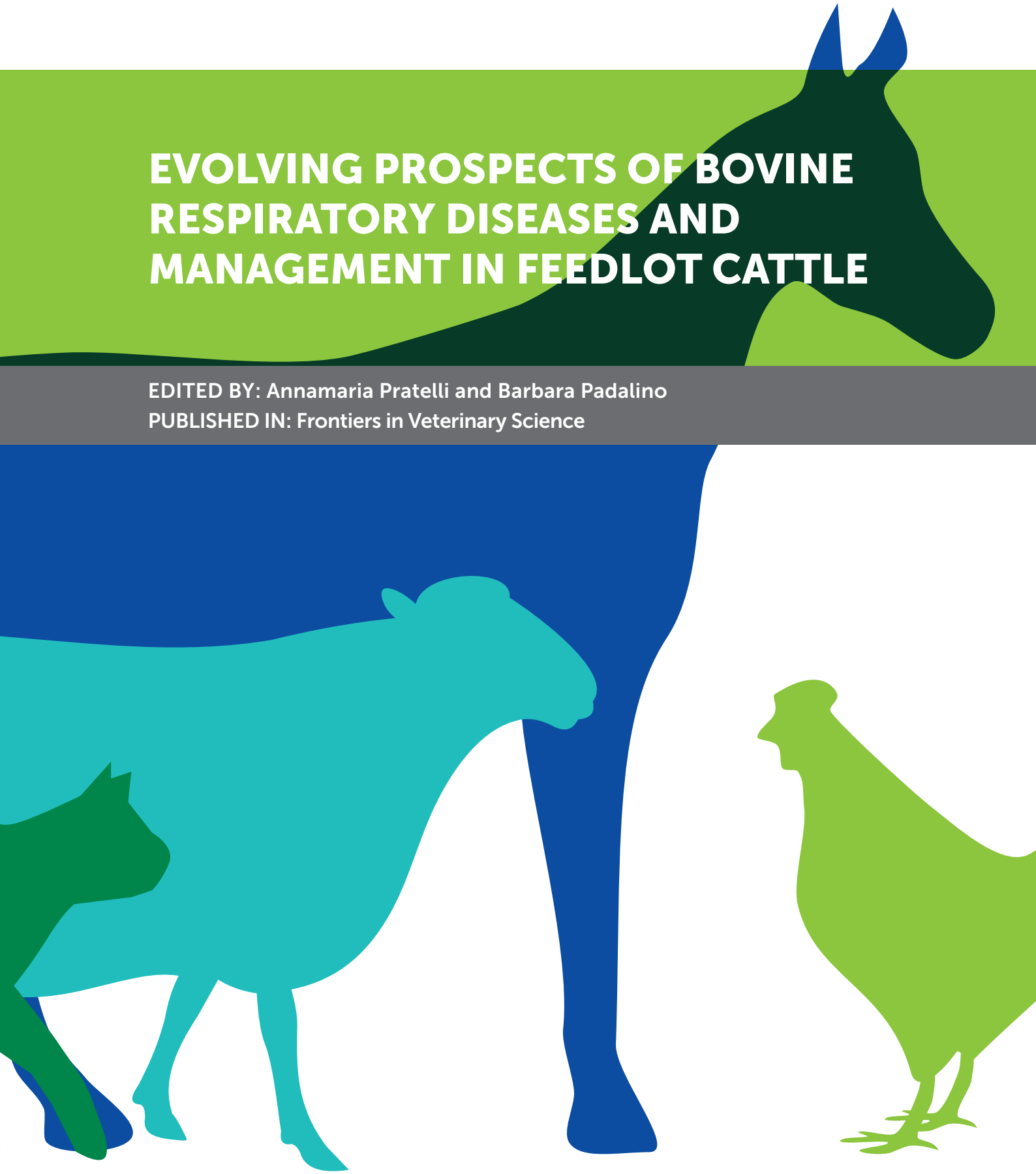
# EVOLVING PROSPECTS OF BOVINE RESPIRATORY DISEASES AND MANAGEMENT IN FEEDLOT CATTLE

EDITED BY: Annamaria Pratelli and Barbara Padalino
PUBLISHED IN: Frontiers in Veterinary Science



frontiers Research Topics

# frontiers



ISSN 1664-8714
ISBN 978-2-88974-708-5
DOI 10.3389/978-2-88974-708-5

## About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

## Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

## Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

## What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

# EVOLVING PROSPECTS OF BOVINE RESPIRATORY DISEASES AND MANAGEMENT IN FEEDLOT CATTLE

Topic Editors:
**Annamaria Pratelli,** University of Bari Aldo Moro, Italy
**Barbara Padalino,** University of Bologna, Italy

**Citation:** Pratelli, A., Padalino, B., eds. (2022). Evolving Prospects of Bovine Respiratory Diseases and Management in Feedlot Cattle.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-708-5

# Table of Contents


05 ***Editorial: Evolving Prospects of Bovine Respiratory Diseases and Management in Feedlot Cattle***
Annamaria Pratelli and Barbara Padalino

08 ***Factors Affecting the Development of Bovine Respiratory Disease: A Cross-Sectional Study in Beef Steers Shipped From France to Italy***
Barbara Padalino, Francesco Cirone, Martina Zappaterra, Daniele Tullio, Gigliola Ficco, Antonio Giustino, Linda Amarachi Ndiana and Annamaria Pratelli

24 ***Prevalence and Risk Factors Associated With Antimicrobial Resistance in Bacteria Related to Bovine Respiratory Disease—A Broad Cross-Sectional Study of Beef Cattle at Entry Into Canadian Feedlots***
Sara Andrés-Lasheras, Reuben Ha, Rahat Zaheer, Catrione Lee, Calvin W. Booker, Craig Dorin, Joyce Van Donkersgoed, Rob Deardon, Sheryl Gow, Sherry J. Hannon, Steve Hendrick, Michele Anholt and Tim A. McAllister

43 ***The Participation of a Malignant Catarrhal Fever Virus and* Mycoplasma bovis *in the Development of Single and Mixed Infections in Beef and Dairy Cattle With Bovine Respiratory Disease***
Thalita Evani Silva Oliveira, Gabriela Sanches Scuisato, Isadora Fernanda Pelaquim, Cristina Wetzel Cunha, Lucas Santana Cunha, Eduardo Furtado Flores, Lucienne Garcia Pretto-Giordano, Júlio Augusto Naylor Lisbôa, Amauri Alcindo Alfieri, João Paulo Elsen Saut, Paulo Henrique Jorge da Cunha and Selwyn Arlington Headley

57 ***Identifying Loci Associated With Bovine Corona Virus Infection and Bovine Respiratory Disease in Dairy and Feedlot Cattle***
Jennifer N. Kiser and Holly L. Neibergs

71 ***Fatal Calf Pneumonia Outbreaks in Italian Dairy Herds Involving* Mycoplasma bovis *and Other Agents of BRD* Complex**
Angela Fanelli, Margie Cirilli, Maria Stella Lucente, Aya Attia Koraney Zarea, Domenico Buonavoglia, Maria Tempesta and Grazia Greco

78 ***Prevalence, Risk Factors, and Antimicrobial Resistance Profile of Respiratory Pathogens Isolated From Suckling Beef Calves to Reprocessing at the Feedlot: A Longitudinal Study***
Diego Nobrega, Sara Andres-Lasheras, Rahat Zaheer, Tim McAllister, Elizabeth Homerosky, R. Michele Anholt and Craig Dorin

94 ***Genomic and Transcriptomic Analysis of Bovine* Pasteurella multocida *Serogroup A Strain Reveals Insights Into Virulence Attenuation***
Li Zhan, Jiaqi Zhang, Boyu Zhao, Xintian Li, Xiqing Zhang, Renge Hu, Emad Mohammed Elken, Lingcong Kong and Yunhang Gao

107 ***The Effects of Ursolic Acid Treatment on Immunopathogenesis Following* Mannheimia haemolytica *Infections***
Jamison R. Slate, Bradley O. Chriswell, Robert E. Briggs and Jodi L. McGill

121 ***Antiviral Effect of Ginsenoside Rb2 and Rb3 Against Bovine Viral Diarrhea Virus and Classical Swine Fever Virus* in vitro**
Bin Tan, Massimo Giangaspero, Na Sun, Yinping Jin, Kexin Liu, Qianying Wang, Shipeng Cheng, Yingping Wang and Shuqin Zhang

130 *Use of Thoracic Ultrasonography to Improve Disease Detection in Experimental BRD Infection*
Madison M. Porter, Paiton O. McDonald, Jamison R. Slate, Amanda J. Kreuder and Jodi L. McGill

145 *Development of a One-Step Multiplex Real-Time PCR Assay for the Detection of Viral Pathogens Associated With the Bovine Respiratory Disease Complex*
Juan Zhang, Wan Wang, Mujiao Yang, Jun Lin, Fei Xue, Yuanmao Zhu and Xin Yin

frontiers in Veterinary Science

EDITORIAL
published: 25 February 2022
doi: 10.3389/fvets.2022.854844

# Editorial: Evolving Prospects of Bovine Respiratory Diseases and Management in Feedlot Cattle

*Annamaria Pratelli*[1]* *and Barbara Padalino*[2]

[1] Department of Veterinary Medicine, University of Bari Aldo Moro, Bari, Italy, [2] Division of Animal Sciences, Department of Agricultural and Food Sciences, Alma Master Studiorum, University of Bologna, Bologna, Italy

**Keywords: bovine, respiratory diseases, health, management, prevention, welfare**

## Editorial on the Research Topic

## Evolving Prospects of Bovine Respiratory Diseases and Management in Feedlot Cattle

Bovine respiratory disease (BRD) is one of the major economical and welfare concerns for the cattle industry worldwide (1). BRD incidence varies enormously according to farm management, prophylaxis measures, involved pathogens, and animal-related predisposing factors since all of them play a decisive role in the development and severity of the disease (2, 3). The Research Topic consequently aims to broaden the current knowledge on BRD etiology, on its pathogenetic mechanisms, on possible hazards related to breeding and transport practices, on immunopathological implications, on new technologies for the diagnosis, on possible prophylaxis and treatment, as well as on risk factors and on emerging antimicrobial resistance (AMR). Due to the multifactorial etiology of the syndrome, the articles encompass within this Research Topic have all a multidisciplinary approach.

Among microorganisms involved in BRD, *Pasteurella multocida* plays a primary role. An interesting study by Zhan et al. elucidated the toxicity targets of *P. multocida* serogroup A providing fundamental information on the pathogenic mechanism and on the antimicrobial drugs resistance of this pathogen of BRD. Similarly, *Mannheimia haemolytica* is involved in BRD onset. Severe infections are often characterized by dysregulated inflammatory responses in the lungs, where IL-17A plays a key role in the inflammatory response activating innate and adaptive immune cells and exacerbating lung congestion. Two independent studies carried out by Slate et al. supported the hypothesis that IL-17A signaling may contribute to lung immunopathology and that further understanding of this inflammatory pathway during BRD could expand therapeutic intervention strategies for managing BRD.

Notoriously, *bovine coronavirus* (BCoV) is one of the most common agents involved in BRD. In the study reported by Kiser and Neibergs several positional candidate genes were identified in association with BRD and BCoV in dairy calves and feedlot cattle in the USA. This study allows to better elucidate the etiology of the disease and allows the identification of loci to be considered for genomic selection, suggesting that selection may reduce susceptibility to BCoV infection and BRD. Oliveira et al. also demonstrated that in addition to the common pathogens, other microorganisms may contribute to the onset of the syndrome. Immunohistochemical assays on formalin-fixed paraffin-embedded pulmonary sections have identified, among the other, *malignant catarrhal fever virus* (MCFV), as well as *Mycoplasma bovis*, in single or mixed infections, as the most frequent pathogen, expanding the list of primary agents in the development of BRD. Moreover, a retrospective study on fatal calf pneumonia in Italy during 2009–2019, allows the identification of *M. bovis* either as the single agent or as a concurrent agent with pathogens correlated with BRD,



***Edited and reviewed by:***
*Michael Kogut,*
*Agricultural Research Service (USDA), United States*

***Correspondence:**
*Annamaria Pratelli*
*annamaria.pratelli@uniba.it*

***Specialty section:***
*This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science*

***Received:*** *14 January 2022*
***Accepted:*** *17 January 2022*
***Published:*** *25 February 2022*

***Citation:***
*Pratelli A and Padalino B (2022) Editorial: Evolving Prospects of Bovine Respiratory Diseases and Management in Feedlot Cattle. Front. Vet. Sci. 9:854844. doi: 10.3389/fvets.2022.854844*

turning the spotlight on *M. bovis* infection and on control measures to apply for the prevention of lethal pneumonia outbreaks in dairy herds (Fanelli et al.).

A 1-year cross-sectional study by Padalino et al. on 169 beef steers documented the prevalence of the aforementioned BRD-related pathogens and the manifested clinical signs before and after a long journey from France to Italy, identifying contextually the possible predisposition factors. The survey demonstrated that the animals displaying clinical signs and positive for the most common pathogens involved in BRD increased dramatically at arrival, that the transport favored coinfections and that the weather conditions were predisposing factors for many of the pathogens. The study also demonstrated that in the majority of the cases co-infections were present and highlighted that understanding of factors responsible for increasing the likelihood of BRD can be useful to reduce or minimize the incidence of the syndrome and to implement animal transport regulations.

Due to its multifactorial etiology, the diagnosis of BRD is still challenging. Since early diagnosis is crucial, in this special issue new diagnostic tools have been presented. A specific one-step multiplex real-time PCR assay was developed for simultaneous detection of five respiratory disease viruses involved in BRD without cross-reaction with others. The test proved to have good specificity and sensitivity allowing rapid detection of pathogens to guide the formulation of BRD prevention and control measures (Zhang et al.). Lastly, in addition to the current laboratory methods for detecting BRD infected calves, thoracic ultrasonography (TUS) was investigated in clinical settings by Porter et al. TUS proved to be able to identify calves with abnormal lung pathology that would have otherwise been misclassified and can provide additional information on calf health due to the high correlation with lung pathology at necropsy. TUS could be consequently recommended on arrival after long-distance journeys to give an early diagnosis and allow timely treatment.

Prevalence and risk factors for the main bacterial pathogens affecting the respiratory tract of calves from the spring processing to the reprocessing at feedlots were also investigated by Nobrega et al. identifying in *P. multocida* the most prevalent species, regardless of time point, but with an increase in prevalence at the weaning/induction sampling. Comingling and co-location of feedlots were not associated with the prevalence of any respiratory pathogen. Contextually, the AMR profile of the four species was characterized phenotypically and genotypically, and limited evidence support increased resistance to respiratory bacteria from the spring processing to reprocessing at feedlots with a few exceptions. On the contrary, parenteral use of macrolides as metaphylaxis at the feedlot induction was associated with an increased minimum inhibitory concentration (MIC) against macrolides in *P. multocida, M. haemolytica*, and *Histophilus somni* contributing to historical changes in macrolides MIC data of respiratory bacteria of beef cattle. Overall, the AMR phenotypes were corroborated by the presence of AMR genes. Prevalence and epidemiology of AMR was also investigated by Andrés-Lasheras et al. on beef cattle upon arrival at Canadian feedlot and before antimicrobials were administered. *M. haemolytica, P. multocida*, and *H. somni* with multidrug-resistant (MDR) profiles were more often isolated from dairy-type than from beef-type cattle, showing that the latter presented higher odds of AMR bacteria as compared to auction-derived calves and that resistance to oxytetracycline was most frequently observed across all *Pasteurellaceae* species and cattle types.

Despite the increasing use of prophylaxis measures, feedlot placement remains a high-risk period for calves to develop respiratory disorders, and new therapy should be proposed. Interestingly, Tan et al. demonstrated that ginsenoside Rb2 and Rb3, major pharmacological ingredients in the plants of ginseng, were able to inhibit the replication and proliferation of bovine and swine pestiviruses, suggesting their potential for effective treatment against infection and thus representing a possible alternative to the use of antibiotics.

These experimental prospective and retrospective studies and critical reviews speculate that the role of the viral infections as the starting point for BRD on which secondary opportunistic bacteria enter, was only a simplistic view of the pathogenesis of the disease, while a primary role of some pathogens rarely detected in the past and generally considered of minor importance, as well as several cofactors such as management of transport and breeding, were identified in eliciting the disease. The potential pathogenetic role for these pathogens and the high frequency with which co-infections occur, make BRD a complex disease difficult to control (4). Despite the increasing use of prophylaxis and treatment measures, feedlot placement remains a high-risk period for calves to develop respiratory disorders, also considering that viral shedding is often greatest even before animals become symptomatic. Consequently, the most important aspects to counteract infections and to restrain the development of BRD and spreading of AMR remain the proper management of cattle, the development of new technologies for early diagnosis, and the application of appropriate prophylaxis measures, since minimizing the risk and the incidence of BRD is crucial to improve cattle productivity, health, and welfare (3, 5, 6). We are proud and highly motivated to promote the development of evidence-based guidelines to prevent BRD in the Frontiers open source venue, and future article collections are needed to further address the gaps of knowledge highlighted in this special issue.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## REFERENCES


1. Earley B, Buckham Sporer K, Gupta S. Invited review: relationship between cattle transport, immunity and respiratory disease. *Anim Int J Anim Biosci.* (2017) 11:486–92. doi: 10.1017/S1751731116001622
2. Cirone F, Padalino B, Tullio D, Capozza P, Lo Surdo M, Lanave G, Pratelli A. Prevalence of pathogens related to bovine respiratory disease before and after transportation in beef steers: preliminary results. *Animals*. (2019) 9:1093. doi: 10.3390/ani9121093
3. Pratelli A, Cirone F, Capozza P, Trotta A, Corrente M, Balestrieri A, Buonavoglia C. Bovine respiratory disease in beef calves supported long transport stress: an epidemiological study and strategies for control and prevention. *Res Vet Sci.* (2021) 135:450–5. doi: 10.1016/j.rvsc.2020.11.002
4. Snowder GD, Van Vleck LD, Cundiff LV, Bennett GL. Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. *J Anim Sci.* (2006) 84:1999–2008. doi: 10.2527/jas.2006-046
5. Moore SJ, O'Dea MA, Perkins N, O'Hara AJ. Estimation of nasal shedding and seroprevalence of organisms known to be associated with bovine respiratory disease in Australian live export cattle. *J Vet Diagn Invest.* (2015) 27:6–17. doi: 10.1177/1040638714559741
6. Holman DB, Timsit E, Amat S, Abbott DW, Buret AG, Alexander TW. The nasopharyngeal microbiota of beef cattle before and after transport to a feedlot. *BMC Microbiol.* (2017) 17:70. doi: 10.1186/s12866-017-0978-6


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 28 June 2021
doi: 10.3389/fvets.2021.627894

# Factors Affecting the Development of Bovine Respiratory Disease: A Cross-Sectional Study in Beef Steers Shipped From France to Italy

*Barbara Padalino[1], Francesco Cirone[2], Martina Zappaterra[1]*, Daniele Tullio[3], Gigliola Ficco[4], Antonio Giustino[5], Linda Amarachi Ndiana[2] and Annamaria Pratelli[2]*

[1] Department of Agricultural and Food Science, University of Bologna, Bologna, Italy, [2] Department of Veterinary Medicine, University of Bari, Bari, Italy, [3] Azienda Sanitaria Locale della Provincia di Bari (ASL BA) – Local Health Authority Veterinary Service, Bari, Italy, [4] Siciliani S.p.A. Industria Lavorazione Carne, Bari, Italy, [5] Self Employed Veterinarian, Bari, Italy

OPEN ACCESS

**Edited by:**
*Beate Conrady, University of Copenhagen, Denmark*

**Reviewed by:**
*Aurélien Madouasse, INRA Biologie, Épidémiologie et Analyse de Risque en santé animale (BIOEPAR), France*
*Selwyn Arlington Headley, State University of Londrina, Brazil*

***Correspondence:**
*Martina Zappaterra*
*martina.zappaterra2@unibo.it*

**Specialty section:**
*This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science*

**Received:** *12 January 2021*
**Accepted:** *24 May 2021*
**Published:** *28 June 2021*

**Citation:**
*Padalino B, Cirone F, Zappaterra M, Tullio D, Ficco G, Giustino A, Ndiana LA and Pratelli A (2021) Factors Affecting the Development of Bovine Respiratory Disease: A Cross-Sectional Study in Beef Steers Shipped From France to Italy. Front. Vet. Sci. 8:627894. doi: 10.3389/fvets.2021.627894*

Bovine respiratory disease (BRD) is a complex, multifactorial syndrome and one of the major welfare and economical concerns for the cattle industry. This 1-year cross-sectional study was aimed at documenting the prevalence of BRD-related pathogens and clinical signs before and after a long journey and at identifying possible predisposition factors. Male Limousine beef steers ($n = 169$) traveling from France to Italy were health checked and sampled with Deep Nasopharyngeal Swabs (DNS) at loading (T0) and 4 days after arrival (T1). Real-time quantitative PCR was used to quantify the presence of bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine alphaherpesvirus 1 (BoHV-1), bovine coronavirus (BCoV), bovine adenovirus (BAdV), bovine parainfluenza virus 3 (BPIV-3), *Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Pasteurella multocida*. Weather conditions at departure and arrival were recorded, and the travel conditions were taken from the travel documentation. At T0, even if no animals displayed clinical signs, some of them were already positive for one or more pathogens. At T1, the number of animals displaying clinical signs and positive for BCoV, BAdV, BRSV, *H. somni*, *M. haemolytica*, *M. bovis*, and *P. multocida* increased dramatically ($p < 0.001$). Transport also significantly increased co-infection passing from 16.0% at T0 to 82.8% at T1 ($p < 0.001$). An extra stop during the journey seemed to favor BRSV, *M. haemolytica*, and *P. multocida* ($p < 0.05$). Weather conditions, in particular sudden climate changes from departure to arrival and daily temperature variance, were found to be predisposing factors for many of the pathogens. The farm of arrival also played a role for BRSV, BAdV, and *H. somni* ($p < 0.05$). BCoV increased dramatically, but no associations were found confirming that it spreads easily during transport phases. Our findings increased our understanding of factors increasing the likelihood of BRD-related pathogens shedding and can be useful to minimize the incidence of BRD and to implement animal transport regulations.

**Keywords: transport, cattle, virus, bacteria, health, welfare**

## INTRODUCTION

Throughout Europe, a population of about 119,357,517 cattle was estimated in 2018, of which 5,923,204 and 18,547,082 were registered in Italy and France, respectively (1). While in France many cattle are kept on pasture, in Italy, they are kept indoors, and every year about 600,000 heads travel from France to Italy for fattening and slaughter purposes (2). Transport is indeed part of the management for most livestock animals, including cattle, having different purposes, such as reaching slaughterhouse, moving to different farms, breeding, fairs, and medical procedures (3). However, transport procedures are known to be stressful for animals, having both short-term and prolonged effects on their health and welfare (4–6). Among transport-related health problems, respiratory diseases are the most common and severe (7).

Bovine respiratory disease (BRD) is a welfare and economic concern in the cattle industry. It affects the lower respiratory tract and is responsible for substantial economic short-term losses due to mortality and costs of treatments and long-term costs that are difficult to assess (7). The syndrome has a multifactorial etiology including infectious agents, host, and environmental factors, such as age, breed, genetic, nutrition, climate, commingling of animals from different sources, marketing, and particularly transport (8, 9). Transportation has been associated with an increased risk of BRD because it is responsible for immune system impairment that favors pathogen prevalence changes and pathogen proliferation (7, 8). Host's immune system suppression and up- and downregulation of the inflammatory responses due to transport stress allow opportunistic pathogens to invade tissues (10). The severity of BRD has furthermore been linked to stressors and primary viral infections, which have been proven to interfere with the mucociliary clearance of the upper respiratory tract and dysregulate the tracheal antimicrobial peptides of the respiratory innate defenses, ultimately enhancing the severity of a secondary bacterial infection (9, 11). A previous study speculated that considering viral infection as the starting point for BRD on which secondary opportunistic bacteria enter was a simplistic view of the pathogenesis of the disease, whereas a primary role of some pathogens rarely detected in the past and generally considered of minor importance was identified in eliciting the disease (12). The potential pathogenetic role for these minor pathogens and the high frequency with which co-infections occur make BRD a complex disease difficult to control.

The prevalence of BRD has been investigated in several studies, and it varies from about 4% to more than 80% (13, 14). In North America, cattle at risk frequently received metaphylactic antimicrobials upon arrival to the feedlot or following feedlot placement, to both mitigate BRD and reduce colonization and proliferation of bacterial pathogens (7). However, considering the need of reducing the use of antibiotics in livestock, it seems to be essential to investigate the factors that favor the development of BRD, and it is essential to minimize them. Preloading handling, journey duration, stocking density, deck levels, season, and environmental factors in the vehicles have all been identified as risk factors for BRD in cattle (15). However, how to transport cattle is still a matter of debate, and regulations on live animal transportation are different among countries and often not respected. For instance, a recent study reported that 437 out of the 979 controlled vehicles transporting cattle across Europe from 2009 to 2013 were found to be not in compliance with the EC 1/2005 during on-road inspections in Italy (16). In Europe, the maximum duration for cattle is set at 29 h, but in recent suggestions for the implementation of the EU 1/2005, the need to reduce the maximum duration was highlighted, and the transport of animals over long journeys should be limited as far as possible (17).

The hypothesis of the study was that a long journey (more than 20 h) from France to Italy would increase the presence of BRD-related viruses and bacteria in the Deep Nasopharyngeal Swabs (DNS), and that the latter would be associated with clinical signs, travel, and weather conditions. The objective of this cross-sectional study was to document the prevalence of the multiple pathogens related to BRD before and after traveling from France to Italy in beef steers over a 1-year time. Potential associations between the different travel conditions, weather, presence of the virus/bacteria, and clinical signs were also investigated.

## MATERIALS AND METHODS

The experimental procedures were approved by the Ethics Committee of the Department of Veterinary Medicine of the University of Bari, Italy (authorization no. 16/18).

### Experimental Design and Sampling

Each year ∼2,000 male beef steers travel from France to the four farms considered in the present study located in Bari Province (data of the co-author GF). These male beef steers are shipped once a week or fortnightly for fattening purposes. A power analysis was conducted using Statulator® (18) to determine the sample size to include in a 1-year cross-sectional study for a target population estimated at 2,000 animals. The number of animals to be assessed was estimated assuming an expected proportion of BRD of 14–15%, with 5% absolute precision and 95% confidence interval (CI). The used proportion of BRD-positive animals was calculated by averaging the apparent BRD prevalences of 8.8 and 21.2% as reported by Timsit et al. (13). It was therefore calculated a sample size of 170–179 animals, taking randomly only few animals (4–7) per vehicle, considering a possible number of vehicles varying from 24 to 48 traveling each year.

The experiment took place during 1 year (from February 22, 2019 to February 21, 2020). In that period, a population of 1,957 male Limousine beef steers traveled from France to the four farms monitored. Out of this total main population, a subpopulation of 1,045 animals traveling in 34 vehicles on a total of 22 different dates was included in the study. The vehicles transported from 20 to 60 animals, and 5 was the median number of animals (ranging from 3 to 6) randomly chosen among the animals traveling each time. Consequently, samples were collected in total from 169 beef steers before loading (T0) and 4 days after arrival (T1). The beef steers were 10–14 months old with a body weight of about 380 ± 20 kg, with Body Condition Scores of 3 (167/169, 98.8%) or 4 (2/169, 1.2%)

(19). They originated from two assembly centers (ACs) located in France: Celmar Siret 31449234900019 (GPS location: 46.222870, 1.497133 DD; Malonze, 23300 La Souterraine, France) (AC1) and Union Altitude Siret 32313877600279 (GPS location: 45.317993, 1.764769 DD; Le Foirail, 19460 Naves) (AC2). In France, before collection at the ACs, animals were reared at pasture on different farms, located close to the ACs (i.e., <200 km). Those farms were officially free of tuberculosis and bovine leucosis. All animals were vaccinated against bluetongue serotype 8 (Merial BTV 8) in conformity with Annex III.A 85 to Regulation (EC) No. 1266/2007. At the ACs, they were grouped respecting the original penning, kept for <6 days, treated with insecticide/repellant (Cydectin® Pour-On for Cattle and Red Deer; Virbac, Carros, France), in conformity with Article 9 of Regulation (EC) No. 1266/2007, and then shipped to Italy.

At T0, official government veterinarians checked the health of each animal, and steers with clinical signs were not loaded onto the trucks. Meanwhile, at T0, two DNS (one swab per nasal cavity) were collected from each of the 169 animals. Before sampling, the nostril was wiped clean with 70% ethanol. Sterile dry swabs 13 cm long were used for sampling, and after collection, DNS were immediately stored on ice. The DNS were transported in refrigerated boxes kept in the cabin of the vehicles where the tested animals were transported. After DNS collection, animals were marked, loaded into the vehicles, and transported to the farm of destination. The beef steers considered for the present study were transported in 34 vehicles throughout all 4 seasons: 9 vehicles traveling on 4 different dates in autumn, 10 vehicles traveling on 7 different dates in winter, 4 vehicles on 4 dates in spring, and 11 vehicles traveling on 7 different dates in summer. The vehicles were 4 × 2 cab over engine prime movers (Scania®, Europe) with a three-axle enclosed flat deck semitrailer configured with two decks (**Supplementary Figure 1**). The vehicles had the certificate of approval according to Article 18 of the EC Regulation No. 1/2005, and belonged to a single transporter, authorized for all journeys including long journeys, according to the same EC Regulation. The vehicles were therefore equipped with suitable drinking systems, temperature sensors, and mechanical ventilation systems (i.e., 28 automatic fans at each site). During the travel, steers received water and were fed at regular intervals, in compliance with the EC Regulation 1/2005. All journeys were performed on a similar route of about 1,700 km, and they were completed in no <20 h and no more than 29 h, as reported in the intended duration on the Trade Control and Expert System (TRACES; URL: https://webgate.ec.europa.eu/sanco/traces/). For this type of journey, based on the EC 1/2005, a stop of 1 h, without unloading the animals, after the first 14 h of the journey is mandatory for permitting animals being watered and, if necessary, fed. However, some vehicles performed an extra stop for resting the animals and other logistic reasons (i.e., change of drivers), and those stops were reported in the TRACES. None of the vehicles included in this study stopped at a control post or took more than 29 h.

The animals were transported to four different farms (F1, 2, 3, 4) in Bari (Southern Italy) of the same breeder, of which two farms (F1 and F2) received the majority of the transported animals. For this reason, F3 and F4 farms were combined in a new category named "others." Upon arrival, the marked animals were separated from the others traveling together and were kept isolated, far from the others already reared at the arrival farm. So, they were located separately in a different pen and fed with alfalfa barley silage mixed diets similar to the ones at the ACs and during the journey. None of the cattle involved was administered antimicrobials or vaccines before or during the study. None of the sampled animals died during the study.

At T1, the marked animals were clinically examined. The presence of the following clinical signs was noted down (present/absent): nasal discharge, lacrimal discharge, coughing, diarrhea, depression, polypnea, lameness, injury, and pain. For the latter three, the type of injury, the score of lameness, and pain were also noted down. Soon after the clinical examinations, two further DNS (one swab per nasal cavity) were collected from each animal, with the same procedures and precautions taken for sample collection at departure. Immediately after collection, all DNS samples were stored on ice and transported to the laboratory of Infectious Diseases of the Department of Veterinary Medicine of Bari (Italy). All the samples per truck were processed within 24 h after the collection of T1 samples.

The epidemiological status of the tested herds was not evaluated, but the animals included in the study were located separately as soon as they arrived at the farm, without any possibility of mixing. However, F1 and F2 were indirectly monitored because, during the study period, veterinarians collected DNS and ocular samples from symptomatic cattle that were tested in the same laboratory for bovine viral diarrhea virus (BVDV), bovine alphaherpesvirus 1 (BoHV-1), bovine coronavirus (BCoV), *Pasteurella multocida*, and *Histophilus somni*. In particular, on F1, two outbreaks of ocular BoHV-1 and *P. multocida* infections were detected in December 2019 and March 2019, respectively. On F2, *P. multocida* and *H. somni* were instead identified in an outbreak that developed in December 2019.

## Real-Time Quantitative PCR

Real-time quantitative PCR (RT-qPCR) was used to quantify the presence in the collected DNS of BVDV, BoHV-1, BCoV, bovine respiratory syncytial virus (BRSV), bovine adenovirus (BAdV), bovine parainfluenza virus 3 (BPIV-3), *H. somni*, *P. multocida*, *Mannheimia haemolytica*, and *Mycoplasma bovis*.

Dry DNS were suspended in tissue-cultured media (10% p/v), and an aliquot was employed for biomolecular analysis. Nucleic acids were extracted using the commercial kit QIAamp® cador® Pathogen Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions, with an elution volume of 100 μl. The extracted samples were stored at −80°C until tested by RT-qPCR. The samples were subjected to reverse transcription in a 10 μl total volume, using random hexamers and MuLV reverse transcriptase, according to the manufacturer's protocol (GeneAmp® RNA PCR; Applied Biosystems, Applera Italia, Monza, Italy). Primers and TaqMan probe for RT-qPCR assay were used as previously described (20, 21), in the same reaction conditions, including reaction mix component and thermal cycling with an annealing temperature of 56°C

**TABLE 1 |** Weather and transport conditions considered as predictive variables, with their definitions and categories, during a 1-year cross-sectional study in beef steers shipped from France to Italy.

| Name | Definition | Categories |
|---|---|---|
| **Weather conditions** | | |
| Season | Season in which the tested animals were transported from France to Italy | Autumn, spring, summer, winter |
| Arrival temperature (AT) | The average temperature recorded in the weather website for the farm of arrival area on the day of arrival | 1 (from 5 to 9°C), 2 (from 10 to 13°C), 3 (from 14 to 19°C), 4 (from 20 to 23°C), 5 (from 24 to 30°C) |
| Arrival humidity (AH) | The average relative humidity recorded in the weather website for the farm of arrival area on the day of arrival | Medium–low (from 60 to 70%), medium–high (from 71 to 80%), very high (from 81 to 100%) |
| Diurnal temperature variation | This range was calculated as the difference between the maximum and the minimum temperature recorded on the day of arrival | 1 (from 0 to 5°C), 2 (from 6 to 8°C), 3 (from 9 to 11°C), 4 (from 12 to 17°C) |
| Delta temperature between arrival and departure (ΔT) | The difference between the average temperature recorded in the weather website for the farm of arrival area on the day of arrival and the average temperature recorded for the assembly point area on the day of departure | Low difference (from −1 to +3°C), medium difference (from +4 to +6°C), high difference (from +7 to +10°C) |
| Delta humidity between arrival and departure (ΔH) | The difference between the average relative humidity recorded in the weather website for the farm of arrival area on arrival and the average relative humidity recorded for the assembly point area on the day of departure | 1 (from −35 to −10%), 2 (from −5 to +10%), 3 (from +15 to +30%) |
| Arrival wind speed | The average wind speed recorded in the weather website for the farm of arrival area on the day of arrival | Low (from 0 to 10 km/h), medium–low (from 11 to 14 km/h), medium (from 15 to 19 km/h), strong (from 20 to 24 km/h), very strong (≥25 km/h) |
| Arrival precipitations | The presence/absence of atmospheric precipitations recorded in the weather website for the farm of arrival area on the day of arrival | Yes, no |
| **Travel conditions** | | |
| Assembly center (AC) | Center where animals from different farms converge before being loaded for transport. The animals in this study come from two assembly centers located in La Souterraine (France) and Naves (France) | AC1, AC2 |
| Vehicles | The vehicles transporting the beef steers from the assembly centers (ACs) to the arrival farms (F) | 34 vehicles |
| Stocking density | Stocking density during transport calculated by splitting the total kg loaded for the space available in each vehicle | Low (from 104.8 to 182.5 kg/m$^2$), high (from 323.5 to 390.6 kg/m$^2$) |
| Extra stop | The presence/absence of an extra stop | Yes, no |
| Farm (F) | Farm of arrival in Bari, Southern Italy | F1, F2, others (F3 + F4) |
| Positivity for a pathogen at T0 | For each animal, the positivity of the DNS for a certain pathogen at T0 | Presence (1), absence (0) |

(**Supplementary Table 1**). Briefly, 10 μl of cDNA or extracted DNA was added to the 15 μl reaction master mix (IQ™ Supermix; Bio-Rad Laboratories Srl, Milan, Italy) containing 0.6 μM of each primer and 0.4 μM probe. Thermal cycling consisted of the activation of iTaq DNA polymerase at 95°C for 10 min and 45 cycles of denaturation at 95°C for 10 s and annealing–extension at 56°C for 30 s. RT-qPCR was performed in an i-Cycler iQTM Real-Time Detection System (Bio-Rad Laboratories Srl), and the data were analyzed with the appropriate sequence detector software (version 3.0). Fluorescence was monitored during the whole RT-qPCR process. A DNS was considered positive if the fluorescence peaked between 7 and 45 cycles, following the settings reported by Kishimoto et al. (21).

## Statistical Analysis

### Predictive and Outcome Variables

The weather parameters (average, minimal and maximal temperatures, humidity, wind speed, and quantity of precipitation) at the cities of departure and arrival were taken from a weather website (meteoblue.com). Diurnal temperature variation on the day of arrival and the changes in temperature and humidity between arrival and departure (delta, ΔT, and ΔH, respectively) were calculated as reported in **Table 1**.

The data related to the vehicle (number of animals traveling, total kg loaded, available space), estimated journey duration, and presence of a resting stop(s) during transport were taken from the TRACES. The estimated journey duration varied from 20 to 28 h depending on the presence of an extra stop. Unfortunately, it was not possible to record the effective journey since many of the vehicles arrived at night at the destination farm and we were not allowed to have the journey log. The estimated journey duration was not considered as a predictive variable for avoiding confounding factors since it was collinear with the presence of more than a resting stop. Stocking density was calculated by splitting the total kg loaded for the space available in each vehicle.

Weather and travel conditions were clustered in categories and thus considered as predictive variables in the regression analysis (**Table 1**). The positivity for each pathogen (positive/negative, 1/0) of the DNS collected from the beef steers at T0 was also used as predictive variable for the regression analyses performed for the positivity for the same pathogen at T1.

For each animal, the total number of positivities to the tested pathogens was calculated at T0 and T1. Depending on the number of pathogens identified in each animal, the following categories were created for both T0 and T1: negative, single positive, and positive for 2, 3, 4, 5, and 6 pathogens. Based on those data, a category named "co-infection" was created with two values (yes/no): (i) animals positive for at least two pathogens (yes) and (ii) animals negative or single positive (no).

### Descriptive Statistics

Descriptive statistics were performed on the weather conditions detected for the 44 sampling dates, with the minimum, maximum, and average values obtained with Microsoft Office Excel. Descriptive statistics of all predictive variables, identified as categorical, were performed using the Statulator® online free software and reported as counts and percentages. The number of animals with clinical signs (presence/absence), positive for the pathogens (positive/negative), and the co-infection category (yes/no) were counted and then compared between T0 and T1 with a two-tailed McNemar's $\chi^2$ test statistics using the online tool of the Center for Clinical Research Biostatistics (CCRB; URL: https://www2.ccrb.cuhk.edu.hk/stat/confidence%20interval/McNemar%20Test.htm). The clinical signs and pathogens that resulted to be significant with the McNemar's $\chi^2$ test and had at least 10 positive observations at T1 (10/169, a prevalence of more than 5%) (22) were further investigated in the subsequent statistical analyses.

### Regression Analysis

Collinearity between categorical variables was tested using Kendall's tau statistic with the *cor.test* function in R environment (23). Categories 4 and 5 of the arrival temperature (AT) variable were collinear with the summer season. Each clinical sign (presence/absence, 1/0) and each pathogen (positive/negative, 1/0) at T1 were used as dependent categorical dichotomous variables (dummy variables) in univariate logistic regression models, in order to identify the predictive variables to be included in the stepwise backward selection and find the final multivariable model for each clinical sign and pathogen. Additionally, only for the dummy dependent variables of the positivity to bacteria at T1, the positivity to the viruses at T1 (positive/negative, 1/0) was used as a predictive variable. No associations were noticed between the AC variable and the dependent variables of the presence of clinical signs and pathogens at T1, nor between the 34 vehicles and the dependent variables of the presence of clinical signs and pathogens at T1. Thus, ACs and vehicles were not further considered in the subsequent statistical analyses. Results of the univariate logistic regression are reported as odds ratio (OR), 95% CI, and *p*-values. *p*-values were calculated using the Wald test, and for each outcome, the variables that showed a $p < 0.25$ were considered for inclusion in the stepwise multiple regression. Due to the collinearity between categories 4 and 5 of the AT and the summer season, if season and AT were both significant in univariate logistic regression, only the AT was further considered for the inclusion in the stepwise multiple regression. A stepwise backward elimination procedure was indeed conducted for each dependent variable (presence/absence of clinical signs and positivity/negativity to the pathogens) to test the combined effect of the predictive variables. The predictive variables were removed until all variables in the final model had a $p < 0.10$ (24). The final multivariable models resulting from the stepwise backwards selection are presented as OR, 95% CI, and *p*-value. The scripts used to perform the univariate logistic and stepwise multiple regressions were a combination of functions in the packages *nlme*, *lsmeans*, *lme4*, and *car* in R environment (23).

Univariate logistic regression was also used to test the associations between the presence/absence of clinical signs (dependent variable) and the dummy variable of the positivity to the pathogens (predictive variable).

### Multiple Correspondence Analysis

Multiple correspondence analysis (MCA) was performed to detect underlying structures in the dataset, using the dummy variables of the clinical signs and the positivity to the pathogens, and the predictive variables defined in **Table 1**. The results are represented by the proportion (in %) of total variance (called "inertia" in MCA) explained by each dimension (Dim), the list of the contributions of the variable categories to each Dim (the highest the contribution of a category to a Dim, the most important it is in explaining the inertia of the dataset), and the coordinates of the categories in each Dim. The absolute value of the coordinates allows identifying the weight of each category in a Dim, and the sign of the coordinates provides also an indication concerning the correlation existing among the categories in each Dim. The MCA was performed using the *FactoMineR* package, and the results were plotted using the packages *factoextra*, *gplots*, and *grDevices* in R environment (23).

## RESULTS

## Descriptive Statistics

The weather conditions recorded on the 44 sampling dates (22 on departure and 22 on arrival) are summarized in **Supplementary Table 2**. The average temperatures noted over the different seasons were generally comparable between the ACs at T0 and the arrival farms at T1, with the average temperatures ranging from 9°C in winter to 22°C in summer at T0 and from 10°C in winter to 24°C in summer at T1. However, the minimum temperatures observed at T1 were on average higher than those observed at T0. In particular, the sampling dates in autumn and spring showed 7°C and 6°C as minimum temperatures, respectively, whereas the minimum temperatures noticed in the same two seasons were near to 0 at T0 (2°C in autumn and 1°C in spring). The greater diurnal temperature variation was observed at T0 during the summer period when the observed minimum temperature was 12°C and the maximum temperature was 35°C. During spring and summer, the average and maximum humidity at T1 were overall higher than those at T0 (in summer 59–80% at T0 vs. 73–80% at T1, in spring 60–75% at T0 vs. 78–85% at T1), whereas in autumn and winter, the humidity percentages were higher at T0 than at T1 (in autumn 82–100% at T0 vs. 73–85% at T1, in winter 78–85% at T0 vs. 76–80% at T1). Average and maximum atmospheric precipitations were notably higher in

autumn, winter, and spring at T0 (6–14 mm in autumn, 4–12 mm in winter, 4–16 mm in spring) than at T1 (1–2 mm in autumn, 1–8 mm in winter, 1–2 mm in spring).

Overall, AC1 and AC2 were equally represented in the sample, with a similar number of steers coming from each AC (78/169, 46.1% from AC1; 91/169, 53.9% from AC2). Most of the transported animals (155/169, 91.7%) were headed to two (F1 and F2) out of the four farms. About half of the steers (74/169, 43.8%) traveled at lower stocking densities (from 104.8 to 182.5 kg/m$^2$), whereas the remaining ones (95/169, 56.2%) were transported at higher stocking densities (from 323.5 to 390.6 kg/m$^2$). The transport distance was comparable among the journeys (about 1,700 km); however, 30.2% (51/169) of the vehicles performed an extra stop during the journey for logistic reasons (**Table 2**). Descriptive statistics of the explanatory variables related to the weather conditions are shown in **Supplementary Table 3**.

**TABLE 2 |** Frequency table of beef steers ($n = 169$) transported from France to Italy for the variables related to travel conditions and co-infection at T0 (departure) and T1 (4 days after arrival).

| Variable | Count | % |
|---|---|---|
| Assembly center (AC) | | |
| AC1 | 78 | 46.1 |
| AC2 | 91 | 53.9 |
| Total | 169 | 100.0 |
| Farm (F) | | |
| F1 | 74 | 43.8 |
| F2 | 81 | 47.9 |
| Others (F3 + F4) | 14 | 8.3 |
| Total | 169 | 100.0 |
| Stocking density | | |
| Low (from 104.8 to 182.5 kg/m$^2$) | 74 | 43.8 |
| High (from 323.5 to 390.6 kg/m$^2$) | 95 | 56.2 |
| Total | 169 | 100.0 |
| Extra stop | | |
| No | 118 | 69.8 |
| Yes | 51 | 30.2 |
| Total | 169 | 100.0 |
| Number of animals positive to pathogens at T0 | | |
| Negative | 90 | 53.2 |
| Single positive | 52 | 30.8 |
| Positive for 2 pathogens | 16 | 9.5 |
| Positive for 3 pathogens | 8 | 4.7 |
| Positive for 4 pathogens | 2 | 1.2 |
| Positive for 5 pathogens | 1 | 0.6 |
| Total | 169 | 100.0 |
| Number of animals positive to pathogens at T1 | | |
| Negative | 4 | 2.4 |
| Single positive | 25 | 14.8 |
| Positive for 2 pathogens | 49 | 29.0 |
| Positive for 3 pathogens | 42 | 24.8 |
| Positive for 4 pathogens | 35 | 20.7 |
| Positive for 5 pathogens | 13 | 7.7 |
| Positive for 6 pathogens | 1 | 0.6 |
| Total | 169 | 100.0 |

## Travel Effect on the Prevalence of Clinical Signs and Pathogens at T1

As reported in **Table 3**, only healthy animals that showed no clinical signs of coughing, depression, diarrhea, injury, lacrimal discharge, lameness, nasal discharge, pain, or polypnea were transported. Despite no clinical signs at T0, some of the DNS were positive. In particular, 48 beef steers (28.4%) were positive for *H. somni*, 21 to *P. multocida* (12.4%), 14 to *M. haemolytica* (8.3%), 7 to *M. bovis* (4.1%), and 4 to BAdV (2.4%).

At T1, while the number of animals showing injury, pain, depression, lameness, or polypnea was low (1/169, 0.6%), the number of animals displaying coughing (20/169, 11.8%), diarrhea (28/169, 16.6%), and lacrimal (33/169, 19.5%) and nasal (90/169, 53.3%) discharges increased dramatically ($p < 0.001$). The number of positive DNS also increased significantly for almost all the investigated pathogens, except for BPIV-3, with only four animals positive at T1 (4/169, 2.4%; $p > 0.050$), and for BVDV and BoHV-1, which were always negative both at T0 and T1. Transport also significantly increased co-infection, which went from 16.0% at T0 to 82.8% at T1 ($p < 0.001$).

## Factors Associated With Clinical Signs at T1

The complete list of Wald test *p*-values calculated between the predictive variables and the presence of clinical signs at T1 is reported in **Supplementary Table 4**. The OR, 95% CI, and *p*-values of the variables significantly associated with the presence of clinical signs at T1 in the univariate logistic regressions are reported in **Supplementary Table 5**.

**Table 4** reports the OR, 95% CI, and *p*-values of the variables retained in the final multiple regression models. The presence of coughing was associated with stocking density ($p = 0.004$), $\Delta$T ($p = 0.002$), and arrival precipitations ($p < 0.001$). In particular, the odds of coughing were increased 10-fold in animals transported at high stocking density, by 11 times when $\Delta$T was of 4–6°C, and by 35 times when there were no arrival precipitations.

Diarrhea was mainly related to $\Delta$T ($p < 0.001$) and $\Delta$H ($p < 0.001$). In particular, high difference category for $\Delta$T (from +7 to +10°C) and $\Delta$H (from +15 to +30%) increased, respectively, by 60 and almost 6 times the presence of diarrhea compared with lower $\Delta$.

Lacrimal discharge was related to the farm of arrival ($p < 0.001$) and to $\Delta$H ($p = 0.022$). Animals transported to F2 were almost 25 times more likely to display lacrimal discharge than beef steers in F1, and $\Delta$H (between +15 and +30% than T0) was associated with a 20-time increase in the likelihood of showing lacrimal discharge compared with the $\Delta$H from −35 to −10%.

Nasal discharge was associated with the farm of arrival ($p < 0.001$) and AT ($p < 0.001$). The animals were eight times more likely to display nasal discharge if transported to F2 than to F1, and if AT was above 10°C.

**TABLE 3 |** Beef steers ($n = 169$) traveling from France to Italy.

| | T0 | | T1 | | McNemar's $\chi^2$ test | |
|---|---|---|---|---|---|---|
| | N | % | N | % | Statistics | *p*-value |
| Injury | 0/169 | 0.0 | 1/169 | 0.6 | n.e. | n.e. |
| Pain | 0/169 | 0.0 | 1/169 | 0.6 | n.e. | n.e. |
| Depression | 0/169 | 0.0 | 1/169 | 0.6 | n.e. | n.e. |
| Polypnea | 0/169 | 0.0 | 1/169 | 0.6 | n.e. | n.e. |
| Coughing | 0/169 | 0.0 | 20/169 | 11.8 | 18.05 | <0.001 |
| Diarrhea | 0/169 | 0.0 | 28/169 | 16.6 | 26.04 | <0.001 |
| Lacrimal discharge | 0/169 | 0.0 | 33/169 | 19.5 | 31.03 | <0.001 |
| Nasal discharge | 0/169 | 0.0 | 90/169 | 53.3 | 88.01 | <0.001 |
| BoHV-1 | 0/169 | 0.0 | 0/169 | 0.0 | n.e. | n.e. |
| BVDV | 0/169 | 0.0 | 0/169 | 0.0 | n.e. | n.e. |
| BRSV | 1/169 | 0.6 | 15/169 | 8.9 | 12.07 | <0.001 |
| BCoV | 27/169 | 16.0 | 110/169 | 65.1 | 67.91 | <0.001 |
| BPIV-3 | 0/169 | 0.0 | 4/169 | 2.4 | 2.25 | 0.133 |
| BAdV | 3/169 | 1.84 | 18/169 | 10.7 | 11.53 | <0.001 |
| *H. somni* | 48/169 | 28.4 | 148/169 | 87.6 | 92.46 | <0.001 |
| *M. haemolytica* | 14/169 | 8.3 | 27/169 | 16.0 | 4.36 | 0.036 |
| *M. bovis* | 7/169 | 4.1 | 78/169 | 46.1 | 65.33 | <0.001 |
| *P. multocida* | 21/169 | 12.4 | 60/169 | 35.5 | 29.47 | <0.001 |
| Co-infection | 27/169 | 16.0 | 140/169 | 82.8 | 101.98 | <0.001 |

*Descriptive statistics and comparison between departure (T0) and 4 days after arrival (T1) of the observed prevalence of the animals displaying clinical signs and positivity for the investigated nasopharyngeal pathogens.*
*n.e., not estimable.*

## Factors Associated With Viral Infections at T1

The complete list of Wald test *p*-values calculated between the predictive variables and the viral infections at T1 is reported in **Supplementary Table 6**. The OR, 95% CI, and *p*-values of the variables significantly associated with the presence of viral infections at T1 in the univariate logistic regressions are reported in **Supplementary Table 7**.

**Table 5** reports the OR, 95% CI, and *p*-values of the variables retained in the final multiple regression models. BRSV was found to be associated with the farm of arrival ($p = 0.029$), the presence of an extra stop during the transport ($p = 0.020$), and the AH ($p < 0.001$). For the variable farm of arrival, the category "others" was more than 20 times more likely to have beef steers positive for BRSV than F1, and this possibility was even higher if at arrival there was a medium–low AH than a medium–high AH.

BAdV was found to associated with the farm of arrival ($p = 0.007$), the stocking density ($p = 0.002$), and the ΔH ($p = 0.006$). The odds of having beef steers positive to BAdV at T1 were 48 times higher if transported to F2, almost 50 times with a ΔH of category 3 (+15 to +30%), and 20 times higher if transported at low stocking densities.

On the other hand, BCoV did not show any significant associations with the investigated factors.

**TABLE 4 |** Final multivariable regression models for the dummy dependent variables of the presence/absence of clinical signs.

| Variable | Category | Final multivariable model | | |
|---|---|---|---|---|
| | | OR | 95% CI | *p* |
| **Dependent variable: coughing** | | | | |
| Stocking density | Low | Ref | | |
| | High | 10.33 | 2.88–46.86 | <0.001 |
| ΔT | Low | Ref | | |
| | Medium | 11.62 | 3.54–44.89 | <0.001 |
| | High | n.e. | n.e. | 0.991 |
| Arrival precipitations | Yes | Ref | | |
| | No | 35.38 | 5.65–707.05 | 0.001 |
| **Dependent variable: diarrhea** | | | | |
| ΔT | Low | Ref | | |
| | Medium | 2.30 | 0.76–7.06 | 0.136 |
| | High | 60.37 | 7.43–1,342.21 | <0.001 |
| ΔH | Medium | Ref | | |
| | Low | 0.45 | 0.02–3.98 | 0.516 |
| | High | 5.84 | 1.74–26.86 | <0.001 |
| **Dependent variable: lacrimal discharge** | | | | |
| F | F1 | Ref | | |
| | F2 | 24.97 | 6.29–170.90 | <0.001 |
| | Others | n.e. | n.e. | 0.992 |
| ΔH | 1 (−35 to −10%) | ref | | |
| | 2 (−5 to +10%) | 4.04 | 0.68–77.56 | 0.202 |
| | 3 (+15 to +30%) | 19.98 | 3.29–389.81 | 0.007 |
| **Dependent variable: nasal discharge** | | | | |
| F | F1 | Ref | | |
| | F2 | 8.03 | 2.10–40.71 | 0.005 |
| | Others | 3.13 | 0.82–13.30 | 0.103 |
| AT | 1 (5–9°C) | Ref | | |
| | 2 (10–13°C) | 13.22 | 2.72–84.56 | 0.003 |
| | 3 (14–19°C) | 140.65 | 20.32–1,250.12 | <0.001 |
| | 4 (20–23°C) | 38.05 | 5.04–361.04 | <0.001 |
| | 5 (24–30°C) | 65.77 | 12.40–448.29 | <0.001 |

*The symptoms were observed 4 days after arrival in 169 beef steers transported from France to Italy. Data are presented as odds ratio (OR), 95% confidence interval (95% CI), and p-value (p).*
*n.e., not estimable; Ref, reference; AT, arrival temperature; ΔT, delta temperature between arrival and departure; ΔH, delta humidity between arrival and departure; F, farm of arrival.*

## Factors Associated With Bacterial Infections at T1

The complete list of Wald test *p*-values calculated between the predictive variables and the bacterial infections at T1 is reported in **Supplementary Table 8**. The OR, 95% CI, and *p*-values of the variables significantly associated with the presence of bacterial infections at T1 in the univariate logistic regressions are reported in **Supplementary Table 9**.

**Table 6** reports the OR, 95% CI, and *p*-values of the variables retained in the final multiple regression models. The positivity to *H. somni* was associated with the farm of arrival ($p < 0.001$) and the diurnal temperature variation ($p < 0.001$). Indeed, the

**TABLE 5 |** Final multivariable regression models for the dummy dependent variables of positivity/negativity to viral infections.

| Variable | Category | Final multivariable model | | |
|---|---|---|---|---|
| | | OR | 95% CI | *p* |
| **Dependent variable: BRSV positivity** | | | | |
| F | F2 | Ref | | |
| | F1 | 2.39 | 0.48–17.68 | 0.321 |
| | Others | 23.08 | 2.14–624.09 | 0.021 |
| Extra stop | No | Ref | | |
| | Yes | 5.52 | 1.30–28.99 | 0.026 |
| AH | Medium–high | Ref | | |
| | Medium–low | 37.03 | 4.70–1,163.15 | 0.006 |
| | Very high | n.e. | n.e. | 0.995 |
| **Dependent variable: BAdV positivity** | | | | |
| F | F1 | Ref | | |
| | F2 | 48.82 | 6.33–1,167.10 | 0.002 |
| | Others | 7.79 | 0.88–174.69 | 0.097 |
| Stocking density | High | Ref | | |
| | Low | 23.60 | 4.99–179.78 | <0.001 |
| ΔH | 1 (−35 to −10%) | Ref | | |
| | 2 (−5 to +10%) | 4.81 | 0.58–103.48 | 0.193 |
| | 3 (+15 to +30%) | 49.46 | 5.98–1,227.89 | 0.002 |

*The positivities were observed 4 days after arrival in the DNS from 169 beef steers transported from France to Italy. Data are presented as odds ratio (OR), 95% confidence interval (95% CI), and p-value.*
*n.e., not estimable; Ref, reference; F, farm of arrival; AH, arrival humidity; ΔH, delta humidity between arrival and departure.*

**TABLE 6 |** Final multivariable regression models for the dummy dependent variables of positivity/negativity to bacterial infections.

| Variable | Category | Final multivariable model | | |
|---|---|---|---|---|
| | | OR | 95% CI | *p* |
| **Dependent variable: *H. somni* positivity** | | | | |
| F | F2 | Ref | | |
| | F1 | 36.28 | 5.72–775.03 | 0.002 |
| | Others | n.e. | n.e. | 0.992 |
| Diurnal temperature variation | 4 (12–17°C) | Ref | | |
| | 1 (0–5°C) | 61.28 | 7.91–1,377.89 | <0.001 |
| | 2 (6–8°C) | 101.45 | 14.67–2,130.59 | <0.001 |
| | 3 (9–11°C) | 53.16 | 6.99–1,175.43 | <0.001 |
| **Dependent variable: *M. haemolytica* positivity** | | | | |
| Extra stop | No | Ref | | |
| | Yes | 6.60 | 1.40–48.02 | 0.028 |
| Diurnal temperature variation | 3 (9–11°C) | Ref | | |
| | 1 (0–5°C) | 29.01 | 4.65–312.96 | 0.001 |
| | 2 (6–8°C) | 2.17 | 0.41–14.57 | 0.380 |
| | 4 (12–17°C) | n.e. | n.e. | 0.992 |
| Arrival precipitations | Yes | Ref | | |
| | No | 17.38 | 2.87–346.72 | 0.011 |
| BRSV positivity at T1 | No | Ref | | |
| | Yes | 9.95 | 1.70–74.32 | 0.015 |
| **Dependent variable: *M. bovis* positivity** | | | | |
| Stocking density | Low | Ref | | |
| | High | 2.39 | 1.06–5.60 | 0.038 |
| AT | 1 (5–9°C) | Ref | | |
| | 2 (10–13°C) | 5.07 | 1.52–20.47 | 0.013 |
| | 3 (14–19°C) | 2.19 | 0.42–1.22 | 0.356 |
| | 4 (20–23°C) | 1.44 | 0.23–9.68 | 0.702 |
| | 5 (24–30°C) | 1.37 | 0.29–6.79 | 0.688 |
| **Dependent variable: *P. multocida* positivity** | | | | |
| Stocking density | High | Ref | | |
| | Low | 2.76 | 1.26–6.14 | 0.028 |
| Extra stop | No | Ref | | |
| | Yes | 3.16 | 1.44–6.99 | 0.002 |
| *P. multocida* positivity at T0 | No | Ref | | |
| | Yes | 5.62 | 1.90–19.40 | 0.002 |

*The positive DNS were observed 4 days after arrival (T1) in 169 beef steers transported from France to Italy. Data are presented as odds ratio (OR), 95% confidence interval (95% CI), and p-value (p).*
*n.e., not estimable; Ref, reference; F, farm of arrival; AT, arrival temperature.*

odds of finding animals positive to *H. somni* were 30 times higher when beef steers arrived at F1 than at F2, and there was the diurnal temperature variation category 4 (from 12 to 17°C).

*M. haemolytica* was associated with the presence of an extra stop during transport ($p = 0.013$), the diurnal temperature variation ($p < 0.001$), and the arrival precipitations ($p < 0.001$). The odds of finding animals positive to *M. haemolytica* increased by over 6 times if an extra stop was performed during transport, 29 times with the category 1 (from 0 to 5°C) of diurnal temperature variation when compared with category 3 (from 9 to 11°C), 17 times if arrival precipitations were absent, and 10 times if the animal was also positive for BRSV.

*M. bovis* positivity was related to the AT ($p = 0.027$) and the stocking density ($p = 0.035$). The odds of finding animals positive to *M. bovis* increased by five times with category 2 (from 10 to 13°C) of AT compared with category 1 (from 5 to 9°C) and by more than two times with high stocking densities.

*P. multocida* was associated with the positivity for *P. multocida* at T0 ($p = 0.002$), the stocking density ($p = 0.029$), and an extra stop during transport ($p = 0.002$). The odds of finding animals positive to *P. multocida* increased by more than five times if they were already positive to this pathogen at T0, by three times with the presence of an extra stop during transport, and by more than two times if transported at low stocking densities.

## Association Between the Presence of Clinical Signs and Pathogens at T1

**Figure 1** shows the results of the univariable regression analysis. Among the observed clinical signs, the presence of nasal discharge was associated with the positivity to *M. haemolytica*; the animals displaying nasal discharges were six times more likely to have resulted to be positive to *M. haemolytica* (OR = 6.44, 95%

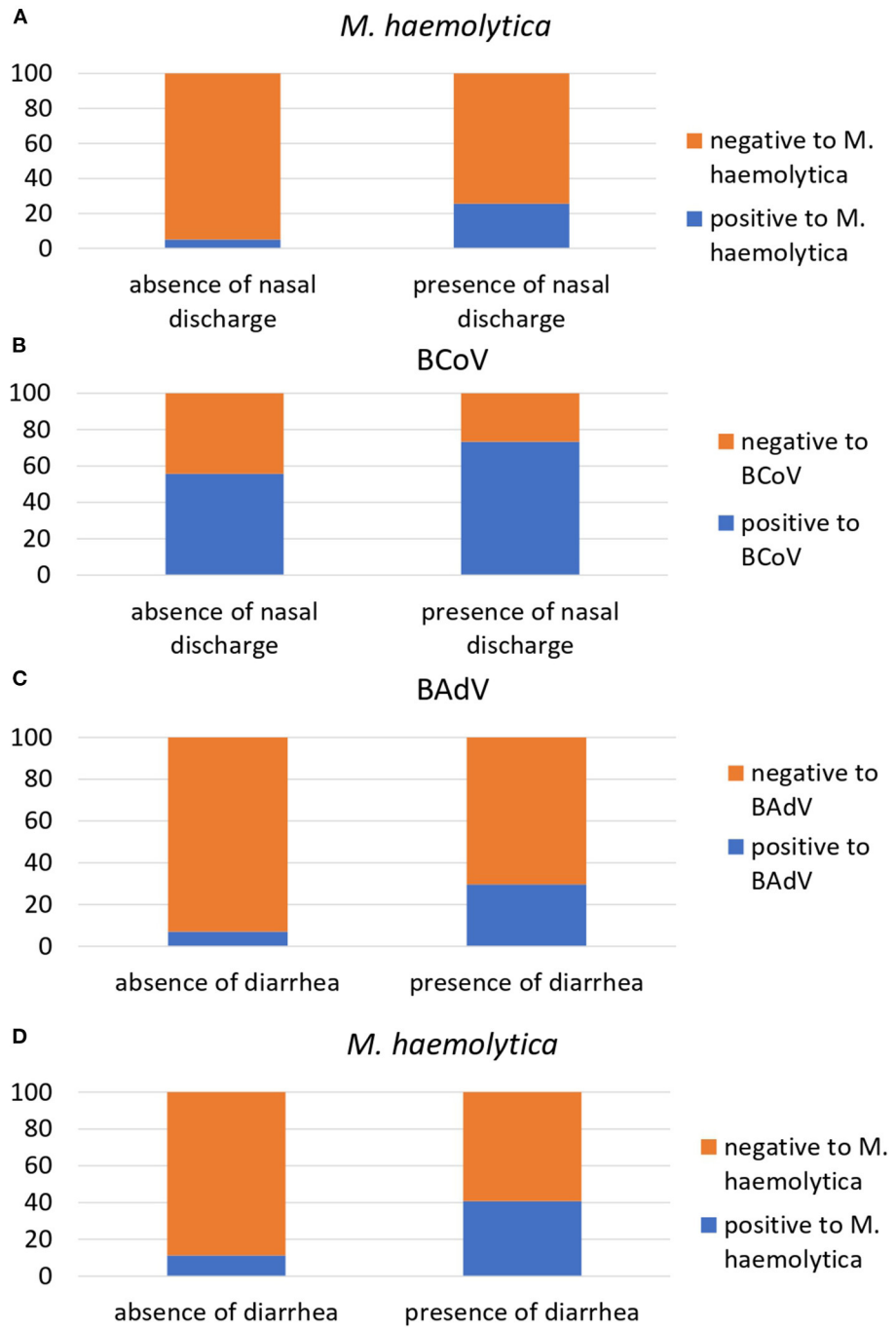



**FIGURE 1 |** Associations between the presence/absence of nasal discharge and *M. haemolytica* at T1 **(A)**, nasal discharge and BCoV at T1 **(B)**, diarrhea and BAdV at T1 **(C)**, and between diarrhea and *M. haemolytica* at T1 **(D)** in 169 beef steers transported from France to Italy. Each table reports the relative prevalence of the pathogens in animals with or without clinical signs.

CI = 2.33–22.79, $p = 0.001$) and two times more likely to have resulted to be positive to BCoV (OR = 2.19, 95% CI = 1.15–4.20, $p = 0.017$). Beef steers with diarrhea were instead five times more likely to have BAdV (OR = 5.56, 95% CI = 1.91–15.92, $p = 0.001$) and *M. haemolytica* infections (OR = 5.41, 95% CI = 2.12–13.76, $p < 0.001$).

## Identification of Variables Shaping the Multivariate Structure of the Dataset

MCA identified the underlying structures in the dataset, associations and correlations linking the categorical variables, and the most important variables shaping the dataset inertia. The top 10 new dimensions identified by MCA explained 66.8% of

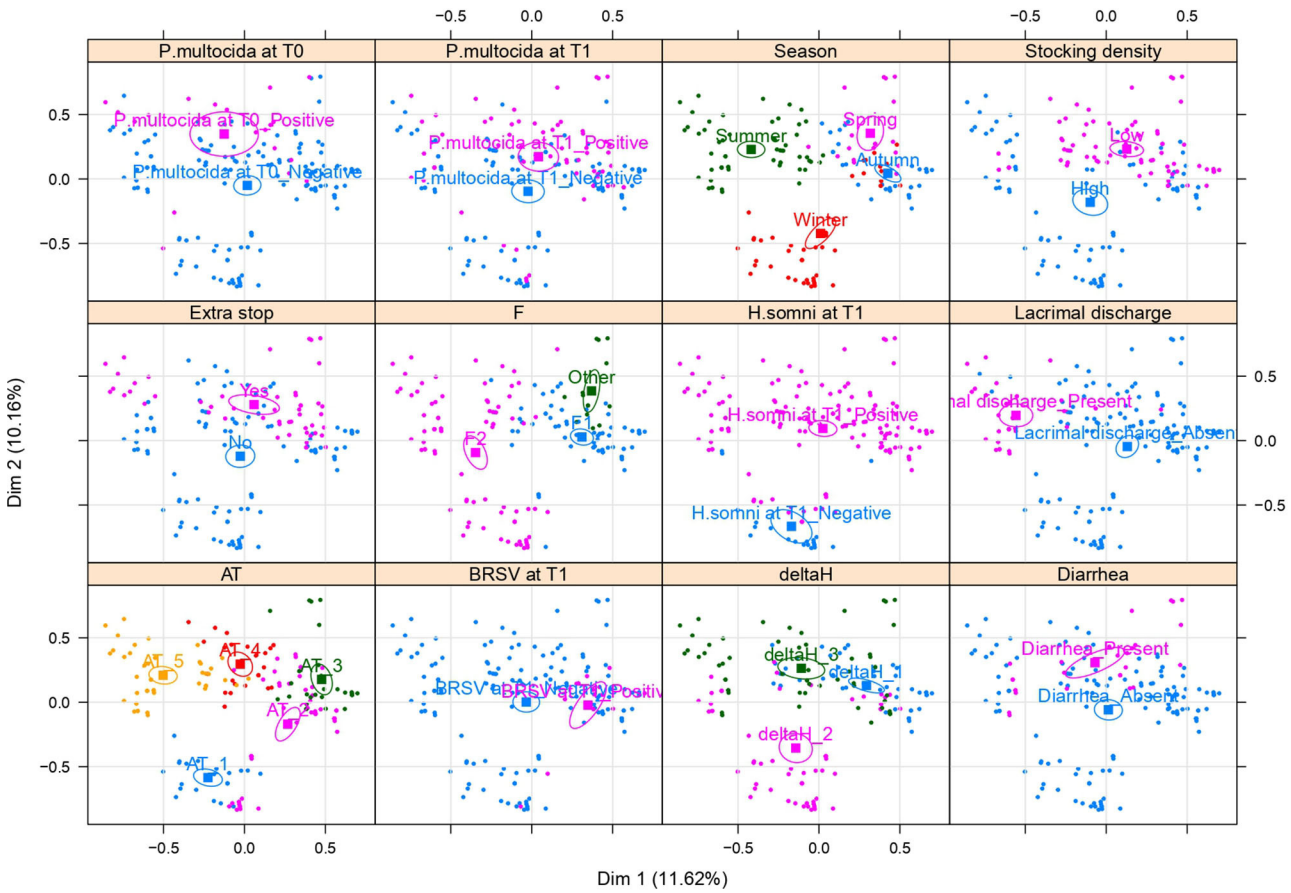



**FIGURE 2 |** The multiple correspondence analysis (MCA) biplot with Dimension 1 (Dim1) as x-axis and Dimension 2 (Dim2) as y-axis, with the explained variance between brackets. Different colors and confidence ellipses are drawn for the main variables entering the first two dimensions and explaining most of the variance noticed in the sample of 169 beef steers transported over a long-distance journey from France to Italy. F, farm of arrival; AT, arrival temperature; ΔH, delta humidity between arrival and departure.

the total inertia (**Supplementary Figure 2**). The two dimensions explaining the highest inertia percentages were Dimension 1 (Dim1) and Dimension 2 (Dim2), accounting for 11.6% and 10.2% of the total inertia, respectively. The contribution of the variable categories (in %) to the definition of the dimensions is reported in **Supplementary Table 10**, whereas the complete list of the coordinates for all the variable categories is reported in **Supplementary Table 11**.

The multivariate structure of the samples is graphically represented in **Figures 2**, **3**, where only the variables contributing the most to the inertia explained by each dimension have been reported. **Figure 2** shows a reproduction of the same MCA biplot with Dim1 as x-axis and Dim2 as y-axis, with different colors and confidence ellipses for the variables showing clusters (and thus defining the two main dimensions). The variable categories contributing the most to the total inertia of the dataset structure were the season and the farm of arrival determining the subsequent clusterization visible in the biplot for the other variables. Comparing the biplots in **Figure 2**, it is possible to note that the animals showing lacrimal discharge at T1 were mainly those that were transported in summer to F2, where temperatures ranging from 24 to 30°C were noticed (category 5 of the AT variable). The animals that were negative to *H. somni* at T1 were those that were transported during winter to F2, where temperatures ranged from 5 to 9°C (category 1 of AT), and with a difference in the humidity comprised in category 2 (from −5% to +10%) of ΔH. The animals displaying DNS positive to BRSV were those that were transported during the winter season and at the time of arrival at F1 or "others" found temperatures comprised in category 3 (from 14 to 19°C) of the AT variable. Furthermore, it is possible to note comparing the biplots in **Figure 2** that the animals positive for *P. multocida* at T1 were those already positive for this pathogen at T0, transported at low stocking densities (from 104.8 to 182.5 kg/m$^2$), and with an extra stop during transport. Finally, beef steers displaying diarrhea at T1 were those subjected to high ATs (categories 4 and 5 of the AT) and an abrupt increase in humidity at arrival (category 3 of the ΔH). **Figure 3** shows the same MCA biplot with Dim2 as x-axis and Dim3 as y-axis, with different colors and confidence ellipses for the variables showing clusters (and thus defining the

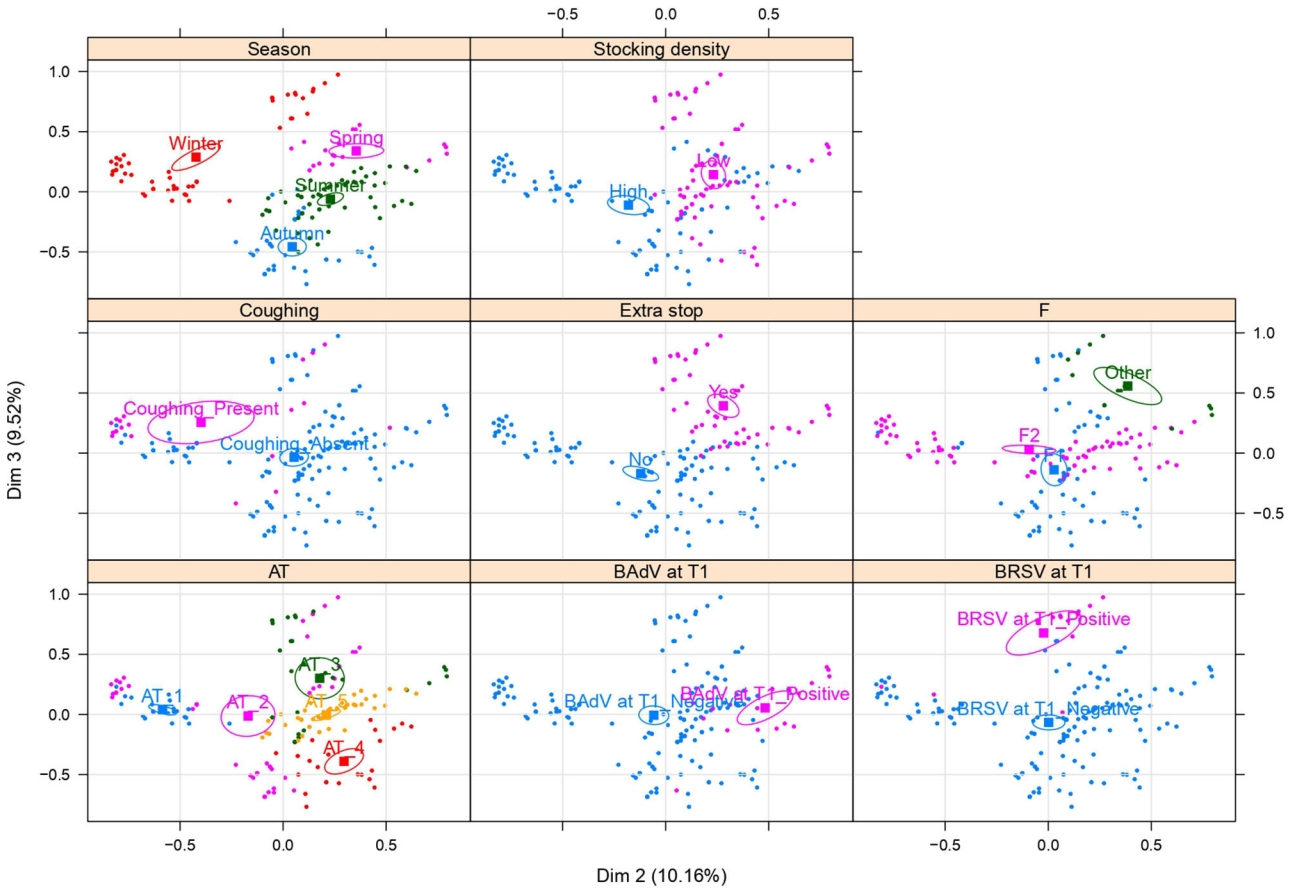



**FIGURE 3 |** The multiple correspondence analysis (MCA) biplot with Dimension 2 (Dim2) as x-axis and Dimension 3 (Dim3) as y-axis, with the explained variance between brackets. Different colors and confidence ellipses are drawn for the main variables entering the first two dimensions and explaining most of the variance noticed in the sample of 169 beef steers transported with a long-distance journey from France to Italy. F, farm of arrival; AT, arrival temperature.

second and the third main dimensions). Comparing the biplots in **Figure 3**, it is possible to note that the animals positive at T1 for BRSV were all transported at low stocking densities and during winter, whereas those positive at T1 for BAdV were transported at low stocking densities but mainly during spring and summer. It is also clear the presence of an association between winter and coughing, as beef steers transported during this season had a greater prevalence of this clinical sign.

## DISCUSSION

This study documented the prevalence of the clinical signs and pathogens related to BRD after a long-distance journey from France to Italy in beef steers. Several associations between the different weather and travel conditions (e.g., season, diurnal temperature variation, stocking density, farms of arrival) and the presence of virus/bacteria and clinical signs recorded 4 days after arrival were identified. Our findings strongly support our hypothesis. They confirmed that the prevalence of BRD-related pathogens increased after the tested journey length and suggested that weather conditions and the presence of an extra stop during transport may also be factors increasing the likelihood of BRD and its clinical signs. To our knowledge, this is the first study on the effects of the difference of temperature and humidity between arrival and departure on the occurrence of BRD-related pathogens and clinical signs in a consistent sample of beef steers (169 animals) traveling on a similar long-distance journey throughout the whole year. Our results may be useful to minimize the incidence of BRD and to propose best practices for cattle transportation.

A valuable observation point of the sanitary conditions of cattle in Europe was provided. In Europe, there are more than 4 million cattle transported each year across the member states, with more than 117,000 cattle consignments registered in 2015 (25). Italy is one of the largest cattle importers in the EU (2), and France is Italy's main supplier of cattle for fattening and slaughter, with more than 600,000 heads imported from France to Italy in 2016 (2). The fitness for travel of the tested animals was performed by official veterinarians, but despite this, cases of positivity were registered before departure. Our results highlight therefore the role of live animal transportation as a possible

way to move pathogens across Europe. However, long-distance journeys are not only a biosecurity hazard (26) but also a major welfare concern worldwide (27, 28). It is well-known that long travels are stressful events affecting the animal immune system and increasing the pathogen shedding, and ultimately result in production efficiency impairment and health problems onset (14, 29, 30). Over the last years, the number of animals transported for long-distance journeys has greatly increased, due to the increased trade in live animals between different countries and the establishment of fewer and larger growing/finishing farms and slaughter facilities often built far from the farms (31). Consequently, minimizing sufferance and poor health and welfare in the transported animals is crucial for lowering the risks of disease spread, and this is achievable only by improving the welfare standards before, during, and after a journey. Our findings are of interest not only for Europe but also for other countries whose dimensions require long journeys, such as North America or Australia.

