective of clinical signs. This method of screening and treating has led to the overuse of antimicrobials and has been linked to the emergence of multidrug resistant (MDR)-*R. equi* in both clinical and environmental *R. equi* isolates. The implementation of judicious use of antimicrobials at endemic farms is crucial for mitigating antimicrobial resistance (AMR); however, the long-term impacts of the overuse of antimicrobials is unknown. Here, we investigate the main challenges to preventing *R. equi* disease, finding alternative treatments, and the potential of long-term impact of antimicrobial residue accumulation in the environment of farms on MDR-*R. equi* mitigation strategies.

Dedication

I dedicate this work to the countless amount of people in my community who have touched my life by nurturing, supporting, and inspiring me, but most of all for believing in me.

To my family and friends who equipped me with a support system that will outlast a lifetime, you gave me the foundation my life is built on today. It is because of this foundation that I am able to work hard for what I believe in and have compassion for others while navigating through the waves of life.

To my mentors who inspired me to dive deep into the microbiome of the world. Mrs. Terri Hathcock and our family in the bacteriology lab, I would not be where I am today without you. It was with your guidance, patience, and confidence in me that nurtured my admiration for all things microbiology. Dr. Mark Liles and our "Planetary Protection Team", without your introduction to the research world, I most certainly would not have found myself conducting research on life's unknowns. You have inspired me to reach for the stars—almost literally—even in times of uncertainty. I extend my deepest gratitude for taking a chance on me and allowing me to be a part of your team.

To Dr. Laura Huber, or Laura, I should say. You are the best mentor I could ever ask for. Your knowledge, kindness, dedication, and compassion has provided me with endless opportunities to grow as a researcher as well as a human being. After all, you do always remind me I am only human, when I need it the most.

Last but not least, to Luna. Some may see you as just a silly cat who thinks she's a dog—actually, everyone knows how wonderful you are—but you are what keeps me going through all life's obstacles. Thank you for being you.

This work is presented in the memory of Dr. Steeve Giguère.

Acknowledgements

I would like to express my deepest gratitude to my major professor, Laura Huber, for trusting me with the opportunity to work on a project that is held so close to her heart. You provided me with all the resources one could ever need to succeed as a researcher and as a student. It was an honor to be your first graduate student.

I would like to thank the members of my committee for their time and mentorship during my degree. Dr. Noah Cohen, thank you for your collaboration on our research projects and especially for your patience in advising me from several states away. Dr. Mark Liles, thank you for sharing your knowledge as a professor and for being an invaluable resource to our lab. Dr. Stuart Price, thank you for sharing your endless knowledge on microorganisms and for the numerous times your lab has saved us when chaos ensues. Dr. Chengming Wang, thank you for always introducing new ideas and helping us troubleshoot our molecular experiments.

Thank you to my lab partner, Pankaj, and our undergraduate students for helping us create an incredible lab environment and for your assistance on my many experiments.

Thank you to Dr. Slovis and the Hagyard Equine Medical Institute for your collaboration on our projects and for providing us with samples and data from horse-breeding farms in Kentucky.

Thank you to Melissa Boersma and the Chemistry Department for your collaboration on our antimicrobial residue experiments.

Thank you to Amelia Pendleton and the Department of Pathobiology for prioritizing the graduate students in our program.

Thank you to Auburn University and the Grayson Jockey Club Research Foundation for funding our research and providing others with opportunities to make our world a better place.

Table of Contents

List of Tables

Page

List of Figures

Page

Chapter 1

Introduction

Rhodococcus equi is a facultative intracellular pathogen commonly found in the environment of horse-breeding farms. *R. equi* is known to cause subclinical to severe pneumonia in foals following inhalation of aerosolized virulent *R. equi* from soil in their environment. The pathogen then invades and replicates within alveolar macrophages ultimately causing pneumonia. While it has been shown that many foals with subclinical signs of *R. equi* pneumonia spontaneously recover, thoracic ultrasonographic screening (TUS) in foals is often used at horse-breeding farms endemic with *R. equi* pneumonia to early detect lung lesions and prevent the development of severe *R. equi* pneumonia. Following TUS, antimicrobials are often administered to foals if lung lesions are detected despite the absence of clinical signs of the disease. The most common antimicrobial treatment for clinical or subclinical rhodococcal pneumonia is the combination of a macrolide and rifampicin. The widespread overuse of antimicrobials to treat foals with subclinical pneumonia is strongly associated with the emergence of antimicrobial resistance (AMR) in the environment of horse-breeding farms and in clinical isolates from foals in central Kentucky. In 2017, efforts were taken by many farms in central Kentucky to reduce reliance on the "screen-and-treat" approach to subclinical pneumonia, but it is unknown if it is possible to mitigate AMR in the environment of horse-breeding farms by reducing antimicrobial use. Recent literature points to the possibility that bacteria evolve to adapt to fitness costs conferred by carrying AMR mechanisms, allowing for the persistence of antimicrobial resistant bacteria in the environment despite selective pressure from antimicrobials. Moreover, the accumulation of antimicrobial residues in the environment following treatment of animals could play an important role on maintaining selective pressure over long periods of time.

Our studies focused first on identifying the main challenges in controlling AMR at horse-breeding farms and second on investigating the effects of farm practices, such as antimicrobial use and use of TUS, and accumulation of antimicrobial residues in the soil on the presence of *R. equi* carrying antimicrobial resistance genes (AMRGs) in the environment of horse-breeding farms. The specific aims and hypotheses are as follows:

- **Specific Aim 1:** to describe current knowledge of the emergence of MDR-*R. equi* in the United States and challenges to providing guidance for antimicrobial use practices (*chapter 2*);
- **Specific Aim 2:** to determine time trends on antimicrobial use in foals, and prevalence of *R. equi* carrying AMRGs and antimicrobial residues in the environment of horse-breeding farms (*chapter 3*);

  Hypothesis 1: antimicrobial use, prevalence of *R. equi* carrying AMRGs, and antimicrobial residues in the environment of horse-breeding farms will be significantly lower in 2021 compared with 2017; and
- **Specific Aim 3:** to evaluate the effects of farm practices, antimicrobial residue in the soil, and their interactions over time on the proportion of *R. equi* carrying AMRGs in the soil of horse-breeding farms (*chapter 3*);

  Hypothesis 2: Farm practices, such as the use of TUS, and number of foals treated with antimicrobials, and the concentration of antimicrobial residues are positively associated with the presence of *R. equi* carrying AMRGs in the soil.

Chapter 2

Rhodococcus equi: Challenges to Treat Infections and to Mitigate Antimicrobial Resistance *

---

*Courtney Higgins, Laura Huber,
Rhodococcus equi: challenges to treat infections and to mitigate antimicrobial resistance,
Journal of Equine Veterinary Science, Volume 127, 2023, 104845, ISSN 0737-0806, https://doi.org/10.1016/j.jevs.2023.104845. Reprinted here with the permission of the publisher.

Abstract

Rhodococcus equi, a gram-positive facultative intracellular pathogen and a soil saprophyte, is one of the most common causes of pneumonia in young foals. It poses a threat to the economy in endemic horse-breeding farms and to animal welfare annually. Many farms use thoracic ultrasonographic screening and antimicrobial treatment of subclinically affected foals as a preventive measure against severe *R. equi* infections. The wide use antimicrobials to treat subclinically affected foals has contributed to the emergence of multidrug resistant (MDR)-*R. equi* in both clinical isolates from sick foals and in the environment of horse-breeding farms. Alternatives to treat foals infected with MDR-*R. equi* are scarce and the impact of the emergence of MDR-*R. equi* in the environment of farms is still unknown. The aim of this review is to discuss the emergence of MDR-*R. equi* in the United States and the challenges faced to guide antimicrobial use practices. Reduction of antimicrobial use at horse-breeding farms is essential for the preservation of antimicrobial efficacy and, ultimately, human, animal, and environmental health.

Keywords: Antimicrobial resistance; Equine; One Health; *Rhodococcus equi*

2.1. Introduction

Rhodococcus equi is an environmental organism with the ability to survive and replicate intracellularly within a host.[1] The gram-positive, soil-born actinobacterium commonly causes respiratory illness in animals and immunocompromised humans.[2,3] The phylum Actinomycetota is composed by mostly gram-positive bacteria that are adapted to terrestrial and aquatic environments and are essential for agriculture due to its contribution to soil ecosystems.[4] The phylum includes members of the genera *Mycobacterium, Rhodococcus, Nocardia*, and *Corynebacterium*, all sharing the characteristics of carrying a mycolic acid cell wall of importance for survival in hostile conditions, including within macrophages.[5–8] Elimination of *R. equi* from the environment is deemed not possible and not recommended given *R. equi* benefits to the ecosystem. Therefore, foals are exposed to potentially virulent *R. equi* present in the environment beginning the first day of life. *R. equi* is known to cause severe pneumonia in foals worldwide. Horse-breeding farms endemic with *R. equi* disease routinely experience economic loss and decline in animal welfare as foals are exposed to the pathogen's natural environment during early development.[1] The morbidity of *R. equi* infections in horse farms is unknown because most diagnosis of *R. equi* infections are presumptive. Foal mortality depends on disease severity and detection time, and studies have reported case-fatality rates as low as 0.5% out of the 2,756 foals diagnosed with pneumonia [9] to 80% in older studies.[10]The lack of effective preventative measures at farms endemic with *R. equi* pneumonia has been an issue in the past and persists in present day. Consequently, endemic farms, as an attempt to prevent severe *R. equi* pneumonia, resort to a method known as "screen-and-treat" to detect and treat subclinical pneumonia in foals.[11–13] This preventative measure involves using periodic thoracic ultrasonographic screening (TUS) for early detection of subclinical pneumonic lesions in foals. If pulmonary lesions are detected,

antimicrobials are then administered to prevent onset of clinical signs.[12,13]The use of antimicrobial therapy to treat subclinically affected foals at horse-breeding farms across the United States has contributed to the emergence of multidrug resistant (MDR)-*R. equi* in the environment which poses a threat worldwide to animal and human health as horse movement may potentially contribute to the spread of antimicrobial resistance (AMR) genes geographically.[14–22] The emergence of AMR in *R. equi* may impair treatment of affected foals because of the lack of antimicrobials that are effective against *R. equi in vivo*,[23] however more studies are needed to understand the impact of emergence of MDR-*R. equi* on clinical outcomes. To the authors' knowledge, only one retrospective observational study[18] has been published demonstrating the association between AMR and the outcome of foals infected with *R. equi*. More studies are needed to evaluate outcomes of foals infected with the different genotypes of MDR-*R. equi* found in the environment and their respective minimum inhibitory concentrations (MIC) for each antimicrobial that could potentially be used to treat these infections. A large-scale epidemiological study in this context, could help better advise antimicrobial use practice in endemic horse-breeding farms.

The purpose of this review is to summarize and put in context the current knowledge about the emergence of MDR-*R. equi* in horse-breeding farms in the United States, the challenges to find alternative treatment and prevention, and potential AMR mitigation strategies.

2.2. *R. equi* Pathogenesis and Immunity in Foals

R. equi most commonly causes pyogranulomatous bronchopneumonia in foals between 3 and 24 weeks of age,[1,24] however extrapulmonary disorders, such as abdominal lesions, polysynovitis, and uveitis can also occur [24–27] and are associated with worse prognosis.[24] Foals are initially exposed to *R. equi* through contact with the farm's environment.[1,3,28] Frequently, infection occurs through inhalation of the pathogen and the time frame between exposure and infection is

highly dependent on the initial dose and virulence of the *R. equi*.[1] Aerosolization and increased concentrations of airborne *R. equi* due to dry climate and density of animals in paddocks are associated with higher infection rates. [29] Concentration of airborne *R. equi* is higher in enclosed spaces, such as in stalls, [30,31] however, the higher density of animals can increase the aerosolization of *R. equi* in paddocks.[32]

Younger foals (<3 weeks of age) are more susceptible to the disease, likely due to the immature neonatal immune system.[33,34] The mechanisms of immunity against *R. equi* in foals remains not fully understood. Adaptative T-cell responses are largely responsible for immunity against intracellular pathogens, however, the complex combination of innate and adaptative immunities is believed to play an important role in responding to *R. equi* infections in foals.[15] After inhalation, the alveolar macrophages engulf and phagocytize *R. equi*. However, virulent strains of *R. equi* are often able to overcome phagocytosis and replicate within the macrophages ultimately causing infection leading to pyogranulomatous pneumonia frequently observed early in the disease development during ultrasound surveillance.[1,2,11] Pathogenicity in *R. equi* is conferred by a virulence gene (*vapA*) inserted in a conjugative virulence plasmid (pVAPA) adapted to equine hosts.[1,35,36] The presence of *vapA* is necessary for establishment of infection by preventing the maturation of the phagosome to the stage of fusion of vacuoles containing *R. equi* with lysosomes.[37] Lung consolidation after heavy intrabronchial challenge can occur as early as within 3 days,[36] however, the incubation period under field conditions is unknown. Clinical signs may develop in later stages of the disease, making the identification and treatment of clinically affected foals challenging.[38] Therefore, the use of TUS has become important for the early identification of presumed *R. equi* infections.[11–13] The use of ultrasonographic screening to detect presumptive *R.*

equi in foals, however, may lead to the increase of antimicrobial use in endemic farms and contribute to emergence of MDR-*R. equi.* [20]

2.3. Antimicrobial Therapy

Macrolides are the most common treatment against *R. equi* infections. The main mechanism of action for macrolides is the inhibition of bacterial protein synthesis by binding to the bacterial 50S ribosomal subunit.[39] Macrolides commonly used in treatment of foals are erythromycin, azithromycin, clarithromycin, tulathromycin and gamithromycin.[15,40–43] In a retrospective study, survival of foals with severe *R. equi* pneumonia was superior when treated with macrolides and rifampicin compared to foals treated with trimethoprim-sulfamethoxazole, chloramphenicol, oxytetracycline, and a combination of penicillin and gentamicin.[26] Therefore, the traditional treatment of choice remains to be macrolide monotherapy or the combination therapy of macrolide and rifampicin since the 1980's.[23] Further, in a retrospective study, the combination of clarithromycin and rifampicin was significantly more effective than erythromycin or azithromycin combined with rifampicin especially in foals with severe pneumonia.[44] Therefore, the combination of clarithromycin or azithromycin with rifampicin has been most commonly used due to convenience of administration, efficacy, and drug distribution.[38,44]

The benefits of using macrolides and rifampicin in combination are under investigation. Evidence in favor of the use of the combined therapy includes the synergy observed *in vitro*, in a case series, and in a study using historical controls.[45–47] Evidence against this combined use is based on more recent *in vitro* studies showing that the use of rifampicin decreases concentration of macrolides in plasma and bronchoalveolar lavage fluids,[48,49] and that there are no differences in disease recovery when using macrolides alone versus in combination with rifampicin.[50–52] Because resistance is more likely to occur with macrolide monotherapy than in combination with rifampicin

in vitro,[53] the combined therapy is still most commonly used, however the role of rifampicin on minimizing the occurrence of macrolide resistance is unclear. Large-scale, randomized, blinded, clinical trials are needed to evaluate the safety and efficacy of combining these antimicrobial classes to treat *R. equi* infections. *In vitro* studies evaluating the drug interactions between macrolides and rifampicin are lacking. The antagonism between rifampicin and macrolides, if exists, is potentially an important factor for *R. equi* AMR. Recent studies have discovered that drug interactions in bacterial disease treatments may affect the evolution of resistance.[54,55] Therefore, studies evaluating not only drug interactions, pharmacokinetics and pharmacodynamics, but also the potential for AMR selection are needed to efficiently assess best antimicrobial therapy for *R. equi* infections in foals.

