Retrospective characterization of *Rhodococcus equi* clinical isolates: Integrating phenotypic antimicrobial resistance with *vapA*, *erm*(46), and *erm*(51) genotyping

by

Alexandra Lauren Awtrey

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
May 2nd, 2026

Keywords: *Rhodococcus equi*, PCR assay, antimicrobial resistance, *erm*(46), MLSB, foal pneumonia, molecular diagnostics

Approved by

Dr. Chengming Wang, Chair, Professor Department of Pathobiology
Dr. Constantinos Kyriakis, Co-Chair, Associate Professor Department of Pathobiology
Dr. Laura Huber, Assistant Professor of Epidemiology Department of Pathobiology
Dr. Subarna Barua, Postdoctoral Fellow Department of Pathobiology

Abstract

Rhodococcus equi pneumonia remains a leading cause of mortality in foals, with treatment outcomes critically dependent on early detection of virulent strains and antimicrobial resistance. The emergence of macrolide-resistant strains carrying *erm*(46) has altered therapeutic success rates, calling for rapid molecular diagnostic approaches. The objective of this study was to develop and validate a multiplex PCR assay for simultaneous detection of *R. equi* (*choE*), and determining virulence (*vapA*), and macrolide resistance (*erm*(46), *erm*(51)), and to characterize resistance prevalence and temporal trends in clinical isolates. A total of 229 clinical *R. equi* isolates collected from February 2021 to December 2023 were analyzed using quantitative PCRs targeting the genes: *choE*, *vapA*, *erm*(46) and *erm*(51). Antimicrobial susceptibility testing was performed using broth microdilution for 12 agents. Sequence validation was conducted using MEGA (MUSCLE) alignment analysis. Geographic distribution, temporal trends, and phenotypic-genotypic concordance were evaluated. The PCRs demonstrated high specificity with 100% sequence identity to reference strains. Among 229 isolates, *vapA* was detected in 82.1% (188/229), confirming the majority were virulent strains. The *erm*(46) gene showed high prevalence (55.9%, 128/229) with significant temporal variation: 48.3% in 2021, peaking at 81.5% in 2022, then declining to 69.6% in 2023 ($p<0.05$). In contrast, *erm*(51) was rare (0.4%, 1/229), consistent with its environmental origin. Phenotypic-genotypic concordance was high for macrolides (>55%) but low for non- MLS_B antibiotics (35-38%), confirming mechanistic specificity. Gentamicin, ceftiofur, minocycline, and doxycycline maintained universal or near-universal susceptibility (97-100%) regardless of *erm*(46) status. This study demonstrates high

erm(46) prevalence with significant temporal fluctuation, while *erm*(51) remains primarily an environmental concern. The PCR assays enabled rapid identification of virulent, resistant strains within 12 hours compared to 72 hours for conventional methods. Concordance between *erm*(46) detection and macrolide resistance validates molecular screening for guiding therapeutic decisions. The sustained efficacy of alternative antimicrobials provides critical treatment options for resistant infections. These findings support integration of molecular diagnostics into routine *R. equi* pneumonia management and highlight the need for enhanced antimicrobial stewardship on endemic farms.

Artificial Intelligence (AI) Use Disclosure Statement

In the preparation of this thesis, the following Artificial Intelligence (AI) tools were used: Claude Sonnet 4 (Anthropic, 2025), Anara Labs (2025), Grammarly, Superhuman Platform Inc. These tools were used primarily to assist with deconstructing papers, checking grammar, paragraph styling, and statistical code. The author acknowledges full responsibility for the intellectual content of this work and has ensured that all AI-assisted sections have been reviewed and revised for accuracy and appropriate academic style. All AI-generated content was reviewed and validated for relevance, appropriateness, and accuracy before incorporation into the final document to maintain scholarly integrity of this research.

Acknowledgments

As I reflect on this journey, I am grateful for how each person acknowledged here has played a major role in bringing me closer to my dreams and goals, fundamentally shaping my perspective and deepening my passion for pursuing a career in veterinary medicine. This research experience has shown me how integral scientific investigation is to advancing patient outcomes and management and will be foundational to my future career as a veterinarian.

I would like to express my deepest gratitude to my mentor and principal investigator, Dr. Chengming Wang, for his valuable guidance, support, and mentorship throughout this research project and my time here in the Department of Pathobiology. His expertise and dedication have been instrumental in shaping both this work and my development as a researcher and a professional. I am extremely grateful for the opportunities he has provided and the knowledge he has shared with me.

Special recognition goes to my laboratory colleagues who provided both technical expertise and invaluable support throughout this journey. I'd like to thank Dr. Subarna Barua, Ms. Nneka Iduu, and Mrs. Kelly Chenoweth for their mentorship and assistance in the laboratory. I am also grateful to our collaborators, Dr. Alinne Santana-Pereira, Ms. Kiranmayee Bhimavarapu, and Dr. Laura Huber. Their expertise and collaborative spirit greatly enhanced the quality and scope of this research.

My sincere appreciation goes to my committee's co-chair, Dr. Constantinos Kyriakis, for his collaborative leadership and encouraging my growth throughout my time in this program. I am also grateful to my committee members, Dr. Laura Huber and Dr. Subarna Barua, for their

expert guidance, thoughtful feedback, and unwavering commitment to the excellence of this research. Their mentorship has been invaluable to my development as a researcher.

Special thanks to my friends from Sugg Laboratory, especially Trinity Bell and Camryn Mattox, for their friendship, encouragement, and the countless ways they made this journey even more enjoyable and meaningful. Their support and camaraderie have made even the most challenging days brighter.

Finally, I am extremely grateful to my family and friends for their unwavering support throughout my graduate studies. To my parents and my brother Camden, thank you for your endless encouragement and belief in me, especially during times of uncertainty. Your constant love and support have been my foundation. To my best friends Alexa and Alex, thank you for your friendship, understanding, and for always being there when I needed you most. Your support has been a constant source of strength and motivation.

This research would not have been possible without the collective contributions and support of all these remarkable individuals. As I continue pursuing my goals in animal and veterinary medicine, I carry with me the knowledge, experiences, and relationships that have made this journey so meaningful.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

R. equi	*Rhodococcus equi*
MDR	Multidrug-resistant
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
MLS_B	Macrolides, lincosamides, and streptogramin B
MIC	Minimum Inhibitory Concentration
CLSI	Clinical Laboratory Standards Institute
AST	Antimicrobial Susceptibility Testing
API	Analytical Profile Index
ATCC	American Type Culture Collection
CFU	Colony Forming Units
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
TW	Tracheal Wash
TBA	Tracheobronchial Aspirate
BALF	Bronchoalveolar Lavage Fluid
ELISA	Enzyme-Linked Immunosorbent Assay
AGID	Agar Gel Immunodiffusion
CAMP	Christie, Atkins, Munch-Petersen (test)
ROC	Receiver Operating Characteristic
LOD	Limit of Detection

TUS	Thoracic Ultrasonographic Screening
pVAPs	Virulence-Associated plasmids
pVAPA	Virulence-associated plasmid A (equine)
pVAPB	Virulence-associated plasmid B (porcine)
pVAPN	Virulence-associated plasmid N (ruminant)
Vaps	virulence-associated proteins
pRErm46	Plasmid carrying Erythromycin resistance methylase (class 46)
TnRErm46	Transposon carrying Erythromycin resistance methylase (class 46)
pRErm51	Plasmid carrying Erythromycin resistance methylase (class 51)
TnRErm51	Transposon carrying Erythromycin resistance methylase (class 51)
choE	Cholesterol Oxidase gene
vapA	Virulence-Associated Protein A gene
vapK1	Virulence-Associated Protein K1 gene
vapK2	Virulence-Associated Protein K2 gene
vapN	Virulence-Associated Protein N gene
traA	Transfer gene A
rpoB	RNA Polymerase Beta subunit gene
tetRA(33)	Tetracycline Resistance gene (class 33)
erm(46)	Erythromycin Resistance Methylase (class 46)
erm(51)	Erythromycin Resistance Methylase (class 51)
MaR	Macrolide-Rifampin (combination)
GaM	Gallium Maltolate
POC-RD	Point-of-Care Resistance Diagnostics

WGS	Whole-Genome Sequencing
CARD	Comprehensive Antibiotic Resistance Database
MEGARes	Megares Antibiotic Resistance Database
NCBI	National Center for Biotechnology Information
ST	Sequence Type

Chapter One: Literature Review

The convergence of rapid disease progression, limited diagnostic speed, therapeutic constraints, and escalating antimicrobial resistance creates an urgent clinical need for improved diagnostic and management strategies to…. . Molecular diagnostics, particularly multiplex PCR assays simultaneously detecting *R. equi* and clinically relevant resistance genes, offer the potential to provide rapid diagnosis and resistance profiling from clinical samples within a few hours, enabling targeted therapy initiation before culture results become available.

This review delves into current knowledge regarding molecular diagnostic innovations for *R. equi* detection and antimicrobial resistance characterization, with emphasis on: multiplex PCR design and validation; virulence gene detection and host tropism determination; molecular mechanisms of macrolide and rifampin resistance; clinical implementation of rapid diagnostics; whole-genome sequencing applications for novel resistance detection; and One Health implications of zoonotic transmission and resistance dissemination. By integrating these advances into routine diagnostic workflows, clinicians can optimize antimicrobial stewardship while improving outcomes for infected foals.

1. Ecology and Epidemiology of *R. equi*

R. equi is a gram-positive, aerobic actinobacterium that exists primarily as a soil saprophyte but has emerged as a significant pathogen across multiple animal species and humans (Barton & Hughes, 1984). The organism is ubiquitous in the environment, particularly in soil and manure, where it multiplies in pasture and farm settings (Barton & Hughes, 1984). *R. equi* exhibits coprophilic characteristics, with populations increasing substantially in equine dung

both during and after deposition, making contaminated fecal material and aerosolized dust the primary sources of transmission (Barton & Hughes, 1984).

The organism demonstrates host adaptability, causing documented infections in foals, , swine, cattle, ruminants, camelids, companion animals, wild animals , and immunocompromised humans (Khurana et al., 2014). Foals between one and four months of age represent the most clinically significant population, with disease occurring rarely in adult horses despite exposure on endemic farms (Khurana et al., 2014). Infection is acquired early in life, typically within the first two weeks after birth through inhalation or ingestion of contaminated material, yet clinical signs often do not manifesting until substantial lung pathology has developed (Sanz, 2023). Humans are considered occasional hosts rather than primary targets, with transmission occurring predominantly among immunocompromised individuals, particularly those with HIV/AIDS (Ghielmetti et al., 2025)(Khurana et al., 2014).

2. Pathogenicity and Clinical Disease Manifestations

In foals, *R. equi* causes multifocal purulent bronchopneumonia with characteristic abscess formation resulting from macrophage necrosis and continuous reinfection of new macrophages (Vázquez-Boland et al., 2022). The pathogen's intracellular lifestyle within macrophages makes it particularly challenging to treat and contributes to the chronic nature of infection. Approximately 50% of foals on endemic farms develop subclinical pulmonary lesions detectable by thoracic ultrasonography, yet 70–85% of untreated foals remain subclinical and spontaneously resolve infection (Sanz, 2023). Only 15–30% progress to severe clinical pneumonia requiring treatment, with mortality rates reaching 19% in complicated cases but exceeding 90% survival in uncomplicated pulmonary infections (Sanz, 2023).

Extrapulmonary manifestations significantly complicate the clinical picture, occurring in approximately 50–74% of affected foals. These include ulcerative enterocolitis, mesenteric lymphadenitis, osteomyelitis, arthritis, and disseminated abscessation, which extend prognosis and treatment duration (Khurana et al., 2014; Vázquez-Boland et al., 2022). The systemic nature of these manifestations reflects the organism's ability to disseminate hematogenously from the primary pulmonary focus. In humans, *R. equi* typically presents as subacute cavitary pneumonia with constitutional symptoms, often requiring differential diagnosis from pulmonary tuberculosis (Vázquez-Boland et al., 2022). Human infection occurs predominantly in immunocompromised individuals, particularly those with HIV/AIDS, where mortality rates range from 25–58% despite appropriate therapy, underscoring the pathogen's intracellular resilience and therapeutic challenges (Song et al., 2022).

3. Virulence Gene Detection in *R. equi*

The pathogenicity of *R. equi* is mediated by host-adapted, plasmid-encoded Virulence-Associated Proteins (pVAPs) that promote intracellular survival within macrophages (Álvarez-Narváez & Sánchez, 2023). Three host-specific plasmid types have been characterized: pVAPA (primarily equine), pVAPB (porcine), and pVAPN (ruminant) (Álvarez-Narváez, Berghaus, et al., 2021; Vázquez-Boland et al., 2022). Each encodes distinct virulence-associated proteins (Vaps) that determine host tropism and pathogenic capacity (Khurana et al., 2014). These three host-specific virulence plasmids carry pathogenicity islands encoding 17 *vap* genes, with only *vapA*, *vapK1*, *vapK2*, and *vapN* supporting macrophage intracellular replication (Yerlikaya et al., 2025).

vapA is essential for equine foal disease and uniquely expressed on pVAPA, with equine isolates worldwide carrying sequence types ST-1 and ST-24 with pVAPA. *vapB*, encoded by pVAPB (porcine origin), surprisingly does not support intracellular growth; instead, *vapK1* and *vapK2* function as *vapA* homologs. *vapN* on linear pVAPN (bovine-associated) enables growth comparable to *vapA*. Human isolates show mixed profiles: 26% carry pVAPB and 52% carry pVAPN, suggesting zoonotic transmission through livestock rather than equine sources (Ghielmetti et al., 2025). This distribution pattern has important implications for understanding transmission pathways and developing targeted surveillance strategies.

Conventional PCR targets *traA* (present on all virulence plasmids) and individual *vap* genes for species and virulence confirmation. Real-time qPCR of fecal *vapA* shows poor predictive value for disease development but aids farm surveillance. Tracheal wash PCR achieves 100% sensitivity and 90.6% specificity, superior to culture and serology, enabling reliable exclusion of *R. equi* pneumonia in culture-negative foals (Sellon et al., 2001). Clinical integration combines PCR with culture for comprehensive assessment, recognizing PCR detects virulence potential rather than infection confirmation. Serial tracheal wash fluid PCR monitors treatment response when cultures become negative due to antimicrobial exposure (Sellon et al., 2001). Plasmid typing reveals distinct lineages adapted to specific hosts, with geographic distribution patterns indicating global transmission of clonal populations like the multidrug-resistant clone 2287 (Ghielmetti et al., 2025). Shared plasmid profiles between farm animals and humans suggest common infection sources, particularly meat consumption pathways (Ghielmetti et al., 2025).

4. Diagnostic and Therapeutic Challenges

Current diagnostic approaches for *R. equi* pneumonia rely on a combination of cytology, bacterial culture, and antimicrobial susceptibility testing that collectively require up to 72 hours for results (Álvarez-Narváez et al., 2022). During this interval, disease progression remains uncontrolled, and prolonged environmental exposure facilitates transmission and selection of resistant strains. (Sellon et al., 2001) Culture sensitivity is compromised by prior antibiotic administration, contamination with competing pathogens, and specimen quality degradation, with only 57–62% sensitivity from tracheal wash fluid even in culture-positive cases at necropsy (Sellon et al., 2001).

Serological methods including ELISA and agar gel immunodiffusion demonstrate poor sensitivity and specificity due to widespread environmental exposure producing antibodies in exposed but unaffected foals (Sellon et al., 2001). Conventional PCR targeting virulence genes provides improved sensitivity (90%) over culture (57.1%) but remains limited for clinical implementation (Sellon et al., 2001).

Therapeutic management is further complicated by the organism's intracellular localization within macrophages, necessitating prolonged antimicrobial courses (2–8 weeks in foals, 2–6 months in immunocompromised humans) with limited antibiotic options demonstrating adequate intracellular and pulmonary penetration (Khurana et al., 2014)(Ranganath et al., 2024). The combination of a macrolide with rifampin has remained the standard of care since the 1980s, yet resistance emergence demands alternative approaches (Vázquez-Boland et al., 2022)(Sanz, 2023).

5. Emergence of Antimicrobial Resistance

The convergence of clinical need and available therapeutics has driven concerning resistance trends. Prior to 2001, macrolide and rifampin resistance in *R. equi* was rarely reported in the United States (Álvarez-Narváez, Berghaus, et al., 2021). However, the adoption of thoracic ultrasonographic screening coupled with prophylactic antimicrobial treatment "screen and treat" of subclinical foals dramatically increased selective pressure (Álvarez-Narváez, Berghaus, et al., 2021). Between 1995–2000, MDR *R. equi* prevalence was <5%, increasing to 13–16% by 2007–2017 in central Kentucky, with some endemic farms documenting resistance in up to 40% of clinical isolates (Álvarez-Narváez, Berghaus, et al., 2021).

Three primary clonal populations dominate the MDR landscape: clone 2287, clone G2016, and clone G2017 (Álvarez-Narváez et al., 2022). Clones 2287 and G2016 carry the *erm*(46) gene conferring macrolide, lincosamide, and streptogramin B (MLS_B) resistance on the conjugative plasmid pRErm46, coupled with *rpoB* mutations (primarily Ser531Phe) conferring rifampin resistance (Huber et al., 2020; Rivera-Velez et al., 2022). Clone G2017 carries the novel *erm*(51) resistance gene on transposon tnRErm51 (Huber et al., 2020; Rivera-Velez et al., 2022). Critically, pRErm46 demonstrates efficient horizontal transfer among *R. equi* strains and related environmental actinobacteria, with documented transfer to Nocardia and Arthrobacter species, presenting substantial risk for resistance dissemination beyond *R. equi* (Álvarez-Narváez, Berghaus, et al., 2021).

MDR infection dramatically reduces survival: foals infected with resistant strains demonstrate only 25% survival compared to 69% survival with susceptible strains when treated with macrolide-rifampin combinations (Sanz, 2023). Treatment limitations necessitate alternative

agents with uncertain intracellular penetration and limited human medicinal applications (Sanz, 2023).

6. Classical Diagnostic Approaches

6.1 Culture and Phenotypic Methods

R. equi infection in foals is most reliably diagnosed from tracheobronchial aspirates (TBA), which are considered the gold standard specimen. Fecal samples are valuable for serial screening because virulent isolates can be shed in the feces, while nasal swabs often reflect environmental contamination and are generally PCR negative, and blood cultures are rarely positive in foals but may be positive in occasional human cases (Cohen et al., 2022; Madrigal et al., 2016; Sellon et al., 2001). Tissue specimens obtained at necropsy (lung, liver, lymph nodes, and abscesses) and respiratory samples such as sputum or bronchoalveolar lavage are also examined in human infections (Erol, Scortti, et al., 2021; Li et al., 2024).

On solid media, *R. equi* initially forms teardrop-shaped, mucoid colonies within 24–48 hours that coalesce, and after four to five days a characteristic salmon pink pigment deepens with age (Khurana et al., 2014). The organism displays a distinctive CAMP-type arrowhead hemolysis adjacent to a Staphylococcus aureus indicator strain after 24–48 hours, and it grows readily on non-selective media such as brain heart infusion broth and Trypticase soy agar at 37°C. Selective isolation is facilitated by NANAT medium (nalidixic acid novobiocin actidione tellurite) and by Columbia agar with 5% sheep blood or CNA plates (Willingham-Lane et al., 2019). An enrichment step using TANP broth (Trypticase soy broth supplemented with cycloheximide, penicillin, and potassium tellurite) incubated for six to seven days at 30°C before subculture further improves recovery (Barton & Hughes, 1984).

Despite these methods, culture sensitivity remains limited; in one study only 62% of foals with necropsy-confirmed *R. equi* yielded a positive TBA culture, and mixed bacterial growth was present in 65% of samples, complicating identification (Sellon et al., 2001). Full culture and antimicrobial susceptibility testing may require up to 72 hours, necessitating empiric therapy while awaiting definitive results (Álvarez-Narváez, Berghaus, et al., 2021). Prior antibiotic administration and overgrowth by competing pathogens also reduce culture yield, leading to false negative results (Sellon et al., 2001). Bacteriologic culture or PCR amplification combined with cytological examination showing suppurative inflammation is required for definitive diagnosis (Giguère et al., 2017).

6.2 Biochemical and Serological Identification

Biochemical identification uses the API Coryne test and classic profiles: catalase-positive, oxidase-negative, and urease-positive (Kaczmarkowska et al., 2023). The CAMP test is diagnostic, producing characteristic arrowhead hemolysis near Staphylococcus aureus (Kaczmarkowska et al., 2023). Relying solely on conventional biochemical and morphological tests risks misidentification with similar organisms like *Dietzia* spp. (Vázquez-Boland et al., 2022).

Serological methods like ELISA and agar gel immunodiffusion (AGID) are generally not useful for diagnosing individual foals due to poor sensitivity and specificity (Bordin et al., 2022; Vázquez-Boland et al., 2022). Widespread environmental exposure results in antibody production in healthy, exposed foals, confounding interpretation. Maternally derived antibodies further complicate results of sensitive ELISA tests in young foals (Sellon et al., 2001). While total VapA-specific IgG assays often lack accuracy, the IgG(T) subclass specifically

demonstrates potential, showing high sensitivity and specificity for identifying infected foals in some studies and correlating with an ineffective Th2-type immune response (Vázquez-Boland et al., 2022).

6.3 Histopathology, Cytology, and Tissue Examination

Cytological examination of respiratory samples provides rapid preliminary diagnosis when culture results are pending. Microscopic examination reveals characteristic inflammatory patterns that can suggest *R. equi* infection, though definitive identification requires culture or molecular confirmation. Bronchoalveolar lavage fluid (BALF) examination reveals higher neutrophil percentages in *R. equi* pneumonia compared to other bacterial causes; however, large overlap in neutrophil ranges limits diagnostic utility as a standalone test (Álvarez-Narváez et al., 2022; Sanz, 2023).

Characteristic necropsy lesions include caseating and suppurative granulomas disseminated throughout lungs, liver, abdominal lymph nodes, and other organs (Bordin et al., 2022). Pulmonary consolidation with nodular areas of abscessation is typical, and Gram-positive coccobacilli are identifiable within macrophage cytoplasm (Bordin et al., 2022). Multiple organ systems are frequently involved in extrapulmonary manifestations, reflecting the systemic nature of advanced disease and the organism's ability to disseminate through the blood stream from the primary pulmonary infection.

6.4 Immunohistochemical Detection

Affinity-purified rabbit IgG can rapidly detect *R. equi* in impression smears from affected organs. This method proved identical in sensitivity to bacterial culture and identified *R. equi* in

some cases where culture failed. However, some lesions are recognized only on postmortem evaluation, and immunohistochemistry may not distinguish virulent strains or detect avirulent isolates (Sanz, 2023; Stefańska et al., 2016). These limitations emphasize the need for complementary molecular diagnostic approaches to achieve definitive pathogen identification and virulence characterization.