Our results reinforce the argument that transport represents a risk factor in the development of BRD (32). The prevalence of BRD-related pathogens (BAdV, BCoV, BRSV, *H. somni*, *M. bovis*, *M. haemolytica*, and *P. multocida*) and clinical signs (coughing, diarrhea, nasal and lacrimal discharges) increased dramatically after travel, and extreme dysbiosis of the nasopharyngeal tract was observed, with a dramatic increase in co-infection (i.e., prevalence of concurrent infections increased by 500% after travel). Our results support the idea that long-distance transportation favors respiratory tract dysbiosis with concurrent bacterial and virus infections leading to BRD (14, 33–35). The drastic increase in co-infection found gets even more interesting when considering that all journeys included in this study complied with EC 1/2005, and all were within the maximum journey duration allowed. Extensive literature exists on the effect that different travel distances may have on stress-related physiological responses (36, 37) and BRD-related pathogens (15, 38). Our findings support the idea that the maximum travel time allowed should be reduced, as already pointed out in previously published activity reports to the European Parliament (17). However, the journey time represents only one of the variables affecting the occurrence of BRD, and other factors are supposed to play roles in the occurrence of this multifactorial disease (33). Consequently, analyzing individually which factors influence the prevalence of pathogens and clinical signs may provide suggestions for further implementing EC 1/2005 and provide best practices for cattle transportation.

Multiple factors affecting the occurrence of this complex disease have been investigated. BRD predisposing factors are weather conditions, including season (39–42), management factors at the ACs or sale barns (43), stocking density (44), human factors (33, 39), and age and gender of the animals (33). Our results strongly indicated that season and its associated variables, such as ΔT, ΔH, AT, AH, diurnal temperature variation, and arrival wind speed, resulted to be crucial for the prevalence of the BRD-related pathogens and clinical signs investigated in the present study. Autumn has been found as one of the possible predisposing factors in the outbreak of this disease in cattle transported across North America (39–41). However, subsequent scientific literature suggested that the association between autumn and BRD occurrence may also be caused by the greater numbers of cattle marketed during that season, with implications on higher crowding, commingling, and competition for feeding at sale barns (33). We were not able to find the same strong association between all tested BRD-related pathogens and autumn, and this could be due to the difference in climate between North America and South Europe and to the different production strategies. Finally, it is worth noting the effect of climate changes on seasons, and consequently, also based on our results, to safeguard the welfare of the cattle during the journey, it seems more appropriate to plan journeys accordingly with the weather forecasting and mitigate the weather conditions than reducing the journeys in a particular season. Overall, our results suggest that different combinations of weather and travel conditions can increase or decrease the odds of animals being positive for a particular virus or bacterium, depending also on the characteristics of the pathogens.

Several bacteria have been linked with BRD. The positivity for *M. bovis* increased 10 times after traveling. *M. bovis* has been isolated from the lungs of animals affected by BRD, and in several cases of feedlot cattle with chronic, non-responsive respiratory disease (45, 46). However, it is often regarded as an opportunist in cattle respiratory tract, whose virulence is enhanced by other concomitant infections (47). Interestingly, in the present study, the prevalence of DNS positive to *M. bovis* increased when the AT was between 10 and 13°C and animals were transported in autumn to F1, with a strong arrival wind speed and a ΔH between −35 and −10%. The positive association between *M. bovis* and autumn agrees with the literature (39–41). This could be due to temperatures that favor bacterial growth and to the fact that the animals were grazed for the summer months on pasture in France. Sharing pasture between cattle and other animal species was proven to be a risk factor in *M. bovis* shedding (48). However, the sanitary condition of the farm (people, equipment, and managing decisions) may play also a primary role in *M. bovis* shedding, as suggested also by previous research on this pathogen (49). Feedlot placement affects the nasopharyngeal microbiota of beef cattle (7), and it is not surprising that the farm of arrival was also one of the major factors affecting the prevalence of *H. somni* (14). In particular, the animals being more likely positive to this pathogen were those transported to F1, suggesting that this bacterium could have been endemic in that farm. An outbreak of *H. somni* infection was found in young male cattle from an autochthonous breed reared in a farm located in the Center of Italy during the first months of 2016 (50). Schiavon et al. (51) reported that in Italy, *H. somni*-related pathologies mainly occur in beef steers aged between 4 and 10 months during autumn and winter seasons and their onset can be affected by the stress resulting from cold and changing weather conditions (52). Our animals belonged to that age category, and we found an increase of positivity in autumn and summer, but not in winter. Despite the association between summer and *H. somni* positivity may appear surprising at first, it is worth mentioning that the tested vehicles crossed the Alps; the temperature curve during these types of journeys in summer may be extremely stressful for the transported animals, with temperatures dropping from 25 to 6°C

(53) and then increasing again to 30°C during the travel. While the role of *H. somni* in BRD onset is still unclear, *P. multocida* is among the key pathogens associated with bovine pneumonia (54). In our study, about 35% of the animals resulted to be positive for *P. multocida* after the journey, and low stocking densities, pre-existing infection, and the presence of an extra stop during transport resulted to be predisposing factors. *P. multocida* transmission mainly occurs *via* direct contact with infectious secretions or excretions or through inhalation of aerosols (55). It is, therefore, possible that the transported beef steers already positive to this pathogen at T0, which were free to move inside the vehicles, may have expressed more exploratory behaviors and social interactions when transported at low stocking densities and during the stop, thus increasing the frequency of contacts between animals and the spreading of *P. multocida*. The presence of an extra stop during the journey should also be seen as an increase in journey duration and as an unuseful delay. It is well-known indeed that travel duration is a risk factor that increases the likelihood of opportunistic infections, such as Pasteurellaceae, in the lower respiratory tract (56) and of pneumonia in the transported animals (28, 57). Finally, MCA highlighted a cluster of animals where *P. multocida* positivity seemed to be related to BRSV positivity, spring season, and others as the category for the farm of arrival. In our study, the presence of BRSV was positively associated with *M. haemolytica*. This is in line with the literature because BRSV acts as a primary pathogen and predisposes the animal to secondary infections by *Pasteurella* spp. (14, 58) and *M. haemolytica* (59). As for *Pasteurella* spp., *M. haemolytica* positivity was more common at T1 among the animals that were subjected to an extra stop during the transport. However, this bacterium resulted to be positively associated also with low precipitations at arrival. A dry climate could be a possible predisposing factor because of the greater presence of dust in the air. This result seems to be consistent also with the evidence by Dubrovsky et al. (60) that reported dust occurring regularly in the calf-raising area was associated with increased BRD prevalence in pre-weaned calves. Dust may cause an inflammatory response in the nasopharyngeal tract, but also may be a carrier of airborne microorganisms, including viruses (61).

The exact role of viruses in BRD occurrence is still unclear. Viruses may produce a clinical syndrome consistent with BRD also in the absence of bacterial co-infection (62), but viral infections are generally considered as previous to, or concurrent with, bacterial infection (15). In our study, no positive DNS at departure and very few positivities at arrival were observed for BVDV, BoHV-1, and BPIV-3. On the other hand, BCoV was the pathogen showing the most dramatic increase from T0 and T1. This result suggests a fast replication of BCoV in the upper respiratory tract of animals in distress after long-distance transport, in agreement with the literature (14). Despite the significant increase, no associations were found. This result suggests that BCoVs spread independently of season, stocking densities, and climate, being extremely contagious no matter the travel and weather conditions. Contrariwise, BRSV resulted to be associated with an extra stop during transport, a particular farm (F3 and F4), a humidity ranging between 60 and 70%, and abnormally high temperatures in winter. This could be due to the fact that air humidity is considered among the main environmental risk factors increasing pathogen density and survival time outside the host (63). As observed for *P. multocida*, lower stocking densities during transport were associated with increased odds of having beef steers positive to BAdV. As already suggested, this may be related to the fact that increased space allowance during transport facilitates social interactions. However, during low stocking density, ventilation may also be stronger, increasing the spreading of the virus. The farm of arrival was associated with BAdV suggesting the epidemiological role of the farm of arrival even if our animals were isolated. Therefore, as the sampled animals did not get in touch with the others already located in the arrival farms, it may be likely that equipment and workers may have played roles. However, this hypothesis needs to be confirmed by further studies.

In our study, the odds of having beef steers with lacrimal and nasal discharges were higher at F2. Nasal discharge was significantly associated with the prevalence of *M. haemolytica* and BCoV, and with AT above 10°C. Both the presence of lacrimal discharge and diarrhea were associated with a higher AH. These results are therefore in agreement with the general view that sudden changes in air humidity represent a stressor possibly triggering a clinical state, and that high humidity favors dampness and disease spread. In addition, high $\Delta T$ and $\Delta H$ resulted in increase in the possibility of transported animals displaying diarrhea and coughing. These results are not surprising when we consider the scientific literature. Several viral agents may survive longer in aerosol with increased relative humidity in the air, and their persistence may also depend on temperature (64). Coughing was also more frequent if no arrival precipitations were noticed. As mentioned before, a dry climate may cause increased amounts of dust in the air, possibly causing an inflammatory response in the respiratory tract epithelium and also playing a role as a carrier of airborne microorganisms. Concerning coughing, this symptom was associated also with higher stocking density during transport. The more the animals, the more the concentration of noxious gases, impurities, and water vapor increases in the air during transport. This may participate in causing inflammatory reactions in the mucosa of the respiratory tract. However, coughing did not result to be associated with any positivity for the tested pathogens. This was expected since this clinical sign is non-specific, and it is not always present in the case of transport pneumonia in both horses (5) and bovine (33). Our result suggests that using cough monitoring to identify an early stage of BRD may not be appropriate. In the present study, the nasal discharge was instead found to be often associated with specific pathogens positivities. Thus, the presence of nasal discharge may be a more accurate marker to start treating the animals.

Antimicrobial metaphylaxis upon feedlot arrival is commonly used to reduce morbidity and mortality (65). However, this mass administration of antibiotics dramatically contributes to the development of antibiotic resistance, one of the major global health threats (66). To limit the spreading of pathogens linked to the transport of live animals and slow the development of antibiotic resistance, it would be desirable to identify more accurate diagnostic methods to test the animals before departure

and other types of preventions. For instance, in our study before departure, many animals resulted to be positive but asymptomatic. The use of accurate diagnostic tools may help in identifying the animals positive to the BRD-related pathogens, preventing them from being transported, and decreasing the risks of pathogen spreading during and after transport. Given the high prevalence of BCoV at T1, it is also advisable to vaccinate animals before departure, to avoid the possibility that this pathogen may act as a trigger to other secondary bacterial infections involved in BRD occurrence. Based on our findings, immunization toward the pathogens present in the destination farms seems also a good practice. Good management practices, such as avoiding abrupt changes in the environmental parameters and the presence of dust inside the feedlot, may help to prevent the outbreak of BRD and should be also be recommended. Better transport practices would also help to reduce the use of antibiotics, thus limiting the exposure of bacteria to antibiotics, therefore slowing the development of antibiotic resistance (14).

Our results need to be interpreted with caution because the study has some limitations. First, this is a preliminary and an opportunistic research, and thus we had not the possibility to balance throughout the year of sampling all the variables possibly affecting BRD-related symptoms and pathogens. For example, as can be noticed by the MCA biplots, some farms received few animals and mainly in one or two seasons. This may have affected the power of the statistical analysis and might have been a confounding factor. Second, it was not possible to download from the vehicles and analyze the data concerning the temperatures and humidity conditions during transport, which may have contributed to explaining part of the variability observed in the considered sample. Third, mortality and morbidity rates of the total population ($n = 1{,}045$) were not recorded, and no other follow-up of the 169 tested beef steers was possible due to logistic problems. Fourth, since only nasal swabs were collected in unhandled animals and some sampling was difficult to perform, it would have been possible that our prevalence was underestimated due to false negative. Fifth, it was impossible to record the real duration of each journey and the rectal temperature. Finally, our methodological approach did not presume to define direct cause–effect relationships, but mainly to investigate possible associations useful to screen possible factors that need further investigation. Notwithstanding these limitations, this manuscript documents for the first time the prevalence of clinical signs and pathogens related to BRD in a consistent sample of beef steers (169 animals) throughout the whole year of long-distance travel from France to Italy with integrated use of regression and multivariate analyses.

Overall, the tested journey led to an increased prevalence of BRD-related pathogens and clinical signs, and we suggested that weather and travel conditions play important roles. It seems that environmental parameters and their abrupt changes, such as those occurring during hot seasons in Italy, are more likely to increase the prevalence of several symptoms and pathogens related to BRD. This paper also raises doubts about the effects of more than one unloading stop during long journeys that seemed to increase the positivity to particular bacteria. Finally, our results confirmed the importance of feedlot management as a factor affecting the prevalence of several BRD-related pathogens, suggesting that more interventions (i.e., more testing and immunizations) should be performed before transport.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The experimental procedures were approved by the Ethics Committee of the Department of Veterinary Medicine of the University of Bari, Italy (authorization no. 16/18).

## AUTHOR CONTRIBUTIONS

BP, GF, AG, AP, and DT: conceptualization. FC, BP, GF, AG, LAN, AP, and DT: methodology. BP and MZ: formal analysis. AP, FC, and MZ: investigation. AP: resources, supervision, and project administration. BP and MZ: data curation and writing—original draft preparation. BP and AP: writing—review and editing. All authors contributed to the article and approved the submitted version.

## ACKNOWLEDGMENTS

The authors are grateful to Siciliani S.p.A. for allowing us to use the animals and the facilities.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.627894/full#supplementary-material

## REFERENCES

1. FAOSTAT. *Live Animals*. Available online at: http://www.fao.org/faostat/en/#data/QA (accessed November 9, 2020).
2. *SustainBeef - Beef Production in Italy*. Available online at: https://www6.inrae.fr/sustainbeef/Publications/Beef-production-in-the-EU/Beef-production-in-Italy (accessed November 9, 2020).
3. Padalino B, Riley CB. Editorial: the implications of transport practices for horse health and welfare. *Front Vet Sci.* (2020) 7:202. doi: 10.3389/fvets.2020.00202
4. Schwartzkopf-Genswein KS, Faucitano L, Dadgar S, Shand P, González LA, Crowe TG. Road transport of cattle, swine and poultry in North America and its impact on animal welfare, carcass and meat quality: a review. *Meat Sci.* (2012) 92:227–43. doi: 10.1016/j.meatsci.2012.04.010

5. Padalino B. Effects of the different transport phases on equine health status, behavior, and welfare: a review. *J Vet Behav.* (2015) 10:272–82. doi: 10.1016/j.jveb.2015.02.002
6. Šímová V, Večerek V, Passantino A, Voslárová E. Pre-transport factors affecting the welfare of cattle during road transport for slaughter – a review. *Acta Vet Brno.* (2016) 85:303–18. doi: 10.2754/avb201685030303
7. Holman DB, Timsit E, Amat S, Abbott DW, Buret AG, Alexander TW. The nasopharyngeal microbiota of beef cattle before and after transport to a feedlot. *BMC Microbiol.* (2017) 17:70. doi: 10.1186/s12866-017-0978-6
8. Griebel P, Hill K, Stookey J. How stress alters immune responses during respiratory infection. *Anim Health Res Rev.* (2014) 15:161–5. doi: 10.1017/S1466252314000280
9. Earley B, Buckham Sporer K, Gupta S. Invited review: relationship between cattle transport, immunity and respiratory disease. *Anim Int J Anim Biosci.* (2017) 11:486–92. doi: 10.1017/S1751731116001622
10. Padalino B, Raidal SL, Carter N, Celi P, Muscatello G, Jeffcott L, et al. Immunological, clinical, haematological and oxidative responses to long distance transportation in horses. *Res Vet Sci.* (2017) 115:78–87. doi: 10.1016/j.rvsc.2017.01.024
11. Guzman E, Taylor G. Immunology of bovine respiratory syncytial virus in calves. *Mol Immunol.* (2015) 66:48–56. doi: 10.1016/j.molimm.2014.12.004
12. Murray GM, O'Neill RG, More SJ, McElroy MC, Earley B, Cassidy JP. Evolving views on bovine respiratory disease: an appraisal of selected key pathogens - part 1. *Vet J Lond Engl.* (2016) 217:95–102. doi: 10.1016/j.tvjl.2016.09.012
13. Timsit E, Dendukuri N, Schiller I, Buczinski S. Diagnostic accuracy of clinical illness for bovine respiratory disease (BRD) diagnosis in beef cattle placed in feedlots: a systematic literature review and hierarchical Bayesian latent-class meta-analysis. *Prev Vet Med.* (2016) 135:67–73. doi: 10.1016/j.prevetmed.2016.11.006
14. Pratelli A, Cirone F, Capozza P, Trotta A, Corrente M, Balestrieri A, et al. Bovine respiratory disease in beef calves supported long transport stress: an epidemiological study and strategies for control and prevention. *Res Vet Sci.* (2021) 135:450–5. doi: 10.1016/j.rvsc.2020.11.002
15. Cusack PMV, McMeniman N, Lean IJ. The medicine and epidemiology of bovine respiratory disease in feedlots. *Aust Vet J.* (2003) 81:480–7. doi: 10.1111/j.1751-0813.2003.tb13367.x
16. Padalino B, Barrasso R, Tullio D, Zappaterra M, Nanni Costa L, Bozzo G. Protection of animals during transport: analysis of the infringements reported from 2009 to 2013 during on-road inspections in Italy. *Anim Open Access J MDPI.* (2020) 10:356. doi: 10.3390/ani10020356
17. *Report on the Implementation of Council Regulation No 1/2005 on the Protection of Animals During Transport Within and Outside the EU*. Available online at: https://www.europarl.europa.eu/doceo/document/A-8-2019-0057_EN.html (accessed November 9, 2020).
18. Khatkar NS Dhand MK. *Statulator: An Online Tool for Basic Statistical Analysis*. Available online at: http://statulator.com/ (accessed November 9, 2020).
19. Ayres H, Ferreira RM, de Souza Torres-Júnior JR, Demétrio CGB, de Lima CG, Baruselli PS. Validation of body condition score as a predictor of subcutaneous fat in Nelore (*Bos indicus*) cows. *Livest Sci.* (2009) 123:175–9. doi: 10.1016/j.livsci.2008.11.004
20. Cirone F, Padalino B, Tullio D, Capozza P, Lo Surdo M, Lanave G, et al. Prevalence of pathogens related to bovine respiratory disease before and after transportation in beef steers: preliminary results. *Anim Open Access J MDPI.* (2019) 9:1093. doi: 10.3390/ani9121093
21. Kishimoto M, Tsuchiaka S, Rahpaya SS, Hasebe A, Otsu K, Sugimura S, et al. Development of a one-run real-time PCR detection system for pathogens associated with bovine respiratory disease complex. *J Vet Med Sci.* (2017) 79:517–23. doi: 10.1292/jvms.16-0489
22. Peduzzi P, Concato J, Kemper E, Holford TR, Feinstein AR. A simulation study of the number of events per variable in logistic regression analysis. *J Clin Epidemiol.* (1996) 49:1373–9. doi: 10.1016/S0895-4356(96)00236-3
23. R Core Team. *R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.* (2020). Available online at: https://www.R-project.org/ (accessed November 10, 2020).
24. Bursac Z, Gauss CH, Williams DK, Hosmer DW. Purposeful selection of variables in logistic regression. *Source Code Biol Med.* (2008) 3:17. doi: 10.1186/1751-0473-3-17
25. European Parliament. Directorate General for Parliamentary Research Services. *Regulation (EC) No 1/2005 on the Protection of Animals During Transport and Related Operations: European Implementation Assessment*. LU: Publications Office (2018). Available online at: https://data.europa.eu/doi/10.2861/15227 (accessed November 9, 2020).
26. Manuja BK, Manuja A, Singh RK. Globalization and livestock biosecurity. *Agric Res.* (2014) 3:22–31. doi: 10.1007/s40003-014-0097-7
27. Broom D. Transport stress in cattle and sheep with details of physiological, ethological and other indicators. *DTW Dtsch Tierarztl Wochenschr.* (2003) 110:83–9.
28. Nielsen BL, Dybkjær L, Herskin MS. Road transport of farm animals: effects of journey duration on animal welfare. *Anim Int J Anim Biosci.* (2011) 5:415–27. doi: 10.1017/S1751731110001989
29. *Long Distance Transport and Welfare of Farm Animals*. CABI.org. Available online at: https://www.cabi.org/bookshop/book/9781845934033/ (accessed November 9, 2020).
30. Padalino B, Tullio D, Cannone S, Bozzo G. Road transport of farm animals: mortality, morbidity, species and country of origin at a Southern Italian control post. *Anim Open Access J MDPI.* (2018) 8:155. doi: 10.3390/ani8090155
31. Schwartzkopf-Genswein K. Cattle transport by road. In: Grandin T, editor. *Livestock Handling and Transport. 4th Edn*. Wallingford, UK: CABI (2015) p. 143–73. doi: 10.1079/9781780643212.0143
32. Cernicchiaro N, White BJ, Renter DG, Babcock AH, Kelly L, Slattery R. Associations between the distance traveled from sale barns to commercial feedlots in the United States and overall performance, risk of respiratory disease, and cumulative mortality in feeder cattle during 1997 to (2009). *J Anim Sci.* (2012) 90:1929–39. doi: 10.2527/jas.2011-4599
33. Taylor JD, Fulton RW, Lehenbauer TW, Step DL, Confer AW. The epidemiology of bovine respiratory disease: what is the evidence for predisposing factors? *Can Vet J.* (2010) 51:1095–102.
34. Richer L, Marois P, Lamontagne L. Association of bovine viral diarrhea virus with multiple viral infections in bovine respiratory disease outbreaks. *Can Vet J.* (1988) 29:713–7.
35. Storz J, Lin X, Purdy CW, Chouljenko VN, Kousoulas KG, Enright FM, et al. Coronavirus and *Pasteurella* infections in bovine shipping fever pneumonia and evans' criteria for causation. *J Clin Microbiol.* (2000) 38:3291–8. doi: 10.1128/JCM.38.9.3291-3298.2000
36. Cook NJ, Veira D, Church JS, Schaefer AL. Dexamethasone reduces transport-induced weight losses in beef calves. *Can J Anim Sci.* (2011) 89:335–9. doi: 10.4141/CJAS09006
37. Schwartzkopf-Genswein KS, Booth-McLean ME, Shah MA, Entz T, Bach SJ, Mears GJ, et al. Effects of pre-haul management and transport duration on beef calf performance and welfare. *Appl Anim Behav Sci.* (2007) 108:12–30. doi: 10.1016/j.applanim.2006.11.012
38. Frank GH, Smith PC. Prevalence of *Pasteurella haemolytica* in transported calves. *Am J Vet Res.* (1983) 44:981–5.
39. Ribble CS, Meek AH, Janzen ED, Guichon PT, Jim GK. Effect of time of year, weather, and the pattern of auction market sales on fatal fibrinous pneumonia (shipping fever) in calves in a large feedlot in alberta (1985-1988). *Can J Vet Res.* (1995) 59:167–72.
40. Loneragan GH, Dargatz DA, Morley PS, Smith MA. Trends in mortality ratios among cattle in US feedlots. *J Am Vet Med Assoc.* (2001) 219:1122–7. doi: 10.2460/javma.2001.219.1122
41. Cernicchiaro N, Renter DG, White BJ, Babcock AH, Fox JT. Associations between weather conditions during the first 45 days after feedlot arrival and daily respiratory disease risks in autumn-placed feeder cattle in the United States. *J Anim Sci.* (2012) 90:1328–37. doi: 10.2527/jas.2011-4657
42. Snowder GD, Van Vleck LD, Cundiff LV, Bennett GL. Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. *J Anim Sci.* (2006) 84:1999–2008. doi: 10.2527/jas.2006-046
43. Step DL, Krehbiel CR, DePra HA, Cranston JJ, Fulton RW, Kirkpatrick JG, et al. Effects of commingling beef calves from different sources and weaning protocols during a forty-two-day receiving period on performance and bovine respiratory disease. *J Anim Sci.* (2008) 86:3146–58. doi: 10.2527/jas.2008-0883
44. Gorden PJ, Plummer P. Control, management, and prevention of bovine respiratory disease in dairy calves and cows. *Vet Clin North Am Food Anim Pract.* (2010) 26:243–59. doi: 10.1016/j.cvfa.2010.03.004

45. Shahriar FM, Clark EG, Janzen E, West K, Wobeser G. Coinfection with bovine viral diarrhea virus and *Mycoplasma bovis* in feedlot cattle with chronic pneumonia. *Can Vet J.* (2002) 43:863–8.
46. Haines DM, Martin KM, Clark EG, Jim GK, Janzen ED. The immunohistochemical detection of *Mycoplasma bovis* and bovine viral diarrhea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. *Can Vet J.* (2001) 42:857–60.
47. Janzen E. The role of *Haemophilus somnus* in bovine respiratory disease (BRD). *Bovine Practitioner.* (1997) 1997:39–45.
48. Spergser J, Macher K, Kargl M, Lysnyansky I, Rosengarten R. Emergence, re-emergence, spread and host species crossing of *Mycoplasma bovis* in the Austrian alps caused by a single endemic strain. *Vet Microbiol.* (2013) 164:299–306. doi: 10.1016/j.vetmic.2013.02.007
49. Gille L. *Mycoplasma bovis: Sources of Infection, Prevalence and Risk Factors [dissertation]*. Ghent University (2018). Available online at: http://hdl.handle.net/1854/LU-8587081 (accessed November 9, 2020).
50. Attili AR, Rossi G, Cavirani S, Preziuso S, Bartolini L, Cuteri V. *Histophilus somni Infection: An Outbreak in Cattle in Central italy*. Hipra. (2017). Available online at: https://www.hipra.com/portal/it/hipra/knowledge/pubdetail/histophilus-somni-outbreak-in-cattle-in-italy (accessed November 9, 2020).
51. Schiavon E, Florian E, Alberton A, Rampin F, Mutinelli F. Infezione da *Histophilus somni* nel bovino: casi clinici. *Large Anim Rev.* (2008) 14:155–60. Available online at: https://www.vetjournal.it/images/archive/pdf_riviste/4186.pdf
52. Blood DC, Radostis OM, Henderson JA. *Patologia Medica Veterinaria*. Padova: Casa Editrice Ambrosiana (1988). Available online at: https://www.libreriauniverso.it/ (accessed November 9, 2020).
53. Hartung J, Springorum AC. Microclimate on transport vehicles. In: Smulders FJM, Algers B, editors. *Welfare of Production Animals: Assessment and Management of Risks*. Wageningen: Wageningen Academic Publishers (2009). p. 162–3.
54. Allen JW, Viel L, Bateman KG, Rosendal S, Shewen PE, Physick-Sheard P. The microbial flora of the respiratory tract in feedlot calves: associations between nasopharyngeal and bronchoalveolar lavage cultures. *Can J Vet Res Rev.* (1991) 55:341–6.
55. Jericho KW, Carter GR. Pneumonia in calves produced with aerosols of *Pasteurella multocida* alone and in combination with bovine herpesvirus 1. *Can J Comp Med.* (1985) 49:138–44.
56. Padalino B, Raidal SL, Knight P, Celi P, Jeffcott L, Muscatello G. Behaviour during transportation predicts stress response and lower airway contamination in horses. *PLoS ONE.* (2018) 13:e0194272. doi: 10.1371/journal.pone.0194272
57. Padalino B, Hall E, Raidal S, Celi P, Knight P, Jeffcott L, et al. Health problems and risk factors associated with long haul transport of horses in Australia. *Anim Open Access J MDPI.* (2015) 5:1296–310. doi: 10.3390/ani5040412
58. Yaman T, Büyükbayram H, Özyildiz Z, Terzi F, Uyar A, Keles ÖF, et al. Detection of bovine respiratory syncytial virus, *Pasteurella multocida*, and *Mannheimia haemolytica* by immunohistochemical method in naturally-infected cattle. *J Vet Res.* (2018) 62:439–45. doi: 10.2478/jvetres-2018-0070
59. Sacco RE, McGill JL, Pillatzki AE, Palmer MV, Ackermann MR. Respiratory syncytial virus infection in cattle. *Vet Pathol.* (2014) 51:427–36. doi: 10.1177/0300985813501341
60. Dubrovsky SA, Van Eenennaam AL, Karle BM, Rossitto PV, Lehenbauer TW, Aly SS. Epidemiology of bovine respiratory disease (BRD) in preweaned calves on California dairies: the BRD 10K study. *J Dairy Sci.* (2019) 102:7306–19. doi: 10.3168/jds.2018-14774
61. Zhao Y, Aarnink AJA, Jong MCMD, Koerkamp PWGG. Airborne microorganisms from livestock production systems and their relation to dust. *Crit Rev Environ Sci Technol.* (2014) 44:1071–128. doi: 10.1080/10643389.2012.746064
62. Decaro N, Campolo M, Desario C, Cirone F, D'Abramo M, Lorusso E, et al. Respiratory disease associated with bovine coronavirus infection in cattle herds in southern italy. *J Vet Diagn Investig Off Publ Am Assoc Vet Lab Diagn Inc.* (2008) 20:28–32. doi: 10.1177/104063870802000105
63. Callan RJ, Garry FB. Biosecurity and bovine respiratory disease. *Vet Clin North Am Food Anim Pract.* (2002) 18:57–77. doi: 10.1016/S0749-0720(02)00004-X
64. Elazhary MA, Derbyshire JB. Effect of temperature, relative humidity and medium on the aerosol stability of infectious bovine rhinotracheitis virus. *Can J Comp Med Rev.* (1979) 43:158–67.
65. Step DL, Engelken T, Romano C, Holland B, Krehbiel C, Johnson JC, et al. Evaluation of three antimicrobial regimens used as metaphylaxis in stocker calves at high risk of developing bovine respiratory disease. *Vet Ther.* (2007) 8:136–47.
66. McEachran AD, Blackwell BR, Hanson JD, Wooten KJ, Mayer GD, Cox SB, et al. Antibiotics, bacteria, and antibiotic resistance genes: aerial transport from cattle feed yards via particulate matter. *Environ Health Perspect.* (2015) 123:337–43. doi: 10.1289/ehp.1408555


**Conflict of Interest:** GF was employed by the company Siciliani S.p.A. Industria Lavorazione Carne.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 01 July 2021
doi: 10.3389/fvets.2021.692646

# Prevalence and Risk Factors Associated With Antimicrobial Resistance in Bacteria Related to Bovine Respiratory Disease—A Broad Cross-Sectional Study of Beef Cattle at Entry Into Canadian Feedlots

*Sara Andrés-Lasheras[1], Reuben Ha[1], Rahat Zaheer[1], Catrione Lee[1], Calvin W. Booker[2], Craig Dorin[3], Joyce Van Donkersgoed[4], Rob Deardon[5,6], Sheryl Gow[7,8], Sherry J. Hannon[2], Steve Hendrick[9], Michele Anholt[5,10] and Tim A. McAllister[1]**

[1] Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada, [2] Feedlot Health Management Services, Okotoks, AB, Canada, [3] Veterinary Agri-Health Systems, Airdrie, AB, Canada, [4] Alberta Beef Health Solutions, Picture Butte, AB, Canada, [5] Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada, [6] Department of Mathematics and Statistics, University of Calgary, Calgary, AB, Canada, [7] Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, [8] Public Health Agency of Canada, Saskatoon, SK, Canada, [9] Coaldale Veterinary Clinic Ltd., Lethbridge, AB, Canada, [10] POV Inc., Airdrie, AB, Canada

A broad, cross-sectional study of beef cattle at entry into Canadian feedlots investigated the prevalence and epidemiology of antimicrobial resistance (AMR) in *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis,* bacterial members of the bovine respiratory disease (BRD) complex. Upon feedlot arrival and before antimicrobials were administered at the feedlot, deep nasopharyngeal swabs were collected from 2,824 feedlot cattle in southern and central Alberta, Canada. Data on the date of feedlot arrival, cattle type (beef, dairy), sex (heifer, bull, steer), weight (kg), age class (calf, yearling), source (ranch direct, auction barn, backgrounding operations), risk of developing BRD (high, low), and weather conditions at arrival (temperature, precipitation, and estimated wind speed) were obtained. *Mannheimia haemolytica*, *P. multocida*, and *H. somni* isolates with multidrug-resistant (MDR) profiles associated with the presence of integrative and conjugative elements were isolated more often from dairy-type than from beef-type cattle. Our results showed that beef-type cattle from backgrounding operations presented higher odds of AMR bacteria as compared to auction-derived calves. Oxytetracycline resistance was the most frequently observed resistance across all *Pasteurellaceae* species and cattle types. *Mycoplasma bovis* exhibited high macrolide minimum inhibitory concentrations in both cattle types. Whether these MDR isolates establish and persist within the feedlot environment, requires further evaluation.

**Keywords: antimicrobial resistance, bovine respiratory disease, cross-sectional study, epidemiology, *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pasteurella multocida*, *Histophilus somni***

OPEN ACCESS

***Edited by:***
*Annamaria Pratelli, University of Bari Aldo Moro, Italy*

***Reviewed by:***
*Tara G. McDaneld, United States Department of Agriculture, United States*
*Getahun E. Agga, United States Department of Agriculture, United States*

****Correspondence:***
*Tim A. McAllister*
*tim.mcallister@agr.gc.ca*

***Specialty section:***
*This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science*

***Received:*** *08 April 2021*
***Accepted:*** *31 May 2021*
***Published:*** *01 July 2021*

***Citation:***
*Andrés-Lasheras S, Ha R, Zaheer R, Lee C, Booker CW, Dorin C, Van Donkersgoed J, Deardon R, Gow S, Hannon SJ, Hendrick S, Anholt M and McAllister TA (2021) Prevalence and Risk Factors Associated With Antimicrobial Resistance in Bacteria Related to Bovine Respiratory Disease—A Broad Cross-Sectional Study of Beef Cattle at Entry Into Canadian Feedlots. Front. Vet. Sci. 8:692646. doi: 10.3389/fvets.2021.692646*

## INTRODUCTION

Bovine respiratory disease (BRD) continues to be a challenging and costly disease in feedlot cattle in North America (1–3), accounting for 70–80% and 40–50% of the total herd-level morbidity and mortality, respectively (4). Moreover, it has been estimated that BRD costs the North American feedlot cattle industry over $3 billion annually (5). *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* are considered the main bacterial species associated with BRD (6). However, viruses, host, environment, and management practices also play important roles in this multi-factorial, complex disease (7). Considerable resources have been invested in the development of technologies and management strategies to mitigate and treat BRD, but the incidence of morbidity and mortality have remained relatively constant over the last 45 years (8). Practices such as preconditioning, improved diagnostics, and new vaccines continue to be developed, investigated, and validated as alternatives to antimicrobials. However, until these alternatives are shown to be cost-effective, practically implementable, and on-par or surpass currently available practices, it is likely that antimicrobial therapy will continue to be an important tool for preventing, treating, and controlling BRD in feedlot cattle.

Previous studies have suggested that antimicrobial resistance (AMR) amongst BRD-related bacteria has been increasing in North America (9–11). However, these studies mostly collected samples from clinical BRD cases. A previous Alberta study found that 30% of *M. haemolytica* and 12.5% of *P. multocida* isolated from BRD cattle mortalities were resistant to macrolides, tetracyclines (TETs), β-lactams, fluoroquinolones (FQs), lincosamides, aminoglycosides (AMGs), and pleuromutilins (12). Resistance to macrolides is of particular concern due to the importance of these drugs in controlling BRD in high risk incoming cattle (13). Unlike clinical BRD cases, studies that targeted beef cattle at feedlot arrival, prior to antimicrobial administration found that *M. haemolytica* and *P. multocida* were susceptible to fluoroquinolones, macrolides, β-lactams, and cephalosporins (14–16). This suggests that AMR increases after feedlot placement. However, previous published studies that assessed AMR prior to administration of antimicrobials at arrival focused on *M. haemolytica* and not all four members of the BRD bacterial complex (14, 15).

Development of AMR threatens access and efficacy of antimicrobials, has the potential to increase antimicrobial use (AMU) (5), threaten animal health and welfare (17), increase production costs (18), and promote dissemination of antimicrobial-resistance genes (ARG) in cattle and the environment (19). Passive AMR surveillance can help to identify emerging AMR trends (20). However, for a global and non-biased vision of current AMR patterns, epidemiologically robust studies are required. The Canadian beef industry in partnership with CIPARS (Canadian Integrated Program for Antimicrobial Resistance Surveillance) has recently developed a national feedlot AMU/AMR surveillance program targeting selected respiratory nasal bacterial pathogens and enteric bacteria (21). To complement the surveillance program, this study was designed to estimate the current prevalence and describe AMR in *M. haemolytica*, *P. multocida, H. somni,* and *M. bovis,* and to provide insight into the epidemiology and possible risk factors for AMR prior to cattle arriving at the feedlot.

## MATERIALS AND METHODS

### Ethics Statement and General Cattle Management

The animal study was reviewed and approved by the Lethbridge Research Centre Animal Care and Use Committee (Protocol #1641, Jan 18th, 2017) and was conducted according to the Canadian Council of Animal Care Guidelines (22).

### Sampling

From August 2017 to May 2018 and from August 2018 to April 2019 (19 months), deep nasopharyngeal swabs (DNPS) were collected from feedlot cattle at processing at feedlot arrival, prior to the administration of antimicrobials. Cattle were housed in ten different commercial feedlots located across southern and central Alberta purposely selected based on their one-time holding capacity, the use of an electronic record keeping system, and willingness to collaborate in the study. Each feedlot (designated A-J) was managed by one of four veterinary practices providing services to ∼80% of Alberta's 1.5 million feedlot cattle. Feedlots were selected based on their one-time holding capacity, the use of an electronic animal health record-keeping system, and their willingness to participate. Six feedlots had a one-time capacity >10,000 head; whereas four housed <10,000 head. For sampling, transport truck was considered the sampling unit. When beef-type cattle from different locations were transported in the same truck, location was also considered to define the sampling unit. For sample size calculation, the isolation percentage of BRD bacteria resistant to at least one antimicrobial was expected to by 15% based on previous pilot studies carried out in the same geographical area (JVD, personal communication). Sample size at animal level was calculated before the participating feedlots were assigned. Assuming $n = Z_{\alpha}^2\ pq/L^2$; $\alpha = 0.05$ ($Z_{\alpha} = 1.96$); $p = 0.15$; and with a precision of $L = 0.05$, a total of 200 head were initially targeted per feedlot per year. A total of 10 cattle from each of the 10 participating feedlots were targeted

**Abbreviations:** A, auction; AF, arrived from; AMG, aminoglycoside; AMP, ampicillin; AMR, antimicrobial resistance; AMU, antimicrobial use; ARG, antimicrobial resistance gene; B, backgrounding; BHI, brain-heart infusion; BRD, bovine respiratory disease; CI, Confidence Interval; CLSI, Clinical an Laboratory Standards Institute; cPCR, conventional PCR; CTET, chlortetracycline; DANO, danofloxacin; DNPS, deep nasopharyngeal swab; ENRO, enrofloxacin; FFN, florfenicol; FQ, fluoroquinolone; GLMM, generalized linear mixed model; GAM, gamithromycin; GEN, gentamycin; MDR, multidrug resistance; ICE, integrative and conjugative element; MGE, mobile genetic element; MIC, minimum inhibitory concentration; NEO, neomycin; OR, odds ratio; OXY, oxytetracycline; PEN, penicillin; qPCR, quantitative PCR; QRDR, quinolone resistant determining region; RD, ranch direct; RE, random effect; RFID, radio frequency identification; SIR, susceptible, intermediate, resistant; SPE, spectinomycin; TC, truck cluster; TET, tetracycline; TIL, tilmicosin; TIO, ceftiofur; TIP, tildipirosin; TMR, total mixed ration; TUL, tulathromycin; TYLT, tylosin tartrate; WGS, whole genome sequencing.

for sampling every 2 weeks for 40 weeks (September to May) over two consecutive years. Animals were selected using cluster sampling, with cattle within a transport truck constituting a cluster. During off-loading, ten animals were randomly selected as they passed through the chute. If two or more transport trucks arrived on the same day, two trucks were randomly selected and five animals were sampled from each truck. A single DNPS was collected from each animal by a trained staff member using a guarded swab (BD CultureSwab™ Plus, Mississauga ON). Swabs were shipped on ice in Amies transport media without charcoal (BD CultureSwab™ Plus) to the Lethbridge Research and Development Centre Microbiology Laboratory. Samples were kept at 4°C for up to 7 d prior to isolation of bacteria.

Upon arrival, the electronic ear tag was scanned and the arrival date, cattle type (beef, dairy), sex (heifer, bull, steer), weight (kg), age class (calf, yearling), source (ranch direct, auction barn, backgrounding operation), weather (temperature, precipitation, estimated wind speed), and BRD risk (high, low) were recorded. Ranch-direct cattle were defined as calves that arrived directly from the birth farm immediately after weaning or following a variable feeding period. Backgrounding operation-sourced cattle arrived directly from the backgrounding feedlot where they had been since weaning and fed a forage-based diet until ready for a finishing diet. Auction-derived cattle may have arrived at auction directly from the birth farm or from a backgrounding operation; this earlier history was unknown. Risk of BRD was predicted by the feedlot as per usual practices based on algorithms that consider age class, body weight, source, comingling prior to arrival, weather, transport distance, and visual health assessment. The geographical location of each feedlot, country of origin, and the point of sale at auction were recorded. Unlike dairy-type calves, beef-type calves were not traced back to their farm of origin given that more than 2/3 of the cattle (67.7%) were auction market derived. Morbidity and mortality data were compiled for all enrolled cattle up to 120 d post-arrival. Morbid animals [e.g., displaying gauntness, inactivity, depression, elevated rectal temperature (typically over 40°C), ocular/nasal discharge, cough, and/or elevated respiration rate] were designated as having BRD by trained feedlot pen riders or animal health personnel. Information on AMU before feedlot placement was unavailable.

## Bacterial Isolation and Species Identification

Upon arrival at the laboratory, swabs were individually immersed in 1 mL of brain-heart infusion (BHI) containing 20% glycerol (Dalynn Biologicals, Calgary AB) and vortexed for 1 min (14). For the isolation of *M. haemolytica* and *P. multocida*, 100 μL suspension was plated onto blood agar (TSA) with 5% sheep blood supplemented with bacitracin at 15 μg/mL (Dalynn Biologicals). An additional 100 μL aliquot was plated onto TSA blood agar (Dalynn) for the isolation of *H. somni*. Additionally, a 1:10 dilution of the initial BHI-glycerol suspension was plated (100 μL) on TSA blood agar plates to enable isolation of *H. somni* without overgrowth. Bacitracin plates were incubated in an aerobic atmosphere at 37°C for 24 h, before selecting *P. multocida* and *M. haemolytica* colonies. *Histophilus somni* plates were incubated at 37°C for 2 d in a 5% $CO_2$ atmosphere. Presumptive *M. haemolytica*, *P. multocida*, or *H. somni* colonies were identified, and three colonies of each bacterial species were sub-cultured onto TSA blood agar. Colonies were picked from TSA and stored at −80°C in BHI supplemented with 20% glycerol. For *M. bovis*, a high throughput PCR-based enrichment process was used for screening for the presence of *M. bovis* followed by bacterial isolation from positive suspensions (23).

All bacterial isolates were confirmed using PCR species-specific primers (**Table 1**) using a *HotStartTaq Plus Master Mix* kit (Qiagen, Toronto ON). For this, 3–5 colonies of pure cultures of *P. multocida*, *M. haemolytica*, or *H. somni* were suspended in 100 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH = 8) and heat-lysed for 5 min at 95°C. The suspension was centrifuged for 10 min, at 18,400 × g at 4°C, and 2 μL of the supernatant was used as a DNA template. Presumptive *M. bovis* cultures were confirmed via direct-culture-PCR using 2 μL of liquid culture directly in the PCR master mix (23). The PCR amplicons were visualized either by agarose gel or capillary electrophoresis (QIAxcel, Qiagen). One randomly selected colony per bacterial species per DNPS was selected for further characterization and analysis. Additionally, *M. haemolytica* isolates were further characterized as serotype A1, A2 or A6 using a multiplex PCR (**Table 1**) (27).

## Antimicrobial Susceptibility Testing

Due to the high recovery of *P. multocida* and the cost of *M. bovis* ASTs, antimicrobial susceptibilities were only performed on a subset of *P. multocida* ($n = 273/703$ beef and $n = 242/463$ dairy isolates) and *M. bovis* ($n = 122/257$ beef and $n = 100/198$ dairy) isolates, whereas all *M. haemolytica* ($n = 281$ beef and $n = 209$ dairy) and *H. somni* ($n = 68$ and $n = 173$ dairy) isolates were tested. *Pasteurella multocida* and *M. bovis* isolates were selected based on proportional stratified random sampling (28) with consideration of feedlot, sampling month, cattle type, and country of origin. The antimicrobial susceptibility testing of *M. haemolytica*, *P. multocida*, and *H. somni* was carried out by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines using a final bacterial inoculum of $\sim 5 \times 10^5$ CFU/mL (29). The Sensititre plate BOPO6F was used (Thermo Fisher Scientific, Mississauga ON), which represents a broad-spectrum of antimicrobials commonly used in beef cattle production. For *M. haemolytica* and *P. multocida*, the final per-well bacterial inoculum volume was 50 μL, whereas for *H. somni,* the volume was doubled to 100 μL as per manufacture's specifications. Therefore, the final antimicrobial concentrations tested for *H. somni* were half that of *M. haemolytica* and *P. multocida* (**Supplementary Tables 1.1–1.3**). Considering that the maximum spectinomycin (SPE) concentration against *H. somni* in BOPO6F plate was only 32 μg/mL, a custom plate with SPE ranging from 8 to 512 μg/mL was prepared for susceptibility testing. Bacterial isolates were designated as susceptible, intermediate, or resistant (SIR) according to CLSI

**TABLE 1 |** Oligonucleotide primers, PCR protocols, and amplicon sizes for each PCR assay used in this study.

| Bacterial species and targeted gene | Primer sequences (5'–3') and PCR cycling conditions | | Amplicon size (bp) | References |
|---|---|---|---|---|
| Mh, *lktC - artJ* intergenic region | F – GTCCCTGTGTTTTCATTATAAG<br>R – ACTCGATAATTATTCTAAATTAG<br>95°C, 5 min; (94°C, 30 s; 58°C, 45 s; 72°C, 60 s) ×35 cycles; 72°C, 10 min | | 385 | (24) |
| Pm, 23S rRNA | F – GGCTGGGAAGCCAAATCAAAG<br>R – CGAGGGACTACAATTACTGTAA<br>95°C, 5 min; (94°C, 30 s; 58°C, 45 s; 72°C, 60 s) ×35 cycles; 72°C, 10 min | | 1,432 | (25) |
| Hs, 16S rRNA | F – GAAGGCGATTAGTTTAAGAG<br>R – TTCGGGCACCAAGTRTTCA<br>95°C, 5 min; (94°C, 30 s; 55°C, 45 s; 72°C, 60 s) ×35 cycles; 72°C, 10 min | | 400 | (26) |
| Mb, *uvrC* | F – CCTGTCGGAGTTGCAATTGT<br>R – GCACTGCGCTCATTTAAAGC<br>95°C, 5 min[a]; (94°C, 30 s; 61.5°C, 30 s; 72°C, 30 s) × 35 cycles, 72°C, 10 min | | 92 | (23) |
| Mh serotype A1, hypothetical protein | F – CATTTCCTTAGGTTCAGC<br>R – CAAGTCATCGTAATGCCT | 95°C, 15 min; (94°C, 30 s; 55°C, 45 s; 72°C, 1 min) × 35 cycles; 72°C, 10 min | 306 | (27) |
| Mh serotype A2, core-2/I-branching enzyme | F – GGCATATCCTAAAGCCGT<br>R – AGAATCCACTATTGGGCACC | | 160 | |
| Mh serotype A6, *TupA* | F – TGAGAATTTCGACAGCACT<br>R – ACCTTGGCATATCGTACC | | 78 | |

[a]*Denaturation time increased from 5 min to 10 min if cell lysis was required during the PCR cycle.*
*Mh, Mannheimia haemolytica; Pm, Pasteurella multocida; Hs, Histophilus somni; Mb, Mycoplasma bovis; F, forward primer; R, reverse primer.*

(30). Additionally, CLSI MIC interpretive criteria for tilmicosin (TIL) in *M. haemolytica* was extrapolated to *P. multocida* and *H. somni* (31). Interpretive criteria for danofloxacin from *M. haemolytica* and *P. multocida* was also extrapolated to *H. somni* (32).

For *M. bovis*, recommendations for broth microdilution antimicrobial susceptibility were followed as official international guidelines are unavailable (11, 33, 34). A custom Sensititre plate (ref. no. YCML2FMBC, Thermo Fisher Scientific) was used to assess isolate sensitivities (**Supplementary Table 1.4**) (11). *Mycoplasma bovis* isolates were grown in PPLO broth and suspensions were adjusted to obtain a final concentration of $10^3$-$10^5$ CFU/mL per well (100 μL final volume). Plates were incubated for 48 h, at 37°C, in a 5% $CO_2$ atmosphere (35). AlamarBlue™ (Cell Viability Reagent. Thermo Fisher Scientific) at a final concentration of 10% was included in each well as indicator of cell viability and growth. *Mycoplasma bovis* ATCC 25523 was included as an internal standard in all susceptibility assays. Since CLSI breakpoints for *M. bovis* have not been developed, only MICs were reported.

Multidrug resistance (MDR) was defined as resistance to three or more antimicrobial drug classes (36). Isolates with intermediate susceptibility to an antimicrobial were not included in this definition. The 50th and 90th percentiles for MIC ($MIC_{50}$ and $MIC_{90}$, respectively) were calculated as the antimicrobial concentrations required to inhibit 50 and 90% of the isolates within each bacterial species. If growth was observed at the highest antimicrobial concentration tested, the MIC was assigned to the next dilution.

## Antimicrobial Resistance Genetic Determinants

A subset of *M. haemolytica* ($n = 87$), *P. multocida* ($n = 64$), and *H. somni* ($n = 24$) isolates were selected for whole-genome sequencing (WGS). Isolates were selected to represent the diversity of feedlot operations, geographical origin, cattle type, sex, time of the year, and AMR phenotypes using proportional stratified random sampling. To isolate genomic DNA, bacterial cultures grown on blood agar plates were suspended in TE buffer (10 mM Tris – 1 mM EDTA, pH 8.0) to an $OD_{600}$ of ∼2, representing ∼$2 \times 10^9$ CFU/ mL. The cell suspension (1 mL) was transferred to a microcentrifuge tube and centrifuged for 2 min at 14,000 × g. Genomic DNA was extracted from the cell pellet using a DNeasy Blood and Tissue kit (Qiagen, Montreal QC). DNA quality and quantity were confirmed using a Nanodrop 2000 spectrophotometer and a fluorometer using PicoGreen (Thermo Fisher Scientific), respectively. Genomic library construction was performed using the NEB Ultra II library preparation kit (New England BioLabs, Ipswich MA). Library quality was assessed using a Bioanalyzer (Agilent, Santa Clara CA). Libraries were sequenced on an Illumina HiSeqX platform to generate 2 × 150 base paired-end reads. Library preparation and Illumina sequencing services were provided by Genome Quebec (McGill, QC). The quality of raw sequence reads was assessed using the quality control tool FastQC and reads were assembled into contigs using the Shovill pipeline (37). This pipeline included a quality trimming step with Trimmomatic (v.0.38) to remove common Illumina adapter sequences followed by *de novo* assembly with SPAdes (v.3.13.0) (38). The assembled

contigs were annotated with Prokka (39) to identify coding genes. The assembled contigs were searched against the NCBI Bacterial Antimicrobial Resistance Reference Gene database (NCBI BioProject ID: PRJNA313047) to identify AMR genes. The genome sequencing data of isolates used in this study have been submitted to the NCBI under BioProject ID: PRJNA720670.

## Data Analysis

Analyses were stratified by cattle type (beef or dairy) and included a descriptive examination of the unadjusted proportion of the following outcomes: DNPS positive for BRD bacteria, *M. haemolytica* serotypes, CLSI-determined resistance percentages, and BRD morbidities and mortalities (**Figure 1**). The unadjusted proportions of these outcomes were compared by cattle type using a chi-square or, when there were <5 samples in a stratum, the Fisher's Exact test (STATCALC, EpiInfo v.7.2.3.1). Antimicrobial susceptibility data were also presented as MIC frequency distributions and as $MIC_{50}$ and $MIC_{90}$ values. For *M. bovis*, only MIC frequency distributions and $MIC_{50}$/$MIC_{90}$ values were reported.

Multivariable logistic regression with mixed effects was used to describe the relationship between epidemiological risk factors and outcomes (**Figure 1**, **Table 2**), with control for confounding and clustered data. Outcomes included the isolation of BRD-related bacteria (yes, no), antimicrobial resistance [resistant bacteria vs. non-resistant (susceptible and intermediate) bacteria], BRD morbidity (yes, no) and BRD mortality (yes, no). Transport trailer was considered the sampling unit with animals from the same truck-load considered a cluster (TC). The majority of beef-type cattle were sourced through auction marts whereas most dairy-type cattle were farm-direct. When auction mart beef cattle were from different locations, but transported in the same truck, the random effect was nested (*arrived from* within TC). For dairy-type cattle models, 2 random effects were included (TC and *arrived from*) since the location of their origin was known. Random effects that did not account for variance within the model were removed. The variable feedlot was collinear with one-time feedlot capacity in morbidity and mortality models and as a result, it was excluded from these analyses. Based on descriptive analyses, bacterial AMR levels were consistently higher in dairy-type cattle as compared to beef-type cattle (**Figure 2**). Therefore, cattle type was used as an explanatory variable in logistic regression analyses in which the whole sampled population was considered to investigate associations between cattle type and AMR. The R package *lme4* (v.1.1-23) was used to fit mixed effects models (RStudio v.4.0.2). Continuous variables (temperature and body weight) were scaled as it was found to improve model convergence. When models were fitted with no random effects because the variance of the RE was equal to zero, the *glm* function (package *stats* v.4.0.2) was used with logit link. A backward elimination (40) approach was used and if the Akaike information criterion (AIC) value increased substantially and/ or the β-coefficient of a variable varied more than 30% upon removal, then it was retained in the model (28). Collinearity was evaluated through the fitted model through the variance inflation factor (VIF) (*car* package v.3.0-7; R Studio v.3.6.3). The possibility of a non-linear relationship between the continuous explanatory variables (weight and temperature) and the probability of the outcome on the log odds (logit) scale was tested by the addition of a quadratic term that was included in the model if statistically significant.

Clinical and Laboratory Standards Institute breakpoints for BRD bacteria have not been developed for all antimicrobials. Consequently, heatmaps with dendrograms were generated to visually explore the associations of MIC across all antimicrobials, except for trimethroprim/ sulfamethoxazole (SXT) and sulfadimethoxine which were tested at a single concentration. For these, antimicrobial MIC frequency distributions were plotted using the *heatmap.2* function from the *gplots* package v.3.0.4 in RStudio. Dendrograms were generated to explore associations across antimicrobials and across the strata of cattle type, monthly intervals, country of origin, source, feedlot, weight range (100 kg intervals: 100–199, 200–299, 300–399, 400–499, and 500–599 kg), age class, risk category, atmospheric temperature range (14°C intervals: −27 to −14, −13 to 0, 1 to 13, and 13 to 27°C), and BRD treatment (yes/no) and BRD mortality (yes/no). Feedlot was used as a clustering factor to explore whether any of the feedlots included in this study purchased cattle from locations containing higher levels of AMR. When non-clonal bacterial populations presenting high MICs were associated with a specific feedlot, individual dendrograms were generated for bacterial species/ feedlot combination using source, arrived from within a truck load, and truck load as clustering factors. Since not all the antimicrobials were tested in the same concentration range and most MIC frequency distributions were not normally distributed, MIC observed values were normalized (the minimum value of the dataset was subtracted to each value and divided by the maximum value in the dataset) bringing the data to the 0–1 scale (42).

# RESULTS

## Sampling

A total of 2,824 DNPS were collected with 1,391 (49.3%) in year 1 and 1,433 (50.7%) in year 2 (**Supplementary Table 2**). Six feedlots provided DNPS only from beef-type cattle (B, D, E, F, H, I), two only from dairy-type cattle (C, J), with a mixture of both cattle types from the remainder (A, G). Four feedlots provided over 300 DNPS, five provided over 200 DNPS, and one feedlot provided <200 DNPS during the study (**Supplementary Table 2**). To optimize sampling numbers related to seasonal inconsistency in cattle availability, more than 10 animals may have been sampled from each truck load. Additionally, a small proportion of the collected DNPS ($n$ = 241) failed to meet the quality standards for processing and were eliminated from the study. For beef-type cattle, the range of animals sampled per truck load was 3–57 (median = 10) whereas for dairy-type cattle, the number of animals sampled per load ranged from 9 to 20 (median = 10).

Two thousand and fifty-five (72.8%) DNPS were collected from beef-type cattle and 769 (27.2%) DNPS were collected from dairy-type cattle. Compared to dairy-type cattle, beef cattle-type were heavier in incoming body weight, older upon arrival,

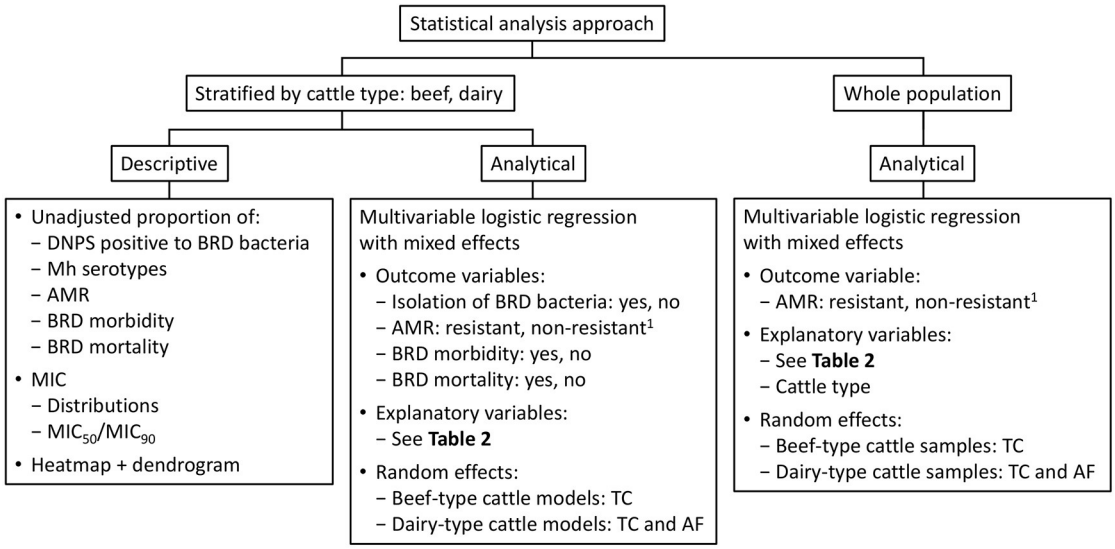



**FIGURE 1 |** Statistical analyses used in the present study. [1]Non-resistant category includes intermediate and susceptible categories. AF, arrived from; AMR, antimicrobial resistance; BRD, bovine respiratory disease; DNPS, deep nasopharyngeal swab; Mh, *Mannheimia haemolytica*; MIC, minimum inhibitory concentration; TC, truck cluster.

and originated more frequently from within Canada (96.1%) (**Table 3**). Beef cattle-type were also more often classified as low BRD risk (60.5%) than dairy-type cattle (24.5%). Beef-type cattle were primarily sourced from auction barns (67.7%), whereas, a greater proportion of dairy-type cattle originated directly from dairy farms (88.4%). Of 2,824 cattle, 338 (12.0%) were clinically diagnosed with BRD over the 120 d feeding period (**Figure 3**) and 29 animals (5.6%) succumbed to BRD. Among the 29 mortalities, 19 (65.5%) were previously treated for BRD at least once.

## Bacterial Isolation and Species Identification

A total of 1,646 out of 2,824 (58.3%) DNPS were positive for at least one of the bacterial species. Overall, *P. multocida* (41.3%) was most commonly isolated, followed by *M. haemolytica* (17.4%), *M. bovis* (16.1%), and *H. somni* (8.5%) (**Figure 4A**). A total of 492 *M. haemolytica* isolates were obtained from beef and dairy-type cattle (**Figure 4B**). For beef-type cattle, 57 isolates (20.1%) were serotype A1, 178 (62.9%) were A2, 45 (15.9%) were A6, and 3 (1.1%) were undetermined. In dairy-type cattle, 59 (28.2%), 99 (47.4%), 32 (15.3%), and 19 (9.1%) of the isolates were serotype A1, A2, A6, and undetermined, respectively.

## Antimicrobial Susceptibility Testing

Overall, the proportions of AMR-resistant *M. haemolytica*, *P. multocida*, and *H. somni* isolates recovered from dairy-type cattle were higher than those from beef-type cattle (**Figures 2A–D**). *Mannheimia haemolytica* isolates from dairy-type cattle had higher $MIC_{90}$ for CTET and neomycin (NEO) (8 and >32 μg/mL, respectively) than those from beef-type cattle (2 and 8 μg/mL, respectively) (**Supplementary Table 1.5**). Likewise, *P. multocida* isolates from dairy-type cattle had higher $MIC_{50}/MIC_{90}$ values for CTET (4/8 μg/mL), NEO (>32/>32 μg/mL), and TYLT (>32/>32 μg/mL) than isolates from beef-type cattle (0.5/1, 16/16, and 16/32 μg/mL, respectively). Among *H. somni* isolates from dairy-type cattle, $MIC_{50}/MIC_{90}$ values were also higher for CTET (2/4 μg/mL) and TYLT (8/16 μg/mL) than those from beef-type cattle (0.25/0.25 and 4/4 μg/mL, respectively).

Antimicrobial susceptibility testing was completed for 490 out of 492 (99.6%) of the *M. haemolytica* isolates (281 from beef and 209 from dairy) (**Supplementary Table 1.1**). The most frequently observed resistances were OXY (10%), TIL (6.4%), and ampicillin (AMP; 4.6%) among isolates from beef-type cattle and OXY (46.4%), TIL (26.8%), and TUL (21.0%) among dairy-type cattle isolates (**Figure 2A**, **Supplementary Figure 1.1**). Interestingly, all *M. haemolytica* isolates exhibited a high MIC for TYLT at 32 μg/mL (1.8% of the isolates) and >32 μg/mL (98.2% of the isolates). A higher proportion of *M. haemolytica* serotype A1 isolates were resistant to ENRO (7.8%), DANO (24.1%), and OXY (61.2%) than serotype A2 (0, 1.5, and 18.5%) and A6 (0, 1.3, and 1.3%) isolates. In contrast, serotype A2 isolates exhibited higher proportion of resistance to penicillin (PEN; 7.6%), TUL (17.1%), and FFN (13.5%) than did A1 and A6 (**Supplementary Figure 1.2**). Resistance to SPE and TIL was similar between A1 and A2 isolates (1.7 and 0.4%, respectively; 19.8 and 18.5%, respectively). Of the three serotypes, A6 showed the overall lowest AMR. Among *P. multocida* isolates, resistance to OXY (8.4%), SPE (8.1%), and AMP (7.7%) was most frequent from beef compared to OXY (89.7%), TIL (73.1%), and DANO (55.8%) from dairy-type cattle (**Figure 2B**). *Pasteurella multocida* also presented high MICs ($MIC_{50}$ and $MIC_{90}$ > 32 μg/mL) for

**TABLE 2 |** Risk factors and outcomes investigated by multivariable logistic regression for beef and dairy-type cattle upon feedlot arrival.

| Risk factor | Outcome | | | |
|---|---|---|---|---|
| | Isolation of BRD-related bacteria[a] | Isolation of AMR BRD bacteria[b] | BRD-related morbidity | BRD-related mortality |
| Country (Canada, US) | • | • | • | • |
| Sampling year (1st, 2nd) | • | • | • | • |
| Monthly interval[c] | • | • | • | • |
| Source (A, B, RD)[d] | • | • | • | • |
| BRD risk category[e] (high, low) | • | • | • | • |
| Weight (kg) | • | • | • | • |
| Sex (female, male)[f] | • | • | • | • |
| Age class (calf, yearling)[g] | • | • | • | • |
| Weather[h] | • | • | NA[j] | NA[j] |
| Co-isolation of other BRD-related bacteria | • | NA | NA | NA |
| Isolation of BRD-related bacteria | NA | NA | • | • |
| *Mannheimia haemolytica* serotype (A1+A6 vs. A2)[i] | NA | • | NA | NA |
| At once feedlot capacity (>10 K, <10 K) | NA | NA | • | • |
| Suffered a previous BRD episode | NA | NA | NA | • |

[a] *One model per bacterial species i.e., Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, and Mycoplasma bovis.*
[b] *One model per bacterial species (i.e., Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni)/antimicrobial combination.*
[c] *Sampling months were grouped from Aug-Nov, Dec-Feb, and Mar-May as an approximation of the seasons of the year.*
[d] *Auction (A) calves were predominant among beef-type cattle when compared to backgrounding operations (B) and ranch direct (RD) calves. In those AMR models in which source was a significant explanatory variable and its SE and β-coefficients were substantially high because of a low number of samples within 1 or more stratus, backgrounding operations and ranch direct samples were grouped and modeled against auction cattle. Likewise, auction samples were eliminated from those dairy AMR models in which <5 auction cattle samples were observed.*
[e] *Risk of suffering a BRD episode during the feeding period.*
[f] *Bulls accounted for a very small proportion of male population i.e., 2.2%.*
[g] *Calf, <1-year-old; yearling, over 1-year old.*
[h] *Weather conditions at feedlot entry included ambient temperature (°C), precipitation - yes (light and heavy rain/snow) or no (none, foggy), and wind speed - high (>20 km/h) or low (<20 km/h).*
[i] *Due to the uneven distribution of the samples across serotype levels, samples with unknown M. haemolytica serotype isolates were removed from the analysis (n = 3 among beef-type cattle and n = 19 among dairy-type cattle) and serotype A1+A6 isolates were grouped separately from commensal A2 isolates due to their well-documented role in BRD.*
[j] *Weather-related risk factors were not included in morbidity and mortality analysis since the published literature does not provide evidence of their relevance on this matter (41). Additionally, weather measures were only related to the day that cattle arrived at the feedlots whereas the reported morbidity and mortality took place during the first 120 days on feed.*
*BRD, bovine respiratory disease; NA, not applicable.*

TYLT, especially in isolates from dairy-type cattle, with only one (0.2%) *P. multocida* isolate exhibiting resistance to ceftiofur (TIO; cephalosporin). *Histophilus somni* isolates from dairy-type cattle were more frequently resistant to OXY (70.5%), SPE (22%), and PEN (11.0%) compared to isolates from beef-type cattle, which only exhibited resistance to AMP (2.9%) (**Figure 2C**). The total percentage of MDR in all *M. haemolytica, P. multocida*, and *Histophilus somni* isolates were 12, 34.2, and 1.25%, respectively (**Figure 2D**).

*Mycoplasma bovis* $MIC_{50}/MIC_{90}$ values were consistently higher for macrolides than for other antimicrobials, regardless of cattle type, especially for TIL (>256 μg/mL both) and TIP (>128 μg/mL both) (**Supplementary Table 1.5**). Likewise, the $MIC_{50}/MIC_{90}$ values of GAM (128/>256 μg/mL), TUL (16/>256 μg/mL), and TYLT (32/>128 μg/mL) were also high. Enrofloxacin (dairy-type cattle isolates), GAM (beef and dairy isolates), TUL (beef and dairy isolates), and TYLT (beef and dairy isolates) MIC distributions exhibited a bimodal pattern. As previously reported, AlamarBlue™ prevented the estimation of the OXY MIC of 2 *M. bovis* isolates from dairy-type cattle when MIC ≥ 32 μg/mL (11).

## Antimicrobial Resistance Genetic Determinants

Overall, the AMR phenotypes for *M. haemolytica*, *P. multocida*, and *H. somni* were corroborated with the presence of related antimicrobial resistance genes (ARG), as inferred from the WGS data (**Supplementary Tables 3.1–3.3**). The *tet*(H) gene was the most abundant determinant identified and corresponded with OXY resistance in *M. haemolytica* and *P. multocida* (**Supplementary Tables 3.1, 3.2**). In *H. somni*, *tet(H)* was detected in isolates with CTET MICs ranging between 1 and >4 μg/mL (**Supplementary Table 3.3**). Genes related to NEO and kanamycin resistance were the second most abundant determinants identified [*aph(3')-Ia*], coinciding with NEO MIC values > 32 μg/mL for *M. haemolytica* and *P. multocida*. Considering that the NEO concentrations were not tested beyond 16 μL/mL for *H. somni*, it was not possible to determine if the presence of *aph(3')-Ia* conferred NEO MIC > 32 μg/mL. Likewise, the ARG for GEN resistance [*ant(2")-Ia*] was present in *M. haemolytica* when MIC values for this drug were >16 μg/mL, whereas in *H. somni*, *aac(3)-Iva*, a gentamycin (GEN) resistance gene, was associated with the GEN phenotype

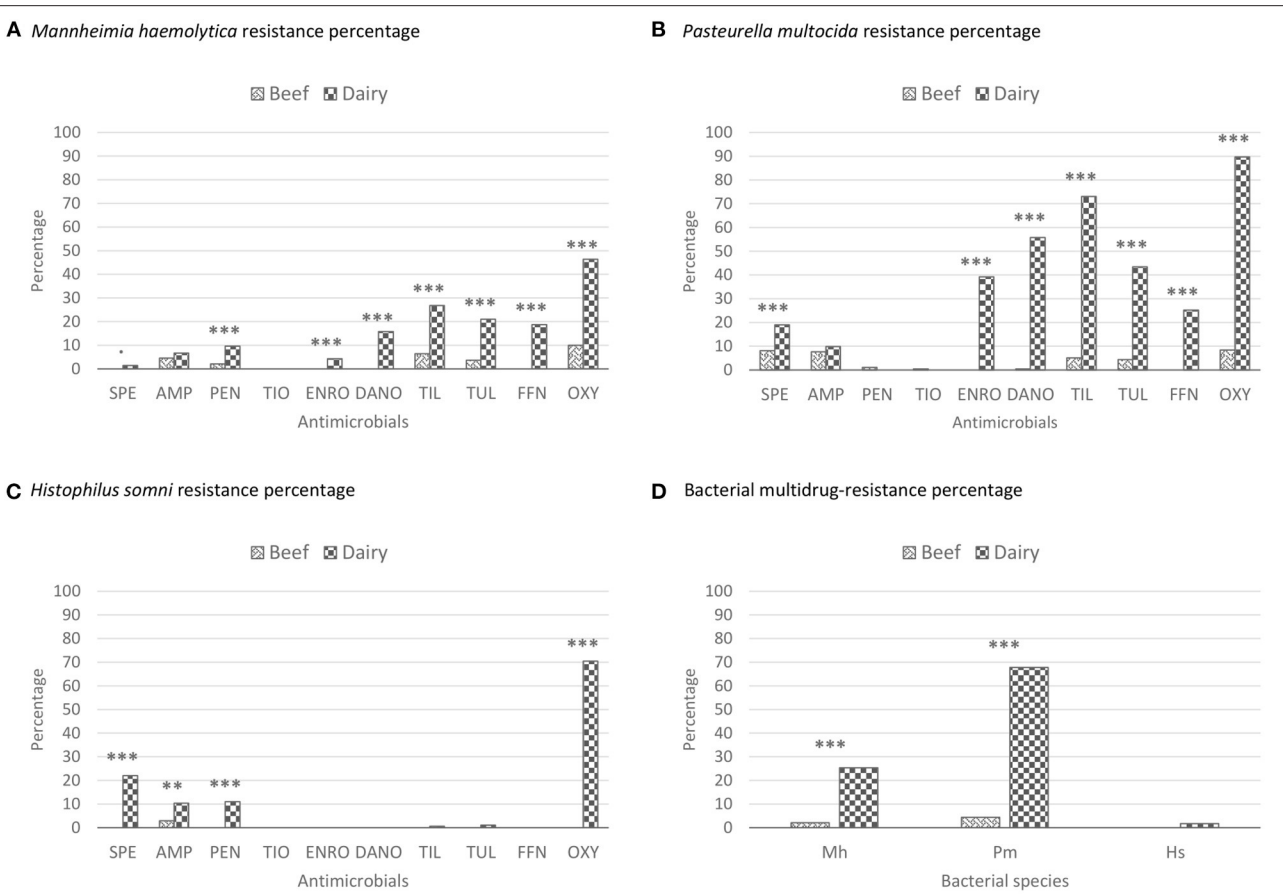



**FIGURE 2 |** Antimicrobial resistance percentages of the BRD-bacterial isolates recovered from beef and dairy-type cattle upon feedlot arrival. These percentages represent unadjusted proportions. The asterisks represent the statistical test significance levels as follows: "." 0.1, "**" 0.01, "***" 0.001. Multidrug resistance was defined as resistance to 3 or more different antimicrobial classes (36). AMP, ampicillin; BRD, bovine respiratory disease; DANO, danofloxacin; ENRO, enrofloxacin; FFN, florfenicol; Hs, *Histophilus somni*; Mb, *Mycoplasma bovis*; Mh, *Mannheimia haemolytica*; OXY, oxytetracycline; PEN, penicillin; Pm, *Pasteurella multocida*; SPE, spectinomycin; TIL, tilmicosin; TIO, ceftiofur; TUL, tulathromycin. **(A)** Beef SPE resistance (R): 0/281; beef AMP-R: 13/281; beef PEN-R: 6/281; beef TIO-R: 0/281; beef ENRO-R: 0/281; beef DANO-R: 0/281; beef TIL-R: 18/281; beef TUL-R: 10/281; beef FFN-R: 0/281; beef OXY-R: 28/281; dairy SPE-R: 3/209; dairy AMP-R: 14/209; dairy PEN-R: 20/209; dairy TIO-R: 0/209; dairy ENRO-R: 9/209; dairy DANO-R: 33/209; dairy TIL-R: 56/209; dairy TUL-R: 44/209; dairy FFN-R: 39/209; dairy OXY-R: 97/209. SPE $X^2$ (1, $n = 409$) = 4.06, $p = 0.0440$; AMP $X^2$ (1, $n = 409$) = 0.99, $p = 0.3201$; PEN $X^2$ (1, $n = 409$) = 13.18, $p < 0.001$; ENRO $X^2$ (1, $n = 409$) = 12.33, $p < 0.001$; DANO $X^2$ (1, $n = 409$) = 47.57, $p < 0.001$; TIL $X^2$ (1, $n = 409$) = 38.86, $p < 0.001$; TUL $X^2$ (1, $n = 409$) = 37.41, $p < 0.001$; FFN $X^2$ (1, $n = 409$) = 56.97, $p < 0.001$; OXY $X^2$ (1, $n = 409$) = 83.79, $p < 0.001$. **(B)** Beef SPE-R: 22/273; beef AMP-R: 21/273; beef PEN-R: 3/273; beef TIO-R: 1/273; beef ENRO-R: 0/273; beef DANO-R: 1/273; beef TIL-R: 14/273; beef TUL-R: 12/273; beef FFN-R: 0/273; beef OXY-R: 23/273; dairy SPE-R: 46/242; dairy AMP-R: 24/242; dairy PEN-R: 0/242; dairy TIO-R: 0/242; dairy ENRO-R: 95/242; dairy DANO-R: 135/242; dairy TIL-R: 177/242; dairy TUL-R: 105/242; dairy FFN-R: 61/242; dairy OXY-R: 217/242. SPE $X^2$ (1, $n = 515$) = 13.42, $p < 0.001$; AMP $X^2$ (1, $n = 515$) = 0.80, $p = 0.3722$; PEN $X^2$ (1, $n = 515$) = 2.67, $p = 0.1482$; TIO $X^2$ (1, $n = 515$) = 0.89, $p = 0.2650$; ENRO $X^2$ (1, $n = 515$) = 131.41, $p < 0.001$; DANO $X^2$ (1, $n = 515$) = 202.73, $p < 0.001$; TIL $X^2$ (1, $n = 515$) = 254.32, $p < 0.001$; TUL $X^2$ (1, $n = 515$) = 111.09, $p < 0.001$; FFN $X^2$ (1, $n = 515$) = 78.06, $p < 0.001$; OXY $X^2$ (1, $n = 515$) = 340.27, $p < 0.001$. **(C)** Beef SPE-R: 0/68; beef AMP-R: 2/68; beef PEN-R: 0/68; beef TIO-R: 0/68; beef ENRO-R: 0/68; beef DANO-R: 0/68; beef TIL-R: 0/68; beef TUL-R: 0/68; beef FFN-R: 0/68; beef OXY-R: 0/68; dairy SPE-R: 38/173; dairy AMP-R: 23/173; dairy PEN-R: 19/173; dairy TIO-R: 0/173; dairy ENRO-R: 0/173; dairy DANO-R: 0/173; dairy TIL-R: 1/173; dairy TUL-R: 2173/; dairy FFN-R: 0/173; dairy OXY-R: 122/173. SPE $X^2$ (1, $n = 241$) = 17.73, $p < 0.001$; AMP $X^2$ (1, $n = 241$) = 5.63, $p = 0.0063$; PEN $X^2$ (1, $n = 241$) = 8.11, $p < 0.001$; TIL $X^2$ (1, $n = 241$) = 0.69, $p = 0.3589$; TUL $X^2$ (1, $n = 241$) = 0.79, $p = 0.2572$; OXY $X^2$ (1, $n = 241$) = 97.12, $p < 0.001$. **(D)** Beef Mh MDR: 6/281; beef Pm MDR: 12/274; beef Hs MDR: 0/68; dairy Mh MDR: 53/209; dairy Pm MDR: 164/242; dairy Hs MDR: 3/173. $X^2$ (1, $n = 409$) = 61.04, $p < 0.001$; $X^2$ (1, $n = 515$) = 137.84, $p < 0.001$; $X^2$ (1, $n = 241$) = 1.19, $p = 0.1810$.

in only 5 of the 8 GEN resistant (MIC > 8 μg/mL) isolates. The presence of *flo*R corresponded with FFN MIC ≥ 8 μg/mL for *M. haemolytica*, MIC ≥ 4 μg/mL for *P. multocida*, and MIC ≥ 0.5 μg/mL for *H. somni*. The presence of $bla_{ROB-1}$ was associated with AMP resistance and the detection of *aadA*25 (*M. haemolytica*) or *aadA*31 (*P. multocida and H. somni*) was associated with SPE resistance. In *M. haemolytica* and *P. multocida*, the presence of mutations in the quinolone resistance-determining region (QRDR) was related to resistance to FQs. In many cases, resistance to macrolides (TIL, TUL) was related to the presence of the *erm*(42) and/or the *mph*(E)-*msr*(E) operon in *M. haemolytica*, and *erm*(42) and/or the *mph*(E)-*msr*(E) operon

**TABLE 3 |** Feedlot cattle demographics from beef and dairy-type cattle upon feedlot arrival.

| Variable | | Beef, $n = 2,055$ | Dairy, $n = 769$ |
|---|---|---|---|
| Weight (kg) | Median | 333 | 159 |
| | Range | 115–683 | 97–542 |
| Age category | Calf | 57% | 92.3% |
| | Yearling | 43% | 7.7% |
| Country | Canada | 96.1% | 27.4% |
| | US | 3.9% | 72.6% |
| BRD risk | Low | 60.5% | 24.5% |
| Category | High | 39.5% | 75.5% |
| Source | Auction market | 67.7% | 1.3% |
| | Backgrounding operation | 13.7% | 10.3% |
| | Ranch direct | 18.5% | 88.4% |

*These percentages represent unadjusted proportions.*

or A2058G mutation in the 23S rRNA gene of *P. multocida*. No corroboration could be established between macrolide phenotype and genotype for *H. somni* isolates as 5 of the 24 sequenced isolates carried *erm*(42) and/or *erm*(F) genes, yet they were not resistant to macrolides (TIL and TUL MIC ranging from 2 to 4 μg/mL). Streptomycin resistance ARGs *aph(3")-Ib* and *aph(6)-Id* frequently co-existed with NEO determinant *aph(3')-Ia* in the three *Pasteurellaceae* species. However, phenotypic streptomycin resistance was not assessed in any of the isolates.