The combination of macrolides and rifampicin is shown to be highly efficient in treating pneumonia in foals as well as other *R. equi* infections, improving the survival rate of affected foals. However, the impact of the emergence of MDR-*R. equi* on treatment outcomes at endemic farms is unknown. The current efficacy of these antimicrobials to treat *R. equi* infections may be impaired due to the increasing AMR prevalence in *R. equi,* potentially increasing mortality of animals due to treatment failure.[18] Alternative antimicrobials to the macrolide and rifampicin combination were found to be effective against *R. equi in vitro*, however no research has been conducted to evaluate the effectiveness of alternative antimicrobials to fight infections with MDR-*R. equi*. Clinical trials are still pending to evaluate the pharmacokinetics and dynamics of antimicrobials to be used against MDR-*R. equi* infections in foals, and to compare outcomes associated with MIC values of isolates. Interpretation criteria for *R. equi* susceptibility is currently unavailable at Clinical & Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, and extrapolations from other species and pathogens are used to

evaluate *R. equi* susceptibility. However, this approach is not ideal, and it may lead to uninformed use of antimicrobial to treat infections. A recent paper has found that the Epidemiological Cutoff (ECOFF) value for isolates of *R. equi* is similar to *Staphylococcus aureus.* [56] While ECOFF values are important to differentiate between wild-type and organisms that acquired AMR mechanisms, clinical decisions should not be based on ECOFF values. Therefore, more epidemiological and clinical studies are needed to better inform antimicrobial use practices. Moreover, many foals that are irresponsive to antimicrobial therapy are infected with susceptible strains of *R. equi* to both macrolides and rifampicin, thus treatment failure should not be used as an indicator of presence of resistant strains.[23] Other factors including antimicrobial concentration on the infection site, contact time, concentration of bacteria, and host characteristics could interfere with the association between antimicrobial treatment and outcome.[57] Therefore, adequate diagnosis of infections and characterization of *R. equi* strains causing the disease is important for optimal treatment and recovery. Additionally, records on proper diagnosis and strain characterization aid in providing guidance for treatment regimens by demonstrating trends of AMR in *R. equi*. The main challenge with studies associating MIC with outcomes in foals lies on the availability of information about the timing of *R. equi* isolation to determine if foals were originally infected with MDR-*R. equi* from the environment or if the *R. equi* isolates have become resistant after initiation of antimicrobial treatment. Close collaboration with the farms and clinicians is necessary to investigate this association.

The definitive diagnosis of rhodococcal disease should be based on *R. equi* culture and amplification of *vapA* from tracheobronchial aspirate samples from foals presenting with clinical signs of lower respiratory tract disease, cytological evidence of septic airway inflammation, or radiographic/ultrasonographic evidence of bronchopneumonia.[38] To determine *R. equi* clinical

isolates susceptibility to antimicrobials, isolates should be subjected to antimicrobial susceptibility testing to determine the level of susceptibility to a panel of antimicrobials.[20] Currently, interpretation for susceptibility to erythromycin and rifampicin is based on human MIC criteria for *S. aureus*.[16,18,20,44] However, the applicability of the breakpoint extrapolation is not always possible because of the lack of breakpoints for some antimicrobials and the fundamental differences of physiology and acquired mechanisms of resistance between *R. equi* and *S. aureus.* The treatment of foals affected with confirmed MDR-*R. equi* strains is challenging. Resistant strains to macrolides and rifampicin were shown to be susceptible to gentamicin, linezolid, fluoroquinolones, vancomycin,[18,58,59] chloramphenicol, minocycline, and trimethoprim-sulfamethoxazole [18] *in vitro,* however, their efficacy to treat affected foals is not yet well described. Additionally, some of the above-mentioned drugs, such as linezolid and vancomycin, are considered critical agents for human health, and their use should be restricted.[60] The use of doxycycline has been suggested as a good alternative to the use of macrolides and rifampicin, and it was found to be adequate to treat infections caused by susceptible strains in foals, however, its safety and effectiveness against MDR-*R. equi* remains unproven in clinical settings.[52] Because of the lack of clinically proven alternative antimicrobials to treat foals infected with MDR-*R. equi*, it is of utmost importance that we preserve the efficacy of macrolides and rifampicin and mitigate the emergence of resistance through implementing the judicious use of these antimicrobial classes. Moreover, few epidemiological studies have collected data on the criteria used to decide when to treat foals with antimicrobials and what antimicrobials and combinations are used across different farms and geographic regions. Information on the treatment criteria in different farms is necessary to evaluate the impact of antimicrobial use practices on strategies to mitigate the spread of AMR in horse-breeding farms.

2.4. Development of Resistance to Macrolides

AMR in *R. equi* has rapidly emerged in clinical strains in the last two decades,[16,18,20] and is highly prevalent in the environment of endemic horse-breeding farms in Kentucky,[14,21] The most relevant epidemiological studies demonstrating the emergence and mechanisms of MDR-*R. equi* are presented in **Fig. 1**. The first report of AMR in *R. equi* was a study demonstrating the development of AMR to rifampicin and erythromycin in a foal treated with antimicrobials for pneumonia caused by *R. equi* in 1994.[61] In that case, increased MIC was observed in isolates recovered from the sick foal after 8 weeks of treatment. Initially, the foal responded positively to the antimicrobial treatment but a relapse of clinical signs occurred 7 weeks after the initiation of treatment and led to euthanasia.[61] In 1996, another report showed emergence of rifampicin resistance in virulent and avirulent *R. equi* isolated from lesions and intestinal contents of foals treated with antimicrobials.[62]

The excessive use of antimicrobials due to the treatment of subclinically affected foals in endemic farms is hypothesized as one of the most important factors contributing to the emergence of AMR in *R. equi*. Subclinical *R. equi* pneumonia is highly prevalent in horse-breeding farms worldwide, reaching up to 100% of the foal population.[12,51,63,64] While most clinically affected foals require antimicrobial therapy to recover from the disease, most subclinically affected foals, *i.e.*, foals that do not present clinical signs of the disease, but have evidence of presumptive *R. equi* pulmonary infection, have been noted to recover spontaneously without antimicrobial intervention.[1,64] Recently, a study demonstrated a decrease in over 30% of the number of foals treated without increasing mortality when treating only foals with large lung lesions instead of treating the all subclinically affected foals. [9] The application of the findings from this study needs to be approached with caution because the standard values for treatment of foals may vary among

farms.[15] Therefore, a careful observation of each farm's characteristics and risk factors for *R. equi* pneumonia such as density of animals and management of newborn foals, should be considered to develop a targeted approach to decrease antimicrobial use while maintaining the health and welfare of the horses. The monitoring of foals with TUS is an important tool to screen for subclinical disease allowing for close observation of foals for clinical signs of rhodococcal pneumonia. But its use as a tool to identify subclinical infections with the purpose of establishing preventative antimicrobial treatment is not recommended due to the risk of AMR development. The first retrospective study exploring the association between antimicrobial use in subclinically affected foals and AMR showed that AMR increased significantly in *R. equi* clinical isolates from foals submitted to diagnostic laboratories in Texas and Florida after 2001, when "screen and treat" was implemented [18]. Supporting this finding, a study in 2013 reported a 40% increase in AMR in isolates from foals 7 years after the implementation of "screen and treat" in one farm [16]. Later, a similar increasing trend in AMR was found in samples from foals submitted to diagnostic laboratories in Kentucky.[20] Other studies including clinical isolates of foals collected between 2011 and 2017, reported that prevalence of AMR to macrolides and rifampicin ranges from 15% to 24% of the isolates.[17,65] Macrolides remains the drug of choice in most treatment plans worldwide. Therefore, the rise in *R. equi* AMR threatens foal welfare and health due to the limited antimicrobial therapy alternatives and because foals infected with antimicrobial resistant-*R. equi* have a higher odds (odds ratio, 6.9; 95% confidence interval, 1.3 to 37) of non-survival compared with foals infected with susceptible isolates.[18]

The presence of the gene *erm*(46) in clinical isolates and both *erm*(46) and the novel *erm*(51) genes in environmental isolates confer resistance to macrolides, lincosamides, and streptogramins B (MLS_B).[66–69] Both *erm*(51) and *erm*(46) are inserted in conjugative elements

capable of horizontal gene transfer (HGT) to susceptible *R. equi* and actinobacterial members of the environmental microbiota.[66,67,69] While most *erm*(51)-positive *R. equi* strains found are avirulent, most *R. equi* carrying *erm*(46) also carry the virulence gene *vapA*, allowing these resistant genotypes to potentially cause disease in foals.[69] *VapA* is inserted in a plasmid, called pVAPA and the HGT of *erm*(46) was shown *in vitro* to become more efficient when inserted in the pVAPA plasmid.[67] Therefore, the potential of rapid HGT of these resistant genes to current susceptible bacterium poses a threat of further emergence of MDR-species of veterinary and public health importance. A recent study revealed that *R. equi* strains that carry pRErm46 had increased MIC to trimethoprim-sulfamethoxazole, doxycycline, tetracycline, streptomycin, and spectinomycin in addition to macrolides.[70] These resistances were attributed to the Class 1 Integron's sulfonamide (*sul1*) and aminoglycoside (*aaA9*) adjacent to the *tetRA* determinant inserted on pRErm46.[35,70] Previously, pRErm46 remained isolated within the clonal strain 2287.[35] However, a new clone (G2016) of MDR-*R. equi* carrying *erm*(46) has emerged,[14] suggesting a shift in antimicrobial pressure over time and migration of MDR-*R. equi* genotypes through the United States.[14,15,66] Moreover, the clonal strain 2287 has been recently isolated from horses in Ireland, suggesting that the MDR-*R. equi* that has been circulating in the United States since the 2000s has spread internationally likely through movement of equids.[22]

The emergence of novel MDR-*R. equi* genotypes in the environment [69] of horse-breeding farms is concerning. MDR-*R. equi* was found in a high prevalence in the environment of farms in central Kentucky (76%; 76/100 farms) and the number of foals treated with macrolides and rifampicin was significantly associated with resistance.[21] Thus, it is believed that the mass antimicrobial treatment of foals has increased the selective pressure in the environment due to post-treatment shedding of MDR-*R. equi* and antimicrobial residues in the soil. Whole genome

sequencing supports this hypothesis, demonstrating that MDR-*R. equi* carrying *erm*(51) were confined in a clonal population.[69] The environment is known to be the main source of infection for foals, and exposure of foals to MDR-*R. equi* in both stalls and paddocks was found to be constant throughout the breeding season in endemic farms.[71] This constant exposure, starting from birth through the most susceptible periods of a foal's life, potentially increases the risk of MDR-*R. equi* infections in foals. The infection with MDR-*R. equi* is known to increase the odds of death due to treatment failure,[18] however, mortality trends in endemic farms and its association with the concentration of MDR-*R.equi* in the soil are yet to be evaluated. Moreover, resistant organisms can adapt to persist in the environment,[72] which allows survival in complex populations, such as the environment of horse-breeding farms, independent of constant selective pressure. *In vitro* and soil plot experiments in different conditions show none to limited fitness cost of carrying *erm*(46), *erm*(51) and *rpoB* mutations on *R. equi.*[73–76] Little is known about the evolutionary dynamics of *R. equi* to compensate for fitness cost. Therefore, it is not possible to predict if MDR-*R. equi* would be outcompeted in the environment in the absence of antimicrobial pressure. In practice, measures to decrease antimicrobial use might fail in mitigating AMR due to these adaptative abilities of MDR-organisms to persist in the environment. Continued surveillance of antimicrobial use and AMR trends in the environment of farms and in clinical isolates of foals is necessary to understand these complex dynamics and to prevent MDR-*R. equi* infections in foals.