7. Molecular Diagnostics: Tools Beyond Culture

7.1 Conventional PCR Methods

Early conventional PCR applications targeted three key molecular markers for species identification and virulence assessment. The 16S rRNA gene serves as a universal bacterial marker amplifying a 441-bp segment, useful for confirming bacterial presence but not species-specific identification (Sellon et al., 2001). The *choE* gene encodes cholesterol oxidase and represents a conserved chromosomal *R. equi* marker that detects all strains regardless of virulence status (Stefańska et al., 2016). Virulence-associated plasmid markers (*vapA*, *vapB*, *vapN*) amplify 301-bp segments and distinguish virulent from avirulent isolates (Madrigal et al., 2016; Żychska et al., 2021). Conventional PCR targets *traA* (present on all virulence plasmids) and individual *vap* genes for species and virulence confirmation. Tracheal wash PCR achieves 100% sensitivity and 90.6% specificity, superior to culture and serology, enabling reliable exclusion of *R. equi* pneumonia in culture-negative foals (Sellon et al., 2001).

Real-time qPCR of fecal *vapA* shows poor predictive value for disease development but aids farm surveillance. Serial tracheal wash fluid PCR monitors treatment response when cultures become negative due to antimicrobial exposure (Sellon et al., 2001). VP primers demonstrated superior diagnostic performance compared to 16S alone in reference laboratory

settings, achieving the same 100% sensitivity and 90.6% specificity in tracheal wash samples (Sellon et al., 2001). However, conventional PCR showed lower overall detection rates (20% positive) versus real-time PCR methods and required post-amplification gel electrophoresis analysis, limiting clinical utility despite good specificity for virulent strains (Stefańska et al., 2016). Clinical integration combines PCR with culture for comprehensive assessment, recognizing that PCR detects virulence potential rather than confirming active infection.

7.2 Real-Time PCR and Quantitative Assays

Real-time PCR (qPCR) and quantitative assays offer increased sensitivity, speed, and the ability to quantify pathogens, making them valuable diagnostic tools beyond conventional methods. The goal of developing qPCR assays for *R. equi* detection is to provide a rapid, accurate, and sensitive method for detection and quantification of the pathogen (Khurana et al., 2014; Stefańska et al., 2016). qPCR assays primarily target the conserved chromosomal *choE* gene, which identifies all *R. equi* strains (virulent and avirulent), or virulence plasmid markers like *vapA* to detect only virulent strains (Álvarez-Narváez et al., 2022; Stefańska et al., 2016). qPCR techniques are significantly faster, typically providing results in the first 12 hours, compared to the 72 hours required for isolation and antimicrobial susceptibility testing via culture (Álvarez-Narváez et al., 2022; Khurana et al., 2014).

A developed assay using *choE* demonstrated a detection rate of 90.0% in clinical specimens compared to 60.0% for culture and 20.0% for conventional PCR (Stefańska et al., 2016). qPCR assays have been validated using various clinical materials, including tissue samples, tracheal wash, abscesses, nasal swabs, and swine submaxillary lymph nodes, showing utility for direct detection in diverse specimens (Stefańska et al., 2016). When applied to rectal

swabs for *vapA* detection, one study showed good accuracy (Area Under the Curve 83.7%), but concluded the test lacked adequate diagnostic accuracy for clinical use as a diagnostic tool (Cohen et al., 2022). Another study evaluating serial fecal qPCR for *vapA* as a screening tool found it was not useful for predicting the development of clinical pneumonia in foals (Madrigal et al., 2016).

7.3 Multiplex PCR

Multiplex PCR enables simultaneous amplification of multiple target sequences in a single reaction, dramatically improving diagnostic efficiency. For *R. equi*, this approach simultaneously detects species identification and antimicrobial resistance profiles, reducing analysis time and sample requirements while increasing clinical utility. A novel multiplex qPCR assay was developed for simultaneous detection of *R. equi* (*choE*) and macrolide resistance genes (*erm*(46) and *erm*(51)) in equine respiratory samples (Álvarez-Narváez et al., 2022). This multiplex assay demonstrated high analytical sensitivity with a limit of detection below 12 complete genome copies per PCR reaction and excellent specificity with 98.5% inclusivity for *R. equi* and 100% for resistance genes. The assay showed no cross-reactivity with normal respiratory flora or common equine pathogens, making it a reliable tool to improve diagnosis and aid in treatment selection (Álvarez-Narváez et al., 2022). The three oligo pairs and probes were designed targeting *choE* (Rhodo_Dlab set), *erm*(46) (Erm46_Dlab set), and *erm*(51) (Erm51_Dlab set). In-silico validation confirmed specificity across 202 *R. equi* genomes and excluded false amplifications from non-target species (Álvarez-Narváez et al., 2022).

Multiplex performance showed slight efficiency decreases but maintained clinically acceptable limit of detection values, with amplification efficiency between 90-115% (Álvarez-

Narváez et al., 2022). This technology represents a significant advancement in point-of-care diagnostics, enabling clinicians to rapidly identify both pathogen presence and resistance patterns to guide immediate treatment decisions. Current research projects continue to expand multiplex capabilities to include additional resistance markers and virulence factors, further enhancing clinical utility.

7.4 Emerging Technologies for *R. equi* Diagnostics

While isothermal amplification methods represent emerging diagnostic approaches, the available literature indicates limited development specifically for *R. equi*. Loop-mediated isothermal amplification (LAMP) technology has been referenced as a potential advance for "detection and surveillance of biosecurity-relevant equine bacterial diseases," offering advantages for field deployment through isothermal reaction conditions that eliminate thermal cycling requirements. However, detailed applications to *R. equi* diagnostics are not extensively documented in current peer-reviewed sources. Portable sequencing technologies are advancing *R. equi* characterization. MinION sequencing has been successfully applied to generate hybrid assemblies of *R. equi* genomes, producing complete circular chromosomes and linear plasmids with genomic annotation.

This technology enables rapid identification of virulence determinants, mobile genetic elements (genomic islands, prophages, CRISPR-Cas systems), and antimicrobial resistance genes directly from clinical isolates (Ghielmetti et al., 2025). CRISPR-based diagnostics (SHERLOCK, DETECTR) and lab-on-chip platforms remain underdeveloped for *R. equi* in available literature. Development of such point-of-care technologies represents a critical gap, particularly given the

need for rapid resistance detection in endemic farm settings to support judicious antimicrobial stewardship and combat emerging multidrug-resistant strains.

8. Antimicrobial Resistance in *R. equi*

8.1 Common Therapies and Historical Standards

Since the 1980s, macrolide-rifampin combinations (erythromycin, azithromycin, or clarithromycin) have been the standard treatment for *R. equi* pneumonia in foals, with rare documented resistance until the early 2000s (Giguère et al., 2011). Between 1995–2006, resistance prevalence was 0.7% (erythromycin) and 2.3% (rifampin), but between 2007–2017, prevalence increased dramatically to 13.6% and 16.1%, respectively (Álvarez-Narváez, Giguère, et al., 2021).

8.2 Emerging Resistance Trends and Epidemiology

Emergence of resistance accelerated dramatically after 2001, coinciding with implementation of mass antimicrobial prophylaxis on endemic farms (Álvarez-Narváez, Giguère, et al., 2021; Giguère et al., 2017). Over the past 15 years, the incidence of macrolide-resistant *R. equi* from foals has increased considerably, particularly in the United States (Álvarez-Narváez et al., 2021). The prevalence of macrolide and rifampin-resistant isolates in Texas and Florida between 1997 and 2008 was 4%, with most resistant isolates identified after 2001 (Giguère et al., 2011, 2017). Notably, the odds of death were approximately 7 times higher in foals infected with resistant isolates (Álvarez-Narváez, Berghaus, et al., 2021). Rare reports of rifampin resistance existed during the early era but were typically associated with monotherapy use (Giguère et al., 1999). Dual resistance to macrolides and rifampin began to emerge in the United States in the

2000s in equine farms where mass macrolide-rifampin antibiotic prophylaxis was systematically practiced (Álvarez-Narváez, Giguère, et al., 2021; Huber et al., 2018). Three primary mechanisms of macrolide resistance include rRNA methylation (*erm* genes), active efflux, and enzymatic inactivation (Pechère, 2001). rRNA methylation and active efflux account for the majority of resistant isolates, and most macrolide-resistance genes are associated with mobile elements, enabling spread among strains, species, and bacterial ecosystems (Pechère, 2001).

8.3 Erythromycin Resistance Methylases: *erm*(46) and *erm*(51)

Two distinct erythromycin resistance methylases have emerged in *R. equi* populations, representing separate evolutionary pathways with different clinical implications. The *erm*(46) gene encodes a predicted methyltransferase targeting the 50S ribosomal subunit and is most similar (68–69% nucleotide sequence identity) to mycobacterial rRNA methyltransferases (*erm*(38), *erm*(39), *erm*(40)) (Álvarez-Narváez, Giguère, et al., 2020). The gene is flanked upstream by an open reading frame encoding a putative AAA-family P-loop ATPase/nucleotide kinase domain, and downstream by an open reading frame encoding a putative integrase/Tra5-like transposase with closely related homologs in other Actinobacteria (Pechère, 2001).

Expression of *erm*(46) in macrolide-susceptible strain 103+ conferred high-level (>256 μg/ml) resistance to all macrolides tested (azithromycin, clarithromycin, erythromycin, gamithromycin, tildipirosin), lincosamides, and streptogramin B (Giguère et al., 2017). The gene conferred lower-level resistance (8–16 μg/ml) to ketolides and quinupristin/dalfopristin, but did not confer resistance to aminoglycosides, tetracyclines, glycopeptides, β-lactams, fluoroquinolones, or rifampin (Giguère et al., 2017). The *erm*(46)-carrying multidrug-resistant clone, designated 2287, is increasingly prevalent across equine farms in the United States and

likely to spread internationally (Álvarez-Narváez & Sánchez, 2023). Clone 2287 harbors pRErm46, a 90 kb conjugative plasmid carrying antimicrobial resistance genes for macrolides, lincosamide, streptogramin B (MLS_B), tetracycline, and sulfamethoxazole (Alvarez Narvaez & Sanchez, 2023). The plasmid also encodes a class 1 integron (C1I) with *sul1* (sulfonamide resistance) and *aadA9* (aminoglycoside resistance) cassettes, plus a *tet*(33) tetracycline resistance element flanked by IS6100 copies (Erol, Scortti, et al., 2021). Isolates of *R. equi* resistant to macrolides and rifampin have been identified from at least five U.S. states and have been documented internationally, including China (EFSA Panel on Animal Health and Welfare (AHAW) et al., 2022). At some farms, up to 40% of clinical isolates demonstrate dual macrolide-rifampin resistance (Baptiste et al., 2025; Huber et al., 2018).

The *erm*(51) is a recently discovered MLS_B resistance gene structurally distinct from *erm*(46), identified exclusively in environmental *R. equi* isolates (Huber et al., 2020). The gene is inserted within TnErm51 transposon and associates with both a putative conjugative plasmid (pRErm51) and mobilizable plasmid (pMobErm51), enabling horizontal gene transfer (Huber et al., 2020). *erm*(51) confers high-level resistance to MLS_B *in vitro* and enables horizontal gene transfer to susceptible organisms (Huber et al., 2020). Clone G2017, the *erm*(51)-positive clonal population, remains restricted to environmental samples and has not been isolated from sick animals (Huber et al., 2020). Isolates carrying both *vapA* (equine virulence plasmid) and *erm*(51) are rarely found, indicating either recent acquisition of *erm(*51) with limited clinical adaptation or impaired survival when both genes coexist (Huber et al., 2020). This genetic separation suggests *erm*(51) currently poses minimal clinical threat but warrants surveillance for potential future virulence acquisition (Huber et al., 2020). *erm*(51) associates with a unique *rpoB* S531Y mutation (tyrosine), distinct from the S531F substitution (phenylalanine) characteristic of

erm(46)-positive clinical clone 2287 (Álvarez-Narváez, Giguère, et al., 2020; Rivera-Velez et al., 2022). This genetic divergence indicates separate evolutionary trajectories for environmental (*erm*(51)-G2017) and clinical (*erm*(46)-clone 2287) MDR populations.

8.4 Rifampin Resistance: *rpoB* Mutations

Rifampin resistance in *R. equi* results from mutations in the *rpoB* gene encoding the RNA polymerase β subunit (Fines et al., 2001). Mutations are concentrated within an 81-bp resistance-determining region (RRDR) corresponding to codons 507 to 533 (*E. coli* numbering), with the most commonly reported substitutions occurring at codons 526 or 531 (Fines et al., 2001; Rivera-Velez et al., 2022). The S531F substitution (serine to phenylalanine, TCG→TTC transversion) is characteristic of (and, to date, unique to) the MDR 2287 clone (Álvarez-Narváez, Berghaus, et al., 2021). The S531Y substitution (serine to tyrosine), by contrast, characterizes *erm*(51)-positive environmental strains (Álvarez-Narváez, Berghaus, et al., 2021). Different isolates with the same substitution have been found to have considerably different MICs (Giguère et al., 2017). Rifampin monotherapy selects for resistance more readily than combination therapy, as demonstrated by mutant prevention concentration (MPC) studies (Berghaus et al., 2013). The S531F mutation in clone 2287 appears not to impose a fitness cost, as it has maintained stability in culture over 45 passages (Alvarez Narvaez & Sanchez, 2023).

Lab-generated *rpoB* mutations in strain 103S showed reversion after 20 passages, suggesting the naturally selected S531F mutation is uniquely stable (Alvarez Narvaez & Sanchez, 2023). Rare isolates of *R. equi* resistant to rifampin do not have mutations within the 81-bp resistance-determining region, suggesting mutations in other regions of the *rpoB* gene or alternative resistance mechanisms (Giguère et al., 2017). A gene encoding a monooxigenase-like

protein was identified in one strain of *R. equi*, conferring low-level rifampin resistance by inactivating the drug (Andersen et al., 1997).

8.5 Additional Resistance Determinants

The *tetRA*(33) efflux pump is located on pRErm46 as an adjacent resistance element and confers tetracycline resistance via active drug efflux (Erol, Scortti, et al., 2021). The *tetA*(33) efflux pump is inactive against minocycline and tigecycline. For doxycycline, the *tetRA*(33) determinant was associated with a statistically significant increase in MIC from 0.5/1 µg/ml to 2 µg/ml, indicating low-level cross-resistance. This elevation may be clinically relevant in horses due to poor oral bioavailability of doxycycline (Erol, Scortti, et al., 2021). Approximately 29% of *erm*(46)-positive isolates show deletion of the *tetRA*(33) locus, causing loss of tetracycline resistance while maintaining C1I-mediated sulfonamide resistance, suggesting genetic dispensability due to lack of antibiotic selection pressure (Erol, Scortti, et al., 2021). The *sul1* gene within the class 1 integron (C1I) encodes sulfonamide resistance, while the aadA9 cassette encodes resistance to spectinomycin and streptomycin via inactivation enzymes (O-nucleotidyltransferase and O-phosphotransferase) (Erol, Scortti, et al., 2021).

There was 100% correlation between the presence of a functional *sul1* and sulfonamide resistance phenotype in *erm*(46)-positive isolates (Erol, Scortti, et al., 2021). However, not all *erm*(46)-positive Ermr isolates exhibited a sulfonamide-resistant (Smxr) phenotype; six (43%) of 14 *erm*(46)-positive isolates showed low TMP-SMX MICs similar to susceptible isolates, suggesting pRErm46 plasmid loss with retention of *erm*(46) via transposition (Erol, Scortti, et al., 2021).

8.6 Other Resistance Mechanisms

Resistance to higher-generation fluoroquinolones (enrofloxacin, ciprofloxacin) remains rare in equine *R. equi*, with <5% classified as resistant (Giguère et al., 2017). In vitro selection for fluoroquinolone resistance is typically associated with single amino acid substitutions in the quinolone resistance-determining region of DNA gyrase subunit A (Niwa & Lasker, 2010). Mutants with amino acid substitutions at Ser-83 of GyrA were particularly resistant (MIC >64 μg/ml) (Niwa & Lasker, 2010). *R. equi* isolates are typically resistant or of intermediate susceptibility to most β-lactam antimicrobial agents, except for carbapenems (imipenem, meropenem) (Giguère et al., 2017)

The *R. equi* 103S genome encodes 10 putative β-lactamase homologs and an array of 11 penicillin-binding proteins, contributing to intrinsic and mutational resistance (Vázquez-Boland & Meijer, 2019). Resistance to carbapenems is rare, with low-level imipenem resistance associated with altered penicillin-binding protein patterns (Nordmann et al., 1993). The *R. equi* genome contains an array of putative antimicrobial-resistance determinants, including a variety of antibiotic-inactivation enzymes, β-lactamases, and multidrug efflux systems (Gressler et al., 2014). These systems enable active extrusion of antimicrobials, contributing to innate and acquired resistance phenotypes.

9. PCR-Based Detection of Antimicrobial Resistance Genes

9.1 Targets and Primer Design for Resistance Gene Detection

PCR-based detection of antimicrobial resistance genes requires carefully designed primer sets targeting conserved sequences within resistance determinants while maintaining specificity to avoid cross-reactivity with related genes. For *erm*(46) detection, primers target the

methyltransferase coding sequence, enabling identification of the dominant clinical resistance determinant in *R. equi* (Álvarez-Narváez et al., 2022). The *erm*(46) gene shows high sequence conservation among clinical isolates, facilitating reliable PCR amplification and subsequent sequencing confirmation of specific variants. Detection of *rpoB* mutations requires primers flanking the resistance-determining region (codons 507-533) to capture key substitutions like S531F (characteristic of clone 2287) and S531Y (associated with *erm*(51)-positive environmental strains) (Rivera-Velez et al., 2022). PCR amplification followed by sequencing enables discrimination between these distinct mutational patterns, providing epidemiological insights into resistance evolution pathways.

Distinguishing *erm*(51) from *erm*(46) requires specific primer design targeting unique sequences in each methyltransferase gene, as both confer similar MLS_B resistance phenotypes but have different clinical implications (Huber et al., 2020). *erm*(51) detection is particularly important for environmental surveillance, as this determinant remains restricted to non-virulent strains and serves as an early warning system for potential resistance gene acquisition by clinical strains. Virulence plasmid typing employs primers targeting conserved regions of *traA* (present on all virulence plasmids) and specific *vap* genes (*vapA*, *vapB*, *vapN*) to determine host-specific virulence profiles (Stefańska et al., 2016). These assays enable simultaneous assessment of pathogenic potential and epidemiological tracking of plasmid lineages across different host species and geographic regions.

9.2 Integrated Diagnostics and Resistance Profiling

Multiplex PCR assays can simultaneously target *R. equi* detection (*choE*), virulence markers (*traA*, *vapA*, *vapB*, *vapN*), macrolide resistance (*erm*(46), *erm*(51)), rifampin resistance

(*rpoB* regions), tetracycline resistance (*tetRA*(33)), and sulfonamide resistance (*sul1*). This comprehensive approach accelerates diagnosis and resistance profiling in clinical samples while reducing analysis time and sample requirements. A PCR assay targeting the *choE* gene enables rapid species-specific identification of *R. equi*, which can be combined with resistance gene amplification in a multiplex format (Álvarez-Narváez, Berghaus, et al., 2021; Val-Calvo et al., 2022). This integrated approach provides both pathogen identification and resistance characterization from a single reaction, significantly improving diagnostic efficiency. When *erm*(46) detection is positive, sequencing of PCR amplicons confirms specific amplification and investigates genetic variability (Ghielmetti et al., 2025). Similarly, PCR confirmation of *rpoB* mutations enables distinction between resistant variants and susceptible isolates, providing definitive resistance characterization beyond phenotypic testing alone.

9.3 Clinical Decision-Making and Treatment Selection

Detection of *erm*(46) with pRErm46 genetic context predicts failure of macrolide-rifampin therapy. In foals with resistant isolates, the odds of death were approximately 7 times higher compared to foals infected with susceptible strains (Álvarez-Narváez et al., 2022). PCR-based identification enables rapid treatment adjustment before clinical deterioration, potentially improving therapeutic outcomes in critically ill foals. Combined detection of *erm*(46), *rpoB mutations*, *sul1*, and *tetRA*(33) via multiplex PCR defines the full resistance spectrum of MDR clone 2287, guiding selection of alternative agents (linezolid, minocycline, tigecycline) (Álvarez-Narváez & Sánchez, 2023; Erol, Scortti, et al., 2021). This comprehensive resistance profiling enables clinicians to avoid ineffective therapies and select appropriate alternatives based on the specific resistance pattern detected. PCR discrimination between *erm*(46) (clinical threat) and

erm(51) (environmental, minimal current threat) informs epidemiological interpretation (Huber et al., 2020). *erm*(51)-positive environmental isolates do not require immediate therapeutic adjustment but warrant continued surveillance to detect potential virulence acquisition, as horizontal gene transfer could eventually create clinically significant resistant strains.

9.4 Surveillance and Farm Management

Environmental monitoring for *erm*(51) and continued PCR-based genotyping of clinical isolates are critical for tracking divergent resistance evolution (Huber et al., 2020). Soil monitoring from 100 horse-breeding farms identified macrolide-resistant *R. equi* in 76 farms (76% prevalence), with erm(46) as the dominant resistance determinant in clinical isolates and *erm*(51) restricted to environmental samples (Huber et al., 2020). This surveillance data guides regional resistance management strategies and informs risk assessments for individual farms. Plasmid typing reveals distinct lineages adapted to specific hosts, with geographic distribution patterns indicating global transmission of clonal populations like the multidrug-resistant clone 2287 (Álvarez-Narváez, Berghaus, et al., 2021; Val-Calvo et al., 2022). Shared plasmid profiles between farm animals and humans suggest common infection sources, particularly meat consumption pathways (Ghielmetti et al., 2025). This epidemiological information supports One Health approaches to resistance containment across species boundaries.