## Multivariable Logistic Regression

### Recovery of BRD-Related Bacteria

Multivariable logistic regression determined that the odds of isolating a BRD pathogens was variably associated with the isolation of other BRD pathogens and the monthly arrival interval (**Table 4**). In the second year, the odds ratio of recovering *M. bovis* from both cattle types was higher than in the first year i.e., 2.3 (95% CI = 1.3–4.0, $p = 0.006$) and 2.5 (95% CI = 1.3–4.74, $p = 0.004$) in both beef and dairy-type cattle, respectively (**Supplementary Tables 4.1, 4.2**).

### Recovery of AMR Bacteria

Depending on the BRD bacterial species isolated from beef-type cattle, the odds of these bacteria being AMR were between 8.5 and 17.5 higher between Mar–May as compared to Aug–Nov (**Tables 5**, **6**). In dairy-type cattle, BRD-related bacteria had lower odds of being AMR during Mar–May than Aug–Nov, and Aug–Nov as compared to Dec–Feb (**Tables 7**, **8**). The country of origin did not affect the odds of isolating AMR BRD bacteria in beef-type cattle (**Supplementary Table 4.1**), whereas in dairy-type cattle it varied depending on the antimicrobial and the bacterial species (**Tables 7**–**9**). The odds of recovering AMR bacteria from backgrounded beef-type feedlot cattle were higher than for those purchased at auction (**Tables 5**, **6**). The BRD risk category showed no effect on AMR among beef-type cattle (**Supplementary Table 4.3**), whereas for dairy-type cattle, it varied depending on the antimicrobial and the bacterial species (**Supplementary Table 4.4**). Overall, a higher ambient temperature was associated with greater odds of recovering resistant bacteria in both cattle types (**Supplementary Tables 4.3, 4.4**). *Mannheimia haemolytica* A1 and A6 serotypes were associated with higher odds of OXY resistance in beef (OR = 38.3, 95% CI = 4.8–304.1, $p < 0.001$) and dairy-type cattle (OR = 4.8, 95% CI = 1.9–11.9, $p < 0.001$) as compared to A2. The A1 and A6 serotypes, presented lower odds of being resistant to almost all antimicrobials tested among dairy-type cattle when compared to A2 (**Tables 5**, **6**). Dairy-type cattle were associated with higher levels of AMR as compared to beef-type cattle (**Supplementary Tables 4.3, 4.4**).

### BRD-Related Morbidity and Mortality

There were no differences in the morbidity or mortality between cattle types sourced in either Canada or the US (**Supplementary Table 4**). The isolation of any BRD-related nasal bacteria upon feedlot arrival was not related to BRD mortalities during the feeding period. Cattle that were treated for BRD at least once were positively associated with dying from BRD. Additionally, different risk factors were associated with morbidity and/or mortality depending on cattle type i.e., BRD risk, monthly interval, weight, and age (**Supplementary Tables 4.6–4.9**).

## Heatmaps

Antimicrobial resistant profiles of isolates from dairy-type cattle presented higher MIC values (values closer to 1), including for antimicrobials with no SIR categories such as CTET or TYLT, in *M. haemolytica*, *P. multocida*, and *H. somni* (**Supplementary Figures 1.3.1–1.3.3**). *Mycoplasma bovis* isolates with high macrolide MIC values were obtained from both cattle types. However, higher ENRO MICs were mainly associated with isolates from dairy-type calves as indicated by $MIC_{50}/MIC_{90}$ values (**Supplementary Table 1.5**, **Supplementary Figure 1.3.4**). Additionally, *M. bovis* isolates obtained from backgrounding beef-type cattle predominated among AMR profiles showing higher MICs as compared to auction and ranch direct beef-type cattle (**Supplementary Figure 1.3.41**). Interestingly, *M. haemolytica* serotypes A1 and A6 isolated from beef-type cattle were mainly distributed among isolates showing higher MICs, whereas A2 typically exhibited lower MICs (**Supplementary Figure 1.3.13**). *Pasteurella multocida* from US dairy-type cattle exhibited higher MIC values as compared to those isolated from Canadian dairy-type cattle (**Supplementary Figure 1.3.17**). Among dairy-type cattle, *M. haemolytica* and *M. bovis* isolates collected at feedlot C also had higher MICs than those from feedlots A, G, and J (**Supplementary Figures 1.3.16, 1.3.49**). Notably, feedlot C purchased dairy-type cattle mainly from different farms located in Alberta, Canada, whereas feedlots A, G, and J purchased dairy-type cattle mainly from the US (**Supplementary Figures 1.3.50, 1.3.51**).

# DISCUSSION

In this study, we described the prevalence of AMR in *M. haemolytica*, *P. multocida*, and *H. somni* in cattle at feedlot

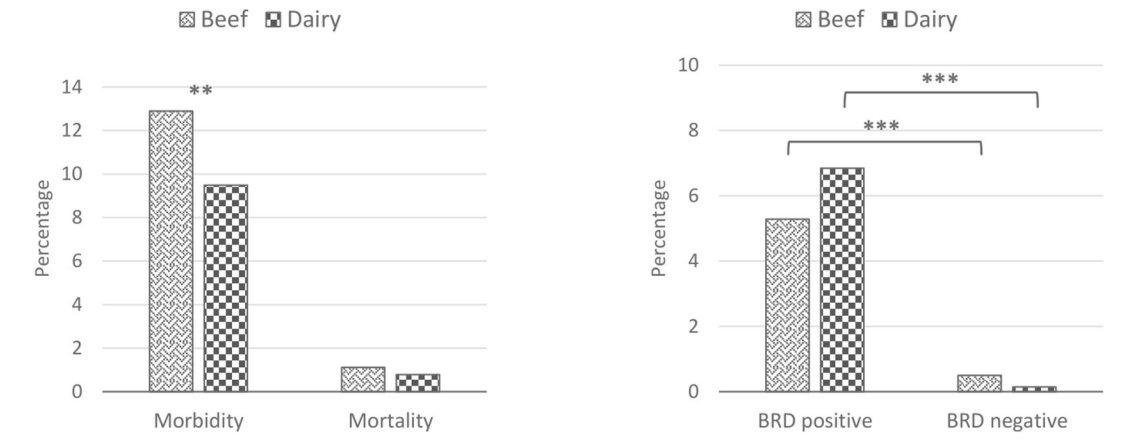



**FIGURE 3 |** Proportion of cattle treated at least once for BRD and/ or succumbed to BRD during the first 120 d of the feeding period for beef and dairy-type cattle. These percentages represent unadjusted proportions. The asterisks represent the statistical test significance level as follows: "**" 0.01, "***" 0.001. BRD, bovine respiratory disease; Tx, treatment. **(A)** Beef morbidity proportion: 265/2,055 DNPS; beef mortality proportion: 23/2,055 DNPS; dairy morbidity proportion: 73/769 DNPS; dairy mortality proportion: 6/769 DNPS. Morbidity $X^2$ (1, $n = 2{,}428$) = 6.15, $p = 0.0132$; mortality $X^2$ (1, $n = 2{,}428$) = 0.63, $p = 0.4264$. **(B)** BRD-treated beef mortality proportion: 14/265; non-BRD treated beef mortality proportion: 9/1,790; BRD-treated dairy mortality proportion: 5/73; non-BRD treated dairy mortality proportion: 1/696. Beef $X^2$ (1, $n = 2{,}055$) = 48.04, $p < 0.001$; dairy *Fisher Exact* (1, $n = 769$) = 35.27, $p < 0.001$.

arrival, and documented that it was higher in dairy than beef-type cattle. Multidrug-resistant *Pasteurellaceae* bacteria have been isolated from BRD clinical cases and mortalities before (43–45), and AMR has been investigated in cattle at feedlot arrival in previous studies (14–16, 46). However, to our knowledge this study is the first in North America to collect all four species of the BRD bacterial complex from a broad cross-sectional feedlot cattle population, and investigate their antimicrobial susceptibilities at feedlot entry. One limitation of this study is the large ORs and wide CIs generated by logistic regression in some instances (**Supplementary Tables 4.1–4.9**) limited the precision of the conclusions obtained in this study.

The prevalence of *P. multocida* and *H. somni* in beef-type cattle was higher compared to previous studies that collected respiratory samples from feedlot cattle at arrival, but comparable for *M. haemolytica* (14–16). The higher prevalence of *M. haemolytica* serotype A2 was expected as compared to A1 and A6, as it is more frequently isolated from healthy cattle (47). Dairy-type cattle tended to harbor more members of the BRD bacterial complex than beef-type cattle. This association may be a consequence of early weaning practices in the dairy industry (17). Most dairy calves are weaned within a day of birth (17), which may limit their ingestion of colostrum. The acquisition of passive immunity at this age is limited during a critical time when the calf's immune system is still immature (48). Most beef calves are raised under extensive conditions, whereas dairy calves are often housed in confinement from birth, a practice that could promote the transmission of BRD bacteria among individuals. As reported by Griffin (49), the odds of isolating *Mycoplasma bovis* from both beef and dairy cattle classified as high-BRD risk were higher than those classified as low risk. However, in the current study, *M. haemolytica*, *P. multocida*, and *H. somni* were not associated with cattle classified as high-BRD risk.

The proportion of cattle that had BRD at least once (12%) and succumbed to it (5.6%) was comparable to those previously reported, 16.2 and 4%, respectively (50). It has been suggested that sourcing cattle directly from ranches reduces exposure to BRD-related bacteria (49), but we did not find greater odds of recovering BRD bacteria from beef-type cattle purchased at auction as compared to those from backgrounding operations or directly from ranches. Nevertheless, auction-sourced cattle did have greater odds of being treated for BRD. Auction market-derived beef-type cattle ($n = 1{,}392$) originated from 69 different locations. Frequent comingling of cattle from different locations can increase the incidence of BRD morbidity during the feeding period (51, 52). Most weaned beef calves enter the feedlot in the fall and at this time a large number of calves are comingled during sale and shipped to feedlots. During this time they are subject to handling and transport stress, resulting in an increase in BRD-morbidities and mortalities (41). Although the fall interval (Aug-Nov) was associated with more BRD morbidity in beef-type cattle, it was not associated with higher isolation of BRD bacteria at arrival. Heavier and older (yearlings) cattle were less likely to develop BRD, an observation that agrees with that of others (41). High-BRD risk beef-type cattle were not associated with greater BRD during the feeding period. It has been reported that high-BRD risk cattle are over 100 times more likely to be administrated metaphylactic macrolides than low-BRD risk cattle (53). It is well-established that the use of metaphylactic antimicrobials in moderate to high risk BRD cattle reduces morbidity and mortality (53) which could account for the lower BRD morbidity in high-BRD risk

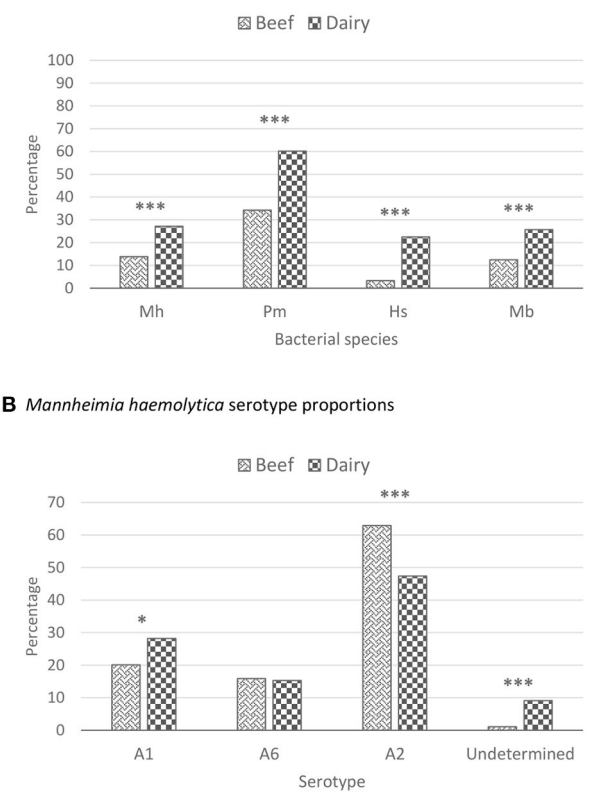



**FIGURE 4 |** Proportion of deep nasopharyngeal swabs that were positive for BRD-related bacteria and *Mannheimia haemolytica* serotype proportions recovered from beef and dairy-type cattle upon feedlot arrival. These percentages represent unadjusted proportions. The asterisks represent the statistical test significance levels as follows: "*" 0.05, "***" 0.001. BRD, bovine respiratory disease; Hs, *Histophilus somni*; Mb, *Mycoplasma bovis*; Mh, *Mannheimia haemolytica*; Pm, *Pasteurella multocida*. **(A)** Beef Mh positive proportion: 283/2,055; beef Pm positive proportion: 703/2,055; beef Hs positive proportion: 68/2,055; beef Mb positive proportion: 257/2,055; dairy Mh positive proportion: 209/769; dairy Pm positive proportion: 463/769; dairy Hs positive proportion: 173/769; dairy Mb positive proportion: 198/769. Mh $X^2$ (1, $n = 2{,}824$) = 69.91, $p < 0.001$; Pm $X^2$ (1, $n = 2{,}824$) = 156.04, $p < 0.001$; Hs $X^2$ (1, $n = 2{,}824$) = 263.94, $p < 0.001$; Mb $X^2$ (1, $n = 2{,}824$) = 72.60, $p < 0.001$. **(B)** Beef Mh A1 positive proportion: 57/283; beef Mh A6 positive proportion: 45/283; beef Mh A2 positive proportion: 178/283; beef Mh unknown positive proportion: 3/283; dairy Mh A1 positive proportion: 59/209; dairy Mh A6 positive proportion: 32/209; dairy Mh A2 positive proportion: 99/209; dairy Mh unknown positive proportion: 19/209. Mh A1 $X^2$ (1, $n = 492$) = 4.36, $p = 0.0367$; Mh A6 $X^2$ (1, $n = 492$) = 0.03, $p = 0.8587$; Mh A2 $X^2$ (1, $n = 492$) = 11.78, $p < 0.001$; Mh unknown *Fisher Exact* (1, $n = 492$) = 18.15, $p < 0.001$.

cattle. Only the isolation of *P. multocida* in dairy-type cattle at feedlot entry was associated with higher BRD morbidity during the feeding period (**Supplementary Table 4.8**). The bovine respiratory tract microbiome undergoes substantial changes in bacterial composition after feedlot placement (54). Bovine respiratory disease is also a complex disease process involving viral co-infections, and host immunity and environmental factors. Therefore, there may not be a causal link between the presence/absence of BRD bacteria in the respiratory tract of cattle upon arrival and morbidity and mortality during the feeding period. This study was limited to considering only the implications of the bacteriological component on arrival for BRD morbidities and mortalities that occurred later in the feeding period. Other factors known to contribute to this disease complex, such as immune and nutritional status, vaccination, housing, management practices, and the respiratory virome (8, 55) were not evaluated.

A higher risk of BRD morbidity has been associated with the purchase of calves in the fall (41). Aug–Nov (for dairy) and Dec–Feb (for beef and dairy) as compared to Mar–May were associated with higher AMR possibly as a result of increased AMU during this time of the year. Higher odds of AMR nasal BRD bacteria recovery were observed in beef-type cattle backgrounded in feedlots as compared to auction-derived calves. This observation most likely reflects the administration of some antimicrobials to cattle at backgrounding feedlots and the confined conditions that may promote transmission of AMR bacteria amongst cattle. However, some auction-derived cattle may also have been in a backgrounding operation prior to transfer to an auction market but were classified as auction-derived due to the impossibility of gathering such information. The odds of isolating AMR-BRD bacteria were consistently higher in dairy-type cattle than beef-type cattle (**Supplementary Table 4.5**). In North America, dairy calves are typically housed indoors in confinement whereas most beef calves are on pasture until weaning. Dairy calves that are fed in feedlots for meat production are moved to calf grower operations as they come out of the hutch at 1 day of age (FHMS personal communication). In calf grower operations, dairy-type calves are housed in pens which may promote the transmission of AMR bacteria as per in beef-type cattle backgrounded in feedlots. It is also possible that these dairy calves were administrated more antimicrobials than beef-type calves of a similar age to assist their immature immune system to fight bacterial infections that may take place in the growing operation.

Low levels of AMR in *M. haemolytica* isolated from newly arrived feedlot cattle has been previously reported, with few studies investigating AMR in *P. multocida* and *H. somni*. *Mannheimia haemolytica* and *P. multocida* resistance to TIL and TUL were very low (up to 6.4%) in beef cattle compared to dairy cattle, most likely because macrolides are less frequently administrated to ranch beef calves than dairy calves (56–58). Even though the use of TYLT is less prevalent than TIL and TUL in ranch beef calves (58), *M. haemolytica* and *P. multocida* had high TYLT MICs, a phenomenon also observed in dairy calves. Previous surveillance studies have also reported high TYLT MICs in these two bacterial species in beef cattle at arrival and during the feeding period (6, 46). Further studies are required to determine if *M. haemolytica* and *P. multocida* exhibit some degree of intrinsic resistance to TYLT as a result of up-regulation of antimicrobial efflux systems (59) or impairment of cellular uptake as per AMGs (60). *Mannheimia haemolytica* obtained from cattle at feedlot entry exhibited no resistance to TUL a year after it was approved for the control of BRD in Canada (46). It is possible that the slightly higher resistance (i.e., from 0 to 3.6%)

**TABLE 4 |** Significant results obtained from logistic regression of recovery of bovine respiratory disease complex bacteria from beef and dairy-type cattle upon feedlot arrival.

| Risk factor | *M. haemolytica* | | | *P. multocida* | | | *H. somni* | | | *M. bovis* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** |
| **Monthly intervals – BEEF** | | | | | | | | | | | | |
| Aug–Nov | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| Dec–Feb | 2.4 | 1.7–3.6 | **<0.001** | 1.45 | 1.0–2.1 | **0.048** | 1.7 | 0.7–4.1 | 0.248 | 12.7 | 5.4–30.1 | **<0.001** |
| Mar–May | 1.2 | 0.8–1.8 | 0.279 | 0.93 | 0.6–1.4 | 0.700 | 3.5 | 1.4–8.5 | **0.005** | 14.5 | 6.1–34.9 | **<0.001** |
| **Monthly intervals - DAIRY** | | | | | | | | | | | | |
| Aug–Nov | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| Dec–Feb | 1.3 | 0.8–2.2 | 0.266 | 1.1 | 0.6–2.0 | 0.716 | na | na | na | na | na | na |
| Mar–May | 2.4 | 1.5–3.9 | **<0.001** | 1.8 | 1.1–3.2 | **0.034** | na | na | na | na | na | na |
| **Isolation of other BRD-related bacteria - BEEF** | | | | | | | | | | | | |
| Mh | ni | ni | ni | na | na | na | 2.2 | 1.2–4.0 | **0.011** | 1.5 | 0.9–2.3 | **0.060** |
| Pm | na | na | na | ni | ni | ni | na | na | na | 1.6 | 1.2–2.2 | **0.006** |
| Hs | 1.9 | 1.1–3.6 | **0.025** | na | na | na | ni | ni | ni | na | na | na |
| Mb | 1.6 | 1.1–2.4 | **0.014** | 1.6 | 1.1–2.2 | **0.005** | na | na | na | ni | ni | ni |
| **Isolation of other BRD-related bacteria - DAIRY** | | | | | | | | | | | | |
| Mh | ni | ni | ni | na | na | na | 0.6 | 0.4–1.0 | **0.048** | na | na | na |
| Pm | na | na | na | ni | ni | ni | na | na | na | 1.5 | 1.0–2.2 | **0.050** |
| Hs | 0.7 | 0.4–1.0 | **0.063** | na | na | na | ni | ni | ni | na | na | na |
| Mb | na | na | na | 1.5 | 1.0–2.3 | **0.038** | na | na | na | ni | ni | ni |

*CI, confident interval; Hs, Histophilus somni; Mb, Mycoplasma bovis; Mh, Mannheimia haemolytica; na, not applicable; ni, not included; OR, odds ratio; Pm, Pasteurella multocida.*
*na, that variable was not significant at the p-value ≤ 0.1 level and was eliminated from the regression.*
*ni, not included as an explanatory variable in the model.*
*Bold p-values indicate statistically significant results at p ≤ 0.1.*

**TABLE 5 |** Significant results obtained from logistic regression of antimicrobial resistant *Mannheimia haemolytica* from beef-type cattle upon feedlot arrival.

| Risk factor | Ampicillin | | | Oxytetracycline | | | Tilmicosin | | | Tulathromycin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** |
| **Monthly intervals** | | | | | | | | | | | | |
| Aug–Nov | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| Dec–Feb | 0.9 | 0.2–5.0 | 0.879 | 0.6 | 0.04–7.7 | 0.689 | 1.0 | 0.1–11.0 | 0.973 | na | na | na |
| Mar–May | 10.0 | 1.3–76.8 | **0.027** | 17.5 | 1.1–275.9 | **0.041** | 11.6 | 1.1–121.2 | **0.040** | na | na | na |
| **Source** | | | | | | | | | | | | |
| A | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| B | na | na | na | 34.0 | 1.5–776.4 | **0.027** | 11.7 | 1.4–100.9 | **0.024** | 85.8 | 4.3–1,715.4 | **0.003** |
| RD | na | na | na | 2.6 | 0.2–34.6 | 0.433 | 4.0 | 0.7–23.6 | 0.121 | | | |
| ***Mannheimia haemolytica* serotype** | | | | | | | | | | | | |
| A2 | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| A1 + A6 | na | na | na | 38.3 | 4.8–304.1 | **<0.001** | 6.6 | 1.5–29.9 | **0.014** | na | na | na |

*A, auction; B, backgrounding operations; CA, Canada; CI, confident interval; OR, odds ratio; RD, ranch direct; RF, risk factor.*
*na, that variable was not significant at the p-value ≤ 0.1 level and was eliminated from the regression.*
*Bold p-values indicate statistically significant results at p ≤ 0.1.*

observed in the present study reflects the increased use of TUL, but more isolates should be collected over a longer period of time to confirm this trend.

Macrolide resistance phenotype (TIL and TUL) appeared to be due to the presence of *erm*(42) and/or the macrolide efflux protein and phosphotransferase gene pair *msr*(E)-*mph*(E) in *M. haemolytica* and *P. multocida* isolates. Three macrolide resistant *P. multocida* isolates possessed the A2058G mutation in 23S rRNA. Macrolide resistance due to rRNA mutations is well-documented in bacteria with a homogeneous or heterogeneous presence in *rrn* operons (61). In *H. somni*, *erm*(42) and/or *erm*(F) genes were present, but this did not appear to be linked to macrolide MICs. Furthermore, TYLT did not seem to be a reliable phenotypic indicator of macrolide resistance for *Pasteurellaceae*

**TABLE 6 |** Significant results obtained from logistic regression of antimicrobial resistant *Pasteurella multocida* from beef-type cattle upon feedlot arrival.

| Risk factor | Ampicillin | | | Oxytetracycline | | | Spectinomycin | | | Tilmicosin | | | Tulathromycin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OR | 95% CI | *p*-value | OR | 95% CI | *p*-value | OR | 95% CI | *p*-value | OR | 95% CI | *p*-value | OR | 95% CI | *p*-value |
| **Monthly interval** | | | | | | | | | | | | | | | |
| Aug–Nov | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| Dec–Feb | na | na | na | na | na | na | 1.3 | 0.1–12.5 | 0.834 | na | na | na | na | na | na |
| Mar–May | na | na | na | na | na | na | 8.5 | 1.1–65.4 | **0.040** | na | na | na | na | na | na |
| **Source** | | | | | | | | | | | | | | | |
| A | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| B | na | na | na | 13.2 | 0.002–0.1 | **0.018** | 13.4 | 1.6–113.8 | **0.017** | 21.9 | 0.7–698.5 | **0.080** | 35.9 | 1.0–1249.4 | **0.048** |
| RD | na | na | na | 1.3 | 0.2–10.4 | 0.807 | 1.8 | 0.3–11.4 | 0.545 | | | | | | |

*A, auction; B, backgrounding operations; CI, confident interval; OR, odds ratio; RD, ranch direct.*
*na, that variable was not significant at the p-value ≤ 0.1 level and was eliminated from the regression.*
*Bold p-values indicate statistically significant results at p ≤ 0.1.*

species isolated in this study due to high MIC values observed (**Supplementary Tables 3.1, 3.2**).

As previously reported, the resistance to TIO was extremely low for *P. multocida* (1/515, 0.2%) and absent in *M. haemolytica* (16, 47) and *H. somni*. These results probably reflect the limited use of TIO in beef cattle (58). Third generation cephalosporins are more frequently administrated to dairy cattle than to beef cattle (56, 57), yet resistance to this drug was not observed in BRD bacteria isolated from dairy calves. The overall resistance to β-lactam antimicrobials was relatively low in beef and dairy cattle, even though this drug class is commonly used in both (56–58). In our study, all *M. haemolytica* isolates that had AMP MICs > 16 μg/mL and all *H. somni* with MICs of 1–2 μg/mL harbored the $bla_{ROB-1}$ gene. Furthermore, one *M. haemolytica* with MIC values of 0.25 μg/mL had $bla_{ROB-1}$, and another isolate with similar MIC value possessed $bla_{OXA-2}$. In *Pasteurellaceae* species, $bla_{ROB-1}$ is typically plasmid-associated (62, 63).

Resistance to OXY was the highest resistance observed across *Pasteurellaceae* isolated from both cattle types (10 and 46.4% in beef and dairy cattle types, respectively), which likely reflects the frequent use of TETs (57, 58). Levels of TET-resistance in *Pasteurellaceae* are even high in feedlot cattle raised without the use of antimicrobials (45). Considering the importance of TETs for the treatment and prevention of histophilosis in western Canada (53), the high level of OXY resistance (70.5%) in *H. somni* isolates from dairy cattle is worrisome. Our results suggest that antimicrobials other than TETs would be more effective for the treatment of *H. somni* infections in feedlot dairy calves. The ARG *tet*(H) was always present in OXY-resistant isolates, a finding consistent with that of others (14, 45). Previous studies have identified *tet*(H) in AMR *M. haemolytica* from clinical BRD cases (64), and it is also frequently found in *P. multocida* (59) and *H. somni* (65).

High FQs resistance was observed in *M. haemolytica* and *P. multocida* isolates from dairy calves. Danofloxacin is not approved for use in lactating dairy cows (56, 57) but it is approved for dairy calves (66). Resistance to FQ was associated with mutations in the QRDR including DNA-gyrase encoded by the *gyrA* and *gyrB* genes and topoisomerase IV encoded by *parC* and *parE* (67–69).

Frequent administration of FFN has been reported in dairy but not in beef cattle (57, 58). Such antimicrobial usage may explain the higher FFN resistance observed in dairy *M. haemolytica* and *P. multocida* isolates, and higher MICs in *H. somni* isolates from dairy compared to beef cattle (**Supplementary Figure 1.3.3**). This resistance and/or high MIC corresponded with the presence of *floR* as inferred from sequenced isolates from these three species.

Aminocyclitols are infrequently used in beef or dairy cattle (56–58) yet, *H. somni* (dairy isolates) and *P. multocida* (beef and dairy isolates) exhibited 22, 8.1, and 19% resistance to SPE, respectively. Co-resistance with other antimicrobials that travel on the same MGE as SPE likely accounts for this resistant phenotype (45). Resistance to SPE was conferred by the *aadA31* gene as previously described (70). In accordance with previous studies (45, 59), linkage of AMG resistance genes *aph(3″)-Ib*, *aph(6)-Id*, *aph(3′)-Ia*, and sulfonamide resistance gene *sul2* was found in *M. haemolytica*, *P. multocida*, and *H. somni*. The GEN-resistance determinant *aac(3)-IVa* also co-occurred with aminoglycoside and sulfonamide resistance genes in *H. somni* depicting GEN MIC > 8 μg/mL. In the isolates harboring these groups of AMR determinants, *floR* and *erm*(42) were also frequently clustered with the AMG-associated ARGs and *sul2* genes. Co-existence of these ARGs along with *tet*(H) is a hallmark of ICE (45, 59).

*Mannheimia haemolytica* serotype A2 isolates from dairy-type cattle were more frequently associated with AMR than serotype A1 and A6 isolate from beef-type cattle. The so-called "virulent *M. haemolytica* serotypes" A1 and A6 have been linked to higher AMR in beef cattle (14, 71). However, it is not known if there is a true association between *M. haemolytica* serotype and AMR or simply virulent serotypes are more frequently exposed to antimicrobials due to treatment for BRD. The results observed in this study may be the consequence of a higher

**TABLE 7 |** Significant results obtained from logistic regression of antimicrobial resistant *Mannheimia haemolytica* from dairy-type cattle upon feedlot arrival.

| Risk factor | Ampicillin | | | Danofloxacin | | | Florfenicol | | | Oxytetracycline | | | Penicillin | | | Tilmicosin | | | Tulathromycin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** |
| **Country** | | | | | | | | | | | | | | | | | | | | | |
| CA | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| US | na | na | na | 0.02 | 0.002–0.2 | **0.001** | 18.7 | 3.3–251.8 | **0.004** | na | na | na | na | na | na | na | na | na | na | na | na |
| **Monthly intervals** | | | | | | | | | | | | | | | | | | | | | |
| Aug–Nov | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| Dec–Feb | 1.0 | 0.2–6.0 | 0.964 | 8.1 | 1.3–50.7 | **0.025** | 9.5 | 1.6–68.3 | **0.017** | 3.33 | 0.9–13.0 | **0.082** | 0.9 | 0.2–3.3 | 0.831 | 3.3 | 0.9–12.4 | **0.077** | 0.3 | 0.02–4.7 | 0.426 |
| Mar–May | 0.2 | 0.03–1.1 | **0.057** | 1.1 | 0.3–3.5 | 0.900 | 1.9 | 0.4–10.8 | 0.465 | 0.95 | 0.3–2.8 | 0.930 | 0.2 | 0.03–0.9 | **0.031** | 0.2 | 0.05–0.9 | **0.041** | 0.1 | 0.01–0.9 | **0.049** |
| ***Mannheimia haemolytica* serotype** | | | | | | | | | | | | | | | | | | | | | |
| A2 | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| A1 + A6 | 0.5 | 0.1–1.8 | 0.259 | 12.1 | 3.4–43.3 | **<0.001** | 0.02 | 0.002–0.1 | **<0.001** | 4.8 | 1.9–11.9 | **<0.001** | 0.1 | 0.01–0.4 | **0.004** | 0.1 | 0.05–0.4 | **<0.001** | 0.03 | 0.01–0.2 | **<0.001** |

*CA, Canada; CI, confident interval; OR, odds ratio; RF, risk factor.*
*na, that variable was not significant at the p-value ≤ 0.1 level and was eliminated from the regression.*
*Bold p-values indicate statistically significant results at p ≤ 0.1.*

**TABLE 8 |** Significant results obtained from logistic regression of antimicrobial resistant *Pasteurella multocida* from dairy-type cattle upon feedlot arrival.

| Risk factor | Ampicillin | | | Danofloxacin | | | Enrofloxacin | | | Florfenicol | | | Oxytetracycline | | | Tilmicosin | | | Tulathromycin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** | **OR** | **95% CI** | ***p*-value** |
| **Country** | | | | | | | | | | | | | | | | | | | | | |
| CA | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| US | na | na | na | 0.003 | 0.0003–0.04 | **<0.001** | 0.01 | 0.001–0.1 | **<0.001** | 0.09 | 0.02–0.4 | **0.002** | na | na | na | na | na | na | 0.01 | 0.001–0.1 | **<0.001** |
| **Monthly intervals** | | | | | | | | | | | | | | | | | | | | | |
| Aug–Nov | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| Dec–Feb | 1.53 | 0.3–6.8 | 0.568 | na | na | na | 0.7 | 0.2–2.3 | 0.588 | 2.5 | 0.9–6.1 | **0.053** | na | na | na | na | na | na | 0.4 | 0.1–1.1 | **0.063** |
| Mar–May | 0.13 | 0.02–0.9 | **0.037** | na | na | na | 0.1 | 0.04–0.4 | **<0.001** | 0.3 | 0.1–0.9 | **0.026** | na | na | na | na | na | na | 1.3 | 0.5–3.2 | 0.647 |

*CA, Canada; CI, confident interval; OR, odds ratio.*
*na, that variable was not significant at the p-value ≤ 0.1 level and was eliminated from the regression.*
*Bold p-values indicate statistically significant results at p ≤ 0.1.*

**TABLE 9 |** Significant results obtained from logistic regression of antimicrobial resistant *Histophilus somni* from dairy-type cattle upon feedlot arrival.

| Risk factor | Ampicillin | | | Oxytetracycline | | | Penicillin | | | Spectinomycin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OR | 95% CI | *p*-value | OR | 95% CI | *p*-value | OR | 95% CI | *p*-value | OR | 95% CI | *p*-value |
| **Country** | | | | | | | | | | | | |
| CA | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – | 1 | 1 | – |
| US | 18.4 | 3.7–92.1 | **<0.001** | na | na | na | 23.22 | 3.6–148.9 | **<0.001** | na | na | na |

*CA, Canada; CI, confident interval; OR, odds ratio.*
*na, that variable was not significant at the p-value ≤ 0.1 level and was eliminated from the regression.*
*Bold p-values indicate statistically significant results at p ≤ 0.1.*

prevalence of serotype A2 among healthy animals under the high selective pressure of AMU in dairy prior to entry into the feedlot environment.

Jelinski et al. (11) proposed an SIR scheme for *M. bovis* isolated from bovine respiratory samples. Application of these criteria to *M. bovis* isolates in our study indicated high levels of resistance to TIP (100% both), TIL (100% beef and 99% dairy), and GAM (97.5% beef and 97% dairy). High MIC values for macrolides in *M. bovis* isolates from dairy and beef have been reported in Europe, Asia, and North America (11, 72). Macrolides are not frequently administrated to ranch calves in beef cow operations (58), which contrasts to the observed high macrolide MICs for *M. bovis* regardless of cattle type in our study. Antimicrobial resistance in *M. bovis* is mediated by mutations and not genes, possibly imposing a lower fitness burden, allowing the persistence of AMR traits regardless of AMU. Based on our results, TUL, ENRO, FFN, and TETs may be more effective against *M. bovis* than TIP, TIL, or GAM. However, the use of TET to treat clinical disease caused by *M. bovis* should be undertaken with caution since other authors have proposed a possible linkage between TETs and chronic pneumonia and polyarthritis syndrome in feedlots (73, 74). Nevertheless, it is not practically known which bacteria are specifically causing BRD in any one individual animal and frequently multiple bacteria can be isolated from fatal BRD cases (75). Therefore, it is difficult for veterinary practitioners to make any specific recommendations as it is not known which pathogen(s) is causing clinical disease. The odds of isolating *M. bovis* from beef and dairy-type cattle were 2.52 and 2.26 times higher, respectively, in the second year as compared to the first year of our study. During the first sampling year, a commercial media for the isolation of *M. bovis* was used (broth and agar; cat. no. TP90 and PM80, Dalynn Biologicals). Since *M. bovis* colonies growing on this commercial media often did not display the typical fried-egg morphology (76), a different media was used during the second sampling year (23). As a consequence, it is not possible to know if the difference in the *M. bovis* recovery was a consequence of inter-year variability.

Twenty-five and 68% of *M. haemolytica* and *P. multocida* isolates from dairy-type calves were MDR. As aforementioned, AMU is greater in dairy-type cattle as compared to beef-type cattle (77). Studies have suggested that feedlots are an environment that promote/amplify AMR via various means including through metaphylaxis (78, 79), serving as a reservoir of AMR bacteria and genes, selection through antimicrobial metabolites in manure (80, 81), and manure enriching the soil microbiome in AMR bacteria (82). It is known that metaphylaxis upon feedlot arrival decreases BRD morbidity and mortality (53) but it may select for AMR bacteria (78). Bovine respiratory disease *Pasteurellaceae* can persist in the farm and feedlot environment (83, 84) and be transmitted amongst different herds (85). In North America, large commercial feedlots receive a constant flow of animals and pens are usually cleaned (not disinfected) of manure/bedding once or twice per year. This constant occupancy of cattle in the feedlot may promote the persistence of AMR bacteria in the environment. Some feedlots feed both beef and dairy-type cattle, resulting in cattle with significantly different AMU backgrounds being housed in close

proximity. Many of the MDR *Pasteurellaceae* harbor ARGs on ICE and it is unknown if bacteria carrying these elements increase and spread among cattle within the feedlot, a possibility that deserves further evaluation.

## CONCLUSION

This is the first published study in Alberta documenting AMR in four major bacterial species involved in the BRD complex isolated from beef and dairy-type cattle on feedlot arrival. Our findings show marked differences in bacterial isolation and AMR levels in bacterial members of the BRD complex between dairy and beef cattle types. Moreover, MDR *M. haemolytica*, *P. multocida*, and *H. somni* isolates presenting AMR phenotypes indicative of the presence of ICE were isolated more often from dairy-type than beef-type cattle. These results raise the question of whether feedlot AMU and AMR should be reported by cattle type which could help to evaluate if the higher prevalence of ICE-related AMR is linked to higher BRD treatment failure and mortality. Additionally, an association between higher AMR and feedlot-backgrounded beef-type cattle was reported. Macrolides may not be an efficacious treatment choice for *M. bovis* (in beef and dairy-type cattle) or *H. somni* (dairy) infections in feedlot cattle in Alberta. Considering that antimicrobial therapy is essential for the prevention, control, and treatment of BRD in feedlot cattle, our study highlights the continued need for AMU and AMR surveillance of BRD-related bacteria in feedlot cattle to help inform veterinarians on treatment protocol decisions that promote prudent drug use.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI repository, accession number PRJNA720670.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Lethbridge Research Centre Animal Care and Use Committee (Protocol #1641, Jan 18th, 2017) and was conducted according to the Canadian Council of Animal Care Guidelines. Written informed consent for participation was not obtained from the owners because Consent was provided verbally as a result of communication between veterinary practitioners and their clients.

## AUTHOR CONTRIBUTIONS

JV, CB, SJH, SH, CD, SG, and TM: concept and design of the study. CB, CD, JV, SH, and SJH: sample collection. CL, SA-L, RH, and RZ: data acquisition. RZ, SA-L, and RD: analysis of data. RZ, SA-L, and MA: interpretation of data. SA-L: drafting the manuscript. CB, CD, CL, JV, MA, RD, RH, RZ, SG, SH, SJH, and TM: critical review of the manuscript including the final version. All authors contributed to the article and approved the submitted version.

## FUNDING

This project was funded by Alberta Beef Producers (ABP) and Alberta Livestock and Meat Agency Ltd. (ALMA). Project number ANH.09.16.

## ACKNOWLEDGMENTS

This work is an excellent illustration of academia and industry collaboration. Its completion could not have been possible without the contribution of all professionals who were involved in it in one way or another. We would like to thank Cheyenne Conrad, Ruth Barbieri, Brendon DeGroot, Sujeema Abeysekara, Wendi Smart (AAFC), Homayoun Zahiroddini (AAF), and Taylor Davedow (University of Manitoba) for their technical assistance in the laboratory; to all the personnel from the veterinary practices and feedlots involved in this project; to Dr. Murray Jelinski, Andrea Kinnear, and Karen Gesy (Western College of Veterinary Medicine, University of Saskatchewan) for their expertise on *Mycoplasma bovis*; and to Dr. Diego Nobrega (University of Guelph) for his expertise on epidemiologic analysis.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.692646/full#supplementary-material

## REFERENCES

1. United States Department of Agriculture. *Part IV: Health and Health Management on U.S. Feedlots With a Capacity of 1,000 or More Head*. Fort Collins, CO: United States Department of Agriculture (2013).
2. Lhermie G, Chiu LV, Kaniyamattam K, Tauer LW, Scott HM, Grohn YT. Antimicrobial policies in United States beef production: choosing the right instruments to reduce antimicrobial use and resistance under structural and market constraints. *Front Vet Sci.* (2019) 6:245. doi: 10.3389/fvets.2019.00245
3. Jim K. Impact of bovine respiratory disease (BRD) from the perspective of the Canadian beef producer. *Anim Health Res Rev.* (2009) 10:109–10. doi: 10.1017/S1466252309990119
4. Hilton WM. BRD in 2014: where have we been, where are we now, and where do we want to go? *Anim Health Res Rev.* (2014) 15:120–2. doi: 10.1017/S1466252314000115
5. Watts JL, Sweeney MT. Antimicrobial resistance in bovine respiratory disease pathogens: measures, trends, and impact on efficacy. *Vet Clin North Am Food Anim Pract.* (2010) 26:79–88. doi: 10.1016/j.cvfa.2009.10.009
6. Anholt RM, Klima C, Allan N, Matheson-Bird H, Schatz C, Ajitkumar P, et al. Antimicrobial susceptibility of bacteria that cause bovine

respiratory disease complex in Alberta, Canada. *Front Vet Sci.* (2017) 4:207. doi: 10.3389/fvets.2017.00207
7. Taylor JD, Fulton RW, Lehenbauer TW, Step DL, Confer AW. The epidemiology of bovine respiratory disease: what is the evidence for preventive measures? *Can Vet J.* (2010) 51:1351–9.
8. Smith RA, Step DL, Woolums AR. Bovine respiratory disease: looking back and looking forward, what do we see? *Vet Clin North Am Food Anim Pract.* (2020) 36:239–51. doi: 10.1016/j.cvfa.2020.03.009
9. DeDonder KD, Apley MD. A literature review of antimicrobial resistance in pathogens associated with bovine respiratory disease. *Anim Health Res Rev.* (2015) 16:125–34. doi: 10.1017/S146625231500016X
10. Portis E, Lindeman C, Johansen L, Stoltman G. A ten-year (2000-2009) study of antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex–*Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*–in the United States and Canada. *J Vet Diagn Invest.* (2012) 24:932–44. doi: 10.1177/1040638712457559
11. Jelinski M, Kinnear A, Gesy K, Andrés-Lasheras S, Zaheer R, Weese S, et al. Antimicrobial sensitivity testing of *Mycoplasma bovis* isolates derived from western Canadian feedlot cattle. *Microorganisms.* (2020) 8:124. doi: 10.3390/microorganisms8010124
12. Klima CL, Zaheer R, Cook SR, Booker CW, Hendrick S, Alexander TW, et al. Pathogens of bovine respiratory disease in North American feedlots conferring multidrug resistance via integrative conjugative elements. *J Clin Microbiol.* (2014) 52:438–48. doi: 10.1128/JCM.02485-13
13. Cameron A, McAllister TA. Antimicrobial usage and resistance in beef production. *J Anim Sci Biotechnol.* (2016) 7:68. doi: 10.1186/s40104-016-0127-3
14. Klima CL, Alexander TW, Read RR, Gow SP, Booker CW, Hannon S, et al. Genetic characterization and antimicrobial susceptibility of *Mannheimia haemolytica* isolated from the nasopharynx of feedlot cattle. *Vet Microbiol.* (2011) 149:390–8. doi: 10.1016/j.vetmic.2010.11.018
15. Noyes NR, Benedict KM, Gow SP, Booker CW, Hannon SJ, McAllister TA, et al. *Mannheimia haemolytica* in feedlot cattle: prevalence of recovery and associations with antimicrobial use, resistance, and health outcomes. *J Vet Intern Med.* (2015) 29:705–13. doi: 10.1111/jvim.12547
16. Guo Y, McMullen C, Timsit E, Hallewell J, Orsel K, van der Meer F, et al. Genetic relatedness and antimicrobial resistance in respiratory bacteria from beef calves sampled from spring processing to 40 days after feedlot entry. *Vet Microbiol.* (2020) 240:108478. doi: 10.1016/j.vetmic.2019.108478
17. McEwen SA, Fedorka-Cray PJ. Antimicrobial use and resistance in animals. *Clin Infect Dis.* (2002) 1:S93–106. doi: 10.1086/340246
18. Jim GK, Booker CW, Ribble CS, Guichon PT, Thorlakson BE. A field investigation of the economic impact of respiratory disease in feedlot calves. *Can Vet J.* (1993) 34:668–73.
19. Boerlin P, Reid-Smith RJ. Antimicrobial resistance: its emergence and transmission. *Anim Health Res Rev.* (2008) 9:115–26. doi: 10.1017/S146625230800159X
20. Mather AE, Reeve R, Mellor DJ, Matthews L, Reid-Smith RJ, Dutil L, et al. Detection of rare antimicrobial resistance profiles by active and passive surveillance approaches. *PLoS ONE.* (2016) 11:e0158515. doi: 10.1371/journal.pone.0158515
21. Hannon SJ, Brault SA, Otto SJG, Morley PS, McAllister TA, Booker CW, et al. Feedlot cattle antimicrobial use surveillance network: a Canadian journey. *Front Vet Sci.* (2020) 7:596042. doi: 10.3389/fvets.2020.596042
22. Olfert E, Cross B, McWilliam A. *Guide to the Care and Use of Experimental Animals.* Ottawa, ON: Canadian Council on Animal Care (1993).
23. Andres-Lasheras S, Zaheer R, Ha R, Lee C, Jelinski M, McAllister TA. A direct qPCR screening approach to improve the efficiency of *Mycoplasma bovis* isolation in the frame of a broad surveillance study. *J Microbiol Methods.* (2019) 169:105805. doi: 10.1016/j.mimet.2019.105805
24. Angen O, Thomsen J, Larsen LE, Larsen J, Kokotovic B, Heegaard PM, et al. Respiratory disease in calves: microbiological investigations on trans-tracheally aspirated bronchoalveolar fluid and acute phase protein response. *Vet Microbiol.* (2009) 137:165–71. doi: 10.1016/j.vetmic.2008.12.024
25. Miflin JK, Blackall PJ. Development of a 23S rRNA-based PCR assay for the identification of *Pasteurella multocida*. *Lett Appl Microbiol.* (2001) 33:216–21. doi: 10.1046/j.1472-765x.2001.00985.x
26. Angen O, Ahrens P, Tegtmeier C. Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. *Vet Microbiol.* (1998) 63:39–48. doi: 10.1016/S0378-1135(98)00222-3
27. Klima CL, Zaheer R, Briggs RE, McAllister TA. A multiplex PCR assay for molecular capsular serotyping of *Mannheimia haemolytica* serotypes 1, 2, and 6. *J Microbiol Methods.* (2017) 139:155–60. doi: 10.1016/j.mimet.2017.05.010
28. Dohoo I, Martin W, Stryhn H. *Veterinary Epidemiologic Research*. 2nd ed. Charlottetown, Prince Edward Island, Canada: Ver Inc. (2014).
29. Institute CaLS. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard.* 4th ed. CLSI document VET01-4. Wayne, PA: Institute CaLS (2013).
30. Institute CaLS. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals*. 4th ed. CLSI document VET08. Wayne, PA: Institute CaLS (2018).
31. (CLSI) CaLSI. *Understanding Susceptibility Test Data as a Component of Antimicrobial Stewardship in Veterinary Settings*. 1st ed. CLSI document VE09. Wayne, PA: CaLSI (2019).
32. Institute CaLS. *Understanding Susceptibility Test Data as a Component of Antimicrobial Stewardship in Veterinary Settings*. 1st ed. CLSI document VET09. Wayne, PA: Institute CaLS (2019).
33. Rosenbusch RF, Kinyon JM, Apley M, Funk ND, Smith S, Hoffman LJ. *In vitro* antimicrobial inhibition profiles of *Mycoplasma bovis* isolates recovered from various regions of the United States from 2002 to 2003. *J Vet Diagn Invest.* (2005) 17:436–41. doi: 10.1177/104063870501700505
34. Hannan PC. Guidelines and recommendations for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary mycoplasma species. *Vet Res.* (2000) 31:373–95. doi: 10.1051/vetres:2000100
35. Ayling RD, Rosales RS, Barden G, Gosney FL. Changes in antimicrobial susceptibility of *Mycoplasma bovis* isolates from Great Britain. *Vet Rec.* (2014) 175:486. doi: 10.1136/vr.102303
36. Institute CaLS. *Generation, Presentation, and Application of Antimicrobial Susceptibility Test Data for Bacteria of Animal Origin; A Report*. CLSI document VET05-R. Wayne, PA: Institute CaLS (2011).
37. Seemann T. *Shovill*. Available online at: https://github.com/tseemann/shovill (accessed March 2021).
38. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol.* (2012) 19:455–77. doi: 10.1089/cmb.2012.0021
39. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics.* (2014) 30:2068–9. doi: 10.1093/bioinformatics/btu153
40. Heinze G, Wallisch C, Dunkler D. Variable selection - a review and recommendations for the practicing statistician. *Biom J.* (2018) 60:431–49. doi: 10.1002/bimj.201700067
41. Taylor JD, Fulton RW, Lehenbauer TW, Step DL, Confer AW. The epidemiology of bovine respiratory disease: what is the evidence for predisposing factors? *Can Vet J.* (2010) 51:1095–102.
42. Galili T, O'Callaghan A. *Introduction to Heatmaply*. (2020). Available online at: https://cran.r-project.org/web/packages/heatmaply/vignettes/heatmaply.html (accessed August 10, 2020).
43. Eidam C, Poehlein A, Leimbach A, Michael GB, Kadlec K, Liesegang H, et al. Analysis and comparative genomics of ICE*Mh1*, a novel integrative and conjugative element (ICE) of *Mannheimia haemolytica*. *J Antimicrob Chemother.* (2015) 70:93–7. doi: 10.1093/jac/dku361
44. Michael GB, Kadlec K, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, et al. ICE*Pmu1*, an integrative conjugative element (ICE) of *Pasteurella multocida*: analysis of the regions that comprise 12 antimicrobial resistance genes. *J Antimicrob Chemother.* (2012) 67:84–90. doi: 10.1093/jac/dkr406
45. Stanford K, Zaheer R, Klima C, McAllister T, Peters D, Niu YD, et al. Antimicrobial resistance in members of the bacterial bovine respiratory disease complex isolated from lung tissue of cattle mortalities managed with or without the use of antimicrobials. *Microorganisms.* (2020) 8:288. doi: 10.3390/microorganisms8020288
46. Alexander TW, Cook S, Klima CL, Topp E, McAllister TA. Susceptibility to tulathromycin in *Mannheimia haemolytica* isolated from feedlot cattle over a 3-year period. *Front Microbiol.* (2013) 4:297. doi: 10.3389/fmicb.2013.00297
47. Klima CL, Alexander TW, Hendrick S, McAllister TA. Characterization of *Mannheimia haemolytica* isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. *Can J Vet Res.* (2014) 78:38–45.

48. Chase CC, Hurley DJ, Reber AJ. Neonatal immune development in the calf and its impact on vaccine response. *Vet Clin North Am Food Anim Pract.* (2008) 24:87–104. doi: 10.1016/j.cvfa.2007.11.001
49. Griffin D. Bovine pasteurellosis and other bacterial infections of the respiratory tract. *Vet Clin North Am Food Anim Pract.* (2010) 26:57–71. doi: 10.1016/j.cvfa.2009.10.010
50. Peel DS. The effect of market forces on bovine respiratory disease. *Vet Clin North Am Food Anim Pract.* (2020) 36:497–508. doi: 10.1016/j.cvfa.2020.03.008
51. Ribble CS, Meek AH, Shewen PE, Guichon PT, Jim GK. Effect of pretransit mixing on fatal fibrinous pneumonia in calves. *J Am Vet Med Assoc.* (1995) 207:616–9.
52. Stroebel C, Alexander T, Workentine ML, Timsit E. Effects of transportation to and co-mingling at an auction market on nasopharyngeal and tracheal bacterial communities of recently weaned beef cattle. *Vet Microbiol.* (2018) 223:126–33. doi: 10.1016/j.vetmic.2018.08.007
53. Brault SA, Hannon SJ, Gow SP, Warr BN, Withell J, Song J, et al. Antimicrobial use on 36 beef feedlots in western Canada: 2008-2012. *Front Vet Sci.* (2019) 6:329. doi: 10.3389/fvets.2019.00329
54. Timsit E, McMullen C, Amat S, Alexander TW. Respiratory bacterial microbiota in cattle: from development to modulation to enhance respiratory health. *Vet Clin North Am Food Anim Pract.* (2020) 36:297–320. doi: 10.1016/j.cvfa.2020.03.001
55. Scott MA, Woolums AR, Swiderski CE, Perkins AD, Nanduri B, Smith DR, et al. Whole blood transcriptomic analysis of beef cattle at arrival identifies potential predictive molecules and mechanisms that indicate animals that naturally resist bovine respiratory disease. *PLoS ONE.* (2020) 15:e0227507. doi: 10.1371/journal.pone.0227507
56. United States Department of Agriculture. *Health Management Practices on U.S. Dairy Operations*. Collins, CO: United States Department of Agriculture (2018).
57. Saini V, McClure JT, Leger D, Dufour S, Sheldon AG, Scholl DT, et al. Antimicrobial use on Canadian dairy farms. *J Dairy Sci.* (2012) 95:1209–21. doi: 10.3168/jds.2011-4527
58. Waldner CL, Parker S, Gow S, Wilson DJ, Campbell JR. Antimicrobial usage in western Canadian cow-calf herds. *Can Vet J.* (2019) 60:255–67.
59. Beker M, Rose S, Lykkebo CA, Douthwaite S. Integrative and conjugative elements (ICEs) in *Pasteurellaceae* species and their detection by multiplex PCR. *Front Microbiol.* (2018) 9:1329. doi: 10.3389/fmicb.2018.01329
60. Lovering A, Reeves D. Aminoglycosides and aminocyclitols. In: Clansey N, editor. *Antibiotic and Chemotherapy*. Elsevier Ltd. (2011). p. 145–69.
61. Vester B, Douthwaite S. Macrolide resistance conferred by base substitutions in 23S rRNA. *Antimicrob Agents Chemother.* (2001) 45:1–12. doi: 10.1128/AAC.45.1.1-12.2001
62. Kadlec K, Watts JL, Schwarz S, Sweeney MT. Plasmid-located extended-spectrum beta-lactamase gene blaROB-2 in *Mannheimia haemolytica*. *J Antimicrob Chemother.* (2019) 74:851–3. doi: 10.1093/jac/dky515
63. San Millan A, Escudero JA, Gutierrez B, Hidalgo L, Garcia N, Llagostera M, et al. Multiresistance in *Pasteurella multocida* is mediated by coexistence of small plasmids. *Antimicrob Agents Chemother.* (2009) 53:3399–404. doi: 10.1128/AAC.01522-08
64. Clawson ML, Murray RW, Sweeney MT, Apley MD, DeDonder KD, Capik SF, et al. Genomic signatures of *Mannheimia haemolytica* that associate with the lungs of cattle with respiratory disease, an integrative conjugative element, and antibiotic resistance genes. *BMC Genomics.* (2016) 17:982. doi: 10.1186/s12864-016-3316-8
65. Bhatt K, Timsit E, Rawlyk N, Potter A, Liljebjelke K. Integrative conjugative element ICE*Hs1* encodes for antimicrobial resistance and metal tolerance in *Histophilus somni*. *Front Vet Sci.* (2018) 5:153. doi: 10.3389/fvets.2018.00153
66. Sawant AA, Sordillo LM, Jayarao BM. A survey on antibiotic usage in dairy herds in Pennsylvania. *J Dairy Sci.* (2005) 88:2991–9. doi: 10.3168/jds.S0022-0302(05)72979-9
67. Ozawa M, Asai T, Sameshima T. Mutations in GyrA and ParC in fluoroquinolone-resistant *Mannheimia haemolytica* isolates from cattle in Japan. *J Vet Med Sci.* (2009) 71:493–4. doi: 10.1292/jvms.71.493
68. Kong LC, Gao D, Gao YH, Liu SM, Ma HX. Fluoroquinolone resistance mechanism of clinical isolates and selected mutants of *Pasteurella multocida* from bovine respiratory disease in China. *J Vet Med Sci.* (2014) 76:1655–7. doi: 10.1292/jvms.14-0240
69. Michael GB, Bosse JT, Schwarz S. Antimicrobial resistance in *Pasteurellaceae* of veterinary origin. *Microbiol Spectr.* (2018) 6:1–33. doi: 10.1128/microbiolspec.ARBA-0022-2017
70. Cameron A, Klima CL, Ha R, Gruninger RJ, Zaheer R, McAllister TA. A novel *aadA* aminoglycoside resistance gene in bovine and porcine pathogens. *mSphere.* (2018) 3:e00568–17. doi: 10.1128/mSphere.00568-17
71. Katsuda K, Kohmoto M, Mikami O. Relationship between serotype and the antimicrobial susceptibility of *Mannheimia haemolytica* isolates collected between 1991 and 2010. *Prev Vet Med.* (2013) 94:205–8. doi: 10.1016/j.rvsc.2012.09.015
72. Lysnyansky I, Ayling RD. *Mycoplasma bovis*: mechanisms of resistance and trends in antimicrobial susceptibility. *Front Microbiol.* (2016) 7:595. doi: 10.3389/fmicb.2016.00595
73. Holman DB, Yang W, Alexander TW. Antibiotic treatment in feedlot cattle: a longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. *Microbiome.* (2019) 7:86. doi: 10.1186/s40168-019-0696-4
74. Hendrick SH, Bateman KG, Rosengren LB. The effect of antimicrobial treatment and preventive strategies on bovine respiratory disease and genetic relatedness and antimicrobial resistance of *Mycoplasma bovis* isolates in a western Canadian feedlot. *Can Vet J.* (2013) 54:1146–56.
75. Booker CW, Lubbers BV. Bovine respiratory disease treatment failure: impact and potential causes. *Vet Clin North Am Food Anim Pract.* (2020) 36:487–96. doi: 10.1016/j.cvfa.2020.03.007
76. Razin S, Oliver O. Morphogenesis of *Mycoplasma* and bacterial L-form colonies. *J Gen Microbiol.* (1961) 24:225–37. doi: 10.1099/00221287-24-2-225
77. Call DR, Davis MA, Sawant AA. Antimicrobial resistance in beef and dairy cattle production. *Anim Health Res Rev.* (2008) 9:159–67. doi: 10.1017/S1466252308001515
78. Snyder E, Credille B, Berghaus R, Giguère S. Prevalence of multi drug antimicrobial resistance in *Mannheimia haemolytica* isolated from high-risk stocker cattle at arrival and two weeks after processing. *J Anim Sci.* (2017) 95:1124–31. doi: 10.2527/jas.2016.1110
79. Woolums AR, Karisch BB, Frye JG, Epperson W, Smith DR, Blanton JJ, et al. Multidrug resistant *Mannheimia haemolytica* isolated from high-risk beef stocker cattle after antimicrobial metaphylaxis and treatment for bovine respiratory disease. *Vet Microbiol.* (2018) 221:143–52. doi: 10.1016/j.vetmic.2018.06.005
80. Agga GE, Schmidt JW, Arthur TM. Effects of in-feed chlortetracycline prophylaxis in beef cattle on animal health and antimicrobial-resistant *Escherichia coli*. *Appl Environ Microbiol.* (2016) 82:7197–204. doi: 10.1128/AEM.01928-16
81. Muller HC, Van Bibber-Krueger CL, Ogunrinu OJ, Amachawadi RG, Scott HM, Drouillard JS. Effects of intermittent feeding of tylosin phosphate during the finishing period on feedlot performance, carcass characteristics, antimicrobial resistance, and incidence and severity of liver abscesses in steers. *J Anim Sci.* (2018) 96:2877–85. doi: 10.1093/jas/sky166
82. Agga GE, Cook KL, Netthisinghe AMP, Gilfillen RA, Woosley PB, Sistani KR. Persistence of antibiotic resistance genes in beef cattle backgrounding environment over two years after cessation of operation. *PLoS ONE.* (2019) 14:e0212510. doi: 10.1371/journal.pone.0212510
83. Burriel AR. Isolation of *Pasteurella haemolytica* from grass, drinking water, and straw bedding used by sheep. *Curr Microbiol.* (1997) 35:316–8. doi: 10.1007/s002849900261
84. Neupane S, Nayduch D, Zurek L. House flies (*Musca domestica*) pose a risk of carriage and transmission of bacterial pathogens associated with bovine respiratory disease (BRD). *Insects.* (2019) 10:358. doi: 10.3390/insects10100358
85. Katsuda K, Kohmoto M, Mikami O, Uchida I. Antimicrobial resistance and genetic characterization of fluoroquinolone-resistant *Mannheimia haemolytica* isolates from cattle with bovine pneumonia. *Vet Microbiol.* (2009) 139:74–9. doi: 10.1016/j.vetmic.2009.04.020


**Conflict of Interest:** CB is part owner and managing partner of Feedlot Health Management Services Ltd. and Southern Alberta Veterinary Services. SJH is an employee at Feedlot Health Management Services Ltd., Okotoks, Alberta, Canada.

Feedlot Health is a private company that provides expert consultation regarding production and management of feedlot cattle and calf grower calves, including developing veterinary protocols to support animal health. They also conduct in-house and contract research related to dairy calf grower and feedlot production.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 22 July 2021
doi: 10.3389/fvets.2021.691448

# The Participation of a Malignant Catarrhal Fever Virus and *Mycoplasma bovis* in the Development of Single and Mixed Infections in Beef and Dairy Cattle With Bovine Respiratory Disease

*Thalita Evani Silva Oliveira[1,2], Gabriela Sanches Scuisato[1], Isadora Fernanda Pelaquim[1], Cristina Wetzel Cunha[3,4], Lucas Santana Cunha[5], Eduardo Furtado Flores[6], Lucienne Garcia Pretto-Giordano[7], Júlio Augusto Naylor Lisbôa[2,8], Amauri Alcindo Alfieri[2,9], João Paulo Elsen Saut[10], Paulo Henrique Jorge da Cunha[11] and Selwyn Arlington Headley[1,2,12]**

[1] Laboratory of Animal Pathology, Department of Veterinary Preventive Medicine, Universidade Estadual de Londrina, Londrina, Brazil, [2] National Institutes of Science and Technology, Dairy Production Chain (INCT-Leite), Universidade Estadual de Londrina, Londrina, Brazil, [3] Animal Disease Research Unit, Agricultural Research Service, United States Department of Agriculture, Pullman, WA, United States, [4] Department of Veterinary Microbiology and Pathology and Paul G. Alan School for Global Animal Health, Washington State University, Pullman, WA, United States, [5] Department of Statistics, Universidade Estadual de Londrina, Londrina, Brazil, [6] Department of Preventive Veterinary Medicine, Universidade Federal de Santa Maria, Santa Maria, Brazil, [7] Laboratory of Veterinary Microbiology and Infectious Diseases, Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina, Londrina, Brazil, [8] Large Animal Internal Medicine, Department of Veterinary Clinics, Universidade Estadual de Londrina, Londrina, Brazil, [9] Laboratory of Animal Virology, Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina, Londrina, Brazil, [10] Large Animal Health Laboratory, Universidade Federal de Uberlândia, Uberlândia, Brazil, [11] Veterinary Medicine Department, Universidade Federal de Goiás, Goiânia, Brazil, [12] Programa de Pós-Graduação em Biociência Animal, Universidade de Cuiabá, Cuiabá, Brazil

The bovine respiratory disease (BRD) complex is a multietiological and multifactorial disease associated with a wide range of viral and bacterial pathogens. This study evaluated the contribution of specific infectious disease agents in the development of BRD in cattle from Brazil and determined if a virus within the malignant catarrhal fever virus (MCFV) group and *Mycoplasma bovis*, acting individually or in conjunction, can be associated with the development of BRD. Formalin-fixed paraffin-embedded pulmonary sections were used in immunohistochemical assays to determine the intralesional presence of six antigens associated with BRD: bovine alphaherpesvirus 1 (BoHV-1), bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), MCFV, and *M. bovis*. Pneumonia was diagnosed in 82.7% (120/145) of all cattle evaluated. Interstitial pneumonia (60%, 72/120) and suppurative bronchopneumonia (25.8%, 31/120) were the most frequent patterns of pneumonia identified. Intralesional antigens of MCFV (53.3%, 64/120) were the most frequently associated with BRD, followed by *M. bovis* (47.5%, 57/120), BVDV (42.5%, 51/120), BoHV-1 (28.3%, 34/120), BRSV (24.2%, 29/120), and BPIV-3 (8.3%, 10/120). Additionally, antigens of BVDV, MCFV, and *M. bovis* were the most frequently identified agents associated with singular and concomitant infections. The MCFV identified during

OPEN ACCESS

**Edited by:**
*Annamaria Pratelli, University of Bari Aldo Moro, Italy*

**Reviewed by:**
*Katarzyna Dudek, National Veterinary Research Institute (NVRI), Poland*
*Liria Okuda, Biological Institute of São Paulo, Brazil*

***Correspondence:**
*Selwyn Arlington Headley*
*selwyn.headley@uel.br*

**Specialty section:**
*This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science*

**Received:** *06 April 2021*
**Accepted:** *20 May 2021*
**Published:** *22 July 2021*

**Citation:**
*Oliveira TES, Scuisato GS, Pelaquim IF, Cunha CW, Cunha LS, Flores EF, Pretto-Giordano LG, Lisbôa JAN, Alfieri AA, Saut JPE, Jorge da Cunha PH and Headley SA (2021) The Participation of a Malignant Catarrhal Fever Virus and Mycoplasma bovis in the Development of Single and Mixed Infections in Beef and Dairy Cattle With Bovine Respiratory Disease. Front. Vet. Sci. 8:691448. doi: 10.3389/fvets.2021.691448*

this study is more likely to be ovine gammaherpesvirus 2 (OvHV-2), since OvHV-2 is the only MCFV identified within the geographical region of this study. Interstitial pneumonia with proliferative vascular lesions may be a useful histologic feature to differentiate MCFV-induced pneumonia from other viral pneumonias of cattle. These results demonstrate that MCFV and *M. bovis*, in single or mixed infections, can produce pneumonia in cattle and should therefore be considered as primary agents in the development of BRD.

**Keywords: diagnostic immunohistochemistry, fibrinoid change, histopathologic patterns, caseonecrotic bronchopneumonia, proliferative vascular alterations, respiratory disease pathogens, ovine gammaherpesvirus 2**

## INTRODUCTION

Bovine respiratory disease (BRD) is a complex multifactorial and multietiological disease entity that is associated with viral and bacterial pathogens coupled with unfavorable management practices and environmental conditions. The principal viral disease pathogens associated with the development of BRD are bovine coronavirus (BCoV), bovine alphaherpesvirus 1 (BoHV-1), bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhea virus (BVDV), and bovine respiratory syncytial virus (BRSV) (1–4). The major bacterial pathogens of BRD include *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* (5–8), and *Mycoplasma bovis*. All of these pathogens have been associated with outbreaks of BRD in beef and dairy cattle herds from Brazil (9).

More recently, ovine gammaherpesvirus 2 (OvHV-2), a virus within the malignant catarrhal fever virus (MCFV) complex, was suggested as a possible infectious disease pathogen associated with the development of BRD (10). Additionally, it must be highlighted that although the MCFV complex is known to be composed of nine members (11), only OvHV-2 was associated with malignant catarrhal fever (MCF) in ruminants from Brazil (10, 12). Although spontaneous cases of interstitial pneumonia associated with the amplification of OvHV-2 DNA were previously described in bison (13, 14) and buffaloes (15), the possible participation of an MCFV in the development of BRD is a novel finding.

Viral disease pathogens of BRD may cause primary infections, acting in either single or mixed infections with other pathogens (3, 16, 17). A significant role for BRD-related viruses is their interaction with bacteria (17–19) and mycoplasma (20–22) in the development of bronchopneumonia. Moreover, studies have shown that *M. bovis* was the only pathogen identified in pneumonic lungs, resulting in fatal pneumonia in calves (3, 23, 24) and adult cattle (16).

In Brazil, information relative to the occurrence of BRD is limited when compared with studies from North America (25–28) and Australia (29, 30). Previous studies done in Brazil were designed to investigate a single (31–37) or several agents (2, 16, 38–43) associated with BRD. These studies focused on the molecular identification of agents, with and without the histopathologic diagnosis of the patterns of pulmonary disease. It must be highlighted that associating the pattern of pulmonary disease with the intralesional presence of the agents is fundamental to confirming infection. Recently, we have identified the occurrence of infectious disease pathogens of BRD by using a combination of the histopathologic patterns and the *in situ* identification of the infectious disease pathogen by immunohistochemistry (IHC) and have shown that *M. bovis* may be a potential primary disease agent of pneumonia in cattle (16).

Consequently, the aims of this study were to determine the occurrence of respiratory infectious disease agents in cattle from several geographical regions of Brazil, evaluate the contribution of specific infectious disease agents in the development of BRD, and determine if an MCFV and *M. bovis*, acting individually or in mixed infections, can be associated with the development of BRD.

## MATERIALS AND METHODS

### Study Design

A cross-sectional study focusing on two units of observation was designed: the first investigated respiratory agents associated with the development of BRD, while the second determined the occurrence of pneumonia. The first was designed to associate the occurrence of pneumonia with specific infectious disease pathogens irrespective of the pattern of pulmonary disease. Subsequently, predetermined patterns of pneumonia were classified and related with specific agent(s) associated with BRD.

### Study Area, Animal Selection, and Histopathologic Analyses

A review of all pulmonary tissues of cattle submitted for histopathologic diagnosis between 2015 and 2018 was done to determine the occurrence of pneumonia. These samples originated from diagnostic laboratories located within the states of São Paulo (Southeastern), Paraná, and Santa Catarina (Southern) Brazil; some of the pulmonary tissues derived from cows from Paraná were part of a larger study that investigated the occurrence of cattle neurological disease (44). Archival records of all animals were retrieved, reviewed, and tabulated to obtain information relative to sex, type of animal (beef, dairy, or mixed), age, and cause of death (natural × slaughter). These data were then associated with the occurrence of pulmonary disease. The age of all cattle was divided into two predetermined categories: calves (cattle up to 15 months old) and adults (animals 16 months or older) (45). Only data relative to the age of cattle with

pulmonary disease, irrespective of the patterns of pneumonia, were included in the analysis.

Formalin-fixed paraffin-embedded (FFPE) pulmonary sections were used to produce new histological slides when necessary. All sections were stained by the hematoxylin-and-eosin (H&E) method and reviewed for histopathologic patterns of pulmonary disease as outlined (16); the histopathologic review was done by two veterinary pathologists (TESO and SAH). In addition, new histological slides were made for all tissues containing intralesional pleomorphic organisms; these were colored with the Giemsa histochemical method for the identification of organisms consistent with *M. bovis* and with the Brown–Brenn Gram histochemical stain to differentiate them from other accumulations of Gram-positive or Gram-negative bacteria; both methods were based on previous protocols (46). Giemsa staining was used to identify *M. bovis*, since we have previously suggested that this histochemical stain may efficiently identify these intralesional organisms (16).

Additionally, the pulmonary tissues evaluated were divided into three categories based on the predominant histologic alterations observed: (1) pulmonary tissues with predominantly cellular and vascular alterations (congestion, reversible, and irreversible cellular lesions); (2) interstitial pneumonia, and (3) bronchopneumonia. These categories were then used as inputs to correlate these histologic findings with the intralesional localization of antigens of the evaluated agents identified by IHC.

## IHC Identification of Infectious Disease Agents Associated With BRD

IHC assays were performed on pulmonary sections of each animal to determine the intralesional presence of six antigens potentially associated with the development of BRD: BoHV-1, BRSV, BVDV, BPIV-3, MCFV, and *M. bovis*. Selected FFPE tissue sections of the lungs were prepared on silanized slides with poly-L-lysine 0.1% (Sigma-Aldrich, St. Louis, MO, USA) and submitted to IHC assays designed to identify the antigens of these agents. The IHC assays to detect antigens of BoHV-1, BVDV, BRSV, BPIV-3, and *M. bovis* were previously described (16). MCFV-specific antigens were identified by using the monoclonal antibody 15A (MAb-15A) (12). Positive controls included FFPE tissue sections known to contain antigens of BoHV-1, BVDV, BRSV, BPIV-3, *M. bovis* (16), and OvHV-2 (12). Two negative controls were used: the first consisted of substituting the primary antibodies with their respective diluents, and the second consisted of utilizing the primary antibodies on FFPE tissues with known negative immunoreactivity to the BRD antigens derived from the studies cited above. Positive and negative controls were included in each IHC assay.

## Data Analysis

The association between the biological data of the cattle and the occurrence of pneumonia and the frequencies of infection (absolute and relative) was determined using descriptive statistics. The determination of the comparative distribution of IHC antigens in normal and affected tissues was obtained due to the comparative frequency relative to each agent. Consequently, the frequency of each agent evaluated in a determined histologic element was obtained by calculating the occurrence of positive immunolabeling within the histologic element with the total IHC identification of each disease pathogen. Additionally, the

**TABLE 2 |** Frequency of pneumonia patterns identified in lungs of affected cattle.

| Patterns of pneumonia | # Number of cattle | % |
|---|---|---|
| Interstitial pneumonia | 72 | 49.7 |
| Suppurative bronchopneumonia | 31 | 21.4 |
| Caseonecrotic bronchopneumonia | 10 | 6.9 |
| Fibrinosuppurative bronchopneumonia | 5 | 3.4 |
| Cuffing pneumonia | 2 | 1.4 |
| Total | 120 | 100 |

**TABLE 1 |** Epidemiological data of pulmonary tissue of cattle submitted for histopathologic diagnosis.

| Variable | Pulmonary tissue without pneumonia (%) | Pulmonary tissue with pneumonia (%) | Chi-square test (*p*-value) |
|---|---|---|---|
| **Sex (*n*)** | | | |
| Male (19) | 6 | 13 | 0.406 |
| Female (54) | 13 | 41 | |
| Not reported (72) | 13 | 59 | |
| **Type of cattle (*n*)** | | | |
| Beef (49) | 10 | 39 | 0.366 |
| Dairy (34) | 5 | 29 | |
| Mixed (62) | 17 | 45 | |
| **Age (*n*)** | | | |
| Calves (51) | 12 | 39 | 0.097 |
| Adult (94) | 20 | 74 | |
| **Form of death (*n*)** | | | |
| Natural (108) | 27 | 81 | 0.146 |
| Slaughter (37) | 5 | 32 | |

**TABLE 3 |** Occurrence of respiratory pathogens, according to the pulmonary categories evaluated ($n = 145$)[a].

| Disease pathogens | Cellular/vascular alterations [n (%)] | Interstitial pneumonia [n (%)] | Bronchopneumonia [n (%)] | Total [n (%)] |
|---|---|---|---|---|
| Malignant catarrhal fever virus | 11 (7.6) | 34 (23.4) | 19 (13.1) | 64 (44.1) |
| *Mycoplasma bovis* | 13 (9) | 14 (9.7) | 30 (20.7) | 57 (39.3) |
| Bovine viral diarrhea virus | 8 (5.5) | 30 (20.7) | 13 (9) | 51 (35.2) |
| Bovine alphaherpesvirus-1 | 3 (2.1) | 21 (14.5) | 10 (6.9) | 34 (23.4) |
| Bovine respiratory syncytial virus | 6 (4.1) | 17 (11.7) | 6 (4.1) | 29 (20) |
| Bovine parainfluenza virus-3 | 4 (2.8) | 4 (2.8) | 2 (1.4) | 10 (6.9) |

[a] *Based on the occurrence of singular and simultaneous pathogens.*

association between the occurrence of pathogens and the number of cattle infected was determined. When appropriated, the association between the presence of pulmonary disease and specific variables was analyzed using the chi-square test, using the free software R 4.0.3 (47); differences were considered significant when the resulting $p$-value was $<0.05$.

# RESULTS

## Occurrence of Pneumonia and Biological Data

During the 4-year period, the lungs from 145 cattle submitted for histopathologic diagnosis were reviewed. Pneumonia was diagnosed in 82.7% (120/145) of these lungs, and 25 animals had no histopathologic lesions consistent with pneumonia. No significant differences ($p > 0.05$) were identified between the type of cattle, sex, and age relative to the occurrence of pneumonia (**Table 1**). The median age of the calves was 2 months (range: 2 days to 13 months), while that of adult animals was 4 years (range: 1 year and 8 months to 10 years).

## Patterns of Pulmonary Disease and Histologic Features of Pneumonia

Most of the lungs evaluated (82.7%, 120/145) had at least one patterns of pneumonia, and some contained more than one patterns of pulmonary disease (**Table 2**). Consequently, from the 120 animals with pneumonia evaluated, 139 patterns of pulmonary disease were observed, with animals presenting one (72.5%, 87/120) or several patterns (27.5%, 33/120) of pneumonia. Nevertheless, interstitial pneumonia (60%, 72/120) was the most predominant pattern observed (**Table 2**), followed by suppurative (25.8%, 31/120), caseonecrotic (8.3%, 10/120), and fibrinosuppurative bronchopneumonia (4.2%, 5/120). A few cows (1.7%, 2/120) had cuffing pneumonia.

Antigens of all agents investigated were identified in all three categories of pulmonary lesions evaluated (**Table 3**). However, antigens of MCFV were more frequently associated with all categories (53.3%, 64/120), including pulmonary tissues with interstitial pneumonia (28.3%, 34/120; **Table 3**) and vascular disease resulting in arterial proliferation (**Figures 1A–C**). Other agents frequently associated with the development of interstitial pneumonia were BVDV (25%, 30/120) and BoHV-1 (17.5%, 21/120; **Table 3**). As expected, antigens of *M. bovis* were more frequently (25%, 30/120) associated with the development of bronchopneumonia (**Table 3** and **Figure 1D**) and suppurative infiltrate predominantly in the terminal and respiratory bronchioles (**Figure 1E**) and frequently associated with obliterative bronchiolitis (**Figure 1F**). Intralesional, Giemsa-positive (**Figures 1G,H**), and bacterial accumulations were observed only in cases of bronchopneumonia (64.6%, 31/48; **Table 2**). Gram-positive or Gram-negative bacteria were not detected by the modified Brown–Brenn stain.

## IHC Identification of Infectious Disease Antigens in Cattle With BRD

The associations between intralesional antigens of infectious disease pathogens of BRD with the specific histologic element of the lung are summarized in **Figure 2**. When IHC results were associated with a single pathogen (**Table 4**), *M. bovis* (31.8%, 14/44) was the most frequently identified agent, followed by BVDV (22.7%, 10/44), MCFV (18.2%, 8/44), BRSV (13.6%, 6/44), BoHV-1 (11.4%, 5/44), and BPIV-3 (2.3%, 1/44).

Positive immunoreactivity to *M. bovis* antigens was widely distributed within the lung during this study (**Figure 2A**). These include the epithelial cells of the bronchiole (**Figures 3A,B**) and bronchus, alveolar macrophages, type I pneumocytes, mixed peribronchial glands (**Figure 3C**), chondrocytes of the bronchial hyaline cartilage (**Figure 3D**), and endothelium (**Figure 3E**). Furthermore, *M. bovis* was the only agent associated with positive immunolabeling on BALT lymphoid tissue (**Figures 3F,G**) and intralesional pleomorphic mollicutes (**Figure 3H**). Antigens of *M. bovis* were also identified within necrotic peribronchial glands (**Figure 3I**) and necrotic bronchial epithelial cells.