2.5. Antimicrobial Resistance Prevention and Discussion

Currently, due to the absence of efficient preventative methods for *R. equi* disease in foals, severe pneumonia caused by *R. equi* remains an extensive issue in endemic horse-breeding farms. There is still a lack of low-risk, high-reward treatment and prevention for *R. equi* pneumonia in foals. However, there are many alternative routes of treatment and prevention that should be

considered. First, the use of the "screen-and-treat" method should be approached with caution as it potentially increases antimicrobial use in farms, is consistently linked to AMR, and does not have consistent data to support the prevention of clinical *R. equi* pneumonia.[9,11,15,19–21,64,66] Moreover, the sole use of ultrasound for subclinical signs of pneumonia is not effective for determining a diagnosis and treatment regimen. Clinical signs, pathogen identification, and susceptibility testing are highly recommended for a definitive diagnosis of *R. equi* pneumonia and to serve as guidance for appropriated treatment.[1,9,20] Regardless of the lack of interpretation criteria for *R. equi*, antimicrobial susceptibility testing is recommended to improve knowledge of the circulating strains and better guide antimicrobial use practices. Antimicrobial use should be reduced and only used in clinically affected foals to preserve the viability of important antimicrobials for both animals and humans. Foals have the potential to spontaneously recover from subclinical pneumonia without antimicrobial use, therefore, close monitoring of lung lesions and the overall clinical assessment of foals would significantly reduce antimicrobial use in endemic farms and the risk of developing MDR-*R. equi* infections.[1,9,64] Travel of horses and people from MDR-*R. equi* endemic farms should be approached with caution, and the adoption of biosafety measures are recommended to reduce the potential spread of resistance genes through fomites within the United States and worldwide.[11,14,22]

Prevention of *R. equi* infections is likely the most effective approach to mitigate AMR and losses due to *R. equi* disease. Vaccination is a promising approach to prevent disease, but most *R. equi* vaccine candidates tested to date have not been sufficiently effective. A recent vaccine candidate based on the highly conserved bacterial polysaccharide PNAG yield immunogenicity in vaccinated mares and protection of their offspring against *R. equi* infection however, the timing and dosing as well as the addition of an adjuvant is still under investigation to improve this

vaccine's efficacy.[77] The use of hyperimmune plasma has also been explored extensively to prevent *R. equi* infections in young foals. Initially, studies demonstrated that the administration of hyperimmune plasma protected foals against experimental and natural infections.[78,79] However, subsequent studies have found contradictory results on its efficacy to protect foals from *R. equi* infections.[80–82] Advantages of using hyperimmune plasma include the potential reduction of *R. equi* shedding in affected foals, resulting in decreased environmental contamination with virulent *R. equi* and the potential decrease of contamination in foals.[82]

Additional alternative treatments include the use of bacteriophages, gallium maltolate (GaM), and the use of alternative antimicrobials. Bacteriophages have been used in the place of antimicrobials in many cases and the *in vitro* experiments against *R. equi* prove worthy of further research in the treatment of foals.[83] The use of GaM has been studied *in vitro* and *in vivo* as a potential treatment of *R. equi* infections in foals. GaM successfully inhibits the proliferation of *R. equi in vitro* extracellularly and within macrophages.[84–86] The administration of GaM as a treatment for subclinical disease in foals was non-inferior to treatment with macrolides.[84] The use of GaM as an alternative to antimicrobials to treat subclinically affected foals would potentially significantly reduce antimicrobial pressure. However, GaM used as a chemoprophylactic failed in decreasing the cumulative incidence of *R. equi* pneumonia in foals.[87] Finally, the use of antimicrobials, such as doxycycline, in place of macrolides and rifampicin suggests potential for reduction of resistance in some *in vitro* studies.[40,52] While the use of other antimicrobials is efficient in treating severe pneumonia in foals and other potential infections caused by MDR-strains, alternative antimicrobial use is not a permanent solution. It is worth noting that overuse of doxycycline could potentially co-select for macrolide resistance because the transferable element carrying *erm*(46) also carries sulfonamide (*sul1*) and aminoglycoside (*aaA9*) resistance genes

adjacent to the *tetRA* determinant inserted on pRErm46.[14,35,68] MDR-*R. equi* strains carrying pRErm46 had increased MIC to trimethoprim-sulfamethoxazole, doxycycline, tetracycline, streptomycin, and spectinomycin in addition to macrolides.[70] Therefore, shifting antimicrobial classes to treat *R. equi* disease in foals with the aim of decreasing AMR should be approached with caution and further investigation of the resistance co-selection is needed.

2.6. Conclusion

Judicious use of antimicrobials in horse-breeding farms should be adopted immediately to prevent the further spread of AMR globally. The use of TUS may be useful to detect and monitor subclinically affected foals but should not be used as a decision factor to treat with antimicrobials without considering clinical presentation. More studies are needed to establish interpretation criteria for *R. equi* susceptibility to antimicrobials and to investigate the association of MIC with the outcome of infected foals to improve guidance for antimicrobial treatment. Challenges to accomplish these important studies can be overcome with close collaboration between veterinarians, diagnostic laboratories, farms, and researchers that would provide temporality and contributing factors for AMR development. Research on the persistence of antimicrobial residue and MDR-*R. equi* in the environment due to shifts in antimicrobials used to treat *R. equi* infections is essential to predict the efficacy of mitigation strategies. Combined efforts to provide alternative measures of prevention and treatment for *R. equi* disease are vital to preserve the efficacy of critically important antimicrobials and to protect the health of humans, animals, and the environment.

Fig 2.1. Timeline representation of important studies showing the emergence of multidrug resistant (MDR)-*R. equi* and discovery of its antimicrobial resistance (AMR) mechanisms. The list is not exhaustive but rather the compilation of studies deemed fundamental on the understanding the epidemiology of MDR-*R. equi* emergence by the authors. Studies are color-coded by their design nature: cyan blue represents case reports; orange, retrospective studies; brown, prospective studies; and purple represents studies focusing on molecular mechanisms of AMR or experimental studies.

2.7. References

1. Giguère, S. *et al. Rhodococcus equi*: Clinical manifestations, virulence, and immunity. *J. Vet. Intern. Med.* **25**, 1221–1230 (2011).
2. Vázquez-Boland, J. A. *et al. Rhodococcus equi*: The many facets of a pathogenic actinomycete. *Vet. Microbiol.* **167**, 9–33 (2013).
3. Prescott, J. F. *Rhodococcus equi*: an animal and human pathogen. *CLIN MICROBIOL REV* **4**, 15 (1991).
4. Servin, J. A., Herbold, C. W., Skophammer, R. G. & Lake, J. A. Evidence Excluding the Root of the Tree of Life from the Actinobacteria. *Mol. Biol. Evol.* **25**, 1–4 (2007).
5. Kartmann, B., Stengler, S. & Niederweis, M. Porins in the Cell Wall of *Mycobacterium tuberculosis*. *J. Bacteriol.* **181**, 6543–6546 (1999).
6. Lichtinger, T. *et al.* The low-molecular-mass subunit of the cell wall channel of the Gram-positive *Corynebacterium glutamicum*: Immunological localization, cloning and sequencing of its gene *porA*. *Eur. J. Biochem.* **268**, 462–469 (2001).
7. Lichtinger, T., Reiss, G. & Benz, R. Biochemical Identification and Biophysical Characterization of a Channel-Forming Protein from *Rhodococcus erythropolis*. *J. Bacteriol.* **182**, 764–770 (2000).
8. Rieß, F. G., Lichtinger, T., Yassin, A. F., Schaal, K. P. & Benz, R. The cell wall porin of the gram-positive bacterium Nocardia asteroides forms cation-selective channels that exhibit asymmetric voltage dependence. *Arch. Microbiol.* **171**, 173–182 (1999).
9. Arnold-Lehna, D., Venner, M., Berghaus, L. J., Berghaus, R. & Giguère, S. Changing policy to treat foals with *Rhodococcus equi* pneumonia in the later course of disease decreases antimicrobial usage without increasing mortality rate. *Equine Vet. J.* **52**, 531–537 (2020).

10. Elissalde, G. S., Renshaw, H. W. & Walberg, J. A. Corynebacterium equi: An interhost review with emphasis on the foal. *Comp. Immunol. Microbiol. Infect. Dis.* **3**, 433–445 (1980).
11. Huber, L. *et al.* Epidemiology and molecular basis of multidrug resistance in *Rhodococcus equi*. *Microbiol. Mol. Biol. Rev.* **85**, (2021).
12. McCracken, J. & Cohen, N. D. Use of thoracic ultrasounds for the prevention of *Rhodococcus equi* pneumonia on endemic farms. *AAEP Proc.* **55**, (2009).
13. Slovis, N. M., McCracken, J. & Mundy, D. How to use thoracic ultrasound to screen foals for *Rhodococcus equi* at affected farms. *Proc Am Assoc Equine Pract* **51**, (2005).
14. Álvarez-Narváez, S., Giguère, S., Cohen, N., Slovis, N. & Vázquez-Boland, J. A. Spread of multidrug-resistant *Rhodococcus equi*, United States. *Emerg. Infect. Dis.* **27**, 529–537 (2021).
15. Bordin, A. L., Huber, L., Sanz, M. & Cohen, N. Rhodococcus equi Foal Pneumonia: Update on Epidemiology, Immunity, Treatment, and Prevention. *Equine Vet. J.* (2022) doi:10.1111/evj.13567.
16. Burton, A. J. *et al.* Macrolide- and rifampin-resistant *Rhodococcus equi* on a horse breeding farm, Kentucky, USA. *Emerg. Infect. Dis.* **19**, 282–285 (2013).
17. Erol, E. *et al.* Antimicrobial susceptibility patterns of *Rhodococcus equi* from necropsied foals with rhodococcosis. *Vet. Microbiol.* **242**, 108568 (2020).
18. Giguère, S. *et al.* Determination of the prevalence of antimicrobial resistance to macrolide antimicrobials or rifampin in *Rhodococcus equi* isolates and treatment outcome in foals infected with antimicrobial-resistant isolates of *R equi*. *J. Am. Vet. Med. Assoc.* **237**, 74–81 (2010).

19. Huber, L. *et al.* Association between antimicrobial treatment of subclinical pneumonia in foals and selection of macrolide- and rifampicin-resistant *Rhodococcus equi* strains at horse-breeding farms in central Kentucky. *J. Am. Vet. Med. Assoc.* **258**, 648–653 (2021).
20. Huber, L. *et al.* Emergence of resistance to macrolides and rifampin in clinical isolates of *Rhodococcus equi* from foals in central Kentucky, 1995 to 2017. *Antimicrob. Agents Chemother.* **63**, e01714-18, /aac/63/1/AAC.01714-18.atom (2018).
21. Huber, L. *et al.* Prevalence and risk factors associated with emergence of *Rhodococcus equi* resistance to macrolides and rifampicin in horse-breeding farms in Kentucky, USA. *Vet. Microbiol.* **235**, 243–247 (2019).
22. Val-Calvo, J. *et al.* International Spread of Multidrug-Resistant *Rhodococcus equi*. *Emerg. Infect. Dis.* **28**, 1899–1903 (2022).
23. Giguère, S. & Cohen, N. D. Controversies in therapy of infections caused by *Rhodococcus equi* in foals. *Equine Vet. Educ.* **30**, 336–341 (2018).
24. Reuss, S. M., Chaffin, M. K. & Cohen, N. D. Extrapulmonary disorders associated with *Rhodococcus equi* infection in foals: 150 cases (1987–2007). *J. Am. Vet. Med. Assoc.* **235**, 855–863 (2009).
25. Huber, L. *et al.* Development of septic polysynovitis and uveitis in foals experimentally infected with *Rhodococcus equi*. *PLOS ONE* **13**, e0192655 (2018).
26. Sweeney, C. R., Sweeney, R. W. & Divers, T. J. *Rhodococcus equi* pneumonia in 48 foals: Response to antimicrobial therapy. *Vet. Microbiol.* **14**, 329–336 (1987).
27. Zink, M. C., Yager, J. A. & Smart, N. L. *Corynebacterium equi* infections in horses, 1958-1984: A Review of 131 cases. *Can. Vet. J. Rev. Veterinaire Can.* **27**, 213–217 (1986).

28. Takai, S. Epidemiology of *Rhodococcus equi* infections: A review. *Vet. Microbiol.* **56**, 167–176 (1997).

29. Muscatello, G., Anderson, G. A., Gilkerson, J. R. & Browning, G. F. Associations between the ecology of virulent *Rhodococcus equi* and the epidemiology of *R. equi* pneumonia on Australian thoroughbred farms. *Appl. Environ. Microbiol.* **72**, 6152–6160 (2006).

30. Cohen, N. D. *et al.* Association of perinatal exposure to airborne *Rhodococcus equi* with risk of pneumonia caused by *R equi* in foals. *Am. J. Vet. Res.* **74**, 102–109 (2013).

31. Muscatello, G. *et al.* Comparison of concentrations of *Rhodococcus equi* and virulent *R. equi* in air of stables and paddocks on horse breeding farms in a temperate climate. *Equine Vet. J.* **38**, 263–265 (2010).

32. Kuskie, K. R. *et al.* Effects of location for collection of air samples on a farm and time of day of sample collection on airborne concentrations of virulent *Rhodococcus equi* at two horse breeding farms. *Am. J. Vet. Res.* **72**, 73–79 (2011).

33. Kachroo, P. *et al.* Age-related changes following *in vitro* stimulation with *Rhodococcus equi* of peripheral blood leukocytes from neonatal foals. *PLoS ONE* **8**, e62879 (2013).

34. Sanz, M. *et al.* The effect of bacterial dose and foal age at challenge on *Rhodococcus equi* infection. *Vet. Microbiol.* **167**, 623–631 (2013).

35. Álvarez-Narváez, S. *et al.* Clonal confinement of a highly mobile resistance element driven by combination therapy in *Rhodococcus equi*. *mBio* **10**, e02260-19, /mbio/10/5/mBio.02260-19.atom (2019).

36. Giguère, S. *et al.* Role of the 85-Kilobase plasmid and plasmid-encoded virulence-associated protein A in intracellular survival and virulence of *Rhodococcus equi*. *Infect. Immun.* **67**, 3548–3557 (1999).

37. von Bargen, K. *et al. Rhodococcus equi* virulence-associated protein A is required for diversion of phagosome biogenesis but not for cytotoxicity. *Infect. Immun.* **77**, 5676–5681 (2009).

38. Giguère, S. *et al.* Diagnosis, treatment, control, and prevention of infections caused by *Rhodococcus equi* in foals. *J. Vet. Intern. Med.* **25**, 1209–1220 (2011).

39. Giguère, S., Prescott, J. F. & Dowling, P. M. *Antimicrobial Therapy in Veterinary Medicine*. (Wiley Blackwell, 2013).

40. Erol, E., Shaffer, C. L. & Lubbers, B. V. Synergistic combinations of clarithromycin with doxycycline or minocycline reduce the emergence of antimicrobial resistance in *Rhodococcus equi*. *Equine Vet. J.* (2021) doi:10.1111/evj.13508.

41. Rutenberg, D., Venner, M. & Giguère, S. Efficacy of tulathromycin for the treatment of foals with mild to moderate bronchopneumonia. *J. Vet. Intern. Med.* **31**, 901–906 (2017).

42. Goebel, B., Freise, F. & Venner, M. Comparison of the efficacy of rifampin/azithromycin and rifampin/tulathromycin for the treatment of foals affected with pneumonia. *Equine Vet. Educ.* **34**, (2022).