Evaluation of fecal samples from mares using quantitative bacteriologic culture and qPCR enables detection of *R. equi* shedding and resistance gene presence, identifying potential sources of foal exposure on endemic farms. This information guides farm-level intervention strategies to reduce disease incidence through targeted management of high-risk animals. Understanding that mass antimicrobial prophylaxis selects for resistance at farms, reducing broad

prophylactic macrolide-rifampin treatment of subclinically affected foals minimizes selection pressure (Álvarez-Narváez, Berghaus, et al., 2020). PCR-guided identification of truly infected animals (tracheal wash PCR positive) versus subclinical colonization refines treatment indications, supporting more judicious antimicrobial use. When PCR surveys reveal ≥40% prevalence of macrolide-rifampin-resistant isolates on a farm (Huber et al., 2018), alternative therapeutic and management strategies become necessary. This threshold guides decision-making regarding purchase of mares, foal placement decisions, and prophylaxis protocols, enabling proactive management before resistance becomes endemic. Detection of pRErm46 transfer from resistant to susceptible strains via conjugation assays (transfer frequencies 3×10^{-3} to 1×10^{-2}) demonstrates that resistance plasmids actively spread in farm environments (Álvarez-Narváez, Berghaus, et al., 2021). Environmental surveillance for shared resistance plasmids among isolates informs infection control priorities and guides implementation of biosecurity measures to limit horizontal gene transfer.

9.5 Alternative Therapeutic Options

Given macrolide-rifampin failure in clone 2287 infections, linezolid, minocycline, and tigecycline demonstrate promising in vitro activity against MDR *R. equi* (Erol, Shaffer, et al., 2021; Giguère et al., 2017). Clarithromycin-doxycycline or clarithromycin-minocycline combinations show synergy with reduced mutant prevention concentrations (Berghaus et al., 2013; Giguère et al., 2017). These combination approaches may help overcome resistance while reducing the likelihood of selecting for additional resistance mechanisms during treatment. Gallium maltolate (GaM) as an alternative to macrolide-rifampin therapy did not alter fecal microbiota diversity or the fecal resistome, nor favored persistence of macrolide-resistant *R. equi*

in soil, offering promise as a resistance-sparing therapeutic option (Álvarez-Narváez, Berghaus, et al., 2020). This novel approach represents an important advancement in therapeutic stewardship, potentially providing effective treatment while preserving antimicrobial susceptibility in farm environments.

10. Current Gaps and Future Perspectives in *R. equi* AMR Surveillance and Control

10.1 Standardization Deficiencies in AMR Testing

No CLSI-approved interpretive criteria currently exist for *R. equi* antimicrobial susceptibility testing. Clinical laboratories rely on breakpoints extrapolated from criteria developed for other bacterial agents or based on therapeutic concentrations in humans or other species, creating significant variability in resistance reporting (Berghaus et al., 2013; Giguère et al., 2017). This lack of standardization compromises the ability to compare resistance data across laboratories and geographic regions, hindering effective surveillance and epidemiological analysis.

Discordance exists among AST methodologies; Etest tends to overestimate clarithromycin MICs relative to broth microdilution, while categorical agreement between methods ranges only 85–100% depending on the drug tested (Berghaus et al., 2013). These methodological inconsistencies create uncertainty in clinical decision-making and may lead to inappropriate treatment selections, particularly when resistance is borderline or when results from different testing methods disagree.

Development of *R. equi*-specific CLSI guidelines require comprehensive multi-laboratory validation studies correlating in vitro susceptibility results with clinical outcomes. Standardized

quality control protocols using validated reference strains are essential to ensure reproducible results across clinical laboratories and support evidence-based resistance surveillance programs.

10.2 Point-of-Care Diagnostic Development Needs

While multiplex qPCR assays demonstrate superior sensitivity (90%) and speed compared to culture-based methods (60%), several critical gaps persist in translating these advances to clinical practice. Current molecular assays require minimum 12 hours processing time—faster than culture but insufficient for true point-of-care applications in acutely deteriorating foals (Álvarez-Narváez et al., 2022; Anastasi et al., 2016). Harmonized cutoff values and consistent interpretation protocols remain undeveloped, limiting widespread clinical adoption.

Limited availability of validated assays in routine veterinary settings creates disparities in diagnostic capabilities between specialized referral centers and primary care practices. Development of ISO-certified, reference-standard assays with quality control protocols comparable to human clinical diagnostics would enhance adoption and ensure consistent performance across diverse clinical settings (Álvarez-Narváez et al., 2022).

Future point-of-care platforms should integrate rapid pathogen detection with resistance gene identification in portable, user-friendly formats suitable for farm-side deployment. Technologies such as CRISPR-based diagnostics or isothermal amplification methods could potentially reduce turnaround times to under one hour, enabling immediate treatment optimization in critical cases.

10.3 Whole-Genome Sequencing and Metagenomics Applications

Whole-genome sequencing (WGS) has revolutionized understanding of *R. equi* population structure and resistance-gene dynamics. Comparative genomic analyses of 251 publicly available sequences combined with MLST revealed host-specific lineages and clonal clustering (Anastasi et al., 2016). Horse isolates predominantly carry VAPA plasmids (ST-1, ST-24), while porcine and human sources share common sequence types (ST-18, ST-36) with VAPB plasmids, indicating potential zoonotic transmission pathways (Ghielmetti et al., 2025).

WGS provides unprecedented resolution for tracking resistance gene evolution and plasmid transfer events in real-time. Environmental soil monitoring using WGS detected *erm*(51) variants before clinical recognition, providing early warning of emerging resistance (Huber et al., 2020). This prospective surveillance capability enables proactive intervention strategies before resistant strains establish clinical prominence.

Metagenomic analysis of foal fecal microbiota revealed that macrolide-rifampin treatment significantly expanded antimicrobial resistance gene abundance and diversity—not just selecting for *R. equi* resistance, but enriching resistance determinants across commensal bacteria (Álvarez-Narváez, Berghaus, et al., 2020). Antibiotic resistance genes for six antimicrobial classes increased after macrolide-rifampin treatment, documenting "collateral resistance" effects that extend beyond the target pathogen. Metagenomics enables detection of novel resistance mechanisms and mobile genetic elements before they establish clinical prominence, supporting comprehensive resistome surveillance.

10.4 Clinical Integration and Diagnostic Workflows

Point-of-care resistance diagnostics (POC-RD) accelerate treatment optimization but require integration into clinical decision-support systems (Álvarez-Narváez, Berghaus, et al., 2021). Effective implementation demands user-friendly interfaces that translate molecular results into actionable therapeutic recommendations, particularly for practitioners without specialized molecular diagnostic training.

While qPCR dominates diagnostic innovation, culture remains essential for comprehensive pathogen characterization. Multiplex qPCR detects 90% of clinical specimens versus 60% for culture, but culture provides antimicrobial susceptibility testing and isolate characterization necessary for epidemiological surveillance (Stefańska et al., 2016). Recommended workflows should combine rapid molecular detection (within 12 hours) with confirmatory culture, enabling simultaneous initiation of targeted therapy and retrospective verification of resistance profiles.

Real-time surveillance systems should automatically flag emergence of novel resistance patterns and trigger epidemiological investigation protocols. Integration with electronic health records can facilitate outbreak detection and support antimicrobial stewardship by tracking prescription patterns and treatment outcomes. Automated alerts when resistance prevalence exceeds predetermined thresholds (such as the 40% farm-level resistance threshold) can prompt immediate intervention strategies.

Clinical decision-support algorithms should incorporate local resistance epidemiology, individual patient risk factors, and molecular diagnostic results to generate personalized treatment recommendations. Machine learning approaches could optimize these algorithms by

continuously analyzing treatment outcomes and resistance patterns to improve predictive accuracy.

10.5 Interdisciplinary Research and Surveillance Coordination

R. equi affects horses, ruminants, swine, dogs, goats, and immuno-compromised humans (Ghielmetti et al., 2025). Phylogenomic analysis reveals host-associated lineages suggesting adaptation mechanisms, yet shared sequence types between farm animals and human isolates indicate potential spillover pathways (Anastasi et al., 2016). Systematic surveillance across veterinary and human medical laboratories could quantify zoonotic transmission frequency and identify high-risk exposure scenarios requiring targeted prevention strategies.

Coordinated surveillance linking equine veterinary clinics, human infectious disease laboratories, and environmental microbiologists would detect *R. equi* transmission pathways and track resistance dissemination across species barriers (Val-Calvo et al., 2022). Standardized sampling protocols and data sharing platforms are essential to enable meaningful inter-institutional collaboration and trend analysis.

One Health approach should integrate environmental monitoring of soil and farm ecosystems with clinical surveillance to capture the full scope of resistance evolution. Regular sampling of farm environments, particularly those with high antimicrobial usage, can provide early warning of emerging resistance before clinical detection. This proactive surveillance enables implementation of prevention strategies while resistance genes remain at low prevalence.

Antimicrobial stewardship programs require evidence-based guidelines that balance therapeutic efficacy with resistance prevention. Widespread prophylactic macrolide-rifampin use selected for MDR clones; changing policy to treat only symptomatic foals decreased resistant-

strain prevalence (Álvarez-Narváez, Berghaus, et al., 2021). Evidence-based alternative therapies like gallium maltolate achieve non-inferiority without selecting macrolide resistance, demonstrating the potential for resistance-sparing treatment strategies.

International collaboration through organizations such as the World Organization for Animal Health (OIE) and World Health Organization (WHO) should establish global surveillance networks for *R. equi* resistance monitoring. Standardized reporting systems and shared databases would enable rapid identification of emerging resistance patterns and facilitate coordinated response strategies across national boundaries. These networks should prioritize resource sharing to support surveillance capabilities in regions with limited diagnostic infrastructure.

11. References

Alvarez Narvaez, S., & Sanchez, S. (2023). Exploring the Accessory Genome of Multidrug-Resistant Rhodococcus equi Clone 2287. *Antibiotics*, *12*(11), 1631. https://doi.org/10.3390/antibiotics12111631

Álvarez-Narváez, S., Berghaus, L. J., Morris, E. R. A., Willingham-Lane, J. M., Slovis, N. M., Giguère, S., & Cohen, N. D. (2020). A Common Practice of Widespread Antimicrobial Use in Horse Production Promotes Multi-Drug Resistance. *Scientific Reports*. https://doi.org/10.1038/S41598-020-57479-9

Álvarez-Narváez, S., Berghaus, R. D., Huber, L. R., Huber, L., Giguère, S., Hart, K. A., Berghaus, R. D., Sánchez, S., & Cohen, N. D. (2021). Epidemiology and Molecular Basis of Multidrug Resistance in Rhodococcus equi. *Microbiology and Molecular Biology Reviews*. https://doi.org/10.1128/mmbr.00011-21

Álvarez-Narváez, S., Fernández, I., Patel, N. V., & Sánchez, S. (2022). Novel Quantitative PCR for Rhodococcus equi and Macrolide Resistance Detection in Equine Respiratory Samples. *Animals*. https://doi.org/10.3390/ani12091172

Álvarez-Narváez, S., Giguère, S., Berghaus, L. J., Dailey, C., & Vázquez-Boland, J. A. (2020). Horizontal Spread of Rhodococcus equi Macrolide Resistance Plasmid pRErm46 across Environmental Actinobacteria. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.00108-20

Álvarez-Narváez, S., Giguère, S., Cohen, N. D., Slovis, N. M., & Vázquez-Boland, J. A. (2021). Spread of Multidrug-Resistant Rhodococcus equi, United States. *Emerging Infectious Diseases*. https://doi.org/10.3201/eid2702.203030

Álvarez-Narváez, S., & Sánchez, S. (2023). Exploring the Accessory Genome of Multidrug-Resistant Rhodococcus equi Clone 2287. *Antibiotics*. https://doi.org/10.3390/ANTIBIOTICS12111631

Anastasi, E., MacArthur, I., Scortti, M., Álvarez-Narváez, S., Giguère, S., & Vázquez-Boland, J. A. (2016). Pangenome and Phylogenomic Analysis of the Pathogenic Actinobacterium Rhodococcus equi. *Genome Biology and Evolution*. https://doi.org/10.1093/GBE/EVW222

Andersen, S. J., Quan, S., Gowan, B., & Dabbs, E. R. (1997). Monooxygenase-like sequence of a Rhodococcus equi gene conferring increased resistance to rifampin by inactivating this antibiotic. *Antimicrobial Agents and Chemotherapy*, *41*(1), 218–221. https://doi.org/10.1128/AAC.41.1.218

Baptiste, K. E., Kyvsgaard, N. C., Ahmed, M. O., Damborg, P., & Dowling, P. M. (2025). Is Rifampin (Rifampicin) Essential for the Treatment of *Rhodococcus equi* Infections in Foals? A Critical Review of the Role of Rifampin. *Journal of Veterinary Pharmacology and Therapeutics*. https://doi.org/10.1111/JVP.70007

Barton, M. D., & Hughes, K. L. (1984). Ecology of Rhodococcus equi. *Veterinary Microbiology*, *9*(1), 65–76. https://doi.org/10.1016/0378-1135(84)90079-8

Barua, S., Tarannum, A., Huber, L., Easterwood, L. A., Velayudhan, B., Da Silveira, B. P., Enyetornye, B., Cohen, N. D., Dimitrov, K. M., Schwarz, E. R., Awtrey, A., Groover, E., Barua, S., Naskou, M., & Wang, C. (2026). Epidemiology and risk factors of equine parvovirus-hepatitis, hepacivirus, Pegivirus caballi, and Pegivirus equi in horses from the Southern United States. *Veterinary Microbiology*, *312*, 110831. https://doi.org/10.1016/j.vetmic.2025.110831

Benoit, S., Taouji, S., Benachour, A., & Hartke, A. (2000). Resistance of Rhodococcus equi to acid pH. *International Journal of Food Microbiology*, *55*(1–3), 295–298. https://doi.org/10.1016/S0168-1605(00)00172-0

Berghaus, L. J., Giguère, S., & Guldbech, K. (2013). Mutant prevention concentration and mutant selection window for 10 antimicrobial agents against Rhodococcus equi. *Veterinary Microbiology*, *166*(3–4), 670–675. https://doi.org/10.1016/j.vetmic.2013.07.006

Bordin, A. I., Huber, L. E., Sanz, M. G., & Cohen, N. D. (2022). Rhodococcus equi Foal Pneumonia: Update on Epidemiology, Immunity, Treatment, and Prevention. *Equine Veterinary Journal*. https://doi.org/10.1111/evj.13567

CLSI. (2021). *CLSI. Performance Standards for Antimicrobial Susceptibility Testing: CLSI Supplement M100. 31st ed. Clinical and Laboratory Standards Institute; 2021.* https://doi.org/10.1128/jcm.00213-21

CLSI. (2022). *Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 32nd ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.*

CLSI. (2023). *Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 33rd ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2023.* (Versions M100-Ed33 (2023)). https://webstore.ansi.org/standards/clsi/clsim100ed33#:~:text=The%20CLSI%20M100%2DEd33%20is%20the%2033rd%20edition,**Non%20printable**%20Some%20documents%20cannot%20be%20printed

Cohen, N. D., Bray, J. M., Flores-Ahlschewde, P., Gonzales, G. M., Kahn, S. K., Silveira, B. P. da, Bray, J. M., King, E. E., Blair, C. C., & Bordin, A. I. (2022). Fecal concentration of Rhodococcus equi determined by quantitative polymerase chain reaction of rectal swab samples to differentiate foals with pneumonia from healthy foals. *Journal of Veterinary Internal Medicine*. https://doi.org/10.1111/jvim.16438

Cohen, N. D., & Moyer, W. (2009). *Control and prevention of Rhodococcus equi foal pneumonia.*

Cohen, N. D., O'Conor, M. S., Chaffin, M. K., & Martens, R. J. (2005). Farm characteristics and management practices associated with development of Rhodococcus equi pneumonia in foals. *Journal of the American Veterinary Medical Association*. https://doi.org/10.2460/JAVMA.2005.226.404

Dawson, T. R. M. Y., Horohov, D. W., Meijer, W. G., & Muscatello, G. (2010). Current understanding of the equine immune response to Rhodococcus equi. An immunological review of R. equi pneumonia. *Veterinary Immunology and Immunopathology*, *135*(1–2), 1–11. https://doi.org/10.1016/j.vetimm.2009.12.004

EFSA Panel on Animal Health and Welfare (AHAW), Nielsen, S. S., Bicout, D. J., Calistri, P., Canali, E., Drewe, J. A., Garin-Bastuji, B., Gonzales Rojas, J. L., Gortázar, C., Herskin, M., Michel, V., Miranda Chueca, M. Á., Padalino, B., Pasquali, P., Roberts, H. C., Spoolder, H., Ståhl, K., Velarde, A., Viltrop, A., … Alvarez, J. (2022). Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): Antimicrobial-resistant Rhodococcus equi in horses. *EFSA Journal*, *20*(2). https://doi.org/10.2903/j.efsa.2022.7081

Erol, E., Scortti, M., Fortner, J., Patel, M., & Vázquez-Boland, J. A. (2021). Antimicrobial Resistance Spectrum Conferred by pRErm46 of Emerging Macrolide (Multidrug)-Resistant Rhodococcus equi. *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.01149-21

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

Fines, M., Pronost, S., Maillard, K., Taouji, S., & Leclercq, R. (2001). Characterization of Mutations in the *rpoB* Gene Associated with Rifampin Resistance in *Rhodococcus equi* Isolated from Foals. *Journal of Clinical Microbiology*, *39*(8), 2784–2787. https://doi.org/10.1128/JCM.39.8.2784-2787.2001

Ghielmetti, G., Stevens, M. J. A., Schmitt, S., Kittl, S., Cernela, N., Biggel, M., Schulthess, B., Keller, P. M., Schrenzel, J., & Stephan, R. (2025). Multi-host distribution of Rhodococcus equi (Prescottella equi) strains and their phylogenomic clustering. *BMC Microbiology*. https://doi.org/10.1186/S12866-025-04152-8

Giguère, S., Berghaus, L. J., & Willingham-Lane, J. M. (2017). Antimicrobial Resistance in Rhodococcus equi. *Microbiology Spectrum*. https://doi.org/10.1128/MICROBIOLSPEC.ARBA-0004-2016

Giguère, S., Cohen, N. D., Chaffin, M. K., Slovis, N. M., Hondalus, M. K., Hines, S. A., & Prescott, J. F. (2011). Diagnosis, treatment, control, and prevention of infections caused by Rhodococcus equi in foals. *Journal of Veterinary Internal Medicine*. https://doi.org/10.1111/J.1939-1676.2011.00835.X

Giguère, S., Wilkie, B. N., & Prescott, J. F. (1999). Modulation of Cytokine Response of Pneumonic Foals by Virulent Rhodococcus equi. *Infection and Immunity*. https://doi.org/10.1128/IAI.67.10.5041-5047.1999

Gressler, L. T., Vargas, A. C. D., Costa, M. M. D., Pötter, L., Silveira, B. P. D., Sangioni, L. A., & Botton, S. D. A. (2014). Genotypic and phenotypic detection of efflux pump in Rhodococcus equi. *Brazilian Journal of Microbiology*, *45*(2), 661–665. https://doi.org/10.1590/S1517-83822014000200040

Hoque, M. M., Valentine, M. J., Kelly, P. J., Barua, S., Murillo, D. F. B., & Wang, C. (2022). Modification of the Folmer primers for the cytochrome c oxidase gene facilitates identification of mosquitoes. *Parasites & Vectors*, *15*(1), 437. https://doi.org/10.1186/s13071-022-05494-2

Huber, L. (2021). Medical management of Rhodococcus equi infections: A clinical epidemiology perspective. *Equine Veterinary Education*. https://doi.org/10.1111/EVE.13417

Huber, L., Giguère, S., Slovis, N. M., Álvarez-Narváez, S., Hart, K. A., Greiter, M., Morris, E. R. A., & Cohen, N. D. (2020). The novel and transferable erm(51) gene confers macrolides, lincosamides and streptogramins B (MLSB) resistance to clonal Rhodococcus equi in the environment. *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.15020

Huber, L., Giguère, S., Slovis, N. M., Carter, C. N., Barr, B. S., Cohen, N. D., Elam, J., Erol, E., Locke, S., Phillips, E. D., & Smith, J. L. (2018). Emergence of Resistance to Macrolides and Rifampin in Clinical Isolates of Rhodococcus equi from Foals in Central Kentucky, 1995 to 2017. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.01714-18

Kaczmarkowska, A., Kwiecień, E., Didkowska, A., Stefańska, I., Rzewuska, M., & Anusz, K. (2023). The Genetic Diversity and Antimicrobial Resistance of Pyogenic Pathogens Isolated from Porcine Lymph Nodes. *Antibiotics*. https://doi.org/10.3390/ANTIBIOTICS12061026

Khurana, S. K., Singha, H., Malik, P., Gulati, B. R., & Singh, R. K. (2014). Molecular characterization of clinical isolates of Rhodococcus equi with PCR assay based on virulence plasmid marker. *Indian Journal of Animal Sciences*.