BVDV antigens were observed in the three categories studied (**Figure 2B**). Immunoreactivity was observed within the epithelial cells of the bronchus and bronchiole (**Figures 4A,B**), type I pneumocytes, alveolar lymphocytes and macrophages, chondrocytes of the bronchial hyaline cartilage (**Figure 4C**), mixed peribronchial glands (**Figures 4D,E**), endothelium cells (**Figure 4F**), and hyperplastic type II pneumocytes.

MCFV-positive intracytoplasmic immunoreactivity (**Figure 2C**) was identified primarily within bronchiolar epithelial cells (**Figures 5A,B**), alveolar macrophages (**Figure 5C**), type I pneumocytes, degenerated bronchial epithelial cells (**Figure 5D**), chondrocytes of the bronchial

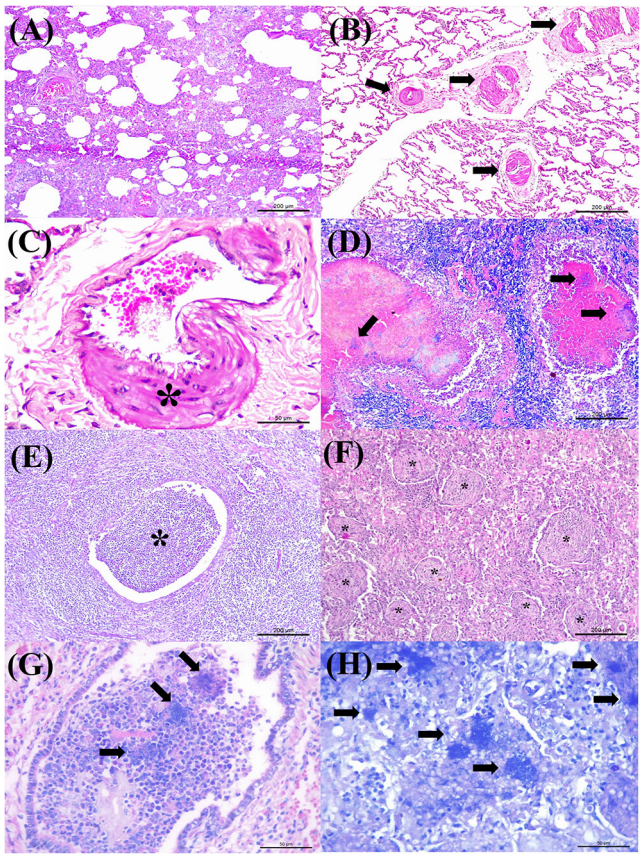



**FIGURE 1 |** Histopathological findings observed in the lungs of cattle with bovine respiratory disease associated with intralesional antigens of MCFV **(A–C)** and *M. bovis* **(D–H)**. **(A)** There is interstitial pneumonia, **(B)** containing areas of arterial proliferation (arrows); **(C)** higher magnification demonstrating proliferation of the tunica media of pulmonary arteries (asterisk). **(D)** Typical demonstration of caseonecrotic bronchopneumonia associated with infection by *M. bovis*; observe the well-demarcated, centrally located, foci of necrosis containing a large necrotic hyper-eosinophilic debris and pleomorphic bacteria (arrows). **(E)** There is a neutrophilic exudate within a terminal bronchiole (asterisk) in a cow with suppurative bronchopneumonia. **(F)** Observe areas of obliterative bronchiolitis (asterisk). **(G)** There are intralesional bacterial aggregates positive for *M. bovis* (arrows). **(H)** Closer view demonstrating intralesional mollicutes (arrows). Hematoxylin and eosin **(A–F)** and Giemsa stain **(G,H)**. Bar: **(A,B,D–F)** 200 μm; **(C,G,H)** 50 μm.

hyaline cartilage (**Figures 5E,F**), endothelial cells of pulmonary venule (**Figure 5G**), hyperplasic type II pneumocytes, mixed peribronchial glands (**Figure 5H**), and alveolar lymphocytes. Positive immunoreactivity to antigens of MCFV was restricted to the pulmonary lesions within the categories classified as cellular and vascular alterations and interstitial pneumonia, without being observed in tissues diagnosed as bronchopneumonia. Additionally, immunoreactivity to MCFV antigens was patchy within the pneumocytes of cows with interstitial pneumonia and was more predominant in type I relative to type II cells.

Immunoreactivity to BRSV (**Figure 2D**), with positive intracytoplasmic immunoreactivity, occurred within bronchial and bronchiolar epithelial cells (**Figures 6A,B**), chondrocytes of the bronchial hyaline cartilage (**Figure 6C**), mixed peribronchial glands, and alveolar macrophages (**Figure 6D**). Similar to MCFV, BRSV antigens were observed only in the categories classified as circulatory changes and interstitial pneumonia.

BoHV-1 antigens (**Figure 2E**) were observed only in cases of interstitial pneumonia and bronchopneumonia, with intracytoplasmic immunoreactivity within bronchial and bronchiolar epithelial cells, pulmonary endothelial cells (**Figures 6E,F**), chondrocytes of the bronchial hyaline cartilage (**Figure 6G**), necrotic bronchial epithelial cells, and type I pneumocytes.

BPIV-3 antigens were observed only in one animal with circulatory changes and revealed cytoplasmic immunoreactivity within the cells of the normal (**Figure 6H**) and degenerated (**Figure 6I**) bronchiolar epithelia.

**Figure 7** illustrates an interesting feature identified in mixed infections due to BVDV and MCFV in 12 animals. Concomitant infections were observed predominantly in bronchopneumonia (16.7%, 2/12) and interstitial pneumonia (75%, 9/12) and were also associated with the development of cellular and vascular alterations (8.3%, 1/12). More interestingly, with simultaneous immunolabeling in bronchopneumonia, antigens of both viruses were identified within bronchial and bronchiole epithelia (**Figures 8A,B**), necrotic bronchiolar cells (**Figures 8C,D**), and alveolar macrophages (**Figure 8E**). Additionally, there was positive immunoreactivity to MCFV antigens within goblet cells (**Figure 8F**), chondrocytes of the bronchial hyaline cartilage, pulmonary endothelia, mixed peribronchial glands, alveolar lymphocytic infiltrates, and degenerated bronchial epithelia (**Figures 8G,H**).

## Frequency and Relationship Between the Occurrence of Infectious Disease Pathogens and Pulmonary Disease

A high occurrence of respiratory disease pathogens was observed in the pulmonary tissues evaluated, with antigens of more than one respiratory disease pathogen detected in 82.7% (120/145) of these, while 36.7% (44/120) of the pulmonary tissues contained only one pathogen associated with the development of BRD. When the occurrence of single infections was evaluated (**Table 4**), *M. bovis* antigens were the most frequently identified (31.8%, 14/44), followed by BVDV (22.7%, 10/44), MCFV (18.2%, 8/44), BRSV (13.6%, 6/44), BoHV-1 (11.4%, 5/44), and BPIV-3 (2.3%, 1/44). However, antigens of infectious disease agents were not identified in 17.2% (25/145) of the pulmonary tissues evaluated. Antigens of MCFV and *M. bovis* (**Table 4**) were associated with singular (18.2%, 8/44; 22.7%, 10/44), dual (70%, 28/40; 45%, 18/40), triple (76%, 19/25; 68%, 17/25), quadruple (88.9%, 8/9; 66.7%, 6/9), and quintuple (100%, 2/2; 100%, 2/2) infections during this study.

Two respiratory disease agents were simultaneously identified within the lungs of 40 cows (**Table 4**), resulting in mixed

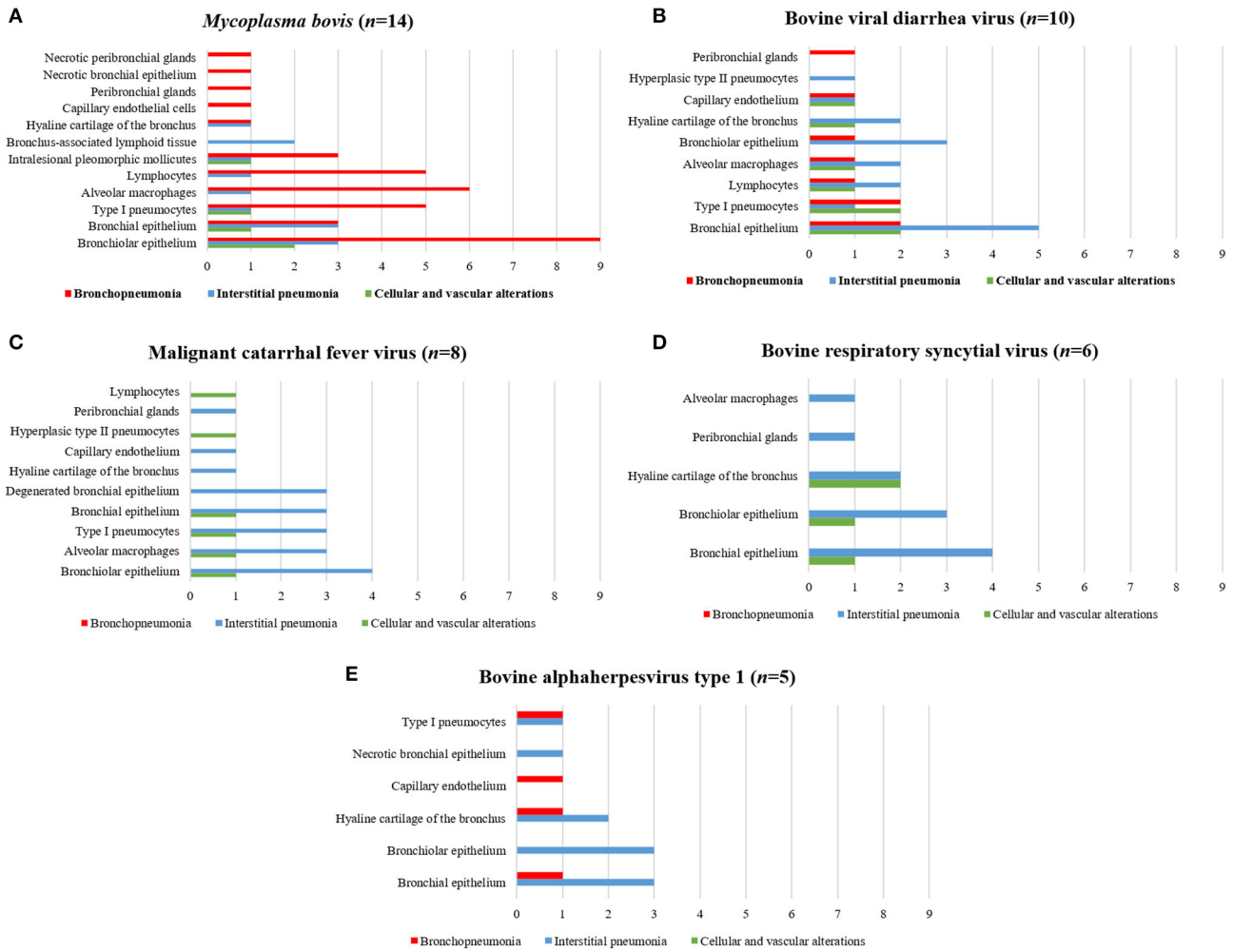



**FIGURE 2 |** Comparative distribution of the IHC identification of antigens of single infectious disease pathogens within the lungs of cattle with BRD due to **(A)** *M. bovis*, **(B)** BVDV, **(C)** MCFV, **(D)** BRSV, and **(E)** BoHV-1. Bar: green, cellular and vascular alterations; blue, interstitial pneumonia; and red, bronchopneumonia.

infections. Antigens of MCFV were the most frequently observed to be associated with BVDV (42.9%, 12/28), followed by *M. bovis* (35.7%, 10/28), BRSV (10.7%, 3/28), and BoHV-1 (10.7%, 3/28). Antigens of BVDV were identified in more than half (53.9%, 41/76; **Table 4**) of these cases, while mixed infections associated with BVDV predominantly included MCFV (40.8%, 31/76) and *M. bovis* (22.4%, 17/76). Additionally, dual infections were more frequently associated with intralesional antigens of MCFV ($n = 28$; **Table 4**), being predominantly associated with antigens of BVDV (42.8%, 12/28) and *M. bovis* (35.7%, 10/28).

In animals infected with a single agent, infection of normal bronchiolar epithelial cells was observed in all *M. bovis*-infected cows (100%, 14/14), followed by BRSV (83.3%, 5/6), MCFV (50%, 4/8), BVDV (40%, 4/10), and BoHV-1 (40%, 2/5). Few pulmonary tissues had positive immunoreactivity at the capillary endothelium, being observed for BVDV (30%, 3/10), BoHV-1 (20%, 1/5), MCFV (12.5%, 1/8), and *M. bovis* (7.1%, 1/14).

Positive immunolabeling at the hyaline cartilage of the bronchus was identified in the pulmonary tissues of 27.3% (12/44) cows; BRSV (66.7%, 4/6) antigens were more frequently identified within chondrocytes of the hyaline cartilage of the bronchus, followed by BoHV-1 (40%, 2/5), BVDV (30%, 3/10), *M. bovis* (14.3%, 2/14), and MCFV (12.5%, 1/8); antigens of BPIV-3 were not observed within chondrocytes. A few cattle had positive immunolabeling at normal peribronchial glands for antigens of BRSV (16.7%, 1/6), MCFV (12.5%, 1/8), and *M. bovis* (7.1%, 1/14). Positive immunoreactivity within lymphocytes was observed in 11 cows and was associated with antigens of *M. bovis* (54.5%, 6/11), BVDV (36.4%, 4/11), and MCFV (9.1%, 1/11); antigens of BRSV and BPIV-3 were not observed within lymphocytes. Intralesional pleomorphic organisms, stained by Giemsa, were identified in 35.7% (5/14; **Figure 3H**) of cows that contained antigens of *M. bovis*.

The most frequent infectious disease pathogen (**Table 3**) identified in association with BRD was MCFV (53.3%, 64/120), followed by *M. bovis* (47.5%, 57/120), BVDV (42.5%, 51/120),

**TABLE 4 |** Distribution of the IHC identification of intralesional antigens in the development of singular and concomitant infections in the lungs of cattle with pneumonia ($n = 120$).

| **Summary of singular ($n = 44$) and dual ($n = 40$) infections** | | | | | | |
|---|---|---|---|---|---|---|
| **Agents** | **MCFV** | ***M. bovis*** | **BVDV** | **BoHV-1** | **BRSV** | **BPIV-3** |
| MCFV | 8 | 10 | 12 | 3 | 3 | 0 |
| *M. bovis* | – | 14 | 2 | 2 | 2 | 2 |
| BVDV | – | – | 10 | 0 | 2 | 0 |
| BoHV-1 | – | – | – | 5 | 1 | 1 |
| BRSV | – | – | – | – | 6 | 0 |
| BPIV-3 | – | – | – | – | – | 1 |

| **Association of intralesional antigens** | **Number of cattle** |
|---|---|
| **Triple infections** | |
| BoHV-1, BPIV-3, BRSV | 1 |
| BoHV-1, BRSV, *M. bovis* | 2 |
| BoHV-1, BVDV, *M. bovis* | 3 |
| BoHV-1, BVDV, MCFV | 6 |
| BoHV-1, *M. bovis*, MCFV | 1 |
| BPIV-3, BRSV, *M. bovis* | 1 |
| BRSV, BVDV, MCFV | 1 |
| BRSV, *M. bovis*, MCFV | 3 |
| BVDV, *M. bovis*, MCFV | 7 |
| Total | 25 |
| **Quadruple infections** | |
| BoHV-1, BPIV-3, BVDV, *M. bovis* | 1 |
| BoHV-1, BRSV, BVDV, MCFV | 3 |
| BoHV-1, BRSV, *M. bovis,* MCFV | 1 |
| BoHV-1, BVDV, *M. bovis*, MCFV | 2 |
| BPIV-3, BRSV, *M. bovis*, MCFV | 1 |
| BRSV, BVDV, *M. bovis*, MCFV | 1 |
| Total | 9 |
| **Quintuple infections** | |
| BoHV-1, BPIV-3, BRSV, *M. bovis*, MCFV | 1 |
| BoHV-1, BPIV-3, BVDV, *M. bovis*, MCFV | 1 |
| Total | 2 |

*BoHV-1, bovine alphaherpesvirus type 1; BPIV-3, bovine parainfluenza virus type 3; BRSV, bovine respiratory syncytial virus; BVDV, bovine viral diarrhea virus; M. bovis, Mycoplasma bovis; MCFV, malignant catarrhal fever viruses.*

BoHV-1 (28.3%, 34/120), BRSV (24.2%, 29/120), and BPIV-3 (8.3%, 10/120). Furthermore, singular (36.7%, 44/120), dual (33.3%, 40/120), triple (20.8%, 25/120), quadruple (7.5%, 9/120), and quintuple (1.7%, 2/120) infections were identified (**Table 4**).

Necrosis was observed at the epithelial cells and peribronchial glands in the lungs of 6.8% (3/44) cows. Of these cases, necrosis affecting bronchial epithelia was associated with antigens of BoHV-1 (20%, 1/5) and *M. bovis* (7.1%, 1/14), with *M. bovis* antigens being observed in 7.1% (1/14) of the necrotic peribronchial glands.

Hyperplastic lesions were identified in few cows (9.1%, 4/44); in two of these, there was bronchus-associated lymphoid tissue (BALT) hyperplasia associated with positive immunoreactivity to *M. bovis* antigens. Hyperplasia of type II pneumonocytes was observed in two (4.5%, 2/44) cows; with positive immunoreactivity to MCFV (2.3%, 1/44) and BVDV (2.3%, 1/44).

## DISCUSSION

The results of this study demonstrated the multietiological nature of BRD, in which 63.3% (76/120) of the lungs of cattle evaluated were infected by two or more infectious disease agents. The IHC identification of infectious disease pathogens on FFPE tissues is a sensitive method to detect intralesional antigens and was previously used to identify BVDV, BoHV-1, BRSV, *M. bovis* (3, 16), BPIV-3 (16, 48), and MCFV (12) antigens in tissues of BRD-affected cattle. Additionally, the *in situ* detection of intralesional tissue antigens is an excellent method for retrospective studies using archival samples. Moreover, this diagnostic method is preferred over

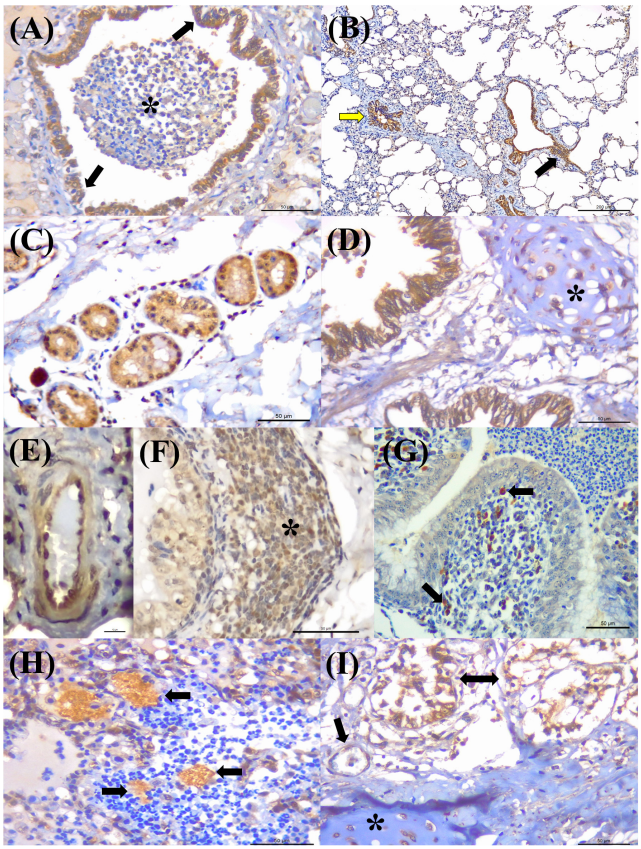



**FIGURE 3 |** Immunohistochemical detection *Mycoplasma bovis* antigens in cattle with bovine respiratory disease. **(A)** There is positive intracytoplasmic immunoreactivity to *M. bovis* antigens in the normal bronchiolar epithelium (arrows) of a cow with bronchopneumonia (asterisk). **(B)** Observe positive immunoreactivity at the respiratory (yellow arrow) and terminal bronchiole (black arrow) of a cow with interstitial pneumonia. **(C)** There is positive reactivity at the mixed peribronchial glands. **(D)** Observe positive immunoreactivity within chondrocytes of the hyaline cartilage (asterisk), **(E)** endothelium cells, **(F)** lymphocytes (asterisk), **(G)** macrophages, (arrow), and bronchus-associated lymphoid tissue. **(H)** There is positive immunolabeling within accumulations of pleomorphic organism (arrows) in the lung with suppurative bronchopneumonia. **(I)** Observe positive immunolabeling in necrotic peribronchial glands (two-headed arrow), normal endothelium cells (arrow), and chondrocytes of the hyaline cartilage (asterisk). Immunoperoxidase counterstained with hematoxylin. Bar: **(A,C,D,F–I)** 50 μm; **(B)** 200 μm; **(E)** 10 μm.

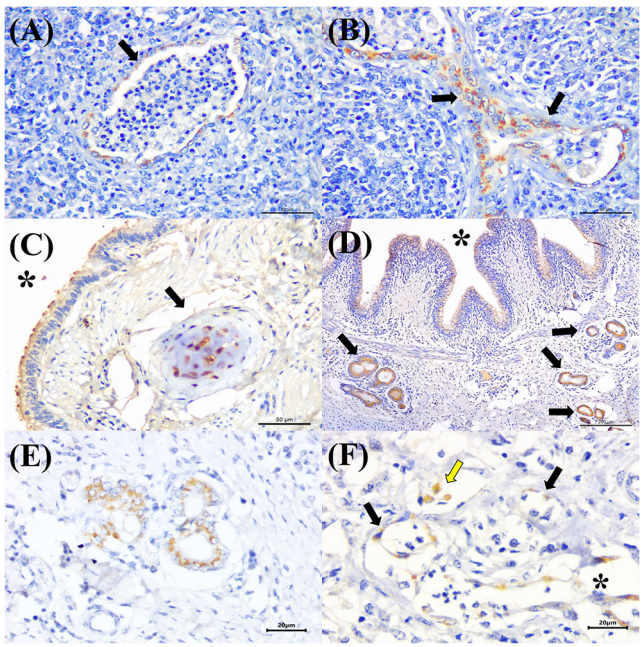



**FIGURE 4 |** Immunohistochemical detection of BVDV antigens in cattle with bovine respiratory disease. There is positive immunoreactivity (arrows) to BVDV in the epithelial cells of the **(A)** terminal and **(B)** respiratory bronchiole of a cow with bronchopneumonia. **(C)** Observe positive immunolabeling at chondrocytes of the hyaline cartilage (asterisk), epithelial cells of the bronchus (arrow), **(D)** mixed peribronchial glands (arrows), and bronchial epithelial cells (asterisk); **(E)** closer view showing positive immunoreactivity within epithelial cells of the mixed peribronchial glands. **(F)** BVDV antigens within alveolar macrophages (yellow arrow), the endothelia of a capillary (black arrows), and venule (asterisk) of the lung. Immunoperoxidase counterstained with hematoxylin. Bar: **(A–C)** 50 μm; **(D)** 200 μm; **(E,F)** 20 μm.

molecular testing, to confirm disease association, since the intralesional identification of disease pathogens within affected tissue clearly demonstrates the association between infectious disease agents and histologic alteration and/or pattern of disease (49, 50).

In a previous study by our group in cattle with neurological manifestation of MCF associated with OvHV-2 but without the classic manifestations of MCF (12), positive immunoreactivity was not observed within the pulmonary tissues available for evaluation. Similarly, in the current study, the cows investigated did not demonstrate the typical clinical manifestations of MCF and were therefore without a clinical diagnosis of MCF. The pulmonary disease associated with MCFV antigens identified during this study can be classified as subacute to chronic interstitial pneumonia due to the accumulated lymphocytes and macrophages (51). Moreover, OvHV-2 is known to produce chronic disease in cattle characterized by proliferating arterial lesions (12, 52, 53); proliferative vascular lesions were observed in the lungs of cattle with interstitial pneumonia associated with intralesional antigens of MCFV and represented 28.3% (34/120) of all interstitial pneumonias identified during this study. These findings may suggest that MCFV produces interstitial pneumonia with vascular proliferating lesions as the prominent histologic feature, which may be useful to distinguish MCFV-induced interstitial pneumonia from other viral pneumonias of cattle. Another interesting feature during this study was the patchy immunoreactivity of pneumocytes in interstitial pneumonia associated with MCFV antigens; similar findings were described in pigs experimentally infected with OvHV-2 (54). Additionally, experimental studies done in sheep (55, 56) and pigs (54) have suggested that the interstitial pneumonia induced by OvHV-2 results in lytic replication predominantly within type II pneumocytes (55). In the present study, intralesional MCFV antigens were observed

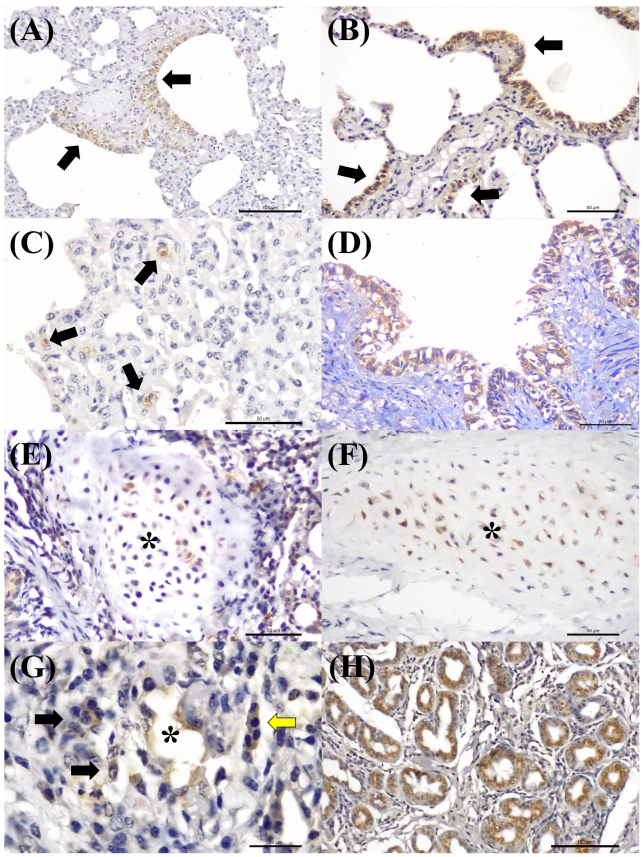



**FIGURE 5 |** Immunohistochemical demonstration of immunoreactivity to MCFV in cattle with bovine respiratory disease. There is positive intracytoplasmic immunoreactivity to antigens of MCFV within the cytoplasm of epithelial cells of the **(A,B)** terminal bronchiole and the patchy immunoreactivity within **(C)** alveolar epithelium (arrows) in a case of interstitial pneumonia. Observe intracytoplasmic immunoreactivity to antigens of MCFV within **(D)** degenerated bronchial epithelial cells, **(E,F)** chondrocytes of the hyaline cartilage (asterisk), **(G)** endothelium cells of a pulmonary venule (asterisk), macrophages (black arrows), lymphocytes (yellow arrow), and **(H)** epithelial cells of the mixed peribronchial glands. Immunoperoxidase counterstained with hematoxylin. Bar: **(A,H)** 100 μm; **(B–F)** 50 μm; and **(G)** 20 μm.

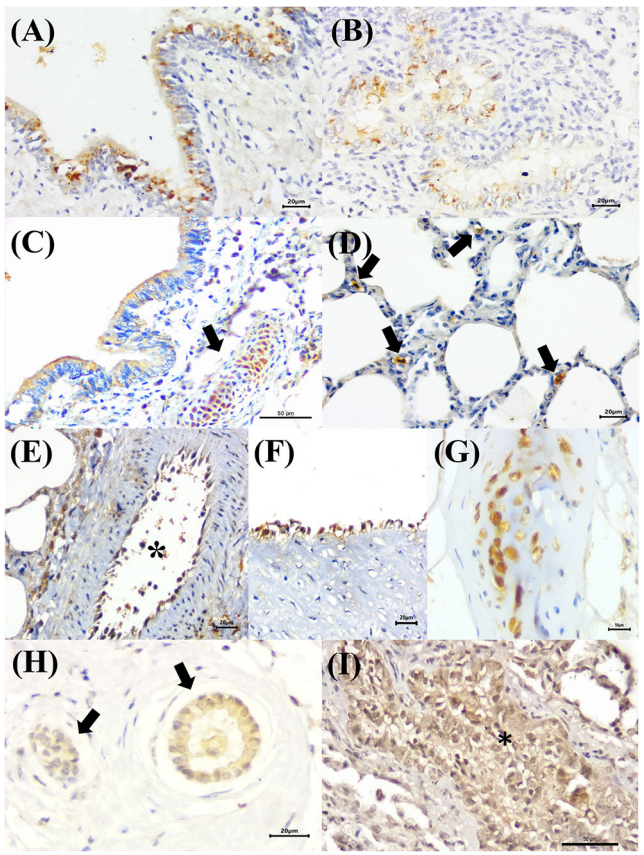



**FIGURE 6 |** Immunohistochemical identification of BRSV, BoHV-1, and BPIV-3 antigens in singular infections of cattle with bovine respiratory disease. **(A)** There is positive intracytoplasmic immunoreactivity to BRSV antigens within bronchial and **(B)** bronchiolar epithelial cells, **(C)** chondrocytes of the hyaline cartilage (arrow), and **(D)** alveolar macrophages (arrows). **(E,F)** Observe positive intracytoplasmic immunolabeling for BoHV-1 antigens within endothelial cells [**(E)**, asterisk] and **(G)** chondrocytes of the hyaline cartilage. **(H)** There is positive intracytoplasmic immunoreactivity to BPIV-3 antigens within epithelial cells of the normal (arrows) and **(I)** vacuolized bronchiolar cells (asterisk). Immunoperoxidase counterstained with hematoxylin. Bar: **(A,B,D–F,H)** 20 μm; **(C,G,I)** 50 μm.

within hyperplastic type II pneumocytes and degenerated and necrotic bronchiolar and bronchial epithelia; these findings herein described corroborate those of experimental studies (54, 56). Consequently, this pathogen may be an innocent bystander, or a primary infectious disease agent, acting individually or in association with other pathogens toward the development of BRD in cattle, and should be considered a possible infectious agent associated with the development of BRD. Moreover, an MCFV, more likely OvHV-2, was related with the occurrence of respiratory disease in a calf that was simultaneously infected with BVDV and *Aspergillus fumigatus* (57).

Furthermore, the MAb-15A antibody that detects epitopes of MCF viruses (58) did not react with common viruses of sheep, goat, and cattle (58, 59) and was shown to effectively identify intralesional antigens of OvHV-2 in FFPE of cattle with MCF (12). The IHC findings observed in this study demonstrated the participation of MCFV in the development of respiratory disease in the cows evaluated. Furthermore, positive immunoreactivity for MCFV was observed within epithelial cells of the lungs of cattle with different categories of pulmonary disease, but principally in cases of interstitial pneumonia. In Brazil, only OvHV-2 is known to be associated with the development of MCF in ruminants (10, 12), suggesting that the MCFV identified in these animals was most likely OvHV-2; similar findings were recently described (57, 60). Previous investigations have shown that OvHV-2 induces infiltrative, degenerative (60), and necrotic changes in the urinary bladder (60, 61), kidney cells (60), salivary gland (61), and the gastrointestinal and respiratory systems of cattle (57, 61). Furthermore, a review of all published cases of MCF in Brazil revealed that interstitial pneumonia is a frequently

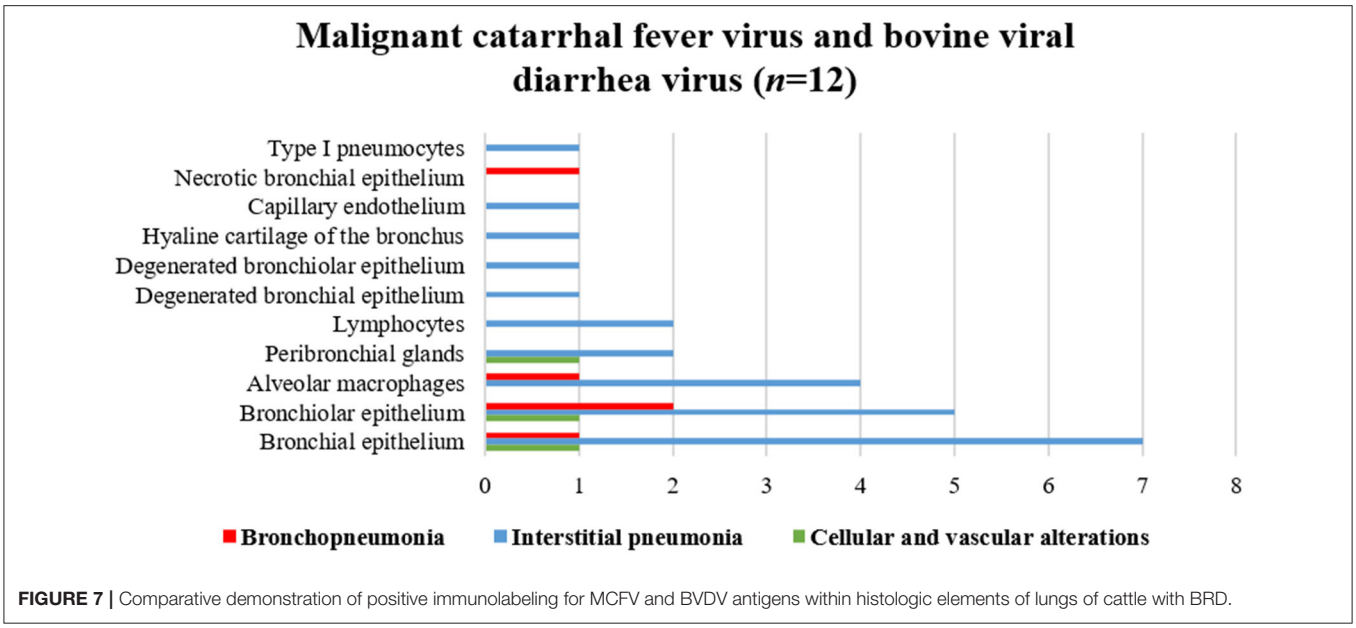



**FIGURE 7 |** Comparative demonstration of positive immunolabeling for MCFV and BVDV antigens within histologic elements of lungs of cattle with BRD.

diagnosed histologic alteration described in cattle infected with OvHV-2 (10).

Only *M. bovis* antigens were observed in the lungs of cattle with BALT hyperplasia, suggesting that this lesion can be used as an indicator of *M. bovis*-induced pneumonia in cattle as previously described (62, 63). It should be emphasized that in most cases of BALT hyperplasia, the pulmonary tissue evaluated had more than one histologic pattern of *M. bovis*-induced pulmonary disease, while only in two animals was cuffing pneumonia the only histologic alteration observed. Other histologic patterns of *M. bovis*-induced pneumonia identified were caseonecrotic and suppurative bronchopneumonia; similar results have been described (21, 62, 63). The caseonecrotic bronchopneumonia (also referred to as necrosuppurative bronchopneumonia) is considered the most important diagnostic histologic feature to differentiate *M. bovis*-induced pneumonia from other bacterial pneumonias of cattle (21, 62).

A limitation of this study was the absence of the IHC analyses to identify members of the *Pasteurellaceae* family associated with the development of BRD. Although *P. multocida*, *M. haemolytica*, and *H. somni* are the most common bacterial pathogens associated with bovine pneumonia (6, 18, 51, 64), these pathogens were not evaluated during this study due to the lack commercial antibodies for IHC. Additionally, attempts to obtain in-house polyclonal antibodies or hyperimmune serum against these bacterial agents were frustrating. Nevertheless, these organisms are commensals of the bovine nasopharynx which, during periods of stress or viral infection, can overwhelm host defense mechanisms establishing infection in the lower respiratory tract and are associated with the development of fibrinosuppurative or suppurative bronchopneumonia, pulmonary abscesses, and necrosis in cattle (6, 49, 51, 64). Consequently, the BALT hyperplasia and caseonecrotic bronchopneumonia associated with intralesional antigens of *M. bovis* can be considered as histologic patterns specific for pulmonary disease by this pathogen as opposed to histologic patterns of pulmonary disease related to *P. multocida*, *M. haemolytica*, and *H. somni*. We had previously postulated that Giemsa staining may be a cheap and adequate method to identify intralesional *Mycoplasma* organisms (16). Similar results were identified in this study, suggesting that this simple histochemical stain may be used to confirm the presence of this organism, principally in cases with the classical histologic presentation of mycoplasma pneumonia.

In this study, 63.3% (76/120) of the pulmonary infections observed were mixed; tissue antigens of MCFV and *M. bovis* were observed simultaneously in two to five infections within the same pulmonary section. These findings suggest that these two organisms can produce pneumonia acting individually or in association with other pathogens of BRD. BVDV is a well-known immunosuppressive agent of cattle (65–72), which could have favored concomitant infections, including MCFV and *M. bovis*. We have previously discussed the relationship between BVDV and *M. bovis* and the synergism between these two organisms (16). In that study, there were four singular infections (11.4%, 4/35) associated with *M. bovis* (16); in the current study, 11.7% (14/120) of the affected cows were infected with *M. bovis*. These results corroborate previous studies that have identified *M. bovis*, in single infections, as a primary contributor toward the development of BRD (3, 20, 73). However, the same does not hold for MCFV or OvHV-2 as described above, since there are few reports associating these pathogens with BRD.

This study demonstrated high frequencies of infections by MCFV (53.3%, 64/120) and *M. bovis* (47.5%, 57/120) in

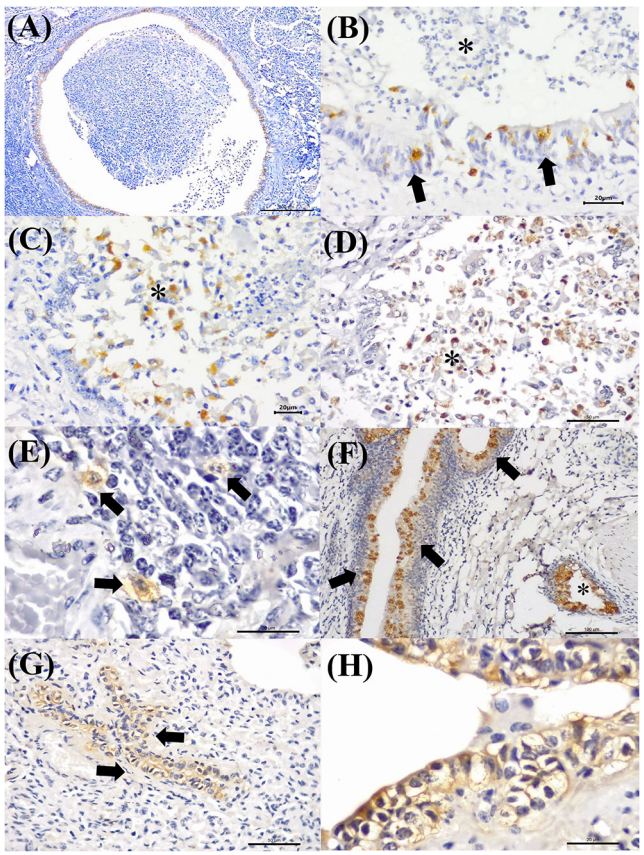



**FIGURE 8 |** Immunohistochemical identification of BVDV and MCFV antigens in cattle with bronchopneumonia with BRD. **(A)** There is positive intracytoplasmic immunoreactivity to BVDV in the bronchial cells of an ectatic bronchiole due to bronchiectasis, **(B)** closer view of epithelial cells (arrows) and negative immunoreactivity in neutrophils (asterisk). **(C,D)** Immunoreactivity to MCFV antigens within necrotic bronchiolar epithelial cells (asterisk), **(E)** alveolar macrophages (arrows), **(F)** goblet cells (arrows), normal cells (asterisk), and **(G)** degenerated bronchiolar epithelial cells (arrows); **(H)** closer view of the degenerated bronchiolar epithelial cells. Immunoperoxidase counterstained with hematoxylin. Bar: **(A)** 200 μm; **(B,C,E,H)** 20 μm; **(D,G)** 50 μm; **(F)** 100 μm.

cattle from geographical locations of Brazil. These elevated occurrences can be related to several conditions, including the absence of a vaccine or specific treatment to effectively control MCFV and the frequently chronic presentation of *M. bovis*-induced pneumonia (21), which results in a late diagnosis. Consequently, farmers must be educated relative to the existence of these diseases, especially concerning the adoption of adequate control and prophylactic measures (74). Another factor that may have contributed to the elevated occurrence of *M. bovis* during this study is the well-established microbial resistance of this organism to common antibiotic therapy (21, 75).

## CONCLUSION

These results suggest that most cattle evaluated presented some form of pulmonary lesions associated with BRD. The occurrence of interstitial pneumonia was most frequently related to antigens of MCFV and BVDV, while *M. bovis* was frequently associated with caseonecrotic bronchopneumonia. These findings suggest that an MCFV, most likely involving OvHV-2, was associated with the development of pulmonary disease in cattle and should be considered as a primary disease pathogen of BRD, acting innocently, singularly, or in association, primarily with BVDV. Moreover, the concomitant occurrence of MCFV and BVDV within the lungs of cattle with pneumonia suggests a possible synergism between these two infectious agents toward the development of BRD. Furthermore, proliferating vascular lesions in the lung may be an important histologic feature to diagnose MCFV-induced interstitial pneumonia in cattle.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICAL STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Universidade Estadual de Londrina (CEUA/UEL; protocol, 835.2019.45). Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

TO contributed substantially to the conception and design of the study, drafted the manuscript, and contributed to the analysis and interpretation of all pathological, IHC, and statistical data. TO, GS, IP, and SH contributed to all histopathological evaluations, histochemical and IHC stains, interpretation of the IHC, and analyses. CC, LP-G, and EF contributed to the interpretation of the IHC analyses. TO, LC, JL, and JS contributed to the interpretation of the statistical analysis. JL, GS, PJ, and AA contributed toward the supply of pulmonary tissues for this study. SH coordinated and supervised the execution of the study. All authors have read, critically analyzed, and approved the final draft of this manuscript and have agreed to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## FUNDING

This work was supported by National Institutes of Science and Technology—Dairy Production Chain (INCT Leite, Brazil); National Council of Scientific and Technological Development (CNPq, Brazil); Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES, Brazil).

## ACKNOWLEDGMENTS

This study forms part of a material transfer agreement between DMVP/UEL and ARS/USA (58-2090-8-068-F). The authors thank the National Institutes of Science and Technology—Dairy Production Chain (INCT-Leite, Brazil)—and the National Council of Scientific and Technological Development (CNPq, Brazil) for financial support. TO, GS, EF, JL, AA, JS, PJ, and SH are recipients of CNPq Fellowships. Part of the PhD thesis of the first author (TO).

## REFERENCES

1. Fulton RW. Viral diseases of the bovine respiratory tract. In:.Anderson DE, Rings DM, editors. *Food Animal Practice,* Saint Louis: W.B. Saunders (2009), p. 171–91. doi: 10.1016/B978-141603591-6.10042-9
2. Headley SA, Okano W, Balbo LC, Marcasso RA, Oliveira TE, Alfieri AF, et al. Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil. *J Vet Diagn Invest*. (2018) 30:249–51. doi: 10.1177/1040638717739945
3. Gershwin LJ, Van Eenennaam AL, Anderson ML, McEligot HA, Shao MX, Toaff-Rosenstein R, et al. Single pathogen challenge with agents of the bovine respiratory disease complex. *PloS ONE*. (2015) 10:e0142479. doi: 10.1371/journal.pone.0142479
4. O'Neill R, Mooney J, Connaghan E, Furphy C, Graham DA. Patterns of detection of respiratory viruses in nasal swabs from calves in Ireland: a retrospective study. *Vet Rec.* (2014) 175:351. doi: 10.1136/vr.102574
5. Fulton RW. Bovine respiratory disease research (1983-2009). *Anim Health Res Rev.* (2009) 10:131–9. doi: 10.1017/S146625230999017X
6. Panciera RJ, Confer AW. Pathogenesis and pathology of bovine pneumonia. *Vet Clin North Am Food Anim Pract.* (2010) 26:191–214. doi: 10.1016/j.cvfa.2010.04.001
7. Grissett GP, White BJ, Larson RL. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. *J Vet Intern Med.* (2015) 29:770–80. doi: 10.1111/jvim.12597
8. Klima CL, Holman DB, Ralston BJ, Stanford K, Zaheer R, Alexander TW, et al. Lower respiratory tract microbiome and resistome of bovine respiratory disease mortalities. *Microb Ecol.* (2019) 78:446–56. doi: 10.1007/s00248-019-01361-3
9. Castro MM, Oliveira TES, Headley SA. Bovine respiratory disease in Brasil: a short review. *Semin Cienc Agrar.* (2021) 42:2081–110. doi: 10.5433/1679-0359.2021v42n3Supl1p2081
10. Headley SA, Oliveira TES, Cunha CW. A review of the epidemiological, clinical, and pathological aspects of sheep-associated malignant catarrhal fever with emphasis on Brazil. *Braz J Microbiol.* (2020) 51:1405–32. doi: 10.1007/s42770-020-00273-6
11. International Committee on Taxonomy of Viruses (2021). Virus Taxonomy: 2019 Release. Genus: *Macavirus*. Subfamily: *Gammaherpesvirinae*. Available online at: https://talk.ictvonline.org/taxonomy/ (acessed March 4, 2021).
12. Headley SA, Oliveira TES, Li H, Lisbôa JAN, Queiroz GR, Fritzen JTT, et al. Immunohistochemical detection of intralesional antigens of Ovine Gamaherpesvirus-2 in cattle with Sheep-Associated Malignant Catarrhal Fever. *J Comp Pathol*. (2020) 174:86–98. doi: 10.1016/j.jcpa.2019.11.002
13. O'Toole D, Li H. The pathology of malignant catarrhal fever, with an emphasis on ovine herpesvirus 2. *Vet Pathol*. (2014) 51:437–52. doi: 10.1177/0300985813520435
14. O'Toole D, Li H, Sourk C, Montgomery DL, Crawford TB. Malignant catarrhal fever in a bison (*Bison bison*) feedlot, 1993–2000. *J Vet Diagn Invest*. (2002) 14:183–93. doi: 10.1177/104063870201400301
15. Costa ÉA, Bastianetto E, Vasconcelos AC, Bomfim MRQ, Fonseca, F.G.d., et al. An outbreak of malignant catarrhal fever in Murrah buffaloes in Minas Gerais, Brazil. *Pesqui Vet Bras.* (2009) 29:395–400. doi: 10.1590/S0100-736X2009000500006
16. Oliveira TES, Pelaquim IF, Flores EF, Massi RP, Valdiviezo MJJ, Pretto-Giordano LG, et al. *Mycoplasma bovis* and viral agents associated with the development of bovine respiratory disease in adult dairy cows. *Transbound Emerg Dis.* (2020) 67:82–93. doi: 10.1111/tbed.13223
17. Zhang M, Hill JE, Godson DL, Ngeleka M, Fernando C, Huang Y. The pulmonary virome, bacteriological and histopathological findings in bovine respiratory disease from western Canada. *Transbound Emerg Dis.* (2020) 67:924–34. doi: 10.1111/tbed.13419
18. Griffin D, Chengappa MM, Kuszak J, McVey DS. Bacterial pathogens of the bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract.* (2010) 26:381–94. doi: 10.1016/j.cvfa.2010.04.004
19. Szeredi L, Janosi S, Palfi V. Microbiological and pathological examination of fatal calf pneumonia cases induced by bacterial and viral respiratory pathogens. *Acta Vet Hung.* (2010) 58:341–56. doi: 10.1556/avet.58.2010.3.7
20. Arcangioli M-A, Duet A, Meyer G, Dernburg A, Bézille P, et al. The role of *Mycoplasma bovis* in bovine respiratory disease outbreaks in veal calf feedlots. *Vet J.* (2008) 177:89–93. doi: 10.1016/j.tvjl.2007.03.008
21. Caswell JL, Bateman KG, Cai HY, Castillo-Alcala F. *Mycoplasma bovis* in respiratory disease of feedlot cattle. *Vet Clin North Am Food Anim Pract.* (2010) 26:365–79. doi: 10.1016/j.cvfa.2010.03.003
22. Nicholas RA, Ayling RD. *Mycoplasma bovis*: disease, diagnosis, and control. *Res Vet Sci.* (2003) 74:105–12. doi: 10.1016/S0034-5288(02)00155-8
23. Margineda CA, Zielinski GO, Jurado S, Alejandra F, Mozgovoj M, Alcaraz AC, et al. *Mycoplasma bovis* pneumonia in feedlot cattle and dairy calves in Argentina. *Braz J Vet Pathol.* (2017) 10:79–86. doi: 10.24070/bjvp.1983-0246.v10i2p79-86
24. Rodriguez F, Bryson DG, Ball HJ, Forster F. Pathological and immunohistochemical studies of natural and experimental *Mycoplasma bovis* pneumonia in calves. *J Comp Pathol.* (1996) 115:151–62. doi: 10.1016/S0021-9975(96)80037-5
25. Francoz D, Buczinski S, Belanger AM, Forte G, Labrecque O, Tremblay D, et al. Respiratory pathogens in Quebec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. *J Vet Intern Med.* (2015) 29:381–7. doi: 10.1111/jvim.12531
26. Gagea MI, Bateman KG, van Dreumel T, McEwen BJ, Carman S, Archambault M, et al. Diseases and pathogens associated with mortality in Ontario beef feedlots. *J Vet Diagn Invest.* (2006) 18:18–28. doi: 10.1177/104063870601800104
27. Klima CL, Zaheer R, Cook SR, Booker CW, Hendrick S, Alexander TW, et al. Pathogens of bovine respiratory disease in North American feedlots conferring multidrug resistance via integrative conjugative elements. *J Clin Microbiol.* (2014) 52:438–48. doi: 10.1128/JCM.02485-13
28. Taylor JD, Fulton RW, Lehenbauer TW, Step DL, Confer AW. The epidemiology of bovine respiratory disease: what is the evidence for predisposing factors? *Can Vet J.* (2010) 51:1095–102.
29. Cusack P, McMeniman N, Lean I. The medicine and epidemiology of bovine respiratory disease in feedlots. *Aust Vet J.* (2003) 81:480–87. doi: 10.1111/j.1751-0813.2003.tb13367.x
30. Hay KE, Morton JM, Mahony TJ, Clements ACA, Barnes TS. Associations between animal characteristic and environmental risk factors and bovine respiratory disease in Australian feedlot cattle. *Prev Vet Med.* (2016) 125:66–74. doi: 10.1016/j.prevetmed.2016.01.013
31. Beuttemmuller EA, Alfieri AF, Headley SA, Alfieri AA. Brazilian strain of bovine respiratory coronavirus is derived from dual enteric and respiratory tropism. *Genet Mol Res.* (2017) 16:gmr16029580. doi: 10.4238/gmr16029580
32. Brasil NDA, Hinnah FL, Fiss L, Sallis ESV, Grecco FB, Ladeira SRL, et al. Doenças respiratórias em bezerros na região sul do Rio Grande do Sul: estudo retrospectivo de 33 surtos. *Pesqui Vet Bras.* (2013) 33:745–51. doi: 10.1590/S0100-736X2013000600010
33. Driemeier D, Gomes MJP, Moojen V, Arns CW, Vogg G, Kessler L, et al. Manifestação clínico-patológica de infecção natural pelo vírus respiratório sincicial bovino (BRSV) em bovinos de criação extensiva no Rio Grande do Sul, Brasil. *Pesqui Vet Bras.* (1997) 17:77–81. doi: 10.1590/S0100-736X1997000200006

34. Flores EF, Weiblen R, Medeiros M, Botton SA, Irigoyen LF, Driemeier D, et al. A retrospective search for bovine respiratory syncytial virus (BRSV) antigens in histological specimens by immunofluorescence and immunohistochemistry. *Pesqui Vet Bras.* (2000) 20:139–43. doi: 10.1590/S0100-736X2000000400002
35. Peixoto PV, Mota RA, Brito MF, Corbellini LG, Driemeier D, Souza MI. Infecção natural pelo vírus sincicial respiratório bovino (BRSV) no estado de Alagoas. *Pesqui Vet Bras.* (2000) 20:171–75. doi: 10.1590/S0100-736X2000000400008
36. Pretto LG, Müller EE, Freitas JC, Mettifoga E, Buzinhani M, Yamaguti M. Isolamento de *Mycoplasma bovis* em bezerros com pneumonia. *Vet Not.* (2001) 7:69–73.
37. Silva MS, Brum MCS, Weiblen R, Flores EF. Identificação e diferenciação de herpesvírus bovino tipos 1 e 5 isolados de amostras clínicas no Centro-Sul do Brasil, Argentina e Uruguai (1987-2006). *Pesqui Vet Bras.* (2007) 27:403–8. doi: 10.1590/S0100-736X2007001000003
38. Baptista AL, Rezende AL, Fonseca PA, Nogueira GM, Headley SA, et al. Bovine respiratory disease complex associated mortality and morbidity rates in feedlot cattle from southeastern Brazil. *J Infect Dev Ctries.* (2017) 11:791–99. doi: 10.3855/jidc.9296
39. Gaeta NC, Ribeiro BLM, Alemán MAR, Yoshihara E, Marques EC, Hellmeister AN, et al. Serological investigation of antibodies against respiratory viruses in calves from Brazilian family farming and their relation to clinical signs of bovine respiratory disease. *Pesqui Vet Bras.* (2018) 38:642–48. doi: 10.1590/1678-5150-pvb-5234
40. Headley SA, Balbo LC, Alfieri AF, Saut JPE, Baptista AL, Alfieri AA. Bovine respiratory disease associated with *Histophilus somni* and bovine respiratory syncytial virus in a beef cattle feedlot from Southeastern Brazil. *Semin Cienc Agrar.* (2017) 38:283–94. doi: 10.5433/1679-0359.2017v38n1p283
41. Magalhães LQ, Baptista AL, Fonseca PA, Menezes GL, Nogueira GM, et al. Use of metaphylactic protocols based on the risk to develop bovine respiratory diseases in feedlot cattle. *Cienc Rural.* (2017) 47:e20161110. doi: 10.1590/0103-8478cr20161110
42. Oliveira VHS, Dall Agnol AM, Fritzen JTT, Lorenzetti E, Alfieri AA, Alfieri AF. Microbial diversity involved in the etiology of a bovine respiratory disease outbreak in a dairy calf rearing unit. *Comp Immunol Microbiol Infect Dis.* (2020) 71:101494. doi: 10.1016/j.cimid.2020.101494
43. Tortorelli G, Carrillo Gaeta N, Mendonça Ribeiro BL, Miranda Marques L, Timenetsky J, Gregory L. Evaluation of *Mollicutes* microorganisms in respiratory disease of cattle and their relationship to clinical signs. *J Vet Intern Med.* (2017) 31:1215–20. doi: 10.1111/jvim.14721
44. Queiroz GR, Oliveira RAM, Flaiban KKMC, Santis GWD, Headley SA. Diagnóstico diferencial das doenças neurológicas dos bovinos no estado do Paraná. *Pesqui Vet Bras.* (2018) 38:1264–77. doi: 10.1590/1678-5150-pvb-5429
45. Vaz RZ, Lobato JFP, Pascoal LL. Desenvolvimento de bezerros de corte desmamados aos 80 ou 152 dias até os 15-16 meses de idade. *R Bras Zootec.* (2011) 40:221–29. doi: 10.1590/S1516-35982011000100030
46. Luna LG. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. New York: McGraw-Hill (1968).
47. R Core Team. *R: A Language and Environment for Statistical Computing.* (2021). R Foundation for Statistical Computing, Vienna, Austria. Available online at: https://www.R-project.org/ (accessed January 9, 2021).
48. Haines DM, Kendall JC, Remenda BW, Breker-Klassen MM, Clark EG. Monoclonal and polyclonal antibodies for immunohistochemical detection of bovine parainfluenza type 3 virus in frozen and formalin-fixed paraffin-embedded tissues. *J Vet Diagn Invest.* (1992) 4:393–9. doi: 10.1177/104063879200400404
49. Fulton RW, Blood KS, Panciera RJ, Payton ME, Ridpath JF, Confer AW, et al. Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. *J Vet Diagn Invest.* (2009) 21:464–77. doi: 10.1177/104063870902100407
50. Maes RK, Langohr IM, Wise AG, Smedley RC, Thaiwong T, Kiupel M. Beyond H&E: integration of nucleic acid-based analyses into diagnostic pathology. *Vet Pathol.* (2014) 51:238–56. doi: 10.1177/0300985813505878
51. López A, Martinson SA. Respiratory system, mediastinum, pleurae. In: Zachary JF, editor. *Pathologic Basis of Veterinary Disease*, St. Louis, Missouri: Elsevier (2017), p. 471–560. doi: 10.1016/B978-0-323-35775-3.00009-6
52. O'Toole D, Li H, Miller D, Williams WR, Crawford TB. Chronic and recovered cases of sheep-associated malignant catarrhal fever in cattle. *Vet Rec.* (1997) 140:519–24. doi: 10.1136/vr.140.20.519
53. O'Toole D, Li H, Roberts S, Rovnak J, DeMartini J, Cavender J, et al. Chronic generalized obliterative arteriopathy in cattle: a sequel to sheep-associated malignant catarrhal fever. *J Vet Diagn Invest.* (1995) 7:108–21. doi: 10.1177/104063879500700118
54. Li H, Brooking A, Cunha CW, Highland MA, O'Toole D, Knowles DP, et al. Experimental induction of malignant catarrhal fever in pigs with ovine herpesvirus 2 by intranasal nebulization. *Vet Microbiol.* (2012) 159:485–89. doi: 10.1016/j.vetmic.2012.04.016
55. Taus NS, Schneider DA, Oaks JL, Yan H, Gailbreath KL, Knowles DP, et al. Sheep (*Ovis aries*) airway epithelial cells support ovine herpesvirus 2 lytic replication *in vivo*. *Vet Microbiol.* (2010) 145:47–53. doi: 10.1016/j.vetmic.2010.03.013
56. Taus NS, Traul DL, Oaks JL, Crawford TB, Lewis GS, Li H. Experimental infection of sheep with ovine herpesvirus 2 via aerosolization of nasal secretions. *J Gen Virol.* (2005) 86:575–9. doi: 10.1099/vir.0.80707-0
57. Headley SA, Müller MC, Oliveira TES, Duarte CABG, Pereira PFV, et al. Diphtheric aspergillosis tracheitis with gastrointestinal dissemination secondary to viral infections in a dairy calf. *Microb Pathog.* (2020) 149:104497. doi: 10.1016/j.micpath.2020.104497
58. Li H, Shen DT, Knowles DP, Gorham JR, Crawford TB. Competitive inhibition enzyme-linked immunosorbent assay for antibody in sheep and other ruminants to a conserved epitope of malignant catarrhal fever virus. *J Clin Microbiol.* (1994) 32:1674–9. doi: 10.1128/JCM.32.7.1674-1679.1994
59. Li H, Gailbreath K, Flach EJ, Taus NS, Cooley J, Keller J, et al. A novel subgroup of rhadinoviruses in ruminants. *J Gen Virol.* (2005) 86:3021–26. doi: 10.1099/vir.0.81238-0
60. Xavier AAC. Ocorrência do vírus da febre catarral maligna em bovinos do estado do Paraná, Sul do Brasil: um estudo imuno-histoquímico retrospectivo [master's thesis]. Universidade Estadual de Londrina, Londrina, Brazil (2021).
61. Liggitt HD, DeMartini JC. The pathomorphology of malignant catarrhal fever: 1. Multisystemic epithelial lesions. *Vet Pathol.* (1980) 17:58–72. doi: 10.1177/030098588001700107
62. Hermeyer K, Buchenau I, Thomasmeyer A, Baum B, Spergser J, Rosengarten R, et al. Chronic pneumonia in calves after experimental infection with *Mycoplasma bovis* strain 1067: characterization of lung pathology, persistence of variable surface protein antigens and local immune response. *Acta Vet Scand.* (2012) 54:9. doi: 10.1186/1751-0147-54-9
63. Gagea MI, Bateman KG, Shanahan RA, van Dreumel T, McEwen BJ, Carman S, et al. Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. *J Vet Diagn Invest.* (2006) 18:29–40. doi: 10.1177/104063870601800105
64. Caswell JL, Williams KJ. Respiratory system. In: Kennedy J, Maxie MG, editors. *Palmer's Pathology of Domestic Animals*, Philadelphia, PA: Saunders/Elsevier (2016), p. 465–591. doi: 10.1016/B978-0-7020-5318-4.00011-5
65. Liebler-Tenorio EM, Ridpath JF, Neill JD. Lesions and tissue distribution of viral antigen in severe acute versus subclinical acute infection with BVDV2. *Biologicals.* (2003) 31:119–22. doi: 10.1016/S1045-1056(03)00026-5
66. Olchowy TWJ, Slauson DO, Bochsler PN. Induction of procoagulant activity in virus infected bovine alveolar macrophages and the effect of lipopolysaccharide. *Vet Immunol Immunop.* (1997) 58:27–37. doi: 10.1016/S0165-2427(96)05746-7
67. Potgieter LN. Immunology of bovine viral diarrhea virus. *Vet Clin North Am Food Anim Pract.* (1995) 11:501–20. doi: 10.1016/S0749-0720(15)30464-3
68. Welsh MD, Adair BM, Foster JC. Effect of BVD virus infection on alveolar macrophage functions. *Vet Immunol Immunop.* (1995) 46:195–210. doi: 10.1016/0165-2427(94)05366-Z
69. Reid E, Juleff N, Windsor M, Gubbins S, Roberts L, Morgan S, et al. Type I and III IFNs produced by plasmacytoid dendritic cells in response to a member of the *Flaviviridae* suppress cellular immune responses. *J Immunol.* (2016) 196:4214–26. doi: 10.4049/jimmunol.1600049
70. Strong R, La Rocca SA, Paton D, Bensaude E, Sandvik T, Davis L, et al. Viral dose and immunosuppression modulate the progression of acute BVDV-1 infection in calves: evidence of long term persistence

after intra-nasal infection. *PloS ONE.* (2015) 10:e0124689–e89. doi: 10.1371/journal.pone.0124689
71. Risalde MA, Molina V, Sanchez-Cordon PJ, Pedrera M, Panadero R, Romero-Palomo F, et al. Response of proinflammatory and anti-inflammatory cytokines in calves with subclinical bovine viral diarrhea challenged with bovine herpesvirus-1. *Vet Immunol Immunop.* (2011) 144:135–43. doi: 10.1016/j.vetimm.2011.07.022
72. Risalde MA, Molina V, Sonchez-Cordon PJ, Pedrera M, Romero-Palomo F, Bautista MJ, et al. Comparison of pathological changes and viral antigen distribution in tissues of calves with and without preexisting bovine viral diarrhea virus infection following challenge with bovine herpesvirus-1. *Am J Vet Res.* (2013) 74:598–610. doi: 10.2460/ajvr.74.4.598
73. Nicholas RA. Bovine mycoplasmosis: silent and deadly. *Vet Rec.* (2011) 168:459–62. doi: 10.1136/vr.d2468
74. MacLachlan NJ, Dubovi EJ. Herpesvirales. In: MacLachlan NJ, Dubovi EJ, editors. *Fenner's Veterinary Virology*, Boston, MA: Academic Press (2017), p. 189–216. doi: 10.1016/B978-0-12-800946-8.00009-X
75. Sulyok KM, Kreizinger Z, Wehmann E, Lysnyansky I, Bányai K, Marton S, et al. Mutations associated with decreased susceptibility to seven antimicrobial families in field and laboratory-derived *Mycoplasma bovis* strains. *Antimicrob Agents Chemother.* (2017) 61:e01983–16. doi: 10.1128/AAC.01983-16

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 02 August 2021
doi: 10.3389/fvets.2021.679074

# Identifying Loci Associated With Bovine Corona Virus Infection and Bovine Respiratory Disease in Dairy and Feedlot Cattle

*Jennifer N. Kiser and Holly L. Neibergs**

*Department of Animal Sciences, Washington State University, Pullman, WA, United States*

Bovine coronavirus (BCoV) is associated with respiratory and enteric infections in both dairy and beef cattle worldwide. It is also one of a complex of pathogens associated with bovine respiratory disease (BRD), which affects millions of cattle annually. The objectives of this study were to identify loci and heritability estimates associated with BCoV infection and BRD in dairy calves and feedlot cattle. Dairy calves from California ($n = 1{,}938$) and New Mexico ($n = 647$) and feedlot cattle from Colorado ($n = 915$) and Washington ($n = 934$) were tested for the presence of BCoV when classified as BRD cases or controls following the McGuirk scoring system. Two comparisons associated with BCoV were investigated: (1) cattle positive for BCoV ($BCoV^+$) were compared to cattle negative for BCoV ($BCoV^-$) and (2) cattle positive for BCoV and affected with BRD ($BCoV^+BRD^+$) were compared to cattle negative for BCoV and BRD ($BCoV^-BRD^-$). The Illumina BovineHD BeadChip was used for genotyping, and genome-wide association analyses (GWAA) were performed using EMMAX (efficient mixed-model association eXpedited). The GWAA for $BCoV^+$ identified 51 loci ($p < 1 \times 10^{-5}$; 24 feedlot, 16 dairy, 11 combined) associated with infection with BCoV. Three loci were associated with $BCoV^+$ across populations. Heritability estimates for $BCoV^+$ were 0.01 for dairy, 0.11 for feedlot cattle, and 0.03 for the combined population. For $BCoV^+BRD^+$, 80 loci ($p < 1 \times 10^{-5}$; 26 feedlot, 25 dairy, 29 combined) were associated including 14 loci across populations. Heritability estimates for $BCoV^+BRD^+$ were 0.003 for dairy, 0.44 for feedlot cattle, and 0.07 for the combined population. Several positional candidate genes associated with BCoV and BRD in this study have been associated with other coronaviruses and respiratory infections in humans and mice. These results suggest that selection may reduce susceptibility to BCoV infection and BRD in cattle.

**Keywords: bovine coronavirus, bovine respiratory disease, cattle, genome-wide association analysis, loci**

OPEN ACCESS

***Edited by:***
*Annamaria Pratelli, University of Bari Aldo Moro, Italy*

***Reviewed by:***
*Carol Geralyn Chitko-McKown, U.S. Meat Animal Research Center, United States*
*Olanrewaju B. Morenikeji, University of Pittsburgh at Bradford, United States*

****Correspondence:***
*Holly L. Neibergs*
*neibergs@wsu.edu*

***Specialty section:***
*This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science*

***Received:*** *10 March 2021*
***Accepted:*** *01 July 2021*
***Published:*** *02 August 2021*

***Citation:***
*Kiser JN and Neibergs HL (2021) Identifying Loci Associated With Bovine Corona Virus Infection and Bovine Respiratory Disease in Dairy and Feedlot Cattle. Front. Vet. Sci. 8:679074. doi: 10.3389/fvets.2021.679074*

## INTRODUCTION

The coronaviridae family, from the Nidoviruse order, consists of enveloped, positive-stranded RNA viruses with some of the largest viral genomes known among all RNA viruses. This family is often split into groups (1–3) based on serological and genetic similarities between the viruses (1). Bovine coronavirus (BCoV) is one of the common viral pathogens associated with bovine respiratory disease (BRD) and is a group 2 coronavirus along with human coronavirus OC43, murine hepatitis

virus, canine respiratory coronavirus, equine coronavirus, and rat sialodacryadenitis virus (1). Several group 2 coronaviruses, including BCoV, have a unique surface glycoprotein, hemagglutinin-esterase (HE), and a spike protein. The expression of the HE glycoprotein has been linked to enhanced virulence of some group 2 coronaviruses (2). In cattle, Popova and Zhang (3) determined that the presence of the spike protein alone, but not the HE protein by itself, was adequate for viral infection in host cells. However, HE is involved in viral attachment of host cells to some extent (4). Interestingly, monoclonal antibodies against HE were able to neutralize BCoV infection *in vitro* (5) and protect the cattle intestinal epithelium from viral infection *in vivo* (6). This suggests that the HE protein may have a role in inducing a protective response during BCoV infection. The spike protein of coronaviruses is involved in viral attachment and viral fusion to host cells during infection, and studies have suggested that natural selection within the spike protein gene is a mechanism BCoV uses to continually adapt to host immune responses to infection (7–9).

The BCoV has a large genome (27–30 kb), and infection with BCoV can result in both respiratory and enteric infections in cattle as well as in wild ruminants. Symptoms of BCoV infection can vary based on age of the infected cattle but commonly include severe diarrhea and respiratory distress (i.e., coughing, nasal discharge). Prevalence of BCoV in both feedlot and dairy cattle is considered widespread, with estimates ranging from 0 to 8.2% in healthy cattle (10–13) and up to 79% in cattle presenting with disease symptoms (12–14). The economic losses associated with BCoV and BRD infection can be quite substantial (15). In feedlot cattle, BCoV infection is associated with decreased weight gain (16, 17). In the dairy industry, neonatal calf diarrhea contributes up to 50% of the mortality seen in preweaned dairy calves, and respiratory illness accounts for an estimated 24% of pre-weaned heifer death and 58.9% of weaned heifer death in the US (18). The exact proportion of these deaths caused by BCoV infection is unknown. However, previous studies worldwide have indicated that the prevalence of BCoV in calves presenting with diarrhea ranged anywhere from 2.8 to 37% (12, 19–21).

Given the large impact and widespread nature of BCoV infection, identifying loci and genes associated with susceptibility to infection could allow for improved selection of cattle. If loci in strong association with BCoV are identified, they could potentially be included on commercially available assays used by producers for genomics selection to allow for the selection of less susceptible cattle. Previous studies have found multiple loci associated with BRD infection in both dairy (12) and feedlot cattle (13). Many of these studies investigated the prevalence of the various viral and bacterial pathogens associated with the BRD complex, but few genome-wide association analyses (GWAA) investigated loci associated with a single pathogen. The goal of the current study is to investigate genomic regions associated with BCoV infection within three cattle groups: a preweaned Holstein calf population, a beef feedlot cattle population, and the combined population of the dairy calves and beef feedlot cattle. The hypothesis of the study was that susceptibility to BCoV infection is associated with loci in feedlot and dairy cattle with and without BRD clinical symptoms. This study was undertaken to better understand the etiology of the disease and for identification of loci for consideration for genomic selection.

# MATERIALS AND METHODS

## Study Populations

The dairy calves studied consisted of calves collected from dairies in California (CA; $n = 1{,}203$ male and 735 female calves) and New Mexico (NM; all female calves, $n = 647$). The preweaned Holstein calves were originally enrolled in a BRD case–control study from July 2011 to January 2012 (12). Only calves with positive or negative BCoV test results were included in the current study.

The feedlot cattle consisted of cattle from Colorado (CO; $n = 915$ steers) and Washington (WA; $n = 934$ heifers). The feedlot population consisted of beef cattle originally enrolled in a BRD case–control study from August 2012 to January 2015 (13, 22). As in the dairy population, only cattle with BCoV test results were included for analysis. Cattle enrolled in the study from the CO feedlot consisted of the following breeds: 837 Angus, 16 Charolais, 20 Hereford, and 42 Red Angus. The cattle enrolled in the study from the WA feedlot consisted of 384 Angus, 96 Charolais, 377 crossbreds, 40 Hereford, and 37 Red Angus.

Cattle in this study were not experimentally challenged with BRD pathogens but were naturally infected. Without performing a pathogen challenge study, it was not possible to be 100% certain that all cattle were exposed to a pathogen. The study's sampling scheme was created in order to maximize the likelihood that case and control calves experienced the same pathogen exposure/environmental stressors. Cattle were enrolled in the original BRD studies based on the McGuirk Health Scoring system [(23); **Supplementary Figure 1**]. Briefly, the McGuirk Health Scoring system evaluates cattle for BRD clinical signs including temperature, cough, nasal discharge, eye discharge, and ear tilt. Cattle are then assigned a numerical score based on the severity (or lack thereof) of each symptom. Scores range from 0 (no signs of disease) to 12 (multiple severe signs of disease). Cattle that score $\leq 4$ were categorized as a control, and cattle with scores $\geq 5$ were categorized as a BRD case. In the dairy populations, as previously described in Neibergs et al. (12), calves were observed daily in their hutches. When a sick calf (clinical score $\geq 5$) was enrolled in the study, researchers scored the calves in adjacent hutches to determine if they met the criteria as a control ($\leq 4$). In an instance where the adjacent calves were also sick, the researcher would continue to look at the next adjacent calf(s) until a control calf was identified. In the beef population, feedlot cattle were observed daily by a pen rider. Whenever a sick animal was pulled out of the home pen and enrolled in a study, the pen rider would also pull a healthy animal from the same pen (thus exposed to the same environment as the sick/case animal) to enroll as a control. This sampling method was implemented in order to increase the exposure of the control animals to BRD pathogens that were infecting the cases and to ensure that case/control cattle had as similar as environments as possible.

## Sample Collection

Samples collected from all cattle included blood for DNA extraction, and mid-nasal and deep pharyngeal swabs collected for viral and bacterial pathogen identification. In addition to the samples collected, all cattle were evaluated for clinical signs of BRD using the McGuirk Health Scoring system (23; **Supplementary Figure 1**). All samples were collected upon enrollment into the original BRD study. This sampling process was described in detail previously (12). Briefly, mid-nasal samples were collected from the nasopharyngeal region using a 6-in. sterile unguarded polyester swab (Puritan Medical Products, Guilford, ME, USA) which was inserted 5 in. into the nostril and rotated against the nasal surface for 15 s. After sampling, the end of the swab was removed and placed in 3 ml of viral transport media (Amphotericin B −250 μg/ml, gentamicin −50 mg/ml, HEPES −1 M, $HCO_3$, and minimum essential media). Deep pharyngeal samples were collected using two 27-inch sterile guarded swabs with polyester tips (Kalajian Industries, Signal Hill, CA, USA). The distance from the nostril to the medial canthus of the eye of each animal was measured and marked on the swabs prior to collection. Then, the swab was inserted into the nostril into the pharyngeal recess until the mark on the swab reached the nostril. The swab was rotated against the pharyngeal recess surface for 15 s before being removed. After collection, the tip of one swab was removed and placed into the same 3-ml viral transport media tube as the mid-nasal swab. The end of the second deep pharyngeal swab was placed into 1 ml of bacterial transport media (Brucella broth, 15% glycerol). Samples were shipped overnight on ice and then underwent diagnostic testing. Bacteriology sample testing began the same day samples were received, while virology samples were stored at −80°C. Virology samples were tested at the diagnostic lab when several hundred were available to be tested at once. Mid-nasal and deep pharyngeal swabs were tested for the presence of the multiple bacterial and viral pathogens common to BRD infection. These pathogens included *Trueperella pyogenes*, *Histophilus somni*, *Mannheimia haemolytica*, *Pasteurella multocida*, BCoV, bovine respiratory syncytial virus, bovine viral diarrhea virus, bovine herpes virus, and various *Mycoplasma* species. The bacteriology samples were tested for the presence of pathogens using aerobic bacterial and mycoplasma culturing while virology samples were tested using quantitative PCR. For in-depth information on testing techniques, please refer to additional file 1 from Neibergs et al. (12) where these methods have been described previously. For the current study, only BCoV results were utilized.

Genotyping for each animal was performed from DNA extracted from ∼3 ml of whole blood collected *via* jugular venipuncture into an EDTA tube (CovidienMonoject, Dublin, Ireland). DNA was isolated using the Puregene DNA extraction kit according to manufacturer's guidelines (Qiagen, Germantown, MD, USA). The DNA was quantified using a NanoDrop 100 spectrophotometer (Wilmington, DE, USA) before genotyping. Genotypes were obtained using the Illumina BovineHD BeadChip (Neogen, Lincoln, NE). The BovineHD BeadChip contained 777,962 SNPs, with an average spacing of 3 kb across the genome. These SNPs were mapped using the ARS-UCD 1.2 assembly (https://www.animalgenome.org/repository/cattle/UMC_bovine_coordinates/).

## Quality Control

Prior to conducting GWAA, a series of quality control filtering steps were applied to the genotypes and the cattle. First, genotypes were filtered by call rate (<90%) which removed 165 cattle. Duplicated genotyped animals were identified, and five duplicated cattle were removed from the analysis. SNPs were removed if the genotyping call rate was <90% (19,983 SNPs removed), if the minor allele frequency was <1% (94,774 SNPs removed), or if they deviated from the Hardy–Weinberg equilibrium ($p < 1 \times 10^{-75}$; 20,212 SNPs removed). Twelve cattle were removed due to discrepancies between genetic and anatomical designations of sex. Finally, 24 cattle were removed for phenotypic inconsistencies or a lack of phenotypic information such as missing diagnostic information or animals misclassified as case or control. After quality control, 4,231 cattle (CA = 1,876; NM = 610; CO = 866; WA = 879) and 642,993 SNPs remained for analysis.

## Phenotypes

Two comparisons associated with BCoV were investigated: (1) cattle positive for BCoV ($BCoV^+$) were compared to cattle negative for BCoV ($BCoV^-$) and (2) cattle positive for BCoV and affected with BRD ($BCoV^+BRD^+$) were compared to cattle negative for BCoV and BRD ($BCoV^-BRD^-$). For the $BCoV^+$ phenotype, the dairy population consisted of 419 $BCoV^+$ calves (262 from NM and 157 from CA) and 2,067 $BCoV^-$ calves (348 from NM and 1,719 from CA), and the feedlot population consisted of 236 $BCoV^+$ cattle (128 from CO and 108 from WA) and 1,509 $BCoV^-$ cattle (738 from CO and 771 from WA). Analyses on a combined dairy and feedlot population analysis were also performed and contained 655 $BCoV^+$ and 3576 $BCoV^-$. For the $BCoV^+BRD^+$ phenotype, the dairy population had 242 $BCoV^+BRD^+$ (88 from CA and 154 from NM) and 1,074 $BCoV^-BRD^-$ (876 from CA and 198 from NM), the feedlot population had $BCoV^+BRD^+$ (82 from CO and 59 from WA) and 790 $BCoV^-BRD^-$ (397 from CO and 392 from WA), and the combined population had 383 $BCoV^+BRD^+$ and 1,864 $BCoV^-BRD^-$.

The dairy and feedlot populations were investigated for loci associated with BRD as previously described (12, 13, 22); however, they were not investigated for susceptibility to BCoV specifically. For the combined dairy and feedlot analysis, the same covariates were assessed as in the individual beef and dairy cattle populations.