43. Hildebrand, F., Venner, M. & Giguère, S. Efficacy of Gamithromycin for the Treatment of Foals with Mild to Moderate Bronchopneumonia. *J. Vet. Intern. Med.* **29**, 333–338 (2015).

44. Giguère, S. *et al.* Retrospective comparison of Azithromycin, Clarithromycin, and Erythromycin for the treatment of foals with *Rhodococcus equi* pneumonia. *J. Vet. Intern. Med.* **18**, 568 (2004).

45. Hillidge, C. J. Use of erythromycin-rifampin combination in treatment of *Rhodococcus equi* pneumonia. *Vet. Microbiol.* **14**, 337–342 (1987).

46. Prescott, J. F. & Nicholson, V. M. The effects of combinations of selected antibiotics on the growth of *Corynebacterium equi*. *J. Vet. Pharmacol. Ther.* **7**, 61–64 (1984).

47. Prescott, J. F. & Sweeney, C. R. Treatment of *Corynebacterium equi* pneumonia of foals: a review. *J. Am. Vet. Med. Assoc.* **187**, 725–728 (1985).

48. Peters, J. *et al.* Clarithromycin is absorbed by an intestinal uptake mechanism that is sensitive to major inhibition by Rifampicin: Results of a short-term drug interaction study in foals. *Drug Metab. Dispos.* **40**, 522–528 (2012).

49. Peters, J. *et al.* Oral absorption of Clarithromycin is nearly abolished by chronic comedication of Rifampicin in foals. *Drug Metab. Dispos.* **39**, 1643–1649 (2011).

50. Venner, M., Credner, N., Lämmer, M. & Giguère, S. Comparison of tulathromycin, azithromycin and azithromycin-rifampin for the treatment of mild pneumonia associated with *Rhodococcus equi*. *Vet. Rec.* **173**, 397–397 (2013).

51. Venner, M., Rödiger, A., Laemmer, M. & Giguère, S. Failure of antimicrobial therapy to accelerate spontaneous healing of subclinical pulmonary abscesses on a farm with endemic infections caused by *Rhodococcus equi*. *Vet. J.* **192**, 293–298 (2012).

52. Wetzig, M., Venner, M. & Giguère, S. Efficacy of the combination of doxycycline and azithromycin for the treatment of foals with mild to moderate bronchopneumonia. *Equine Vet. J.* **52**, 613–619 (2020).

53. Berghaus, L. J., Giguère, S. & Guldbech, K. Mutant prevention concentration and mutant selection window for 10 antimicrobial agents against *Rhodococcus equi*. *Vet. Microbiol.* **166**, 670–675 (2013).

54. Chait, R., Craney, A. & Kishony, R. Antibiotic interactions that select against resistance. *Nature* **446**, 668–671 (2007).

55. Michel, J.-B., Yeh, P. J., Chait, R., Moellering, R. C. & Kishony, R. Drug interactions modulate the potential for evolution of resistance. *Proc. Natl. Acad. Sci.* **105**, 14918–14923 (2008).

56. Kalinowski, M., Jarosz, Ł. & Grądzki, Z. Assessment of Antimicrobial Susceptibility of Virulent Strains of *Rhodococcus equi* Isolated From Foals and Soil of Horse Breeding Farms With and Without Endemic Infections. *J. Equine Vet. Sci.* **91**, 103114 (2020).

57. Li, J. *et al.* Antimicrobial Activity and Resistance: Influencing Factors. *Front. Pharmacol.* **8**, 364 (2017).

58. Berghaus, L. J. *et al.* Comparison of Etest, disk diffusion, and broth macrodilution for *in vitro* susceptibility testing of *Rhodococcus equi*. *J. Clin. Microbiol.* **53**, 314–318 (2015).

59. Carlson, K. *et al.* Antimicrobial activity of Tulathromycin and 14 other antimicrobials against virulent *Rhodococcus equi in vitro* . *Vet Ther* **11**, E1-9 (2010).

60. World Health Organization. WHO list of critically important antimicrobials for human medicine. (2018).

61. Kenney, D. G., Robbins, S. C., Prescott, J. F., Kaushik, A. & Baird, J. D. Development of reactive arthritis and resistance to erythromycin and rifampin in a foal during treatment for *Rhodococcus equi* pneumonia. *Equine Vet. J.* **26**, 246–248 (1994).

62. Takai, S. *et al.* Emergence of rifampin-resistant *Rhodococcus equi* in an infected foal. *J. Clin. Microbiol.* **35**, 1904–1908 (1997).

63. Huber, L. *et al.* Monitoring foals by thoracic ultrasonography, bacterial culture, and PCR: Diagnostic of *Rhodococcus equi* subclinical pneumonia in south of Brazil. *J. Equine Vet. Sci.* **60**, 104-108.e1 (2018).

64. Venner, M., Astheimer, K., Lämmer, M. & Giguère, S. Efficacy of mass antimicrobial treatment of foals with subclinical pulmonary abscesses associated with *Rhodococcus equi*. *J. Vet. Intern. Med.* **27**, 171–176 (2013).

65. Lord, J. *et al.* Antimicrobial resistance among *Streptococcus equi* subspecies *zooepidemicus* and *Rhodococcus equi* isolated from equine specimens submitted to a diagnostic laboratory in Kentucky, USA. *PeerJ* **10**, e13682 (2022).

66. Álvarez-Narváez, S. *et al.* Epidemiology and molecular basis of multidrug resistance in *Rhodococcus equi*. *Microbiol. Mol. Biol. Rev.* **85**, e00011-21, /mmbr/85/2/MMBR.00011-21.atom (2021).

67. Álvarez-Narváez, S., Giguère, S., Berghaus, L. J., Dailey, C. & Vázquez-Boland, J. A. Horizontal spread of *Rhodococcus equi* macrolide resistance plasmid pRErm46 across environmental *Actinobacteria*. *Appl. Environ. Microbiol.* **86**, e00108-20, /aem/86/9/AEM.00108-20.atom (2020).

68. Anastasi, E. *et al.* Novel transferable *erm* (46) determinant responsible for emerging macrolide resistance in *Rhodococcus equi*. *J. Antimicrob. Chemother.* dkv279 (2015) doi:10.1093/jac/dkv279.

69. Huber, L. *et al.* The novel and transferable *erm*(51) gene confers macrolides, lincosamides and streptogramins B resistance to clonal *Rhodococcus equi* in the environment. *Environ. Microbiol.* **22**, 2858–2869 (2020).

70. Erol, E., Scortti, M., Fortner, J., Patel, M. & Vázquez-Boland, J. A. Antimicrobial Resistance Spectrum Conferred by pRErm46 of Emerging Macrolide (Multidrug)-Resistant *Rhodococcus equi*. *J. Clin. Microbiol.* **59**, e01149-21 (2021).

71. Huber, L. *et al.* Identification of macrolide- and rifampicin-resistant *Rhodococcus equi* in environmental samples from equine breeding farms in central Kentucky during 2018. *Vet. Microbiol.* **232**, 74–78 (2019).

72. Levin, B. R., Perrot, V. & Walker, N. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* **154**, 985–997 (2000).

73. Álvarez-Narváez, S. *et al.* A common practice of widespread antimicrobial use in horse production promotes multi-drug resistance. *Sci. Rep.* **10**, 911 (2020).

74. Rivera-Velez, A., Huber, L., Sinha, S. & Cohen, N. D. Fitness cost conferred by the novel *erm*(51) and *rpoB* mutation on environmental multidrug resistant-*Rhodococcus equi*. *Vet. Microbiol.* 109531 (2022) doi:10.1016/j.vetmic.2022.109531.

75. Willingham-Lane, J. M., Berghaus, L. J., Berghaus, R. D., Hart, K. A. & Giguère, S. Effect of macrolide and rifampin resistance on the fitness of *Rhodococcus equi*. *Appl. Environ. Microbiol.* **85**, e02665-18, /aem/85/7/AEM.02665-18.atom (2019).

76. Willingham-Lane, J. M., Berghaus, L. J., Berghaus, R. D., Hart, K. A. & Giguère, S. Effect of macrolide and rifampin resistance on fitness of *Rhodococcus equi* during intramacrophage replication and *in vivo*. *Infect. Immun.* **87**, e00281-19, /iai/87/10/IAI.00281-19.atom (2019).

77. Cywes-Bentley, C. *et al.* Antibody to Poly-N-acetyl glucosamine provides protection against intracellular pathogens: Mechanism of action and validation in horse foals challenged with *Rhodococcus equi*. *PLOS Pathog.* **14**, e1007160 (2018).

78. Madigan, J. E., Hietala, S. & Muller, N. Protection against naturally acquired *Rhodococcus equi* pneumonia in foals by administration of hyperimmune plasma. *J. Reprod. Fertil. Suppl.* **44**, 571–578 (1991).

79. Martens, R. J., Martens, J. G., Fiske, R. A. & Hietala, S. K. *Rhodococcus equi* foal pneumonia: Protective effects of immune plasma in experimentally infected foals. *Equine Vet. J.* **21**, 249–255 (1989).

80. Giguère, S., Gaskin, J. M., Miller, C. & Bowman, J. L. Evaluation of a commercially available hyperimmune plasma product for prevention of naturally acquired pneumonia caused by *Rhodococcus equi* in foals. *J. Am. Vet. Med. Assoc.* **220**, 59–63 (2002).

81. Higuchi, T. *et al.* Effect of prophylactic administration of hyperimmune plasma to prevent *Rhodococcus equi* infection on foals from endemically affected farms. *J. Vet. Med. Ser. B* **46**, 641–648 (1999).

82. Sanz, M. G., Bradway, D. S., Horohov, D. W. & Baszler, T. V. *Rhodococcus equi* -specific hyperimmune plasma administration decreases faecal shedding of pathogenic *R. equi* in foals. *Vet. Rec.* **185**, 19–19 (2019).

83. Summer, E. J. *et al.* Genomic and functional analyses of *Rhodococcus equi* phages ReqiPepy6, ReqiPoco6, ReqiPine5, and ReqiDocB7von ba. *Appl. Environ. Microbiol.* **77**, 669–683 (2011).

84. Cohen, N. D. *et al.* Gallium Maltolate as an Alternative to Macrolides for Treatment of Presumed *Rhodococcus equi* Pneumonia in Foals. *J. Vet. Intern. Med.* **29**, 932–939 (2015).

85. Coleman, M. *et al. In vitro* antimicrobial activity of gallium maltolate against virulent *Rhodococcus equi*. *Vet. Microbiol.* **146**, 175–178 (2010).

86. Harrington, J. R., Martens, R. J., Cohen, N. D. & Bernstein, L. R. Antimicrobial activity of gallium against virulent *Rhodococcus equi in vitro* and *in vivo*. *J. Vet. Pharmacol. Ther.* **29**, 121–127 (2006).

87. Chaffin, M. K., Cohen, N. D., Martens, R. J., O'Conor, M. & Bernstein, L. R. Evaluation of the efficacy of gallium maltolate for chemoprophylaxis against pneumonia caused by *Rhodococcus equi* infection in foals. *Am. J. Vet. Res.* **72**, 945–957 (2011).

Chapter 3

Antimicrobial Residue Accumulation Contributes to Higher Levels of *Rhodococcus equi* Carrying Resistance Genes in the Environment of Horse-Breeding Farms. *

---

*Courtney Higgins, Noah D. Cohen, Nathan Slovis, Melissa Boersma, Pankaj P. Gaonkar, Daniel R. Golden, Laura Huber.
Manuscript in preparation.

Abstract

Recent studies have focused on understanding the role of the environment on the selection and maintenance of antimicrobial resistance (AMR). Antimicrobial residues excreted following antimicrobial treatment enhance resistant microbial communities in the environment and have long-term effects on the selection and maintenance of antimicrobial resistance genes (AMRGs). The environment is the main source of *Rhodococcus equi* infections of foals. Consequently, an increase in *R. equi* carrying transferable AMRGs in the environment is potentially a major contributor to the increase in infections with multidrug resistant (MDR)-*R. equi* in foals. In this study, we found that the presence and concentration of macrolide residues in the environment at horse-breeding farms and the use of thoracic ultrasonographic screening (TUS) for early detection of subclinically affected foals with *R. equi* infections were strongly associated with the presence of *R. equi* carrying AMRGs in the environment of horse-breeding farms. Our findings indicate that the use of TUS contributed to historically higher antimicrobial use in foals, leading to the accumulation of antimicrobial residues in the environment, and to enhancing *R. equi* populations carrying AMRGs. Because of the potentially long-term effects of antimicrobial residue contamination in the environment, it is likely that the persistence of MDR-*R. equi* infections in foals will continue.

3.1. Introduction

Rhodococcus equi is a soil saprophyte commonly found in the environment of horse-breeding farms. This facultative intracellular pathogen is known to cause severe pneumonia in foals.[1,2] Foals contract *R. equi* after inhalation of aerosolized virulent bacteria which then invade and replicate within the alveolar macrophages ultimately causing pneumonia.[3] *R. equi* infections are endemic at many horse-breeding farms. Consequently, many farms choose to use thoracic ultrasonographic screening (TUS) to identify foals with subclinical pneumonia to prevent severe disease and death from this insidious disease. [2,4–6] Detection of subclinical *R. equi* pneumonia using TUS has become a routine procedure at many horse-breeding farms in central Kentucky, USA. Foals that are found to have subclinical lesions in the lungs are treated with antimicrobials, a practice known as the "screen-and-treat" method. The combination of rifampicin with a macrolide such as azithromycin, clarithromycin, or erythromycin is the most common treatment for subclinical (and clinical) pneumonia in foals.[7–9] While many foals with subclinical pneumonia are treated with antimicrobials prophylactically, most foals with subclinical rhodococcal pneumonia recover spontaneously. [4,10–13,37,39]

In the past decade, emergence of multidrug resistant (MDR)-*R. equi* isolated both from foals[14] and their environment[15] at horse-breeding farms has been reported, likely due to the increase in antimicrobial use to prevent *R. equi* infections. Since then, investigators have shown that treating subclinically affected foals might not be necessary to prevent severe *R. equi* disease in an effort to curb the "screen-and-treat" practice at horse-breeding farms. It is believed that efforts to reduce antimicrobial use at horse-breeding farms may result in decreased prevalence of MDR-*R. equi*, reducing the risk of MDR-infections in foals.[12-13,39-40] However, MDR-*R. equi* might persist in the environment by compensatory mutations [16–19] or by constant pressure due to the accumulation of

antimicrobial residues[20–22] in the environment following historical overuse of antimicrobials; even small concentrations of antimicrobials in the soil can drive the spread antimicrobial resistance genes (AMRGs) between bacteria.[23] If this persistence of *R. equi* carrying AMRGs and antimicrobials in the environment occurs at endemic farms, reducing antimicrobial use at farms might not be sufficient to prevent MDR-*R. equi* infections in foals. The purposes of this study are to compare rates of antimicrobial use, prevalence of *R. equi* carrying AMRGs, and the concentration of antimicrobial residues at horse-breeding farms in Kentucky over time and to determine the effect of antimicrobial use and antimicrobial residue accumulation on the prevalence of *R. equi* carrying AMRGs found in the environment at horse-breeding farms.