Landis, J. R., & Koch, G. G. (1977). The Measurement of Observer Agreement for Categorical Data. *Biometrics*, *33*(1), 159. https://doi.org/10.2307/2529310

Li, N., Wu, C., Cao, P., Chen, D., Chen, F., & shen, X. (2024). Multiple systemic infections caused by Rhodococcus equi: A case report. *Access Microbiology*. https://doi.org/10.1099/ACMI.0.000600.V4

Madrigal, R., Shaw, S., Witkowski, L., Sisson, B. E., Blodgett, G. P., Chaffin, M. K., & Cohen, N. D. (2016). Use of Serial Quantitative PCR of the vapA Gene of Rhodococcus equi in Feces for Early Detection of R. equi Pneumonia in Foals. *Journal of Veterinary Internal Medicine*. https://doi.org/10.1111/JVIM.13828

Nordmann, P., Nicolas, M. H., & Gutmann, L. (1993). Penicillin-binding proteins of Rhodococcus equi: Potential role in resistance to imipenem. *Antimicrobial Agents and Chemotherapy*, *37*(7), 1406–1409. https://doi.org/10.1128/AAC.37.7.1406

Pechère, J.-C. (2001). Macrolide resistance mechanisms in Gram-positive cocci. *International Journal of Antimicrobial Agents*, *18*, 25–28. https://doi.org/10.1016/S0924-8579(01)00407-1

Ranganath, N., Mendoza, M. A., Stevens, R. W., Kind, D. R., Wengenack, N., & Shah, A. S. (2024). Rhodococcus infection: A 10-year retrospective analysis of clinical experience and antimicrobial susceptibility profile. *Journal of Clinical Microbiology*. https://doi.org/10.1128/jcm.01537-23

Rivera-Velez, A. G., Huber, L., Sinha, S., & Cohen, N. D. (2022). Fitness cost conferred by the novel erm(51) and rpoB mutation on environmental multidrug resistant-Rhodococcus equi. *Veterinary Microbiology*. https://doi.org/10.1016/J.VETMIC.2022.109531

Sanz, M. G. (2023). Rhodococcus equi–What is New This Decade? *Veterinary Clinics of North America: Equine Practice*. https://doi.org/10.1016/J.CVEQ.2022.11.002

Scarpellini, G. (n.d.). *Cohen's Kappa Free Calculator. 2022* [Computer software]. Idostatistics. Retrieved https://idostatistics.com/cohen-kappa-free-calculator/

Sellon, D. C., Besser, T. E., Vivrette, S. L., & McConnico, R. S. (2001). Comparison of Nucleic Acid Amplification, Serology, and Microbiologic Culture for Diagnosis of *Rhodococcus equi* Pneumonia in Foals. *Journal of Clinical Microbiology*, *39*(4), 1289–1293. https://doi.org/10.1128/JCM.39.4.1289-1293.2001

Song, Y., Jiang, M., Xu, X., Huang, Z., Xiao, Y., Jiang, M., Yin, S., Zheng, M., Zheng, M.-X., Meng, H., Han, Y., Wang, Y., Wang, D., & Wei, Q. (2022). Genomic Characteristics Revealed Plasmid-Mediated Pathogenicity and Ubiquitous Rifamycin Resistance of Rhodococcus equi. *Frontiers in Cellular and Infection Microbiology*. https://doi.org/10.3389/fcimb.2022.807610

Stefańska, I., Witkowski, L., Rzewuska, M., & Dzieciątkowski, T. (2016). Development and evaluation of the internal-controlled real-time PCR assay for Rhodococcus equi detection

in various clinical specimens. *Journal of Veterinary Medical Science*. https://doi.org/10.1292/jvms.15-0516

Val-Calvo, J., Buckley, T., Darcy, J., Gibbons, J., Gibbons, J. F., Creighton, A., Egan, C., Buckley, T., Schmalenberger, A., Fogarty, U., Scortti, M., & Vázquez-Boland, J. A. (2022). International Spread of Multidrug-Resistant Rhodococcus equi. *Emerging Infectious Diseases*. https://doi.org/10.3201/eid2809.220222

Vázquez-Boland, J. A., & Meijer, W. G. (2019). The pathogenic actinobacterium *Rhodococcus equi*: What's in a name? *Molecular Microbiology*, *112*(1), 1–15. https://doi.org/10.1111/mmi.14267

Vázquez-Boland, J. A., Sanz, M. G., & Prescott, J. F. (2022). Rhodococcus equi. In *Pathogenesis of Bacterial Infections in Animals* (1st ed., pp. 715–735). Wiley. https://doi.org/10.1002/9781119754862.ch33

Yerlikaya, Z., Miranda-CasoLuengo, R., Yin, Y., Cheng, C., & Meijer, W. G. (2025). Clade-1 Vap virulence proteins of Rhodococcus equi are associated with the cell surface and support intracellular growth in macrophages. *PLoS ONE*. https://doi.org/10.1371/JOURNAL.PONE.0316541

Żychska, M., Witkowski, L., Klementowska, A., Rzewuska, M., Kwiecień, E., Stefańska, I., Czopowicz, M., Szaluś-Jordanow, O., Mickiewicz, M., Moroz, A., Bonecka, J., & Kaba, J. (2021). Rhodococcus equi-Occurrence in Goats and Clinical Case Report. *Pathogenetics*. https://doi.org/10.3390/PATHOGENS10091141

Chapter Two: Retrospective characterization of *Rhodococcus equi* clinical isolates: Integrating phenotypic antimicrobial resistance with *vapA*, *erm*(46), and *erm*(51) genotyping

1. Abstract

Rhodococcus equi pneumonia remains a leading cause of mortality in foals, with treatment outcomes critically dependent on early detection of virulent strains and antimicrobial resistance. The emergence of macrolide-resistant strains carrying *erm*(46) has altered therapeutic success rates, calling for rapid molecular diagnostic approaches. The objective of this study was to develop and validate a multiplex PCR assay for simultaneous detection of *R. equi* (*choE*), virulence (*vapA*), and macrolide resistance determinants (*erm*(46), *erm*(51)), and to characterize resistance prevalence and temporal trends in clinical isolates. A total of 229 clinical *R. equi* isolates collected from February 2021 to December 2023 were analyzed using quantitative PCRs targeting the genes: *choE*, *vapA*, *erm*(46) and *erm*(51). Antimicrobial susceptibility testing was performed using broth microdilution for 12 agents. Sequence validation was conducted using MEGA (MUSCLE) alignment analysis. Geographic distribution, temporal trends, and phenotypic-genotypic concordance were evaluated. The PCRs demonstrated high specificity with 100% sequence identity to reference strains. Among 229 isolates, *vapA* was detected in 82.1% (188/229), confirming the majority were virulent strains. The *erm*(46) gene showed high prevalence (55.9%, 128/229) with significant temporal variation: 48.3% in 2021, peaking at 81.5% in 2022, then declining to 69.6% in 2023 ($p<0.05$). In contrast, *erm*(51) was rare (0.4%, 1/229), consistent with its environmental origin. Phenotypic-genotypic concordance was high for macrolides (>55%) but low for non- MLS_B antibiotics (35-38%), confirming mechanistic

specificity. Gentamicin, ceftiofur, minocycline, and doxycycline maintained universal or near-universal susceptibility (97-100%) regardless of *erm*(46) status. This study demonstrates high *erm*(46) prevalence with significant temporal fluctuation, while *erm*(51) remains primarily an environmental concern. The PCR assays enabled rapid identification of virulent, resistant strains within 12 hours compared to 72 hours for conventional methods. Concordance between *erm*(46) detection and macrolide resistance validates molecular screening for guiding therapeutic decisions. The sustained efficacy of alternative antimicrobials provides critical treatment options for resistant infections. These findings support integration of molecular diagnostics into routine *R. equi* pneumonia management and highlight the need for enhanced antimicrobial stewardship on endemic farms.

2. Introduction

R. equi is a gram-positive, aerobic actinobacterium and the leading bacterial cause of bronchopneumonia in foals aged one to four months, with mortality rates reaching 19% in complicated cases despite appropriate treatment (Sanz, 2023).The organism exists extensively as a soil saprophyte, particularly in equine fecal material, where its coprophilic characteristics enable rapid population expansion in contaminated pastures and stables (Barton & Hughes, 1984). Foals acquire infection through inhalation of aerosolized bacteria or ingestion of contaminated material within the first two weeks of life, yet clinical signs often do not manifest until substantial pulmonary pathology has developed, creating critical diagnostic and therapeutic challenges (Sanz, 2023).

The pathogenicity of *R. equi* is mediated by host-adapted, conjugative virulence plasmids (pVAPs) that encode virulence-associated proteins (VAPs) enabling intracellular survival within

macrophages (Álvarez-Narváez & Sánchez, 2023).The equine-associated plasmid pVAPA, carrying the *vapA* gene, is the primary virulence determinant in foals and is present in approximately 80-90% of clinical isolates (Álvarez-Narváez et al., 2022). Beyond its veterinary significance, *R. equi* represents an emerging opportunistic pathogen in immunocompromised humans, particularly HIV/AIDS patients, with mortality rates ranging from 25-58% despite appropriate therapy (Song et al., 2022). The detection of pVAPB and pVAPN plasmids in human isolates suggests zoonotic transmission pathways through livestock contact or consumption, underscoring the One Health implications of *R. equi* infection and resistance (Ghielmetti et al., 2025).

Current diagnostic approaches rely on bacterial culture and antimicrobial susceptibility testing, which collectively require up to 72 hours for definitive results (Álvarez-Narváez et al., 2022). During this window, disease progression in affected foals remains uncontrolled, and antimicrobial therapy must be initiated without knowledge of strain virulence or resistance profiles. Culture sensitivity is further compromised by prior antibiotic administration, contamination with competing respiratory pathogens, and specimen quality degradation, with only 57-62% sensitivity from tracheal wash fluid even in culture-confirmed cases at necropsy (Sellon et al., 2001). Serological methods including ELISA and agar gel immunodiffusion demonstrate poor sensitivity and specificity due to widespread environmental exposure producing antibodies in exposed but clinically unaffected foals, rendering these approaches unsuitable for individual case diagnosis (Vázquez-Boland et al., 2022).

The therapeutic landscape for *R. equi* pneumonia has been dominated by macrolide-rifampin combinations since the 1980s, yet the emergence of multidrug-resistant (MDR) strains represents a critical threat to both equine and public health (Álvarez-Narváez, Berghaus, et al.,

2021). Prior to 2001, macrolide and rifampin resistance in *R. equi* isolates from foals was rarely documented in the United States. However, the implementation of ultrasonographic screening coupled with prophylactic antimicrobial treatment of subclinical foals dramatically increased selective pressure on endemic farms (Álvarez-Narváez, Berghaus, et al., 2021; Bordin et al., 2022; Huber, 2021). Between 1995-2000, MDR *R. equi* prevalence was <5%, increasing to 13-16% by 2007-2017 in major breeding regions, with some endemic farms documenting resistance in up to 40% of clinical isolates(Álvarez-Narváez, Berghaus, et al., 2021). Foals infected with resistant strains demonstrate only 25% survival compared to 69% survival with susceptible strains when treated with macrolide-rifampin combinations, highlighting the clinical urgency of rapid resistance detection (Sanz, 2023).

Two primary genetic determinants drive macrolide resistance in *R. equi: erm*(46) and *erm*(51), both encoding rRNA methyltransferases that confer resistance to macrolides, lincosamides, and streptogramin B (MLS_B) antibiotics. The *erm*(46) gene is carried on the conjugative plasmid pRErm46 in the dominant MDR clone 2287, which also harbors *rpoB* mutations (primarily Ser531Phe) conferring rifampin resistance (Álvarez-Narváez, Berghaus, et al., 2021). This clone has been identified across multiple U.S. states and internationally, demonstrating efficient horizontal gene transfer among *R. equi* strains and related environmental actinobacteria, including documented transfer to Nocardia and Arthrobacter species (Álvarez-Narváez et al., 2021). The novel *erm*(51) gene, associated with transposon TnRErm51 and a distinct *rpoB* S531Y mutation, has been detected exclusively in environmental isolates to date and appears to represent a separate evolutionary trajectory from the clinical clone 2287 (Huber et al., 2020; Rivera-Velez et al., 2022).

Molecular diagnostic approaches, particularly multiplex quantitative PCR (qPCR), offer potential to overcome limitations of conventional culture-based methods by providing rapid, simultaneous detection of *R. equi* and clinically relevant resistance genes directly from clinical specimens. Currently, commercial PCR assays are available for *R. equi* species detection (Genetic PCR Solutions) and *vapA* virulence gene identification (Antech Diagnostics), providing clinicians with tools for species identification and rapid diagnosis of pathogenic strains. A previously developed multiplex qPCR assay targeting *choE* (species identification), *erm*(46), and *erm*(51) demonstrated high sensitivity (>99%) and specificity with no cross-reactivity to normal respiratory flora, enabling results within 12 hours compared to 72 hours for culture-based antimicrobial susceptibility testing (Álvarez-Narváez et al., 2022). However, the integration of species identification, virulence assessment, and resistance gene detection into a single comprehensive multiplex platform would provide enhanced diagnostic utility for complete pathogen characterization.

The objectives of this study were to: (1) establish and validate a multiplex PCR assay for simultaneous detection of *R. equi* (*choE*), virulence (*vapA*), and macrolide/rifampin resistance determinants (*erm*(46), *erm*(51)) in a collection of clinical bacterial isolates; (2) determine the prevalence and distribution of these genetic markers among isolates from clinical samples with *R. equi* infections between 2021 and 2023; (3) evaluate trends in *erm*(46) prevalence to assess the evolving resistance landscape; and (4) correlate genotypic resistance markers with phenotypic antimicrobial susceptibility profiles. By integrating rapid molecular diagnostics with resistance profiles and surveillance, this study aims to improve understanding of *R. equi* epidemiology and antimicrobial resistance dynamics, ultimately supporting evidence-based antimicrobial stewardship and optimized clinical management of affected foals.

3. Material & Methods

3.1 *R. equi* Strains

A total of 311 clinical *R. equi* isolates were cultured and subjected to PCR analysis during the study period. Of these, 229 isolates also had corresponding minimum inhibitory concentration (MIC) data available. Only isolates with both PCR and MIC results were included in analyses.

Isolates were recovered from a variety of clinical specimen types. The most common sources included transtracheal wash cultures (n = 127), abscess cultures (n = 40), and wound cultures (n = 26). Additional isolation sites included eye cultures, fecal aerobic cultures, incision site cultures, joint fluid cultures, nasal cultures, peritoneal fluid cultures, pleural fluid cultures, sinus cultures, sterile body fluid cultures, *Streptococcus* screen cultures, uterine flush cultures, and other uterine cultures. All remaining specimen types were represented by fewer than eight isolates each.

Among the total isolates analyzed, 91 isolates had available farm location data. Most of these originated from locations within the hospital catchment area. The isolates were collected over a period spanning February 11, 2021, to December 28, 2023. Farm status information was available for a subset of isolates, with 72 classified as endemic and 30 classified as rare; farm status data were not available for the remaining isolates. Metadata regarding animal sex, breed, or age were not available for the isolates included in this study.

3.2 Culture of *R. equi* Isolates

Brain Heart Infusion broth (BHI broth) was prepared according to the manufacturer's instructions (HiMedia, India). The standard formulation per liter consisted of Hm infusion powder (12 g), BHI powder (5 g), proteose peptone (10 g), dextrose (2 g), sodium chloride (5 g),

and disodium hydrogen phosphate (2.5 g), with a final pH of 7.4 ± 0.2 at 25 °C. For each batch, 29.6 g of BHI broth powder was dissolved in 800 mL of distilled water in an autoclaved 1 L flask. The medium was mixed thoroughly until fully dissolved and free of clumps, then sterilized by autoclaving. Following sterilization, the medium was cooled and stored at 4 °C until use. Antibiotics were not required.

R. equi isolates were stored at −80 °C in glycerol stocks until use. Frozen *R. equi* were thawed and handled under aseptic conditions. Each isolate was inoculated (100 µl of isolate) into 10 mL of sterile BHI broth in a 50 mL conical tube. Cultures were incubated at 35 °C with shaking at 90 rpm for 48 hours.

3.3 Preparation of Bacterial Pellets for DNA Extraction

Following incubation, bacterial growth was assessed visually. For isolates exhibiting sufficient growth, 2 mL of broth culture was transferred to a microcentrifuge tube and centrifuged at 8,000 rpm for 1 minute, or until a visible pellet formed. The supernatant was carefully removed without disturbing the pellet, and the pellet was resuspended in 200 µL of sterile 1× phosphate-buffered saline (PBS). The remaining culture volume was retained at 4 °C as backup material for repeat extraction if necessary.

3.4 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by a diagnostic laboratory using broth microdilution in accordance with "Performance Standards for Antimicrobial Susceptibility Testing. CLSI supplement M100" 31st, 32nd, and 33rd edition established by the Clinical and Laboratory Standards Institute (CLSI). Minimum inhibitory concentration (MIC) values were determined for 12 antimicrobial agents: azithromycin, ceftiofur, chloramphenicol,

clarithromycin, doxycycline, enrofloxacin, erythromycin, gentamicin, marbofloxacin, minocycline, rifampin, and tetracycline.

For isolates collected from 2021 through 2022, MIC values were estimated using the BIOMIC V3 imaging system (Giles Scientific, Santa Barbara, CA), an automated plate reader that captures digital images of broth microdilution plates and determines growth patterns to estimate MIC values (CLSI, 2021, 2022). Beginning in 2023, MIC determination was performed using the Sensititre ARIS HiQ system (Thermo Fisher Scientific, Waltham, MA), which provides true MIC values through automated incubation, reading, and interpretation of Sensititre broth microdilution plates (CLSI, 2023).

Susceptibility interpretations (susceptible, intermediate, or resistant) were assigned based on CLSI-approved breakpoints for the respective antimicrobial agents and bacterial species. Quality control procedures were performed in accordance with CLSI standards to ensure accuracy and reproducibility of results (CLSI, 2021, 2022, 2023).

3.5 PCRs

3.5a Extraction of nucleic acids

DNA was extracted using the IndiMag® Pathogen Prefill system (Indical Bioscience, Leipzig, Germany) according to the manufacturer's instructions with minor adaptations. Prior to use, sealed consumables were inverted several times to ensure resuspension of magnetic beads. The sealed 32-well blocks were briefly centrifuged at 500 × g for 1 minute at room temperature to collect reagents at the bottom of the wells.

For each sample, 200 µL of bacterial suspension was added to the lysis strip. When sample volume was insufficient, sterile 1× phosphate-buffered saline (PBS) was added to achieve a final volume of 200 µL.

Consumables, including the 32-well block, lysis strip, and elution strip, were placed into the IndiMag instrument according to the manufacturer's configuration guidelines. Automated extraction was performed using the preinstalled IndiMag Pathogen Lysis/Block/Elution protocol, with a total run time of approximately 35 minutes. Following completion of the run, eluted DNA was recovered from the elution strip into Cryotubes and stored at -20°C until downstream PCR analysis. A UV decontamination cycle (15min) was performed between runs to minimize cross-contamination.

3.5b Primers and Probes

Representative genome sequences of *choE*, *vapA*, *erm*(46) and *erm*(51) were retrieved from GenBank and aligned using Vector NTI software v11.5 (Invitrogen, Carlsbad, CA, USA). Primers and probes were designed to target conserved regions of the cholesterol oxidase enzyme gene for *choE* (amplicon size = 172 bp), the VAMP-associated protein A gene for *vapA* (amplicon size = 161 bp), the transposon TNRerm46 gene for *erm*(46) (amplicon size = 169 bp), and the transposon TnRerm51 gene for *erm*(51) (amplicon size = 166 bp) (Table 1).

3.5c PCR Thermal Protocol

Real-time PCR amplification was performed using two detection platforms to accommodate different assay requirements. TaqMan-based assays for species identification (*choE*) and macrolide resistance detection (*erm*(46)) were performed on the LightCycler® 96

real-time PCR system (Roche Diagnostics, Mannheim, Germany). The *choE* assay utilized FAM fluorophore detection, while the *erm*(46) assay utilized VIC fluorophore detection. The thermal protocol for the FRET PCR was described previously (Barua et al., 2026).

SYBR Green-based assays for virulence (*vapA*) and the novel resistance gene(*erm*(51)) detection were conducted on the LightCycler® 480 Instrument II (Roche Diagnostics, Mannheim, Germany). For SYBR Green assays, melting curve analysis was performed immediately following amplification to enable target differentiation based on distinct melting temperatures, allowing *vapA* and *erm*(51) to be run as a duplex reaction without cross-reactivity. The thermal protocol for the SYBR Green PCR was described previously (Hoque et al., 2022)

Reactions were performed in duplicate or triplicate to ensure reproducibility, with ten-fold serial dilutions of DNA standards ranging from 10^4 to 10^0 copies. Amplification conditions and primer/probe concentrations were optimized for each platform to achieve maximum sensitivity and specificity for the respective target genes.

3.5d DNA Sequencing and Alignment

PCR products derived from clinical samples were submitted to ELIM Biopharmaceuticals (Hayward, CA, USA) for bidirectional Sanger sequencing. A total of 76 target positive isolates were selected for sequencing: 20 samples for *choE*, 35 samples for *vapA*, 19 samples for *erm*(46), and 2 samples for *erm*(51). Sequencing was performed using the same forward and reverse primers used for PCR amplification. NCBI BLASTn analysis was conducted to compare sequences with known *R. equi* reference genomes: *choE* (GenBank accession: AJ242746.1), *vapA* (GenBank accession: AP038828.1), *erm*(46) (GenBank accession: NG_054941.1), and *erm*(51) (GenBank accession: MN928789.1). Multiple sequence alignments were generated

using MEGA software with the MUSCLE algorithm (Kumar et al., 2024) to assess sequence conservation and validate assay specificity. Query coverage and sequence identity percentages were calculated for each gene target. One *erm*(51)-positive sample lacking corresponding MIC data was excluded from phenotypic-genotypic correlation analysis.

3.6 Genotypic and Phenotypic Correlation Analysis

Agreement between phenotypic MIC data and genotypic WGS data was statistically evaluated using Cohen's kappa (κ) test (https://idostatistics.com/cohen-kappa-free-calculator/#risultati, (Scarpellini, n.d.). Isolates with intermediate resistance were not considered in this analysis. The results were interpreted as 0.01–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement, 0.81–1.00 almost perfect or perfect agreement (Landis & Koch, 1977)

3.7 Statistical Analysis

To evaluate temporal trends in antimicrobial resistance, the distribution of identified AMR genes was compared across different time periods using the Chi-square test of independence. This analysis assessed whether the frequency of specific resistance markers in *R. equi* isolates significantly shifted over time. In cases where expected cell frequencies were less than five, Fisher's exact test was utilized to ensure statistical robustness. All statistical tests were two-tailed, and a p-value < 0.05 was established as the criterion for statistical significance. Data organization and frequency calculations were managed within Microsoft Excel prior to comparative analysis.

4. Results

4.1 Antimicrobial Resistance (AMR) Phenotypes

Antimicrobial susceptibility testing based on MIC interpretation was performed on all 229 isolates, with testing completion varying by antibiotic (Table 2; Figure 1). Gentamicin demonstrated universal susceptibility (229/229 isolates, 100%), establishing it as the most reliable antimicrobial agent against *R. equi* in this population. Similarly, ceftiofur (157/157 tested, 100%) and minocycline (223/223 tested, 100%) showed complete susceptibility with no intermediate or resistant phenotypes detected.

Among macrolides, resistance patterns varied across the class. Erythromycin resistance was observed in 43/224 tested isolates (19.2%), with the vast majority remaining susceptible (180/224, 80.4%) and only one isolate (0.4%) classified as intermediate. Azithromycin demonstrated resistance in 29/160 tested isolates (18.1%), with all remaining isolates susceptible and no intermediate phenotypes observed. Clarithromycin resistance was detected in 38/223 isolates (17.0%), with 179 isolates (80.3%) susceptible and 6 isolates (2.7%) intermediate.

Rifampin resistance was observed in 33/223 tested isolates (14.8%), with 189 isolates (84.8%) susceptible and one isolate (0.4%) intermediate.

Fluoroquinolone resistance was also prevalent, with enrofloxacin exhibiting the highest overall resistance rate (61/222 tested isolates, 27.5%). Most enrofloxacin-tested isolates were susceptible (153/222, 68.9%), while 9 isolates (4.1%) were intermediate susceptible. Marbofloxacin, another fluoroquinolone, demonstrated substantially lower resistance (0/223 tested, 0%), though 15 isolates (6.7%) showed intermediate susceptibility.

Tetracycline class antibiotics showed varied susceptibility profiles. Tetracycline resistance was present in 22/228 tested isolates (9.6%), with 200 isolates (87.7%) susceptible and

6 isolates (2.6%) intermediate. Doxycycline susceptibility was notable with 219/226 tested isolates (96.9%) susceptible, 7 isolates (3.1%) intermediate, and no resistant phenotypes detected. Minocycline, as noted above, showed complete susceptibility.