## Statistical Analysis

A Student's *T*-test ($p < 0.05$) was used to test for effects of location and sex on the presence of BCoV prior to the GWAA. If sex or location was significant, it was subsequently used as a covariate for the GWAA. The GWAA for the three populations (dairy, feedlot, and combined) were performed using the SNP & Variation Suite version 8 from Golden Helix

Inc[1]. Statistical analyses were conducted using the efficient mixed-model association expedited (EMMAX) method (25) and three genotypic models (additive, dominant, and recessive). The general mixed model was described by $\mathbf{y} = \mathbf{X}\beta + \mathbf{Z}u + \epsilon$, where y is the n × 1 vector of observed phenotypes, X was the n × f matrix of fixed effects (f), β was a f × 1 vector containing the fixed effect coefficients, Z was a n × t matrix relating the random effects (t) to the phenotype, and $u$ was the random effect of the mixed model. The model assumes residuals to be independent with an identical distribution such that $Var(u) = \sigma_g{}^2\mathbf{K}$ and $(\epsilon) = \sigma_e{}^2\mathbf{I}$, and $Var(y) = \sigma_g{}^2\mathbf{ZKZ}' + \sigma_e{}^2\mathbf{I}$ (26). For this study, K was a matrix of pairwise genomic relationships and Z was the identity matrix, I (26). Given that the model of inheritance for BCoV infection and BRD is unknown, all three genotypic models were analyzed. Heritability was estimated using a genomic-best linear unbiased prediction (GBLUP) model (27, 28) and an average information, restricted maximum likelihood (AI-REML) algorithm (29, 30). More detailed information about the SVS methods for EMMAX and AI-REML/GBLUP can be found at http://doc.goldenhelix.com/SVS/latest/svsmanual/mixedModelMethods/overview.html.

The Wellcome Trust Consortium (24) significance threshold for uncorrected $p$-values was used to determine if SNP were strongly ($p < 5 \times 10^{-7}$) or moderately ($p$ between $1 \times 10^{-5}$ and $5 \times 10^{-7}$) associated with infection. The false discovery rate (FDR) was also calculated for all $p$-values within SVS. FDR used the following model: FDR $= \Sigma$ (V/R | R > 0) Pr (R > 0), where R is the number of rejected hypotheses and V is the number of reject hypotheses that are truly false positive (type I error). Positional candidate genes were identified within a 36-kb region (18 kb 3′ and 5′ of the SNP) in dairy cattle or 24 kb (12 kb 3′ and 5′ of the SNP) in feedlot cattle around significant SNP. In the combined population, positional candidate genes were identified within a 30-kb region surrounding the significant SNP. This region was based on the average haplotype block size of the dairy and feedlot populations in this study and was determined following the method proposed by Gabriel et al. (31).

## RESULTS

For the dairy calf population, the average age of BCoV positive ($BCoV^+$) calves (49.3 days) and BCoV negative ($BCoV^-$) calves (48.5 days) did not differ ($p = 0.14$). As only the CA calves had both male and female calves, the effect of sex was tested based on BCoV status ($p = 0.05$) and on BCoV and BRD ($BCoV^+BRD^+$) status ($p = 0.04$). When the CA calves were combined with the NM calves, sex remained significant with BCoV ($p = 2 \times 10^{-30}$) and in $BCVoV^+BRD^+$ ($p = 7 \times 10^{-22}$). Dairy location also played a role in the infection of BCoV ($p = 2 \times 10^{-76}$) as more calves were $BCoV^+$ in NM (63%) than in CA (37%). Therefore, for the dairy population analyses, location and sex were included as covariates. In the feedlot populations, there was no significant influence of breed ($p = 0.09$), sex ($p = 0.12$), or location ($p = 0.12$) on BCoV status so these potential covariates were excluded from the beef analyses. In the combined beef and dairy population, there was no significant influence of breed ($p = 0.20$) on BCoV status; however, sex ($p = 1.6 \times 10^{-10}$) and location ($p = 2.6 \times 10^{-41}$) did influence the phenotypes. When location was used as a covariate in the combined population, sex was no longer a significant factor in BCoV or BRD status. Therefore, location alone was used as a covariate in the analysis of the combined population.

*Phenotype 1: bovine coronavirus cases* ($BCoV^+$) *vs. controls* ($BCoV^-$).

Heritability estimates for BCoV infection status varied by population. The heritability estimate was $0.11 \pm 0.06$ in the feedlot population and $0.01 \pm 0.02$ in the dairy population. As the heritability estimate with the standard deviation encompassed zero for the dairy population, one must consider the heritability estimate for dairy as zero. As would be expected, the heritability estimate for the combined feedlot and dairy population was intermediate ($0.03 \pm 0.02$) to the two individual feedlot and dairy populations.

Twenty-three unique SNPs (16 loci) were associated ($p < 1 \times 10^{-5}$) with $BCoV^+$ in the additive, dominant, and recessive models for the dairy population (**Supplementary Table 1**). The additive model identified four moderately ($1 \times 10^{-5} < p > 5 \times 10^{-7}$) and one strongly ($p < 5 \times 10^{-7}$) associated SNP (five loci; **Figure 1A**), while the dominant model identified eight moderately and one strongly associated SNP (five loci; **Figure 1B**). The recessive model identified 14 moderately associated SNPs (eight loci; **Figure 1C**). All of the SNPs associated with $BCoV^+$ in the additive model were also associated with $BCoV^+$ in the dominant model. Eleven positional candidate genes were identified in the dairy population, with significant SNPs located within eight of the genes and the remaining three genes were located within the haplotype block region of the significant SNPs such as *MSI2* (**Supplementary Table 1**).

The GWAA for the feedlot population identified 39 unique SNPs (24 loci) associated with $BCoV^+$ (**Supplementary Table 1**). There were nine moderately and one strongly associated SNP (seven loci) in the additive model (**Figure 2A**), while the dominant model identified seven moderately and two strongly associated SNPs (four loci) associated with $BCoV^+$ (**Figure 2B**). The recessive model in the feedlot cattle, as with the dairy cattle, had more associations with $BCoV^+$ than in the dominant and additive models. There were 23 moderately and 2 strongly associated SNPs (17 loci) in the recessive model (**Figure 2C**). Five SNPs were shared across multiple models, as three SNPs were common to the additive and dominant models, and two SNPs were common to the additive and recessive models. A total of 18 positional candidate genes were identified in the feedlot population, with the significant SNP located within 13 of the positional candidate genes including *PRKCA* and *WWOX* (**Supplementary Table 1**).

The combined dairy and feedlot GWAA identified 15 unique SNPs (11 loci) associated with $BCoV^+$ (**Supplementary Table 1**).

[1]Golden Helix, Inc. SNP & Variation Suite (Version 8.). Available online at: http://www.goldenhelix.com.

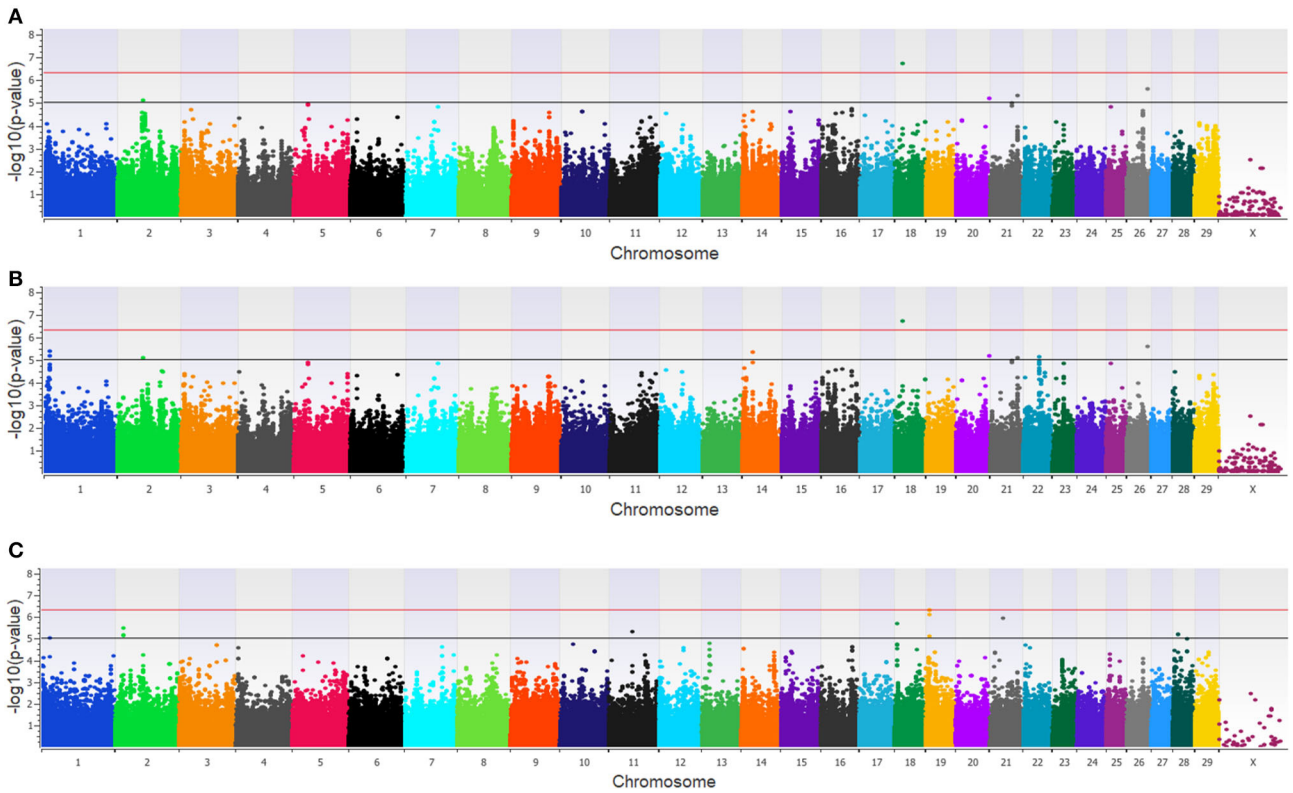



**FIGURE 1 |** Manhattan plots identifying loci associated with bovine coronavirus infection in additive **(A)**, dominant **(B)**, and recessive **(C)** EMMAX models for the dairy population. Single-nucleotide polymorphisms (SNP) are represented by a single dot. Bovine chromosomes are listed on the x-axis. SNPs located between the black and red line provide evidence of moderate association ($p$ between $1 \times 10^{-5}$ and $5 \times 10^{-7}$), and SNPs above the red line provide evidence of strong association ($p < 5 \times 10^{-7}$) based on the Wellcome Trust Case Control Consortium (24) guidelines.

A single SNP was moderately associated in the additive model (**Figure 3A**), and this SNP was also moderately associated with BCoV$^+$ in the dominant model. The dominant model further identified four moderately associated SNPs (three loci) (**Figure 3B**), and the recessive model identified 11 moderately associated SNPs (seven loci) with BCoV$^+$ (**Figure 3C**). Eight positional candidate genes, including *MSI2*, *PRKCA*, and *WWOXX* which harbored the SNP associated with BCoV$^+$, were identified in the combined population (**Supplementary Table 1**).

Three loci were identified in more than one of the feedlot, dairy, and combined cattle populations in the recessive model (**Table 1**), and each of these loci were defined by one or more SNPs that were located within a positional candidate gene *(MSI2*, *PRKCA*, and *WWOX*). No locus was found to be associated with BCoV$^+$ across all three populations.

*Phenotype 2: bovine coronavirus and bovine respiratory disease cases* (BCoV$^+$BRD$^+$) *vs. bovine coronavirus and bovine respiratory disease controls* (BCoV$^-$BRD$^-$).

Heritability estimates for BCoV$^+$BRD$^+$ was highest in the feedlot populations at 0.44 ± 0.13. The heritability estimate for BCoV$^+$BRD$^+$ was (0.005 ± 0.04) for the dairy, which included zero when the standard error was considered, similar to the BCoV$^+$ heritability estimate. The combined population's heritability estimate was intermediate to the feedlot and dairy heritability estimate for BCoV$^+$BRD$^+$ at 0.07 ± 0.04.

The GWAA for the dairy population identified 83 unique SNPs (25 loci) associated with BCoV$^+$BRD$^+$ (**Supplementary Table 2**). The additive model identified nine SNPs (six loci) moderately associated with BCoV$^+$BRD$^+$ (**Figure 4A**), while the dominant model identified eight SNPs (five loci) moderately associated with BCoV$^+$BRD$^+$ (**Figure 4B**). The recessive model identified 60 moderately and 12 strongly associated SNPs with BCoV$^+$BRD$^+$ (15 loci; **Figure 4C**). There were six shared SNPs associated with BCoV$^+$BRD$^+$ in the additive and dominant models. No SNP was shared between the recessive and additive models. Within the dairy population, 31 positional candidate genes were identified (**Supplementary Table 2**). Of these, 17 genes contained the significant SNP within the gene while the remaining 14 positional candidate genes were located in the region surrounding the significant SNP including *CA10*, *CWC22*, *LOC107131482*, *LOC789077*, *LOC524702*, and *NOS1AP*.

In the feedlot population, a total of 44 unique SNP (26 loci) were identified by the three GWAA models (**Supplementary Table 2**). The additive GWAA identified

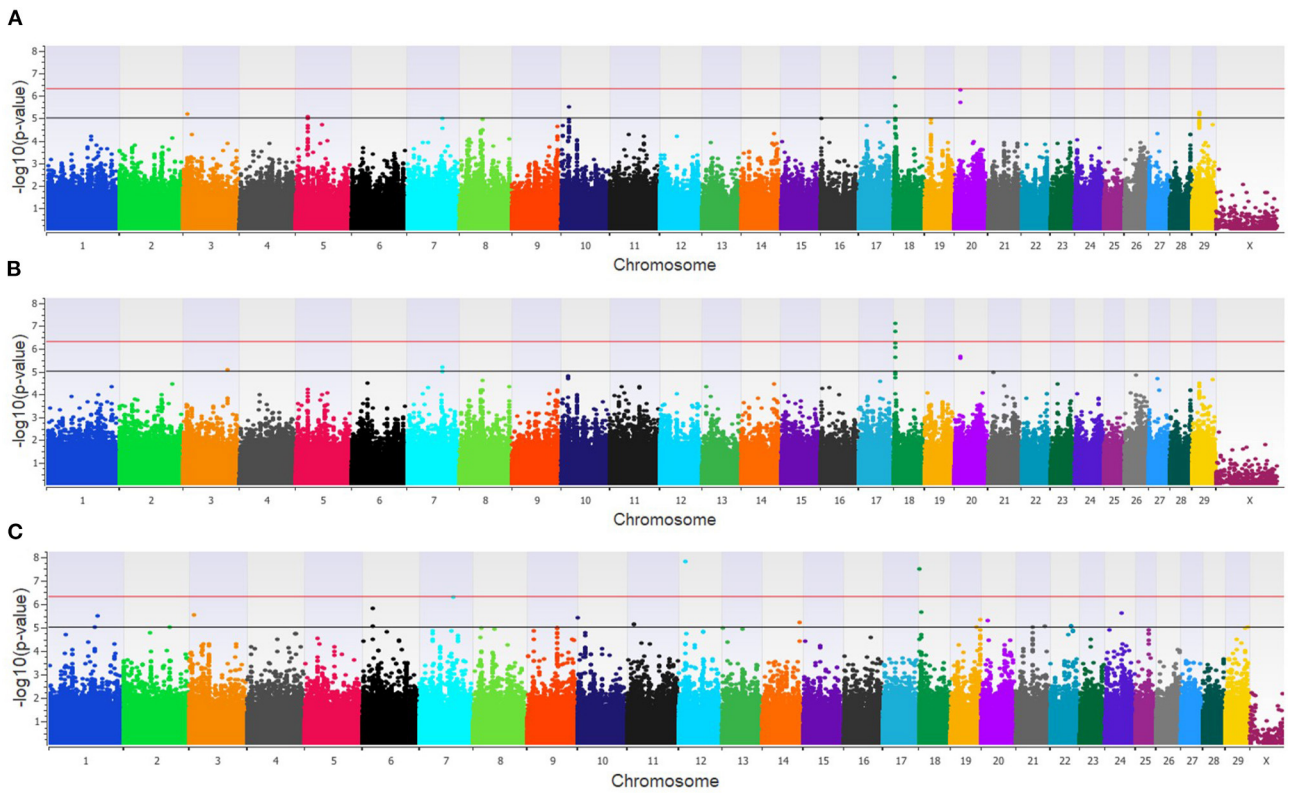



**FIGURE 2 |** Manhattan plots identifying loci associated with bovine coronavirus infection in additive **(A)**, dominant **(B)**, and recessive **(C)** EMMAX models for the feedlot population. Single-nucleotide polymorphisms (SNP) are represented by a single dot. Bovine chromosomes are listed on the x-axis. SNPs located between the black and red lines provide evidence of moderate association ($p$ between $1 \times 10^{-5}$ and $5 \times 10^{-7}$), and SNPs above the red line provide evidence of strong association ($p < 5 \times 10^{-7}$) based on the Wellcome Trust Case Control Consortium (24) guidelines.

23 significant SNP (eight loci; **Figure 5A**), including one that was strongly associated, while the dominant model identified seven significant SNPs including two that were strongly associated (six loci; **Figure 5B**). The recessive model identified 19 associated SNP including two in strong association (16 loci; **Figure 5C**). Between models, three SNPs were associated with $BCOV^{+}BRD^{+}$ in both the additive and dominant models while two SNPs were associated in the recessive and additive models. Eighteen positional candidate genes were identified in the feedlot population (**Supplementary Table 2**). Thirteen of the 18 genes harbored the significant SNP within the gene, while five genes were located in the regions near associated SNP such as *AKAP9, DISC1, LOC100849043, LOC104969525, PRSS48*, and *SH3D19*.

Within the combined population, a total of 81 unique SNPs (29 loci) were associated with $BCoV^{+}BRD^{+}$ in the three GWAA models (**Supplementary Table 2**). The additive model identified 15 SNPs (nine loci) that were in moderate association with $BCoV^{+}BRD^{+}$ (**Figure 6A**), while the dominant model identified nine SNPs (five loci) that were moderately associated with $BCoV^{+}BRD^{+}$ (**Figure 6B**). As in the $BCoV^{+}$ phenotype, the recessive model identified the greatest number of SNPs associated with $BCoV^{+}BRD^{+}$. There were 45 moderately and 15 strongly associated SNPs (18 loci) with $BCoV^{+}BRD^{+}$ (**Figure 6C**). Three associated SNPs were shared between models, two between the additive and dominant models, and one between the additive and recessive models. A total of 30 positional candidate genes were found in the combined population (**Supplementary Table 2**). Fourteen of the positional candidate genes were identified by having an associated SNP within their haplotype block, whereas 16 positional candidate genes harbored SNP associated with $BCoV^{+}BRD^{+}$ within the gene including *AKAP9, CA10, CWC22, DISC1, LOC104969525, LOC107131482, LOC789077, LOC524702, LOC100849043, MSI2, NOS1AP, PRSS48*, and *SH3D19*.

Across the three populations, 14 loci were associated with $BCoV^{+}BRD^{+}$ in more than one of the populations with 13 harboring positional candidate genes (**Table 2**). These loci were associated with $BCoV^{+}BRD^{+}$ in additive and dominant or additive and recessive models and contained between one and 18 SNPs. No loci were associated with $BCoV^{+}BRD^{+}$ in all three populations.

When loci associated with $BCoV^{+}$ and $BCoV^{+}BRD^{+}$ were compared, a single region on BTA19 ranging from 8,294,507 to 8,360,868 was identified with both phenotypes. Associations for both phenotypes in this region were identified in the recessive models in the dairy and combined populations. This region also contained a single positional candidate gene, Musashi RNA-binding protein 2 (*MSI2*), which contained an SNP associated

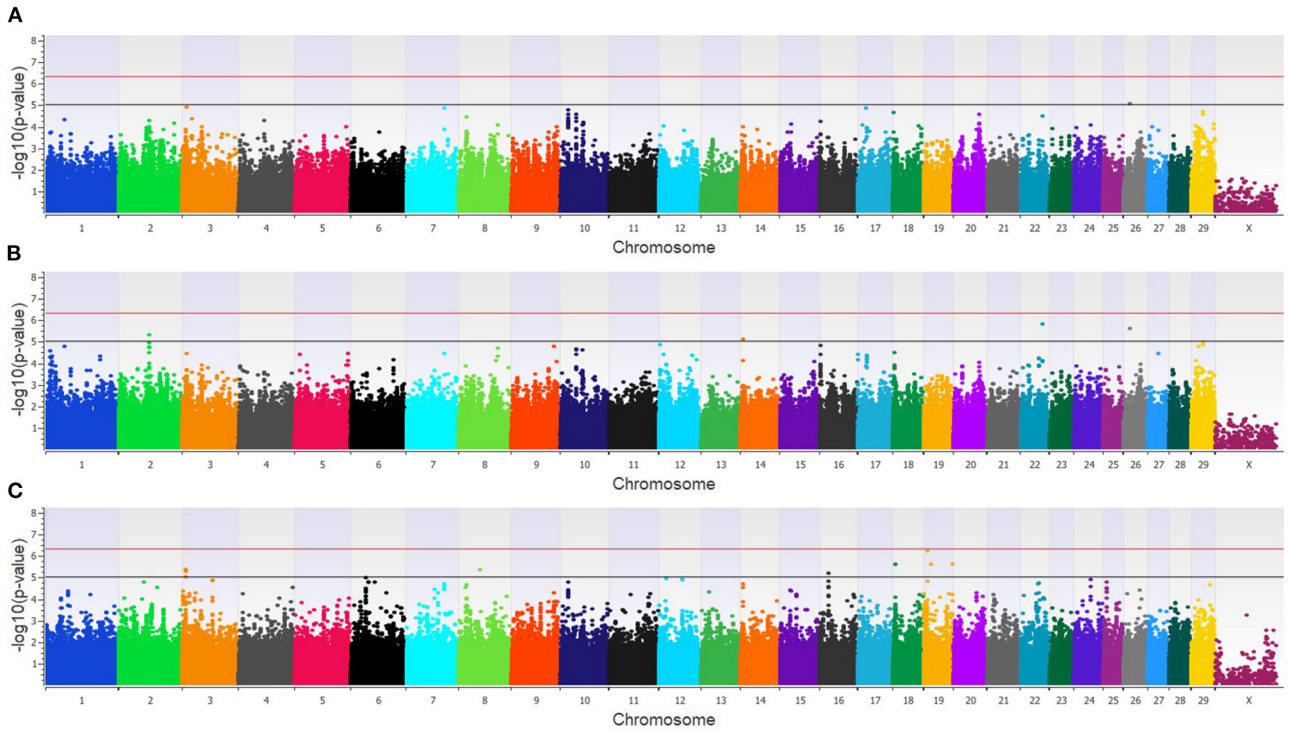



**FIGURE 3 |** Manhattan plots identifying loci associated with bovine coronavirus infection in additive **(A)**, dominant **(B)**, and recessive **(C)** EMMAX models for the combined dairy and feedlot population. Single-nucleotide polymorphisms (SNP) are represented by a single dot. Bovine chromosomes are listed on the x-axis. SNPs located between the black and red lines provide evidence of a moderate association ($p$ between $1 \times 10^{-5}$ and $5 \times 10^{-07}$), and SNPs above the red line provide evidence of a strong association ($p < 5 \times 10^{-7}$) based on the Wellcome Trust Case Control Consortium (24) guidelines.

**TABLE 1 |** Loci associated with bovine coronavirus infection across populations.

| BTA[a] | Locus range (Bb) | Lead SNP ID[b] | # SNP in locus | Population(s)[c] | Model(s) | Positional candidate gene(s)[d] |
|---|---|---|---|---|---|---|
| 18 | 5,420,736–5,749,984 | *rs109736099* | 3 | C & F | Recessive | ***WWOX*** |
| 19 | 8,294,507–8,360,868 | *rs134736482* | 3 | C & D | Recessive | ***MSI2*** |
| 19 | 62,757,064–62,763,041 | *rs41926279* | 2 | C & F | Recessive | ***PRKCA*** |

[a] *Single-nucleotide polymorphism (SNP) location as measured by numbered nucleotides in reference to the ARS 1.2 genome assembly (https://www.animalgenome.org/repository/cattle/UMC_bovine_coordinates/; accessed 21, January 2020).*

[b] *Most significant SNP in each locus is identified by rs number which is a reference number assigned to markers submitted to the National Center for Biotechnology Information SNP database.*

[c] *Populations abbreviated as follows: C, combined feedlot and dairy; F, feedlot; D, dairy.*

[d] *Positional candidate genes are defined as genes that are located within the average haplotype block range (combined = 15 kb; feedlot = 12 kb; dairy = 18 kb) on either side of the associated SNP(s) or had the significant SNP located within the gene itself (bolded).*

with the phenotypes. The positional candidate genes identified across populations for $BCoV^+$ (3; **Table 1**) and for $BCoV^+BRD^+$ (13; **Table 2**) were further analyzed to determine if there were involved in any similar biological pathways. Two genes, *AKAP9* ($BCoV^+$) and *PRKCA* ($BCoV^+BRD^+$), were involved in nine of the same pathways: activation of cAMP-dependent PKA, mitotic cell cycle, DAG and IP3 signaling, developmental biology, dopamine-DARPP32 feedback onto cAMP pathway, innate immune system, RET signaling, signaling by G-coupled protein receptors (GPCR), and transmission of chemical synapses. One of these pathways, signaling by GPCR, also involved a third positional candidate gene WWOX, which was associated with $BCoV^+$.

## DISCUSSION

The heritability estimates described in this study measure the additive genetic variance explained for $BCoV^+$ and

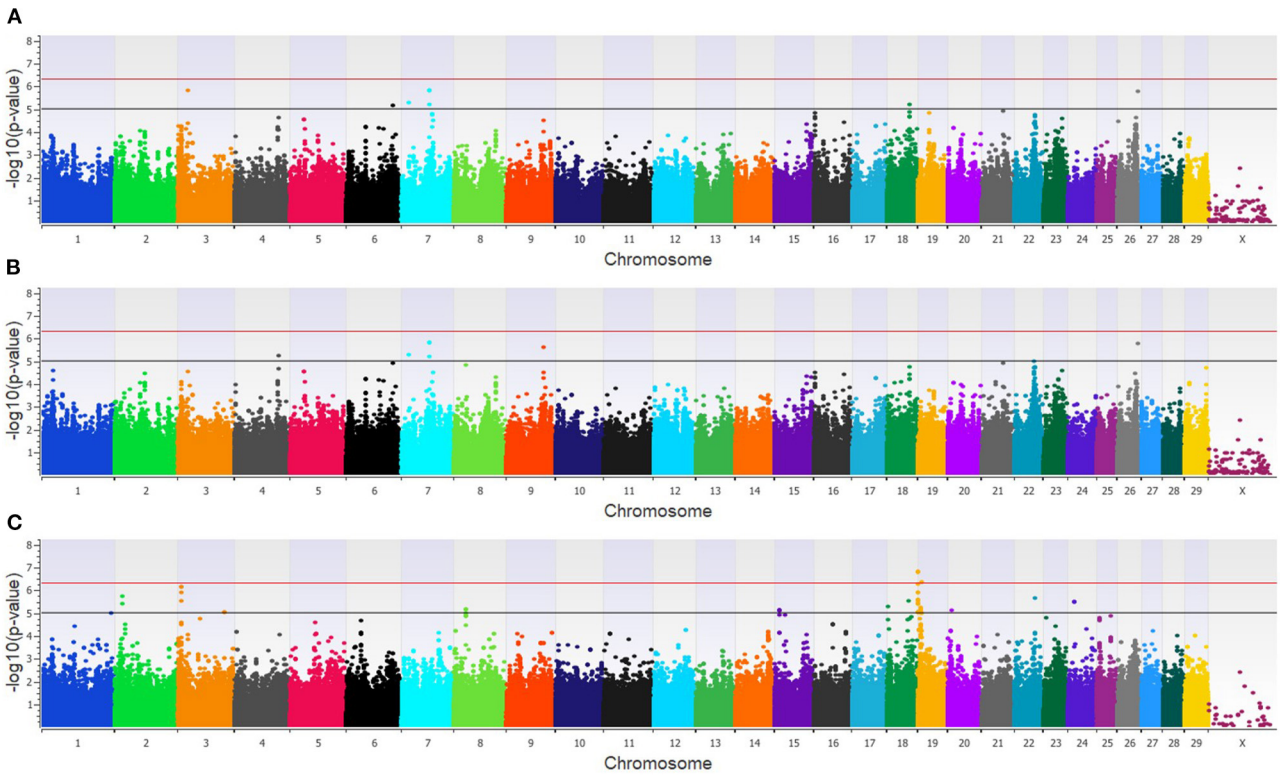



**FIGURE 4 |** Manhattan plots identifying loci associated with bovine coronavirus infection and bovine respiratory disease in additive **(A)**, dominant **(B)**, and recessive **(C)** EMMAX models for the dairy population. Single-nucleotide polymorphisms (SNP) are represented by a single dot. Bovine chromosomes are listed on the x-axis. SNPs located between the black and red lines provide evidence of moderate association ($p$ between $1 \times 10^{-5}$ and $5 \times 10^{-7}$), and SNPs above the red line provide evidence of a strong association ($p < 5 \times 10^{-7}$) based on the Wellcome Trust Case Control Consortium (24) guidelines.

$BCoV^+BRD^+$ infection in cattle. The heritability estimate for the dairy population was low and spanned zero when the standard deviation was included. In dairy, more loci were associated with $BCoV^+$ or $BCoV^+BRD^+$ in the non-additive (recessive and dominant) models than in the additive models. This suggests that the additive gene effects were overshadowed by the genetic variance explained by the non-additive (dominant and recessive) loci. This was particularly true in the diary population for the recessive model, as none of the recessive loci were identified in the additive model, and so did not contribute to the heritability estimate. However, in the dominant model, some of the loci associated with $BCoV^+$ or $BCoV^+BRD^+$ were also identified in the additive model and did contribute to the heritability estimates. In contrast to the dairy populations, in the feedlot populations the heritability estimate was moderate even when including the range of the standard deviations. Although the feedlot population was similar to the dairy population in that more non-additive loci were associated with $BCoV^+$ or $BCoV^+BRD^+$, the proportion of variance explained by the additive loci was slightly greater than in the dairy populations, thereby increasing the heritability estimates. The incidence of $BCoV^+$ was disparate between the NM (42%) and CA (8%) dairy calves, whereas the incidence was similar between the CO (15%) and WA (12%) feedlots. The differences between the incidence of $BCoV^+$ may reflect environmental and genetic differences in these calves, which can also reduce heritability estimates. Although the heritability estimates for $BCoV^+$ were low in dairy cattle, it is possible to make genetic improvement in traits with low heritabilities. Multiple studies have shown that selection for lowly heritable traits is possible and justifiable (32, 33).

Around 100 positional candidate genes were identified within regions associated with $BCoV^+$ and/or $BCoV^+BRD^+$. To allow for a more detailed discussion, the discussion will focus on the potential roles of 15 positional candidate genes associated with these phenotypes in multiple populations.

Of the 15 positional candidate genes identified in multiple populations, only one (from BTA19 at 8.3 kb) was associated with $BCoV^+$ and $BCoV^+BRD^+$. This locus harbors *MSI2*, an RNA-binding protein associated with cell-cycle regulation. While its counterpart *MSI1* has been widely studied for its roles in translation (34, 35), fewer studies have investigated *MSI2*. *MSI2* regulates hematopoiesis, and dysregulation of *MSI2* can impact cellular proliferation and apoptosis (36). A loss of *MSI2* function negatively impacts a host's innate immune responses in response to infection by affecting hematopoietic cell homeostasis and thus leukocyte development in both humans and mice (37, 38). Overexpression of *MSI2* leads to

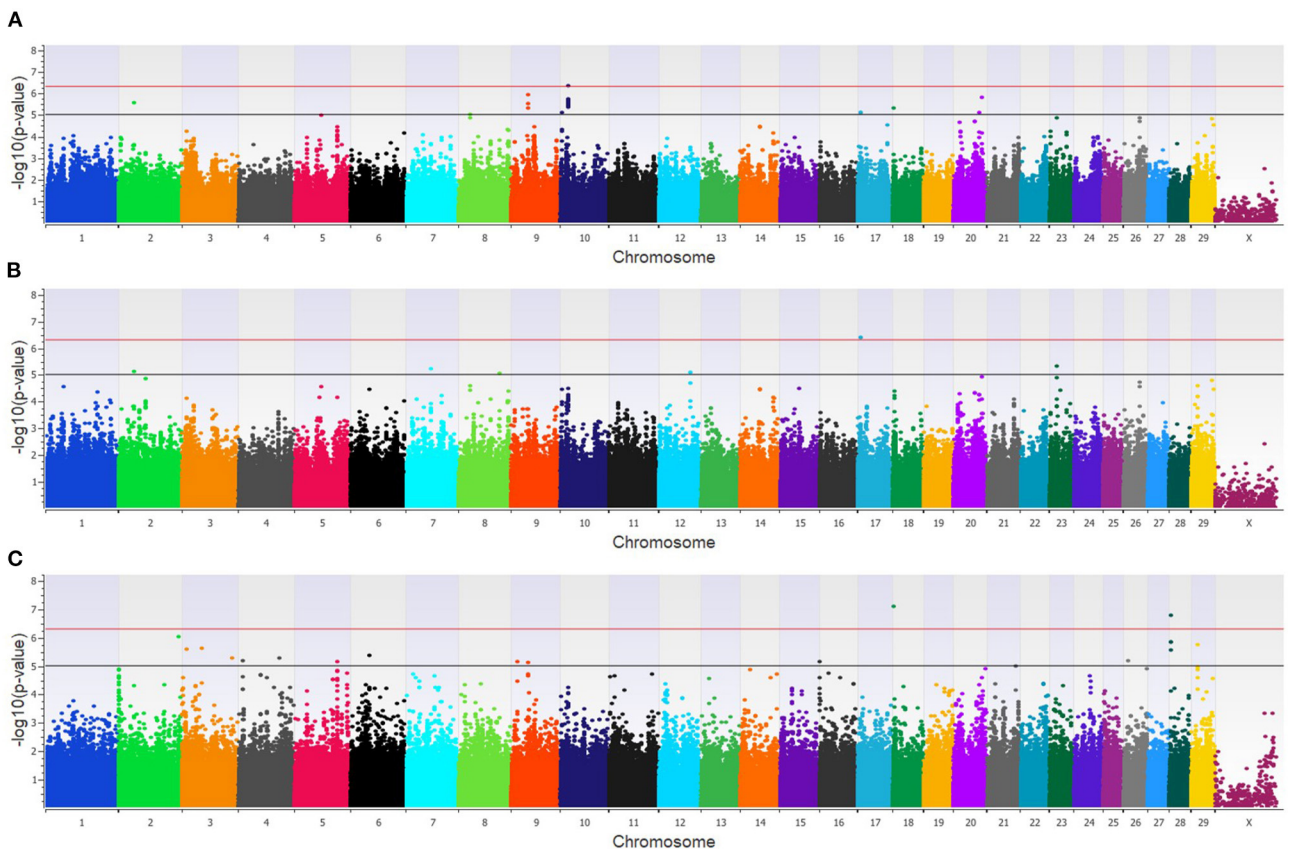



**FIGURE 5 |** Manhattan plots identifying loci associated with bovine coronavirus infection and bovine respiratory disease in additive **(A)**, dominant **(B)**, and recessive **(C)** EMMAX models for the feedlot population. Single-nucleotide polymorphisms (SNP) are represented by a single dot. Bovine chromosomes are listed on the x-axis. SNPs located between the black and red lines provide evidence of moderate association ($p$ between $1 \times 10^{-5}$ and $5 \times 10^{-7}$), and SNPs above the red line provide evidence of strong association ($p < 5 \times 10^{-7}$) based on the Wellcome Trust Case Control Consortium (24) guidelines.

increased hematopoietic cell expansion and self-renewal and can lead to increased pathogenesis of certain hematopoietic cell diseases (36, 39). Previous studies on human bronchial epithelial cells found that during infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative coronavirus associated with COVID-19, expression of *MSI2* was downregulated in infected cells suggesting cells were more prone to infection due to issues with the innate immune response to infection (40). That *MSI2* is involved in other coronavirus infections provides support for a possible role for *MSI2* in modulating BCoV infection.

Two additional positional candidate genes, protein kinase C alpha (*PRKCA*) and WW domain-containing oxidoreductase (*WWOX*), were associated with $BCoV^{+}$ in more than one population (**Table 1**). *PRKCA* is a member of a kinase family that has roles in many cellular processes including cell permeability and cell signaling. In patients with pneumococcal pneumonia, PRKCA has a role in the activation antibiotic-induced release of pneumolysin (41). The presence of pneumolysin in the lungs leads to increased pulmonary endothelial permeability and potentially increases edema in the lungs (41). Similarly, *PRKCA* alters pulmonary endothelial cell permeability in humans and mice in response to lung injury *via* reactive oxygen species, alpha-thrombin, and TNF-alpha (42). Multiple studies have also implicated that *PRKCA* has a role in respiratory syncytial virus (RSV) infection. Upon initial infection with RSV, *PRKCA* is activated and co-localizes with the virus. The binding of PRKCA and RSV is needed to facilitate the fusion of RSV with host cell membranes (43, 44). Both BCoV and bovine RSV are part of the BRD complex of pathogens. Bovine respiratory disease is common to dairy and beef cattle and causes considerable economic loss to the cattle industry. Similar to *MSI2*, *PRKCA* has also been linked to severe acute respiratory syndrome (SARS) in humans, which is caused by a SARS-CoV-1. Liu et al. (45) found that the SARS-CoV spike protein stimulates *PRKCA* to modulate a NFKB pathway through the influx of calcium ions. Ultimately, the activation of this pathway and other calcium-independent pathways induces inflammation and tissue damage within the lungs leading to symptoms of SARS-CoV (45).

The *WWOX* gene is a tumor suppressor which can interact with a variety of transcription factors in inflammation and cancer. A knockdown study in mice found that decreased

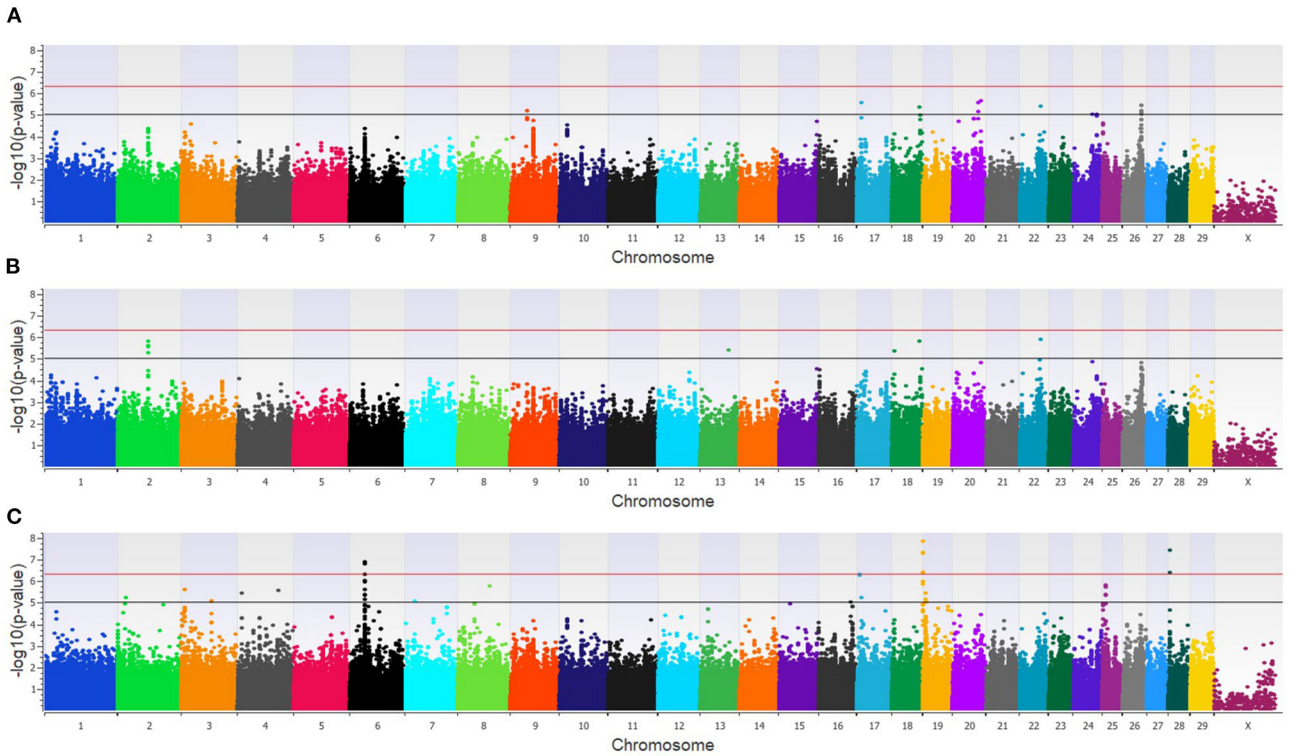



**FIGURE 6 |** Manhattan plots identifying loci associated with bovine coronavirus infection and bovine respiratory disease in additive **(A)**, dominant **(B)**, and recessive **(C)** EMMAX models for the combined dairy and feedlot population. Single-nucleotide polymorphisms (SNP) are represented by a single dot. Bovine chromosomes are listed on the x-axis. SNPs located between the black and red lines provide evidence of a moderate association ($p$ between $1 \times 10^{-5}$ and $5 \times 10^{-7}$), and SNPs above the red line provide evidence of a strong association ($p < 5 \times 10^{-7}$) based on the Wellcome Trust Case Control Consortium (24) guidelines.

expression of WWOX leads to an influx of neutrophils, increased vascular leakage, and increased inflammatory cytokine production (46). Ultimately, these increased immune responses triggered acute respiratory distress syndrome (ARDS) in mice (46). This increase in neutrophils and vascular leakage can negatively impact the host increasing the chances of damage during an infection.

Twelve additional positional candidate genes were identified across populations in $BCoV^{+}BRD^{+}$ cattle (**Table 2**). These genes can be grouped by function such as those that have roles related to olfactory receptors, pulmonary diseases, and viral infections. Three of the positional candidate genes are olfactory receptor like-genes (*LOC10713482*, *LOC524702*, and *LOC789077*). These genes have no known function relating to BCoV or BRD infection and are not fully characterized in cattle. However, the olfactory epithelium and olfactory neurons have been linked to early immune responses to SARS-CoV2 infection and loss of smell is a common symptom of COVID-19 (47). It is possible that cattle also experience a loss of smell during BCoV or BRD infection, and this could contribute to the decrease in appetite seen in some cattle.

Four additional positional candidate genes, carbonic anhydrase 10 (*CA10*), CWC22 spliceosome-associated protein (*CWC22*), DISC1 scaffold protein (*DISC1*), and nitric oxide synthase 1 adaptor protein (*NOS1AP*), have roles in pulmonary dysfunction, viral infections, or both. A recent study in humans determined that dysregulation of carbonic anhydrases, along with membrane metallo-endopeptidase and angiotensin-converting enzyme 2, during a SARS-CoV2 infection results in increased levels of carbon dioxide in the bloodstream as well as pulmonary edema and eventually heart failure (48). Hypermethylation of *NOS1AP* in patients with chronic obstructive pulmonary disorder results in gene silencing in lung tissues (49). Gene silencing of *NOS1AP* expression could disrupt downstream pathways within the lung related to autophagy and apoptosis (49). Like *CA10*, *DISC1* has ties to both pulmonary disease and viral infections (50, 51). *CWC22*, a regulator of mRNA splicing and RNA metabolism, does not have a direct link to BCoV or BRD infection. However, a study on how influenza viruses, like H1N1, harness host cells' ability to replicate during respiratory illness found that the expression of *CWC22* was linked with a decreased cellular infection rate (52). How these genes might be influencing BCoV infection in cattle is unclear, but the overlap with other coronavirus studies and pulmonary health factors suggest the connections are worth further investigation.

Little is known about the functions of the last five positional candidate genes associated with $BCoV^{+}BRD^{+}$ infection in two of the cattle populations. For example, *LOC100849043* and

**TABLE 2 |** Loci associated with bovine coronavirus and bovine respiratory disease infections across populations.

| BTA[a] | Locus range (Bb) | Lead SNP ID[b] | # SNP in locus | Population(s)[c] | Model(s) | Positional candidate gene(s)[d] |
|---|---|---|---|---|---|---|
| 2 | 16,887,183–16,892,466 | *rs136233359* | 2 | C & D | Recessive | *CWC22* |
| 3 | 7,228,232–7,236,300 | *rs109804510* | 4 | C & D | Recessive | ***NOS1AP*** |
| 4 | 9,439,592 | *rs41587962* | 1 | C & F | Recessive | ***AKAP9*** |
| 4 | 88,516,050 | *rs42043233* | 1 | C & F | Recessive | – |
| 9 | 35,944,485–35,956,425 | *rs110498854* | 3 | C & F | Additive & recessive | *LOC104969525* |
| 17 | 6,515,432–6,516,072 | *rs135350640* | 2 | C & F | Additive, dominant, & recessive | ***SH3D19***, *PRSS48* |
| 19 | 154,015–317,404 | *rs135692084* | 6 | C & D | Recessive | ***LOC107131482***, *LOC789077, LOC524702* |
| 19 | 340,070–468,681 | *rs110355574* | 28 | C & D | Recessive | - |
| 19 | 853,741–868,633 | *rs134561123* | 2 | C & D | Recessive | ***CA10*** |
| 19 | 6,846,738–6,855,449 | *rs110565142* | 3 | C & D | Recessive | - |
| 19 | 8,294,507–8,360,868 | *rs133988665* | 3 | C & D | Recessive | ***MSI2*** |
| 20 | 56,000,163–56,003,536 | *rs137256606* | 2 | C & F | Additive | ***LOC100849043*** |
| 20 | 60,955,121 | *rs133738096* | 1 | C & F | Additive | - |
| 28 | 4,224,198–4,534,637 | *rs110583329* | 4 | C & F | Recessive | ***DISC1*** |

[a]*Single nucleotide polymorphism (SNP) location as measured by numbered nucleotides in reference to the ARS 1.2 genome assembly (https://www.animalgenome.org/repository/cattle/UMC_bovine_coordinates/; accessed 21, January 2020).*

[b]*Most significant SNP in each locus is identified by rs number which is a reference number assigned to markers submitted to the National Center for Biotechnology Information SNP database.*

[c]*Populations abbreviated as follows: C, combined feedlot and dairy; F, feedlot; D, dairy.*

[d]*Positional candidate genes are defined as genes that are located within the average haplotype block range (combined = 15 kb; feedlot = 12 kb; dairy = 18 kb) on either side of the associated SNP(s) or had the significant SNP located within the gene itself (bolded).*

*LOC104969525* are listed as uncharacterized genes in cattle. After comparing the bovine sequences for *LOC100849043* and *LOC104969525* to the nucleotide sequences of other species, the only similarities with this sequence in other species were identified in water buffalo, bison, and sheep. In these species, as in cattle, the gene was uncharacterized but predicted to be a non-coding RNA. The serine protease 48 (*PRSS48*) and SH3 domain-containing 19 (*SH3D19*) genes do not have known functions relating to viral infections or respiratory illness. However, studies have shown that *PRSS48* and *SH3D19* have roles in disease (53, 54). For example, a previous study in brain endothelial cells exposed to *Cryptococcus neoformans* indicated that infection caused expression differences in *PRSS48* (53), suggesting a potential role in endothelial cells during infection. This is in keeping with a recent study that compared bronchi alveolar lavage fluid of healthy patients and patients infected with SARS-CoV2. Zhou et al. (54) reported that *SH3D19* was part of a network of genes that was differentially expressed in SARS-CoV2 patients. Similar to *PRSS48*, A-kinase anchoring protein 9 (*AKAP9*) has functions relating to endothelial cells. Sehrawat et al. (55) reported that *AKAP9* is capable of directly regulating *EPAC1*, which is involved in the regulation of the inflammatory response within umbilical and dermal endothelial cells. How this gene might function within pulmonary endothelial cells has not been investigated.

Several positional candidate genes were associated with GPCR signaling pathways (*AKAP9*, *PRKCA*, and *WWOX*). GPCR signaling is involved in a plethora of physiological processes, including immune responses (56). Within the immune system, GPCR signaling pathways are associated with vascular inflammation, through the promotion of proinflammatory cytokine signaling and through the disruption of the endothelial barrier in smooth muscles (57). Limited research is available on the role of GPCR in BRD or other lung infections in cattle. However, a previous study, utilizing the same cattle populations as this study, investigated gene sets associated with BRD susceptibility (22). Neupane et al. found that within the feedlot population, the regulation of a G protein-coupled receptor signaling pathway was enriched (normalized enrichment score = 3.07) for BRD susceptibility. There is considerably more knowledge on the function of GPCR signaling pathways with other coronaviruses. For example, a recent study indicated that SARS-CoV-2 may hijack the GPCR signaling pathway to alter fluid and ion transport within the lungs, which leads to lung edema—a deadly clinical sign of COVID-19 infection (58). Similarly, Hammoudeh et al. (59) suggested that SARS-CoV-2 manipulation of the GPCR pathways could facilitate viral infection within the host leading to dysregulation of intracellular transport within the livers of infected patients. Given its role in infectivity of other coronaviruses, further investigation into GPCR signaling and its association with BCoV and BRD in cattle is warranted.

In conclusion, the identification of loci associated with BCoV+ as well as BCoV+BRD+ could allow for the future selection of cattle with decreased susceptibility to both BCoV infection and BRD. Given the high economic cost as well as the animal welfare issues associated with

both diseases, selecting for cattle that are less likely to be infected with BCoV and BRD is advantageous. Determining how positional candidate genes could be influencing the disease process is also of importance as coronaviruses become more prevalent worldwide in cattle and may be helpful in understanding the disease process of other coronaviruses. Possible shared mechanism across species could provide additional insight into the disease etiology and potentially future treatment options.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: CattleQTLdb : https://www.animalgenome.org/QTLdb/supp/?t=QzYj1V9CmR.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committees from—Washington State University, University of California—Davis, New Mexico State University, and Texas A&M University. Written informed consent for participation was not obtained from the owners because the need for written consent forms from each dairy and feedlot was deemed unnecessary according to the Washington State University Institutional Review Board 45 CFR 46.102(e) (1). However, each dairy and feedlot provided written agreement to participate in the study.

## AUTHOR CONTRIBUTIONS

HN designed the study, collected the samples, reviewed, and edited the manuscript. JK performed the experiments, wrote, and edited the manuscript. All the authors read and approved the final manuscript.

## FUNDING

This project was supported by the Agriculture and Food Research Initiative competitive Grant no. 2011-68004-30367 of the USDA National Institute of Food and Agriculture.

## ACKNOWLEDGMENTS

The authors would like to thank the dairies and feedlots that provided access to the cattle used in this study.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.679074/full#supplementary-material

**Supplementary Figure 1 |** Example of the McGuirk scoring system used to classify cattle as bovine respiratory disease cases and controls.

**Supplementary Table 1 |** Significant loci associated with bovine coronavirus infection in a dairy, a feedlot, and a combined dairy and feedlot population.

**Supplementary Table 2 |** Significant loci associated with bovine coronavirus and bovine respiratory disease infections in a dairy, a feedlot, and a combined dairy and feedlot population.

## REFERENCES

1. González JM, Gomez-Puertas P, Cacanagh D, Gorbalenya AE, Enjuanes L. A comparative sequence analysis to revise the current taxonomy of the family Coronaviridae. *Arch Virol.* (2003) 148:2207–35. doi: 10.1007/s00705-003-0162-1
2. Kazi L, Lissenberg A, Watson R, de Groot RJ, Weiss SR. Expression of hemagglutinin esterase protein from recombinant mouse hepatitis virus enhances neurovirulence. *J Virol.* (2005) 79:15064–73. doi: 10.1128/JVI.79.24.15064-15073.2005
3. Popova R, Zhang X. The spike but not the hemagglutinin/esterase protein of bovine coronavirus is necessary and sufficient for viral infection. *Virology.* (2002) 294:222–36. doi: 10.1006/viro.2001.1307
4. Zeng Q, Langereis MA, van Vliet ALW, Huizinga EG, de Groot RJ. Structure of coronavirus hemagglutinin-esterase offers insight into corona and influenza virus evolution. *Proc Nat Acad Sci USA.* (2008) 105:9065–9. doi: 10.1073/pnas.0800502105
5. Deregt D, Babiuk LA. Monoclonal antibodies to bovine coronavirus: characteristics and topographical mapping of neutralizing epitopes on the E2 and E3 glycoproteins. *Virology.* (1987) 161:410–20. doi: 10.1016/0042-6822(87)90134-6
6. Dereget D, Gifford GA, Ijaz MK, Watts TC, Gilchrist JE, Haines DM, et al. Monoclonal antibodies to bovine coronavirus glycoproteins E2 and E3: demonstration of *in vivo* virus-neutralizing activity. *J Gen Virol.* (1989) 70:993–8. doi: 10.1099/0022-1317-70-4-993
7. Bidokhti MRM, Tråvén M, Krishna NK, Munir M, Belák S, Alenius S, et al. Evolutionary dynamics of bovine coronaviruses: natural selection pattern of the spike gene implies adaptive evolution of the strains. *J Gen Virol.* (2013) 94:2036–49. doi: 10.1099/vir.0.054940-0
8. Shin J, Tark D, Le VP, Choe SE, Cha RM, Park GN, et al. Genetic characterization of bovine coronavirus in Vietnam. *Virus Genes.* (2019) 55:415–20. doi: 10.1007/s11262-019-01647-1
9. Franzo G, Drigo M, Legnardi M, Grassi L, Pasotto D, Menandro ML, et al. Bovine coronavirus: variability, evolution, and dispersal patterns of a no longer neglected betacoronavirus. *Viruses.* (2020) 12:1285. doi: 10.3390/v12111285
10. Uhde FL, Kaufmann T, Sager H, Albini S, Zanoni R, Schelling E, et al. Prevalence of four enteropathogens in the faeces of young diarrhoeic dairy calves in Switzerland. *Vet Record.* (2008) 163:363–6. doi: 10.1136/vr.163.12.362
11. Izzo MM, Kirkland PD, Mohler VL, Perkins NR, Gunn AA, House JK. Prevalence of major enteric pathogens in Australian dairy calves with diarrhoea. *Aust Vet J.* (2011) 89:167–73. doi: 10.1111/j.1751-0813.2011.00692.x
12. Neibergs HL, Seabury CM, Wojtowicz AJ, Wang Z, Scraggs E, Kiser JN, et al. Susceptibility loci revealed for bovine respiratory disease complex in pre-weaned holstein calves. *BMC Genomics.* (2014) 15:1164. doi: 10.1186/1471-2164-15-1164
13. Kiser JN, Lawrence TE, Neupane M, Seabury CM, Taylor JF, Womack JE, et al. Rapid communication: subclinical bovine respiratory disease - loci and pathogens associated with lung lesions in feedlot cattle. *J Anim Sci.* (2017) 95:2726–31. doi: 10.2527/jas2017.1548
14. Lojkić I, Krešić N, Šimić I, Bedeković T. Detection and molecular characterisation of bovine corona and toroviruses from Croatian cattle. *BMC Vet Res.* (2015) 11:202. doi: 10.1186/s12917-015-0511-9

15. Boileau M, Kapil S. Bovine coronavirus associated syndromes. Veterinary clinics of North America. *Food Anim Pract.* (2010) 26:123–46. doi: 10.1016/j.cvfa.2009.10.003
16. Hasoksuz M, Hoet AE, Loerch SC, Wittum TE, Nielsen PR, Saif LJ. Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in an Ohio feedlot. *J Vet Diagnostic Investig.* (2002) 14:308–13. doi: 10.1177/104063870201400406
17. Thomas CJ, Hoet AE, Sreevatsan S, Wittum TE, Briggs RE, Duff GC, et al. Transmission of bovine coronavirus and serologic responses in feedlot calves under field conditions. *Am J Vet Res.* (2006) 67:1412–20. doi: 10.2460/ajvr.67.8.1412
18. USDA. *Dairy 2014 Health and Management Pracitives on the U.S. Dairy Operations, 2014.* Fort Collins, CO: USDA-APHIS-VS-CEAH-NAHMS (2018).
19. Hasoksuz M, Kayar A, Dodurka T, Ilgaz A. Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in Northwestern Turkey. *Acta Vet Hung.* (2005) 53:137–46. doi: 10.1556/avet.53.2005.1.13
20. Bartels CJM, Holzhauer M, Jorritsma R, Swart AJM, Lam TJGM. Prevalence, prediction, and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. *Prev Vet Med.* (2010) 93:162–9. doi: 10.1016/j.prevetmed.2009.09.020
21. Lotfollahzadeh S, Madadgar O, Mohebbi MR, Dezfouli MRM, Watson DG. Bovine coronarvirus in neonatal calf diarrhea in Iran. *Vet Med Sci.* (2020) 6:696–4. doi: 10.1002/vms3.277
22. Neupane M, Kiser JN, Neibergs HL. Gene set enrichment analysis of SNP data in dairy and beef cattle with bovine respiratory disease. *Anim Genet.* (2018) 49:527–38. doi: 10.1111/age.12718
23. McGuirk SM. Disease management of dairy calves and heifers. *Vet Clin North AM Food Anim Pract.* (2008) 24:139–53. doi: 10.1016/j.cvfa.2007.10.003
24. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature.* (2007) 447:661–78. doi: 10.1038/nature05911
25. Kang HM, Sul JH, Service SK, Zaitlen NA, Kong SY, Freimer NB, et al. Variance component model to account for sample structure in genome-wide association studies. *Nat Genet.* (2010) 42:348–54. doi: 10.1038/ng.548
26. Kang HM, Zaitlen NA, Wade CM, Kirby A, Heckerman D, Daly MJ, et al. Efficient control of population structure in model organism association mapping. *Genetics.* (2008) 178:1709–23. doi: 10.1534/genetics.107.080101
27. VanRanden PM. Efficient methods to compute genomic predications. *J Dairy Sci.* (2008) 91:4414–23. doi: 10.3168/jds.2007-0980
28. Taylor J. Implementation and accuracy of genomic selection. *Aquaculture.* (2014) 4:420–1. doi: 10.1016/j.aquaculture.2013.02.017
29. Yang J, Lee SH, Goddard ME, Visscher PM. GCTA: a tool for genome-wide complex trait analysis. *Am J Hum Genet.* (2011) 88:76–82. doi: 10.1016/j.ajhg.2010.11.011
30. Lee SH, Yang J, Goddard ME, Visscher PM, Wray NR. Estimation of pleiotropy between complex diseases using single-nucleotide polymorphism-derived genomic relationships and restricted maximum likelihood. *Bioinformatics.* (2012) 28:2540–2. doi: 10.1093/bioinformatics/bts474
31. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, et al. The structure of haplotype blocks in the human genome. *Science.* (2002) 296:2225–9. doi: 10.1126/science.1069424
32. Togashi K, Lin CY. Theoretical efficiency of multiple-trait quantitative trait loci-assisted selection. *J Anim Breed Genet.* (2010) 127:53–63. doi: 10.1111/j.1439-0388.2009.00817.x
33. Kenji T, Kurogi K, Adachi K, Tokunaka K, Yasumori T, Watanabe T, et al. Asymptotic response to four-path selection due to index and single trait selection according to genomically enhanced breeding values. *Livest Sci.* (2020) 231:103846. doi: 10.1016/j.livsci.2019.103846
34. Imai T, Tokunaga A, Yoshia T, Hashimoto M, Mikoshiba K, Weinmaster G, et al. The neural RNA-binding protein mushashi1translationally regulates mammalian numb gene expression by interaction with its mRNA. *Mol Cell Biol.* (2001) 21:3888–900. doi: 10.1128/MCB.21.12.3888-3900.2001
35. Kawahara H, Imai T, Imataka H, Tsujimoto M, Matsumoto K, Okano H. Neural RNA-binding musashi1 inhibits translation initiation by competing with eIF4G for PABP. *J Cell Biol.* (2008) 181:639–53. doi: 10.1083/jcb.200708004
36. Kharas MG, Lengner CJ, Al-Shahrour F, Bullinger L, Ball B, Zaidi S, et al. Musashi-2 regulates normal hematopoiesis and promotes aggressive myeloid leukemia. *Nat Med.* (2010) 16:903–8. doi: 10.1038/nm.2187
37. Sawai CM, Babovic S, Upadhaya S, D., Knapp JHF, Lavin Y, et al. Hematopoietic stem cells are the major source of multilineage hematopoiesis in adult animals. *Immunity.* (2016) 45:597–609. doi: 10.1016/j.immuni.2016.08.007
38. Belew MS, Bhatia S, Chahi AK, Rentas S, Draper JS, Hope KJ. PLAG1 and USF2 co-regulate expression of musashi-2 in human hematopoietic stem and progenitor cells. *Stem Cell Rep.* (2018) 10:1384–97. doi: 10.1016/j.stemcr.2018.03.006
39. Park S, Gönen M, Vu L, Minuesa G, Tivnan P, Barlowe TS, et al. Musashi2 sustains the mixed-lineage leukemia-driven stem cell regulatory program. *J Clin Invest.* (2015) 125:1286–98. doi: 10.1172/JCI78440
40. Emameh RZ, Nosrati H, Eftekhari M, Falak R, Khoshmirsafa M. Expansion of single cell transcriptomics data of SARS-CoV infection in human bronchial epithelial cells to COVID-19. *Biol Proc Online.* (2020) 22:16. doi: 10.1186/s12575-020-00127-3
41. Lucas R, Yang G, Gorshkov BA, Zemskov EA, Sridhar S, Umapathy NS, et al. Protein kinase c-α and arginase I mediate pneumolysin-induced pulmonary endothelial hyperpermeability. *Am J Respir Cell Mol Biol.* (2012) 47:445–53. doi: 10.1165/rcmb.2011-0332OC
42. Siflinger-Birnbom A, Johnson A. Protein kinase C modulates pulmonary endotherial permeability: a paradigm for acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* (2003) 284:L435–51. doi: 10.1152/ajplung.00106.2002
43. Monick M, Staber J, Thomas K, Hunninghake G. Respiratory syncytial virus infection results in activation of multiple protein kinase C isoforms leading to activation of mitogen-activated protein kinase. *J Immunol.* (2001) 166:2681–7. doi: 10.4049/jimmunol.166.4.2681
44. San-Juan-Vergara H, Peeples ME, Lockey RF, Mohapatra SS. Protein kinase C-alpha activity is required for respiratory syncytial virus fusion to human bronchial epithelial cells. *J Virol.* (2004) 78:13717–26. doi: 10.1128/JVI.78.24.13717-13726.2004
45. Liu M, Yang Y, Gu C, Yue Y, Wu KK, Wu J, et al. Spike protein of SARS-CoV stimulates cyclooxygenase-2 expression via both calcium-dependent and calcium-independent protein kinase C pathways. *FASEB J.* (2007) 21:1586–96. doi: 10.1096/fj.06-6589com
46. Singla S, Chen J, Sethuraman S, Sysol JR, Gampa A, Zhao S, et al. Loss of lung WWOX expression causes neutrophilic inflammation. *Am J Physiol Lung Cell Mol Physiol.* (2017) 312:L903–11. doi: 10.1152/ajplung.00034.2017
47. Butowt R, Bilinska K. SARS-COV-2: olfaction, brain infection, and the urgent need for clinical samples allowing earlier virus detection. *ACS Chem Neurosci.* (2020) 11:1200–3. doi: 10.1021/acschemneuro.0c00172
48. Emameh RZ, Falak R, Bahreini E. Application of system biology to explore the association of neprilysin, angiotensin-converting enzyme 2 (ACE2), and carbonic anhydrase (CA) in pathogenesis of SARS-CoV-2. *Biol Proc Online.* (2020) 22:11. doi: 10.1186/s12575-020-00124-6
49. Sundar IK, Yin Q, Baier BS, Yan L, Mazur W, Li D, et al. DNA methylation profiling in peripheral lung tissues of smokers and patients with COPD. *Clin Epigenetics.* (2017) 9:38. doi: 10.1186/s13148-017-0335-5
50. Wang S, Chen YY, Li YP, Gu J, Gu SD, Shi H, et al. DISC1 overexpression promotes non-small cell lung cancer lung proliferation. *Oncotarget.* (2017) 8:65199–210. doi: 10.18632/oncotarget.18055
51. Marreiros R, Prikulis I, Müller-Schiffmann A, Moreira AR, Sahu S, Soloviev I, et al. P.1.016 - Molecular linking of influenza infection to cellular pathology of protein missassembly: the case of Disrupted-in-Schizophrenia 1 (DISC1). *Eur Neuropsychopharmacol.* (2018) 28:S15. doi: 10.1016/j.euroneuro.2017.12.034
52. Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, et al. IFITM proteins mediate the innate immune response to influenza A H1N1 virus, west nile virus, Dengue virus. *Cell.* (2009) 139:1243–54. doi: 10.1016/j.cell.2009.12.017
53. Aaron PA, Jamklang M, Uhrig JP, Gelli A. The human blood-brain barrier internalizes *Cryptococcus neoformans* via the EphA2-tyrosine kinase receptor. *Cell Microbiol.* (2018) 20:12811. doi: 10.1111/cmi.12811
54. Zhou Z, Ren L, Zhang L, Zhong J, Xiao Y, Jia Z, et al. Heightened innate immune responses in the respiratory tract of COVID-19 patients. *Cell Host Microbe.* (2020) 27:883–90. doi: 10.1016/j.chom.2020.04.017

55. Sehrawat S, Ernandez T, Cullere X, Takahashi M, Ono Y, Komarova Y, et al. AKAP9 regulation of microtubule dynamics promotes Epac1-induced endothelial barrier properties. *Blood.* (2011) 117:708–18. doi: 10.1182/blood-2010-02-268870
56. Woodruff TM, Shukla AK. The complement C5a-C5aR1 GPCR Axis in COVID-19 therapeutics. *Trends Immunol.* (2020) 41:965–7.
57. Birch CA, Molinar-Inglis O, Trejo J. Subcellular hot spots of GPCR signaling promote vascular inflammation. *Curr Opin Endocrine Metabo Res.* (2021) 16:37–42. doi: 10.1016/j.coemr.2020.07.011
58. Hameid RA, Cormet-Boyaka E, Keubler WM, Uddin M, Berdiev BK. SARS-CoV-2 may hijack GPCR signaling pathways to dysregulate lung ion and fluid transport. *Am J Physiol Lung Cell Mol Physiol.* (2021) 320:L430–5. doi: 10.1152/ajplung.00499.2020
59. Hammoudeh SA, Hammoudeh AM, Bhamidimarri PM, Mahboub B, Halwani R, Hamid Q, et al. Insight into molecular mechanisms underlying hepatic dysfunction in severe COVID-19 patients using systems biology. *World J Gastroenterol.* (2021) 27:2850–70. doi: 10.3748/wjg.v27.i21.2850

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 10 September 2021
doi: 10.3389/fvets.2021.742785

# Fatal Calf Pneumonia Outbreaks in Italian Dairy Herds Involving *Mycoplasma bovis* and Other Agents of BRD *Complex*

*Angela Fanelli*[1†], *Margie Cirilli*[1], *Maria Stella Lucente*[1], *Aya Attia Koraney Zarea*[1,2], *Domenico Buonavoglia*[1], *Maria Tempesta*[1] *and Grazia Greco*[1*†]

[1] Department of Veterinary Medicine, University of Bari Aldo Moro, Bari, Italy, [2] Department of Microbiology and Immunology, National Research Centre, Cairo, Egypt

OPEN ACCESS

**Edited by:**
Rohana P. Dassanayake, National Animal Disease Center (USDA ARS), United States

**Reviewed by:**
Carol Geralyn Chitko-McKown, United States Department of Agriculture, United States
Fulvio Marsilio, University of Teramo, Italy

***Correspondence:**
Grazia Greco
grazia.greco@uniba.it

**†ORCID:**
Angela Fanelli
orcid.org/0000-0002-8204-1230
Grazia Greco
orcid.org/0000-0002-4338-6746

**Specialty section:**
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

**Received:** 16 July 2021
**Accepted:** 10 August 2021
**Published:** 10 September 2021

**Citation:**
Fanelli A, Cirilli M, Lucente MS, Zarea AAK, Buonavoglia D, Tempesta M and Greco G (2021) Fatal Calf Pneumonia Outbreaks in Italian Dairy Herds Involving Mycoplasma bovis and Other Agents of BRD Complex. Front. Vet. Sci. 8:742785. doi: 10.3389/fvets.2021.742785

*Mycoplasma bovis* is increasingly recognized worldwide as an important cause of disease with major welfare and production impairments on cattle rearing. Although it was detected in veal calves and beef cattle, little is known on the infection impact and on its temporal morbidity pattern in Italian dairy herds. Thus, this study aimed to investigate the involvement of *M. bovis* on fatal calf pneumonia outbreaks that occurred during 2009–2019 in 64 Italian dairy farms. Furthermore, a deeper diagnostic workup of concurrent infection with other viral and bacterial respiratory pathogens was assessed. Out of the investigated fatal pneumonia cases, *M. bovis* was frequently detected (animal prevalence, 16.16%; 95%CI, 11.82–21.33; herd prevalence, 26.56; 95%CI, 16.29–39.08) either as the single agent of the disease in more than half of the positive samples (20/37) or in concurrent infections with *Histophilus somni* (9/37, 24.3%), *Mannheimia haemolytica* (6/37, 16.621%), *Trueperella pyogenes* (1/37, 2.70%), *Pasteurella multocida* (1/37, 2.70%), bovine respiratory syncytial virus (5/37, 13.51%), and bovine viral diarrhea virus (2/37, 5.55%). Based on time-series analysis, *M. bovis* was recorded in the area since 2009 with outbreaks displaying a clear morbidity seasonal pattern with peaks in April (43.21%) and in September (13.51%). This might be due to the stressing conditions during spring and late summer periods. Results of this study highlight that *M. bovis* infection warrants consideration, and control measures are needed given its involvement in lethal pneumonia outbreaks in dairy herds from an extended area.

**Keywords: *Mycoplasma bovis*, bovine respiratory disease (BRD), fatal pneumonia, seasonal-trend decomposition based on loess (STL), Italy**

## INTRODUCTION

*Mycoplasma bovis* is a cell-wall-less bacterial pathogen, included in the class of *Mollicutes* (1). It is recognized as a causative agent of several diseases in cattle that have severe economic consequences for producers (1). In dairy cattle, *M. bovis* is probably the most common causative agent of mycoplasma mastitis, with arthritis and otitis media sometimes observed in infected animals (2). Noteworthy, it contributes to the occurrence of the bovine respiratory disease complex (BRD), a multifactorial syndrome consisting of chronic bronchopneumonia and pharyngitis, although often the infection can remain subclinical (3).

Under natural condition, *M. bovis* interanimal transmission occurs mainly via colostrum, milk, air-borne, intrauterine, and contaminated semen (4). Moreover, *M. bovis* has the capability to produce a biofilm, making it possible for the bacterium to survive in the environment while withstanding the action of heat or desiccation (5). It was shown that *M. bovis* is able to maintain viability in the environment for months at low temperatures and weeks at room temperature on a variety of substrates in infected herds (5).

After infection, *M. bovis* can spread through the bloodstream, establishing a long-term persistent infection through escaping the immune response. *M. bovis*, as primary agent or under the action of concomitant stressing factors such as weaning, transport, or relocation to feedlots, may impair the host immune system efficiency resulting in the onset of the disease (6) including severe, often fatal, pneumonia (7, 8). Case fatality is estimated to be 5–10% or higher in more severe cases, with morbidity reaching 35% (3).

Although the real economic burden of the disease has not yet been evaluated, in the last few years, *M. bovis* has been increasingly recognized as a significant concern for the cattle industry due to milk loss, decreased weight gain, and cost for veterinary and drug treatments. Furthermore, *M. bovis* infection is particularly important from an animal welfare perspective being the persistent debilitating disease that is unresponsive to therapy (9). Indeed, the antibiotic resistance, the presence of asymptomatic carriers, and the lack of effective vaccines are identified as the major constraints in the control of the disease (10).

*M. bovis* was included in the EU-funded DISCONTOOLS project (https://www.discontools.eu/), which provides a decision tool for diseases prioritization in order to improve the application of preventive and control measures (11). According to epidemiological investigations, the most effective way to prevent the infection spread is based on the strict adoption of biosecurity measures and restrictions on animal movements (12). Currently, although in Europe, *M. bovis* occurrence does not incur official restrictions on livestock trade, nevertheless, some importing countries are requesting cattle to test free from the infection (10).

In Italy, *M. bovis* was detected in veal calves and beef cattle with pneumonic lesions at slaughter (13) and in batches of imported bulls stabled in farms in northern Italy (14). The infection was also recently reported in healthy and BRD symptomatic beef cattle imported from France in Southern Italy (15). Nonetheless, information on *M. bovis* infection in dairy herds is scarce.

In this study, we document the involvement of *M. bovis* in fatal calf pneumonia outbreaks in Italian dairy herds together with its spatial–temporal distribution. Additionally, concurrent infections with other viral and bacterial respiratory pathogens were recorded.

## MATERIALS AND METHODS

From 2009 to 2019, 229 lung samples of calves (<11 months) from 64 dairy farms experiencing outbreaks of fatal calf pneumonia were submitted for postmortem diagnostic workup to the Laboratory of Infectious Diseases of the Department of Veterinary Medicine of the University of Bari (Italy). The farms were from Apulia, Basilicata, Campania, and Calabria regions and managed under either semi-intensive production systems farming, characterized by access to pasture grazing during certain periods of the year or intensive farming, in case of larger herds, where animals were housed in a free stabling system.

*M. bovis* and a panel of concurrent infectious agents involved in BRD were investigated. In detail, bacteria and viruses including *Histophilus somni*, *Mannheimia haemolytica*, *Pasteurella multocida*, *Trueperella pyogenes*, bovine herpesvirus-1 (BoHV-1), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), and bovine coronavirus (BCoV) were included. Samples were submitted for DNA and RNA extraction using commercial kits (Qiagen, Milan, Italy) and subsequently analyzed for the infectious agents by using quantitative PCR (qPCR) or conventional PCR (cPCR) assays as already described (**Supplementary Table S1**). For each pathogen, prevalence was computed at animal (AP) and herd (HP) levels along with the 95% confidence interval (95%CI). The spatial distribution of farms that tested positive for *M. bovis* was mapped by using QGIS software version 3.6.0 (16). To respect farmers' privacy, herds were represented at municipality level. A heatmap was built to better visualize the case distribution. The analysis of pathogen species co-occurrence was performed to detect pairs of pathogens that infect hosts more or less frequently than expected. A probabilistic model, as developed by Veech (17), was used to test for pairwise patterns of species co-occurrence, with the significance level $\alpha$, set at $\leq$0.05.

With the aim of providing valuable insights on disease patterns, time-series analysis was performed, which is being widely implemented in the field of epidemiology (18–21). For the purpose of this study, an outbreak was defined as one of more cases occurring in the same epidemiological unit and month. The number of *M. bovis* outbreaks by calendar month was formatted into time series, and a seasonal trend decomposition based on loess (STL) was used to identify relevant seasonal patterns. Records from 2019 were excluded in the temporal analysis, as data for some months was missing. To extract the seasonality, the loess window was assigned to 13, as it is recommended to use the next odd number following the number of observations in each seasonal cycle (22). Scale bars were included in the plots to describe the range of each component of the decomposition. The relative interquartile range (IQR) was used to measure the variability in the data explained by each component. The relative IQR is computed as the IQR of each component of the decomposition compared to the IQR of the raw data. Quantile plot of the residuals was drawn to ensure that they approximate a normal distribution. All the statistics were done using R software 3.5.2 (23).

## RESULTS

The prevalence values at animal (AP) and herd (HP) level for all the detected bacteria and viruses are displayed in **Table 1**. *M. bovis* was the most frequently detected pathogen in the study area (AP, 16.16%; 95%CI, 11.82–21.33; HP, 26.56%; 95%CI, 16.29%−39.08%) (**Figure 1**). Furthermore, *M. bovis* was detected

**TABLE 1 |** Prevalence at animal and herd levels for *Mycoplasma bovis* and other pathogens associated with fatal pneumonia in farms from Southern Italy.

| Pathogen | Animal prevalence (%) (95%CI) | Herd prevalence (%) (95%CI) |
|---|---|---|
| *M. bovis* | 16.16 (11.82–21.33) | 26.56 (16.29–39.08) |
| *P. multocida* | 4.80 (2.42–8.43) | 6.25 (1.72–15.23) |
| *M. haemolytica* | 13.53 (9.38–18.66) | 20.31 (11.28–32.22) |
| *H. somni* | 7.86 (4.72–12.13) | 7.81 (2.58–17.29) |
| *T. pyogenes* | 1.31 (0.03–3.78) | 3.12 (0.38–10.87) |
| BoHV-1 | 4.80 (2.42–8.43) | 12.50 (5.55–23.15) |
| BVDV | 9.17 (5.76–13.67) | 12.50 (5.55–23.15) |
| BRSV | 6.98 (4.04–11.09) | 9.37 (3.51–19.29) |
| BCoV | 11.79 (7.91–16.68) | 3.12 (0.38–10.83) |

*BoHV-1, bovine herpesvirus-1; BVDV, bovine viral diarrhea virus; BRSV, bovine respiratory syncytial virus; BCoV, bovine coronavirus.*

as single pathogen in more than half of the positive lung samples (20/37). Details of the coinfections ($n = 17$) are provided in **Supplementary Table S2**. In particular, *M. bovis* was detected with *P. multocida* ($n = 1$, 2.70%), *M. haemolytica* ($n = 6$, 16.21%), *H. somni* ($n = 9$, 24.3%), and *T. pyogenes* ($n = 1$, 2.70%), and BVDV ($n = 2$, 5.54%), and BRSV ($n = 5$, 13.51%). No mixed infection of *M. bovis* with BCoV or BoHV-1 was recorded.

Out of 36 potential species pairs (**Figure 2**), 23 were considered in the co-occurrence analysis after removing the other 13 (36.11 %) with the expected outcomes lower than one. The majority of the pathogen pairs had random associations, while non-random associations were recorded for a few pairs including *M. bovis*–*H. somni*, *P. multocida*–BVDV, BVDV–BRSV, and BRSV–*H. somini* (**Figure 2**). Furthermore, a negative significant association was detected for the *M. bovis* and BCoV pair, as these two species co-occurred at a frequency less than expected.

From 2009 to 2018, 37 outbreaks associated with *M. bovis* infection were recorded, with the majority occurring in 2015 ($n = 6$) and 2018 ($n = 5$). Considering the STL decomposition, the scale bars included in the plots showed that the remainder component of the decomposition had the highest IQR (**Figure 3**). This is mainly due to the considerable peak of outbreaks observed in 2015 and 2018, for which trend and seasonality components were not able to explain the variability of the data. The relative IQR measures, which exclude extreme values, were 37.66% for the seasonal component, 19.23% for the trend component, and 40.59% for the remainder. The seasonality pattern was constant over the period of analysis. Out of 37 *M. bovis* outbreaks, 16 (43.21%) occurred in April, 5 (13.51%) in September, and 4 (10.81%) in December, with only sporadic events spread over other months (i.e., January, March, October, or November) (**Supplementary Figure S1**).

## DISCUSSION

In this study, *M. bovis* was the agent most frequently detected from fatal calf pneumonia cases (AP, 16.16%; 95%CI, 11.82–21.33) occurring in dairy farms (HP, 26.56; 95%CI, 16.29–39.08) from Southern Italy during a 10-year period. *M. bovis* was detected as the single agent in half of positive lungs or in association with *H. somni* (9/37, 24.3%), followed by *M. haemolytica* (6/37, 16.21%), *T. pyogenes* (1/37, 2.70%), *P. multocida* (1/37, 2.70%), BRSV (5/37, 13.51%), and BVDV (2/37, 5.54%) (**Figure 2**). However, as the samples all came from fatal cases, it was not possible to determine how prevalent this pathogen is in non-fatal cases.