3.2. Materials and Methods

3.2.1. Farm Selection

The horse-breeding farms located in Kentucky that previously participated in an epidemiological study conducted by our group[15] were re-enrolled for this study. From the original 100 farms enrolled in 2017, 83 agreed to participate in this study. Client consent for participation was obtained from each farm.

3.2.2. Questionnaire Collection

A survey was administered to each farm gathering information about overall farm demographics, such as location, number of foals born, acreage, number of years raising horses; and on overall farm history pertinent to *R. equi* infections, such as antimicrobial treatment rates, incidence of pneumonia in foals, mortality, and management practices to prevent the disease. Information was collected for each year during the period from 2018 to 2021.

3.2.3. Sample Collection

Sample collection took place between June and July of 2017[15] and 2021. A total of 9 surface soil samples from 3 paddocks commonly used to house mares and foals in each farm were collected with a hand shovel that was cleaned and disinfected with alcohol wipes between farms. The soil samples from each location of the individual farms were evenly combined in a sterile collection bag for each respective farm totaling a composite sample of approximately 140 grams per farm. The collection bag was then sealed in a larger plastic bag to avoid cross-contamination between farms. The soil samples were stored at -80ºC before transportation to Auburn University and sample processing.

3.2.4. Sample Processing

3.2.4.1. *R. equi* Quantification

For each individual soil sample from 2017 and 2021 of each farm, the soil samples were thawed and 1 gram was measured in preparation for 10-fold serial dilutions using a 10-mM solution of phosphate buffered saline (Ultra-Pure PBS-Tween® pH 7.5, VWR Life Science). The serial dilutions were then plated on a series of selective media for *R. equi* quantification. The series of agar plates consisted of NANAT agar, NANAT agar with erythromycin (8 μg/mL), and NANAT agar with erythromycin (8 μg/mL) and rifampicin (50 μg/mL).[15] *R. equi* colonies from each plate were identified with characteristic colony morphology[24] and the colony forming units (CFU) were recorded. The colonies recorded from the NANAT agar plates represented the CFU of the total population of *R. equi* in 1 gram of soil. The colonies selected in the NANAT agar plates with added erythromycin and/or rifampicin represented the *R. equi* potentially carrying AMRGs in 1 gram of soil. The *R. equi* colonies from the NANAT agar plates containing erythromycin

and/or rifampicin were tested for the detection the species-specific gene, *choE,*[24] and the possible presence of the virulence gene, *vapA*[3] using conventional PCR. The presence of macrolide AMRGs was determined by PCR-amplification of *erm*(46)[25] and *erm*(51).[26] ETEST® strips were used to measure minimum inhibitory concentration (MIC) for the following antimicrobials: azithromycin, clarithromycin, clindamycin, doxycycline, erythromycin, quinupristin-dalfopristin, rifampicin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin.

3.2.4.2. Antimicrobial Residue Quantification

The presence of antimicrobial residue in the soil samples for each farm and year was evaluated through antimicrobial extraction and mass spectrometry. Antimicrobial extraction was modified from a previously described protocol.[27] Briefly, 1 gram of farm soil was combined with 3 milliliters of methanol in a 15 milliliters conical tube and mixed well for 10 minutes. The sample was then wrapped in foil to avoid light degradation and placed in a shaking incubator overnight at room temperature. After incubation, the sample was centrifuged at 10,000 G for 30 minutes. The supernatant was placed into a glass vial and dried down with nitrogen gas dispensed from an evaporator (Reacti-Vap®, Thermo Fisher Scientific, USA) in a dark room. The dried sample was reconstituted with 150 microliters of a 50% methanol solution and vortexed for 5 minutes. Once reconstituted, the sample was transferred to a 2-milliliters microcentrifuge tube and centrifuged at 10,000 G for 30 minutes. The supernatant was then transferred to a new 2-milliliters microcentrifuge tube. The reconstituted samples were then submitted for mass spectrometry. Standard curves were constructed for each antimicrobial of interest by spiking a known gradient concentration into sterile soil. Standards were purchased commercially, and specifications for each antimicrobial are included in Supplementary Table 3.1.

Analysis was performed on a Vanquish UHPLC system (Thermo Fisher Scientific, USA) coupled with a quadrupole orbitrap mass spectrometer (Orbitrap Exploris 120, Thermo Fisher Scientific, USA) with electrospray ionization (H-ESI using Xcalibur software (V4.4.16.14)). Injection of 10 microliters of the sample or standard was made on a C18 column (SymmetryShield RP18, 3.5 µm, 4.6x150mm, Waters) held at room temperature with a 1 mL/min flow rate of mobile phase solution A (99.9% water with 0.1% formic acid) and solution B (99.9% methanol with 0.1% formic acid). The gradient began at 25% B, held for 2 minutes followed by a linear ramp to 65% B at 7 minutes, 95% B at 8 minutes and held for 1 minute, then back to 25% B for a total analysis time of 14 minutes. The flow was diverted to waste for the 1.8 minutes of analysis and at 9 minutes. The mass spectrometer utilized positive mode for the entire analysis time and negative mode was used from 8.3 to 9.3 minutes. The positive mode scan range was 100-1000 m/z with resolution of 120,000, standard AGC target, 70% RF lens, maximum injection time 100 ms, mild trapping enabled, with EASY-IC on at the run start. The negative mode was used for analysis of 2,5-dichlorothiophene-3-sulfonamide and resolution was set to 60,000 and the maximum injection time was set to auto, all other parameters were the same as the positive mode settings. The spray voltage was 3400 V in positive mode and 2800 V in negative mode, the ion transfer tube temperature was 350°C, and the vaporizer temperature was 320°C. The processing method required a mass tolerance of less than 5.1 ppm.

3.2.5. Data Analysis

Data were analyzed using descriptive and inferential methods. For descriptive purposes, categorical data were presented as fractions and proportions, and non-normally distributed data were presented as median and interquartile ranges (IQR). For inferential purposes, paired comparisons of non-parametric data were performed using Wilcoxon signed-rank tests;

generalized mixed-effects models were used to evaluate the effect of macrolide residues and year on *R. equi* carrying AMRGs in soil samples with farm identification number as the random factor to account for repeated measurements (2017 and 2021). In the first model, in which the log-transformed *R. equi* carrying AMRGs was the outcome variable, a negative binomial mixed-effects model was used; and in the second model, in which the categorical presence or absence of *R. equi* carrying AMRGs was the outcome variable, a logistic mixed-effects model was used. All variables were transformed to categorical: antimicrobial residue was transformed to the binary presence or absence of residue, and the outcome was transformed to binary presence or absence of *R. equi* carrying AMRGs. No interaction terms were included in the best fit model. Odds ratios (OR) were calculated by exponentiating model coefficients and standard errors (SE). For all analysis, statistical significance was set to p-value < 0.05.

3.3. Results

Of the 83 farms included in the study, 86% (71/83) completed a questionnaire in 2017, and 29 out of these 71 farms (41%) completed a questionnaire in 2021. Loss-of-follow-up was dependent on the outcome of antimicrobial use in this study, therefore, questionnaire data are only represented descriptively. Because TUS is a variable of high interest based on previous epidemiological studies,[66] we contacted farms that did not originally respond to a questionnaire in 2021 and were able to collect data about TUS practices for further inferential analyses. This second follow-up effort yielded complete paired data for TUS from 56 farms.

When including only farms that fully responded to questionnaires in both 2017 and 2021 (n = 29 farms), the proportion of foals treated with antimicrobials and the number of farms treating foals with antimicrobials increased from 10 to 12.4% and from 66 to 79%, respectively (**Table 3.1**). Density of horses and foals per acre decreased slightly and mortality of foals from any cause

remained virtually inexistent (**Table 3.1**). Considering the 56 farms that provided data regarding TUS from both years, the number of farms using TUS decreased from 77% (43/56) in 2017 to 66% (37/56) in 2021. The majority of farms (57%; 32/56) reported using TUS during the preceding decade, 14% (8/56) reported not using TUS in the preceding decade, 27% (15/56) reported using TUS in the period between 2014 and 2017 but not since 2017, and 2% (1/56) indicated they started using TUS after 2017 (**Table 3.1**).

Of the 83 farms for which soil samples were obtained and processed, no differences in the overall percentage of *R. equi* carrying AMRGs were found between 2017 (median, 0.05%; IQR, 0-0.3%) and 2021 (median, 0.03%; IQR 0-0.3%; p = 0.76; **Table 3.2**). The number of farms with *R. equi* carrying AMRGs identified in soil samples was 63% (52/83) in 2017 and 55% (46/83) in 2021, and this difference was not significant (p = 0.43; **Table 3.2, Figure 3.1**). Regarding change in proportion of *R. equi* carrying AMRGs in the soil from 2017 to 2021, 39% of the farms (32/83) have an increased *R. equi* carrying AMRGs proportion, 36% (30/83) have decreased and 25% (21/83) have remained free of *R. equi* carrying AMRGs ; 11% (9/83) of the *R. equi* carrying AMRGs proportion increased from zero in 2017, and 18% (15/83) of the *R. equi* carrying AMRGs proportion decreased to zero in 2021 (**Table 3.2, Figure 3.1**).

Mass spectrometry was used to measure the concentration of ampicillin, azithromycin, clarithromycin, cycloheximide, doxycycline, erythromycin, sulfonamide, tetracycline, and tylosin in soil samples from 2017 and 2021 from 83 farms. Antimicrobial traces were found for azithromycin, clarithromycin, and erythromycin only. A combined sum of the macrolide residue amount found for each farm and each time-period was used as the primary outcome variable representing antimicrobial residue contamination in the environment. The combined amount of macrolide residue was not statistically significantly different between 2017 (median, 1.6×10^{-5}

μg/μL; IQR $2.0 \times 10^{-6} - 2.9 \times 10^{-4}$ μg/μL) and 2021 (median, 1.3×10^{-5} μg/μL; IQR $0 - 9.5 \times 10^{-5}$ μg/μL; p = 0.08) (**Table 3.3, Figure 3.2)**.

The odds of finding *R. equi* carrying AMRGs at farms increased significantly by 3.6-fold (95% CI, 1.2-10.5, p = 0.03) when macrolide residue is present in the environment versus absent. The odds of finding *R. equi* carrying AMRGs in 2021 compared with 2017 was 0.75 (95% CI, 0.3-1.7, p = 0.45) and not statistically significant (**Table 3.4**). Macrolide residue was positively and statistically significantly (estimated OR, 1.1, SE, 0.5, p = 0.02) associated with log-transformed percentage of *R. equi* carrying AMRGs, and year was not statistically significantly associated with log-transformed percentage of *R. equi* carrying AMRGs (estimate, 0.2, SE, 0.3, p = 0.48; **Table 3.4, Figure 3.3**).

The odds of finding *R. equi* carrying AMRGs at a farm increased significantly by 5.4-fold (95% CI, 1.2- to 23.9-fold, p = 0.03) at farms that used TUS for at least 4 years versus farms that reported not using TUS during the preceding 4 years. The odds of having AMRGs did not change significantly in 2021 compared with 2017 (OR, 0.8; 95% CI, 0.3-2.1; p = 0.63; **Table 3.5**). The use of TUS and year was not statistically significantly associated with log-transformed percentage of *R. equi* carrying AMRGs (estimate, 0.7; SE, 0.4, p = 0.08; and estimate, 0.03; SE, 0.3, p = 0.99, respectively; **Table 3.5, Figure 3.4**).

Moreover, the odds of finding antimicrobial residues in a farm increased 6.4-fold (95% CI, 0.8-52.5, p = 0.09) at farms that used TUS for at least 4 years versus farms that reported never using TUS or at least in the last 4 years. The odds of having of antimicrobial residues did not change significantly in 2021 compared with 2017 (OR, 0.4, 95% CI, 0.1-1.5, p = 0.18; **Table 3.6**).

3.4. Discussion

In this study, the presence of macrolide residues was positively associated with the environmental presence of *R. equi* carrying AMRGs at horse-breeding farms. These AMRGs are known to cause resistance to first-line antimicrobials used for the treatment of rhodococcosis in foals. Interest has been growing for investigating the environment as a potential source of selection for AMR and as an important contributor to the global AMR crisis. Recent reports show that the contamination of the environment with antimicrobial residues is an important driver of AMR[28–30] and the role of the environment as a reservoir for AMR is generally underrepresented in action plans to mitigate AMR.[31] The development of AMR in microbials is considered to be a natural evolutionary process, but the presence of antimicrobials stimulates the AMR mechanisms of pathogens.[32] Antimicrobials, including macrolides, can persist in the environment for long periods of time, establishing an antimicrobial pressure sufficient in maintaining the selection of MDR-organisms.[20–22] Studies have shown that animals treated with antimicrobials can shed antimicrobial metabolites for weeks after discontinuing therapy, and the antimicrobial residues in soil can drive the selection of resistant organisms.[33] Therefore, mitigating AMR without understanding the role of environmental contamination with antimicrobial residues might not be possible. The environment is the main source of infection for foals with *R. equi*.[41-43] Therefore, the selection of MDR-*R. equi* in the environment of horse-breeding farms is likely a strong contributor to the increase of MDR-*R. equi* infections seen in foals in recent decades.[14] Because of the potential long-term effect of antimicrobial residues in the environments of microbial communities reported in this study, infections from MDR-*R. equi* will likely continue to increase. Apart for the potential long-term selection of MDR-*R. equi* and its detrimental effect on equine health, the accumulation of antimicrobial residues establishes constant antimicrobial pressure for the enhancement and

spread of MDR-pathogens with the potential to threaten humans and other animals through contact or consumption of contaminated food and water.[34] Moreover, the accumulation of residues can have ecotoxicological effects, interfering with the normal microbiome of soils and plants and affecting agricultural yield.[35,36] Therefore, the existence, persistence, and impact of antimicrobial residues in diverse systems and its potential to cause adverse health effects is an emerging global crisis, one worthy of collaborative, interdisciplinary mitigation efforts.