Chloramphenicol exhibited some resistance (19/229, 8.3%), with 192 isolates (83.8%) susceptible and 18 isolates (7.9%) intermediate, representing the highest proportion of intermediate interpretations among all antibiotics tested.

Overall, the antimicrobial resistance profile revealed fluoroquinolone, macrolide, and rifampin resistance, with enrofloxacin and the macrolide class showing the highest resistance rates. In contrast, aminoglycosides (gentamicin), cephalosporins (ceftiofur), and tetracycline derivatives (minocycline, doxycycline) maintained good activity against *R. equi* isolates in this population.

4.2 Prevalence of AMR genes

Real-time PCR validation was conducted using two PRC platforms. TaqMan-based assays for *choE* (FAM) and *erm*(46) (VIC) were performed on the LightCycler® 96 system, while SYBR Green assays for *vapA* and *erm*(51) were conducted on the LightCycler® 480 II system. Ten-fold serial dilutions of DNA standards ranging from 10^4 to 10^0 copies produced consistent amplification curves across both platforms (Figures 3 and 4). All assays demonstrated analytical sensitivity down to single copy detection, confirming high sensitivity for species identification (*choE*), virulence detection (*vapA*), and resistance gene screening (*erm*(46), *erm*(51)) (Figures 3 and 4). Duplicate or triplicate reactions showed minimal variability, supporting assay reproducibility across the tested concentration range. (Figures 3 and 4) TaqMan assays enabled simultaneous detection of *choE* and *erm*(46) in a duplex using different

fluorophore channels (Figure 2). For SYBR Green assays, melting curve analysis demonstrated clear differentiation between *erm*(51) -positive and *vapA*-positive samples based on distinct melting temperatures, confirming that these targets can be effectively used in a duplex format without cross-reactivity or interference. (Figure 3) The two PCR platforms successfully validated all four gene targets with optimal sensitivity and specificity for molecular detection of *R. equi* virulence and antimicrobial resistance markers.

Bidirectional sequencing of PCR amplicons from 76 selected isolates successfully confirmed the identity of all four gene targets. Forward and reverse sequencing reads demonstrated 100% sequence identity for all gene targets, confirming accurate PCR amplification. BLAST analysis revealed perfect sequence matches to reference genomes: *choE* sequences matched the *R. equi* type strain (AJ242746.1), *vapA* sequences aligned with the equine virulence plasmid reference (AP038828.1), *erm*(46) sequences matched the macrolide resistance gene reference (NG_054941.1), and the *erm*(51) sequence matched the environmental resistance determinant (MN928789.1).

Query coverage ranged from 97-100% for *choE*, 94-100% for *vapA*, 94-100% for *erm*(46), and 100% for *erm*(51). The high coverage percentages confirm that the short amplicon design successfully captured targeted regions while maintaining sequencing quality.

Sequence alignments revealed high sequence conservation among clinical isolates for each gene target (Figures 4-7). *choE* sequences (171 bp amplicon, first 55 bp shown) from 20 isolates showed complete sequence identity, confirming species-specific target identification (Figure 4). *vapA* alignment (141 bp amplicon, first 55 bp shown) from 35 isolates showed complete sequence identity, validating virulence plasmid detection (Figure 5). *Erm*(46) sequences (170 bp amplicon, first 55 bp shown) from 19 isolates showed complete sequence

identity with no polymorphisms, confirming macrolide resistance gene detection (Figure 6). The *erm*(51)-positive isolate (169 bp amplicon, first 55 bp shown) showed complete sequence identity to the reference, validating detection of this rare environmental resistance determinant (Figure 7).

These results showed PCR assay specificity and validated the molecular diagnostic approach for rapid *R. equi* identification, virulence assessment, and resistance screening.

4.3 Antimicrobial Resistance (AMR) Genotypes

PCR detection revealed distinct prevalence patterns among 229 isolates (Figure 8; Table 3). The virulence-associated protein A gene (*vapA*) was most prevalent (188/229, 82.1%), confirming most isolates were pathogenic strains. The macrolide resistance gene *erm*(46) was detected in 128/229 isolates (55.9%), while *erm*(51) was found in only one isolate (0.4%), indicating this environmental resistance gene is exceptionally rare in clinical isolates (Figure 8A; Table 3).

Gene co-occurrence analysis revealed that 127/229 isolates (55.5%) harbored two gene targets, representing *vapA/ erm*(46)-positive strains (Figure 8B; Table 4). Single-gene-positive isolates (60/229, 26.2%) were exclusively *vapA*-positive/ *erm*(46)-negative, representing virulent strains lacking macrolide resistance genes. Triple-negative isolates comprised 41/229 (17.9%), likely representing environmental strains. Only one isolate (0.4%) carried all three targets. (Figure 8B; Table 4).

Among *vapA*-positive isolates, 67.6% (127/188) also harbored *erm*(46), suggesting strong co-selection of virulence and macrolide resistance determinants, potentially due to antimicrobial pressure. The remaining 32.4% (61/188) of virulent strains lacked *erm*(46), indicating

susceptible pathogenic strains persist in the population (Table 3). The low prevalence of *erm*(51)(0.4%) is not surprising since it is normally found in soil isolates.
erm(46) prevalence (55.9%) exceeded the average macrolide resistance rate (18.1%), suggesting incomplete *erm*(46) mediated resistance or presence of additional resistance mechanisms (Table 3, Table 2).

These findings indicate that over half (55.5%) of *R. equi* isolates carry both virulence and macrolide resistance genes, representing a clinically significant threat to standard combination therapy regimens.

4.4 *erm*(46) Prevalence Trends Across Years

Analysis revealed variation in *erm*(46) prevalence across the three-year surveillance period (2021-2023). Annual positivity rates increased substantially from 48.3% in 2021 (42/87 isolates) to 81.5% in 2022 (53/65 isolates), representing a 68.7% relative increase, before declining moderately to 69.6% in 2023 (32/46 isolates) (Figure 9; Table 5). Chi-square test of independence demonstrated significant temporal variation ($p < 0.001$), with post-hoc analysis showing that 2022 prevalence was significantly higher than 2021 (group letters: b vs. a), while 2023 showed an intermediate prevalence that was not significantly different from either year (group letters: ab), suggesting a peak in macrolide resistance gene prevalence during 2022 (Figure 9; Table 5).

Biannual analysis revealed complex within-year variation (Figure 10; Table 6). In 2021, *erm*(46) positivity remained relatively stable, with a slight non-significant decrease from 49.2% in the first half (January-June, 31/63 isolates) to 45.8% in the second half (July-December, 11/24 isolates) ($p = 0.777$, Chi-square test). In contrast, 2022 demonstrated an increase across both

periods, rising from 74.4% in the first half (January-June, 29/39 isolates) to 92.3% in the second half (July- December, 24/26 isolates) ($p = 0.068$, Chi-square test), representing the highest six-month prevalence observed prior to 2023. The 2023 pattern showed an increase from 56.3% in the first half (January-June, 18/32 isolates) to 100% in the second half (July-December, 14/14 isolates) ($p = 0.013$, Fisher's exact test), though the latter period represented the smallest sample size in the dataset and should be interpreted with caution. (Figure 10; Table 6).

Comparison of first half to second-half positivity rates within each year revealed divergent patterns: 2021 showed minimal change (-3.4 percentage points), 2022 exhibited substantial increase (+17.9 percentage points), and 2023 demonstrated dramatic increase (+43.7 percentage points), though the 2023-July-Dec sample size limits definitive conclusions. The peak prevalence periods occurred in 2022-July-Dec (92.3%) and 2023-July-Dec (100%), with the latter representing complete saturation of *erm*(46) among tested isolates during that period. (Figure 10; Table 6). The sustained high prevalence in 2022-2023 compared to 2021 suggests a shift in the resistance landscape.

4.5 Correlation of Phenotypic AMR and Genotypes

Concordance analysis between *erm*(46) detection and MIC-based antimicrobial susceptibility phenotypes revealed antibiotic class-specific patterns (Table 7). The highest concordance rates were observed for macrolide antibiotics, with clarithromycin showing 56.2% agreement (109/194 isolates), erythromycin 55.9% (109/195), and azithromycin 55.2% (85/154) (Table 7). These concordance rates closely approximated the overall *erm*(46) prevalence (55.9%, Table 3), indicating that *erm*(46) presence is a strong predictor of macrolide resistance

phenotypes. The close concordance rates across all three macrolides (range: 55.2-56.2%) reflect the expected cross-resistance pattern conferred by mechanisms.

Rifampin demonstrated intermediate concordance at 49.0% (95/194 isolates, Table 7). This indicates that half of *erm*(46)-positive isolates also exhibit rifampin resistance, while the remainder remain rifampin-susceptible.

Fluoroquinolones showed lower concordance, with enrofloxacin at 47.9% (93/194) and marbofloxacin at 39.2% (76/194) (Table 7). These reduced rates are expected, as fluoroquinolone resistance is mediated by distinct mechanisms (gyrase mutations, efflux pumps). Tetracycline class antibiotics exhibited variable concordance: tetracycline 46.5% (92/198), doxycycline 38.8% (76/196), and minocycline 36.1% (70/194) (Table 7). Chloramphenicol showed 42.7% concordance (85/199). These moderate-to-low concordance rates are consistent with tetracycline and chloramphenicol resistance being mediated by independent mechanisms (efflux pumps, acetyltransferases).

The lowest concordance rates were observed for antibiotics with universal or near-universal susceptibility: gentamicin (35.7%, 71/199), minocycline (36.1%), ceftiofur (38.4%, 58/151), and doxycycline (38.8%) (Table 7). These antimicrobials demonstrated minimal phenotypic resistance (0-3.1% resistant isolates, Table 2), *erm*(46) presence does not confer resistance to these agents and that concordance values likely reflect random co-occurrence.

Overall, the data demonstrates that *erm*(46) detection is highly predictive of macrolide resistance phenotypes (concordance >55%), moderately associated with rifampin patterns (49%), and likely unrelated to non- MLS_B antimicrobials (concordance 36-47%), confirming the expected narrow resistance spectrum conferred by resistance genes. The strong macrolide concordance validates *erm*(46) PCR as a reliable predictor of macrolide resistance in *R. equi*,

while the lack of concordance with aminoglycosides, cephalosporins, and tetracyclines supports their continued utility in antimicrobial therapy.

5. Discussion

R. equi pneumonia remains a leading cause of morbidity and mortality in foals on endemic breeding farms worldwide, with treatment outcomes critically dependent on early detection and appropriate antimicrobial therapy. The emergence of multidrug-resistant strains, particularly those carrying the *erm*(46) gene conferring macrolide resistance and *rpoB* mutations conferring rifampin resistance, has fundamentally altered the therapeutic landscape and increased mortality in affected foals (Álvarez-Narváez, Giguère, et al., 2020; Álvarez-Narváez & Sánchez, 2023; Huber et al., 2018, 2020).

The development and spread of antimicrobial resistance are major concerns for human and animal health. The effects of the overuse of antimicrobials in domestic animals on the dissemination of resistant microbes to humans and the environment are of concern worldwide. *R. equi* is an ideal model to illustrate the spread of antimicrobial resistance at the animal-human-environment interface because it is a natural soil saprophyte that is an intracellular zoonotic pathogen that produces severe bronchopneumonia in many animal species and humans. Globally, *R. equi* is most often recognized as causing severe pneumonia in foals that results in animal suffering and increased production costs for the many horse-breeding farms where the disease occurs. Because highly effective preventive measures for *R. equi* are lacking, thoracic ultrasonographic screening and antimicrobial chemotherapy of subclinically affected foals have been used for controlling this disease during the last 20 years. The resultant increase in antimicrobial use attributable to this “screen-and-treat" approach at farms where the disease is

endemic has likely driven the emergence of multidrug-resistant (MDR) *R. equi* in foals and their environment.

This study characterized 229 clinical *R. equi* isolates between 2021-2023 using a multiplex PCR assay targeting species identification (*choE*), virulence (*vapA*), and resistance determinants (*erm*(46) and *erm*(51)), combined with comprehensive antimicrobial susceptibility testing and temporal surveillance. The key findings demonstrate high *erm*(46) prevalence (55.9% overall, peaking at 81.5% in 2022), strong concordance between genotypic and phenotypic macrolide resistance (~56%), and low prevalence of *erm*(51) in clinical isolates (0.4%). These findings have important implications for diagnostic approaches, antimicrobial stewardship, and understanding of resistance dynamics in this major equine breeding region.

Simultaneous detection of species identification (*choE*) and virulence markers (*vapA*) enables rapid confirmation of clinically relevant *R. equi* infections, as only *vapA*-positive strains carrying the equine virulence plasmid pVAPA are required for the bacteria to survive and replicate within macrophages (Alvarez Narvaez & Sanchez, 2023). The 82.1% *vapA* positivity observed in this study is consistent with previous reports documenting that 80-90% of clinical isolates from foals harbor pVAPA (Álvarez-Narváez et al., 2022), confirming that most cultured isolates represent virulent strains capable of causing disease. The 17.9% of isolates negative for all targets likely represent avirulent environmental strains or specimens with degraded DNA, emphasizing the diagnostic value of virulence gene detection for distinguishing pathogenic from commensal *R. equi.*

The incorporation of *erm*(46) and *erm*(51) resistance gene detection provides immediate resistance profiling that would otherwise require 72 hours for culture-based antimicrobial susceptibility testing (Álvarez-Narváez et al., 2022). This capability is critical given that foals

infected with macrolide-rifampin resistant strains have approximately 7 times higher odds of death compared to those infected with susceptible strains (Álvarez-Narváez, Giguère, et al., 2021). Early identification of *erm*(46) -positive isolates would enable clinicians to implement alternative therapeutic regimens before clinical deterioration occurs, potentially improving survival outcomes.

The low prevalence of *erm*(51)(0.4%, 1/229 isolates) in this clinical collection is consistent with findings by Huber et al. (2020), who identified *erm*(51) predominantly in environmental soil isolates from horse-breeding farms. This novel resistance determinant, which confers MLS_B resistance through ribosomal methylation and is associated with transposon TnRErm51 on conjugative plasmid pRErm51, represents a distinct evolutionary lineage characterized by a previously unidentified *rpoB* S531Y mutation conferring rifampin resistance (Huber et al., 2020). The rarity of isolates carrying both *vapA* and *erm*(51) suggests either recent acquisition of *erm*(51) by virulent strains with limited clinical adaptation, or impaired fitness when both genetic elements coexist (Huber et al., 2020). While *erm*(51) appears to remain primarily an environmental concern rather than an immediate clinical threat in this population, the demonstrated horizontal transferability through conjugative plasmids warrants continued surveillance, particularly given that antimicrobial selection pressure in soil environments could facilitate its spread to virulent clinical strains.

The observed temporal variation in *erm*(46) prevalence, particularly the peak at 81.5% in 2022 followed by moderate decline to 69.6% in 2023, likely reflect multiple interacting factors rather than a single causative mechanism. The sharp increase from 48.3% (2021) to 81.5% (2022) suggests either clonal expansion of *erm*(46) -carrying strains or increased horizontal transfer of the pRErm46 plasmid among endemic farm populations during this period. Temporal

dynamics of antimicrobial resistance in *R. equi* are strongly influenced by antimicrobial usage patterns on breeding farms, particularly the widespread adoption of prophylactic macrolide-rifampin treatment for ultrasonographically-detected subclinical pneumonia (Huber et al., 2018a). Variation in treatment protocols across farms and breeding seasons may have contributed to fluctuating selective pressure, with intensive antimicrobial use in 2021-2022 potentially driving the observed resistance peak.

The consistent pattern of increased *erm*(46) prevalence during the second half of each year, particularly the significant rise in 2023 (56.3% to 100%, $p = 0.013$), may be linked to seasonal foaling patterns and disease epidemiology in the regional breeding industry. The typical foaling season for American Quarter Horses and Thoroughbreds spans January through May, with peak foaling occurring in April and May (American Quarter Horse Association; The Jockey Club). *R. equi* pneumonia typically manifests in foals between 2-4 months of age (peak incidence at 6-12 weeks), although infection occurs within the first days to weeks of life through inhalation of environmental bacteria (Cohen et al., 2005; Cohen & Moyer, 2009; Giguère et al., 2011). This temporal relationship suggests that foals born during the peak spring foaling season would be most susceptible to clinical disease during summer months (June-September), coinciding with the second-half periods when higher *erm*(46) prevalence was observed. This seasonal pattern is consistent with observations from other regions in the northern hemisphere, where *R. equi* pneumonia incidence peaks during July-September months (Benoit et al., 2000; Dawson et al., 2010). Environmental *R. equi* loads typically increase during warmer, drier conditions common in temperate summer climates, further contributing to increased exposure and potential for resistant strain selection during this period.

The moderate decline in 2023, while encouraging, should be interpreted cautiously given the limited sample size in the second half of that year (n=14) and may represent either reduced antimicrobial pressure, displacement of resistant clones by susceptible strains, or sampling bias rather than a sustained epidemiological trend. Continued surveillance with larger sample sizes and detailed farm-level antimicrobial use data will be essential to determine whether this represents genuine resistance decline or statistical artifact.

The phenotypic-genotypic concordance patterns observed in this study provide valuable insights into both the mechanistic basis of *erm*(46) -mediated resistance and the clinical utility of molecular diagnostics. The high concordance for macrolides (clarithromycin 56.2%, erythromycin 55.9%, azithromycin 55.2%) reflects the mechanistic specificity of *erm*(46), which encodes an rRNA methyltransferase conferring cross-resistance to MLS_B antibiotics. The near-identical concordance values across macrolide subclasses confirm that *erm*(46) detection is a reliable predictor of phenotypic macrolide resistance, validating its use as a molecular diagnostic target.

The moderate rifampin concordance (49.0%) reflects the association between *erm*(46) and *rpoB* mutations in the dominant MDR clone 2287, which carries both the pRErm46 plasmid and chromosomal *rpoB* S531F substitutions (Álvarez-Narváez, Berghaus, et al., 2021). The incomplete concordance indicates that not all e *erm*(46) -positive isolates belong to this multidrug-resistant lineage, suggesting independent circulation of macrolide-resistant but rifampin-susceptible strains in the population.

The low concordance rates observed for antimicrobials with universal or near-universal susceptibility; gentamicin (35.7%), ceftiofur (38.4%), minocycline (36.1%), and doxycycline (38.8%), do not represent diagnostic limitations but rather confirm the expected mechanistic

specificity *erm*(46). These agents employ resistance mechanisms independent of ribosomal methylation: aminoglycosides (gentamicin) target the 30S ribosomal subunit, β-lactams (ceftiofur) inhibit peptidoglycan synthesis, and tetracyclines (minocycline, doxycycline) bind the 30S subunit at sites distinct from macrolide targets. The minimal phenotypic resistance observed for these agents (0-3.1% resistant isolates) demonstrates that *R. equi* populations remain broadly susceptible to non- MLS_B antimicrobials regardless of *erm*(46) carriage. This finding has immediate clinical implications: gentamicin, ceftiofur, minocycline, and doxycycline combinations represent reliable alternative therapeutic options for *erm*(46) -positive cases, maintaining therapeutic efficacy even in populations with high macrolide resistance prevalence. The sustained universal susceptibility to these agents across all tested isolates underscores their critical role in antimicrobial stewardship strategies, particularly as macrolide resistance continues to increase in endemic farm populations.

Several limitations must be acknowledged when interpreting these findings. First, the multiplex PCR assay was not fully developed, and two genes had to be run on the LightCycler® 480 II. Being able to run all four targets in a multiplex on LightCycler® 96 would be very useful in a clinical setting.

Sample collection was restricted to clinical isolates from a single geographic region. This geographic limitation may not represent *R. equi* resistance patterns in other regions or internationally, where different clonal populations, antimicrobial use practices, and selective pressures may predominate. Additionally, the hospital-based sampling likely overrepresents severe or complicated cases requiring referral, potentially biasing prevalence estimates upward compared to the true population-level resistance burden. Environmental sampling from soil and pastures was not included in analysis.

Metadata completeness was limited, with farm location data available for only 91 of 229 isolates (39.7%), farm endemic status known for 102 isolates (44.5%), and no data available regarding animal age, sex, breed, or treatment history. This lack of epidemiological metadata prevented analysis of risk factors for resistant infections, correlation between farm management practices and resistance prevalence, or adjustment for potential confounding variables.

The study period spanned only three years (February 2021 to December 2023), providing limited depth for evaluating long-term resistance trends or cyclical patterns. Sample sizes varied across time periods, with only 14 isolates available from the second half of 2023, limiting statistical power for detecting differences in biannual prevalence estimates. Longer-term surveillance with standardized sampling strategies would provide more robust data for trend analysis and evaluation of stewardship intervention effectiveness.

The study examined pure bacterial isolates rather than direct clinical specimens, limiting assessment of the assay's true diagnostic sensitivity and specificity in the intended clinical context. Detection limits and performance characteristics may differ when applied directly to tracheal fluid or other respiratory specimens containing mixed bacterial populations, host DNA, mucus, and other factors. Previous validation studies using mock equine respiratory samples have demonstrated good performance (Álvarez-Narváez et al., 2022), but prospective clinical validation comparing PCR results from direct specimens remains necessary before routine diagnostic implementation.

In conclusion, this study reveals high prevalence of *erm*(46) among clinical isolates and variation in resistance dynamics. The 55.9% overall *erm*(46) prevalence, with peak rates exceeding 80% in 2022, indicates that macrolide resistance has become firmly established in the virulent *R. equi* population affecting foals in this major breeding region. The moderate

concordance (~56%) between *e erm*(46) detection and macrolide resistance phenotypes shows the value of combining rapid molecular screening with comprehensive culture-based antimicrobial susceptibility testing.

From a clinical perspective, these findings support integration of molecular diagnostics into routine workflows for *R. equi* pneumonia cases. Early *erm*(46) detection enables clinicians to avoid macrolide-rifampin combinations in favor of alternative agents (Doxycycline, Ceftiofur, Minocycline, or Gentamicin combinations), potentially improving survival in foals infected with MDR strains. The near absence of *erm*(51) in clinical isolates (0.4%) suggests this resistance determinant remains an environmental concern rather than an immediate clinical threat, though continued surveillance is warranted given its association with conjugative plasmids capable of horizontal transfer.

From an epidemiological perspective, the trends and high resistance prevalence documented here emphasize the urgent need for enhanced antimicrobial stewardship on endemic farms. Reducing prophylactic macrolide-rifampin treatment of sub-clinically affected foals, implementing targeted therapy based on molecular diagnostics, and exploring alternative therapeutic agents can collectively reduce selective pressure driving further resistance. The demonstration of significant year-to-year variation in *erm*(46) prevalence suggests that interventions can meaningfully impact resistance trajectories, providing optimism that evidence-based stewardship can help mitigate this evolving threat.