*M. bovis* occurrence has been described worldwide with an increasing number of reports of calf pneumonia cases from Britain (24), Ireland (25), Canada (26, 27), and Switzerland (28). In Italy, *M. bovis*, together with other BRD agents, was recently reported both in healthy and sick animals (14, 15, 29), stressing the importance of the agent as a primary concern in the Italian feedlot system. To date, no studies investigated fatal pneumonia outbreaks involving *M. bovis* with regard their temporal pattern. Thus, this work provides an important contribution to the knowledge on *M. bovis*, representing the first report investigating a series of fatal calf pneumonia outbreaks that occurred in dairy herds during a 10-year period in Southern Italy.

It is well-known that BRD is a multifactorial syndrome involving different bacterial or viral infectious agents and management factors, thus posing a real challenge in the diagnose without laboratory investigations (28, 30). As reported in previous studies, *M. bovis* was found to be positively associated with BVDV infection (**Figure 2**), although at low frequency, thus supporting for the synergistic role of the two pathogens in the occurrence of fatal bronchopneumonia (27, 31). Furthermore, there was evidence for a significant association between co-occurrence of *M. bovis* and *H. somni* and fatal pneumonia cases. This finding is of particular interest, *H. somni* being an emerging pathogen in the study area (15). Furthermore, *H. somni* was also shown to be widely implicated in pneumonia cases of cattle under 12 months of age in Ireland (32). Finally, in line with previous studies, coinfection of *M. bovis* and *M. haemolytica* was found (33), although the probabilistic model analysis suggests that this was a random association.

The main objective of the study was to investigate the occurrence of *M. bovis* in fatal calf pneumonia cases. Nevertheless, this work also highlighted the spread of other viral and bacterial respiratory pathogens in Italian dairy herds. Indeed, in line with previous studies, significant associations between the pairs of pathogens, including BVDV–BRSV (34) and BRSV–*H. somni* (35), were recorded. These pairs occurred at a frequency higher than expected, hinting that different distinct synergism of pairwise pathogens may occur in BRD pathogenesis.

Based on STL analysis, the occurrence of the *M. bovis* outbreaks revealed a time-dependent behavior with peaks in April (43.21%) and September (13.51%) (**Figure 3**). This is of particular importance, as detecting the relevant risk periods provides useful information for disease preparedness. Nevertheless, the seasonal pattern described in this work did not overlap those ones reported in previous studies performed in indoor systems that recorded the highest incidence

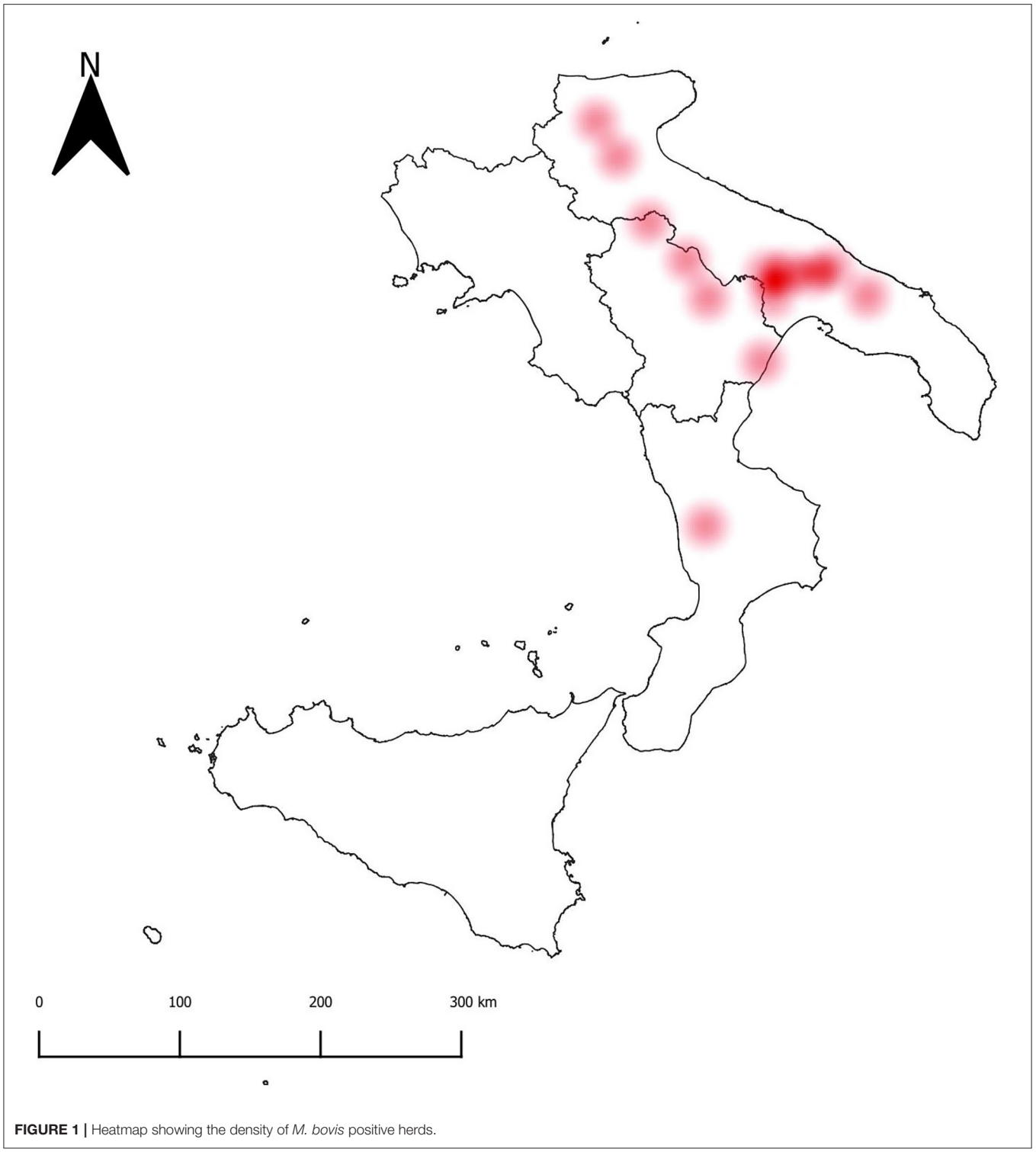



**FIGURE 1 |** Heatmap showing the density of *M. bovis* positive herds.

of pneumonia during colder seasons (14). Possibly, harsh temperature fluctuations rather than the cold temperatures may have played a role in the outbreak occurrence due to the remarkable changes in weather condition experienced during springtime and late summer in our study area. Given the seasonal importance, the effect of climatic stress on the hosts' disease susceptibility should be better investigated with further research.

Based on the time-series analysis, *M. bovis* occurrence was recorded in the area since 2009 (**Figures 2**, **3**), but the sources

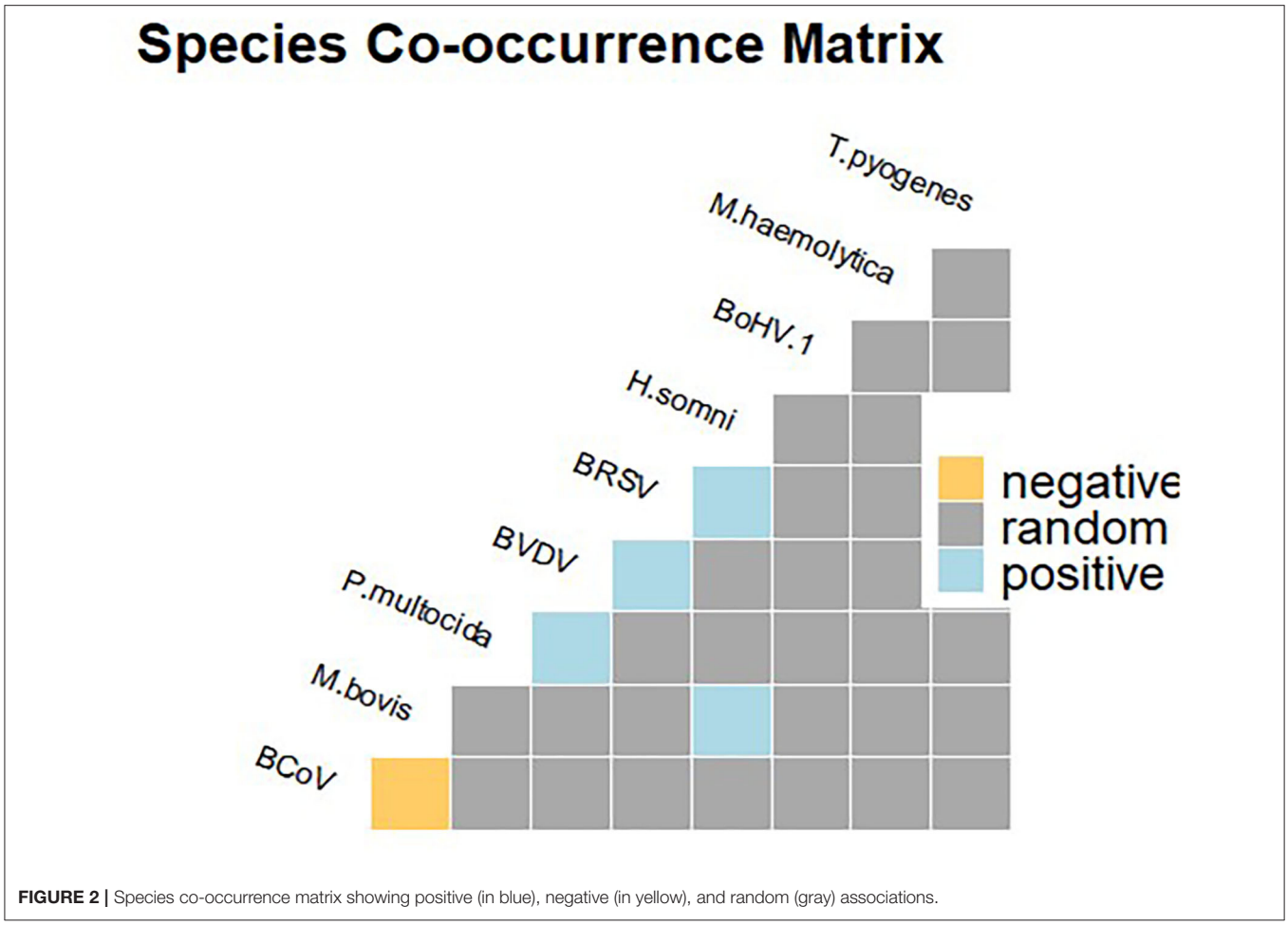



**FIGURE 2 |** Species co-occurrence matrix showing positive (in blue), negative (in yellow), and random (gray) associations.

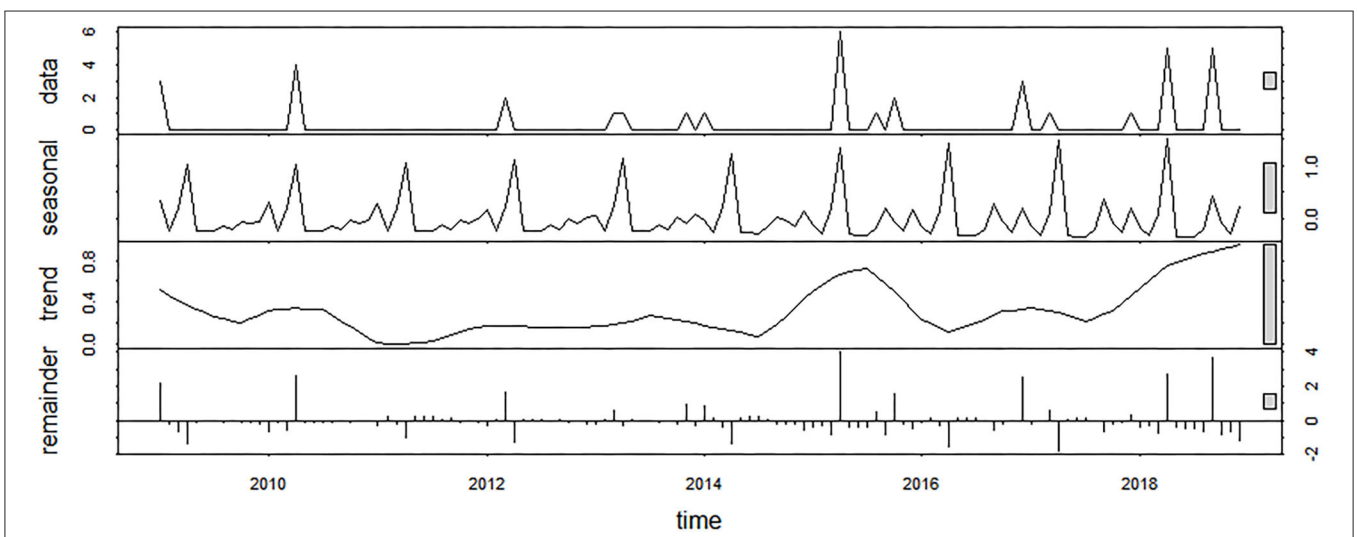



**FIGURE 3 |** Seasonal decomposition of *M. bovis* outbreaks using an additive model (raw data, seasonal component, trend component, and remainder).

for the outbreaks could not be identified, although the putative role of subclinical infected livestock introduced via the trade cannot be ruled out. Indeed, previous studies documented the introduction of the common BRD pathogens via imported cattle from France (15). On the other hand, other studies identified the presence of *M. bovis* infection in subclinical adult animals

as the potential source of infection to the newly imported calves (14).

To conclude, based on the time-series analysis, *M. bovis* was in the area since 2009, with outbreaks displaying a clear morbidity seasonal pattern with peaks in April (43.21%) and in September (13.51%), which might be due to unknown stress conditions during spring and late summer periods. Results of this study highlight that *M. bovis* infection warrants consideration, and control measures are needed given its involvement in lethal pneumonia outbreaks in dairy herds from an extended area. One limitation of the present study is the lack of anamnestic data on the infected animals. Additionally, samples were not tested for bovine adenovirus (BAdV) and bovine parainfluenza virus 3 (BPIV-3), which have been recently found in BRD-affected beef steers in Southern Italy (36). Despite these limitations, results from this study made an important contribution from the animal health perspective.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

Ethical review and approval was not required for the animal study because The study included samples that were voluntary submitted from the animal owners to the diagnostic laboratory for diagnostic workup. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

GG: conceptualization, data curation and analysis, methodology, investigation and supervision, and writing. AF: data curation, formal analysis, and writing—original draft preparation. MC and ML: laboratory analysis. AF, GG, MC, ML, AZ, DB, and MT: reviewing and editing. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.742785/full#supplementary-material

## REFERENCES


1. Brown DR, Whitcomb RF, Bradbury JM. Revised minimal standards for description of new species of the class Mollicutes (division Tenericutes). *Int J Syst Evol Microbiol.* (2007) 57:2703–19. doi: 10.1099/ijs.0.64722-0
2. Nicholas RAJ, Ayling RD. *Mycoplasma bovis*: disease, diagnosis, and control. *Res Vet Sci.* (2003) 74:105–12. doi: 10.1016/S0034-5288(02)00155-8
3. Nicholas R, Ayling R, McAuliffe L. Bovine respiratory disease. In: Nicholas R, Ayling R, McAuliffe L, editors. *Mycoplasma Diseases of Ruminants*. Oxfordshire: CABI Wallingford. p. 132–68. doi: 10.1079/9780851990125.0132
4. Dudek K, Nicholas RAJ, Szacawa E, Bednarek D. *Mycoplasma bovis* infections—occurrence, diagnosis and control. *Pathogens.* (2020) 9:1–21. doi: 10.3390/pathogens9080640
5. McAuliffe L, Ellis RJ, Miles K, Ayling. RD, Nicholas RAJ. Biofilm formation by mycoplasma species and its role in environmental persistence and survival. *Microbiology.* (2006) 152:913–22. doi: 10.1099/mic.0.28604-0
6. Perez-Casal J. Pathogenesis and virulence of *Mycoplasma bovis*. *Vet Clin North Am Pract.* (2020) 36:269–78. doi: 10.1016/j.cvfa.2020.02.002
7. Askar H, Chen S, Hao H, Yan X, Ma L, Liu Y, et al. Immune evasion of *Mycoplasma bovis*. *Pathogens.* (2021) 10:297. doi: 10.3390/pathogens10030297
8. Khodakaram-Tafti A, Lopez A. Immunohistopathological findings in the lungs of calves naturally infected with *Mycoplasma bovis*. *J Vet Med Ser A.* (2004) 51:10–4. doi: 10.1111/j.1439-0442.2004.00596.x
9. Maunsell FP, Donovan GA. *Mycoplasma bovis* infections in young calves. *Vet Clin North Am Food Anim Pract.* (2009) 25:139–77. doi: 10.1016/j.cvfa.2008.10.011
10. Calcutt MJ, Lysnyansky I, Sachse K, Fox LK, Nicholas RAJ, Ayling RD. Gap analysis of Mycoplasma bovis disease, diagnosis and control: an aid to identify future development requirements. *Transbound Emerg Dis.* (2018) 65:91–109. doi: 10.1111/tbed.12860
11. Discontools. Diseases Databases. Available online at: https://www.discontools.eu/ (accessed April 14, 2021).
12. Nicholas RAJ, Fox LK, Lysnyansky I. Mycoplasma mastitis in cattle: to cull or not to cull. *Vet J.* (2016) 216:142–7. doi: 10.1016/j.tvjl.2016.08.001
13. Radaelli E, Luini M, Loria GR, Nicholas RAJ, Scanziani E. Bacteriological, serological, pathological and immunohistochemical studies of *Mycoplasma bovis* respiratory infection in veal calves and adult cattle at slaughter. *Res Vet Sci.* (2008) 85:282–90. doi: 10.1016/j.rvsc.2007.11.012
14. Catania S, Gastaldelli M, Schiavon E, Matucci A, Tondo A, Merenda M, et al. Infection dynamics of mycoplasma bovis and other respiratory mycoplasmas in newly imported bulls on italian fattening farms. *Pathogens.* (2020) 9:537. doi: 10.3390/pathogens9070537
15. Pratelli A, Cirone F, Capozza P, Trotta A, Corrente M, Balestrieri A, et al. Bovine respiratory disease in beef calves supported long transport stress: an epidemiological study and strategies for control and prevention. *Res Vet Sc.* (2021) 135:450–5. doi: 10.1016/j.rvsc.2020.11.002
16. QGIS Development Team. *QGIS Geographic Information System*. Noosa: Open Source Geospatial Foundation (2019).
17. Veech JA. A probabilistic model for analysing species co-occurrence. *Glob Ecol Biogeogr.* (2013) 22:252–60. doi: 10.1111/j.1466-8238.2012.00789.x
18. Fanelli A, Tizzani P, Ferroglio E, Belleau E. *Cheilospirura hamulosa* in the Rock Partridge (*Alectoris graeca saxatilis*): epidemiological patterns and prediction of parasite distribution in France. *Diversity.* (2020) 12:484. doi: 10.3390/d12120484
19. Fanelli A, Tizzani P. Spatial and temporal analysis of varroosis from 2005 to 2018. *Res Vet Sci.* (2020) 131:215–21. doi: 10.1016/j.rvsc.2020.04.017
20. Iacopelli F, Fanelli A, Tizzani P, Berriatua E, Prieto P, Martínez-Carrasco C, et al. Spatio-temporal patterns of sarcoptic mange in red deer and Iberian ibex in a multi-host natural park. *Res Vet Sci.* (2020) 128:224–9. doi: 10.1016/j.rvsc.2019.11.014
21. Fanelli A, Perrone A, Ferroglio E. Spatial and temporal dynamics of wild boars *Sus scrofa* hunted in Alpine environment. *Eur J Wildl Res.* (2021) 67:47. doi: 10.1007/s10344-021-01484-4
22. Cleveland RB, Cleveland WS, McRae JE, Terpenning I. STL: a seasonaltrend decomposition procedure based on loess. *J Off Stat.* (1990) 6:3–37.
23. R Core Team. *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing (2018). Available online at: https://www.r-project.org/ (accessed September 10, 2020).
24. Ayling RD, Bashiruddin SE, Nicholas RAJ. *Mycoplasma* species and related organisms isolated from ruminants in Britain between 1990 and 2000. *Vet Rec.* (2004) 155:413–6. doi: 10.1136/vr.155.14.413
25. Bell CJ, Blackburn P, Elliott M, Patterson TIAP, Ellison S, Lahuerta-Marin A, et al. Investigation of polymerase chain reaction assays to improve detection of bacterial involvement in bovine respiratory disease.

*J Vet Diagnostic Investig.* (2014) 26:631–4. doi: 10.1177/1040638714540166
26. Booker CW, Abutarbush SM, Morley PS, Jim GK, Pittman TJ, Schunicht OC, et al. Microbiological and histopathological findings in cases of fatal bovine respiratory disease of feedlot cattle in western Canada. *Can Vet J.* (2008) 49:473–81.
27. Gagea MI, Bateman KG, Shanahan RA, Van Dreumel T, McEwen BJ, Carman S, et al. Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. *J Vet Diagnostic Investig.* (2006) 18:29–40. doi: 10.1177/104063870601800105
28. Tschopp R, Bonnemain P, Nicolet J, Burnens A. Epidemiological study of risk factors for Mycoplasma bovis infections in fattening calves. *Schweiz Arch Tierheilkd.* (2001) 143:461–7.
29. Cirone F, Padalino B, Tullio D, Capozza P, Losurdo M, Lanave G, et al. Prevalence of pathogens related to bovine respiratory disease before and after transportation in beef steers: preliminary results. *Animals.* (2019) 9:1–7. doi: 10.3390/ani9121093
30. Szeredi L, Jánosi S, Pálfi V. Microbiological and pathological examination of fatal calf pneumonia cases induced by bacterial and viral respiratory pathogens. *Acta Vet Hung.* (2010) 58:341–56. doi: 10.1556/avet.58.2010.3.7
31. Haines DM, Martin KM, Clark EG, Kee Jim G, Janzen ED. The immunohistochemical detection of Mycoplasma bovis and bovine viral diarrhea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. *Can Vet J.* (2001) 42:857–60.
32. Murray GM, More SJ, Sammin D, Casey MJ, McElroy MC, O'Neill RG, et al. Pathogens, patterns of pneumonia, and epidemiologic risk factors associated with respiratory disease in recently weaned cattle in Ireland. *J Vet Diagnostic Investig.* (2017) 29:20–34. doi: 10.1177/1040638716674757
33. Caswell JL, Bateman KG, Cai HY, Castillo-Alcala F. Mycoplasma bovis in respiratory disease of feedlot cattle. *Vet Clin North Am Food Anim Pract.* (2010) 26:365–79. doi: 10.1016/j.cvfa.2010.03.003
34. Liu L, Hägglund S, Hakhverdyan M, Alenius S, Larsen LE, Belák S. Molecular epidemiology of bovine coronavirus on the basis of comparative analyses of the S gene. *J Clin Microbiol.* (2006) 44:957–60. doi: 10.1128/JCM.44.3.957-960.2006
35. Corbeil LB. Histophilus somni host–parasite relationships. *Anim Heal Res Rev.* (2007) 8:151–60. doi: 10.1017/S1466252307001417
36. Padalino B, Cirone F, Zappaterra M, Tullio D, Ficco G, Giustino A, et al. Factors affecting the development of bovine respiratory disease: a cross-sectional study in beef steers shipped from France to Italy. *Front Vet Sci.* (2021) 8:672. doi: 10.3389/fvets.2021.627894

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 02 November 2021
doi: 10.3389/fvets.2021.764701

# Prevalence, Risk Factors, and Antimicrobial Resistance Profile of Respiratory Pathogens Isolated From Suckling Beef Calves to Reprocessing at the Feedlot: A Longitudinal Study

*Diego Nobrega*[1]*, *Sara Andres-Lasheras*[2], *Rahat Zaheer*[2], *Tim McAllister*[2], *Elizabeth Homerosky*[3], *R. Michele Anholt*[4] and *Craig Dorin*[3]

[1] Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada, [2] Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada, [3] Veterinary Agri-Health Services, Rocky View County, AB, Canada, [4] One Health at UCalgary, University of Calgary, Calgary, AB, Canada

OPEN ACCESS

**Edited by:**
*Annamaria Pratelli, University of Bari Aldo Moro, Italy*

**Reviewed by:**
*Patrick Blackall, The University of Queensland, Australia*
*Conny Turni, The University of Queensland, Australia*

***Correspondence:**
*Diego Nobrega*
*dnobrega@uoguelph.ca*

**Specialty section:**
*This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science*

**Received:** *25 August 2021*
**Accepted:** *29 September 2021*
**Published:** *02 November 2021*

**Citation:**
*Nobrega D, Andres-Lasheras S, Zaheer R, McAllister T, Homerosky E, Anholt RM and Dorin C (2021) Prevalence, Risk Factors, and Antimicrobial Resistance Profile of Respiratory Pathogens Isolated From Suckling Beef Calves to Reprocessing at the Feedlot: A Longitudinal Study. Front. Vet. Sci. 8:764701. doi: 10.3389/fvets.2021.764701*

Here, we investigated the prevalence and risk factors for the presence of *Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Pasteurella multocida* in the respiratory tract of calves from the spring processing to the reprocessing at feedlots. Additionally, we characterized, phenotypically and genotypically, the antimicrobial resistance (AMR) profile of the four species. Calves from 22 cow–calf operations were enrolled in the study ($n$ = 30 calves per operation) and sampled by deep nasopharyngeal swabs at three time points: spring processing, weaning, or induction into feedlots, and at reprocessing at the feedlot. Isolates were tested for susceptibility using the minimum inhibitory concentration (MIC) test against commonly administered antimicrobials. Additionally, a subset of isolates underwent whole-genome sequencing to infer presence of AMR genes and resistance determinants. Among studied pathogens, *P. multocida* was the most prevalent species, regardless of time point, followed by *M. haemolytica*, *M. bovis*, and *H. somni*. For *M. bovis*, a sharp increase in prevalence was detected at the reprocessing sampling, whereas for *P. multocida*, an increase in prevalence was observed at the weaning/induction sampling. Comingling and co-location of feedlots were not associated with prevalence of any respiratory pathogen. In terms of AMR, resistance against macrolides was prevalent in *M. bovis*, with most isolates resistant against tildipirosin, tilmicosin, and tylosin. In general, there was limited evidence to support an increase in resistance rates of respiratory bacteria from the spring processing to reprocessing at feedlots, with the exception of florfenicol resistance in *M. bovis*, which increased at reprocessing. Metaphylactic administration of tetracyclines at feedlot induction was not associated with the MIC of tetracyclines in any respiratory bacteria. Conversely, there were clear associations between the parenteral use of macrolides as metaphylaxis at the feedlot induction, and increased MIC against

macrolides in *P. multocida*, *M. haemolytica*, and *H. somni*. Overall, the AMR phenotypes were corroborated by presence of AMR genes. We hypothesize that the administration of macrolides such as tulathromycin at feedlot induction contributes to historical changes in macrolides MIC data of respiratory bacteria of beef cattle.

**Keywords: antimicrobial resistance, beef calves, bovine respiratory disease, *Mycoplasma bovis*, *Pasteurella multocida***

## INTRODUCTION

Antimicrobial resistance (AMR) has emerged as one of the most important threats facing public health globally. By 2050, it is estimated that AMR will claim 10 million human lives per year (1). The rapid dissemination of AMR is aggravated by indiscriminate use of antimicrobials in humans and animals. It is increasingly recognized that the administration of antimicrobials in food-producing animals can contribute to the emergence and spread of antimicrobial resistant strains in animals as well as in humans (2). Accordingly, use of antimicrobials in livestock is under increasing scrutiny.

Bovine respiratory disease (BRD) is one of the leading causes of morbidity and mortality for North American beef cattle (3), and a frequent reason for the use of antimicrobials at feedlots (4). A number of factors can predispose to BRD, including host (age, genetics, and co-infections), agent (causative pathogen), and environmental factors such as transportation of animals, comingling, and extreme weather (5). Among causative agents, *Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Pasteurella multocida* are prevalent in clinical BRD (6). BRD is often polymicrobial, with complex interactions between pathogens and the host immune system. The complex nature of BRD infections challenges the accurate identification of cases (7), which when identified are commonly treated with antimicrobials.

Cow–calf operations in Western Canada are mostly extensive and characterized by large pastures in which animals are housed. Antimicrobials commonly administered to treat respiratory disease in Canadian cow–calf cattle include phenicols, tetracyclines, and macrolides (8); the latter are classified as critically important antimicrobials of the highest priority to human health according to the World Health Organization (WHO) (9). Regional increases in resistance rates of BRD pathogens against specific antimicrobial classes have been reported in North America and France (10, 11), which may have been fueled by the administration of antimicrobials and spread of resistant clones (12).

The use of antimicrobials has been implicated as a cause of decreased susceptibility in BRD bacteria from cattle (13). In Western Canadian cow–calf operations, antimicrobials are commonly administered for treatment of lameness in cow and bulls, and for respiratory disease and diarrhea in calves (14). It remains unknown whether and to what extent early-life exposure to antimicrobials can impact pathogen carriage and AMR at the feedlot. At feedlots, antimicrobials are frequently administered to calves as metaphylaxis; e.g., the antimicrobial treatment of a group of animals to prevent or control infectious diseases in high-risk animals at feedlot entry. Metaphylaxis is a highly effective practice to reduce morbidity and mortality of feedlot cattle (15). Arguably, limitations of BRD diagnostics and the ease in which antimicrobials can be administered to calves at feedlots largely contribute to the widespread adoption of metaphylaxis. In terms of administration, many antimicrobial formulations can be used for mass medication either in feed or in drinking water. Alternatively, parenteral (injectable) antimicrobials are also available and used routinely in Western Canadian operations as metaphylaxis (8). Altogether, routine metaphylaxis has led to substantial antimicrobial use as healthy, sometimes low-risk animals will also be treated. Given the increasing recognition of an AMR One Health framework, it is important to increase our understanding of potential impacts of metaphylaxis in AMR of BRD pathogens, which includes the study of potential effects of different antimicrobial classes toward AMR. Such assessment can later incorporate informed discussions on the risks and benefits of metaphylaxis in beef cattle, followed by the establishment of best practices related to the practice.

Our overarching goals were to identify factors associated with prevalence of respiratory pathogens, and to infer potential effects of antimicrobials on AMR in BRD-associated bacteria isolated from suckling beef calves. Specific objectives of this longitudinal study were to (1) estimate prevalence of respiratory pathogens in beef calves from branding through to reprocessing at the feedlot; (2) study potential risk factors for increased carriage of respiratory pathogens; and (3) investigate the AMR profile of respiratory pathogens at different time points, including the study of factors associated with resistance such as the metaphylaxic administration of antimicrobials.

## MATERIALS AND METHODS

### Ethics Statement

This study followed strict recommendations of the Canadian Council of Animal Care. The research protocol was reviewed and approved by the Lethbridge Research and Development Center's Animal Care Committee (Protocol Review #1639).

### Cow–Calf Operations and Feedlots

Producers were recruited using a client database from a beef cattle veterinary practice in the province of Alberta. Cow–calf operations were eligible for enrolment based on the following criteria: (i) physically located in the province of Alberta; (ii) a minimum herd size of 30 cows; (iii) expected retained ownership of calves after feedlot induction; and (iv) agreement to provide detailed information about the health and antimicrobial use in enrolled calves. Producers were informed that their

participation would be anonymous, and they would be financially compensated for the use of their cattle.

Twenty-two cow–calf operations that met the eligibility criteria agreed to participate. Cow–calf operations ranged from 200 to 3,200 cows and were a mixture of pure-bred and mixed-breed commercial operations. From each operation, 30 calves were randomly selected during spring processing (branding) from April to June 2017. Calves were selected using a systematic random approach, where every *k*th calf pulled for processing at a cow–calf operation was selected. *k* was defined as the nearest integer of the quotient of *N* over 30, where *N* stands for the number of calves to be processed in the operation. Calves selected were clinically healthy and had not been exposed to antimicrobials. Assuming a low within-herd prevalence of respiratory pathogens in pre-weaned calves (16), an intraclass correlation coefficient of 0.25, and an error rate of 5%, sampling 30 animals per operation allowed us to estimate a prevalence of 7.65% of any BRD-associated pathogen with 90% power at any given time point. The study had 76% power to detect an odds ratio of 2 associated with an increased risk of AMR in respiratory pathogens isolated from calves that received antibiotics at feedlot induction. This was based on the assumption that 6.3% of feedlot cattle would be BDR-positive (17), half of feedlots employed metaphylaxis (18), a baseline prevalence of any given resistance of 20% in respiratory pathogens, and a standard deviation of operation-level random effects of 2.

Management protocols were determined by each producer. The most common procedures at spring processing included vaccination [modified-live BVD type 1 (Bovine Viral Diarrhea Virus) and 2/IBR/PI3/BRSV (Infectious Bovine Rhinotracheitis Virus, Parainfluenza Virus 3, Bovine Respiratory Syncytial Virus) vaccine, seven- or eight-way clostridial, *H. somni*, and *M. haemolytica* vaccines] and castration of males. The spring processing branding protocol of some operations included dehorning, growth implants, and/or administration of meloxicam. Calves were fed on the dam's milk and had access to forage. Calves were weaned in the fall and moved to a feedlot. All calves underwent "hard" weaning where they were abruptly separated from dams. Calves from six operations were moved to a distant feedlot (<1–3 h of driving). In the remaining 16 operations, calves were placed in a feedlot co-located with the cow–calf operation. A typical processing protocol for fall-placed, high-risk calves included a modified-live BVD type 1 and 2/IBR/PI3/BRSV vaccine, seven- or eight-way clostridial bacterin, *H. somni* bacterin, *M. haemolytica* bacterin, endectocide, and an anabolic implant. At feedlots, adoption of metaphylaxis varied by producer, and this information was recorded. Animals were housed according to sex in large outdoor dirt-floor pens with porosity fencing. Calves were fed rations once or twice daily, which were formulated to meet standard nutritional requirements of backgrounding or finishing feedlot cattle.

## Sampling Protocol

Samples were collected at three time points: (i) spring processing (BRANDING) when calves were from 2 to 8 weeks old; (ii) at weaning or feedlot induction (WEANING/INDUCTION), with animals aged from 5 to 8 months old; and (iii) at reprocessing at the feedlot (REPROCESSING) when calves were from 9 months to 1 year of age. From each animal, deep nasopharyngeal swabs (DNPS) were collected as described (**Supplementary Methods**). Following sample collection, guarded culture swabs (CP Group, Newmarket, ON) were placed in Amies culture media (ThermoFisher Scientific, Mississauga, ON) and transported to the laboratory.

At WEANING/INDUCTION, timing of sampling was not consistent between herds. As rapid changes in nasopharyngeal microbiota following arrival at the feedlot are expected (19), it was important to distinguish samples collected at weaning at the cow–calf environment from samples collected post-weaning after arrival at the feedlot. Producers were asked if calves were comingled with calves from other sources at the feedlot and, if so, when the DNPS was collected relative to the time of transport and comingling. Calves sampled at weaning, prior to or within 24 h of feedlot arrival were considered to be "not comingled" at WEANING/INDUCTION. Additionally, calves from producers that did not introduce animals from other sources at their own feedlots were also considered to be "not comingled" at WEANING/INDUCTION. All other calves were considered "comingled." At REPROCESSING, all animals were considered "comingled" except in operations where producers fed their own animals without introducing animals from other sources.

Number of days on feed (DOF) at feedlots was obtained for each calf. Additionally, all antimicrobial treatments were recorded, from birth up to REPROCESSING, including the reason for treatment and the type of antimicrobial administered. Pasture (pre-weaning) treatments, metaphylactic antimicrobial use at feedlot induction, and therapeutic antimicrobial use during feeding were recorded. Antimicrobial use data at feedlots were recovered from electronic management systems. Where producer records clearly stated "no antimicrobials," AMU exposure was considered as "none." Where farm records were either not provided or unclear regarding exposure to antimicrobials, the associated AMU history of an animal was classified as "unknown." Decisions involving antimicrobial use were made by producers with support from veterinarians.

## Bacteriology

After arrival at the laboratory, each swab was individually immersed in 1 ml of brain–heart infusion (BHI; BD) containing 20% glycerol and vortexed for 1 min. A 50-µl aliquot was plated onto tryptic soy agar with 5% sheep blood (BAP; Dalynn Biologicals, Calgary, AB, Canada) for the isolation of *H. somni*. A second aliquot (100 µl) was plated on blood agar supplemented with 15 µg/ml bacitracin (BAC; Dalynn) for the isolation of *M. haemolytica* and *P. multocida*. BAC plates were incubated in an aerobic atmosphere at 37°C for 24 h, and examined for the presence of suspected *P. multocida* and *M. haemolytica* colonies, whereas BAP plates were incubated for 2 days in a 5% $CO_2$ atmosphere before examination of *H. somni* colonies. When *M. haemolytica*, *P. multocida*, or *H. somni* suspected colonies were observed in primary cultures (20), three colonies were first sub-cultured onto separate BAP plates, from which one plate per sample/species was

randomly selected for further characterization. Bacteria were stored at −80°C in BHI supplemented with 20% glycerol for further analysis.

For the isolation of *M. bovis*, a 150-µl aliquot of the initial BHI-glycerol suspension was inoculated into 1.5 ml of PPLO broth (Dalynn Biologicals) (21) containing 500 µg/ml of ampicillin (Millipore Sigma, Oakville ON). The mixture was filter sterilized and incubated at 37°C in a 5% $CO_2$ atmosphere. After 5 days of incubation, 100 µl of enrichment was plated onto PPLO Agar + ampicillin plates. Plates were incubated for an additional 5 days under the same conditions and primary agar cultures were observed under a stereoscopic microscope. When bacterial growth was observed, an isolated colony was randomly selected and transferred to 1.5 ml PPLO + ampicillin broth. *M. bovis* positive PPLO broth cultures were stored at −80°C in PPLO supplemented with 20% glycerol and 0.5% pyruvate for further analysis.

Species confirmation was carried out for all isolates using PCR following protocols that were internally validated at the Lethbridge Research and Development Center (**Table 1**). Colonies were suspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and heated for 5 min at 95°C for DNA extraction. The lysate was vortexed and centrifuged, and 2 µl of the supernatant was used as DNA template in separate reactions for each species (**Table 1**). In all reactions, the HotStartTaq Plus Master Mix kit (Qiagen, Toronto, ON) was used.

## Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was carried out using broth microdilution. For *M. haemolytica* and *H. somni*, all isolates were tested, whereas 150 *P. multocida* isolates were randomly selected for AST. Commercial antimicrobial panels (Thermo Scientific, Mississauga, ON) were used, and tests were carried out according to the manufacturer guidelines. In brief, bacterial inocula were concentration-adjusted in either saline solution (*P. multocida* and *M. haemolytica*) (26) or Mueller-Hinton and yeast extract broth (*H. somni*). The working solutions were inoculated into 96-microwell commercial plates (50 µl per well for *P. multocida* and *M. haemolytica*; 100 µl per well for *H. somni*) that contained a series of two-fold dilutions of antimicrobials of interest (SensititreTM bovine/porcine plate format BOPO6F for *H. somni* and *M. haemolytica*; SensititreTM bovine/porcine plate format BOPO7F for *P. multocida*). Following incubation (35°C for 20 h for *M. haemolytica* and *P. multocida*; 35°C for 24 h in a 5% $CO_2$ incubator for *H. somni*), plates were visually examined for presence of bacterial growth. The minimum inhibitory concentration (MIC) was defined on a pathogen and antimicrobial basis according to the Clinical and Laboratory Standards Institute guidelines (27). Additionally, isolates were classified as susceptible, intermediate, or resistant against antimicrobials for which cattle-specific breakpoints have been defined (26). For the purpose of analysis, intermediate and resistant isolates were classified as resistant (or non-susceptible). Antimicrobials, concentrations tested, and breakpoints adopted are listed as a Supplementary Material (**Supplementary Table 1**).

For *M. bovis*, antimicrobial susceptibility testing was performed as suggested elsewhere (28, 29). Customized antimicrobial plates (30) (Trek Diagnostics, Oakwood, GA, USA) were used to test those antimicrobials that are most relevant for the treatment of *M. bovis* infections in feedlot cattle in western Canada (**Supplementary Table 2**). The AST custom plate contained 50 µl of a PPLO (21) suspension with antimicrobials in each well. *M. bovis* colonies were grown in PPLO broth with pyruvate for 72 h, and 500 µl of this culture was transferred to 500 µl of fresh PPLO and incubated for another 48 h. A 50-µl aliquot from this final solution was inoculated into each well of the AST plate yielding a concentration of $1 \times 10^3$-$1 \times 10^5$ CFU/ml per well. Plates were incubated for 48 h at 37°C and 5% $CO_2$. Results were recorded after 48 h and used to estimate the MIC (31). *M. bovis* ATCC 25523 was used as an internal quality control strain in all assays. Breakpoints were used to classify isolates as susceptible, intermediate, or resistant, as described elsewhere (30) (**Supplementary Table 2**).

**TABLE 1 |** Oligonucleotide primers, PCR protocols, and amplicon sizes for each cPCR assay.

| Bacteria, amplified gene | Primer sequences (5′-3′)[a] | Cycling conditions | Amplicon size (bp) | Primer reference |
|---|---|---|---|---|
| *Mannheimia haemolytica*, *lkt* | F: GTCCCTGTGTTTTCATTATAAG<br>R: CACTCGATAATTATTCTAAATTAG | 95°C, 5 min; (94°C, 30 s; 58°C, 45 s; 72°C, 60 s) ×35 cycles; 72°C, 10 min | 385 | (22) |
| *Pasteurella multocida*, 23S rRNA | F: GGCTGGGAAGCCAAATCAAAG<br>R: CGAGGGACTACAATTACTGTAA | 95°C, 5 min; (94°C, 30 s; 58°C, 45 s; 72°C, 60 s) ×35 cycles; 72°C, 10 min | 1,432 | (23) |
| *Histophilus somni*, 16S rRNA | F: GAAGGCGATTAGTTTAAGAG<br>R: TTCGGGCACCAAGTRTTCA | 95°C, 5 min; (94°C, 30 s; 55°C, 45 s; 72°C, 60 s) ×35 cycles; 72°C, 10 min | 400 | (24) |
| *Mycoplasma bovis*, *uvrC* | F: TTACGCAAGAGAATGCTTCA<br>R: TCATCCAAAAGCAAAATGTTAAA | 95°C, 5 min; (94°C, 30 s; 56°C, 45s; 72°C, 60 s) ×35 cycles, 72°C, 10 min | 171 | (25) |
| *Mycoplasma bovis*, 16S | F: GGGAGCAAACAGGATTAGATACCCT<br>R: TGCACCATCTGTCACTCTGTTAACCT | 95°C, 5 min; (94°C, 30 s; 56°C, 45s; 72°C, 60 s) ×35 cycles, 72°C, 10 min | 269 | |

[a] *F, forward primer; R, reverse primer.*

## Sequencing

Eighty-three isolates from BRD *Pasteurellaceae* species [*M. haemolytica* ($n = 25$), *P. multocida* ($n = 29$), and *H. somni* ($n = 29$)] were selected for sequencing. The selection protocol was based on inclusion of isolates of varying MIC levels (low, intermediate, and high) against selected antimicrobials (macrolides, fluoroquinolones, tetracyclines, and phenicols), from different operations and sampling points in order to ensure that all operations were represented. Isolates were streaked onto BAC plates for *M. haemolytica* and *P. multocida*, and BAP plates for *H. somni*. Plates were incubated overnight at 37°C (BAC), or for 48 h at 37°C in 5% $CO_2$ (BAP). A single colony was then sub-cultured onto BAC or BAP plates, and incubated as described above. Bacteria were diluted in TE (10 mM Tris, 1 mM EDTA), pH 8.0 buffer to an OD 600 of ≈2, equivalent to ≈2 × $10^9$ cells/ml. The cell suspension (1 ml) was transferred to a microcentrifuge tube and centrifuged for 2 min at 14,000 *g*. Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen, Montreal, QC, Canada) following manufacturer's instructions. DNA quality and quantity were estimated using a Nanodrop 2000 spectrophotometer and a Qubit Fluorometer with PicoGreen (Thermo Fisher Scientific, Mississauga, ON), respectively. Genomic library construction was performed using the Illumina Nextera XT DNA sample preparation kit (Illumina Inc., San Diego, CA, USA). Libraries were sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit V3 to generate 2 × 300 base paired-end reads.

Sequencing reads were *de novo* assembled into contigs using SPAdes version 3.13.0 with a multi-sized de Bruijn graph approach (32). Draft genome assemblies were annotated with Prokka (33). ABRicate version 0.8.7 (34) was used to screen contigs against the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (NCBI BioProject ID: PRJNA313047) for presence of AMR genes. The sequencing data of isolates used in this study have been submitted to the NCBI (under BioProject ID: PRJNA720670).

## Statistical Analysis

All analyses were carried out in R (35) using the following packages: *brms*, *lme4*, *mice*, and *runjags* (36–39).

### MIC50 and MIC90

Minimum inhibitory concentration results (in μg/ml) were summarized for each pathogen, sampling point, and antimicrobial tested using distribution tables. Likewise, the 50th and 90th MIC percentiles, defined as the MIC capable of inhibiting the growth of 50 and 90% of isolates ($MIC_{50}$ and $MIC_{90}$, respectively), were estimated for each species individually.

### Prevalence of Respiratory Pathogens and Associated Risk Factors

Prevalence of respiratory pathogens (*P. multocida*, *M. haemolytica*, *H. somni*, and *M. bovis*) was estimated at each sampling point (BRANDING, WEANING/INDUCTION, and REPROCESSING). Prior to model estimation, preliminary assessments were carried out to check for missing values. In 21 out of the 22 operations enrolled, at least 1 animal was lost to follow-up either at WEANING/INDUCTION or REPROCESSING (**Supplementary Table 3**). All calves from five cow–calf operations were lost to follow-up at REPROCESSING. Operation-conditional comparisons of prevalence of respiratory pathogens at WEANING/INDUCTION (the second sampling point) indicated no difference between calves from operations with at least one sample collected at REPROCESSING and calves from the five operations with no REPROCESSING data, meaning that the probability of missing values was most likely independent from the observed values (results not shown). Regardless, data from the five operations were excluded from prevalence estimates at the last sampling point (no imputation was attempted). For the remaining data, we used a multivariate imputation method based on chained equations for handling missing values. Imputation models were used to generate a set of 20 datasets with complete outcome information after 100 iterations each. Imputation was based on the "*2l.bin*" method from the *mice* package in R (38). Imputation models contained operation-level random effects, sex, and results from previous sampling(s) introduced as fixed effects. All Markov chains were visually inspected for convergence, where absence of trends for any chain was deemed adequate. If trends were detected for any inputted parameter, the number of iterations was increased until convergence was achieved. As samples were mostly collected in batches, DOF of missing samples were deemed as the most frequently observed value for samples that were collected at the same feedlot at that sampling. Likewise, a similar approach was used to infer the comingling status of missing samples. Thereafter, generalized linear mixed models with logit link and operation-specific random effects at the intercept level were fit in a Bayesian framework to estimate prevalence of respiratory pathogens, and to study associations between prevalence and potential risk factors [presence of feedlot onsite, sex, comingling status at weaning and results from previous sampling point(s)]. Binary indicators for potential risk factors were generated and introduced as predictors in multivariable models. Risk factor effects were assumed to be common to all levels of remaining model terms (no two- or three-way terms were considered). A minimum of five observations per factor was required for risk assessment; for instance, effects of previous sampling points at WEANING/INDUCTION were not assessed in absence of at least five calves harboring the bacteria at BRANDING. To account for potential deviations from original sampling protocols, DOF centered at 0 and 139 days (median DOF of REPROCESSING samples) were forced in WEANING/INDUCTION and REPROCESSING models, respectively. The relationship between the log odds of the presence of a respiratory pathogen and DOF was assumed to be linear. As DNPS is not a perfect test, latent class models, containing sensitivity estimates based on findings from previous research (40) (**Supplementary Table 4**), were used to account for potential misclassification of DNPS to detect respiratory pathogens in cattle. For *P. multocida* and *H. somni*, DNPS sensitivity was assumed to be identical to that of *M. haemolytica*. To allow for some degree of uncertainty in sensitivity parameters, beta distributions were truncated at ±5 percentual points from

distribution modes. Specificity of DNPS was deemed to be 100%, as results were PCR-confirmed. Non-informative priors were used for all other parameters. Overlapping of credible intervals (95% CI) were used for statistical inference. Predictors not associated with presence of a respiratory pathogen were excluded and simpler models were attempted. A full Bayesian statistical inference framework with Markov chains based on Hamiltonian Monte Carlo sampling was used for model estimation. This scheme generates proposal distributions that are pulled toward the posterior distribution mode instead of being symmetrical around the current position. Two parallel chains per dataset were used with a total of 100,000 post-warmup samples. Effective sample sizes (minimum of 1,000 for each parameter), Rhat estimates per dataset, and visual inspection of chains were used to evaluate efficacy. Posterior distribution plots were generated and distribution modes as well as respective 95% CIs were reported as proportions. Analysis were done in R with use of functions from the *brms* package (36).

A second approach was attempted with the use of models for longitudinal data (three-level models [operation, animal, and sample] with random slopes for time at the second level) and time splines that would allow for the evaluation of non-linear time changes and comingling effects when DOF $>$ 0. In this second approach, DOF was used as a metric of time, where DOF $=$ 0 represented the day when animals transitioned to feeding. However, Markov chains failed to converge. Therefore, results from the first approach were kept for presentation.

### Antimicrobial Resistance of Respiratory Pathogens

Frequency tables were built to summarize AMR rates for each species and sampling point. Next, two distinct approaches were used to compare resistance rates between sampling points and estimate comingling effects: mixed models and exact logistic regression models. For these analyses, only antimicrobials for which animal-specific breakpoints were available were retained.

Generalized linear mixed effects models were used to estimate effects of comingling and time in the AMR of *P. multocida* at the isolate level. A binary indicator related to presence or absence of resistant *P. multocida* in the sample was considered an outcome. The logit link was used for analysis, and models contained operation-specific random effects. Models were fit for each antimicrobial separately. Comingling effects were analyzed at WEANING/INDUCTION exclusively; only two producers did not comingle their calves at feedlots and it was impossible to distinguish operation-specific effects from comingle effects at REPROCESSING. As calves could not have been comingled prior to BRANDING, models were fit separately to assess time and comingle effects. For time models, results obtained at BRANDING were used as a reference. Models were fit using maximum likelihood, using the adaptive Gauss-Hermite quadrature with 50 quadrature points per scalar (37), providing existence of at least one resistant and one susceptible isolate per strata (sampling point or comingle status), as well as a minimum of five isolates per strata.

For *M. haemolytica*, *H. somni*, and *M. bovis*, exact logistic regression models were used to compare the prevalence of AMR between sampling points and estimate comingling effects. Exact logistic regression models for clustered data were carried out according to Troxler et al. (41) in R. Pairwise comparisons between sampling points were carried out on a pathogen basis for each antimicrobial in separate analyses. Comingling effects at WEANING/INDUCTION were also tested using exact logistic regression models. Models were attempted providing existence of at least one resistant and one susceptible isolate per species, as well as a minimum of five isolates per strata. Statistical significance was set at the 5% level.

### Effects of Administration of Antimicrobials in the MIC of Respiratory Pathogens

The goal of this analysis was to compare the MIC of respiratory pathogens sampled from animals treated with antimicrobials to corresponding values from animals not treated. In short, throughout the study, antimicrobials were administered as one of the following: (i) prior to weaning to treat clinical diseases; (ii) metaphylactic injections at weaning or immediately after placement at feedlots; (iii) in feed as BRD metaphylaxis to high-risk groups at feedlot induction; (iv) after feedlot induction, as treatment or prevention of clinical diseases at the feedlot; (v) a second injection to treat clinical diseases at feedlots. Prior to any assessment, records containing unreliable information about antimicrobial exposure status (e.g., where AMU was classified as "unknown") were excluded. Administration of antimicrobials prior to BRANDING and after WEANING/INDUCTION was uncommon and therefore not analyzed due to limited statistical power.

For the assessment of effects of metaphylactic use of antimicrobials at feedlot induction toward AMR at REPROCESSING, analyses were carried out at the isolate level. A preliminary assessment was carried out at the operation level to compare the distribution of DOF of samples collected at REPROCESSING according to the type of metaphylaxis adopted using general linear models. No differences were detected in DOF of samples collected at REPROCESSING when contrasting operations according to main categories of metaphylaxis (mean DOF of 135, 139, 138, and 135 for calves that were fed chlortetracycline, were not fed chlortetracycline, were treated parenterally with macrolides, and were not treated parenterally with macrolides, respectively; $p > 0.05$). Thereafter, DOF was omitted from further analysis. Isolates obtained at REPROCESSING were retained, and effects of parenteral administration of macrolides or in-feed administration of tetracyclines toward antimicrobial-specific MIC data were assessed using Bayesian models (42). Minor modifications were implemented to adapt the WinBUGS code to JAGS. In brief, log-transformed MIC values (logMIC) were assumed to follow a normal distribution with a common variance. As the true underlying MICs were not observed, logMIC values were considered censored at the maximum and minimum antimicrobial concentrations tested (interval censored). Unlike the original WinBUGS code used for analysis of MIC data (42), the distributions were not truncated in JAGS using the *I* operator. Instead, the *dinterval* function was used to represent censored outcomes, as recommended (43). Models contained frailty terms to account for the variability due to unobserved

operation-level effects. Models were fit for tetracyclines and macrolides separately. Fixed-effects included the parenteral administration of macrolides (macrolide models), or the in-feed use of tetracyclines at feedlot induction (tetracycline models). To evaluate effects of other routes of administration that were adopted throughout the study (parenteral administration of tetracyclines or in-feed administration of macrolides), we performed sensitivity analysis. Estimates and conclusions obtained from models with and without observations from selected operations were contrasted. A sensitivity analyses was undertaken due to the absence or low number of operations in each stratum defined according to type of antimicrobial therapy adopted, as this would preclude the proper estimation of operation-level random effects if a second herd-level exposure was added to the models.

Models were attempted when a minimum degree of variability in MIC values was apparent and not analyzed for antimicrobials with the same MIC for all isolates. Models were fit for each pathogen (*M. haemolytica, P. multocida, H. somni,* and *M. bovis*) and antimicrobial [(oxy)tetracycline, tylosin, tulathromycin, tilmicosin, and tildipirosin] combination in a Bayesian framework using a Markov Chain Monte Carlo approach based on Gibbs sampling. Four chains were run in parallel with a total of 500,000 iterations using the *runjags* package (39). Posterior distribution plots were visualized, and 95% CIs were used for statistical inference. Iteration plots were visually inspected for proper mixing of Markov chains. Autocorrelation values and ESS were used as measures of efficacy, where an ESS of 10,000 or higher was deemed as adequate.

# RESULTS

In total, 660 calves were enrolled consisting of 301 heifers and 359 steers. Nearly 40% of calves ($n = 265$) were not sampled at all times (**Supplementary Table 3**), with the sale of calves as the most common reason for missingness (65% or 173 missing animals), including all calves from five operations, which were sold prior to the third sampling. Additionally, in large operations, study animals were distributed among several pens. In those settings, there were challenges involved in gathering calves for sampling, which accounted for another 87 animals (32% of 265 missing animals) lost to follow-up (**Supplementary Figure 1**).

Six operations (27%) had a feedlot co-located to the cow–calf operation. At WEANING/INDUCTION, seven producers sampled calves prior to arrival at the feedlot. Nine operations sampled calves after comingling participants with calves from other sources. Following feedlot induction, two producers did not practice comingling, meaning that no animals from other sources were introduced at their feedlots. REPROCESSING samples were collected from 81 to 228 DOF, with an average of 142.6 DOF.

Antimicrobial administration prior to or at BRANDING was limited. A total of four calves from two operations were treated with florfenicol for pneumonia. Additionally, one calf with pneumonia was treated with tilmicosin, and a calf with a navel infection was treated with enrofloxacin. Finally, a calf with footrot was treated with tulathromycin. In total, treatment with antimicrobials prior to or at BRANDING were reported from four operations.

At WEANING/INDUCTION, animals from five operations were categorized as "low risk" and were not treated with antimicrobials. Calves from remaining operations were treated with tetracyclines, macrolides, and a combination of the two classes (**Table 2**).

After induction, calves from seven operations received no antimicrobials. Two operations that induced with macrolides also administered tylosin in feed to prevent or reduce the incidence of liver abscesses (110 mg/hd-day). Fourteen of the 392 (3.6%) calves that remained in the study were treated individually with antimicrobials at least once after induction. From this total, nine calves from four feedlots were treated for BRD. Other reasons for treatment included lameness ($n = 3$), footrot ($n = 2$), atypical interstitial pneumonia ($n = 1$), and infectious keratoconjunctivitis ($n = 1$). The most commonly used antimicrobials were florfenicol ($n = 8$ animals; four operations), ceftiofur ($n = 4$; two operations), and tilmicosin ($n = 3$; one operation).

## Prevalence of Respiratory Pathogens and Associated Risk Factors

Among studied BRD-associated pathogens, *P. multocida* was the most frequently recovered species ($n = 424$), followed

**TABLE 2 |** Antimicrobial treatment protocols adopted at WEANING/INDUCTION at each cow–calf operation.

| Operation[a] | Feed[b] | | Parenteral[b] | |
|---|---|---|---|---|
| | Antimicrobial | Dosage | Antimicrobial | Dosage |
| 1 | –[c] | – | – | – |
| 2 | Chlortetracycline | 1 g/100 lbs[d] | Gamithromycin | 6 mg/kg |
| 3 | – | – | Tulathromycin | 2.5 mg/kg |
| 5 | – | – | – | – |
| 7 | Chlortetracycline | 1 g/100 lbs[d] | Tulathromycin | 2.5 mg/kg |
| 9 | – | – | – | – |
| 11 | – | – | – | – |
| 12 | Chlortetracycline | 1 g/100 lbs[d] | Tulathromycin | 2.5 mg/kg |
| 13 | – | – | Tilmicosin | 10 mg/kg |
| 15 | – | – | Oxytetracycline | 20 mg/kg |
| 16 | Chlortetracycline | 1 g/100 lbs[d] | Tilmicosin[e] | 10 mg/kg[e] |
| 17 | – | – | – | – |
| 18 | Chlortetracycline | 1 g/100 lbs[d] | | |
| 19 | – | – | Tulathromycin | 2.5 mg/kg |
| 20 | Chlortetracycline | 1 g/100 lbs[d] | Oxytetracycline | 20 mg/kg |
| 21 | Chlortetracycline | 1 g/100 lbs[d] | – | – |
| 22 | Chlortetracycline | 1 g/100 lbs[d] | – | – |

[a] *Five operations with no antimicrobial use data were excluded.*
[b] *Protocols listed are those adopted in most calves from each operation.*
[c] *A dash (–) indicates that antimicrobials were not used at WEANING/INDUCTION by that route of administration.*
[d] *Body weight, daily for 15 days.*
[e] *Protocol administered to 15 calves. Another 14 calves were treated with tulathromycin (2.5 mg/kg).*

by *M. haemolytica* ($n = 61$), *M. bovis* ($n = 49$), and *H. somni* ($n = 30$). From a total of 660 samples collected at BRANDING, 7.4% ($n = 49$) were positive for at least one respiratory pathogen. This percentage increased to 52.3% (310/608) at WEANING/INDUCTION, and 50.3% (197/392) at REPROCESSING at feedlots. Accordingly, prevalence of *P. multocida*, *M. bovis*, and *M. haemolytica* were significantly higher at REPROCESSING than at BRANDING (**Table 3**). Additionally, for *M. haemolytica* there was no difference between prevalence at WEANING/INDUCTION, and prevalence at either BRANDING or REPROCESSING. Conversely, in *M. bovis*, there was a significant difference between prevalence at WEANING/INDUCTION and at REPROCESSING, suggesting that animals were infected at feedlots. For *P. multocida*, a sharp increase in prevalence was observed at WEANING/INDUCTION, which persisted at REPROCESSING (**Figure 1**). *M. bovis* was the second most prevalent respiratory pathogen at feedlots (**Figure 1**). Comingling, co-location of feedlot, and cow–calf operation, and results from previous sampling point did not have an impact on the prevalence of respiratory pathogens at WEANING/INDUCTION.

## Antimicrobial Resistance of Respiratory Pathogens

On average, 14 isolates per operation were tested for susceptibility, ranging from 5 to 28 isolates per operation. $MIC_{50}$ and $MIC_{90}$ according to species and sampling point are available as Supplementary Material (**Supplementary Tables 5–8**). Overall, low levels of AMR were detected in BRD-associated *Pasteurellaceae* species isolates. Considering antimicrobials for which resistance breakpoints have been established, resistance against spectinomycin was observed in 12, 11, and 3% of *M. haemolytica, P. multocida*, and *H. somni* isolates, respectively. In *M. bovis*, resistance against macrolides was prevalent, with the majority of isolates resistant to tildipirosin, tilmicosin, and tylosin. In contrast, resistance to enrofloxacin and ceftiofur was absent or rare, regardless of species (**Table 4**). Resistance to tetracyclines was observed in all species, ranging from 3.3% of oxytetracycline resistance in *H. somni* to 18.4% in *M. bovis*. Resistance to tetracyclines and spectinomycin appeared to co-exist in *M. haemolytica, P. multocida*, and *H. somni*.

There was limited evidence to support an increase in resistance rates from BRANDING to REPROCESSING in BRD-associated bacteria isolated from cattle (**Table 4**). Most pairwise comparisons indicated no significant differences in AMR rates among sampling points. The exception was resistance against florfenicol in *M. bovis*; 68% of isolates at REPROCESSING were resistant against florfenicol vs. 37% of *M. bovis* isolated at WEANING/INDUCTION ($p = 0.02$). Florfenicol-resistant *M. bovis* were isolated from eight feedlots. From this total, only a single feedlot reported one incidence of treatment with florfenicol. Finally, there were no differences in resistance rates at WEANING/INDUCTION among bacteria isolated from comingled and non-comingled calves.

As inferred from the WGS, 2 out of 25 sequenced *M. haemolytica* isolates harbored the *aph(3′)-Ia*, *aph(6)-Id*, *aph(3″)-Ib*, *sul2*, and *tet(H)* antimicrobial resistance genes (ARGs). Additionally, 9 out of 29 sequenced *P. multocida* had the *aph(3′)-Ia*, *aph(6)-Id*, *aph(3″)-Ib*, *sul2*, *aadA31*, and *tet(H)* genes, with two isolates also carrying a A2058G mutation in the 23S rRNA gene. We did not detect any ARG in *H. somni* sequenced isolates. Overall, the AMR phenotypes were corroborated by presence of ARGs (**Supplementary Table 9**). The *tet*(*H*) gene was present in all tetracycline-resistant *M. haemolytica* and *P. multocida*. *P. multocida* and *M. haemolytica* isolates harboring the neomycin and kanamycin resistance gene *aph(3′)-Ia* had neomycin MIC values >32 μg/ml. *aadA*31 was associated with spectinomycin resistance in *P. multocida* (**Supplementary Table 9**). The streptomycin resistance ARGs *aph(3″)-Ib* and *aph(6)-Id* were detected in all *M. haemolytica* and *P. multocida* strains harboring the neomycin resistance determinant *aph(3′)-Ia*. Unfortunately, streptomycin resistance was not assessed for any species. In all isolates, the MIC of sulphadimethoxime was ≥256 μl/ml. Yet, *sul2* was detected in

**TABLE 3 |** Prevalence per 100 animals of *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, and *Mycoplasma bovis* at BRANDING ($n = 660$ samples), WEANING/INDUCTION ($n = 608$ samples), and REPROCESSING ($n = 392$ samples).

| Bacteria | BRANDING | | WEANING/ INDUCTION | | REPROCESSING | |
|---|---|---|---|---|---|---|
| | Prevalence | 95% CI[1] | Prevalence | 95% CI | Prevalence | 95% CI |
| *M. haemolytica* | 0.87[a] | 0.68; 1.47 | 3.06[a,b] | 1.20; 6.19 | 5.31[b] | 2.28; 9.96 |
| *P. multocida* | 3.59[a] | 1.34; 7.71 | 40.0[b] | 27.9; 49.2 | 30.74[b] | 22.04; 40.36 |
| *H. somni* | 0.83 | 0.67; 1.35 | 1.53 | 0.72; 3.52 | 2.70 | 0.88; 7.13 |
| *M. bovis* | 1.29[a] | 0.70; 2.80 | 1.65[a] | 0.71; 4.71 | 9.37[b] | 4.92; 14.99 |

[1]*95% Credible interval (95% CI).*

[a,b]*Within row prevalences followed by different letters denote statistically significant differences between sampling points for each species.*

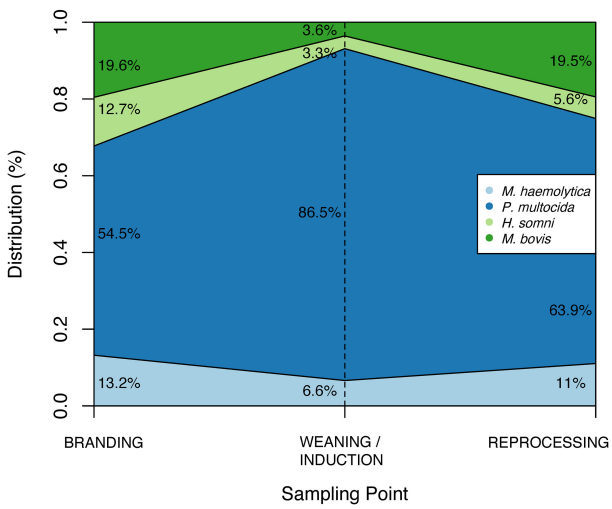



**FIGURE 1 |** Distribution of respiratory pathogens according to sample point.

9 *P. multocida* and 2 *M. haemolytica* isolates (31.0 and 8.0% of total sequenced *P. multocida* and *M. haemolytica*, respectively). The A2058G mutation in the 23S rRNA gene was detected in two *P. multocida* isolates and linked to resistance against macrolides (**Supplementary Table 9**). For the remaining sequenced isolates, no relevant 23S rRNA mutation was detected. One *P. multocida* isolate had the A2059G mutation in ∼35% of the sequence reads mapped to 23S rRNA. The MICs for tilmicosin and tulathromycin were at 4 μg/ml (both susceptible). No mutations were detected in genes encoding the ribosomal proteins L4 and L22 (*rplD* and *rplV*, respectively). No other macrolide resistance mechanism was evident in the sequenced isolates.

**TABLE 4 |** Antimicrobial resistance rates (%) according to antimicrobial, sampling point (B, BRANDING; I, WEANING/INDUCTION; R, REPROCESSING), and bacteria species.

| Class | Antimicrobial | *Mannheimia haemolytica* | | | *Histophilus somni* | | |
|---|---|---|---|---|---|---|---|
| | | B | I | R | B | I | R |
| | | *n* = 1 | *n* = 30 | *n* = 27 | *n* = 0 | *n* = 10 | *n* = 20 |
| Aminocyclitol | Spectinomycin | 0 | 20 | 3.7 | –[1] | 10 | 0 |
| Cephalosporin | Ceftiofur | 0 | 0 | 0 | – | 0 | 0 |
| Fluoroquinolone | Danofloxacin | 0 | 3.3 | 0 | – | 10 | 5 |
| | Enrofloxacin | 0 | 0 | 0 | – | 10 | 0 |
| Macrolide | Tilmicosin | 0 | 13.3 | 14.8 | – | 10 | 0 |
| | Tulathromycin | 0 | 0 | 3.7 | – | 0 | 0 |
| Penicillin | Penicillin | 0 | 3.3 | 14.8 | – | 0 | 0 |
| Phenicol | Florfenicol | 0 | 0 | 0 | – | 10 | 0 |
| Tetracycline | Oxytetracycline | 0 | 0 | 7.4 | – | 0 | 5 |
| **Antimicrobial** | **Antimicrobial** | ***Pasteurella multocida*** | | | ***Mycoplasma bovis*** | | |
| | | B | I | R | B | I | R |
| | | *n* = 24 | *n* = 88 | *n* = 38 | *n* = 2 | *n* = 19 | *n* = 28 |
| Aminocyclitol | Spectinomycin | 8.3 | 10.2 | 15.8 | – | – | – |
| Cephalosporin | Ceftiofur | 0 | 0 | 0 | – | – | – |
| Fluoroquinolone | Danofloxacin | 0 | 0 | 0 | – | – | – |
| | Enrofloxacin | 0 | 0 | 0 | 0 | 0 | 0 |
| Macrolide | Gamithromycin | 0 | 1.1 | 5.3 | 100 | 84.2 | 100 |
| | Tildipirosin | 0 | 0 | 5.3 | 100 | 94.7 | 100 |
| | Tilmicosin | 4.2 | 3.4 | 7.9 | 100 | 94.7 | 100 |
| | Tulathromycin | 0 | 0 | 5.3 | 100 | 31.6 | 60.7 |
| | Tylosin | – | – | – | 100 | 78.9 | 85.7 |
| Penicillin | Penicillin | 0 | 0 | 0 | – | – | – |
| Phenicol | Florfenicol | 0 | 0 | 0 | 50 | 36.8[a] | 67.9[b] |
| Tetracycline | Chlortetracycline | – | – | – | 0 | 15.8 | 14.3 |
| | Oxytetracycline | – | – | – | 0 | 15.8 | 21.4 |
| | Tetracycline | 8.3 | 9.1 | 15.8 | – | – | – |

[1]*A dash (–) denotes either absence of clinical breakpoints for that specific pathogen–antimicrobial combination or absence of tested isolates.*

[a,b]*Within row percentages followed by different letters denote statistically significant differences between resistance rates for each species ($p < 0.05$).*

## Effects of Administration of Antimicrobials in the MIC of Respiratory Pathogens

Administration of tetracyclines at WEANING/INDUCTION was not linked to the MIC of tetracyclines (oxytetracycline, chlortetracycline, and tetracycline) in any respiratory bacteria at REPROCESSING (**Figure 2**). Effects were independent on the parenteral use of tetracyclines. In contrast, there were clear associations between the use of and reduced susceptibility to macrolides in *P. multocida*, *M. haemolytica*, and *H. somni* (**Figure 3**). The parenteral administration of macrolides at WEANING/INDUCTION was linked to an increased MIC of at least one macrolide for each species. In *M. bovis*, the tylosin MIC was on average 1.44 logs higher in bacteria isolated from calves treated parenterally with macrolides at WEANING/INDUCTION vs. the MIC of calves that did not receive macrolides. Yet, no statistically significant association was detected, as the 95% CI ranged from −0.05 to 2.93 (**Figure 3**). Some of the effects were dependent on inclusion of observations from the two operations that fed tylosin; effects of the parenteral administration of macrolides toward the MIC of tylosin and gamithromycin in *P. multocida* as well as tulathromycin in *H. somni* were detected only when observations from the two operations that fed tylosin after induction were kept in models. The MICs of tilmicosin and tildipirosin in *M. bovis*, and tylosin in *M. haemolytica* were either constant or had a very low variability. Therefore, no modeling was attempted for these antimicrobials.

## DISCUSSION

Here, we report on the prevalence of BRD-associated bacteria in calves from the spring processing to the reprocessing at feedlots.

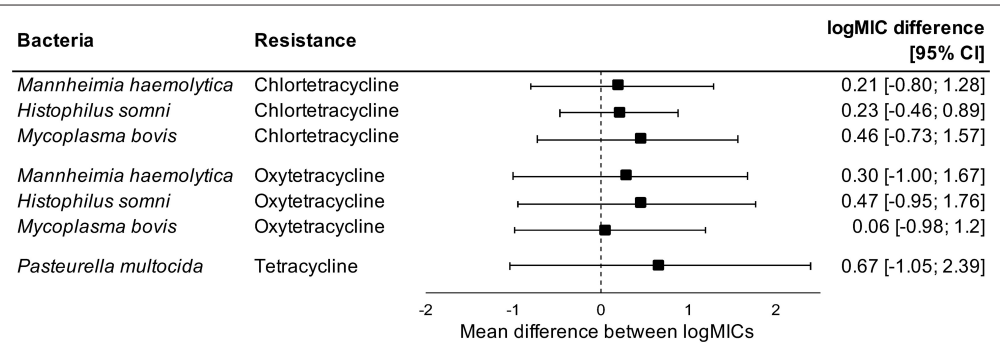



**FIGURE 2 |** Mean difference between antimicrobial-specific logMICs of bacteria isolated at REPROCESSING from the respiratory tract of calves fed, and not fed tetracyclines at feedlot induction. Model includes observations from all operations, including ones that administered tetracyclines parenterally at induction. Sensitivity analysis indicated no effects of removing observations from operations that administered tetracyclines parenterally at induction.

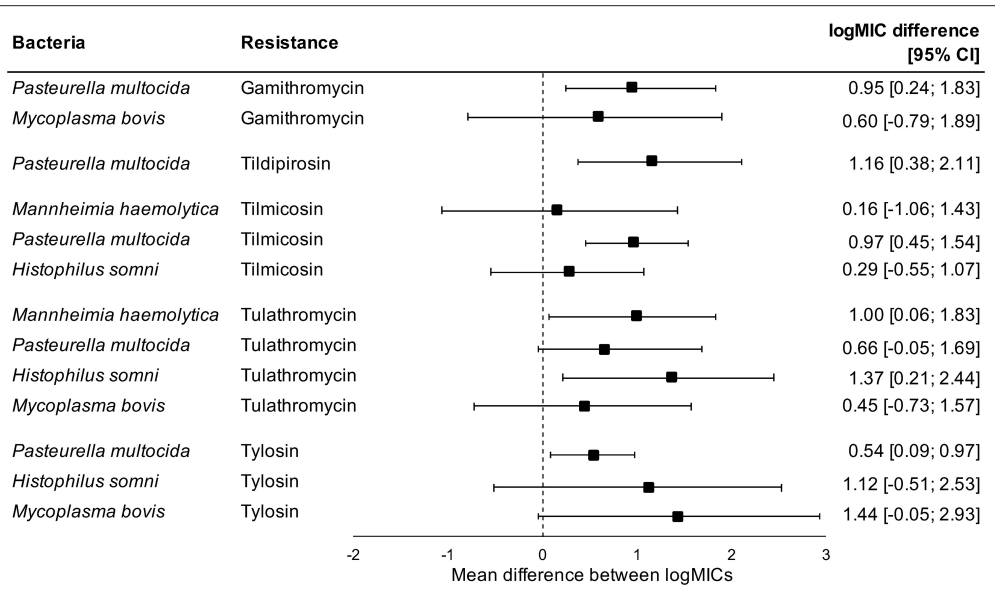



**FIGURE 3 |** Mean difference between antimicrobial-specific logMICs of bacteria isolated at REPROCESSING from the respiratory tract of animals treated, and not treated parenterally with macrolides at feedlot induction. Model includes observations from all operations, including ones that fed tylosin to control liver abscesses. Sensitivity analysis indicated that effects of parenteral administration of macrolides toward the logMIC of tylosin and gamithromycin in *P. multocida*, and tulathromycin in *H. somni* were statistically significant only when observations from operations that fed tylosin after induction were kept in models.

We also determine the AMR profile of respiratory pathogens and explore factors linked to it such as the use and type of antimicrobial used in BRD metaphylaxis. We provide a summary that will integrate the existing literature reporting on AMR of BRD bacteria and explain trends that have been observed in BRD-associated bacteria from feedlot cattle in North America.

There are a number of factors that contribute to clinical BRD in beef cattle, including stressors such as weaning, transportation, and feedlot entry (44), and presence of pathogenic bacteria in the respiratory tract of animals (45). Here, we demonstrate that prevalence of three respiratory pathogens (*P. multocida*, *M. bovis*, and *M. haemolytica*) increases from spring processing (BRANDING) to reprocessing at feedlots. At least one respiratory pathogen was recovered from >50% of calves at WEANING/INDUCTION and at REPROCESSING, whereas <10% of calves were colonized at BRANDING. *P. multocida* was the most abundant species identified at all three time points, with a marked increase in prevalence at WEANING/INDUCTION that persisted at feedlots. Prevalence of *P. multocida* in clinical BRD appears to be increasing (46). In Canada, *P. multocida* was more prevalent in feedlot cattle with BRD than in healthy control animals (47). Interestingly, prevalence of *P. multocida* at WEANING/INDUCTION was independent of comingling status, which means that an increased prevalence of *P. multocida* was detected for calves sampled early at feedlots as well as for those still at the cow–calf environment (e.g., at weaning). Based on our findings, we hypothesize that animals become increasingly colonized by *P. multocida* prior to or immediately after feedlot placement rather than around 40 days after placement as previously suggested (48). In contrast, *M. bovis* had the highest relative increase in prevalence from WEANING/INDUCTION to REPROCESSING at the feedlot. At BRANDING, prevalence of *M. bovis* was low, in agreement with previous North American studies where the prevalence ranged from 0 to 7% (49, 50). The increased relative abundance of *Mycoplasma* spp. after feedlot placement has been previously described (51), as well as their role in BRD development (45). The highly contagious nature of *M. bovis* among cattle and increased animal density in feedlots have been implicated as the most likely reasons for sharp increases in prevalence of *M. bovis* at feedlots (52).

Comingling of cattle from multiple sources can increase the incidence of respiratory disease (53). We observed some variability of the time of sampling at WEANING/INDUCTION between operations, which reflected producer-specific practices relative to the feedlot induction. This variability was explored in our models by assessing comingling effects. Step et al. (54) reported on associations between commingling and animal health indicators such as antibodies titers to *M. haemolytica* and *P. multocida* and incidence of clinical BRD during a 42-day receiving period. Comingled calves had increased BRD morbidity and were treated earlier and more often with antimicrobials than non-comingled calves, which suggests that comingling can be a risk factor for development of AMR among BRD bacteria. In our study, comingling, results from previous sampling, and co-location of cow–calf operations and feedlots were not associated with prevalence of respiratory pathogens at WEANING/INDUCTION. Additionally, bacteria from comingled and non-comingled cattle had comparable AMR rates at WEANING/INDUCTION. As comingled calves were sampled relatively close to the arrival at feedlots (mean DOF = 9 days, range 2–21 days), we believe that comingling effects in

AMR, if present, would manifest later at feeding. Indeed, Step et al. (54) estimated an average of 10.6 days after placement for the first antimicrobial treatment of comingled cattle due to clinical BRD. In contrast, commingling for short periods of time did not significantly impact the nasopharyngeal or tracheal bacterial communities of recently weaned beef calves (51).

*M. haemolytica*, *P. multocida*, and *H. somni* isolates were susceptible to most antimicrobials tested for which standardized clinical breakpoints were available. None or very few isolates were resistant to florfenicol, fluoroquinolones, and ceftiofur, in agreement to previous findings involving clinically healthy feedlot cattle in Canada (47). Resistance to aminoglycosides was reported in bacteria causing BRD in feedlot cattle in Alberta (55). As inferred from WGS data, ARG profiles of resistant isolates indicated the co-existence of tetracycline [*tet(H)*], neomycin [*aph(3′)-Ia*], streptomycin [*aph(6)-Id* and *aph(3″)-Ib*], and sulfadimethoxine (*sul2*) determinants in multidrug resistant (MDR) *M. haemolytica* and *P. multocida*. Presence of multiple ARGs in the same isolates suggests existence of integrative and conjugative elements (ICE), which have been previously detected in BRD bacteria (56–58). We confirmed that ICE-associated recombination and conjugation genes were present in MDR isolates. It is unclear what exactly is driving presence of ICE-harboring MDR bacteria in the nasopharynx of beef cattle. In our study, ICE-positive MDR bacteria were isolated from 8 to 17% of sequenced isolates obtained at WEANING/INDUCTION and REPROCESSING, respectively, which suggests that antimicrobial use at induction could be driver of their presence. Nevertheless, it is intriguing that 2 out of 10 sequenced isolates obtained at BRANDING also harbored ICE and were MDR.