Studying the association between antimicrobial use and resistance is challenging due to lack of data on antimicrobial use and potential response bias to questionnaires. Therefore, independent methods to assess the antimicrobial pressure in certain settings are needed. In this study, the recovery of data about antimicrobial use in foals from enrolled farms through the collection of questionnaires was limited, but antimicrobial residue measurements in the soil served as an unbiased source of information on existing antimicrobial pressure in the environment of farms. Antimicrobial residue was a strong predictor of AMR in the environment at horse-breeding farms in this study and our results are consistent with previously reported effects of antimicrobial residues on the amplification of antimicrobial resistant communities in the environment.[28] Several previous studies have shown irrefutable evidence of the increase in resistant *R. equi* likely driven by the overuse of antimicrobials in foals.[14,15,37-38] Thus, prudent antimicrobial use at horse-breeding farms has been recommended to mitigate AMR and protect foal health. However, reducing antimicrobial use alone might not be sufficient to fight the spread of MDR-*R. equi* at horse-breeding farms. Because our data on antimicrobial use in this study were limited, it was not possible to establish if antimicrobial residues persisted over time without antimicrobial use in animals, or if the presence of antimicrobials in the environment was due to constant antimicrobial use. However, the snapshot of this strong association between antimicrobial residues and AMR

indicates that management of environments and herds at horse-breeding farms can help control and limit the spread of MDR-*R. equi*. Practices, such as delimiting antimicrobial contaminated areas and avoiding contact of horses, establishing a washout time after antimicrobial therapy is discontinued before animals are reintroduced to the herd, and choosing effective antimicrobials that have a shorter half-life in the environment might contribute to AMR mitigation at horse-breeding farms. However, information on the shedding rate and persistence of the various antimicrobial residues in the environment after therapy in foals is unknown. It is also important to note that previous studies have reported environmental factors such as soil texture and mineral components can affect the recovery rate and persistence of antimicrobial residues and AMRGs in the environment.[44-46] Therefore, enforcing such practices is not yet indicated because more studies investigating the rate of residue shedding, its persistence, its potential to establish antimicrobial pressure and drive AMR, and if the contact with MDR-*R. equi* in the soil is a risk factor for its acquisition by foals are needed.

In this study, we found that farms continue to use TUS as a screening method for *R. equi* infections. TUS was previously identified as a risk factor for the presence of MDR-*R. equi* in the environment at horse-breeding farms[37] and in this study, TUS continues to be an important risk factor for AMR at horse-breeding farms. Due to the limited access to data about antimicrobial use at participating farms, it was not possible to establish whether TUS was associated with increased rate of antimicrobial treatment at farms. However, the use of TUS at farms has been associated with overuse of antimicrobials in foals in past studies.[37] In this study, we found that the use of TUS during the preceding 4-year period may have contributed to the presence of antimicrobial residues in the soil of farms, albeit not significantly ($p = 0.09$). Considering the long half-life of macrolides in the environment, it is possible that the use of TUS and consequently the historic use of

antimicrobials is sufficient to maintain antimicrobial pressure over the subsequent years, increasing AMR rates in farms that have used TUS compared to farms that have never used TUS in the past. Recent studies have shown that the use of TUS is associated with higher rates of antimicrobial use at farms[37] and does not contribute to better survival and recovery of foals subclinically affected with *R. equi* pneumonia.[13] Therefore, the evidence demonstrates that TUS not only has no proven effect of preventing severe *R. equi* disease in endemic horse-breeding farms, but also may contribute to the long-term accumulation of antimicrobial residues and selection of MDR-*R. equi* in the environment, increasing the risk of infections with MDR-*R. equi*.

3.5. Conclusion

We report that antimicrobial residues in the soil are an unbiased measurement of antimicrobial pressure and are strongly associated with AMR of *R. equi* at horse-breeding farms. Antimicrobial residues might persist in the environment establishing continuous selection of MDR-*R. equi* in the soil of farms. Farms have continued to rely on TUS, a practice known to increase antimicrobial use in foals at farms where *R. equi* infections of foals are endemic. The historic use of antimicrobials at these farms may have set an existing antimicrobial pressure that has persisted over time, contributing to the selection of *R. equi* carrying AMRGs. Given the current prevalence of MDR-*R. equi* and antimicrobial residues in the environment of farms, the prevalence of MDR-*R. equi* infections in foals will likely continue to increase due to the constant exposure to foals. However, more studies are needed to better predict the effect of historic use of antimicrobials on selection of MDR-*R. equi* and if the exposure of foals to MDR-*R. equi* in the soil is a risk factor for infections with these resistant pathogens.

Tables

Data from Farms with Questionnaire (29 farms)	**2017**	**2021**
Proportion of foals treated with any antimicrobial (median, IQR)	10.00 (0-20.00%)	12.38 (3.50-27.21%)
Proportion of foals treated macrolides (median, IQR)	0.00 (0-10%)	1.66 (0-6.95%)
Proportion of foals treated rifampicin (median, IQR)	7.14 (0-19.59%)	6.64 (0.83-18.09%)
Number of farms treating foals with any antimicrobials (%)	19/29 (65.5%)	23/29 (79.3%)
Number of farms treating foals with macrolides (%)	11/29 (37.9%)	20/29 (68.9%)
Number of farms treating foals with rifampicin (%)	18/29 (62.1%)	22/29 (75.86%)
Number of farms using TUS (%)	21/29 (72%)	24/29 (83%)
Density of animals/acre (median, IQR)	0.85 (0.61-1.15)	0.62 (0.44-1.06)
Foal mortality (median, IQR)	0.00 (0-0.00)	0.00 (0-0.002)
TUS in Farms Over Time (56 farms)		**N (%)**
Farms that use TUS in 2017		43/56 (77%)
Farms that use TUS in 2021		37/56 (66%)
Farms that have used TUS in the last decade		32/56 (57%)
Farms that have not used TUS in the last decade		8/56 (14%)
Farms that **did use** TUS from 2014-2017 but discontinued use of TUS after 2017		15/56 (27%)
Farms that **did not use** TUS from 2014-2017 but began use of TUS after 2017		1/56 (2%)

Table 3.1. Overall antimicrobial treatment, farm density, mortality, and use of TUS over time in 29 farms from which questionnaire was available for both years (2017 and 2021). TUS over time from recovered questionnaire data in 56 farms.

MDR-*R. equi* in Farms	**N (%)**	**Proportion MDR-*R. equi* (median, IQR)**
Farms with MDR-*R. equi* in 2017	52/83 (63%)	0.05 (0.00 – 0.30)
Farms with MDR-*R. equi* in 2021	46/83 (55%)	0.03 (0.00 – 0.33)
Farms with increased MDR-*R. equi*	32/83 (39%)	0.30 (0.10 – 1.11)
Farms with decreased MDR-*R. equi*	30/83 (36%)	0.00 (0.00 – 0.30)
Farms remained MDR-*R. equi* free	21/83 (25%)	0.00 (0.00 – 0.00)
Farms that increased **from zero** MDR-*R. equi*	9/83 (11%)	0.20 (0.07 – 0.90)
Farms that decreased **to zero** MDR-*R. equi*	15/83 (18%)	0.20 (0.05 – 0.30)

Table 3.2. Descriptive data of the percentage of farms where MDR-*R. equi* was found, decreased or increased over time. For each farms categorization, the median and interquartile range (IQR) proportion MDR-*R. equi* is shown.

Antimicrobial Residue in Farms	**N (%)**	**Macrolide Residue (median, IQR; μg/μL)**
Farms with macrolide residue in 2017	64/83 (77%)	1.6×10^{-5} (2.0×10^{-6} – 2.9×10^{-4})
Farms with macrolide residue in 2021	53/83 (64%)	1.3×10^{-5} (0.00 – 9.5×10^{-5})
Farms with increased macrolide residue	26/83 (31%)	7.0×10^{-5} (2.0×10^{-5} – 2.6×10^{-4})
Farms with decreased macrolide residue	43/83 (52%)	3.0×10^{-5} (1.0×10^{-5} – 9.0×10^{-5})
Farms remained macrolide residue free	14/83 (17%)	0.00 (0.00 – 0.00)
Farms that increased from zero macrolide residue	5/83 (6%)	1.0×10^{-5} (1.0×10^{-5} – 2.0×10^{-5})
Farms that decreased to zero macrolide residue	16/83 (19%)	3.0×10^{-5} (0.00 – 4.0×10^{-5})

Table 3.3. Descriptive data of the percentage of farms where macrolide residue was found, decreased or increased over time. For each farms categorization, the median and interquartile range (IQR) concentration of macrolide residue is shown.

Negative Binomial Model	Estimate	SE	P-value
Year 2021 (reference: 2017)	0.22	0.30	0.48
Presence of residue (reference: absence)	1.12	0.51	0.02*
Logistic Model	**OR**	**95% CI**	**P-value**
Year 2021 (reference: 2017)	0.75	0.33-1.65	0.45
Presence of residue (reference: absence)	3.55	1.19-10.52	0.03*

Table 3.4. Estimates, standard errors (SE), and p-values generated from negative binomial mixed-effects model with log proportion MDR-*R. equi* as outcome and farm identification as random variable. Odds ratios (OR) and 95% confidence intervals (CI) generated from logistic mixed-effects model with presence or absence of MDR-*R. equi* as outcome and farm identification as random variable. * represents p-value < 0.05.

Negative Binomial Model	Estimate	SE	P-value
Year 2021 (reference: 2017)	0.003	0.34	0.99
Have used TUS at least in the last 4 years (reference: never used TUS or stopped this practice for at least 4 years)	0.73	0.43	0.08
Logistic Model	**OR**	**95% CI**	**P-value**
Year 2021 (reference: 2017)	0.79	0.31-2.05	0.63
Have used TUS at least in the last 4 years (reference: never used TUS or stopped this practice for at least 4 years)	5.43	1.24-23.89	0.03*

Table 3.5. Estimates, standard errors (SE), and p-values generated from negative binomial mixed-effects model with log proportion MDR-*R. equi* as outcome and farm identification as random variable. Dataset contains 56 farms that have provided information on TUS use for both periods (2014-2017 and 2018-2021). Odds ratios (OR) and 95% confidence intervals (CI) generated from

logistic mixed-effects model with presence or absence of MDR-*R. equi* as outcome and farm identification as random variable. * represents p-value < 0.05.

Logistic Model	OR	95% CI	P-value
Year 2021 (reference: 2017)	0.44	0.14-1.46	0.18
Have used TUS at least in the last 4 years (reference: never used TUS or stopped this practice for at least 4 years)	6.36	0.76-52.52	0.09

Table 3.6. Odds ratios (OR) and 95% confidence intervals (CI) generated from logistic mixed-effects model with presence or absence of macrolide residues as outcome and farm identification as random variable. Dataset contains 56 farms that have provided information on TUS use for both periods (2014-2017 and 2018-2021). * represents p-value < 0.05.

Antimicrobial Standard	CAS-No.	Brand
2,5-Dichlorothiophene-3-sulfonamide	53595-68-9	Aldrich Chemistry
Ampicillin	69-53-4	Sigma-Aldrich
Azithromycin	117772-70-0	European Pharmacopeia
Clarithromycin	81103-11-9	European Pharmacopeia
Cycloheximide	66-81-9	VWR Life Science
Doxycycline hyclate	24390-14-5	U. S. Pharmacopeia
Erythromycin	114-07-8	U. S. Pharmacopeia
Tetracycline hydrochloride	64-75-5	Sigma-Aldrich
Tylosin	1401-69-0	Sigma-Aldrich

Supplementary Table 3.1. List of antimicrobial standards used to create standard curves for mass spectrometry analysis. The antimicrobial concentrations used for the construction of standard curves were adjusted to the following gradient: 5 μg/mL, 1 μg/mL, 0.5 μg/mL, 0.1 μg/mL, 0.05 μg/mL, 0.01 μg/mL.