Future research should focus on expanding multiplex assays, conducting prospective clinical validation of molecular diagnostics in direct respiratory specimens, implementing standardized surveillance programs across multiple geographic regions with enhanced epidemiological metadata collection, evaluating the cost-effectiveness of molecular diagnostic-

guided therapy. Through new knowledge, the veterinary community can improve patient outcomes while mitigating the ongoing evolution and spread of antimicrobial resistance in this important equine pathogen.

6. References

Alvarez Narvaez, S., & Sanchez, S. (2023). Exploring the Accessory Genome of Multidrug-Resistant Rhodococcus equi Clone 2287. *Antibiotics*, *12*(11), 1631. https://doi.org/10.3390/antibiotics12111631

Álvarez-Narváez, S., Berghaus, L. J., Morris, E. R. A., Willingham-Lane, J. M., Slovis, N. M., Giguère, S., & Cohen, N. D. (2020). A Common Practice of Widespread Antimicrobial Use in Horse Production Promotes Multi-Drug Resistance. *Scientific Reports*. https://doi.org/10.1038/S41598-020-57479-9

Álvarez-Narváez, S., Berghaus, R. D., Huber, L. R., Huber, L., Giguère, S., Hart, K. A., Berghaus, R. D., Sánchez, S., & Cohen, N. D. (2021). Epidemiology and Molecular Basis of Multidrug Resistance in Rhodococcus equi. *Microbiology and Molecular Biology Reviews*. https://doi.org/10.1128/mmbr.00011-21

Álvarez-Narváez, S., Fernández, I., Patel, N. V., & Sánchez, S. (2022). Novel Quantitative PCR for Rhodococcus equi and Macrolide Resistance Detection in Equine Respiratory Samples. *Animals*. https://doi.org/10.3390/ani12091172

Álvarez-Narváez, S., Giguère, S., Berghaus, L. J., Dailey, C., & Vázquez-Boland, J. A. (2020). Horizontal Spread of Rhodococcus equi Macrolide Resistance Plasmid pRErm46 across Environmental Actinobacteria. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.00108-20

Álvarez-Narváez, S., Giguère, S., Cohen, N. D., Slovis, N. M., & Vázquez-Boland, J. A. (2021). Spread of Multidrug-Resistant Rhodococcus equi, United States. *Emerging Infectious Diseases*. https://doi.org/10.3201/eid2702.203030

Álvarez-Narváez, S., & Sánchez, S. (2023). Exploring the Accessory Genome of Multidrug-Resistant Rhodococcus equi Clone 2287. *Antibiotics*. https://doi.org/10.3390/ANTIBIOTICS12111631

Anastasi, E., MacArthur, I., Scortti, M., Álvarez-Narváez, S., Giguère, S., & Vázquez-Boland, J. A. (2016). Pangenome and Phylogenomic Analysis of the Pathogenic Actinobacterium Rhodococcus equi. *Genome Biology and Evolution*. https://doi.org/10.1093/GBE/EVW222

Andersen, S. J., Quan, S., Gowan, B., & Dabbs, E. R. (1997). Monooxygenase-like sequence of a Rhodococcus equi gene conferring increased resistance to rifampin by inactivating this antibiotic. *Antimicrobial Agents and Chemotherapy*, *41*(1), 218–221. https://doi.org/10.1128/AAC.41.1.218

Baptiste, K. E., Kyvsgaard, N. C., Ahmed, M. O., Damborg, P., & Dowling, P. M. (2025). Is Rifampin (Rifampicin) Essential for the Treatment of *Rhodococcus equi* Infections in Foals? A Critical Review of the Role of Rifampin. *Journal of Veterinary Pharmacology and Therapeutics*. https://doi.org/10.1111/JVP.70007

Barton, M. D., & Hughes, K. L. (1984). Ecology of Rhodococcus equi. *Veterinary Microbiology*, *9*(1), 65–76. https://doi.org/10.1016/0378-1135(84)90079-8

Barua, S., Tarannum, A., Huber, L., Easterwood, L. A., Velayudhan, B., Da Silveira, B. P., Enyetornye, B., Cohen, N. D., Dimitrov, K. M., Schwarz, E. R., Awtrey, A., Groover, E., Barua, S., Naskou, M., & Wang, C. (2026). Epidemiology and risk factors of equine parvovirus-hepatitis, hepacivirus, Pegivirus caballi, and Pegivirus equi in horses from the Southern United States. *Veterinary Microbiology*, *312*, 110831. https://doi.org/10.1016/j.vetmic.2025.110831

Benoit, S., Taouji, S., Benachour, A., & Hartke, A. (2000). Resistance of Rhodococcus equi to acid pH. *International Journal of Food Microbiology*, *55*(1–3), 295–298. https://doi.org/10.1016/S0168-1605(00)00172-0

Berghaus, L. J., Giguère, S., & Guldbech, K. (2013). Mutant prevention concentration and mutant selection window for 10 antimicrobial agents against Rhodococcus equi. *Veterinary Microbiology*, *166*(3–4), 670–675. https://doi.org/10.1016/j.vetmic.2013.07.006

Bordin, A. I., Huber, L. E., Sanz, M. G., & Cohen, N. D. (2022). Rhodococcus equi Foal Pneumonia: Update on Epidemiology, Immunity, Treatment, and Prevention. *Equine Veterinary Journal*. https://doi.org/10.1111/evj.13567

CLSI. (2021). *CLSI. Performance Standards for Antimicrobial Susceptibility Testing: CLSI Supplement M100. 31st ed. Clinical and Laboratory Standards Institute; 2021.* https://doi.org/10.1128/jcm.00213-21

CLSI. (2022). *Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 32nd ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.*

CLSI. (2023). *Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 33rd ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2023.* (Versions M100-Ed33 (2023)). https://webstore.ansi.org/standards/clsi/clsim100ed33#:~:text=The%20CLSI%20M100%2DEd33%20is%20the%2033rd%20edition,**Non%20printable**%20Some%20documents%20cannot%20be%20printed

Cohen, N. D., Bray, J. M., Flores-Ahlschewde, P., Gonzales, G. M., Kahn, S. K., Silveira, B. P. da, Bray, J. M., King, E. E., Blair, C. C., & Bordin, A. I. (2022). Fecal concentration of Rhodococcus equi determined by quantitative polymerase chain reaction of rectal swab samples to differentiate foals with pneumonia from healthy foals. *Journal of Veterinary Internal Medicine*. https://doi.org/10.1111/jvim.16438

Cohen, N. D., & Moyer, W. (2009). *Control and prevention of Rhodococcus equi foal pneumonia.*

Cohen, N. D., O'Conor, M. S., Chaffin, M. K., & Martens, R. J. (2005). Farm characteristics and management practices associated with development of Rhodococcus equi pneumonia in foals. *Journal of the American Veterinary Medical Association*. https://doi.org/10.2460/JAVMA.2005.226.404

Dawson, T. R. M. Y., Horohov, D. W., Meijer, W. G., & Muscatello, G. (2010). Current understanding of the equine immune response to Rhodococcus equi. An immunological review of R. equi pneumonia. *Veterinary Immunology and Immunopathology*, *135*(1–2), 1–11. https://doi.org/10.1016/j.vetimm.2009.12.004

EFSA Panel on Animal Health and Welfare (AHAW), Nielsen, S. S., Bicout, D. J., Calistri, P., Canali, E., Drewe, J. A., Garin-Bastuji, B., Gonzales Rojas, J. L., Gortázar, C., Herskin, M., Michel, V., Miranda Chueca, M. Á., Padalino, B., Pasquali, P., Roberts, H. C., Spoolder, H., Ståhl, K., Velarde, A., Viltrop, A., … Alvarez, J. (2022). Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): Antimicrobial-resistant Rhodococcus equi in horses. *EFSA Journal*, *20*(2). https://doi.org/10.2903/j.efsa.2022.7081

Erol, E., Scortti, M., Fortner, J., Patel, M., & Vázquez-Boland, J. A. (2021). Antimicrobial Resistance Spectrum Conferred by pRErm46 of Emerging Macrolide (Multidrug)-Resistant Rhodococcus equi. *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.01149-21

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

Fines, M., Pronost, S., Maillard, K., Taouji, S., & Leclercq, R. (2001). Characterization of Mutations in the *rpoB* Gene Associated with Rifampin Resistance in *Rhodococcus equi* Isolated from Foals. *Journal of Clinical Microbiology*, *39*(8), 2784–2787. https://doi.org/10.1128/JCM.39.8.2784-2787.2001

Ghielmetti, G., Stevens, M. J. A., Schmitt, S., Kittl, S., Cernela, N., Biggel, M., Schulthess, B., Keller, P. M., Schrenzel, J., & Stephan, R. (2025). Multi-host distribution of Rhodococcus equi (Prescottella equi) strains and their phylogenomic clustering. *BMC Microbiology*. https://doi.org/10.1186/S12866-025-04152-8

Giguère, S., Berghaus, L. J., & Willingham-Lane, J. M. (2017). Antimicrobial Resistance in Rhodococcus equi. *Microbiology Spectrum*. https://doi.org/10.1128/MICROBIOLSPEC.ARBA-0004-2016

Giguère, S., Cohen, N. D., Chaffin, M. K., Slovis, N. M., Hondalus, M. K., Hines, S. A., & Prescott, J. F. (2011). Diagnosis, treatment, control, and prevention of infections caused by Rhodococcus equi in foals. *Journal of Veterinary Internal Medicine*. https://doi.org/10.1111/J.1939-1676.2011.00835.X

Giguère, S., Wilkie, B. N., & Prescott, J. F. (1999). Modulation of Cytokine Response of Pneumonic Foals by Virulent Rhodococcus equi. *Infection and Immunity*. https://doi.org/10.1128/IAI.67.10.5041-5047.1999

Gressler, L. T., Vargas, A. C. D., Costa, M. M. D., Pötter, L., Silveira, B. P. D., Sangioni, L. A., & Botton, S. D. A. (2014). Genotypic and phenotypic detection of efflux pump in Rhodococcus equi. *Brazilian Journal of Microbiology*, *45*(2), 661–665. https://doi.org/10.1590/S1517-83822014000200040

Hoque, M. M., Valentine, M. J., Kelly, P. J., Barua, S., Murillo, D. F. B., & Wang, C. (2022). Modification of the Folmer primers for the cytochrome c oxidase gene facilitates identification of mosquitoes. *Parasites & Vectors*, *15*(1), 437. https://doi.org/10.1186/s13071-022-05494-2

Huber, L. (2021). Medical management of Rhodococcus equi infections: A clinical epidemiology perspective. *Equine Veterinary Education*. https://doi.org/10.1111/EVE.13417

Huber, L., Giguère, S., Slovis, N. M., Álvarez-Narváez, S., Hart, K. A., Greiter, M., Morris, E. R. A., & Cohen, N. D. (2020). The novel and transferable erm(51) gene confers macrolides, lincosamides and streptogramins B (MLSB) resistance to clonal Rhodococcus equi in the environment. *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.15020

Huber, L., Giguère, S., Slovis, N. M., Carter, C. N., Barr, B. S., Cohen, N. D., Elam, J., Erol, E., Locke, S., Phillips, E. D., & Smith, J. L. (2018). Emergence of Resistance to Macrolides

and Rifampin in Clinical Isolates of Rhodococcus equi from Foals in Central Kentucky, 1995 to 2017. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.01714-18

Kaczmarkowska, A., Kwiecień, E., Didkowska, A., Stefańska, I., Rzewuska, M., & Anusz, K. (2023). The Genetic Diversity and Antimicrobial Resistance of Pyogenic Pathogens Isolated from Porcine Lymph Nodes. *Antibiotics*. https://doi.org/10.3390/ANTIBIOTICS12061026

Khurana, S. K., Singha, H., Malik, P., Gulati, B. R., & Singh, R. K. (2014). Molecular characterization of clinical isolates of Rhodococcus equi with PCR assay based on virulence plasmid marker. *Indian Journal of Animal Sciences*.

Landis, J. R., & Koch, G. G. (1977). The Measurement of Observer Agreement for Categorical Data. *Biometrics*, *33*(1), 159. https://doi.org/10.2307/2529310

Li, N., Wu, C., Cao, P., Chen, D., Chen, F., & shen, X. (2024). Multiple systemic infections caused by Rhodococcus equi: A case report. *Access Microbiology*. https://doi.org/10.1099/ACMI.0.000600.V4

Madrigal, R., Shaw, S., Witkowski, L., Sisson, B. E., Blodgett, G. P., Chaffin, M. K., & Cohen, N. D. (2016). Use of Serial Quantitative PCR of the vapA Gene of Rhodococcus equi in Feces for Early Detection of R. equi Pneumonia in Foals. *Journal of Veterinary Internal Medicine*. https://doi.org/10.1111/JVIM.13828

Nordmann, P., Nicolas, M. H., & Gutmann, L. (1993). Penicillin-binding proteins of Rhodococcus equi: Potential role in resistance to imipenem. *Antimicrobial Agents and Chemotherapy*, *37*(7), 1406–1409. https://doi.org/10.1128/AAC.37.7.1406

Pechère, J.-C. (2001). Macrolide resistance mechanisms in Gram-positive cocci. *International Journal of Antimicrobial Agents*, *18*, 25–28. https://doi.org/10.1016/S0924-8579(01)00407-1

Ranganath, N., Mendoza, M. A., Stevens, R. W., Kind, D. R., Wengenack, N., & Shah, A. S. (2024). Rhodococcus infection: A 10-year retrospective analysis of clinical experience

and antimicrobial susceptibility profile. *Journal of Clinical Microbiology*. https://doi.org/10.1128/jcm.01537-23

Rivera-Velez, A. G., Huber, L., Sinha, S., & Cohen, N. D. (2022). Fitness cost conferred by the novel erm(51) and rpoB mutation on environmental multidrug resistant-Rhodococcus equi. *Veterinary Microbiology*. https://doi.org/10.1016/J.VETMIC.2022.109531

Sanz, M. G. (2023). Rhodococcus equi–What is New This Decade? *Veterinary Clinics of North America: Equine Practice*. https://doi.org/10.1016/J.CVEQ.2022.11.002

Scarpellini, G. (n.d.). *Cohen's Kappa Free Calculator. 2022* [Computer software]. Idostatistics. Retrieved https://idostatistics.com/cohen-kappa-free-calculator/

Sellon, D. C., Besser, T. E., Vivrette, S. L., & McConnico, R. S. (2001). Comparison of Nucleic Acid Amplification, Serology, and Microbiologic Culture for Diagnosis of *Rhodococcus equi* Pneumonia in Foals. *Journal of Clinical Microbiology*, *39*(4), 1289–1293. https://doi.org/10.1128/JCM.39.4.1289-1293.2001

Song, Y., Jiang, M., Xu, X., Huang, Z., Xiao, Y., Jiang, M., Yin, S., Zheng, M., Zheng, M.-X., Meng, H., Han, Y., Wang, Y., Wang, D., & Wei, Q. (2022). Genomic Characteristics Revealed Plasmid-Mediated Pathogenicity and Ubiquitous Rifamycin Resistance of Rhodococcus equi. *Frontiers in Cellular and Infection Microbiology*. https://doi.org/10.3389/fcimb.2022.807610

Stefańska, I., Witkowski, L., Rzewuska, M., & Dzieciątkowski, T. (2016). Development and evaluation of the internal-controlled real-time PCR assay for Rhodococcus equi detection in various clinical specimens. *Journal of Veterinary Medical Science*. https://doi.org/10.1292/jvms.15-0516

Val-Calvo, J., Buckley, T., Darcy, J., Gibbons, J., Gibbons, J. F., Creighton, A., Egan, C., Buckley, T., Schmalenberger, A., Fogarty, U., Scortti, M., & Vázquez-Boland, J. A. (2022). International Spread of Multidrug-Resistant Rhodococcus equi. *Emerging Infectious Diseases*. https://doi.org/10.3201/eid2809.220222

Vázquez-Boland, J. A., & Meijer, W. G. (2019). The pathogenic actinobacterium *Rhodococcus equi*: What's in a name? *Molecular Microbiology*, *112*(1), 1–15. https://doi.org/10.1111/mmi.14267

Vázquez-Boland, J. A., Sanz, M. G., & Prescott, J. F. (2022). Rhodococcus equi. In *Pathogenesis of Bacterial Infections in Animals* (1st ed., pp. 715–735). Wiley. https://doi.org/10.1002/9781119754862.ch33

Yerlikaya, Z., Miranda-CasoLuengo, R., Yin, Y., Cheng, C., & Meijer, W. G. (2025). Clade-1 Vap virulence proteins of Rhodococcus equi are associated with the cell surface and support intracellular growth in macrophages. *PLoS ONE*. https://doi.org/10.1371/JOURNAL.PONE.0316541

Żychska, M., Witkowski, L., Klementowska, A., Rzewuska, M., Kwiecień, E., Stefańska, I., Czopowicz, M., Szaluś-Jordanow, O., Mickiewicz, M., Moroz, A., Bonecka, J., & Kaba, J. (2021). Rhodococcus equi-Occurrence in Goats and Clinical Case Report. *Pathogenetics*. https://doi.org/10.3390/PATHOGENS10091141

Figures and Tables

Table 1: Oligonucleotides used in this study

Targets		Oligonucleotide sequences (5'-3')	Gene & amplicon size
choE	Forward primer	CGAGTCCACCGAGTGGTCAAG	cholesterol oxidase enzyme; 172 bp
	reverse primer	GTTRCCGTAGATGACCTCACCG	
	probe	6-FAM/AC GTG TAC G/ZEN/A CTT CGA GTA CAT GAA GAA GG/3IABkFQ	
vapA	Forward primer	ACGTCCGAAAGGAGAAGTAAGATGA	VAMP-Associated Protein A; 161 bp
	reverse primer	GCCCCACTATTGAGAATCGCA	
erm(46)	Forward primer	AGTCGATGCAGGGCTGATGAC	transposon (TnRErm46); 169 bp
	reverse primer	CTTAACCGCGGCCTTCTCAG	
	probe	CAAGGGCCACGGYCTGCAT/5YakYel/CA AGG GCC A/ZEN/C GGY CTG CAT /3IABkFQ/	
erm(51)	Forward primer	CGACGCCGRTGTCCTGGT	transposon (TnRErm51); 166 bp
	reverse primer	AGCACACCGTCGTTCACGC	

This table lists the primers and probes used for the detection of *choE, vapA, erm*(46), and *erm*(51) by PCR. Oligonucleotide sequences are shown in the 5′–3′ orientation. Gene targets and corresponding amplicon sizes are indicated.

Table 2: Antimicrobial susceptibility profiles of isolates based on MIC testing

Antibiotic	Susceptible	Intermediate	Resistant	Not Available
Ceftiofur	157	0	0	72
Chloramphenicol	192	18	19	0
Clarithromycin	179	6	38	6
Doxycycline	219	7	0	3
Enrofloxacin	153	9	61	6
Erythromycin	180	1	43	5
Gentamicin	229	0	0	0
Marbofloxacin	208	15	0	6
Minocycline	223	0	0	6
Rifampin	189	1	33	6
Tetracycline	200	6	22	1
Azithromycin	131	0	29	69

Table 3: PCR detection frequency of individual gene targets among isolates

Gene	Positive	Negative	Positivity (%)
vapA	188	41	82.1%
erm(46)	128	101	55.9%
erm(51)	1	228	0.4%

Table 4. Distribution of isolates by number and combination of PCR-detected gene targets

Number of Genes present	Number of Isolates	Percentage, %
0	41	17.9%
1	60	26.2%
2	127	55.5%
3	1	0.4%

Table 5: Prevalence of *erm(*46)-positive isolates from 2021 to 2023

Year	Total Isolates	Positive	Positivity (%)
2021	87	42	48.3%
2022	65	53	81.5%
2023	46	32	69.6%

Table 6: Biannual distribution of *erm*(46)-positive isolates from 2021 to 2023.

Period	Total Isolates	Positive	Negative	Positivity, %
2021-Jan-June	63	31	32	49.2%
2021-July-Dec	24	11	13	45.8%
2022-Jan-June	39	29	10	74.4%
2022-July-Dec	26	24	2	92.3%
2023-Jan-June	32	18	14	56.3%
2023-July-Dec	14	14	0	100.0%

Table 7: Concordance between *erm(*46) PCR detection and phenotypic antimicrobial susceptibility results

Antibiotic	Isolates	Matched isolates	
		Number	Percentage
Clarithromycin	194	109	56.2%
Erythromycin	195	109	55.9%
Azithromycin	154	85	55.2%
Rifampin	194	95	49.0%
Enrofloxacin	194	93	47.9%
Tetracycline	198	92	46.5%
Chloramphenicol	199	85	42.7%
Marbofloxacin	194	76	39.2%
Doxycycline	196	76	38.8%
Ceftiofur	151	58	38.4%
Minocycline	194	70	36.1%
Gentamicin	199	71	35.7%

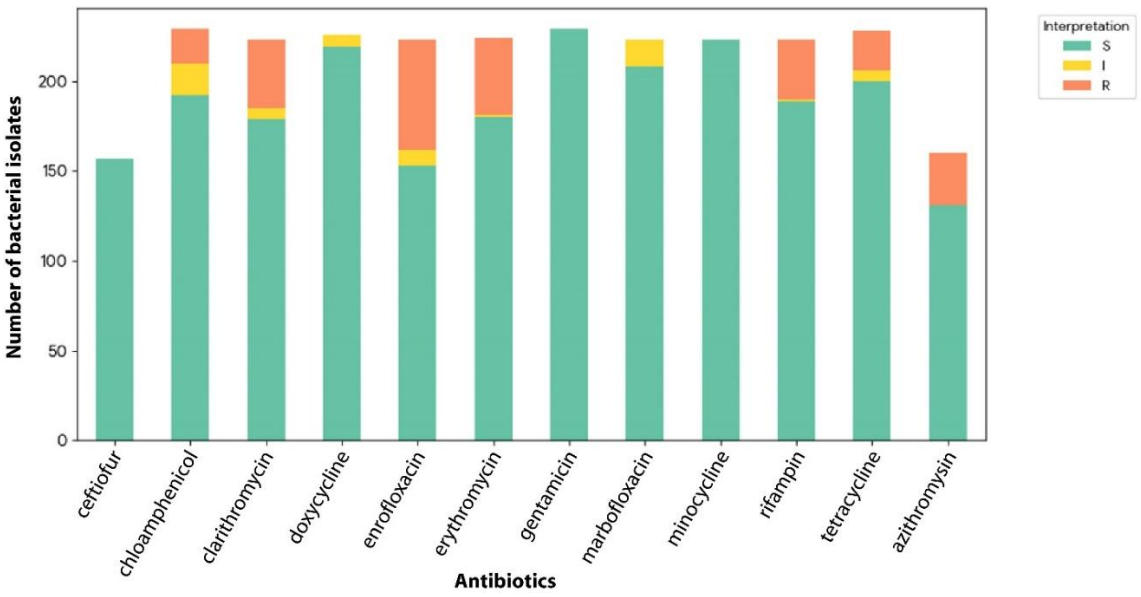

Figure 1: MIC-based Antibiotic susceptibility interpretation distribution across Bacterial Isolates. For each antibiotic, stacked bars represent the antimicrobial susceptibility profiles for 12 antibiotics tested against bacterial isolates (n=229 total isolates). For each antibiotic, bars are segmented to show the number of isolates classified as susceptible (green), intermediate (yellow), or resistant (red) according to Clinical and Laboratory Standards Institute (CLSI) interpretive criteria. The x-axis lists the antibiotics tested, and the y-axis indicates the total number of isolates evaluated for each antimicrobial agent. Note that not all isolates were tested against every antibiotic; "Not Available" data (see Table 2) indicate isolates for which MIC testing was not performed for a given antimicrobial. Gentamicin showed universal susceptibility (229/229 isolates), and no intermediate or resistant isolates were identified for ceftiofur(157 isolates) and minocycline(233 isolates). Enrofloxacin had the highest resistance rate (61/222 tested isolates, 27.5%), followed by erythromycin (43/224, 19.2%), azithromycin (29/160, 18.1%), clarithromycin (38/223, 17.0%), and rifampin (33/223, 14.8%). Corresponding numerical values and detailed susceptibility data are provided in Table 2.