We detected the A2058G mutation in the 23S rRNA of two macrolide-resistant *P. multocida*. Macrolide resistance due to rRNA mutations is well-documented in bacteria with a single or multiple rrn operons (59). Bacteria from the *Pasteurellaceae* family associated with BRD typically contain five to six copies of the rrn operon. As more copies harbor the A2058G mutation, a greater increase in the MIC against macrolides is expected. Indeed, a single mutated operon confers only a slight selective advantage in the presence of macrolides, which can be followed by homologous recombination between rrn operons amplifying the proportion of mutant ribosomes, with a corresponding larger increase in macrolide resistance (60). An in-depth analysis of the sequence read data mapped to the wild-type 23S rRNA from *P. multocida* revealed that ∼80% of reads from the two macrolide-resistant isolates contained the A2058G SNP, whereas the remaining 20% had the wild-type sequence at the locus, which suggests that four out of five copies of the rrn operons encoded the mutation. Accordingly, the two isolates displayed a high MIC (64 μg/ml) for tilmicosin and tulathromycin.

In *M. bovis*, there was an increased rate of resistance against florfenicol at REPROCESSING in comparison to isolates obtained at WEANING/INDUCTION. Interestingly, florfenicol was the most frequently used antimicrobial after feedlot induction, with four operations reporting at least one treatment with florfenicol. Yet, florfenicol-resistant *M. bovis* were detected in eight feedlots, from which only one reported use of florfenicol. There are two likely explanations to our findings. First, the increase in prevalence of florfenicol-resistant *M. bovis* at REPROCESSING might not be linked to the use of florfenicol at feedlots. Florfenicol-resistant strains could harbor mutations linked to resistance to other antimicrobial classes that were in use at feedlots. Indeed, florfenicol and macrolides target the same ribosomal subunit, and changes in the MIC of tylosin and tilmicosin were noted in florfenicol-resistant strains (61). Unfortunately, as we did not sequence *M. bovis* isolates, we could not infer whether mutations were shared between florfenicol and macrolide-resistant strains. Alternatively, we might have failed to detect antimicrobial therapies with florfenicol at feedlots. From 2008 to 2012, use of phenicols has nearly doubled in Western Canada feedlots, with an estimated use of approximately 1 daily dose for every 10 feedlot cattle in 2012 (62). Additionally, phenicols were among the most frequently used antimicrobials to treat cattle that succumbed to BRD at western Canadian feedlots, where nearly 80% of diseased animals were treated with phenicols (63). Conversely, we observed eight treatments with florfenicol during feeding in nearly 400 feedlot-placed calves. It is conceivable that the use of florfenicol was higher than reported by feedlots, and responsible for increased florfenicol resistance rates in *M. bovis* isolated at REPROCESSING. Our study was done before increased veterinary oversight of antimicrobial use in Canada, as well as prior to the launch of a Canadian fed-cattle antimicrobial surveillance program (64). Under new regulations, medically important antimicrobials are sold under prescription, which facilitates the gathering and recording of antimicrobial usage data, providing a much more reliable metric of antimicrobial exposure for future studies.

High levels of resistance to macrolides in *M. bovis* were detected in our study, in agreement to previous reports (30, 65). A western Canadian study reporting on resistance rates of *M. bovis* recovered from feedlot cattle also demonstrated elevated macrolide-resistance rates, with resistance to various macrolides detected in over 95% of isolates (55). These results suggest that resistance against macrolides in *M. bovis* isolated from the respiratory tract of feedlot cattle in western Canada is widespread. A word of caution is necessary, as currently the CLSI has no approved breakpoints for *M. bovis* isolated from the respiratory tract of beef cattle. Breakpoints adopted herein were defined based on a combination of historical MIC data, previous reports and current breakpoints for other respiratory pathogens of cattle (30). When $MIC_{50}$ of macrolides were inspected, our estimates were comparable to ones reported from France and Japan in 2008–2012 (11, 66), and higher than values reported from the US in 2002–2003 (29).

Susceptibility of *M. bovis* from feedlot cattle to macrolides has decreased over the last decades in France (11). In Canada, although we currently lack definitive data to support a sustained increase in AMR rates of *M. bovis*, there is some evidence suggesting an increase in resistance against tulathromycin over the last decade (30). Additionally, a study done in 2007 and 2008 involving *M. bovis* from dead and sick cattle from a single feedlot in western Canada reported lower $MIC_{50}$ values of tulathromycin than those observed in our study (67), which also suggests that macrolide resistance of *M. bovis* from western Canadian feedlots

is on the rise. Such trends could be driven by the increased use of macrolides in western Canadian feedlots for metaphylaxis and the prevention of liver abscesses (68). The link between use of macrolides in feedlot cattle and resistance against tulathromycin in *M. bovis* isolated from the respiratory tract of feedlot cattle was recently explored. *M. bovis* isolated from cattle raised without antimicrobials had diminished MIC of tulathromycin in comparison to bacteria from cattle exposed to macrolides (12). In our study, the incidence of tulathromycin-resistant *M. bovis* at REPROCESSING was 5.3 times higher in feedlots that treated calves parenterally with macrolides at induction in comparison to feedlots not using macrolides ($p = 0.04$ in operation-conditional Poisson models; results not shown).

We detected significant associations between parenteral use of and increased MICs against macrolides in *P. multocida*, *H. somni*, and *M. haemolytica* at REPROCESSING. We were not able to infer whether effects were also evident for the in-feed administration of macrolides; the two operations that fed tylosin after induction as prevention of liver abscesses also inducted with macrolides. Nonetheless, by excluding observations from operations in which tylosin was administered to calves *via* feed, some of the associations previously detected were lost, which suggests that the in-feed administration of tylosin will also play a role in the selection of respiratory bacteria with increased MIC against macrolides. Macrolides are antimicrobials of critical importance to human health according to the WHO (9). It is unknown if and to what extent the presence of respiratory bacteria with increased resistance to macrolides in beef cattle represents a threat to humans or to the environment. Furthermore, we are not able to estimate the likely impacts of increased MICs of macrolides on the therapeutic effectiveness of antimicrobials against BRD, especially if increases incur under breakpoints used to define clinical resistance. Nevertheless, our findings are extremely valuable to interpret trends of susceptibility in BRD bacteria from North American feedlots. Decreases in susceptibility to macrolides in *P. multocida*, *H. somni*, and *M. haemolytica* have been documented (10). Based on our findings, we hypothesize that the use of macrolides at feedlots is impacting the susceptibility of respiratory bacteria against macrolides.

Conversely, the in-feed administration of tetracyclines as metaphylaxis was not associated with antimicrobial-specific MICs in respiratory bacteria isolated from feedlot cattle at REPROCESSING. Levels of AMR are expected to increase after antimicrobial use, but this pattern was not evident when chlortetracycline was fed in our study. The lack of an association could be due to an already widespread prevalence of resistance to tetracyclines in respiratory bacteria of feedlot cattle in western Canadian feedlots. Timsit et al. (47) studied the prevalence of AMR in *P. multocida*, *H. somni*, and *M. haemolytica* recovered from the respiratory tract of healthy and sick cattle in Canadian feedlots. Resistance to oxytetracycline was widespread, regardless of animal category or bacteria species. Yet, most of our isolates were susceptible to tetracyclines, and resistance levels were much lower than reported previously in western Canada (55). Alternatively, we could have missed rapid, transient increases of AMR following use of chlortetracycline in feed. Indeed, cattle exposed to a 5-day therapy with chlortetracycline in feed had increased levels of tetracycline-resistant *Escherichia coli* in feces 5 days post-treatment in comparison to unexposed animals, with no differences between groups detected >27 days post-treatment (69). Our median DOF at REPROCESSING was 139 days, which means that transient increases of AMR in respiratory bacteria, if any, were probably not captured by our study.

Our findings suggest that, under One Health lens, tetracyclines fed at induction will have a minimal long-term impact in prevalence of AMR in respiratory bacteria, which could support the sustained use of tetracyclines in feedlots as BRD metaphylaxis. From an animal health perspective, two meta-analysis explored the effects of metaphylaxis on BRD in beef cattle. Abell et al. (15) identified differences between classes of antimicrobials used as metaphylaxis, with macrolides outperforming tetracyclines, regardless of the duration of the feeding period. In contrast, Baptiste and Kyvsgaard (70) did not detect differences in efficacy of these antimicrobial classes against BRD; macrolides were superior to tetracyclines only when used as a prophylaxis (medication of asymptomatic cattle upon arrival at feedlot), but not as metaphylaxis. Altogether, further consideration should be given to the in feed use of tetracyclines as metaphylaxis, including the identification of specific circumstances in which these antimicrobials should be recommended as first choice in prevention of BRD.

As the use of macrolides at feedlot induction was linked to the MIC of bacteria isolates at REPROCESSING, we were surprised by the lack of differences in prevalence of macrolide resistance between sampling points. This contradiction is a consequence of at least four factors. First, not all feedlots administered macrolides at induction, and resistance to macrolides could have been dependent on the use of macrolides. Indeed, the incidence of tulathromycin-resistant *M. bovis* was statistically higher at REPROCESSING in comparison to WEANING/INDUCTION only in feedlots that induced with macrolides (1.4 resistant isolates/100 calves at WEANING/INDUCTION vs. 5.2 resistant isolates/100 calves at REPROCESSING in feedlots that used macrolides at induction, $p < 0.05$ in operation-conditional Poisson models; results not shown). Second, increases in MIC could have occurred under clinical breakpoints used to define macrolide resistance, particularly for species with low resistance levels (*M. haemolytica*, *H. somni*, and *P. multocida*). Surveillance data have demonstrated substantial year-to-year variation in the MIC of some macrolides, but with values generally below the specific criteria for defining resistance (71). The clinical implications of sub-breakpoints MIC increases remain to be demonstrated, but such increases could partially account for historical trends observed in MIC data. Third, we could have lacked power to detect specific differences in AMR rates between sampling points. Despite lack of statistical significance, all macrolides resistance rates in *P. multocida* isolated at REPROCESSING were higher in comparison to resistance rates at other sampling points. Finally, Bayesian models for MIC data can be used even in absence of clinical or epidemiological susceptibility breakpoints. For antimicrobials in which breakpoints have not been defined (e.g., tylosin in *P. multocida*, *H. somni*, and *M. haemolytica*), we were not able to

compare resistance rates between sampling points, which could contrast with findings obtained from Bayesian models.

We provided important results from a longitudinal study that can be used to inform strategies to optimize use of antimicrobials in beef cattle. Nevertheless, our findings should be considered in the face of study limitations and caveats. In a cohort study, loss to follow-up can negatively affect the internal validity of the study if the probability of missingness is dependent on the observed values, values that would have been observed, or both. Producers were selected, in part, by their usual management strategy of retaining ownership of their cattle through to slaughter. However, more cattle than expected were sold before the end of the study, or producers divided study calves among a number of pastures or pens and animals were not available for sampling. Such challenges are not uncommon when working with commercial operations. Regardless, we believe our findings are internally valid for at least three reasons. First, decisions to sell cattle or divide the study calves among several pastures were based on economics and management terms and not on animal health. There is limited evidence to suggest that the probability of not being sampled was dependent on presence of respiratory bacteria that were either observed or that would have been observed. Our missing data were most likely generated at random, also given results from the comparison between some of the completers and non-completers (discussed in the Statistical Analysis). Second, we used operation-conditional models, meaning that estimates and comparisons reported were conditional on the operation. These models are efficient at dealing with missing data that are generated randomly (e.g., missing completely at random or missing at random). Hence, any impact arising from imbalances in the number of samples collected per operation was minimized, assuming that, within each operation, the probability of not being sampled was independent of observed values. Third, for prevalence estimates, we used multiple imputation models that included results from previous sampling point(s) and operation random effects. This approach is very efficient to increase the study power and therefore minimize impacts of missing data. A second limitation arises from the use of sensitivity and specificity estimates in our prevalence models. It is unclear whether the sensitivity and specificity values of DNPS were adequate for detection of *H. somni* and *P. multocida* in the respiratory tract of calves. Unfortunately, to our knowledge there is no study reporting on the characteristics of DNPS to detect the two species in the respiratory tract of beef cattle.

In summary, *P. multocida* was the most prevalent bacteria in the respiratory tract of beef calves from spring processing to reprocessing at feedlots, followed by *M. haemolytica*, *M. bovis*, and *H. somni*. For *M. bovis*, a sharp increase in prevalence was detected at REPROCESSING, whereas for *P. multocida*, an increase in prevalence occurred at WEANING/INDUCTION and persisted during feeding. Comingling was not associated with prevalence of any respiratory pathogen at feedlot induction. Resistance levels were generally low, with a few exceptions. In *M. bovis*, resistance against macrolides was prevalent, with the majority of isolates resistant against tildipirosin, tilmicosin, and tylosin. There was limited evidence to support an increase in resistance rates from the spring processing to reprocessing at feedlots in *M. haemolytica*, *P. multocida*, and *H. somni*, although macrolide resistance rates were consistently higher in *P. multocida* at REPROCESSING. In *M. bovis*, increased florfenicol resistance rates were detected at REPROCESSING. Metaphylactic administration of tetracyclines at feedlot induction was not linked to the MIC of tetracyclines in any respiratory bacteria at REPROCESSING. Conversely, there were clear associations between the parenteral use of macrolides as metaphylaxis, and increased MIC in *P. multocida*, *M. haemolytica*, and *H. somni*. We hypothesize that the use of macrolides such as tulathromycin at feedlot induction is responsible for historical changes in macrolides MIC data of respiratory bacteria isolated from post-arrival cattle in feedlots.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The sequencing data of isolates used in this study have been submitted to the NCBI (BioProject ID: PRJNA720670/PRJNA313047).

## ETHICS STATEMENT

The animal study was reviewed and approved by Lethbridge Research and Development Centre's Animal Care Committee. Written informed consent for participation was not obtained from the owners because consent was provided verbally as a result of communication between veterinary practitioners and their clients.

## AUTHOR CONTRIBUTIONS

RA, TM, EH, and CD conceived and designed the study. CD, RA, SA-L, and EH were involved in sample and data collection. RZ worked on the sequencing data. DN performed statistical analysis and worked in the interpretation of results, and wrote the first draft of the manuscript. RA, TM, EH, SA-L, RZ, and CD revised the document for important intellectual content. All authors gave approval of the final version to be published and agreed to be accountable for all aspects of the work.

## FUNDING

This study received funding from Alberta Beef Producers (ABP) and Alberta Livestock and Meat Agency Ltd. (ALMA). The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

## ACKNOWLEDGMENTS

We thank producers, veterinarians, technicians, and professionals involved in this work. We would like to thank Cheyenne Conrad, Ruth Barbieri, Brendon DeGroot, Sujeema Abeysekara, Wendi Smart (AAFC), Homayoun Zahiroddini

(AAF), and Taylor Davedow (University of Manitoba) for their assistance in the laboratory analyses; and Dr. Murray Jelinski, Andrea Kinnear, and Karen Gesy (Western College of Veterinary Medicine, University of Saskatchewan) for their expertise in microbiology.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.764701/full#supplementary-material

## REFERENCES


1. O'Neill J. *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. The Review on Antimicrobial Resistance.* (2016). Available online at: https://amr-Review.org/Sites/Default/Files/160518_Final%20paper_With%20cover.pdf (accessed July 18, 2021).
2. Nobrega DB, Tang KL, Caffrey NP, De Buck J, Cork SC, Ronksley PE, et al. Prevalence of antimicrobial resistance genes and its association with restricted antimicrobial use in food-producing animals: a systematic review and meta-analysis. *J Antimicrob Chemother.* (2021) 76:561–75. doi: 10.1093/jac/Dkaa443
3. Loneragan GH, Dargatz DA, Morley PS, Smith MA. Trends in mortality ratios among cattle in US feedlots. *J Am Vet Med Assoc.* (2001) 219:1122–7. doi: 10.2460/Javma.2001.219.1122
4. Ives SE, Richeson JT. Use of antimicrobial metaphylaxis for the control of bovine respiratory disease in high-risk cattle. *Vet Clin North Am Food Anim Pract.* (2015) 31:341–50. doi: 10.1016/j.Cvfa.2015.05.008
5. Taylor JD, Fulton RW, Lehenbauer TW, Step DL, Confer AW. The epidemiology of bovine respiratory disease: what is the evidence for predisposing factors? *Can Vet J.* (2010) 51:1095–102.
6. Griffin D, Chengappa MM, Kuszak J, McVey DS. Bacterial pathogens of the bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract.* (2010) 26:381–94. doi: 10.1016/j.Cvfa.2010.04.004
7. White BJ, Renter DG. Bayesian estimation of the performance of using clinical observations and harvest lung lesions for diagnosing bovine respiratory disease in post-weaned beef calves. *J Vet Diagn Invest.* (2009) 21:446–53. doi: 10.1177/104063870902100405
8. Waldner CL, Parker S, Gow S, Wilson DJ, Campbell JR. Attitudes towards antimicrobial use and factors associated with antimicrobial use in Western Canadian cow-Calf Herds. *Can Vet J.* (2019) 60:391–8.
9. World Health Organization. *Critically Important Antimicrobials for Human Medicine, 6th Revision.* Geneva: World Health Organization (2019). Available online at: https://www.who.int/Publications/I/Item/9789241515528 (accessed September 22, 2021).
10. Portis E, Lindeman C, Johansen L, Stoltman G. A ten-year (2000-2009) study of antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex–*Mannheimia Haemolytica, Pasteurella Multocida*, and *Histophilus Somni*–in the United States and Canada. *J Vet Diagn Invest.* (2012) 24:932–44. doi: 10.1177/1040638712457559
11. Gautier-Bouchardon AV, Ferre S, Le Grand D, Paoli A, Gay E, Poumarat F. Overall decrease in the susceptibility of *Mycoplasma Bovis* to antimicrobials over the past 30 years in France. *PLoS ONE.* (2014) 9:e87672. doi: 10.1371/Journal.Pone.0087672
12. Stanford K, Zaheer R, Klima C, McAllister T, Peters D, Niu YD, et al. Antimicrobial resistance in members of the bacterial bovine respiratory disease complex isolated from lung tissue of cattle mortalities managed with or without the use of antimicrobials. *Microorganisms.* (2020) 8:288. doi: 10.3390/Microorganisms8020288
13. Magstadt DR, Schuler AM, Coetzee JF, Krull AC, O'Connor AM, Cooper VL, et al. Treatment history and antimicrobial susceptibility results for *Mannheimia Haemolytica*, *Pasteurella Multocida*, and *Histophilus Somni* isolates from bovine respiratory disease cases submitted to the Iowa State University Veterinary Diagnostic Laboratory from 2013 to 2015. *J Vet Diagn Invest.* (2018) 30:99–104. doi: 10.1177/1040638717737589
14. Waldner CL, Parker S, Gow S, Wilson DJ, Campbell JR. Antimicrobial usage in Western Canadian cow-Calf herds. *Can Vet J.* (2019) 60:255–67.
15. Abell KM, Theurer ME, Larson RL, White BJ, Apley M. A mixed treatment comparison meta-analysis of metaphylaxis treatments for bovine respiratory disease in beef cattle. *J Anim Sci.* (2017) 95:626–35. doi: 10.2527/jas.2016.1062
16. Woolums AR. Editor BRD in preweaned calves: what's new in risk factors? In: *46th Annual Conference of the American Association of Bovine Practitioners*. Milwaulkee, WI (2013).
17. Davis-Unger J, Schwartzkopf-Genswein KSG, Pajor EA, Hendrick S, Marti S, Dorin C, et al. Prevalence and lameness-associated risk factors in Alberta feedlot cattle. *Transl Anim Sci.* (2019) 3:595–606. doi: 10.1093/tas/Txz008
18. Checkley SL, Campbell JR, Chirino-Trejo M, Janzen ED, Waldner CL. Associations between antimicrobial use and the prevalence of antimicrobial resistance in fecal *Escherichia Coli* from feedlot cattle in Western Canada. *Can Vet J.* (2010) 51:853–61.
19. Holman DB, Timsit E, Amat S, Abbott DW, Buret AG, Alexander TW. The nasopharyngeal microbiota of beef cattle before and after transport to a feedlot. *BMC Microbiol.* (2017) 17:70. doi: 10.1186/S12866-017-0978-6
20. Klima CL, Zaheer R, Cook SR, Booker CW, Hendrick S, Alexander TW, et al. Pathogens of bovine respiratory disease in North American feedlots conferring multidrug resistance via integrative conjugative elements. *J Clin Microbiol.* (2014) 52:438–48. doi: 10.1128/JCM.02485-13
21. Register KB, Thole L, Rosenbush RF, Minion FC. Multilocus sequence typing of *Mycoplasma Bovis* reveals host-specific genotypes in cattle versus bison. *Vet Microbiol.* (2015) 175:92–8. doi: 10.1016/j.Vetmic.2014.11.002
22. Angen O, Thomsen J, Larsen LE, Larsen J, Kokotovic B, Heegaard PM, et al. Respiratory disease in calves: microbiological investigations on trans-tracheally aspirated bronchoalveolar fluid and acute phase protein response. *Vet Microbiol.* (2009) 137:165–71. doi: 10.1016/j.Vetmic.2008.12.024
23. Miflin JK, Blackall PJ. Development of a 23S RRNA-based PCR assay for the identification of *Pasteurella multocida*. *Lett Appl Microbiol.* (2001) 33:216–21. doi: 10.1046/j.1472-765x.2001.00985.x
24. Angen O, Ahrens P, Tegtmeier C. Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. *Vet Microbiol.* (1998) 63:39–48. doi: 10.1016/S0378-1135(98)00222-3
25. Gioia G, Werner B, Nydam DV, Moroni P. Validation of a mycoplasma molecular diagnostic test and distribution of mycoplasma species in bovine milk among New York State dairy farms. *J Dairy Sci.* (2016) 99:4668–77. doi: 10.3168/jds.2015-10724
26. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard,* 4th ed. VET01-A4. Wayne, PA Clinical and Laboratory Standards Institute (2013).
27. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing.* 26th ed. Wayne, PA: Clinical and Laboratory Standards Institute (2016).
28. Hannan PC. Guidelines and recommendations for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary mycoplasma species. International research programme on comparative mycoplasmology. *Vet Res.* (2000) 31:373–95. doi: 10.1051/Vetres:2000100
29. Rosenbusch RF, Kinyon JM, Apley M, Funk ND, Smith S, Hoffman LJ. *In vitro* antimicrobial inhibition profiles of *mycoplasma bovis* isolates recovered from various regions of the United States from 2002 to 2003. *J Vet Diagn.* (2005) 17:436–41. doi: 10.1177/104063870501700505
30. Jelinski M, Kinnear A, Gesy K, Andres-Lasheras S, Zaheer R, Weese S, et al. Antimicrobial sensitivity testing of *Mycoplasma bovis* isolates derived from western Canadian feedlot cattle. *Microorganisms.* (2020) 8:124. doi: 10.3390/Microorganisms8010124
31. Ayling RD, Rosales RS, Barden G, Gosney FL. Changes in antimicrobial susceptibility of *Mycoplasma bovis* isolates from Great Britain. *Vet Rec.* (2014) 175:486. doi: 10.1136/vr.102303
32. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol.* (2012) 19:455–77. doi: 10.1089/cmb.2012.0021

33. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics.* (2014) 30:2068–9. doi: 10.1093/Bioinformatics/Btu153
34. Feldgarden M, Brover V, Haft DH, Prasad AB, Slotta DJ, Tolstoy I, et al. Validating the AMRFinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. *Antimicrob Agents Chemother.* (2019) 63:e00483. doi: 10.1128/AAC.00483-19
35. R Core Team. *R: A Language and Environment for Statistical Computing.* Vienna: R Foundation for Statistical Computing (2017).
36. Bürkner P-C. Brms: an R package for Bayesian multilevel models using stan. *J Stat Soft.* (2017) 80:28. doi: 10.18637/jss.V080.i01
37. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using Lme4. *J Stat Soft.* (2015) 67:48. doi: 10.18637/jss.V067.i01
38. van Buuren S, Groothuis-Oudshoorn K. Mice: multivariate imputation by chained equations in R. *J Stat Soft.* (2011) 45:67. doi: 10.18637/jss.V045.i03
39. Denwood MJ. Runjags: an R package providing interface utilities, model templates, parallel computing methods and additional distributions for MCMC models in JAGS. *J Stat Softw.* (2016) 71:1–25. doi: 10.18637/jss.V071.i09
40. Godinho KS, Sarasola P, Renoult E, Tilt N, Keane S, Windsor GD, et al. Use of deep nasopharyngeal swabs as a predictive diagnostic method for natural respiratory infections in calves. *Vet Rec.* (2007) 160:22–5. doi: 10.1136/vr.160.1.22
41. Troxler S, Lalonde T, Wilson JR. Exact logistic models for nested binary data. *Stat Med.* (2011) 30:866–76. doi: 10.1002/sim.4157
42. van de Kassteele J, van Santen-Verheuvel MG, Koedijk FD, van Dam AP, van der Sande MA, de Neeling AJ. New statistical technique for analyzing MIC-based susceptibility data. *Antimicrob Agents Chemother.* (2012) 56:1557–63. doi: 10.1128/AAC.05777-11
43. Plummer M. *JAGS Version 4.3.0 User Manual*. (2017). Available online at: https://People.Stat.sc.edu/Hansont/Stat740/Jags_User_Manual.pdf (accessed July 18, 2021).
44. Snowder GD, Van Vleck LD, Cundiff LV, Bennett GL. Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. *J Anim Sci.* (2006) 84:1999–2008. doi: 10.2527/jas.2006-046
45. McMullen C, Alexander TW, Orsel K, Timsit E. Progression of nasopharyngeal and tracheal bacterial microbiotas of feedlot cattle during development of bovine respiratory disease. *Vet Microbiol.* (2020) 248:108826. doi: 10.1016/j.Vetmic.2020.108826
46. Dabo SM, Taylor JD, Confer AW. *Pasteurella multocida* and bovine respiratory disease. *Anim Health Res Rev.* (2007) 8:129–50. doi: 10.1017/S1466252307001399
47. Timsit E, Hallewell J, Booker C, Tison N, Amat S, Alexander TW. Prevalence and antimicrobial susceptibility of *Mannheimia Haemolytica*, *Pasteurella Multocida*, and *Histophilus Somni* isolated from the lower respiratory tract of healthy feedlot cattle and those diagnosed with bovine respiratory disease. *Vet Microbiol.* (2017) 208:118–25. doi: 10.1016/j.Vetmic.2017.07.013
48. Guo Y, McMullen C, Timsit E, Hallewell J, Orsel K, van der Meer F, et al. Genetic relatedness and antimicrobial resistance in respiratory bacteria from beef calves sampled from spring processing to 40 days after feedlot entry. *Vet Microbiol.* (2020) 240:108478. doi: 10.1016/j.Vetmic.2019.108478
49. Caswell JL, Bateman KG, Cai HY, Castillo-Alcala F. *Mycoplasma bovis* in respiratory disease of feedlot cattle. *Vet Clin North Am Food Anim Pract.* (2010) 26:365–79. doi: 10.1016/j.Cvfa.2010.03.003
50. Castillo-Alcala F, Bateman KG, Cai HY, Schott CR, Parker L, Clark ME, et al. Prevalence and genotype of *Mycoplasma bovis* in beef cattle after arrival at a feedlot. *Am J Vet Res.* (2012) 73:1932–43. doi: 10.2460/Ajvr.73.12.1932
51. Stroebel C, Alexander T, Workentine ML, Timsit E. Effects of transportation to and co-mingling at an auction market on nasopharyngeal and tracheal bacterial communities of recently weaned beef cattle. *Vet Microbiol.* (2018) 223:126–33. doi: 10.1016/j.Vetmic.2018.08.007
52. Timsit E, Arcangioli MA, Bareille N, Seegers H, Assie S. Transmission dynamics of *Mycoplasma bovis* in newly received beef bulls at fattening operations. *J Vet Diagn Invest.* (2012) 24:1172–6. doi: 10.1177/1040638712463211
53. Ribble CS, Meek AH, Shewen PE, Guichon PT, Jim GK. Effect of pretransit mixing on fatal fibrinous pneumonia in calves. *J Am Vet Med Assoc.* (1995) 207:616–9.
54. Step DL, Krehbiel CR, DePra HA, Cranston JJ, Fulton RW, Kirkpatrick JG, et al. Effects of commingling beef calves from different sources and weaning protocols during a forty-two-day receiving period on performance and bovine respiratory disease. *J Anim Sci.* (2008) 86:3146–58. doi: 10.2527/jas.2008-0883
55. Anholt RM, Klima C, Allan N, Matheson-Bird H, Schatz C, Ajitkumar P, et al. Antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex in Alberta, Canada. *Front Vet Sci.* (2017) 4:207. doi: 10.3389/fvets.2017.00207
56. Klima CL, Cook SR, Zaheer R, Laing C, Gannon VP, Xu Y, et al. Comparative genomic analysis of *Mannheimia Haemolytica* from bovine sources. *PLoS ONE.* (2016) 11:e0149520. doi: 10.1371/Journal.Pone.0149520
57. Eidam C, Poehlein A, Leimbach A, Michael GB, Kadlec K, Liesegang H, et al. Analysis and comparative genomics of ICEMh1, a novel integrative and conjugative element (ICE) of *Mannheimia haemolytica*. *J Antimicrob Chemother.* (2015) 70:93–7. doi: 10.1093/jac/Dku361
58. Michael GB, Kadlec K, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, et al. ICEPmu1, an integrative conjugative element (ICE) of *Pasteurella multocida*: analysis of the regions that comprise 12 antimicrobial resistance genes. *J Antimicrob Chemother.* (2012) 67:84–90. doi: 10.1093/jac/Dkr406
59. Vester B, Douthwaite S. Macrolide resistance conferred by base substitutions in 23S RRNA. *Antimicrob Agents Chemother.* (2001) 45:1–12. doi: 10.1128/AAC.45.1.1-12.2001
60. Olsen AS, Warrass R, Douthwaite S. Macrolide resistance conferred by RRNA mutations in field isolates of *Mannheimia haemolytica* and *Pasteurella multocida*. *J Antimicrob Chemother.* (2015) 70:420–3. doi: 10.1093/jac/Dku385
61. Sulyok KM, Kreizinger Z, Wehmann E, Lysnyansky I, Banyai K, Marton S, et al. Mutations associated with decreased susceptibility to seven antimicrobial families in field and laboratory-derived *Mycoplasma bovis* strains. *Antimicrob Agents Chemother.* (2017) 61:e01983-16. doi: 10.1128/AAC.01983-16
62. Brault SA, Hannon SJ, Gow SP, Warr BN, Withell J, Song J, et al. Antimicrobial use on 36 Beef Feedlots in Western Canada: 2008-2012. *Front Vet Sci.* (2019) 6:329. doi: 10.3389/Fvets.2019.00329
63. Kinnear A. *An investigation of antimicrobial susceptibility and genotypes of Mycoplasma bovis isolates derived from Western Canadian feedlot cattle* (Dissertation), Saskatoon: University of Saskatchewan (2021).
64. Hannon SJ, Brault SA, Otto SJG, Morley PS, McAllister TA, Booker CW, et al. Feedlot cattle antimicrobial use surveillance network: a Canadian journey. *Front Vet Sci.* (2020) 7:596042. doi: 10.3389/Fvets.2020.596042
65. Andrés-Lasheras S, Ha R, Zaheer R, Lee C, Booker CW, Dorin C, et al. Prevalence and risk factors associated with antimicrobial resistance in bacteria related to bovine respiratory disease—a broad cross-sectional study of beef cattle at entry into Canadian feedlots. *Front Vet Sci.* (2021) 8:692646. doi: 10.3389/Fvets.2021.692646
66. Uemura R, Sueyoshi M, Nagatomo H. Antimicrobial susceptibilities of four species of *Mycoplasma* isolated in 2008 and 2009 from cattle in Japan. *J Vet Med Sci.* (2010) 72:1661–3. doi: 10.1292/Jvms.10-0165
67. Hendrick SH, Bateman KG, Rosengren LB. The effect of antimicrobial treatment and preventive strategies on bovine respiratory disease and genetic relatedness and antimicrobial resistance of *Mycoplasma bovis* isolates in a Western Canadian feedlot. *Can Vet J.* (2013) 54:1146–56.
68. Davedow T, Narvaez-Bravo C, Zaheer R, Sanderson H, Rodas-Gonzalez A, Klima C, et al. Investigation of a reduction in tylosin on the prevalence of liver abscesses and antimicrobial resistance in enterococci in feedlot cattle. *Front Vet Sci.* (2020) 7:90. doi: 10.3389/Fvets.2020.00090
69. Agga GE, Schmidt JW, Arthur TM. Effects of in-feed chlortetracycline prophylaxis in beef cattle on animal health and antimicrobial-resistant *Escherichia coli*. *Appl Environ Microbiol.* (2016) 82:7197–204. doi: 10.1128/AEM.01928-16
70. Baptiste KE, Kyvsgaard NC. Do antimicrobial mass medications work? a systematic review and meta-analysis of randomised clinical trials investigating antimicrobial prophylaxis or metaphylaxis against naturally occurring bovine respiratory disease. *Pathog Dis.* (2017) 75:ftx083. doi: 10.1093/femspd/ftx083

71. Watts JL, Sweeney MT. Antimicrobial resistance in bovine respiratory disease pathogens: measures, trends, and impact on efficacy. *Vet Clin North Am Food Anim Pract.* (2010) 26:79–88. doi: 10.1016/j.Cvfa.2009.10.009

**Conflict of Interest:** CD and EH are part owner and managing partners of Veterinary Agri-Health Services. Veterinary Agri-Health Services is a health management oriented veterinary practice based in Alberta that provides professional services to feedlot and cow/calf operations across western Canada.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 10 November 2021
doi: 10.3389/fvets.2021.765495

# Genomic and Transcriptomic Analysis of Bovine *Pasteurella multocida* Serogroup A Strain Reveals Insights Into Virulence Attenuation

*Li Zhan*[1†], *Jiaqi Zhang*[1†], *Boyu Zhao*[1], *Xintian Li*[1], *Xiqing Zhang*[1], *Renge Hu*[2], *Emad Mohammed Elken*[3], *Lingcong Kong*[1,4*] *and Yunhang Gao*[1,4*]

[1] College of Animal Science and Technology, Jilin Agricultural University, Changchun, China, [2] Marine College, Shandong University, Weihai, China, [3] Animal Production Department, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt, [4] The Key Laboratory of Animal Production, Product Quality and Security, Ministry of Education, Jilin Agricultural University, Changchun, China

OPEN ACCESS

**Edited by:**
Annamaria Pratelli,
University of Bari Aldo Moro, Italy

**Reviewed by:**
Yibao Chen,
Huazhong Agricultural University, China
Nengzhang Li,
Southwest University, China

***Correspondence:**
Yunhang Gao
gaoyunhang@163.com
Lingcong Kong
congwbs@126.com

[†]These authors have contributed equally to this work and share first authorship

**Specialty section:**
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

**Received:** 27 August 2021
**Accepted:** 15 September 2021
**Published:** 10 November 2021

**Citation:**
Zhan L, Zhang J, Zhao B, Li X, Zhang X, Hu R, Elken EM, Kong L and Gao Y (2021) Genomic and Transcriptomic Analysis of Bovine Pasteurella multocida Serogroup A Strain Reveals Insights Into Virulence Attenuation. Front. Vet. Sci. 8:765495. doi: 10.3389/fvets.2021.765495

*Pasteurella multocida* is one of the primary pathogens of bovine respiratory disease (BRD), and causes huge losses in the cattle industry. The Pm3 strain was a natural isolate, which is a strong form of pathogen and is sensitive to fluoroquinolones antibiotics. A high fluoroquinolone resistant strain, Pm64 (MIC = 64 μg/mL), was formed after continuous induction with subinhibitory concentration (1/2 MIC) of enrofloxacin, with the enhanced growth characteristics and large attenuation of pathogenicity in mice. This study reports the whole genome sequence and the transcription profile by RNA-Seq of strain Pm3/Pm64. The results showed an ineffective difference between the two strains at the genome level. However, 32 genes could be recognized in the gene islands (GIs) of Pm64, in which 24 genes were added and 8 genes were lost. Those genes are involved in DNA binding, trehalose metabolism, material transportation, capsule synthesis, prophage, amino acid metabolism, and other functions. In Pm3 strain, 558 up-regulated and 568 down-regulated genes were found compared to Pm64 strain, from which 20 virulence factor-related differentially expressed genes (DEGs) were screened. Mainly differentially transcribed genes were associated with capsular polysaccharide (CPS), lipopolysaccharide (LPS), lipooligosaccharide (LOS). Iron utilization, and biofilm composition. We speculated that the main mechanism of virulence attenuation after the formation of resistance of Pm64 comes from the change of the expression profile of these genes. This report elucidated the toxicity targets of *P. multocida* serogroup A which provide fundamental information toward the understanding of the pathogenic mechanism and to decreasing antimicrobial drugs resistance.

**Keywords: *P. multocida* serogroup A, complete genome sequencing, transcriptomic sequencing, gene islands, virulence gene**

## INTRODUCTION

In recent years, the high incidence of bovine respiratory diseases (BRD) has seriously affected the cattle breeding industry worldwide, and these diseases mainly occur in fattening cattle and calves during long distance transportation, and cause a lack of energy and appetite, accompanied by cough, runny nose, and dyspnea (1). In North America, BRD accounts for ∼75% of all disease incidence and 50% of all mortality in cattle farms, with an even higher prevalence (nearly 90%) in calves (2).

Bacterial pathogens which are associated with BRD include *Pasteurella multocida*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Histophilus somni* (3). Among these bacteria, *Pasteurella multocida* (*P. multocida*), a pathogenic gram-negative bacterium, has been divided into three subspecies, five capsular serogroups, and 16 serotypes. *P. multocida* serogroup A is commonly isolated from both enzootic calf pneumonia of young dairy calves and shipping fever of weaned and stressed beef cattle (4). In Belgium between 2016 and 2018, analysis of the cattle samples from 128 BRD outbreaks found *P.multocida* with a detection rate of 89.1% (5). Several researchers confirmed that *P. multocida* serogroup A was the primary pathogen causing BRD in China, and in our previous study, we found it has a higher risk of fluoroquinolone resistance during antibiotic therapy (6, 7).

After acquiring antibiotic resistance, bacteria are prone to incurring fitness costs, which are manifested in survival inhibition, transformation rate retardation, and virulence attenuation in resistant strains compared with wild type strains without antibiotic treatments (8, 9). But the cost is not absolute; some bacteria do not experience this phenomenon, while others have a compensative evolution to maintain their competitive advantage and levels of resistance without antibiotic pressure (10–12). It blows a hole in the theory that reducing the use of antibiotics can reduce antibiotic resistance. Developing targeted drugs at the molecular level to coordinate antibiotic use to control antibiotic resistance and virulence factors may lead to more effective control.

During the evaluation of antimicrobial susceptibility test and antibiotic resistance risk of *P. multocida* isolates in our research, one *P. multocida* serogroup A isolate (Pm3) showed a strong virulence to mice and resistance developed rapidly with the increase of fluoroquinolones *in vitro*. Hence, a fluoroquinolone-resistant strain, Pm64, was obtained from fluoroquinolone-sensitive strain Pm3 induced by enrofloxacin at a subinhibitory concentration (Increasingly 1/2 MIC). And we found that the virulence of the Pm64 strain to enrofloxacin decreased significantly. This study aimed to further analyze the mechanism of toxicity attenuation of *P. multocida* serogroup A with the development of fluoroquinolone drug resistance. In this study, the difference between *P. multocida* Pm3 and Pm64 strains were compared on genomic and transcriptome levels. We hypothesized that genes and gene expression patterns investigations will show the differences between the two strains which may elucidate the underlying molecular virulence attenuation mechanisms of *P. multocida*. We found several candidate genes that may be highly important to the virulence attenuation of *P. multocida* serogroup A strains and may facilitate the design of new and improved vaccines and target drugs to overcome the rapid growth of antibiotic resistance.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

*P. multocida* serogroup A Pm3 strain used in the present study was isolated from the nasal cavity of cattle with BRD in Henan China. Previous studies have identified the molecular type of Pm3 (7). The Pm64 strain was induced from Pm3 strain by gradually increasing subinhibitory concentration (1/2 MIC) enrofloxacin in a liquid culture environment. Every 12 h was recorded as one generation, and the growth was observed to determine whether enrofloxacin concentration doubled or not. All the strains were streaked on Brain Heart Infusion (BHI) agar plates (Qingdao Hope Biol-Technology Co., Ltd., Qingdao, China). One colony of each strain was inoculated into 5 mL BHI broth at 37°C with shaking (160 rpm).

### Median Lethal Dose ($LD_{50}$) Determination

The $LD_{50}$ of Pm3 and Pm64 strains were determined by the modified Kirschner method, and the colony count of each strain was carried out (13). Then, four graded doses of $1 \times 10^6$-$1 \times 10^9$ and $1 \times 10^{11}$-$1 \times 10^{14}$ colony-forming units (CFU)/mL of the two strains were set through the pre-experiment. Eight mice, with equal numbers of males and females ($20 \pm 3$ g), were randomly selected from each group. Mice by intraperitoneal injection of 0.2 mL bacterial suspension of different concentration gradients and the control group was injected with equal pH 7 phosphate buffer solution (PBS). The mice in each group were kept isolated, the death rate was observed for 3 days, and the $LD_{50}$ was counted and calculated (14). The data were statistically analyzed using SPSS (19.0) software. The lungs of dead mice were fixed in formalin, embedded in paraffin, and then stained with H&E. Observe the pathological changes under a microscope. All the methods were carried out in accordance with the US NIH guidelines and protocols for laboratory animal use and proper care, approved by the Animal Care and Use Committee of Jilin Agricultural University.

### Growth Curves Measurement

Pm3 and Pm64 strains were aerobically subcultured twice in the BHI medium. 4% cultures were selected to inoculate fresh BHI medium for monitoring growth at 37°C with shaking(160 rpm). The growth rates of Pm3 and Pm64 cultures were plotted by recording OD changes at 0, 2, 4, 6, 8, 10, and 12 h.

### Whole Genome Sequencing

Genome DNA of Pm3 and Pm64 was extracted using a genomic DNA kit (A&A Biotechnology, Gdansk, Poland). The harvested DNA was detected by the agarose gel electrophoresis and quantified by Qubit® 2.0 Fluorometer (Thermo Scientific). Then the large fragments of DNA were recovered by Blue Pippin automatic nucleic acid fragment recovery system, and repaired. Barcode was added by PCR-free method of EXP-NBD104 kit (Oxford Nanopore Technologies Company). The size of fragments was detected by AATI automatic

capillary electrophoresis instrument, and the samples were isomolar mixed.After that the SQK-LSK109 connection kit was used to link the adapter. The Nanopore PromethION platform Libraries for sequencing were constructed with an insert size of 10 kb. Next, Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA). The whole genome of Pm-3 and Pm-64 was sequenced using Nanopore PromethION platform and Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd. Genome sequences were assembled using Unicycler to combine PE150 data and Nanopore data, then the reads were compared to the assembled sequence, the distribution of sequencing depth was couned, whether the assembled sequence is a chromosomal sequence or a plasmid sequence was distinguished according to sequence length and alignment, and it was checkedwhether it was a circular genome. The Pm3 DNA sequence was deposited in GenBank with the accession number CP081486 (BioSample SAMN20842242). The Pm64 DNA sequence was deposited in GenBank with the accession number CP081487 (BioSample SAMN20845833).

## Genomic Analysis

Related coding genes were retrieved using the GeneMarkS program and the interspersed repetitive sequences were predicted using the RepeatMasker (perl 5.8.0). The tandem Repeats were analyzed by the TRF (Tandem repeats finder). Genomics Islands were predicted using IslandPath-DIOMB program. Gene function of the whole genome sequences was subsequently annotated with GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Cluster of Orthologous Groups of proteins), and NR (Non-Redundant Protein Database). The secretory proteins were predicted by the Signal P database, and the prediction of Type I-VII proteins secreted by the pathogenic bacteria was based on the EffectiveT3 software. Pathogenicity and drug resistance were analyzed using the PHI (Pathogen Host Interactions), VFDB (Virulence Factors of Pathogenic Bacteria), and ARDB (Antibiotic Resistance Genes Database).

## RNA Sequencing

Total RNA of Pm3 and Pm64 bacterial samples were extracted using Trizol reagent (Invitrogen Life Technologies, USA), following the manufacturer's protocol, each treatment was conducted in triplicate. RNA degradation and contamination of samples was monitored on 1% agarose gels. RNA integrity of samples were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). After the RNA quality testing, the ribosomal RNA (rRNA) in total RNA was removed to obtain mRNA. Subsequently, the obtained mRNA was broken into short fragments randomly by fragmentation buffer, and the library was built in a chain-specific way (15). After that, Qubit2.0 Fluorometer was used for preliminary quantification, and the library was diluted to 1.5 ng/ul. Then the Agilent 2100 bioanalyzer was used to detect the insert size of the library. qRT-PCR was used to accurately quantify the effective concentration of the library (>2 nM) when the insert size meets expectations (StepOnePlus Real-time PCR Systems, Thermo Science). Finally, samples were sequenced on the Illumina Novaseq platform, and 150-bp paired-end reads were generated. The sequencing data were deposited in GenBank, with accession number GSE182406.

## RNA-Seq Analysis

Clean data (clean reads) that all the downstream analyses were based on were obtained from Raw data (raw reads) by removing reads containing adapter, reads containing ploy-N, and low quality reads. At the same time, quality scores Q20, Q30, and GC content of the clean data were calculated. Reads were mapped onto the genes of *P. multocida* 36950. Both building index of the reference genome and aligning clean reads to reference genome used Bowtie2-2.2.3 (16). HTSeq v0.6.1 was used to count the read numbers mapped to each gene. And then FPKM (Fragments Per Kilobase of transcript per Million mapped reads) of each gene was calculated based on the length of the gene and read count mapped to the gene. Differential expression analysis (DEGs) of two strains was performed using the DESeq2 (17). Genes with an adjusted log2(FoldChange)| > 0 & Padj < 0.05 found by DESeq2 were assigned as differentially expressed. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias was corrected. And the statistical enrichment of differential expression genes in KEGG pathways (http://www.genome.jp/kegg/) was tested using KOBAS software.

## Quantitative Real-Time PCR

Take 1ml of logarithmic growth phase bacterial liquid and use EZ gene bacterial RNA kit (BIOMIGA, California, USA) for total RNA extraction. cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, RR047A, Japan), and qRT-PCR was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, RR820A, Japan). The premixing system is 20ul (10 μL 2×TB Green Premix Ex Taq II, 0.8 μM for each primer, 2 μg cDNA, add water to 20 μL). The PCR reaction conditions were as follows: 95°C for 30 s, 45 cycles at 95°C for 5 s, and 55°C for 60 s). Each target gene was individually normalized by the reference gene 16S rRNA using the quantification method $2^{-\Delta\Delta Ct}$ (18). The specific primers were designed according to the reference sequences in NCBI with Primer-BLAST and the qRT-PCR primer sequences were listed in **Supplementary Table 1**.

# RESULTS

## Induction and Biological Characteristics of Pm64

Pm3 strain was isolated from the nasal cavity of cattle with BRD in the previous study (6). In this research, under the stimulation of a gradually increasing 1/2 MIC concentration of enrofloxacin culture environment, the MIC of enrofloxacin on Pm3 was increased from 0.05 to 64 μg/mL (continued culture for 21 generations *in vitro*). After more than two generations of continuous passage culture, Pm64 strain was obtained (**Figure 1A**).Then, the pathogenicity in mice ($LD_{50}$)

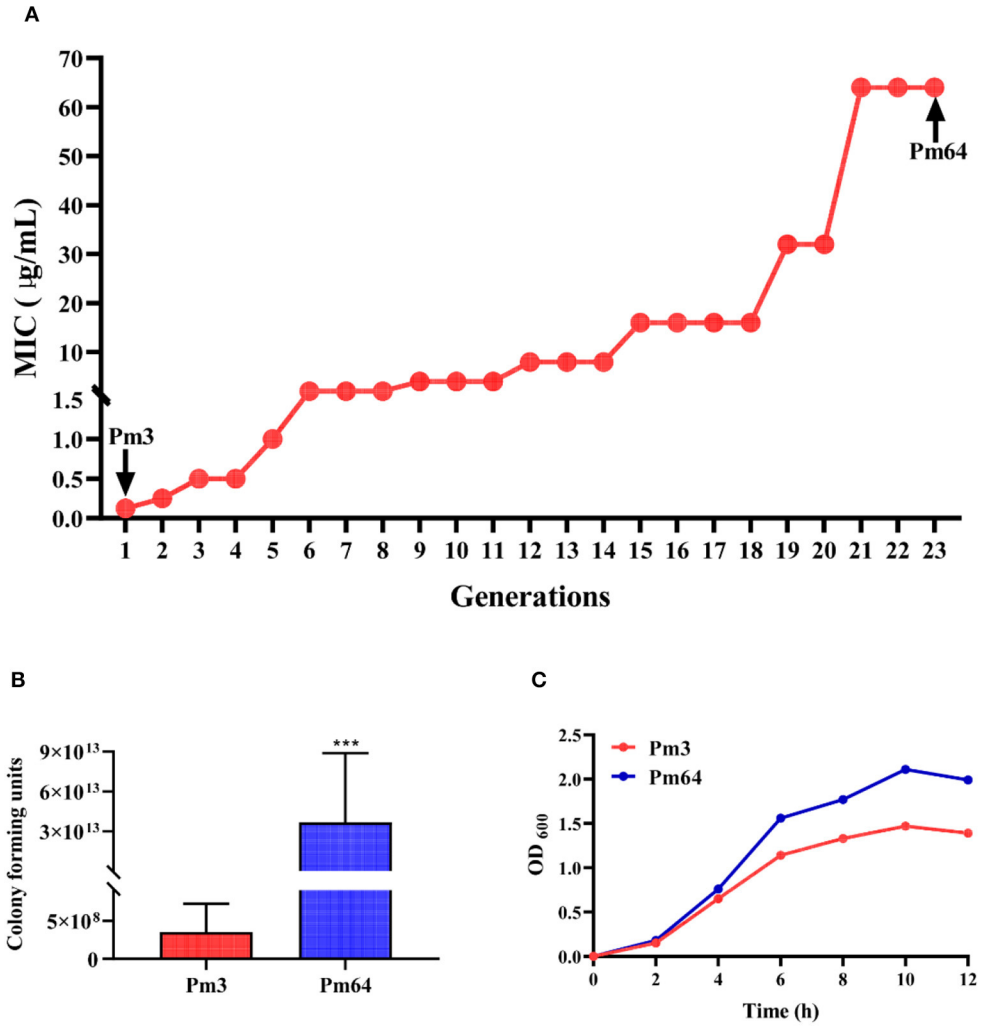



**FIGURE 1 |** Biological characteristics of Pm3 and Pm64. **(A)** Induction process of Pm64 from Pm3 strain under continuous increasing subinhibitory concentration (1/2MIC) enrofloxacin culture condition. **(B)** $LD_{50}$ of Pm3 and Pm64 to mice. **(C)** Growth curve of Pm3 and Pm64 (***$p < 0.001$).

and culture characteristics *in vitro* of the two strains were examined (**Figures 1B,C**). Results showed Pm3 strain showed a strong virulence to mice ($3.532 \times 10^8$ CFU/mL per mice). Observing the pathological changes under a microscope, it can be seen that the alveolar wall capillaries are dilated; a large number of neutrophils infiltrate, which is consistent with the infection characteristics of P. multocida (**Supplementary Figure 1**). The LD50 for the Pm64 showed a significant reduction (3.682×1013 CFU/mL per mice) with an increased growth rate and plateau peak concentration on the growth curve. The virulence of the Pm64 strain showed significant attenuation during the formation of quinolone resistance..

## Whole-Genome Features of Pm3 and Pm64

Based on the significant differences in biological characteristics of *P. multocida* serogroup A isolates P3 after being induced by fluoroquinolones, Pm3 and Pm64 were selected for whole-genome sequencing to elucidate potential mechanisms of virulence attenuation. Circos circular representation of the Pm3 and Pm64 genome with annotated genes were constructed (**Supplementary Figures 2A,B**). Lengths of the obtained genomes were, respectively, 2,386,471 bp (Pm3) and 2,424,216 bp (Pm64) and contained 2,265 (Pm3) and 2,378 (Pm64) predicted coding genes. The GC content of the Pm3 and Pm64 genome was 40.28 and 40.3%, respectively, which showed a higher similarity (**Supplementary Table 1**). The complete nucleotide sequence of Pm3 and Pm64 sequenced in this study were submitted to GenBank with accession number CP081486 and CP081487.

## Gene Islands Comparison

Gene islands (GIs) contribute to lateral gene transfer and bacterial evolution. Thus, the phylogenetically biased and mobility genes (such as transposases or integrases) of the Pm3 and Pm64 genome were detected to determine the GIs and the potential horizontal genes transfer. Eightand seven GIs were

**TABLE 1 |** Gene islands (GIs) prediction results statistics.

| Sample | Pm3 | Pm64 |
|---|---|---|
| GIs number | 8 | 7 |
| GIs total length (bp) | 148,520 | 131,993 |
| Average length (bp) | 18,565 | 18,856 |
| Predicted genes | 162 | 138 |

predicted in Pm3 and Pm64 genome, respectively (**Table 1**; **Figure 2**). Then population analysis was performed to analyze core and specific genes, and 38 and 14 genes were recognized as specific genes of Pm3 and Pm64 GIs (**Figure 3**); 24 and 8 genes of these could be realized with a potential function. We found some specific genes changed in the composition of GIs; those specific genes were involved in DNA binding, trehalose metabolism, material transportation, capsule synthesis, prophage, amino acid metabolism, and other functions (**Supplementary Tables 3**, **4**).

## Genomic Annotation and Annotation Comparison

The genome sequencing results were compared and annotated with GO, KEGG, and COG databases. The annotation results between Pm3 and Pm64 strains were compared (**Supplementary Figures 3–5**). It was found that no new genes were obtained during the formation of enrofloxacin resistance in Pm64. Although there are numerical differences in the annotated results, most of them are the recurrence of some genes. Similarly, comparing annotations results from VFDB, ARDB, and CARD database, no new genes were obtained in Pm64. In order to explain the causes of the formation of fluoroquinolone resistance in Pm64, the quinolone resistance determining region (QRDR) genes *gyrA*, *gyrB*, *parC,* and *parE* were selected for sequence alignment (**Figure 4**). The results showed that all four genes had partial mutations of the QRDR sequence but only three gene mutations led to the replacement amino acid mutations (GyrA R88I, GyrB S467F, and ParC L84S).

## RNA-Seq Analysis and DEGs Identification of Pm3 and Pm64

All data on the quality of RNA-Seq are shown in **Supplementary Table 5**. The average percentages mapped to the reference genome were all above 98%. The correlation analysis of gene expression patterns (**Figure 5A**) showed significant differences among samples and good repeatability between groups ($R^2 > 0.9$). The gene expressions' data were statistically analyzed to screen the genes with significant change in their expression in the samples of different states, and the significance of each gene in all comparison combinations was assessed by *P*-value test and FDR (False discovery rate) correction. Finally, a complete set of differential genes were identified. Based on the transcriptome analysis of Pm3 and Pm64 [$|\log2(\text{FoldChange})| > 0$ & $\text{Padj} < 0.05$], a total of 1,126 DEGs were observed, including 558 up-regulated and 568 down-regulated genes (**Figures 5B,C**). The top 10 up and down regulated DEGs were selected and shown in **Figure 6**.

## Virulence Related DEGs of Pm3 and Pm64

Functional enrichment of filtered DEGs of the virulent Pm3 strain and virulence attenuation Pm64 strain were carried out based on GO and KEGG database. By GO enrichment analysis, 508 DEGs were matched to three parts of the gene function: BP (Biological Process), CC (Cellular Component), and MF (Molecular Function). The top 30 enriched GO functions was shown in **Figure 7** and **Supplementary Figure 6**. In the overall DEGs function enrichment, CC is the most enriched, mainly including associated functions of cell, intracellular, organelle, periplasmic space, and ribosome. However, the up-regulated DEGs were more concentrated in BP, mainly nitrogen, sulfur, phosphorus metabolism, and phospholipid biosynthesis. At the same time, the down-regulated genes were concentrated in MF, which was mainly reflected primarily in the weakened activities of some transferases, oxidoreductases, kinases, and some molecules' binding ability. By KEGG enrichment analysis, a total of 504 DEGs were identified. The top 20 significantly enriched KEGG pathways were shown in **Figure 7B**, including Citrate cycle (TCA cycle), Sulfur metabolism, Biosynthesis of secondary metabolites, Oxidative phosphorylation, Metabolic pathways, Amino acids biosynthesis and metabolism, Ribosome, RNA degradation, Fatty acid biosynthesis and metabolism, Carbon metabolism, 2–Oxocarboxylic acid metabolism, Butanoate metabolism, Fructose and mannose metabolism, Propanoate metabolism, Porphyrin and chlorophyll metabolism, and Glutathione metabolism. Moreover, 20 virulence factors annotated in VFDB and related to five lipopolysaccharide and lipooligosaccharide (LPS/LOS), four pilis (including Flp pili and Type IV pili), five iron utilization, and six others were significantly changed in the formation of virulence attenuated Pm64 strain (**Supplementary Table 6**).

To validate the transcriptomic analysis results, three significantly down-regulated genes (*speF*, *grcA*, *potE*) and three significantly up-regulated genes (*L31*, *ftsH*, *dnaK*) were selected for qRT-PCR validation. The relative expression levels($2^{-\Delta\Delta Ct}$) of these genes with the transcriptome quantification results (log2FoldChange) were shown together in **Figure 8**, which shows a consistent trend from qRT-PCR results to RNA-seq results.

# DISCUSSION

Based on previous studies, *P. multocida* serogroup A is considered the major pathogen of BRD (2, 19, 20). The persistent respiratory tract infection caused by *P. multocida* serogroup A has always been a huge issue for the cattle industry. The prevention of and treatment measures for *P. multocida* are still based on broad-spectrum antibiotics, but the increasing use of antibiotics (such as fluoroquinolones and macrolides) has led to the increase of resistance and resistance spectrum of *P. multocida* (6). In view of this situation, while standardizing antibiotics, new strategies should be sought to slow down the emergence of *P. multocida* resistance and effectively suppress the harm of *P. multocida* to the host. While much progress has been made in

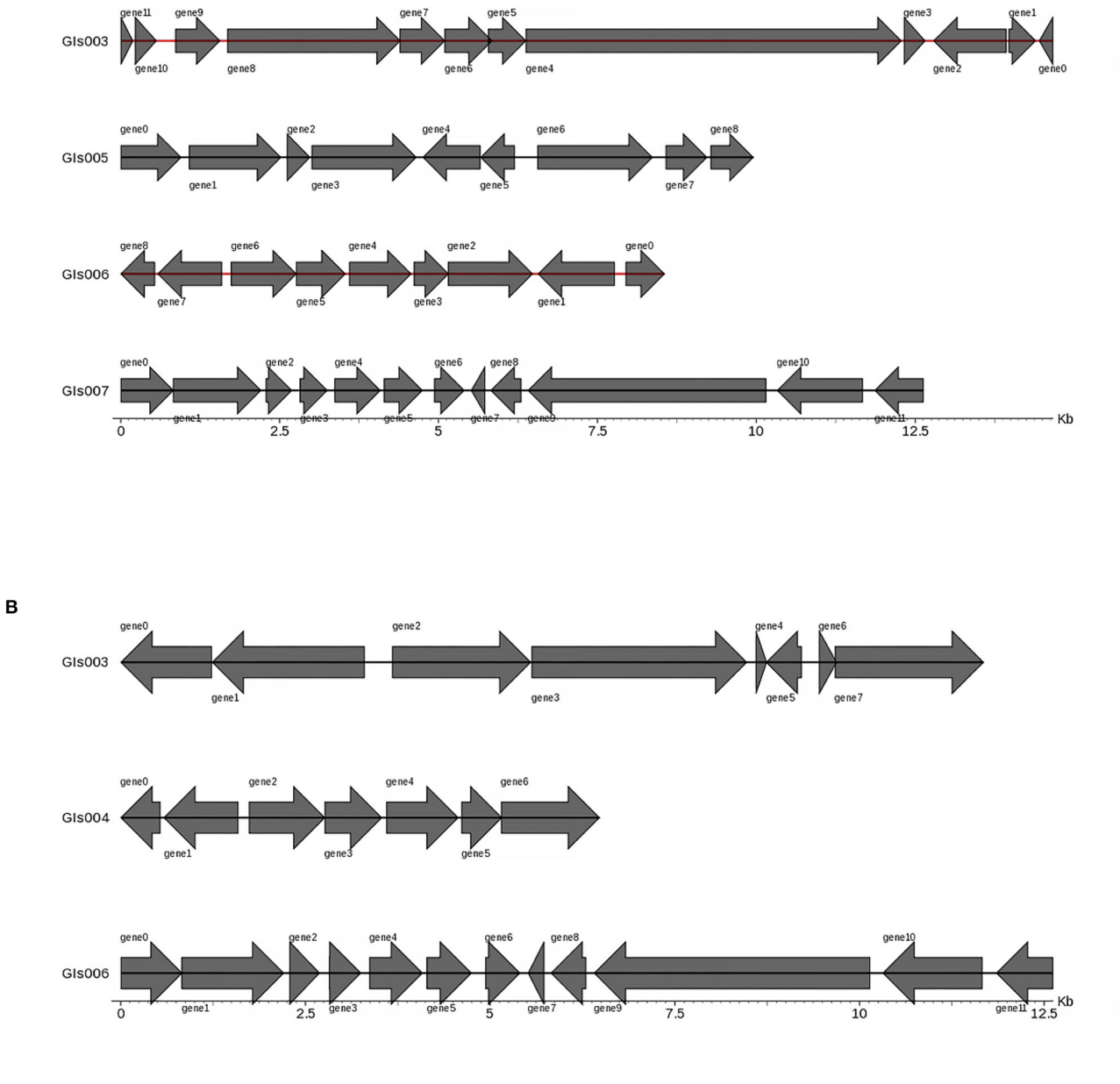



**FIGURE 2 |** Statistical representation of gene distribution in GIs of Pm3 and Pm64. **(A)** Predicted GIs structure in Pm3 chromosome. **(B)** Predicted GIs structure in Pm64 chromosome. The horizontal coordinates are length scales and the lengh of gene islands shown in the figure are <15 kb.

understanding bacterial resistance and virulence development, major gaps remain in our knowledge of the potential adaptive evolution of virulence and resistance mechanisms. We used global transcriptome and genome analyses to address theses gaps to identify the underlying regulatory and genetic adaptations in acquired fluoroquinolone-resistant *P. multocida* serogroup A. The results of our study revealed the multiple pathways in DNA replication recombination and repair, amino acid transport and metabolism, inorganic ion transport, and cell wall and membrane biogenesis that is associated with the attenuation of virulence following acquired resistance of *P. multocida* serogroup A.

The original intention of the study was to simulate the formation of fluoroquinolone resistance of *P. multocida* serogroup A *in vitro* to analyze the genetic changes in the development of resistance phenotypes in *P. multocida*. Then the study aimed to find the target of antibiotic resistant inhibitors that can be used in the follow-up screening of synergistic drug use with antibiotics.

Resistance mutations often map to genes that encode antibiotics targeted proteins; these proteins are mostly involved in some essential functions such as DNA replication, transcription and translation, or cell wall biosynthesis (21). The mutations that endowed resistance to antibiotics often provide a selective advantage in the presence of a hostile environment, but these mutations tend to alter the biochemistry of the target protein, which will adversely affect the biological function of mutants, sometimes resulting in reduced fitness of the microorganism (also known as fitness costs) (22, 23). However, in some cases, compensatory mutations ameliorate fitness costs of resistance mutations from secondary mutation, gene duplication, ectopic dominance, and metabolic compensation (12, 24–27). It was suggested that fitness costs and compensatory evolution occurred in fluoroquinolone resistance acquisition in Pm64, which allowed us to move beyond the target mutation from quinolone resistance to the mechanisms of virulence attenuation due to acquired resistance. Thus, the whole genome sequences and transcriptome expression profiles related to highly virulent quinolone sensitive strain Pm3 and virulent attenuated quinolone-resistant strain Pm64 are explored in this study.

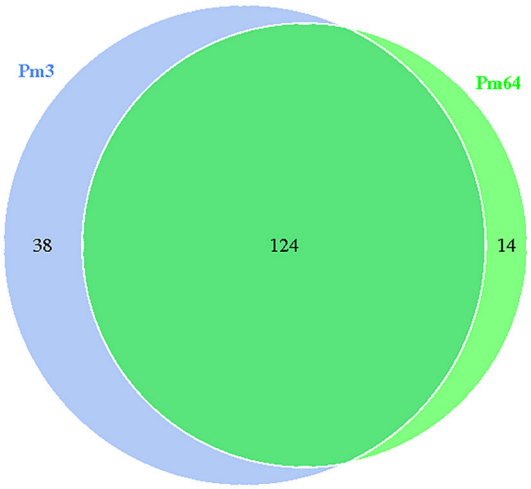



**FIGURE 3 |** Venn diagram of core and specific gene cluster of Pm3 and Pm64 gene islands. Each ellipse represents a sample, the data on each region represents the groups number occur in and only in the samples. A group represents a set of genes with more than 50% similarity and sequence length difference < 0.7.

Resistance development against fluoroquinolones can occur via various mechanisms ranging from chromosomal gene mutations to the specific transferable genes acquisition (28). The mutations in the chromosomal elements encoding the target enzyme (quinolone resistance determining region, QRDR) DNA gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*parC*, *parE*) can considerably alter the susceptibility of the isolates, while the acquisition specific genes are always involved in overexpression of efflux pumps, alteration of the membrane permeability, and the expression of inactivation enzymes (28–30). Since foreign plasmids were not acquired in the genome, the acquired horizontal transfer of quinolone resistant genes was excluded. Only chromosome-mediated spontaneous mutations of QRDR genes were detected in this study. The complete ORF genes (*gyrA*, *gyrB*, *parC,* and *parE*) of Pm3 and Pm64 were compared by sequence alignment. It was shown from comparison that the QRDR genes had incurred varying degrees of base mutation in Pm64 induced from Pm3, which leads to the amino acid substation GyrA R88I, GyrB S467F, and ParC L84S (**Figure 5**). It suggests that these site mutations may be the vital cause of quinolone resistance of *P. multocida* serogroup A, which requires further in-depth verification.

Genomic islands (GIs) of prokaryotes are discrete inserted DNA segments obtained by horizontal transfer which carries genes that could affect pathogenicity, antibiotic resistance, metabolism, and adaptability (31–33). Compared with Pm3, the GIs of Pm64 had lost some genes involved in trehalose metabolism (*treB*, *treC* and *treR*). They added several capsular polysaccharide syntheses and transport-related genes (*lipA*, *lipB*) and a gene *folD* that mediates tetrahydrofolate metabolism. Trehalose is a sugar widely distributed in bacteria, fungi, and other higher organisms, playing different biological functions. In some plant bacterial pathogens, it plays a important role in enhancing colonization and enhancing virulence (34, 35).The trehalose-monomycolate is a precursor for the synthesis of

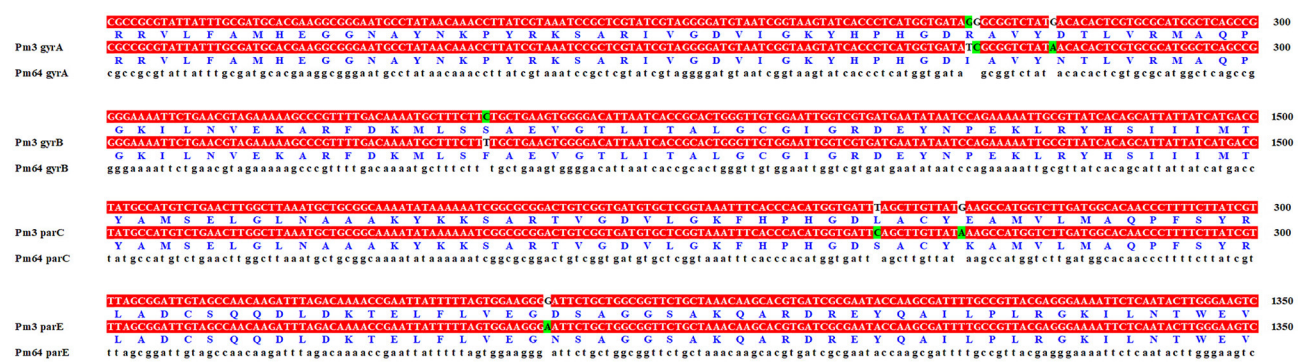



**FIGURE 4 |** Quinolone resistance determining region (QRDR) comparison of Pm3 and Pm64. Only part of the base mutations sequence (labeled in green) was shown.

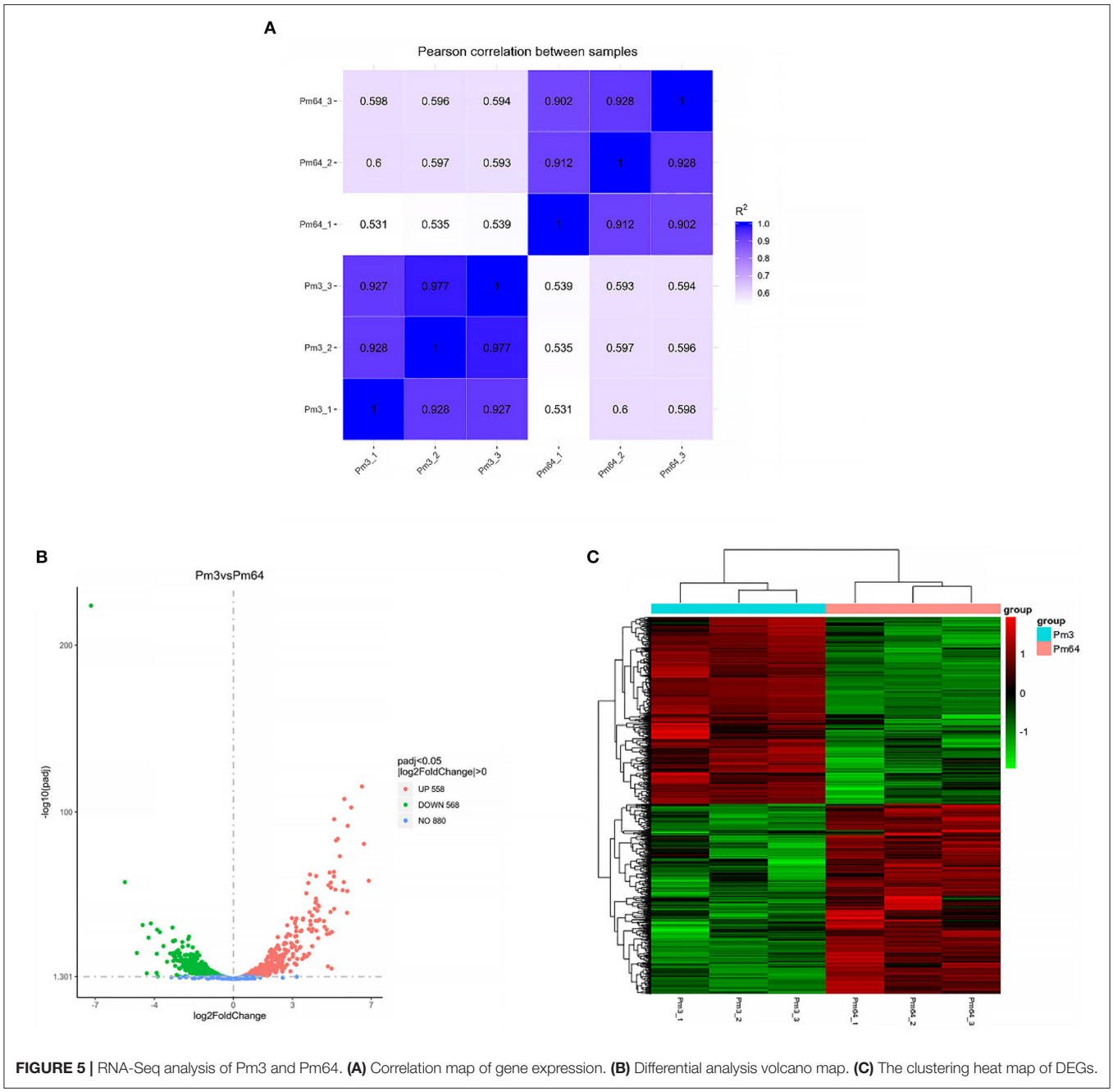



**FIGURE 5 |** RNA-Seq analysis of Pm3 and Pm64. **(A)** Correlation map of gene expression. **(B)** Differential analysis volcano map. **(C)** The clustering heat map of DEGs.

mycolic acid essential for the synthesis of the bacterial cell wall and is pathogenic (36). Capsule plays the most critical role of *P. multocida* serotype A, which is a critical structural component and a virulence factor (37). Hyaluronic acid (HA) is a component of some types of capsules of *P. multocida serotype A*, which endows the strain with anti-phagocytosis ability and the bactericidal action defense from antimicrobial agents (38, 39). FolD occupies a central position in the folate-dependent C1 metabolism, and the folate-dependent C1 metabolism provides the key building blocks for growth, most importantly nucleic acids, amino acids, provitamines, and formylated methionine tRNA for translation initiation (40, 41). Therefore, we speculated that these genetic changes in GIs were of great significance for virulence attenuation, fluoroquinolone resistance, and growth performance enhancement of *P. multocida* serotype A.

In microbial infections, the virulence genes are the major determinants of disease severity (33). In explaining the mechanism of Pm64 virulence attenuation, more attention should be attached to the level of gene expression in addition to the presence or absence of virulence genes. Therefore, the

differences in the expression levels of virulence factor related genes were analyzed in transcriptome sequencing results.

Fimbrial low-molecular-weight protein (Flp) pili are assembled and secreted by a complex of proteins encoded by the tad operon,which plays an important role in the establishment and resistance of *Vibrio vulnificus* biofilms to mechanical clearance (42). The Type IV pili, meanwhile, contributes to biofilm formation and auto agglutination (43). It was reported that the amount of capsular polysaccharide (CPS) produced by *P. multocida* is inversely proportional to the amount of biofilm formed. Through mutation or *in vitro* passage, CPS can be lost or reduced. Finally the deficient biofilm-forming strains can be transformed into robust biofilm-forming strains (32). Four pili-related genes (*tadD*, *tadF*, *comE,* and *flp1*) were up-regulated in Pm3/Pm64, which increased in biofilm formation and may lead to the decrease of CPS; in turn this leads to the attenuation of virulence to a certain extent.

Lipid A-anchored lipopolysaccharide (LPS) or lipooligosaccharide (LOS) in the outer leaflet of the outer membrane are also major virulence factors of *Gram-negative bacteria*, which are essential for bacterial viability and fitness in the host (44). And the inflammatory response to the endotoxic lipid A component is a significant cause of infection pathogenesis (45). There are two up-regulated (lpxB and lpxC) and three down-regulated (galE, rfaC, and msbA) LPS related genes in RNA Sequencing. In Gram-negative bacteria, LpxB and LpxC are essential for the lipid A biosynthesis, so maintaining the structure of the bacterial cell envelope and its growth (44, 46, 47). GalE was associated with exopolysaccharides (EPS) and LOS. The disruption of *galE* gene in *Glaesserella parasuis* could produce more biofilms and increase the sensitivity to porcine serum (48). RfaC is necessary for oligosaccharide synthesis, which is a core block of LPS formation. It was involved in flagella

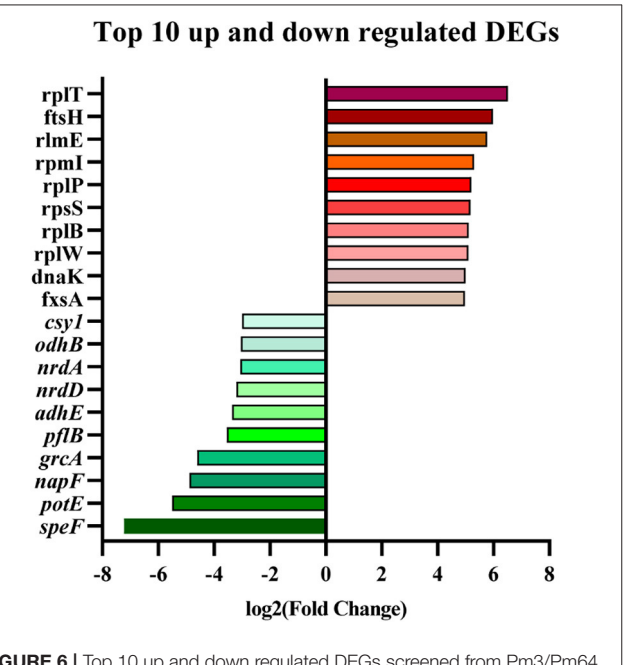



**FIGURE 6 |** Top 10 up and down regulated DEGs screened from Pm3/Pm64.

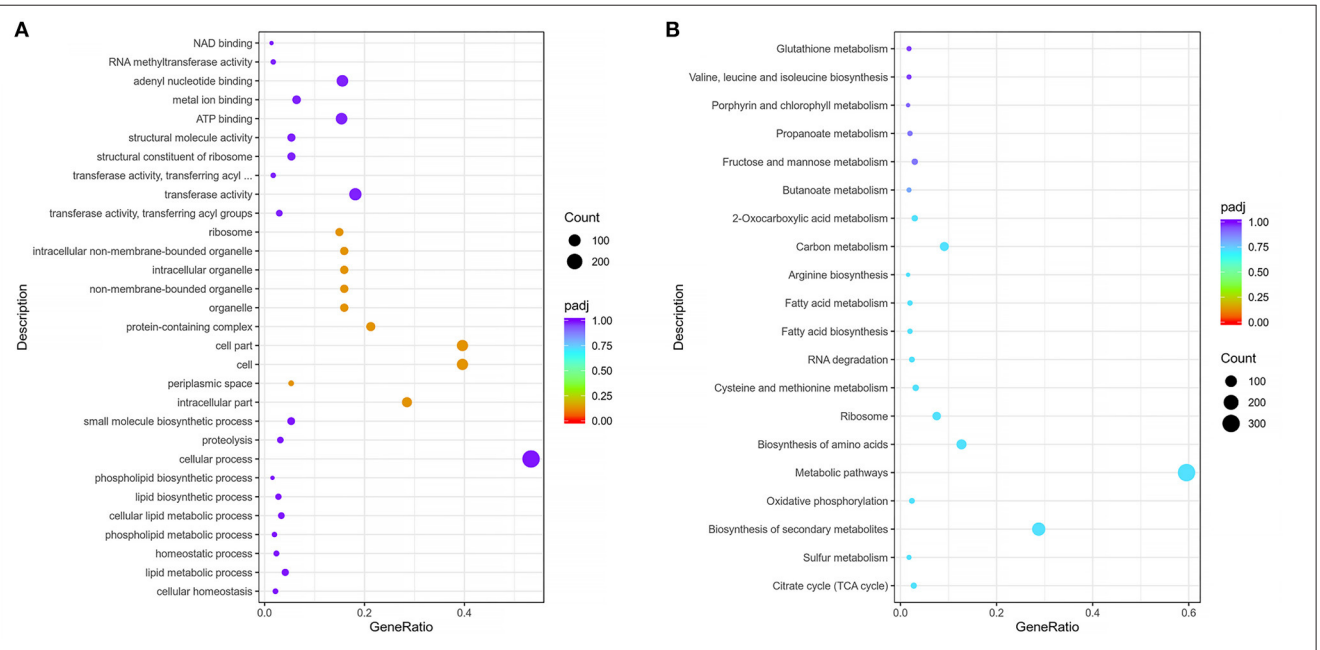



**FIGURE 7 |** Scatter plot of GO and KEGG enrichmen. **(A)** Top 30 enriched GO function and DEGs numbers enriched in each function of Pm3/Pm64. **(B)** Top 20 enriched KEGG pathways and the number of DEGs in of Pm3/Pm64. The abscissa represents the ratio of DEGs annotated to the total differential genes; the ordinate represents the GO Term and KEGG pathway, respectively; the size of the point represents the number of annotated genes; the color from red to purple represents the decreasing significance of enrichment.