Figures

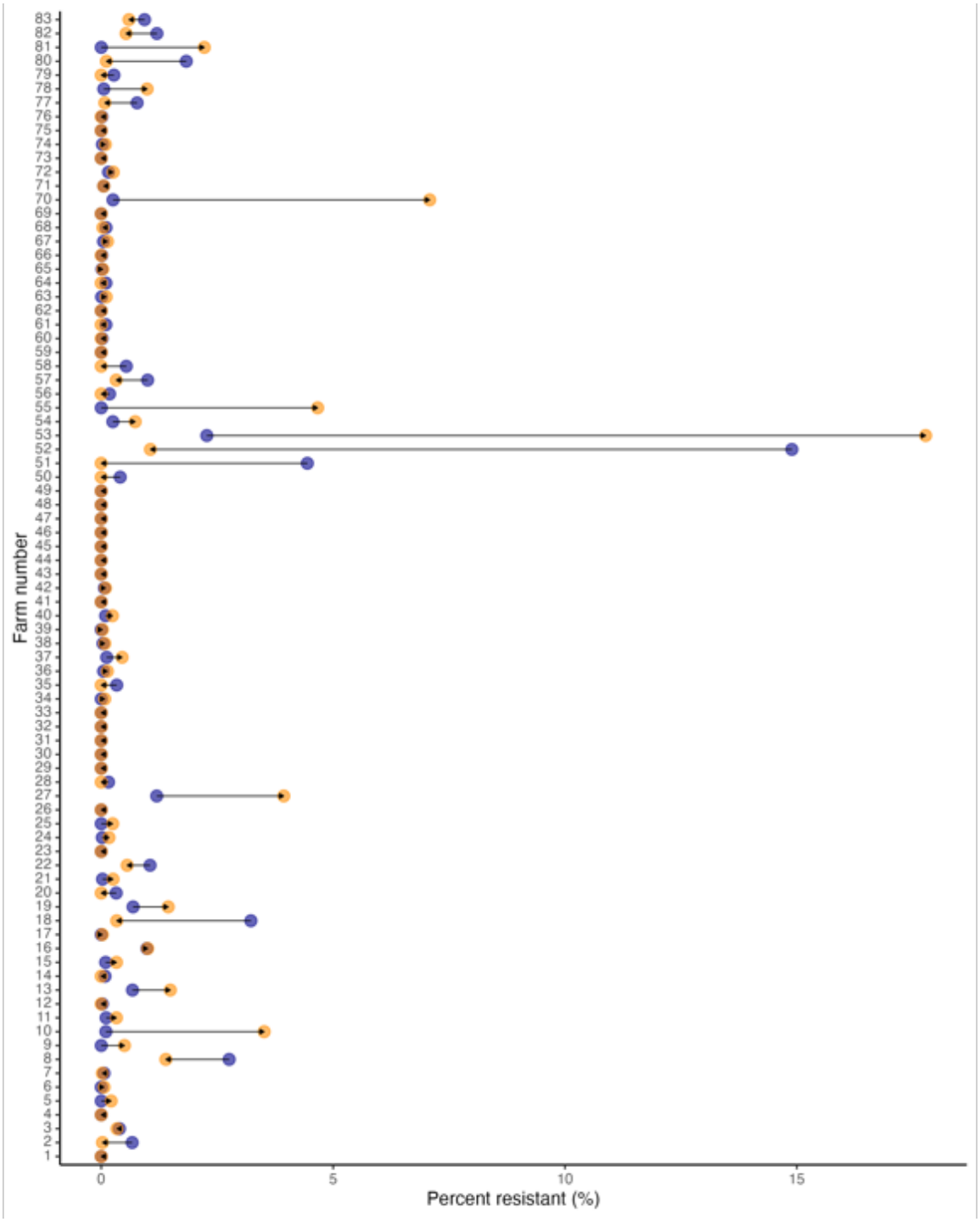

Figure 3.1. Dumbbell plots with the change in proportion of resistant MDR-*R. equi* in each farm between 2017 (orange) and 2021 (blue).

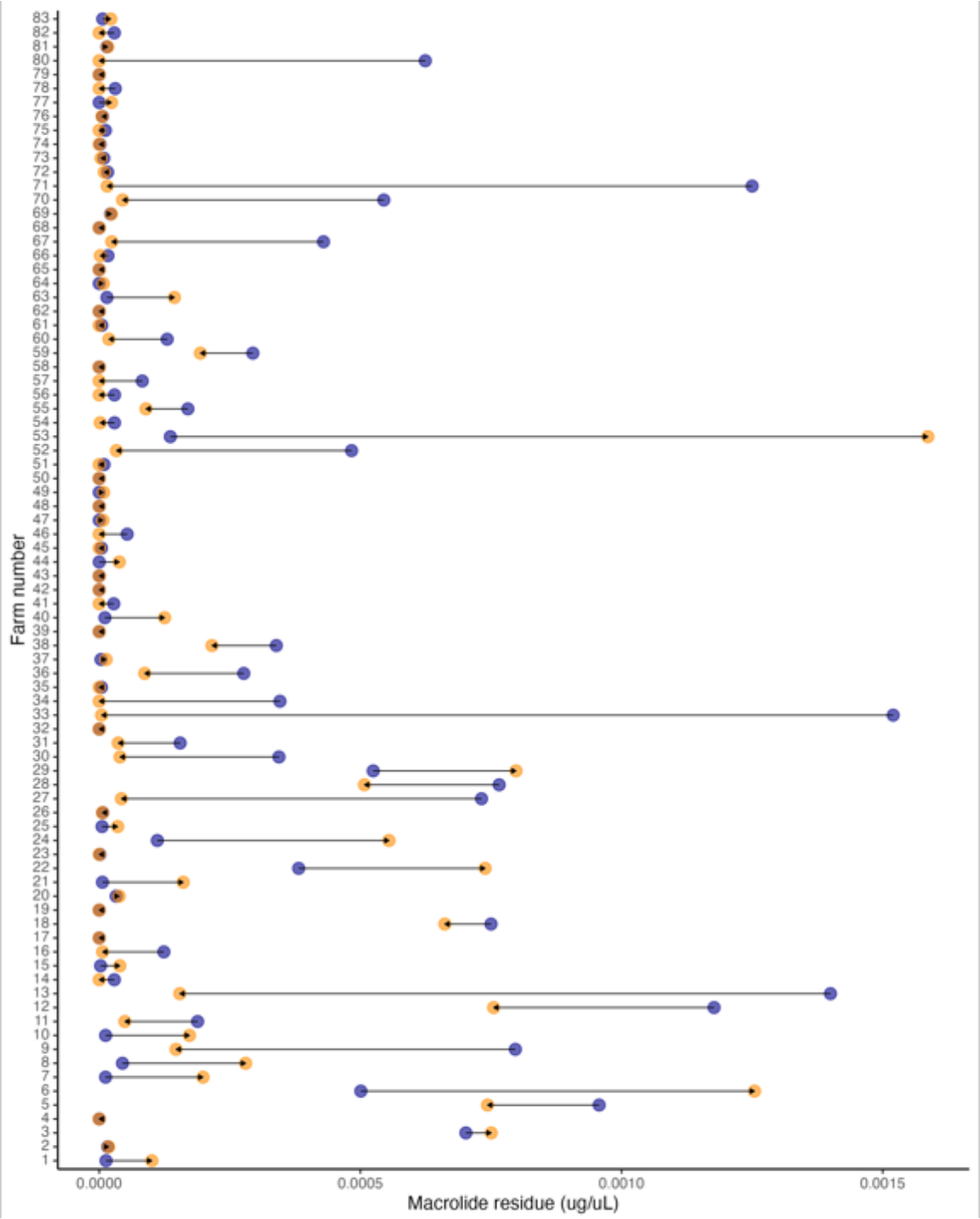

Figure 3.2. Dumbbell plots with the change in concentration of macrolide residue in the soil from each farm between 2017 (orange) and 2021 (blue).

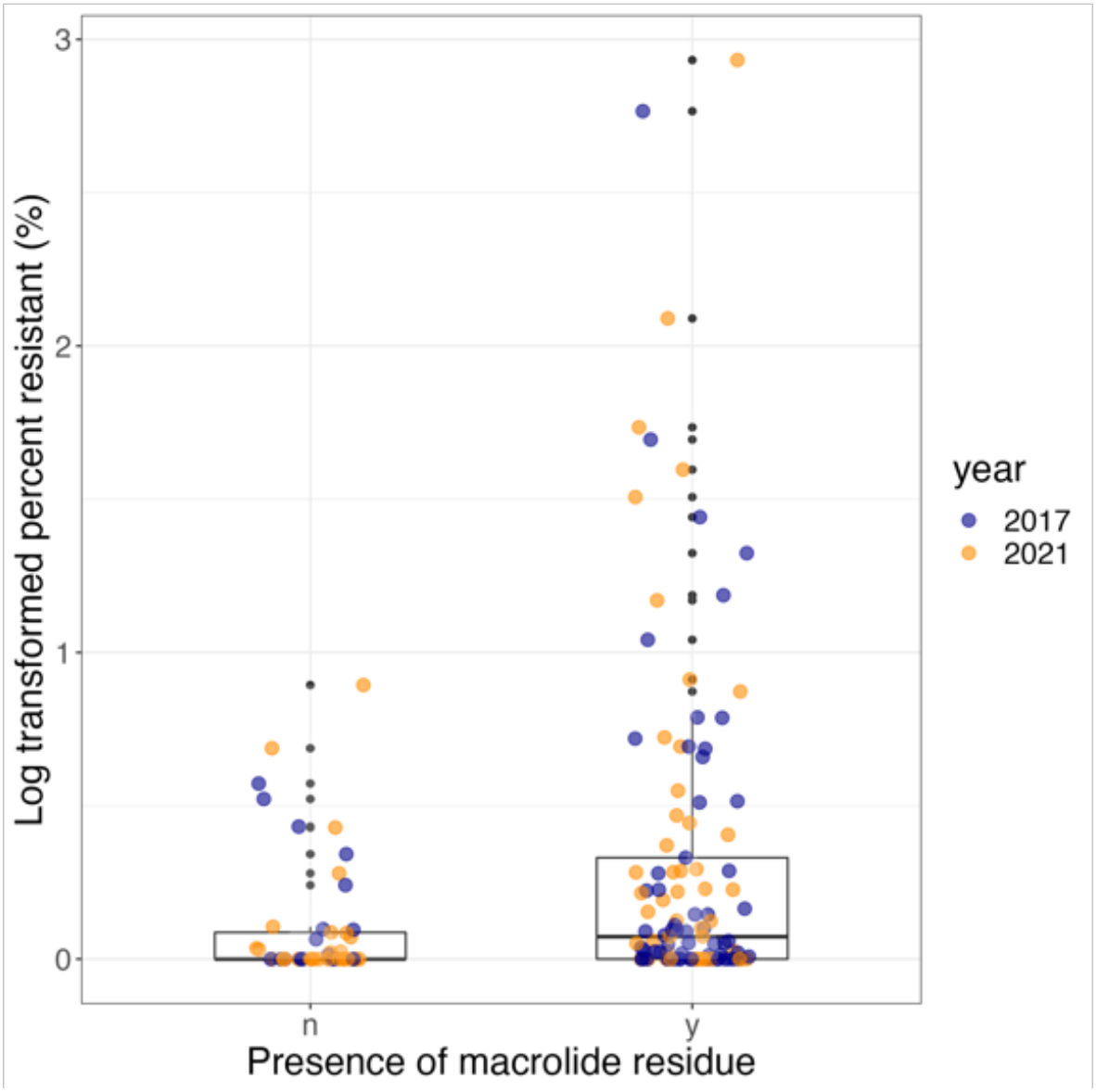

Figure 3.3. Boxplot showing the proportion of MDR-*R. equi* in farms where macrolides were present or absent color-coded by year, 2017 (blue) and 2021 (orange).

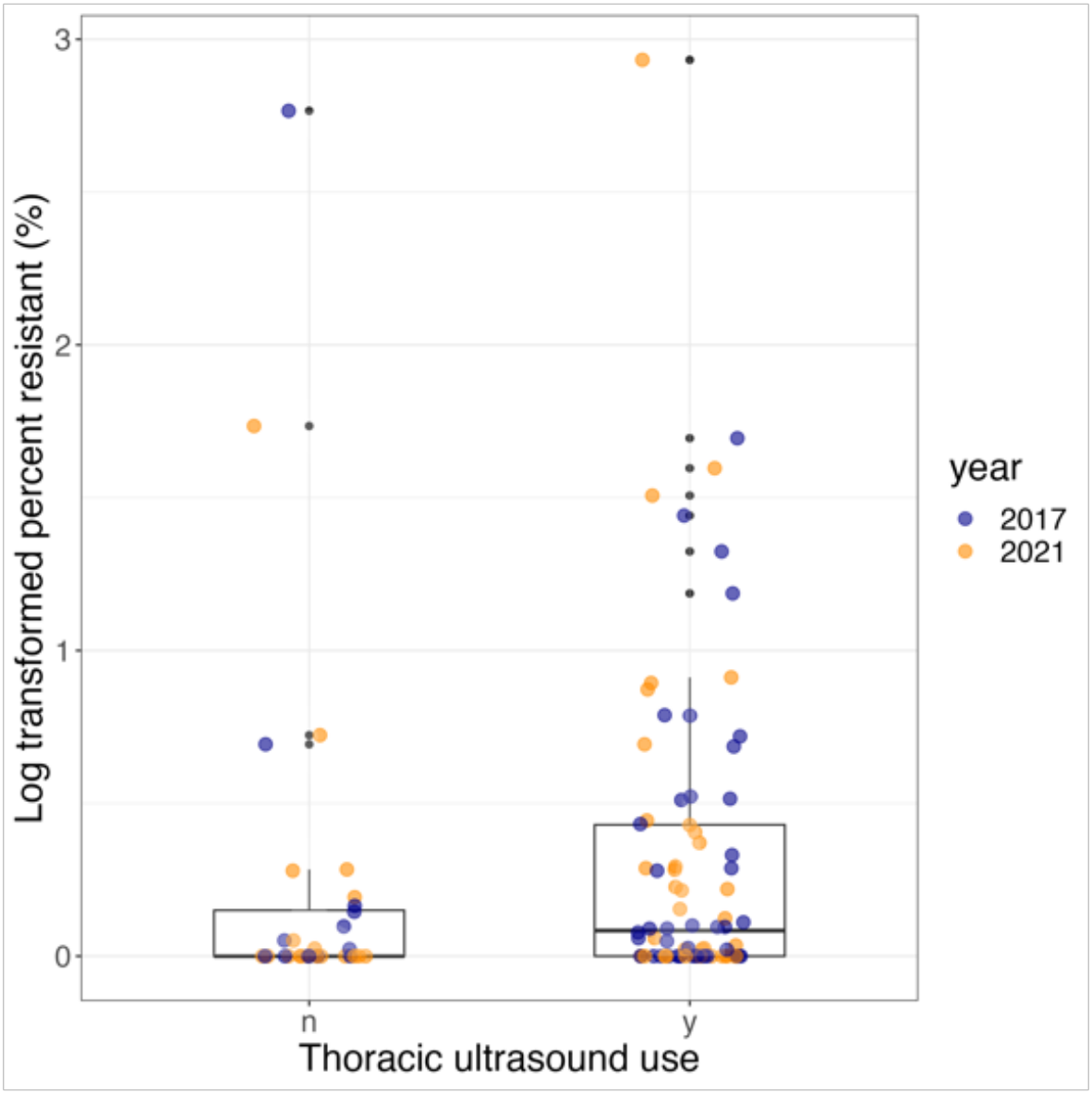

Figure 3.4. Boxplot showing the proportion of MDR-*R. equi* in farms that use or do not use TUS color-coded by year, 2017 (blue) and 2021 (orange).