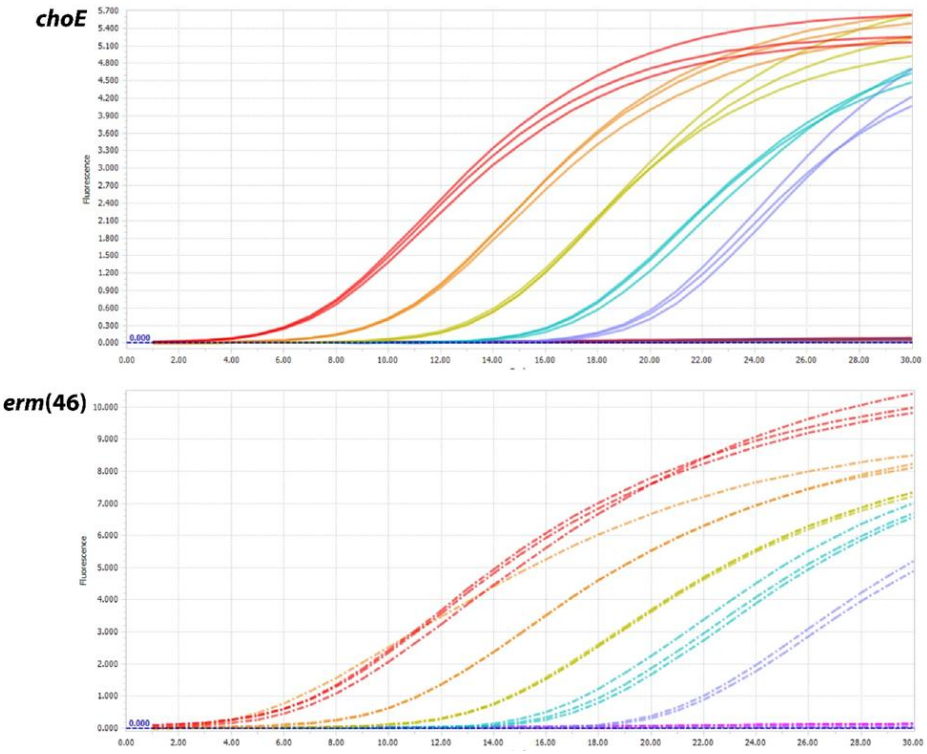

Figure 2: TaqMan-based real-time PCR amplification of choE and erm(46) standards.

TaqMan real-time PCR amplification curves for *choE* and *erm*(46) targets are shown. Panel A (*choE*) displays amplification curves using FAM fluorophore detection, while panel B (*erm*(46)) shows amplification curves using VIC fluorophore detection. Both panels show triplicate runs of ten-fold serial dilutions of DNA standards ranging from 10^4 to 10^0 copies. Both TaqMan assays demonstrated analytical sensitivity down to single copy detection, confirming high assay sensitivity for species identification (*choE*) and macrolide resistance detection (*erm*(46)). The use of different fluorophore channels (FAM for *choE*, VIC for *erm*(46) enables simultaneous detection in a duplex format. Amplification was performed using the LightCycler® 96 real-time PCR system (Roche Diagnostics, Indianapolis, IN, USA). The consistent amplification patterns across the dilution series validate quantitative performance and reproducibility.

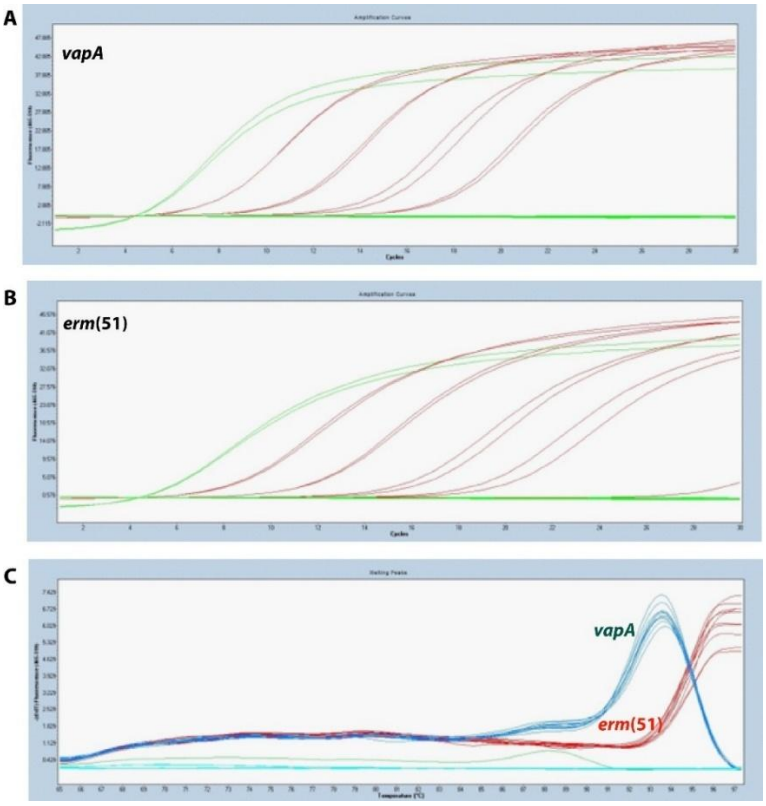

Figure 3: SYBR Green real-time PCR amplification curve and melting curve of Standards for *erm*(51) and *vapA*. SYBR Green real-time PCR amplification and melting curve analysis for *vapA* and *erm*(51) targets. Panels A (*vapA*) and B (*erm*(51)) display amplification curves from duplicate runs of ten-fold serial dilutions of DNA standards ranging from 10^4 to 10^0 copies, with both targets achieving single copy detection sensitivity. Panel C shows melting curve analysis, demonstrating clear differentiation between *vapA* and *erm*(51) targets based on distinct melting temperatures, confirming that these targets can be effectively used in a duplex format without cross-reactivity. Amplification and melting curve analysis were performed using the LightCycler® 480 II real-time PCR platform (Roche Diagnostics, Indianapolis, IN, USA). The differential melting profiles validate the specificity of SYBR Green-based detection and support the simultaneous virulence (*vapA*) and resistance (*erm*(51)) screening in a single reaction.

Figure 4: Multiple sequence alignment of *choE* gene amplicons from *R. equi* isolates.

MEGA (MUSCLE) sequence alignment of 171 bp (first 55 bp shown) *choE* amplicon sequences from 20 *R. equi* clinical isolates compared to a *choE* reference sequence (GenBank accession: AJ242746.1). The alignment demonstrates 100% sequence identity across all isolates with 97-100% query coverage, confirming species-specific target identification and validating PCR assay specificity for *R. equi* detection. This high degree of conservation validates the reliability of *choE* as a molecular diagnostic target for rapid *R. equi* identification in clinical specimens.

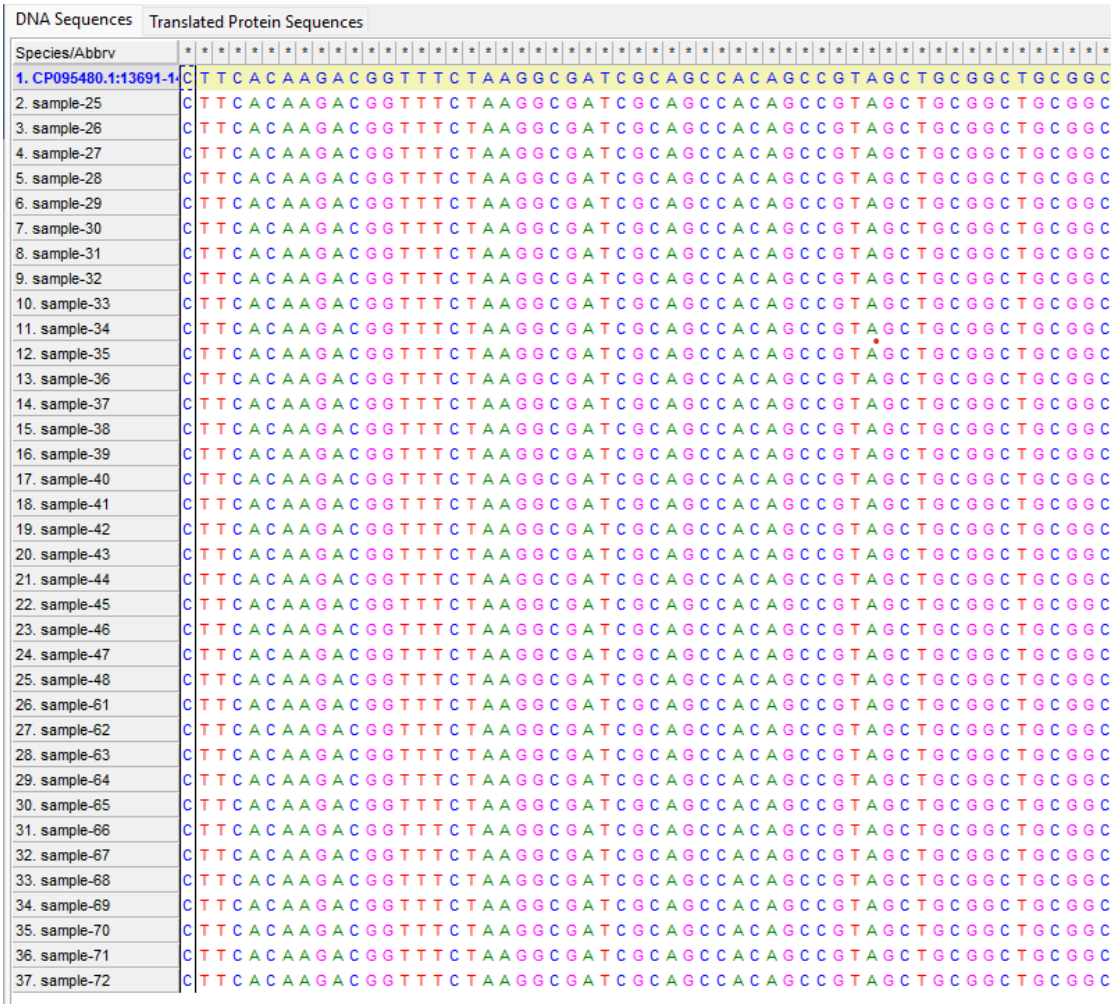

Figure 5: Multiple sequence alignment of *vapA* gene amplicons from *R. equi* isolates.

MEGA (MUSCLE) sequence alignment of 141 bp (first 55 bp shown) *vapA* amplicon sequences from 35 representative virulent *R. equi* clinical isolates compared to the equine virulence plasmid pVAPA reference sequence (GenBank accession: AP038828.1). The alignment shows 100% sequence identity across all isolates with 94-100% query coverage, confirming virulence plasmid detection and validating molecular identification of pathogenic strains. This sequence conservation validates *vapA* detection as a reliable marker for distinguishing virulent clinical strains from avirulent environmental *R. equi*, supporting its use in molecular diagnostics for identifying clinically relevant infections in foals.

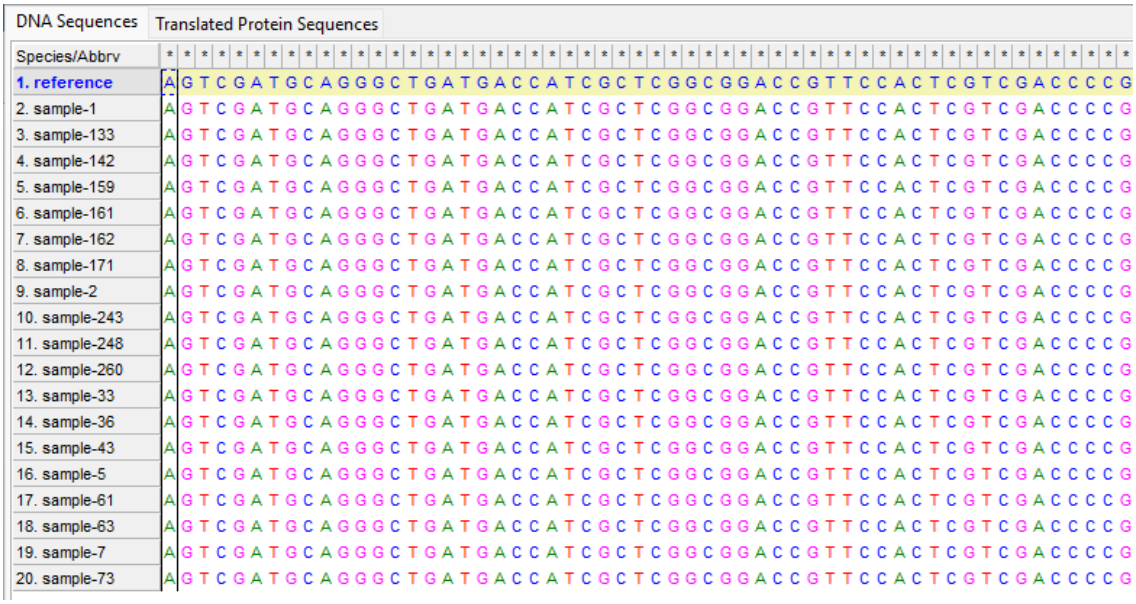

Figure 6: Multiple sequence alignment of *erm*(46) gene amplicons from *R. equi* isolates. MEGA (MUSCLE) sequence alignment of 170 bp (first 55 bp shown) *erm*(46) amplicon sequences from 19 representative *erm*(46) positive *R. equi* clinical isolates compared to a reference sequence (GenBank accession: NG_054941.1). The alignment demonstrates 100% sequence identity across all isolates with 94-100% query coverage, confirming *erm*(46) gene detection and validating molecular identification of the dominant macrolide resistance determinant. The identical sequences validate *erm*(46) PCR as a reliable predictor of macrolide resistance phenotypes, supporting its use for rapid resistance screening to guide antimicrobial therapy decisions in *R. equi* pneumonia cases.

Figure 7: Multiple sequence alignment of *erm*(51) gene amplicon from *R. equi* isolates.

MEGA (MUSCLE) sequence alignment of the 169 bp (first 55 bp shown) *erm*(51) amplicon sequence from the positive clinical isolates compared to the novel environmental resistance determinant reference sequence (GenBank accession: MN928789.1). The alignment shows 100% sequence identity with 100% query coverage, confirming detection of the rare macrolide resistance gene associated with transposon TnRErm51 and conjugative plasmid pRErm51. This confirms molecular identification of this novel resistance determinant and validates assay specificity for *erm*(51) detection distinct from other erm family genes. The rarity of this resistance gene in clinical isolates (0.4% prevalence) combined with its association with horizontal gene transfer elements highlights the importance of continued surveillance for emerging resistance mechanisms that could potentially spread from environmental reservoirs to virulent clinical strains.

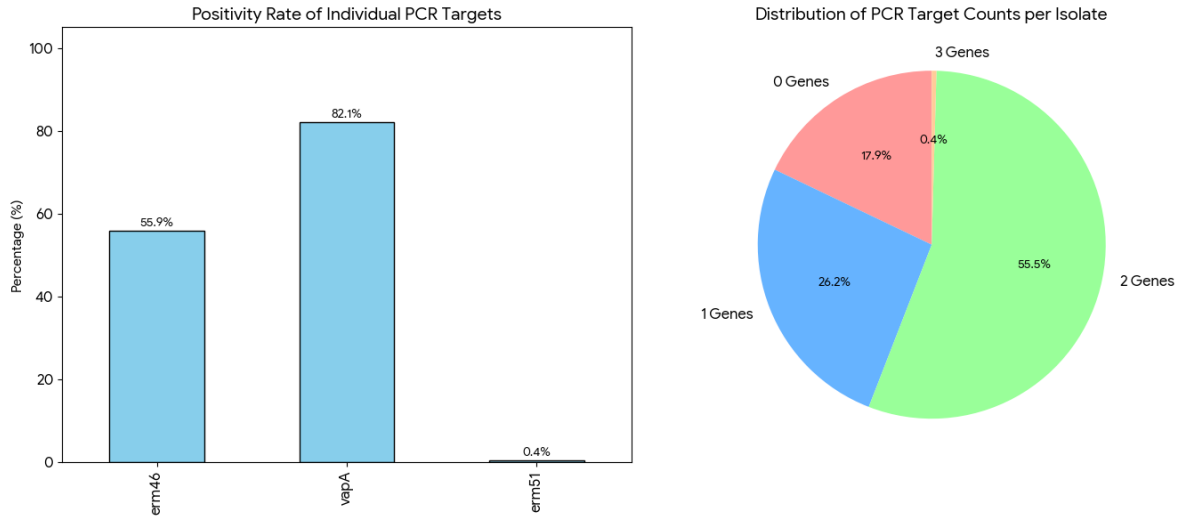

Figure 8: PCR positivity rates for individual gene targets and distribution of multiple gene target positivity among isolates. (A)Bar graph showing the detection frequency of individual gene targets among all isolates (n=229). The y-axis represents the percentage of PCR-positive isolates, and the x-axis displays the three gene targets examined: *vapA* , *erm*(46), and *erm*(51). The *vapA* gene was detected in most isolates (188/229, 82.1%), followed by *erm*(46) (128/229, 55.9%), while *erm*(51) was rarely detected (1/229, 0.4%). (B) Pie chart illustrates the distribution of isolates according to the quantity of gene targets detected per isolate. 127 isolates(55.5%) tested positive for two gene targets, 60 isolates (26.2%) harbored a single gene target, 41 isolates (17.9%) were negative for all three tested genes, and only one isolate (0.4%) was positive for all three gene targets. The high co-occurrence of *vapA* and *erm*(46) suggests these genes may be commonly co-selected in this population of isolates. Detailed numerical data and gene target combinations are provided in Tables 3 and 4.

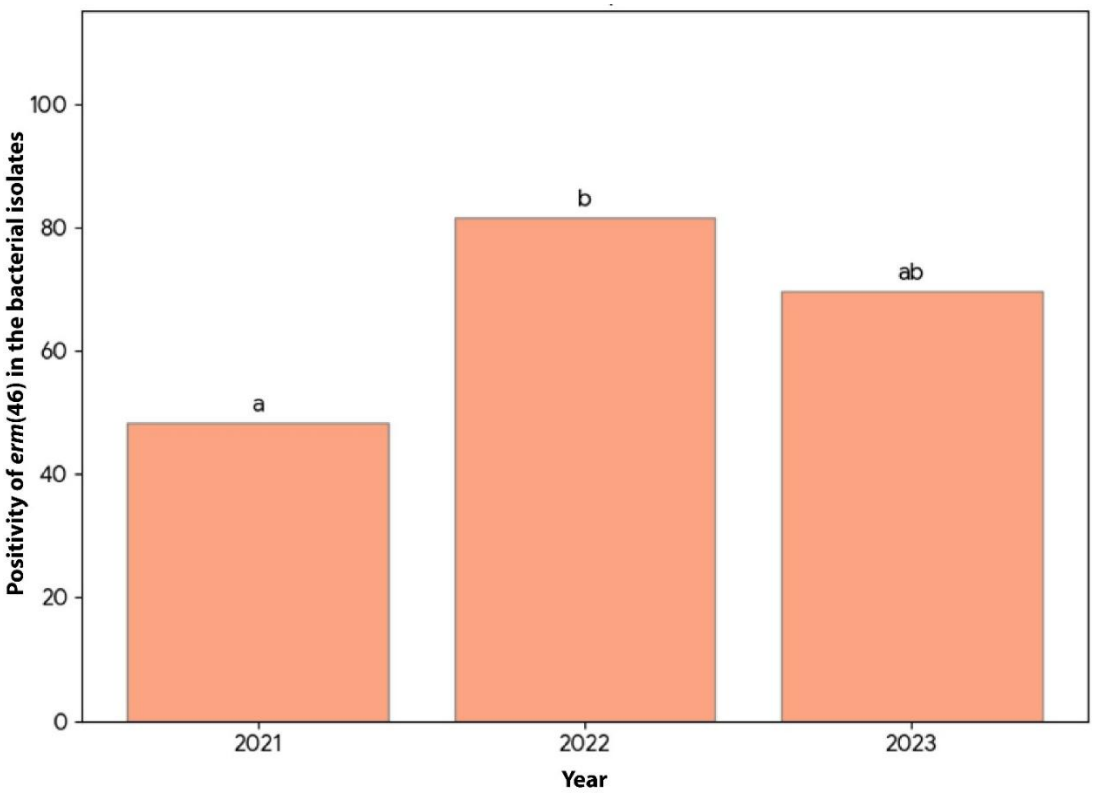

Figure 9: Annual prevalence of *erm*(46)-positive isolates from 2021 to 2023. Bars represent the annual percentage of isolates positive for *erm*(46) by PCR. The y-axis indicates the positivity rate (%), and the x-axis shows the collection year. Chi-square test of independence revealed significant temporal variation in *erm*(46) prevalence across the study period ($\chi^2 = 18.64$, df = 2, $p < 0.001$, two-tailed), with prevalence rising from 48.3% (42/87 isolates) in 2021 to 81.5% (53/65 isolates) in 2022, followed by a moderate decline to 69.6% (32/46 isolates) in 2023. This analysis assessed whether the frequency of *erm*(46) resistance markers in *R. equi* isolates significantly shifted over time. The observed temporal variation may reflect changes in antimicrobial selection pressure, clonal expansion of resistant strains, or shifts in the circulating bacterial population. Numerical data, isolate counts, and statistical groupings are provided in Table 5. Statistical significance was established at $p < 0.05$.

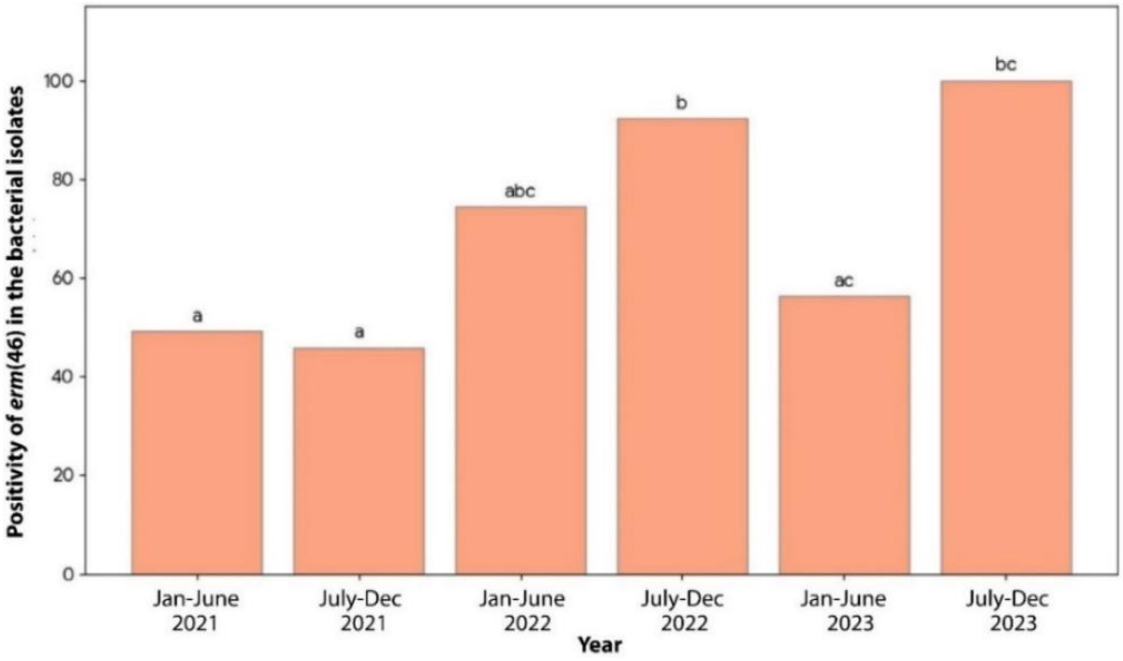

Figure 10: Biannual *erm*(46) positivity rates among isolates from 2021 to 2023. Bars represent the percentage of isolates positive for the *erm*(46) gene by PCR during each six-month period. The x-axis displays biannual periods, where the first half (January–June) and second half (July–December) of each year are shown separately. The y-axis indicates the *erm*(46) positivity rate. In 2021, positivity rates remained relatively stable with a slight decrease from the first half (31/63 isolates, 49.2%) to the second half (11/24 isolates, 45.8%) ($p = 0.777$, Chi-square test). In contrast, 2022 showed a marked progressive increase, rising from 74.4% (29/39 isolates) in the first half to 92.3% (24/26 isolates) in the second half ($p = 0.068$, Chi-square test). In 2023, positivity increased significantly from 56.3% (18/32 isolates) in the first half to 100% (14/14 isolates) in the second half ($p = 0.013$, Fisher's exact test), though the latter period had the smallest sample size. The peak prevalence occurred in 2022 July-Dec and 2023 July-Dec, with the latter showing complete saturation. This biannual analysis provides a more detailed picture than annual trends (Figure 9) and reveals complex within-year and between-year dynamics that

may reflect seasonal influences, changing antimicrobial use patterns, clonal expansion events, or epidemiological shifts in the bacterial population. Detailed numerical data including isolate counts are provided in Table 6.

Cumulative References

Alvarez Narvaez, S., & Sanchez, S. (2023). Exploring the accessory genome of multidrug-resistant Rhodococcus equi clone 2287. Antibiotics, 12(11), 1631. https://doi.org/10.3390/antibiotics12111631

Álvarez-Narváez, S., Berghaus, L. J., Morris, E. R. A., Willingham-Lane, J. M., Slovis, N. M., Giguère, S., & Cohen, N. D. (2020). A common practice of widespread antimicrobial use in horse production promotes multi-drug resistance. Scientific Reports, 10(1), 3045. https://doi.org/10.1038/s41598-020-57479-9

Álvarez-Narváez, S., Berghaus, R. D., Huber, L., Giguère, S., Hart, K. A., Sánchez, S., & Cohen, N. D. (2021). Epidemiology and molecular basis of multidrug resistance in Rhodococcus equi. Microbiology and Molecular Biology Reviews, 85(2), e00011-21. https://doi.org/10.1128/mmbr.00011-21

Álvarez-Narváez, S., Fernández, I., Patel, N. V., & Sánchez, S. (2022). Novel quantitative PCR for Rhodococcus equi and macrolide resistance detection in equine respiratory samples. Animals, 12(9), 1172. https://doi.org/10.3390/ani12091172

Álvarez-Narváez, S., Giguère, S., Berghaus, L. J., Dailey, C., & Vázquez-Boland, J. A. (2020). Horizontal spread of Rhodococcus equi macrolide resistance plasmid pRErm46 across environmental actinobacteria. Applied and Environmental Microbiology, 86(6), e00108-20. https://doi.org/10.1128/aem.00108-20

Álvarez-Narváez, S., Giguère, S., Cohen, N. D., Slovis, N. M., & Vázquez-Boland, J. A. (2021). Spread of multidrug-resistant Rhodococcus equi, United States. Emerging Infectious Diseases, 27(2), 529-537. https://doi.org/10.3201/eid2702.203030

Anastasi, E., MacArthur, I., Scortti, M., Álvarez-Narváez, S., Giguère, S., & Vázquez-Boland, J. A. (2016). Pangenome and phylogenomic analysis of the pathogenic actinobacterium Rhodococcus equi. Genome Biology and Evolution, 8(10), 3140-3148. https://doi.org/10.1093/gbe/evw222

Andersen, S. J., Quan, S., Gowan, B., & Dabbs, E. R. (1997). Monooxygenase-like sequence of a Rhodococcus equi gene conferring increased resistance to rifampin by inactivating this antibiotic. Antimicrobial Agents and Chemotherapy, 41(1), 218-221. https://doi.org/10.1128/aac.41.1.218

Baptiste, K. E., Kyvsgaard, N. C., Ahmed, M. O., Damborg, P., & Dowling, P. M. (2025). Is rifampin (rifampicin) essential for the treatment of Rhodococcus equi infections in foals? A critical review of the role of rifampin. Journal of Veterinary Pharmacology and Therapeutics, 48(1), 1-15. https://doi.org/10.1111/jvp.70007

Barton, M. D., & Hughes, K. L. (1984). Ecology of Rhodococcus equi. Veterinary Microbiology, 9(1), 65-76. https://doi.org/10.1016/0378-1135(84)90079-8

Barua, S., Tarannum, A., Huber, L., Easterwood, L. A., Velayudhan, B., Da Silveira, B. P., Enyetornye, B., Cohen, N. D., Dimitrov, K. M., Schwarz, E. R., Awtrey, A., Groover, E., Naskou, M., & Wang, C. (2026). Epidemiology and risk factors of equine parvovirus-hepatitis, hepacivirus, Pegivirus caballi, and Pegivirus equi in horses from the Southern

United States. Veterinary Microbiology, 312, 110831. https://doi.org/10.1016/j.vetmic.2025.110831

Benoit, S., Taouji, S., Benachour, A., & Hartke, A. (2000). Resistance of Rhodococcus equi to acid pH. International Journal of Food Microbiology, 55(1-3), 295-298. https://doi.org/10.1016/s0168-1605(00)00172-0

Berghaus, L. J., Giguère, S., Guldbech, K., Warner, E., Ugorji, U., & Berghaus, R. D. (2015). Comparison of Etest, disk diffusion, and broth macrodilution for in vitro susceptibility testing of Rhodococcus equi. Journal of Clinical Microbiology, 53(1), 314-318. https://doi.org/10.1128/jcm.02618-14

Berghaus, L. J., Giguère, S., & Guldbech, K. (2013). Mutant prevention concentration and mutant selection window for 10 antimicrobial agents against Rhodococcus equi. Veterinary Microbiology, 166(3-4), 670-675. https://doi.org/10.1016/j.vetmic.2013.07.006

Bordin, A. I., Huber, L. E., Sanz, M. G., & Cohen, N. D. (2022). Rhodococcus equi foal pneumonia: Update on epidemiology, immunity, treatment, and prevention. Equine Veterinary Journal, 54(5), 810-826. https://doi.org/10.1111/evj.13567

CLSI. (2021). *CLSI. Performance Standards for Antimicrobial Susceptibility Testing: CLSI Supplement M100. 31st ed. Clinical and Laboratory Standards Institute; 2021.* https://doi.org/10.1128/jcm.00213-21

CLSI. (2022). *Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 32nd ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.*

CLSI. (2023). *Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 33rd ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2023.* (Versions M100-Ed33 (2023)). https://webstore.ansi.org/standards/clsi/clsim100ed33#:~:text=The%20CLSI%20M100%2DEd33%20is%20the%2033rd%20edition,**Non%20printable**%20Some%20documents%20cannot%20be%20printed

Cohen, N. D., Flores-Ahlschewde, P., Gonzales, G. M., Kahn, S. K., da Silveira, B. P., Bray, J. M., King, E. E., Blair, C. C., & Bordin, A. I. (2022). Fecal concentration of Rhodococcus equi determined by quantitative polymerase chain reaction of rectal swab samples to differentiate foals with pneumonia from healthy foals. Journal of Veterinary Internal Medicine, 36(3), 1146-1151. https://doi.org/10.1111/jvim.16438

Cohen, N. D., & Moyer, W. (2009). Control and prevention of Rhodococcus equi foal pneumonia. Veterinary Clinics of North America: Equine Practice, 25(2), 203-220.

Cohen, N. D., O'Conor, M. S., Chaffin, M. K., & Martens, R. J. (2005). Farm characteristics and management practices associated with development of Rhodococcus equi pneumonia in foals. Journal of the American Veterinary Medical Association, 226(3), 404-413. https://doi.org/10.2460/javma.2005.226.404

Dawson, T. R., Horohov, D. W., Meijer, W. G., & Muscatello, G. (2010). Current understanding of the equine immune response to Rhodococcus equi. An immunological review of R. equi pneumonia. Veterinary Immunology and Immunopathology, 135(1-2), 1-11. https://doi.org/10.1016/j.vetimm.2009.12.004

EFSA Panel on Animal Health and Welfare, Nielsen, S. S., Bicout, D. J., Calistri, P., Canali, E., Drewe, J. A., Garin-Bastuji, B., Gonzales Rojas, J. L., Gortázar, C., Herskin, M., Michel, V., Miranda Chueca, M. Á., Padalino, B., Pasquali, P., Roberts, H. C., Spoolder, H., Ståhl, K., Velarde, A., Viltrop, A., & Alvarez, J. (2022). Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): Antimicrobial-resistant Rhodococcus equi in horses. EFSA Journal, 20(2), 7081. https://doi.org/10.2903/j.efsa.2022.7081

Erol, E., Scortti, M., Fortner, J., Patel, M., & Vázquez-Boland, J. A. (2021). Antimicrobial resistance spectrum conferred by pRErm46 of emerging macrolide (multidrug)-resistant Rhodococcus equi. Journal of Clinical Microbiology, 59(8), e01149-21. https://doi.org/10.1128/jcm.01149-21

Erol, E., Shaffer, C. L., & Lubbers, B. V. (2021). Synergistic combinations of clarithromycin with doxycycline or minocycline reduce the emergence of antimicrobial resistance in Rhodococcus equi. Equine Veterinary Journal, 53(6), 1278-1285. https://doi.org/10.1111/evj.13508

Fines, M., Pronost, S., Maillard, K., Taouji, S., & Leclercq, R. (2001). Characterization of mutations in the rpoB gene associated with rifampin resistance in Rhodococcus equi isolated from foals. Journal of Clinical Microbiology, 39(8), 2784-2787. https://doi.org/10.1128/jcm.39.8.2784-2787.2001

Ghielmetti, G., Stevens, M. J. A., Schmitt, S., Kittl, S., Cernela, N., Biggel, M., Schulthess, B., Keller, P. M., Schrenzel, J., & Stephan, R. (2025). Multi-host distribution of Rhodococcus equi (Prescottella equi) strains and their phylogenomic clustering. BMC Microbiology, 25(1), 15. https://doi.org/10.1186/s12866-025-04152-8

Giguère, S., Berghaus, L. J., & Willingham-Lane, J. M. (2017). Antimicrobial resistance in Rhodococcus equi. Microbiology Spectrum, 5(5), ARBA-0004-2016. https://doi.org/10.1128/microbiolspec.arba-0004-2016

Giguère, S., Cohen, N. D., Chaffin, M. K., Slovis, N. M., Hondalus, M. K., Hines, S. A., & Prescott, J. F. (2011). Diagnosis, treatment, control, and prevention of infections caused by Rhodococcus equi in foals. Journal of Veterinary Internal Medicine, 25(6), 1209-1220. https://doi.org/10.1111/j.1939-1676.2011.00835.x

Giguère, S., Wilkie, B. N., & Prescott, J. F. (1999). Modulation of cytokine response of pneumonic foals by virulent Rhodococcus equi. Infection and Immunity, 67(10), 5041-5047. https://doi.org/10.1128/iai.67.10.5041-5047.1999

Gressler, L. T., Vargas, A. C. D., Costa, M. M. D., Pötter, L., Silveira, B. P. D., Sangioni, L. A., & Botton, S. D. A. (2014). Genotypic and phenotypic detection of efflux pump in Rhodococcus equi. Brazilian Journal of Microbiology, 45(2), 661-665. https://doi.org/10.1590/s1517-83822014000200040

Hoque, M. M., Valentine, M. J., Kelly, P. J., Barua, S., Murillo, D. F. B., & Wang, C. (2022). Modification of the Folmer primers for the cytochrome c oxidase gene facilitates identification of mosquitoes. Parasites & Vectors, 15(1), 437. https://doi.org/10.1186/s13071-022-05494-2

Huber, L. (2021). Medical management of Rhodococcus equi infections: A clinical epidemiology perspective. Equine Veterinary Education, 33(12), 658-667. https://doi.org/10.1111/eve.13417

Huber, L., Giguère, S., Slovis, N. M., Álvarez-Narváez, S., Hart, K. A., Greiter, M., Morris, E. R. A., & Cohen, N. D. (2020). The novel and transferable erm(51) gene confers macrolides, lincosamides and streptogramins B (MLSB) resistance to clonal Rhodococcus equi in the environment. Environmental Microbiology, 22(6), 2137-2152. https://doi.org/10.1111/1462-2920.15020

Huber, L., Giguère, S., Slovis, N. M., Carter, C. N., Barr, B. S., Cohen, N. D., Elam, J., Erol, E., Locke, S., Phillips, E. D., & Smith, J. L. (2018). Emergence of resistance to macrolides and rifampin in clinical isolates of Rhodococcus equi from foals in Central Kentucky, 1995 to 2017. Antimicrobial Agents and Chemotherapy, 62(12), e01714-18. https://doi.org/10.1128/aac.01714-18

Kaczmarkowska, A., Kwiecień, E., Didkowska, A., Stefańska, I., Rzewuska, M., & Anusz, K. (2023). The genetic diversity and antimicrobial resistance of pyogenic pathogens isolated from porcine lymph nodes. Antibiotics, 12(6), 1026. https://doi.org/10.3390/antibiotics12061026

Khurana, S. K., & Khurana, P. (2015). Rhodococcus equi: A review of epidemiology, diagnosis, and treatment. Veterinary World, 8(12), 1478-1490.

Khurana, S. K., Singha, H., Malik, P., Gulati, B. R., & Singh, R. K. (2014). Molecular characterization of clinical isolates of Rhodococcus equi with PCR assay based on virulence plasmid marker. Indian Journal of Animal Sciences, 84(8), 900-905.

Landis, J. R., & Koch, G. G. (1977). The measurement of observer agreement for categorical data. Biometrics, 33(1), 159-174. https://doi.org/10.2307/2529310

Li, N., Wu, C., Cao, P., Chen, D., Chen, F., & Shen, X. (2024). Multiple systemic infections caused by Rhodococcus equi: A case report. Access Microbiology, 6(1), acmi000600. https://doi.org/10.1099/acmi.0.000600.v4

Madrigal, R. G., Shaw, S. D., Witkowski, L. A., Sisson, B. E., Blodgett, G. P., Chaffin, M. K., & Cohen, N. D. (2016). Use of serial quantitative PCR of the vapA gene of Rhodococcus equi in feces for early detection of R. equi pneumonia in foals. Journal of Veterinary Internal Medicine, 30(2), 664-670. https://doi.org/10.1111/jvim.13828

Niwa, H., & Lasker, B. A. (2010). In vitro fluoroquinolone resistance development in clinical isolates of Rhodococcus equi. Antimicrobial Agents and Chemotherapy, 54(12), 5218-5221.

Nordmann, P., Nicolas, M. H., & Gutmann, L. (1993). Penicillin-binding proteins of Rhodococcus equi: Potential role in resistance to imipenem. Antimicrobial Agents and Chemotherapy, 37(7), 1406-1409. https://doi.org/10.1128/aac.37.7.1406

Pechère, J. C. (2001). Macrolide resistance mechanisms in Gram-positive cocci. International Journal of Antimicrobial Agents, 18(Suppl 1), S25-S28. https://doi.org/10.1016/s0924-8579(01)00407-1

Ranganath, N., Mendoza, M. A., Stevens, R. W., Kind, D. R., Wengenack, N., & Shah, A. S. (2024). Rhodococcus infection: A 10-year retrospective analysis of clinical experience and antimicrobial susceptibility profile. Journal of Clinical Microbiology, 62(4), e01537-23. https://doi.org/10.1128/jcm.01537-23

Rivera-Velez, A. G., Huber, L., Sinha, S., & Cohen, N. D. (2022). Fitness cost conferred by the novel erm(51) and rpoB mutation on environmental multidrug resistant-Rhodococcus

equi. Veterinary Microbiology, 271, 109531. https://doi.org/10.1016/j.vetmic.2022.109531

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J. A., & Pla, M. (2006). Quantitative detection of Listeria monocytogenes and Listeria innocua by real-time PCR: Assessment of hly, iap, and lin02483 targets and AmpliFluor technology. Applied and Environmental Microbiology, 72(2), 1321-1326.

Sanz, M. G. (2023). Rhodococcus equi—What is new this decade? Veterinary Clinics of North America: Equine Practice, 39(1), 1-14. https://doi.org/10.1016/j.cveq.2022.11.002

Scarpellini, G. (2022). Cohen's kappa free calculator [Computer software]. Idostatistics. https://idostatistics.com/cohen-kappa-free-calculator/

Sellon, D. C., Besser, T. E., Vivrette, S. L., & McConnico, R. S. (2001). Comparison of nucleic acid amplification, serology, and microbiologic culture for diagnosis of Rhodococcus equi pneumonia in foals. Journal of Clinical Microbiology, 39(4), 1289-1293. https://doi.org/10.1128/jcm.39.4.1289-1293.2001

Song, Y., Jiang, M., Xu, X., Huang, Z., Xiao, Y., Jiang, M., Yin, S., Zheng, M., Zheng, M. X., Meng, H., Han, Y., Wang, Y., Wang, D., & Wei, Q. (2022). Genomic characteristics revealed plasmid-mediated pathogenicity and ubiquitous rifamycin resistance of Rhodococcus equi. Frontiers in Cellular and Infection Microbiology, 12, 807610. https://doi.org/10.3389/fcimb.2022.807610

Stefańska, I., Witkowski, L., Rzewuska, M., & Dzieciątkowski, T. (2016). Development and evaluation of the internal-controlled real-time PCR assay for Rhodococcus equi detection

in various clinical specimens. Journal of Veterinary Medical Science, 78(4), 651-654. https://doi.org/10.1292/jvms.15-0516

Val-Calvo, J., Buckley, T., Darcy, J., Gibbons, J. F., Creighton, A., Egan, C., Schmalenberger, A., Fogarty, U., Scortti, M., & Vázquez-Boland, J. A. (2022). International spread of multidrug-resistant Rhodococcus equi. Emerging Infectious Diseases, 28(9), 1847-1856. https://doi.org/10.3201/eid2809.220222

Vázquez-Boland, J. A., & Meijer, W. G. (2019). The pathogenic actinobacterium Rhodococcus equi: What's in a name? Molecular Microbiology, 112(1), 1-15. https://doi.org/10.1111/mmi.14267

Vázquez-Boland, J. A., Sanz, M. G., & Prescott, J. F. (2022). Rhodococcus equi. In F. Van Immerseel, J. F. Prescott, J. D. Baggott, & R. J. Yancey (Eds.), Pathogenesis of bacterial infections in animals (5th ed., pp. 715-735). Wiley. https://doi.org/10.1002/9781119754862.ch33

Willingham-Lane, J. M., Berghaus, L. J., Berghaus, R. D., Hurley, D. J., & Giguère, S. (2019). Influence of growth medium on antibiogram results for Rhodococcus equi. Veterinary Microbiology, 236, 108367. https://doi.org/10.1016/j.vetmic.2019.07.017

Yerlikaya, Z., Miranda-CasoLuengo, R., Yin, Y., Cheng, C., & Meijer, W. G. (2025). Clade-1 Vap virulence proteins of Rhodococcus equi are associated with the cell surface and support intracellular growth in macrophages. PLoS ONE, 20(1), e0316541. https://doi.org/10.1371/journal.pone.0316541

Żychska, M., Witkowski, L., Klementowska, A., Rzewuska, M., Kwiecień, E., Stefańska, I., Czopowicz, M., Szaluś-Jordanow, O., Mickiewicz, M., Moroz, A., Bonecka, J., & Kaba,

J. (2021). Rhodococcus equi—Occurrence in goats and clinical case report. Pathogens, 10(9), 1141. https://doi.org/10.3390/pathogens10091141