3.7. References

1. Hondalus, M. K., Diamond, M. S., Rosenthal, L. A., Springer, T. A. & Mosser, D. M. The intracellular bacterium *Rhodococcus equi* requires Mac-1 to bind to mammalian cells. *Infection and Immunity* **61**, 2919–2929 (1993).
2. Giguère, S. *et al.* Diagnosis, treatment, control, and prevention of infections caused by *Rhodococcus equi* in foals. *J Vet Intern Med* **25**, 1209–1220 (2011).
3. Giguère, S. *et al.* Role of the 85-Kilobase plasmid and plasmid-encoded virulence-associated protein A in intracellular survival and virulence of *Rhodococcus equi*. *Infect. Immun.* **67**, 3548–3557 (1999).
4. McCracken, J. & Cohen, N. D. Use of thoracic ultrasounds for the prevention of *Rhodococcus equi* pneumonia on endemic farms. *AAEP Proceedings* **55**, (2009).
5. Slovis, N. M., McCracken, J. & Mundy, D. How to use thoracic ultrasound to screen foals for *Rhodococcus equi* at affected farms. *Proc Am Assoc Equine Pract* **51**, (2005).
6. Venner, M., Kerth, R. & Klug, E. Evaluation of tulathromycin in the treatment of pulmonary abscesses in foals. *The Veterinary Journal* **174**, 418–421 (2007).
7. Giguère, S., Lee, E. A., Guldbech, K. M. & Berghaus, L. J. In vitro synergy, pharmacodynamics, and postantibiotic effect of 11 antimicrobial agents against *Rhodococcus equi*. *Veterinary Microbiology* **160**, 207–213 (2012).
8. Nordmam, P. & Ronco, E. In-vitro antimicrobial susceptibility of *Rhodococcus equi*. *J Antimicrob Chemother* **29**, 383–393 (1992).
9. Prescott, J. F. & Nicholson, V. M. The effects of combinations of selected antibiotics on the growth of *Corynebacterium equi*. *J Vet Pharmacol Ther* **7**, 61–64 (1984).

10. Huber, L. *et al.* Monitoring foals by thoracic ultrasonography, bacterial culture, and PCR: Diagnostic of *Rhodococcus equi* subclinical pneumonia in south of Brazil. *Journal of Equine Veterinary Science* **60**, 104-108.e1 (2018).
11. Venner, M., Rödiger, A., Laemmer, M. & Giguère, S. Failure of antimicrobial therapy to accelerate spontaneous healing of subclinical pulmonary abscesses on a farm with endemic infections caused by *Rhodococcus equi*. *The Veterinary Journal* **192**, 293–298 (2012).
12. Venner, M., Astheimer, K., Lämmer, M. & Giguère, S. Efficacy of mass antimicrobial treatment of foals with subclinical pulmonary abscesses associated with *Rhodococcus equi*. *J Vet Intern Med* **27**, 171–176 (2013).
13. Arnold-Lehna, D., Venner, M., Berghaus, L. J., Berghaus, R. & Giguère, S. Changing policy to treat foals with *Rhodococcus equi* pneumonia in the later course of disease decreases antimicrobial usage without increasing mortality rate. *Equine Vet J* **52**, 531–537 (2020).
14. Huber, L. *et al.* Emergence of Resistance to Macrolides and Rifampin in Clinical Isolates of *Rhodococcus equi* from Foals in Central Kentucky, 1995 to 2017. *Antimicrob Agents Chemother* **63**, e01714-18, /aac/63/1/AAC.01714-18.atom (2018).
15. Huber, L. *et al.* Prevalence and risk factors associated with emergence of *Rhodococcus equi* resistance to macrolides and rifampicin in horse-breeding farms in Kentucky, USA. *Veterinary Microbiology* **235**, 243–247 (2019).
16. Andersson, D. I. & Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* **8**, 260–271 (2010).
17. Borman, A. M., Paulous, S. & Clavel, F. Resistance of human immunodeficiency virus type 1 to protease inhibitors: selection of resistance mutations in the presence and absence of the drug. *Journal of General Virology* **77**, 419–426 (1996).

18. Levin, B. R., Perrot, V. & Walker, N. Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* **154**, 985–997 (2000).

19. Paulander, W., Maisnier-Patin, S. & Andersson, D. I. Multiple mechanisms to ameliorate the fitness burden of mupirocin resistance in *Salmonella typhimurium*. *Mol Microbiol* **64**, 1038–1048 (2007).

20. Schlüsener, M. P. & Bester, K. Persistence of antibiotics such as macrolides, tiamulin and salinomycin in soil. *Environmental Pollution* **143**, 565–571 (2006).

21. Hamscher, G., Sczesny, S., Höper, H. & Nau, H. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal. Chem.* **74**, 1509–1518 (2002).

22. Jacobsen, A. M., Halling-Sørensen, B., Ingerslev, F. & Honoré Hansen, S. Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A* **1038**, 157–170 (2004).

23. Grenni, P., Ancona, V. & Barra Caracciolo, A. Ecological effects of antibiotics on natural ecosystems: A review. *Microchemical Journal* **136**, 25–39 (2018).

24. Ladrón, N. *et al.* Rapid identification of *Rhodococcus equi* by a PCR assay targeting the *choE* gene. *J Clin Microbiol* **41**, 3241–3245 (2003).

25. Anastasi, E. *et al.* Novel transferable *erm* (46) determinant responsible for emerging macrolide resistance in *Rhodococcus equi*. *J. Antimicrob. Chemother.* dkv279 (2015) doi:10.1093/jac/dkv279.

26. Huber, L. *et al.* The novel and transferable *erm*(51) gene confers macrolides, lincosamides and streptogramins B resistance to clonal *Rhodococcus equi* in the environment. *Environ Microbiol* **22**, 2858–2869 (2020).
27. Mishra, A. *et al.* Rapid and simultaneous analysis of multiple classes of antimicrobial drugs by liquid chromatography-tandem mass spectrometry and its application to routine biomedical, food, and soil analyses. *ACS Omega* **5**, 31584–31597 (2020).
28. Tu, Z. *et al.* Exploring the abundance and influencing factors of antimicrobial resistance genes in manure plasmidome from swine farms. *Journal of Environmental Sciences* **124**, 462–471 (2023).
29. Avillan, J. J. *et al.* Excreted Antibiotics May Be Key to Emergence of Increasingly Efficient Antibiotic Resistance in Food Animal Production. *Appl Environ Microbiol* **88**, e00791-22 (2022).
30. Liu, J., Zhao, Z., Orfe, L., Subbiah, M. & Call, D. R. Soil-borne reservoirs of antibiotic-resistant bacteria are established following therapeutic treatment of dairy calves: Antibiotic residues. *Environ Microbiol* **18**, 557–564 (2016).
31. Williams-Nguyen, J. *et al.* Antibiotics and Antibiotic Resistance in Agroecosystems: State of the Science. *J. Environ. Qual.* **45**, 394–406 (2016).
32. Davies, J. & Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol Mol Biol Rev* **74**, 417–433 (2010).
33. Subbiah, M., Shah, D. H., Besser, T. E., Ullman, J. L. & Call, D. R. Urine from Treated Cattle Drives Selection for Cephalosporin Resistant Escherichia coli in Soil. *PLoS ONE* **7**, e48919 (2012).

34. Chee-Sanford, J. C. *et al.* Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste. *J. Environ. Qual.* **38**, 1086–1108 (2009).
35. Wang, X., Ryu, D., Houtkooper, R. H. & Auwerx, J. Antibiotic use and abuse: A threat to mitochondria and chloroplasts with impact on research, health, and environment. *BioEssays* **37**, 1045–1053 (2015).
36. Minden, V., Deloy, A., Volkert, A. M., Leonhardt, S. D. & Pufal, G. Antibiotics impact plant traits, even at small concentrations. *AoB PLANTS* **9**, (2017).
37. Huber, L. *et al.* Association between antimicrobial treatment of subclinical pneumonia in foals and selection of macrolide- and rifampicin-resistant *Rhodococcus equi* strains at horse-breeding farms in central Kentucky. *Journal of the American Veterinary Medical Association* **258**, 648–653 (2021).
38. Burton, A. J. *et al.* Macrolide- and rifampin-resistant *Rhodococcus equi* on a horse breeding farm, Kentucky, USA. *Emerg. Infect. Dis.* **19**, 282–285 (2013).
39. Bordin, A. L., Huber, L., Sanz, M. & Cohen, N. Rhodococcus equi Foal Pneumonia: Update on Epidemiology, Immunity, Treatment, and Prevention. *Equine Vet. J.* (2022) doi:10.1111/evj.13567.
40. Higgins, C. & Huber, L. *Rhodococcus equi*: challenges to treat infections and to mitigate antimicrobial resistance, Journal of Equine Veterinary Science, Volume 127, 2023, 104845, ISSN 0737-0806, https://doi.org/10.1016/j.jevs.2023.104845.
41. Prescott, J. F. *Rhodococcus equi*: an animal and human pathogen. *CLIN MICROBIOL REV* **4**, 15 (1991).
42. Giguère, S. *et al. Rhodococcus equi*: Clinical manifestations, virulence, and immunity. *J. Vet. Intern. Med.* **25**, 1221–1230 (2011).

43. Takai, S. Epidemiology of *Rhodococcus equi* infections: A review. *Vet. Microbiol.* **56**, 167–176 (1997).

44. Shi H, Hu X, Li W, Zhang J, Hu B, Lou L. Soil Component: A Potential Factor Affecting the Occurrence and Spread of Antibiotic Resistance Genes. Antibiotics (Basel). 2023 Feb 4;12(2):333. doi: 10.3390/antibiotics12020333. PMID: 36830244; PMCID: PMC9952537.

45. Macedo G, Hernandez-Leal L, van der Maas P, Heederik D, Mevius D, Schmitt H. The impact of manure and soil texture on antimicrobial resistance gene levels in farmlands and adjacent ditches. Sci Total Environ. 2020 Oct 1;737:139563. Doi: 10.1016/j.scitotenv.2020.139563. Epub 2020 May 20. PMID: 32512295.

46. Cycoń M, Mrozik A, Piotrowska-Seget Z. Antibiotics in the Soil Environment-Degradation and Their Impact on Microbial Activity and Diversity. Front Microbiol. 2019 Mar 8;10:338. doi: 10.3389/fmicb.2019.00338. PMID: 30906284; PMCID: PMC6418018.

Chapter 4

Conclusion

The opportunistic pathogen, *R. equi*, is a robust soil saprophyte present in the environment of horse-breeding farms. Its ability to survive and replicate within the alveolar macrophages of foals with immature immune systems commonly leads to *R. equi* pneumonia at *Rhodococcus*-endemic farms. Even with the possibility of spontaneous recovery from *R. equi* infections in foals without antimicrobial intervention, many endemic farms rely on the "screen-and-treat" method in the absence of alternative efficient methods of disease prevention. This method consists of the use of thoracic ultrasonographic screening (TUS) of foals and preventative use of antimicrobials if lung lesions are detected regardless of the presence of clinical signs of disease. This approach has led to the historical overuse of prophylactic antimicrobials which have been proven to be a major contributor to the rise in antimicrobial resistance (AMR) in both clinical isolates cultured from sick foals and soil from horse-breeding farms. While the reduction of nonessential antimicrobial use is imperative to prevent further spread of AMR, the identification of safe, cost-efficient, and effective alternatives to prevent and treat rhodococcal disease are needed to maintain the health and wellness of foals. Possible alternatives of prevention not associated with the increase of antimicrobial use include the implementation of proper biosafety procedures during equine transport, vaccine development, and the administration of hyperimmune plasma. Additionally, treatment alternatives to help mitigate AMR include bacteriophage therapy, gallium maltolate, and judicious use of antimicrobials. Future studies on the above-mentioned alternatives are needed to provide evidence of their efficiency level in the prevention of *R. equi* infections and therefore, their ability to mitigate the spread of multidrug resistant (MDR)-*R. equi* in horse-breeding farms. In addition to the scarcity of effective alternative prevention and treatment methods for foals, the

environmental impact the historical overuse of antimicrobials has on the maintenance and persistence of MDR-*R.equi* is not fully understood. Is reducing antimicrobial use and relying on alternative methods enough to mitigate MDR-*R. equi* persistence in horse-breeding farms?

As an effort to explore the possibility of mitigation of MDR-*R. equi* infections, we collected soil samples and questionnaires from 2017 to 2021 after advising farms to use caution when using TUS and administering antimicrobials to foals with subclinical cases of suspected *R. equi* infection. Due to loss of follow-up on antimicrobial use data in questionnaires, we retroactively collected as many responses on TUS practices as possible. In our studies, we found that macrolide residue was positively associated with the presence of *R. equi* carrying AMRGs in the soil of horse-breeding farms. Measuring antimicrobial residues found in the soil provided an unbiased estimation of antimicrobial pressure in the environment. This finding is consistent with a previous study demonstrating animals treated with antimicrobials shed antimicrobial metabolites into the environment. Therefore, the persistence of antimicrobial residue in the farm environment may establish a continuous and long-term selection for MDR-*R. equi*. It is known that foals are exposed to *R. equi* through the inhalation of the aerosolized bacterium from the soil in horse-breeding farms. Therefore, the continuous selection for MDR-*R. equi* may contribute to the maintenance and potential increase of MDR-*R. equi* infections in foals. Additionally, antimicrobial residue has also been shown to disrupt the existing microbiomes of soils and plants potentially causing a negative impact of yield in the agriculture sector. Therefore, the existence, persistence, and impact of antimicrobial residues in diverse systems and its potential to cause adverse health effects is an emerging global crisis worthy of collaborative, interdisciplinary mitigation efforts.

This study is the first to provide evidence that the accumulation of antimicrobials in the environment following the treatment of foals might be an important factor contributing to the long-

term maintenance of MDR-*R. equi* in the soil, indicating that mitigation of MDR-*R. equi* without understanding the role of environmental contamination with antimicrobial residues may not be possible. Moving forward, this study could help inform measures to reduce the spread of MDR-*R. equi* in horse-breeding farms. For example, in addition to prudent use of antimicrobials to reduce antimicrobial metabolites being shed into the environment, animals requiring antimicrobial therapy could be quarantined to an area separate from other farm animals for an antimicrobial washout period before returning to the unaffected herd. Consideration of the environmental half-life of antimicrobials could also lead to a decrease in antimicrobial contamination in the environment. Further studies will be needed to determine appropriate antimicrobial washout periods in addition to determining the environmental half-life of appropriate antimicrobial therapy before these practices are implemented in farms.