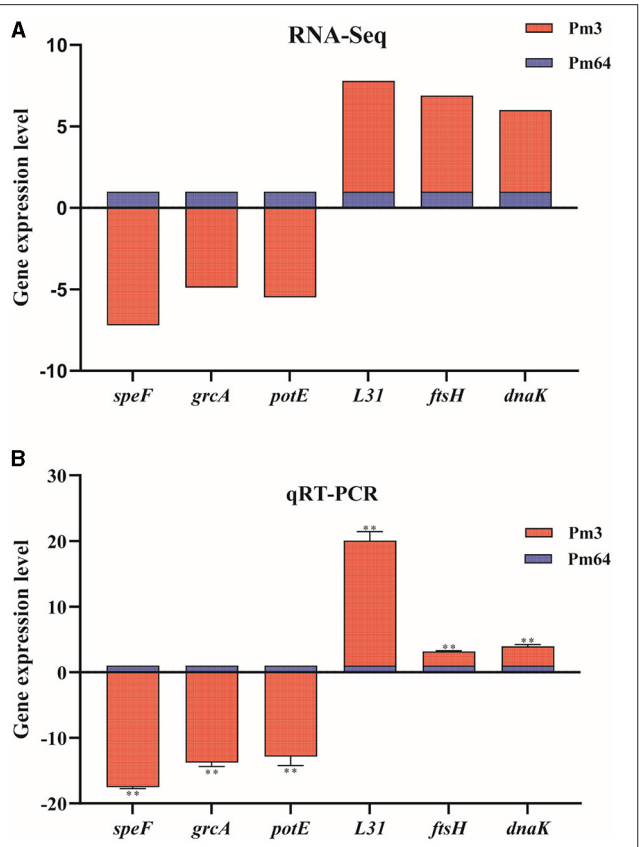



**FIGURE 8 |** Expression level validation of part of virulence factor-related DEGs ($n = 3$). **(A)** The mRNA expression (RNA-Seq) of virulence factor-related DEGs in Pm3/Pm64. **(B)** The mRNA expression (RNA-Seq) of virulence factor-related DEGs in Pm3/Pm64. Results were representative of two independent experiments, with three replicates per group, analyzed by multiple comparative analysis and expressed as means ± SD (*$p < 0.05$, **$p < 0.01$).

assembly, T3SS secretion mechanism, and protein secretion (49). Some studies have reported that it is related to the fitness cost of *E. coli* (50, 51). LPS ABC transporter MsbA is associated with the transbilayer movement of lipid A-core molecules from the cytoplasmic to the periplasmic face of the inner membrane,which is also a putative determinant of tetracycline resistance (52, 53). The changes in expression levels of LPS and LOS related gene in Pm64 not only affect the host viability and antibiotics adaptation mediated by LPS and LOS, but also greatly affect the biofilm and potentially attenuate CPS production.

Iron assimilation and its utilization are crucial for the cells' biological functions, which are not only involved in pathogenesis but also the resistance of strains (54). For pathogenic bacteria, heme is the major source of nutritional iron. Heme biosynthesis is an important cofactor of peroxidases, catalases, sensor molecules, and cytochromes, indirectly coupled to respiration (55). For the DEGs associated with heme biosynthesis, *hemG* (protoporphyrinogen oxidase), *hemH* (ferrochelatase), and *hemE* (uroporphyrinogen decarboxylase) were down-regulated and two heme transportation related genes (*ccmB* and *ccmE*) were up-regulated in Pm3/Pm64. It suggests that iron assimilation and its utilization may be the key mechanisms for the formation of quinolone resistance and the resulting attenuation of virulence.

The major outer membrane protein DnaK plays a key role in native protein folding, which was also recognized as a potential therapeutic targets in *Mycobacterium smegmatis* and *Helicobacter pylori* (23, 56). Other studies have shown that *dnaK* enhances the virulence of *Listeria monocytogenes* (57). Compared with Pm64, the *dnaK* gene in Pm3 was the most significantly up-regulated among all the screened virulence factors' DEGs. This indicates that *dnaK* may play an essential role in stress survival and virulence attenuation in *P. multocida* serogroup A.

In addition, we found some special genes that might influence the virulence but are not annotated toVFDB. These include *speF* (ornithine decarboxylase), *potE* (putrescine-ornithine antiporter), and *ftsH* (Atp-dependent zinc metalloproteinase). The polyamines have been described as key signals of virulence in pathogenic bacteria. Putrescine is the most important polyamine in bacterial cells, which could be synthesized by ornithine decarboxylation of redundant enzymes encoded by the *speC* and *speF* genes (58). The *potE* gene encodes a membrane protein that was associated with exchange reactions of putrescine and ornithine (59). The *speF* and *potE* are the two most obvious down-regulated genes in Pm3/Pm64. It is speculated that they may have a negative regulatory effect on pathogenicity. FtsH is a membrane protease that is critical for degrading membrane proteins. The decrease of proteolytic activity of FtsH protease could promote the pathogenicity of *Salmonella* in phagocytic cells, as well as the negative effects on growth, stress tolerance, and biofilm formation on *Lactobacillus plantarum* (60, 61). Moreover, the overexpression of *ftsH* in *Mycobacterium tuberculosis* causes growth retardation (62). These phenotypes were consistent with what we observed in Pm3/Pm64, suggesting an important role of these genes in the virulence attenuation mechanism of *P. multocida* serogroup A. Furthermore, some 30/50s ribosome proteins were down-regulated in Pm64 strain, suggesting more translational modification changes in the mechanism of *P. multocida* virulence attenuation, which needs more in-depth studies in the future.

While deeply exploring the mechanism of *P. multocida* virulence attenuation, an important question that needs to be discussed is whether virulence-virulence reduced strains can revert to a highly virulent phenotype if they infect animals and lose the inhibition of drugs for a long time. Similar results were not found in our study, and may require more *in vivo* data.

In conclusion, the study showed that, after being induced by enrofloxacin at the subinhibitory concentration, the virulence of the *P. multocida* serogroup A resistant strain decreased significantly. The potential mechanisms may be related to the loss genes of GIs at the genomic level and the expression changes of some virulence and drug-resistance related genes at the transcriptional level. Several candidate genes that may be highly important for the virulence attenuation of *P. multocida* serogroup A strains were found in this study, which may facilitate the design of new and improved vaccines. The data presented here provide fundamental background knowledge that

would help follow-up research on pathogenesis and antimicrobial drugs development.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, CP081486; https://www.ncbi.nlm.nih.gov/genbank/, CP081487; https://www.ncbi.nlm.nih.gov/, GSE182406.

## AUTHOR CONTRIBUTIONS

LZ and JZ: collected the samples. LZ, JZ, BZ, and XL: performed the experiments. RH, LK, XZ, LZ, and JZ: wrote the manuscript. All authors read and approved the final manuscript.

## FUNDING

This research was supported by the National Natural Science Foundation of China Youth Fund (31702293) and the China Agriculture Research System of MOF and MARA (CARS-37).

## ACKNOWLEDGMENTS

The authors would like to thank Novogene Technology Co., Ltd., Beijing, China, for high throughput sequencing.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.765495/full#supplementary-material

## REFERENCES

1. Dabo SM, Taylor JD, Confer AW. Pasteurella multocida and bovine respiratory disease. *Anim Health Res Rev.* (2007) 8:129–50. doi: 10.1017/S1466252307001399
2. Snyder E, Credille B. Mannheimia haemolytica and *Pasteurella multocida* in bovine respiratory disease: how are they changing in response to efforts to control them? *Vet Clin North Am Food Anim Pract.* (2020) 36:253–68. doi: 10.1016/j.cvfa.2020.02.001
3. Grissett GP, White BJ, Larson RL. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. *J Vet Intern Med.* (2015) 29:770–80. doi: 10.1111/jvim.12597
4. Fanelli A, Cirilli M, Lucente MS, Zarea AAK, Buonavoglia D, Tempesta M, et al. Mycoplasma bovis fatal calf pneumonia outbreaks in Italian dairy herds involving and other agents of BRD. *Front Vet Sci.* (2021) 8:742785. doi: 10.3389/fvets.2021.742785
5. Pardon B, Callens J, Maris J, Allais L, Van Praet W, Deprez P, et al. Pathogen-specific risk factors in acute outbreaks of respiratory disease in calves. *J Dairy Sci.* (2020) 103:2556–66. doi: 10.3168/jds.2019-17486
6. Kong LC, Gao D, Gao YH, Liu SM, Ma HX. Fluoroquinolone resistance mechanism of clinical isolates and selected mutants of *Pasteurella multocida* from bovine respiratory disease in China. *J Vet Med Sci.* (2014) 76:1655–7. doi: 10.1292/jvms.14-0240
7. Kong LC, Wang Z, Wang YM, Dong WL, Jia BY, Gao D, et al. Antimicrobial susceptibility and molecular typing of *Pasteurella multocida* isolated from six provinces in China. *Trop Anim Health Prod.* (2019) 51:987–92. doi: 10.1007/s11250-018-1754-9
8. Roux D, Danilchanka O, Guillard T, Cattoir V, Aschard H, Fu Y, et al. Fitness cost of antibiotic susceptibility during bacterial infection. *Sci Transl Med.* (2015) 7:297ra114. doi: 10.1126/scitranslmed.aab1621
9. Guo B, Abdelraouf K, Ledesma KR, Nikolaou M, Tam VH. Predicting bacterial fitness cost associated with drug resistance. *J Antimicrob Chemother.* (2012) 67:928–32. doi: 10.1093/jac/dkr560
10. Baker S, Duy PT, Nga TV, Dung TT, Phat VV, Chau TT, et al. Fitness benefits in fluoroquinolone-resistant Salmonella Typhi in the absence of antimicrobial pressure. *Elife.* (2013) 2:e01229. doi: 10.7554/eLife.01229
11. Song T, Park Y, Shamputa IC, Seo S, Lee SY, Jeon HS, et al. Fitness costs of rifampicin resistance in *Mycobacterium tuberculosis* are amplified under conditions of nutrient starvation and compensated by mutation in the beta' subunit of RNA polymerase. *Mol Microbiol.* (2014) 91:1106–19. doi: 10.1111/mmi.12520
12. Meftahi N, Namouchi A, Mhenni B, Brandis G, Hughes D, Mardassi H. Evidence for the critical role of a secondary site rpoB mutation in the compensatory evolution and successful transmission of an MDR tuberculosis outbreak strain. *J Antimicrob Chemother.* (2016) 71:324–32. doi: 10.1093/jac/dkv345
13. Zhang Y, Zeng J, Liu W, Zhao F, Hu Z, Zhao C, et al. Emergence of a hypervirulent carbapenem-resistant Klebsiella pneumoniae isolate from clinical infections in China. *J Infect.* (2015) 71:553–60. doi: 10.1016/j.jinf.2015.07.010
14. Almofti YA, Dai M, Sun Y, Haihong H, Yuan Z. Impact of erythromycin resistance on the virulence properties and fitness of *Campylobacter jejuni*. *Microb Pathog.* (2011) 50:336–42. doi: 10.1016/j.micpath.2011.02.009
15. Parkhomchuk D, Borodina T, Amstislavskiy V, Banaru M, Hallen L, Krobitsch S, et al. Transcriptome analysis by strand-specific sequencing of complementary DNA. *Nucleic Acids Res.* (2009) 37:e123. doi: 10.1093/nar/gkp596
16. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* (2012) 9:357–9. doi: 10.1038/nmeth.1923
17. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* (2014) 15:550. doi: 10.1186/s13059-014-0550-8
18. He F, Zhao Z, Wu X, Duan L, Li N, Fang R, et al. Transcriptomic analysis of high- and low-virulence bovine *Pasteurella multocida in vitro* and *in vivo*. *Front Vet Sci.* (2021) 8:616774. doi: 10.3389/fvets.2021.616774
19. Magstadt DR, Schuler AM, Coetzee JF, Krull AC, O'Connor AM, Cooper VL, et al. Treatment history and antimicrobial susceptibility results for *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* isolates from bovine respiratory disease cases submitted to the Iowa State University Veterinary Diagnostic Laboratory from 2013 to 2015. *J Vet Diagn Invest.* (2018) 30:99–104. doi: 10.1177/1040638717737589
20. Portis E, Lindeman C, Johansen L, Stoltman G. A ten-year (2000-2009) study of antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex–*Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*–in the United States and Canada. *J Vet Diagn Invest.* (2012) 24:932–44. doi: 10.1177/1040638712457559
21. Balbontin R, Frazao N, Gordo I. DNA breaks-mediated fitness cost reveals RNase HI as a new target for selectively eliminating antibiotic-resistant bacteria. *Mol Biol Evol.* (2021) 38:3220–34. doi: 10.1093/molbev/msab093
22. Durao P, Balbontin R, Gordo I. Evolutionary mechanisms shaping the maintenance of antibiotic resistance. *Trends Microbiol.* (2018) 26:677–91. doi: 10.1016/j.tim.2018.01.005
23. Fay A, Philip J, Saha P, Hendrickson RC, Glickman MS, Burns-Huang K. The DnaK chaperone system buffers the fitness cost of antibiotic resistance mutations in mycobacteria. *mBio.* (2021) 12:e00123–21. doi: 10.1128/mBio.00123-21

24. Hughes D, Brandis G. Rifampicin resistance: fitness costs and the significance of compensatory evolution. *Antibiotics.* (2013) 2:206–16. doi: 10.3390/antibiotics2020206
25. Zampieri M, Enke T, Chubukov V, Ricci V, Piddock L, Sauer U. Metabolic constraints on the evolution of antibiotic resistance. *Mol Syst Biol.* (2017) 13:917. doi: 10.15252/msb.20167028
26. Olivares Pacheco J, Alvarez-Ortega C, Alcalde Rico M, Martinez JL. Metabolic compensation of fitness costs is a general outcome for antibiotic-resistant pseudomonas aeruginosa mutants overexpressing efflux pumps. *mBio.* (2017) 8:e00500–17. doi: 10.1128/mBio.00500-17
27. Borrell S, Teo Y, Giardina F, Streicher EM, Klopper M, Feldmann J, et al. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evol Med Public Health.* (2013) 2013:65–74. doi: 10.1093/emph/eot003
28. Juraschek K, Deneke C, Schmoger S, Grobbel M, Malorny B, Kasbohrer A, et al. Phenotypic and genotypic properties of fluoroquinolone-resistant, qnr-carrying *Escherichia coli* isolated from the German food chain in 2017. *Microorganisms.* (2021) 9:1308. doi: 10.3390/microorganisms9061308
29. Hooper DC, Jacoby GA. Mechanisms of drug resistance: quinolone resistance. *Ann N Y Acad Sci.* (2015) 1354:12–31. doi: 10.1111/nyas.12830
30. Chang MX, Zhang JF, Sun YH, Li RS, Lin XL, Yang L, et al. Contribution of different mechanisms to ciprofloxacin resistance in Salmonella spp. *Front Microbiol.* (2021) 12:663731. doi: 10.3389/fmicb.2021.663731
31. Nell S, Estibariz I, Krebes J, Bunk B, Graham DY, Overmann J, et al. Genome and methylome variation in helicobacter pylori with a cag pathogenicity island during early stages of human infection. *Gastroenterology.* (2018) 154:612–23.e7. doi: 10.1053/j.gastro.2017.10.014
32. Bolinger H, Kathariou S. The current state of macrolide resistance in Campylobacter spp.: trends and impacts of resistance mechanisms. *Appl Environ Microbiol.* (2017) 83:e00416–17. doi: 10.1128/AEM.00416-17
33. Yang F, Zhang J, Wang S, Sun Z, Zhou J, Li F, et al. Genomic population structure of *Helicobacter pylori* Shanghai isolates and identification of genomic features uniquely linked with pathogenicity. *Virulence.* (2021) 12:1258–70. doi: 10.1080/21505594.2021.1920762
34. Alexandrino AV, Goto LS, Novo-Mansur MT. treA codifies for a trehalase with involvement in *Xanthomonas citri* subsp. citri pathogenicity. *PLoS ONE.* (2016) 11:e0162886. doi: 10.1371/journal.pone.0162886
35. Hamilton CD, Steidl OR, MacIntyre AM, Hendrich CG, Allen C. Ralstonia solanacearum depends on catabolism of myo-inositol, sucrose, and trehalose for virulence in an infection stage-dependent manner. *Mol Plant Microbe Interact.* (2021) 34:669–9. doi: 10.1094/MPMI-10-20-0298-R
36. Umare MD, Khedekar PB, Chikhale RV. Mycobacterial membrane protein large 3 (MmpL3) inhibitors: a promising approach to combat tuberculosis. *ChemMedChem.* (2021) 16:1–14. doi: 10.1002/cmdc.202100359
37. Guan L, Zhang L, Xue Y, Yang J, Zhao Z. Molecular pathogenesis of the hyaluronic acid capsule of *Pasteurella multocida*. *Microb Pathog.* (2020) 149:104380. doi: 10.1016/j.micpath.2020.104380
38. Pasomboon P, Chumnanpuen P, E-kobon T. Comparison of Hyaluronic acid biosynthetic genes from different strains of *Pasteurella multocida*. *Bioinform Biol Insights.* (2021) 15:11779322211027406. doi: 10.1177/11779322211027406
39. Petruzzi B, Briggs RE, Tatum FM, Swords WE, De Castro C, Molinaro A, et al. Capsular polysaccharide interferes with biofilm formation by *Pasteurella multocida* serogroup A. *mBio.* (2017) 8:e01843–17. doi: 10.1128/mBio.01843-17
40. Fu C, Sikandar A, Donner J, Zaburannyi N, Herrmann J, Reck M, et al. The natural product carolacton inhibits folate-dependent C1 metabolism by targeting FolD/MTHFD. *Nat Commun.* (2017) 8:1529. doi: 10.1038/s41467-017-01671-5
41. Green JM, Matthews RG. Folate biosynthesis, reduction, and polyglutamylation and the interconversion of folate derivatives. *EcoSal Plus.* (2007) 2. doi: 10.1128/ecosalplus.3.6.3.6
42. Pu M, Rowe-Magnus DA. A Tad pilus promotes the establishment and resistance of Vibrio vulnificus biofilms to mechanical clearance. *NPJ Biofilms Microbiomes.* (2018) 4:10. doi: 10.1038/s41522-018-0052-7
43. Masada CL, LaPatra SE, Morton AW, Strom MS. An *Aeromonas salmonicida* type IV pilin is required for virulence in rainbow trout *Oncorhynchus mykiss*. *Dis Aquat Organ.* (2002) 51:13–25. doi: 10.3354/dao051013
44. Zhou P, Hong J. Structure- and ligand-dynamics-based design of novel antibiotics targeting lipid A enzymes LpxC and LpxH in gram-negative bacteria. *Acc Chem Res.* (2021) 54:1623–34. doi: 10.1021/acs.accounts.0c00880
45. Harper M, Boyce JD. The myriad properties of *Pasteurella multocida* lipopolysaccharide. *Toxins.* (2017) 9:254. doi: 10.3390/toxins9080254
46. Bohl TE, Shi K, Lee JK, Aihara H. Crystal structure of lipid A disaccharide synthase LpxB from *Escherichia coli*. *Nat Commun.* (2018) 9:377. doi: 10.1038/s41467-017-02712-9
47. Biernacka D, Gorzelak P, Klein G, Raina S. Regulation of the first committed step in lipopolysaccharide biosynthesis catalyzed by LpxC requires the essential protein LapC (YejM) and HslVU protease. *Int J Mol Sci.* (2020) 21:9088. doi: 10.3390/ijms21239088
48. Zou Y, Feng S, Xu C, Zhang B, Zhou S, Zhang L, et al. The role of galU and galE of *Haemophilus parasuis* SC096 in serum resistance and biofilm formation. *Vet Microbiol.* (2013) 162:278–84. doi: 10.1016/j.vetmic.2012.08.006
49. Crhanova M, Malcova M, Mazgajova M, Karasova D, Sebkova A, Fucikova A, et al. LPS structure influences protein secretion in *Salmonella enterica*. *Vet Microbiol.* (2011) 152:131–7. doi: 10.1016/j.vetmic.2011.04.018
50. Linkevicius M, Anderssen JM, Sandegren L, Andersson DI. Fitness of *Escherichia coli* mutants with reduced susceptibility to tigecycline. *J Antimicrob Chemother.* (2016) 71:1307–13. doi: 10.1093/jac/dkv486
51. Linkevicius M, Sandegren L, Andersson DI. Mechanisms and fitness costs of tigecycline resistance in *Escherichia coli*. *J Antimicrob Chemother.* (2013) 68:2809–19. doi: 10.1093/jac/dkt263
52. Mamat U, Meredith TC, Aggarwal P, Kuhl A, Kirchhoff P, Lindner B, et al. Single amino acid substitutions in either YhjD or MsbA confer viability to 3-deoxy-d-manno-oct-2-ulosonic acid-depleted *Escherichia coli*. *Mol Microbiol.* (2008) 67:633–48. doi: 10.1111/j.1365-2958.2007.06074.x
53. Lucassen K, Muller C, Wille J, Xanthopoulou K, Hackel M, Seifert H, et al. Prevalence of RND efflux pump regulator variants associated with tigecycline resistance in carbapenem-resistant *Acinetobacter baumannii* from a worldwide survey. *J Antimicrob Chemother.* (2021) 76:1724–30. doi: 10.1093/jac/dkab079
54. Sharma D, Bisht D. Role of bacterioferritin & ferritin in *M. tuberculosis* pathogenesis and drug resistance: a future perspective by interactomic approach. *Front Cell Infect Microbiol.* (2017) 7:240. doi: 10.3389/fcimb.2017.00240
55. Heinemann IU, Jahn M, Jahn D. The biochemistry of heme biosynthesis. *Arch Biochem Biophys.* (2008) 474:238–51. doi: 10.1016/j.abb.2008.02.015
56. Gollapalli P, Manjunatha H, Shetty P. Network topology analysis of essential genes interactome of *Helicobacter pylori* to explore novel therapeutic targets. *Microb Pathog.* (2021) 158:105059. doi: 10.1016/j.micpath.2021.105059
57. Hanawa T, Yamanishi S, Murayama S, Yamamoto T, Kamiya S. Participation of DnaK in expression of genes involved in virulence of Listeria monocytogenes. *FEMS Microbiol Lett.* (2002) 214:69–75. doi: 10.1111/j.1574-6968.2002.tb11326.x
58. Guerra PR, Herrero-Fresno A, Pors SE, Ahmed S, Wang D, Thofner I, et al. The membrane transporter PotE is required for virulence in avian pathogenic *Escherichia coli* (APEC). *Vet Microbiol.* (2018) 216:38–44. doi: 10.1016/j.vetmic.2018.01.011
59. Kashiwagi K, Kuraishi A, Tomitori H, Igarashi A, Nishimura K, Shirahata A, et al. Identification of the putrescine recognition site on polyamine transport protein PotE. *J Biol Chem.* (2000) 275:36007–12. doi: 10.1074/jbc.M006083200
60. Choi E, Kwon K, Lee EJ. A single amino acid of a Salmonella virulence protein contributes to pathogenicity by protecting from the FtsH-mediated

proteolysis. *FEBS Lett.* (2015) 589:1346–51. doi: 10.1016/j.febslet.2015.04.014

61. Bove P, Capozzi V, Garofalo C, Rieu A, Spano G, Fiocco D. Inactivation of the ftsH gene of *Lactobacillus plantarum* WCFS1: effects on growth, stress tolerance, cell surface properties and biofilm formation. *Microbiol Res.* (2012) 167:187–93. doi: 10.1016/j.micres.2011.07.001
62. Kiran M, Chauhan A, Dziedzic R, Maloney E, Mukherji SK, Madiraju M, et al. Mycobacterium tuberculosis ftsH expression in response to stress and viability. *Tuberculosis.* (2009) 89:S70–3. doi: 10.1016/S1472-9792(09)70016-2

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 18 November 2021
doi: 10.3389/fvets.2021.782872

# The Effects of Ursolic Acid Treatment on Immunopathogenesis Following *Mannheimia haemolytica* Infections

*Jamison R. Slate*[1], *Bradley O. Chriswell*[2], *Robert E. Briggs*[2] *and Jodi L. McGill*[1]*

[1] *Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, United States,*
[2] *Agricultural Research Service, United States Department of Agriculture, National Animal Disease Center, Ames, IA, United States*

Bovine respiratory disease complex (BRDC) is a costly economic and health burden for the dairy and feedlot cattle industries. BRDC is a multifactorial disease, often involving viral and bacterial pathogens, which makes it difficult to effectively treat or vaccinate against. *Mannheimia haemolytica* (MH) are common commensal bacteria found in the nasopharynx of healthy cattle; however, following environmental and immunological stressors, these bacteria can rapidly proliferate and spread to the lower respiratory tract, giving rise to pneumonic disease. Severe MH infections are often characterized by leukocyte infiltration and dysregulated inflammatory responses in the lungs. IL-17A is thought to play a key role in this inflammatory response by inducing neutrophilia, activating innate and adaptive immune cells, and further exacerbating lung congestion. Herein, we used a small molecule inhibitor, ursolic acid (UA), to suppress IL-17A production and to determine the downstream impact on the immune response and disease severity following MH infection in calves. We hypothesized that altering IL-17A signaling during MH infections may have therapeutic effects by reducing immune-mediated lung inflammation and improving disease outcome. Two independent studies were performed (Study 1 = 32 animals and Study 2 = 16 animals) using 4-week-old male Holstein calves, which were divided into 4 treatment group including: (1) non-treated and non-challenged, (2) non-treated and MH-challenged, (3) UA-treated and non-challenged, and (4) UA-treated and MH-challenged. Based on the combined studies, we observed a tendency ($p = 0.0605$) toward reduced bacterial burdens in the lungs of UA-treated animals, but did not note a significant difference in gross ($p = 0.3343$) or microscopic ($p = 0.1917$) pathology scores in the lungs. UA treatment altered the inflammatory environment in the lung tissues following MH infection, reducing the expression of IL-17A ($p = 0.0870$), inflammatory IL-6 ($p = 0.0209$), and STAT3 ($p = 0.0205$) compared to controls. This reduction in IL-17A signaling also appeared to alter the downstream expression of genes associated with innate defenses (BAC5, DEFB1, and MUC5AC) and lung remodeling (MMP9 and TIMP-1). Taken together, these results support our hypothesis that IL-17A signaling may contribute to lung immunopathology following MH infections, and further understanding of this inflammatory pathway could expand therapeutic intervention strategies for managing BRDC.

**Keywords: *Mannheimia haemolytica*, bovine respiratory disease complex, innate immunity, inflammation, interleukin-17A, immunomodulation, ursolic acid**

OPEN ACCESS

***Edited by:***
*Annamaria Pratelli, University of Bari Aldo Moro, Italy*

***Reviewed by:***
*Chuck Czuprynski, University of Wisconsin-Madison, United States*
*Robin James Flynn, Technological University South-East Ireland, Ireland*

***Correspondence:**
*Jodi L. McGill*
*jlmcgill@iastate.edu*

***Specialty section:***
*This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science*

***Received:*** *24 September 2021*
***Accepted:*** *22 October 2021*
***Published:*** *18 November 2021*

***Citation:***
*Slate JR, Chriswell BO, Briggs RE and McGill JL (2021) The Effects of Ursolic Acid Treatment on Immunopathogenesis Following Mannheimia haemolytica Infections. Front. Vet. Sci. 8:782872. doi: 10.3389/fvets.2021.782872*

## INTRODUCTION

Bovine respiratory disease complex (BRDC) is a leading cause of morbidity and mortality within the North American feedlot (1) and dairy (2) industries, and its economic burden is estimated to cost in excess of 1 billion dollars annually due to increased labor and production-loss expenses (3, 4). Current vaccinations strategies are limited in their efficacy (5), and BRDC is often difficult to treat because it is a multifactorial disease. These infections typically involve a combination of environmental and host factors, as well as multiple viral and bacterial pathogens. Unfortunately, many BRDC pathogens are highly pervasive, and early production practices, like weaning and shipping, can leave young cattle immunologically stressed and susceptible to primary infections. *Mannheimia haemolytica* (MH) is a common commensal bacteria found in the nasopharynx of healthy cattle, where its proliferation is generally limited by other commensal flora and the host's immune system (3, 4). However, following immunological stressors or a primary viral infection, MH can migrate deeper into the lower respiratory tract, eventually culminating in severe bacterial pneumonia (4). Although BRDC is a syndrome involving multiple viral and bacterial pathogens, MH is often the most prevalent bacteria isolated from pneumonic bovine lungs, and it is considered the principal bacterial agent involved in BRDC (6). Severe MH infections are often characterized by fibrinous exudate and an accumulation of activated macrophages and neutrophils, which further contribute to lung congestion through inflammatory cytokine signaling and excretion of cytotoxic compounds (6, 7).

Interleukin-17A (IL-17A) is an inflammatory cytokine known to aid in the infiltration and activation of neutrophils, and recent studies have shown that MH infections induce an upregulation of IL-17A expression in the lungs (8). Our recent studies have shown that IL-17A is also upregulated in the lungs of calves infected with bovine respiratory syncytial virus (BRSV), a common viral agent associated with BRDC. Inhibiting IL-17A production during BRSV infection was shown to reduce disease severity and lung damage in neonatal calves (9). Considering that severe cases of MH infection are often associated with increased lung inflammation and cytotoxic neutrophil infiltration (10, 11), IL-17A signaling following MH infection may be contributing to immune-mediated tissue damage and pathogenesis (8). Therefore, we hypothesized that inhibiting IL-17A signaling during MH infection may have beneficial effects on immune-mediated lung inflammation and disease outcome.

Previous studies in our lab have used a small molecule drug, digoxin, to inhibit the production of IL-17A in neonatal calves; however, digoxin treatment can have considerable cytotoxic effects, limiting its potential as a therapeutic inhibitor of IL-17A (8, 9). Recent research has suggested that another small molecule drug, ursolic acid (UA), may have similar inhibitory capabilities with fewer cytotoxic effects (12, 13). Herein, we confirmed that UA treatment can reduce IL-17A production at non-cytotoxic levels using bovine peripheral blood mononuclear cells (PBMCs). We subsequently employed prophylactic UA treatment as an *in vivo* tool to disrupt IL-17A signaling following MH infection of neonatal calves.

UA-treated calves showed a favorable trend toward reduced bacterial burdens in the lungs compared to control calves. Additionally, UA treatment reduced IL-17A expression *in vivo* in pneumonic lungs, and also significantly reduced expression of inflammatory interleukin-6 (IL-6) and the regulatory transcription factor, signal transducer and activator of transcription 3 (STAT3). Interestingly, untreated control calves had a higher expression ratio of matrix metalloproteinase 9 (MMP9) to tissue inhibitor of metalloproteinase 1 (TIMP-1) in their lungs compared to UA-treated calves, which indicates ongoing extracellular matrix destruction is not being dampened for tissue repair and remodeling. This result is consistent with the increased inflammatory response and more severe lung scores observed in control animals compared to UA-treated calves. Taken together, our results suggest that inhibiting inflammatory IL-17A signaling may decrease lung bacterial burden and alter host immune responses to potentially reduce disease severity following MH infection. By further exploring the role of inflammatory IL-17A signaling in MH infections, we hope to expand therapeutic intervention strategies for severe respiratory infections that contribute to the pathogenesis of BRDC.

## MATERIALS AND METHODS

### MTT Cell Viability Assay

The CyQUANT MTT Cell Viability Assay (Invitrogen) was used according to manufacturer's recommendations. Varying concentrations of UA (AstaTech) in DMSO were prepared using serial dilutions, and 5 μg/ml of Concanavalin A (ConA, MP Biomedicals) in complete RPMI media (cRPMI) was prepared for cellular stimulation. The cRPMI was composed of RPMI-1640 (Gibco) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 1% antibiotic-antimycotic solution, 1% sodium pyruvate, 1% non-essential amino acids, 2% essential amino acids, 50 μM 2-mercaptoethanol, and 10% (volume/volume) fetal bovine serum as outlined in McGill et al. (14). Bovine PBMCs were suspended in cRPMI and plated into 96-well round-bottomed plates at 250,000 cells per well, and then corresponding UA, ConA, DMSO (carrier control), and cRPMI solutions were added to the wells for a total volume of 200 μl in each well. Cells were incubated in the dark at 37°C and 5% $CO_2$ for 72 h. Cell cultures were then centrifuged to pellet the cells and supernatants were collected for ELISA analysis. Cells were re-suspended in the CyQUANT MTT reagent and then returned to the incubator for an additional 4 h. MTT crystals were vigorously mixed with SDS-HCl solubilization mixture and returned to the incubator for another 4–8 h. The solubilized MTT mixture was transferred to a flat-bottomed 96-well plate for microplate spectrophotometer analysis at 570 nm.

### ELISA for IL-17A Production

IL-17A production by bovine PBMCs was determined using the Bovine IL-17A Do-It-Yourself ELISA kit (Kingfisher Biotech, Inc) following the manufacturer's published ELISA Technical Guide (Kingfisher Biotech, Inc). In summary, anti-bovine IL-17A capture antibody was coated overnight at 4°C in 96-well plates (Themo Scientific MaxiSorp, Immulon 4 HBX) at

a concentration of 2.5 μg/ml. Plates were then blocked with a 4% bovine serum albumin in PBS solution for 1 h at room temperature. Plates were washed with a 0.05% Tween 20 in PBS buffer, and bovine IL-17A standard or stimulated PBMC cell culture supernatants (from the MTT cell viability assays) were incubated at room temperature for 2 h. The plates were washed and then incubated with biotinylated anti-bovine IL-17A detection antibody at a concentration of 0.1 μg/ml for 1 h at room temperature. The plates were washed and then incubated with 1:100 HRP-Streptavidin (Kingfisher Biotech) in assay diluent for an additional hour at room temperature. Plates were washed a final time, and then incubated with 1-Step Ultra TMB ELISA Substrate (ThermoFisher Scientific) until sufficient color change was detected in the standards. The reaction was then stopped with 2 M $H_2SO_4$ stop solution and the sample absorbance was measured at 450 nm using an ELISA microplate reader (Fisher Scientific accuSkan™ FC Filter-Based Microplate Photometer).

## Animal Husbandry

All animal procedures were conducted in accordance with institutional guidelines, and protocols were approved by Iowa State University's Institutional Animal Care and Use Committee (IACUC-19-081).

The fall 2019 (F2019) study enrolled 32, male, 4-week-old Holstein and Holstein/cross calves which were randomly divided into 4 treatment groups ($n = 8$ animals per group): non-treated and non-challenged, UA-treated and non-challenged, non-treated and MH-challenged, and UA-treated and MH-challenged. The summer 2020 (S2020) study enrolled 16 male Holstein and Holstein/cross calves, all ∼4 weeks old at the time of receiving, which were randomly divided into 2 treatment groups ($n = 8$ animals per group): non-treated and MH-challenged, and UA-treated and MH-challenged. Calves were housed by treatment group, with 4 animals per room, under climate controlled ABSL2 conditions in the Livestock Infectious Disease and Isolation Facility at Iowa State University.

Animals had *ad libitum* access to water, hay, and calf starter. Calves were fed 3 quarts of milk twice daily (once in the morning and once in the evening) in buckets for the duration of the study. Beginning 3 days prior to MH infection and for the remainder of the trial, UA-treated animals received 0.5 g of UA mixed into their milk at each feeding, resulting in a total of 1 gram of UA powder per day. The selection of UA was determined based on the doses used in the literature for mice and allometric scaling calculations (12, 13, 15, 16); the equation and theories for these allometric scaling calculations have been previously summarized in West and Brown (17).

## MH Intratracheal Infection

Intratracheal inoculation was performed as previously described (18). F2019 animals were challenged intratracheally with $4.15 \times 10^8$ of MH (D153 and D174 strains) in 50 ml of Earle's balanced salt solution (EBSS), followed by a 50 ml intratracheal wash of EBSS: the MH inoculation mixture was comprised of $2.05 \times 10^8$ of MH strain D153 and $2.10 \times 10^8$ of MH strain D174 suspended in EBSS. F2020 animals were challenged intratracheally with $1.98 \times 10^8$ of MH (D153 and D174 strains) in 50 ml of EBSS, followed by a 50 ml intratracheal wash of EBSS: the MH inoculation mixture was comprised of $1.05 \times 10^8$ of strain D153 and $9.25 \times 10^7$ of strain D174 suspended in EBSS.

## Clinical Scoring

Calves were monitored for signs of clinical illness and scored every day beginning on the day of MH infection, or 0 days post infection (0 dpi). Calves were scored using a modified University of Wisconsin Calf Health Respiratory Scoring Chart (9). Scoring categories include nasal discharge, eye discharge, ear tilt, spontaneous or handler-induced coughing, lung sounds detected by auscultation, expiratory effort and rectal temperature (**0**, temperature is <101.0°F; **1**, temperature is 101.1–101.9°F; **2**, temperature is 102.0–102.9°F; **3**, temperature is ≥103.0°F).

The study protocols included provisions for a humane endpoint. Eleven calves from the F2019 study were euthanized early after reaching a humane endpoint: 3 animals in the non-treated and non-challenged group, 0 animals in the UA-treated and non-challenged group, 5 animals in the non-treated and MH-challenged group, and 3 animals in the UA-treated and MH-challenged group. Two calves from the S2020 study were euthanized after reaching a humane endpoint. This included 1 calf from the non-treated and MH-challenged group, which was euthanized before MH infection, and 1 calf from the UA-treated and MH-challenged group, which was euthanized within 24 h of MH infection.

## Sample Collections

Nasal swabs were collected by swabbing both nostrils with sterile polyester-tipped applicators (Puritan) on three separate occasions through the course of each study; 3 days prior to infection (−3 dpi), on the day of MH infection but before intratracheal inoculation (0 dpi), and at the time of necropsy (4 dpi, or humane endpoint).

Blood and serum samples were collected through the jugular vein; whole blood for immediate use in assays was collected in acid citrate dextrose (ACD, yellow-top) tubes, while blood for serum isolation was collected in silica clot activator (marbled red-gray-top) tubes. Blood collected in serum tubes was allowed to clot, centrifuged for serum separation, and aliquoted for storage in −80°C.

## Necropsy

Calves were euthanized by barbiturate overdose and necropsied on 4 dpi or upon reaching humane endpoint. At necropsy, the lungs of each calf were cleaned, photographed, and scored based on the observable lung consolidation and lesion area. The lungs were divided into nine sections and each section was visually evaluated and palpated for the degree of pneumonic consolidation. Scored sections included the cranial half of left cranial, caudal half of left cranial, left caudal, accessory, cranial half of right cranial, caudal half of right cranial, right middle, and right caudal. These sectional scores were then weighted based on air exchange: cranial half of left cranial (4%), caudal half of left cranial (6%), left caudal (32%), accessory (4%), cranial half of right cranial (6%), caudal half of right cranial (5%), right

middle (7%), and right caudal (35%) and each lung was assigned a final score (18).

Bronchoalveolar lavage (BAL) fluid was collected at the time of necropsy. A total of 500 mL of ice-cold saline containing 1% antibiotic-antimycotic solution was washed through the trachea and massaged into the lungs. The BAL fluid was then transferred into sterile collection bottles and stored on ice before processing in the lab. BAL fluid was filtered through sterile gauze, and aliquots were submitted for cytospin and cytology analysis at the Iowa State University Veterinary Diagnostic Laboratory. The remaining BAL fluid was centrifuged and washed twice with cold (4°C) PBS, and then live cells were enumerated using trypan blue exclusion. Cells were suspended in cRPMI for cellular assays or freezing media (10% DMSO in FBS) for storage in liquid nitrogen.

Representative tissue sections were excised from the trachea and lungs (right cranial, upper-right caudal, lower-right caudal, lower-left caudal, upper-left caudal, left cranial, and accessory) of each animal. Samples were fixed in formalin and submitted to the Iowa State University Veterinary Diagnostic Lab for pathological evaluation by a certified veterinary pathologist, stored in either Qiagen RNAlater solution for quantitative polymerase chain reaction (qPCR) analysis, or snap frozen over dry ice for bacterial quantification. Samples of liver and spleen were also snap frozen for bacterial quantification. All samples were stored at −80°C until analysis.

## Bacterial Recovery

Bacterial lung loads were determined by quantitative culture and reported as total colony forming units (CFUs) as previously described (18). Briefly, lung tissue samples were ground in EBSS to produce a homogenized suspension that was then diluted 10-fold in EBSS. The dilutions were then spread on blood agar base plates containing 5% defibrinated bovine blood and incubated overnight at 37°C. Colonies with typical MH morphology were enumerated, and representative colonies were selected for plate agglutination. Swabs were rolled on half of a fresh blood agar plate, then a sterile loop was used to semi-quantitatively streak for isolation on the remaining two quarters. In addition to rapid plate agglutination (19), PCR analyses were performed. In MH, primers targeting Lkt D (all MH) and capsule specific regions (MH st1 and MH st6) were used to confirm the lung isolates produced the same PCR products as the challenge strains (Briggs, unpublished-sequences available upon request). In Pasteurella, KMT primers were used (20). Isolates were sent to NVSL diagnostics for MALDI-ToF identification.

## Dihydrorhodamine 123 (DHR) Reactive Oxygen Species Assay

Approximately 3 mL of fresh whole blood collected in ACD tubes was transferred to a 15 mL conical. Each tube of whole blood was volumed up to the top with warm (35°C) red blood cell lysis buffer and incubated in a 35°C water bath for 5 min, agitating the tubes halfway through the incubation. Cells were centrifuged and washed with warm PBS, then re-suspended in warm Hanks' balanced salt solution (Gibco, 14175-095) and plated in 96-well round-bottomed plates. The cells were re-suspended in a 75 μM solution of DHR (FITC+) in HBSS and incubated at 37°C for 20 min. After 20 min, cells were stimulated with 100 ng of PMA and returned to the incubator for an additional 25 min. Following incubation, the cells were placed on ice for 5 min, centrifuged, and re-suspended in FACS buffer (10% fetal bovine serum, 0.1% sodium azide, and PBS). The cells were surface stained with 10 μg/mL mouse anti-bovine CD14 to detect monocytes (clone CAM36A, IgG1, Kingfisher Biotech, Inc.) and mouse-anti-bovine granulocytes to detect neutrophils (clone CH138A, IgM, Kingfisher Biotech, Inc.). Cells were incubated for 30 min at 4°C. Cells were washed 2 times with FACS buffer and stained with 5 μg/mL mouse anti-IgG1-PE (BD Pharmingen) and goat anti-mouse IgM-AF647 (Invitrogen) for 30 min at 4°C. Finally, cells were fixed in 1x BD FACS lysing solution (BD Bioscience) at room temperature for 10 min and stored in FACS buffer for flow cytometry analysis. **Supplementary Figure 1** includes a representative FACS gating strategy. Flow cytometry data were collected on BD FACS Canto II (BD Biosciences) and analyzed using FlowJo software version 10.6.1 (BD Biosciences).

## RT-qPCR Analysis of Lung Tissues

Pneumonic (lesion) and healthy (non-lesion) control (non-treated, non-challenged) lung tissue samples were stored in RNAlater at −80°C until processing for PCR analysis. RNA isolation, cDNA preparation, and quantitative PCR were performed as previously reported (8). Primer sequences are compiled in **Table 1**. Primers for RORC, IL-8, and DEFB1 were designed using Integrated DNA Technologies' (IDT) PrimerQuest™ Tool. Primers for BAC5 were a kind gift from Dr. Corwin D. Nelson, University of Florida. Relative expression (RE) for each gene was determined using the $2^{-\Delta\Delta CT}$ method (28), with RPS9 used as a reference housekeeping gene from which Δ cycle thresholds (CT) were determined. Briefly, the RE was calculated as $RE = 2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ of pneumonic lung – Average ΔCT of healthy control lungs. These RT-qPCR data were depicted with the Average RE of a particular gene along with the individual RE values for each animal.

## Statistical Analysis

All statistical analyses were made using Prism v9.1.0 (GraphPad Software, Inc.).

The treatment means ± the standard error of the means (SEM) were plotted for the *in vitro* MTT and ELISA assays, and significant differences between these groups were determined using one-way ANOVA with Tukey's multiple comparisons. Clinical scoring data was plotted as the mean clinical score ± SEM for each treatment group, and the data was analyzed by two-way ANOVA (time and treatment) mixed-effects model with multiple comparisons. Pneumonic consolidation (lung lesion) scoring and pathology scoring data were plotted with the mean score ± SEM along with individual scores for each animal amongst the two studies; outliers were removed from these data sets using the ROUT method (Prism v9.1.0), and significant differences between treatment effects were determined using a standardized unpaired *t*-test. Similarly, bacterial burden data were reported on a log scale with mean CFUs ± SEM, as well as CFUs for each animal; outliers for these data sets were

**TABLE 1 |** Primers for RT qPCR Analysis.

| Primer Table | | | |
|---|---|---|---|
| **Gene target** | **NCBI reference sequence** | **Primer sequence** | **References** |
| RPS9 | NM_001101152.2 | F: 5′ CGCCTCGACCAAGAGCTGAAG 3′<br>R: 5′ CCTCCAGACCTCACGTTTGTTCC 3′ | (21) |
| IL-6 | NM_173923.2 | F: 5′ CTGAAGCAAAAGATCGCAGATCTA 3′<br>R: 5′ CTCGTTTGAAGACTGCATCTTCTC 3′ | (21) |
| STAT3 | NM_001012671.2 | F: 5′ GACCGGTGTCCAGTTCACAA 3′<br>R: 5′ AAATTTCCGGGACCCTCTGA 3′ | (22) |
| RORC | NM_001083451.2 | F: 5′ GTCAGCGCTCCAATATCTTCTC 3′<br>R: 5′ CTTAGCGAACTCCACCACATA 3′ | |
| IL-17A | NM_001008412.2 | F: 5′ CACAGCATGTGAGGGTCAAC 3′<br>R: 5′ GGTGGAGCGCTTGTGATAAT 3′ | (23) |
| IL-8 | NM_173925.2 | F: 5′ CGCTGGACAGCAGAGCTCACAAG 3′<br>R: 5′ GCCAAGAGAGCAACAGCCAGCT 3′ | |
| BAC5 (CATHL2) | NM_174826.3 | F: 5′ TTCAAGGAGAATGGGCTGGT 3′<br>R: 5′ GATCGGTGGGAAGATCGGTG 3′ | |
| DEFB1 | NM_001324544.1 | F: 5′ GTCAGGAATAAGTGATTTTGCAAGC 3′<br>R: 5′ GCCGGAAACAGATGCCAATC 3′ | (24) |
| MUC5AC | XM_024987596.1 | F: 5′ CAGACCCTCCACCTTCTTCA 3′<br>R: 5′ GGTCCTCGAAGCTGTTCTTG 3′ | (25) |
| IFNγ | NM_174086.1 | F: 5′ AGAATCTCTTTCGAGGCCGGAG 3′<br>R: 5′ TATTGCAGGCAGGAGGACCATTAC 3′ | (21) |
| MMP9 | NM_174744.2 | F: 5′ GACCAGGACAAGCTCTACGG 3′<br>R: 5′ CAGAAGCCCCACTTCTTGTC 3′ | (26) |
| TIMP-1 | NM_174471.3 | F: 5′ GATGTCGTCATCAGGGCC 3′<br>R: 5′ TCGCTCTGCAGTTTGCAG 3′ | (27) |

removed using the ROUT method (Prism v9.1.0) and significant differences between treatment effects were determined using a standardized unpaired *t*-test. The reactive oxygen species (DHR) assays were reported as the mean change in fluorescent intensity (ΔMFI) ± SEM, and significant differences between treatments were determined using an unpaired *t*-test. RT-qPCR data were reported as the Average RE ± SEM for each gene, and included individual REs for each animal; once again, outliers were removed using the ROUT method (Prism v9.1.0), and significant treatment differences were determined using a standard unpaired *t*-test.

# RESULTS

## UA Treatment Inhibits IL-17A Cytokine Production by Bovine PBMCs *in vitro*

Previous studies in our lab have shown that reducing IL-17A production using digoxin (a small molecule inhibitor of the RORγt transcription factor) can ameliorate a viral respiratory disease in cattle but with potential cytotoxic effects. However, recent investigations have shown that another small molecule inhibitor of RORγt, ursolic acid (UA), can similarly suppress IL-17A production without cytotoxic effects in mouse and human cells (12, 13). To assess the cytotoxicity and verify the inhibitory capabilities of UA within the context of a bovine model, we incubated bovine PBMCs with escalating concentrations of UA and concanavalin A (ConA) for 72 h. We then used an MTT assay to test cell viability, and an enzyme-linked immunosorbent assay (ELISA) to screen for IL-17A production in the cell supernatants. The MTT assay (**Figure 1A**) demonstrated that the three lowest concentrations of UA (dissolved in DMSO) were not significantly different in their absorbance values from the carrier control (DMSO + ConA), indicating no loss of cell viability due to UA treatment. However, the two highest concentrations of UA treatment (12.5 and 25 μM) did have a negative impact on cell viability compared to the carrier control ($p = 0.0901$ and 0.0064, respectively). As seen in **Figure 1B**, all three of the lowest UA treatment concentrations had a substantial impact on IL-17A production, with the 3.13 and 6.25 μM concentrations causing a significant reduction in cytokine production compared to the carrier control ($p = 0.0362$ and 0.0350 respectively), while the 1.56 μM UA treatment also showed notable inhibitory potential ($p = 0.0562$). Although both the 12.5 and 25 μM treatments showed significant reductions in IL-17A production ($p = 0.0112$ and 0.0057, respectively), we cannot determine if the reduced cytokine production was due to inhibitory effects from the treatment or reduced cell viability (**Figure 1A**). Taken together, these data show that UA treatment can effectively reduce IL-17A production at concentrations that are not cytotoxic to bovine lymphocytes *in vitro*.

## Impact of Prophylactic UA Treatment on Clinical Disease and Lung Pathology Following *in vivo* MH Infection in Calves

Based on our *in vitro* data from **Figure 1**, we next chose to use UA treatment to evaluate the role of IL-17A on the outcome of

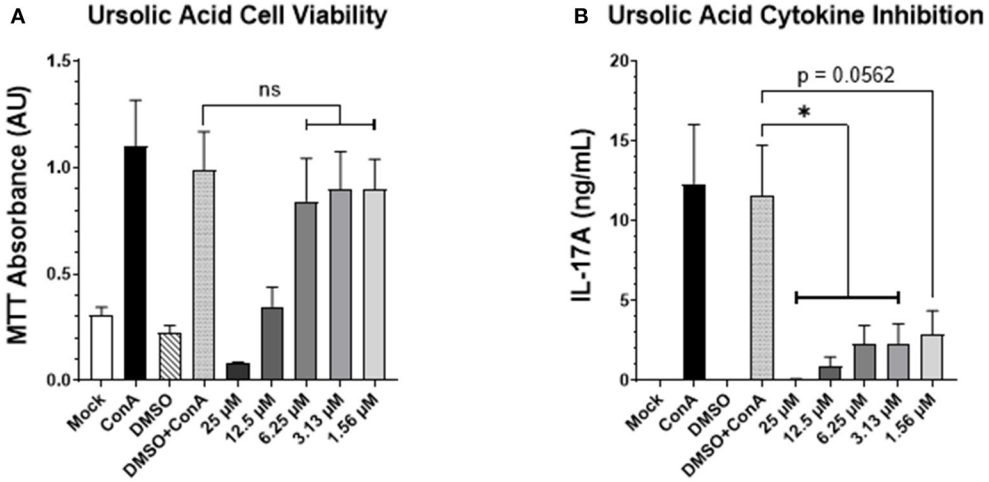



**FIGURE 1 |** UA suppresses mitogen-induced IL-17A production by bovine PBMCs. Peripheral blood mononuclear cells from three healthy bovids were plated at $3 \times 10^5$ cells per well in a 96-well plate and incubated with Concanavalin A, vehicle control (DMSO), or escalating concentrations of UA dissolved in DMSO for 3 days. On day three, cell supernatants were collected, and the remaining cell pellet was re-suspended with an MTT solution using the manufacturer's protocol to subsequently analyze the viability of the cells after the incubation period. **(A)** Mean absorbance values at 570 nm ± SEM following the MTT incubation and solubilization. Significance was determined using a one-way ANOVA with Tukey's multiple comparisons; (ns) indicates no significant differences were observed. **(B)** The supernatants from the 3-day incubation were analyzed for IL-17A concentration by ELISA. Data represent mean IL-17A concentration (in ng/mL) ± SEM detected in the supernatants. The data were analyzed using a standard one-way ANOVA with Tukey's multiple comparisons; $^*p < 0.05$.

MH infection *in vivo* in calves. Full study details are provided in section Animal Husbandry, but in brief, a total of 48, 4-week-old, male Holstein calves were enrolled in two independent studies (F2019 $n = 32$ calves; S2020 $n = 16$ calves). Additionally, calves were either treated with 1 g/head/day of UA mixed into milk replacer, or received no treatment in their milk replacer at feedings. Treatment was initiated 72 h prior to MH infection and continued for the duration of the trials. On day 0, calves were challenged intratracheally with MH. The calves were monitored daily for clinical signs of infection until the study endpoint on 4 dpi. As seen in **Figure 2A**, clinical scores quickly rose within the first 24 h of infection and stabilized between 1 and 4 dpi with no significant differences between the two treatment groups. Several animals in the F2019 and S2020 cohorts reached a humane endpoint prior to 4 dpi and were removed from the trial (**Figure 2B**). In total, 8 animals were removed early from the F2019 study: 5 calves from the non-treated group and 3 calves from the UA-treated group. There were only 2 animals removed early from the S2020 study: 1 calf from the non-treated group was removed before the MH challenge, and 1 calf from the UA-treated group was removed shortly after MH challenge. We observed no differences in mortality between treatment groups.

Necropsies were performed on 4 dpi, or upon reaching a humane endpoint. At necropsy, lungs were photographed (**Figures 2C,D**), scored for consolidated lung area (**Figure 2E**), and tissue sections were fixed in formalin for histopathology scoring (**Figure 2F**). **Figure 2C** shows a representative image of lung consolidation from a non-treated calf. **Figure 2D** shows a representative image of lung consolidation from a UA-treated calf. The lung consolidation scores were combined from both studies, and the average lesion area is depicted in **Figure 2E**. We did not detect a statistically significant difference in lung lesions in UA-treated animals compared to controls ($p = 0.3343$); however, the mean area of pneumonic lung in UA-treated calves (10.31%) was lower than the mean area of pneumonic lung in non-treated calves (14.91%). The S2020 animals tended to cluster lower in lung lesions compared to the F2019 animals.

Histopathology scores for each individual animal are shown in **Figure 2F**. Histopathology scores were not significantly different between treatment groups ($p = 0.1917$); however, the mean histopathology score of UA-treated animals (69.42) was lower than the mean score of non-treated controls (109.90). Similar to the lung lesion scoring in **Figure 2E**, the F2019 animals tended to have the highest histopathology scores in **Figure 2F** compared to the S2020 animals. Taken together, the reduced means of both lung consolidation scoring and histopathology scoring suggest that IL-17A signaling may contribute to lung pathology following MH infection.

## Prophylactic UA Treatment Maintains Immune Cell Populations and Functionality While Reducing Bacterial Burden Following MH Infection

We next performed quantitative culture for MH on lung tissue sections collected at necropsy to determine bacterial loads in UA-treated and non-treated calves. As seen in **Figure 3A**, the average MH CFUs recovered by quantitative culture tended to be lower in the UA-treated animals compared to the non-treated animals ($p = 0.0605$).

Since IL-17A signaling is known to be a chemoattractant for neutrophils, we sampled the cellular populations of the lung environment by collecting BAL fluid for cytospin analysis. **Figure 3B** depicts the relative frequencies of macrophages,

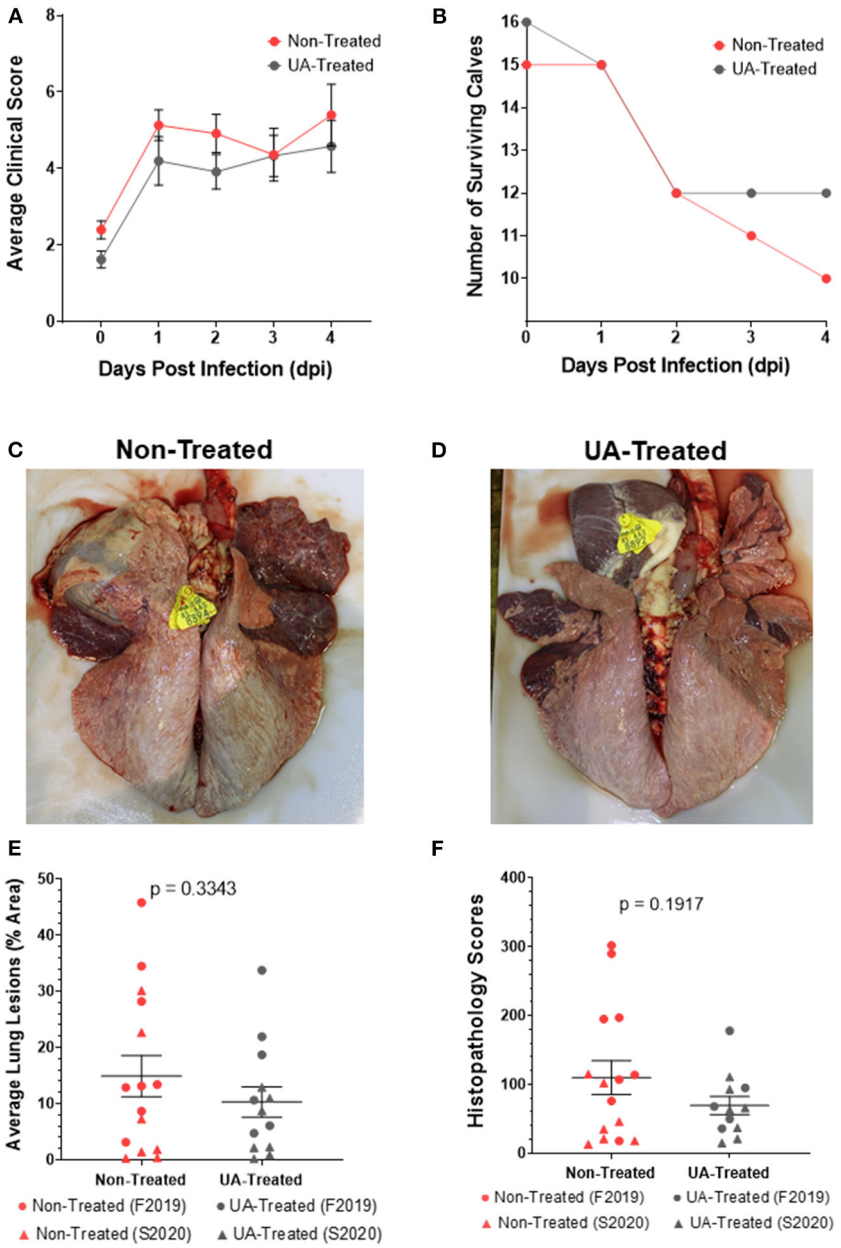



**FIGURE 2 |** Effects of prophylactic UA treatment on clinical disease and lung lesions following *Mannheimia haemolytica* infection. In both studies (F2019 and S2020) animals were divided into non-treated and UA-treated groups ($n = 8$ calves per group). UA-treated animals were given 1 g per head per day of UA powdered supplement with milk feedings beginning 3 days before infection (-3 dpi) and through the duration of the study. Non-treated animals were given no additional supplements with their milk feedings. Animals were challenged through intratracheal instillation with *Mannheimia haemolytica* ($\sim 1 \times 10^8$ each of strains D153 and D174) (0 dpi). **(A)** Calves were monitored and scored daily using a modified Wisconsin scoring system. The data depict the average daily clinical score ± SEM pooled from both studies ($n = 15$ per group). The data were analyzed by two-way ANOVA mixed-effects model with multiple comparisons. **(B)** Calves were removed from their respective studies upon reaching a humane endpoint, and the number of surviving calves in each treatment group were charted over the 4 day challenge period. **(C)** Representative image of a non-treated lung (S2020) at 4 dpi. **(D)** Representative image of a UA-treated lung (S2020) at 4 dpi. **(E)** At necropsy, lungs were photographed and scored based on the percentage of that area that was affected with pneumonic consolidation. Individual animal data from F2019 has been represented as a circle, while S2020 animal data has been represented as a triangle. This figure shows the average lung consolidation ± the SEM for each treatment group (non-treated $n = 15$, UA-treated $n = 13$); the data were analyzed using a standardized unpaired *t*-test. **(F)** Samples from each lung were collected and submitted to a blinded pathologist for scoring as described in McGill et al. (9). Individual animal data from F2019 has been represented as a circle, while S2020 animal data has been represented as a triangle. The graph depicts the mean histopathology ± SEM for each treatment group (non-treated $n = 15$, UA-treated $n = 12$). Outliers in the data were first identified using the ROUT method (Prism v9.1.0), and the data was subsequently analyzed using a standardized unpaired *t*-test to determine significant differences between the two treatments.

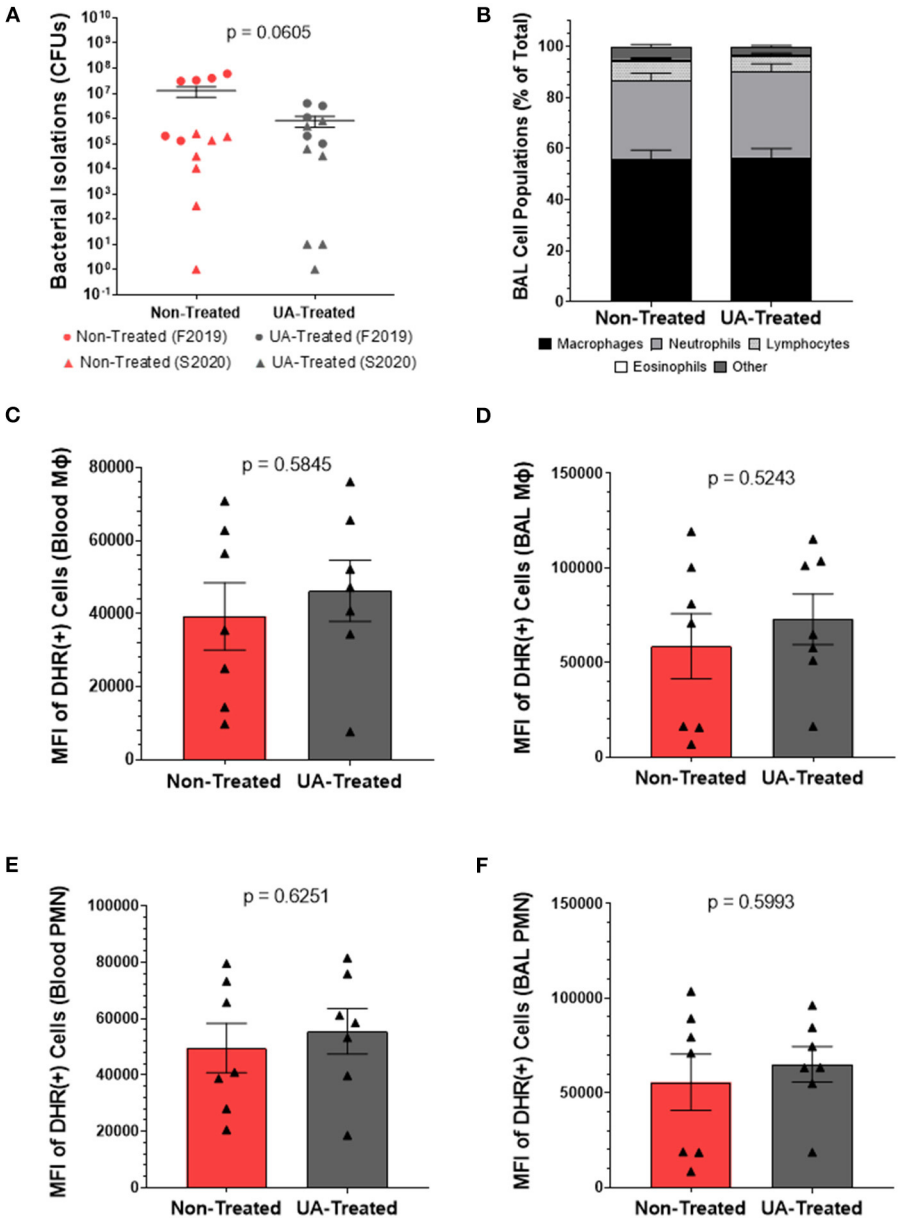



**FIGURE 3 |** Prophylactic UA treatment reduces bacterial burden following MH infection but does not affect the oxidative burst potential of infiltrating or circulating innate immune cells. **(A)** Lung sections were collected from 7 predetermined locations around the lung and assayed by quantitative culture to determine the bacterial burden at 4 dpi as described in Briggs et al. (18). Individual animal data from F2019 has been represented as a circle, while S2020 animal data has been represented as a triangle. The average number of colony forming units (CFUs) determined from each animal are plotted on the graph above along with the average CFUs ± the SEM for each treatment group (non-treated $n = 13$, UA-treated $n = 12$). **(B)** BAL fluid was collected at 4 dpi and subjected to cytology analysis to quantify neutrophils, macrophages, lymphocytes, eosinophils, and other cell types infiltrating the lung airways. The graph depicts the mean relative frequencies of cell types ± SEM for each treatment group (non-treated $n = 14$, UA-treated $n = 13$). **(C–F)** Whole blood and BAL fluid were collected for DHR assays at 4 dpi to determine the oxidative burst potential of circulating macrophages **(C)**, BAL macrophages **(D)**, circulating neutrophils **(E)**, and BAL neutrophils **(F)** in the two treatment groups; these data were only collected during the S2020 study, thus individual animal data is represented with a triangle symbol (▲). Cells were stimulated with PMA or remained unstimulated for 30 min. Cells were then surface stained with monoclonal antibodies to detect monocytes (anti-bovine CD14) and granulocytes (anti-bovine CH138A) and fixed for flow cytometry analysis. These figures depict the average ΔMFI (MFI of PMA stimulated minus MFI of unstimulated wells) ± SEM of DHR labeled and PMA stimulated cells. Data represent animals from the S2020 study only (non-treated $n = 7$, UA-treated $n = 7$).

neutrophils, lymphocytes, eosinophils, and other cell types infiltrating the lung airways. We observed no significant differences in the relative proportions of these cell populations between non-treated and UA-treated animals.

We next chose to determine the impact of UA treatment on the oxidative burst capacity of neutrophils and macrophages from MH infected calves. Functional immune cells undergoing oxidative burst release reactive oxygen species (ROS) that oxidize

dihydrorhodamine 123 (DHR) into fluorescent rhodamine 123; thus, the mean fluorescence intensity (MFI) reported in **Figures 3C–F** is a measure of ROS generated from oxidative burst in cells collected 4 dpi. This assay was conducted only on samples from the S2020 study. **Figures 3C,D** show ROS production generated from circulating and BAL infiltrating macrophages ($CD14^+$ cells), respectively. **Figures 3E,F** show ROS production generated from circulating and BAL infiltrating peripheral mononuclear cells (PMNs, $CH138A^+$ cells), respectively. ROS production from circulating (3C) and lung infiltrating (3D) macrophages was similar between non-treated and UA-treated animals ($p = 0.5845$ and $p = 0.5243$, respectively). Likewise, there were no significant treatment differences in ROS production from circulating (3E) and lung infiltrating (3F) PMNs ($p = 0.6251$ and $p = 0.5993$, respectively).

Irrespective of cell type or location, we did not observe any treatment differences in ROS production, which suggests altered IL-17 signaling is not impacting the oxidative burst functionality of innate immune cells following MH infection. We also noted that UA treatment did not alter the frequencies of host cells infiltrating the BAL fluid; however, UA-treated animals tended to have a lower MH burden ($p = 0.0605$) compared to non-treated animals. Therefore, we speculate that altered IL-17 signaling may be altering other aspects of the immune response to limit bacterial colonization and pathogenesis following infection.

## Prophylactic UA Treatment Modulates Inflammatory Signaling Following MH Infection

The immune response to MH infection includes production of the chemokine IL-8 (neutrophil chemotactic factor, or CXCL-8) and the inflammatory cytokine IL-6. IL-6 binds to surface receptors of other immune cells to induce STAT3 signaling and subsequent activation of the RORC gene; RORC encodes the transcription factor (RORγt) for IL-17A production. IL-17A subsequently acts in a positive feedback loop to induce further upregulation of IL-8. Therefore, we next chose to investigate the impact of UA treatment, and its suppression of IL-17A signaling, on this inflammatory pathway during MH infection.

**Figures 4A–E** show the relative mRNA expressions of genes associated with the IL-17A signaling pathway; each gene's RE was determined using the $2^{-\Delta\Delta CT}$ method by comparing ΔCTs from pneumonic lung tissue to healthy control lung tissues obtained during the F2019 study. **Figure 4A** shows significantly ($p = 0.0209$) higher IL-6 expression in the lungs of non-treated animals compared to UA-treated animals following MH infection. As seen in **Figure 4B**, UA treatment also significantly ($p = 0.0205$) down-regulated STAT3 expression compared to non-treated animals. Intracellular STAT3 signaling has been shown to transcriptionally regulate other immune activating and inflammatory genes (29, 30). Interestingly, the relative mRNA expression for the RORC gene (**Figure 4C**) does not appear to differ ($p = 0.7399$) between treatment groups; since this gene is tightly regulated at multiple levels its expression may be more immutable. Downstream from the RORC gene, IL-17A expression (**Figure 4D**) was reduced ($p = 0.0870$) in UA-treated animals compared to non-treated animals. However, IL-17A may not be the only neutrophil activating signal being differentially expressed during MH infection. Subsequently, we investigated the impact of the IL-17A inhibitor (UA) on IL-8 expression (**Figure 4E**, $p = 0.2603$), but did not observe any significant differences in treated compared to non-treated animals.

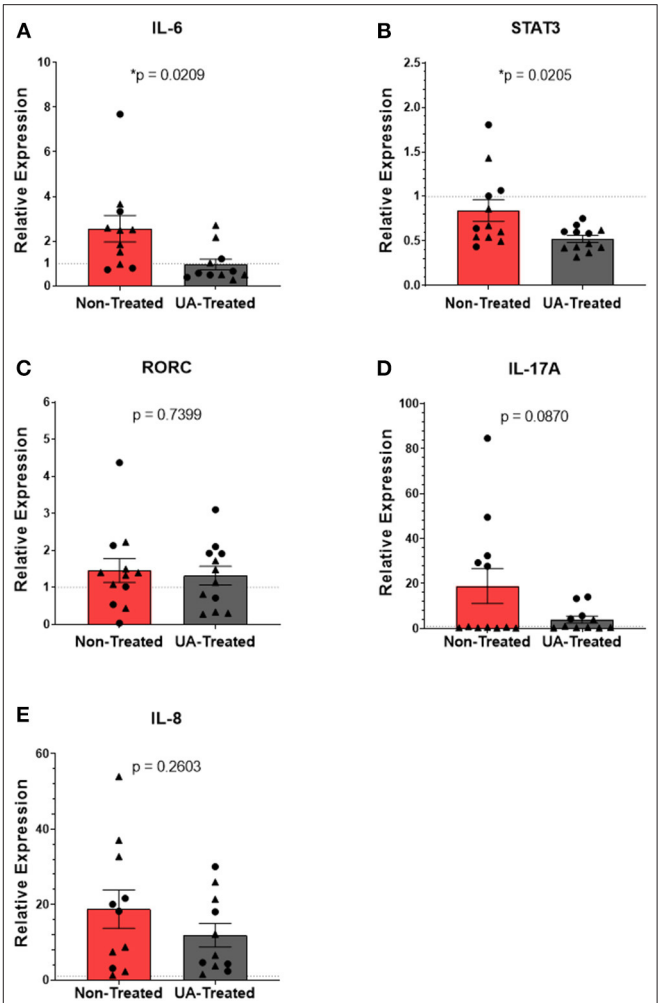



**FIGURE 4 |** Prophylactic UA treatment modulates inflammatory IL-17A signaling in the lungs following MH infection. Samples of lung tissue were collected at necropsy and stored in RNAlater solution for qPCR analysis of **(A)** IL-6, **(B)** STAT3, **(C)** RORC, **(D)** IL-17A, and **(E)** IL-8 mRNA expression; individual animal data from F2019 has been represented as a circle (•), while S2020 animal data has been represented as a triangle (▲). The relative mRNA expression in pneumonic lung tissue from non-treated and UA-treated animals was compared to healthy lung tissue from control animals (F2019 group) for both studies. The average relative expression (RE) ± the SEM for each treatment group across both studies is depicted in **(A–E)**. Outliers were identified using the ROUT method (Prism v9.1.0), and the data were analyzed using a standardized unpaired *t*-test (*$p < 0.05$). **(A)** Non-treated $n = 11$, UA-treated $n = 11$; **(B)** non-treated $n = 12$, UA-treated $n = 12$; **(C)** non-treated $n = 12$, UA-treated $n = 12$; **(D)** non-treated $n = 12$, UA-treated $n = 11$; **(E)** non-treated $n = 11$, UA-treated $n = 11$.

## Prophylactic UA Treatment Alters Expression of Innate Defense Genes in Response to MH Infection

Neutrophilic granules contain a multitude of antimicrobial peptides (AMPS) that can be weaponized against invading pathogens. In addition to its role in the inflammatory cascade, IL-17A also plays an important role in activating neutrophil expression of AMPS and other innate defense molecules (31, 32). **Figures 5A–D** show the relative mRNA expressions of genes associated with the host's innate defenses; each gene's RE was determined using the $2^{-\Delta\Delta CT}$ method by comparing ΔCTs from pneumonic lung tissue to healthy control lung tissues obtained during the F2019 study. Bactenecin-5 (BAC5) is an AMP found in bovine neutrophils that is effective at inhibiting the growth of and killing Gram-negative bacteria (33), and its expression is controlled under the BAC5 (CATHL2) gene. Interestingly, relative expression of BAC5 (**Figure 5A**) tended to be downregulated ($p = 0.0519$) in the lungs of UA-treated animals compared to those of untreated controls. β-defensin 1 is an AMP capable of permeabilizing membranes and inducing neutrophil extracellular traps (NETs) to control bacterial pathogens (34). Its expression is regulated under the DEFB1 gene in epithelial cells that line the respiratory tract. In **Figure 5B**, we do not see a significant difference in the expression of lung DEFB1 ($p = 0.1212$) between non-treated and UA-treated animals. MUC5AC is another innate defense gene commonly expressed in the lungs during respiratory infections, and its expression leads to the production of gel-forming mucins that can trap extracellular pathogens. However, the buildup of these mucins can block airways and contribute to lung congestion, so managing mucin production is important for both mucociliary function and pathogen clearance (3, 35). **Figure 5C** shows that the relative expression of MUC5AC is down regulated in non-treated and UA-treated animals alike, but UA-treated animals tend to express lower levels compared to untreated controls ($p = 0.0913$), which may be indicative of improved infection-resolution in their lungs. Interferon-γ (IFNγ) signaling can play an overlapping role in both the induction of AMPs and inhibition of some mucins. As seen in **Figure 5D**, the relative expression of IFNγ tends to be reduced in UA-treated calves compared to control calves ($p = 0.0889$), and is thus not likely a driving factor for the observed increase in AMP production.

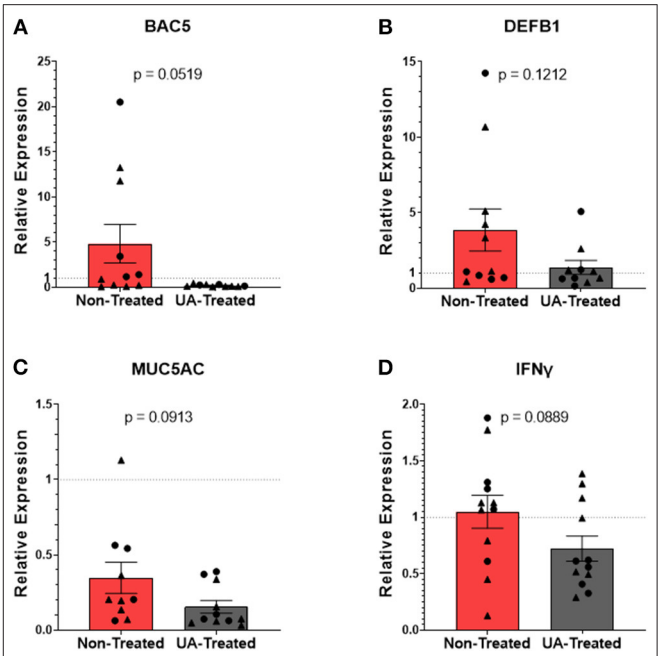



**FIGURE 5 |** Prophylactic UA treatment alters expression of innate defense genes in the lungs following MH infection. Samples of lung tissue were collected at necropsy and stored in RNAlater solution for qPCR analysis of **(A)** BAC5, **(B)** DEFB1, **(C)** MUC5AC, and **(D)** IFNγ mRNA expression; individual animal data from F2019 has been represented as a circle (•), while S2020 animal data has been represented as a triangle (▲). The relative mRNA expression in pneumonic lung tissue from non-treated and UA-treated animals was compared to healthy lung tissue from control animals (F2019 group) for both studies. The average relative expression (RE) ± the SEM for each treatment group across both studies is depicted. Outliers in the data sets were first identified using the ROUT method (Prism v9.1.0), and the data were analyzed using a standardized unpaired *t*-test (*$p < 0.05$). **(A)** Non-treated $n = 11$, UA-treated $n = 10$; **(B)** non-treated $n = 11$, UA-treated $n = 10$; **(C)** non-treated $n = 10$, UA-treated $n = 11$; **(D)** non-treated $n = 12$, UA-treated $n = 12$.

## Prophylactic UA Treatment May Influence Lung Tissue Remodeling Following MH Infections

Matrix metalloproteinases (MMPs) are produced to degrade the extracellular matrix and allow for more efficient leukocyte infiltration while clearing away damaged and necrotic tissues. MMP9 (gelatinase B, a type IV collagenase) has been implicated in lung tissue damage following MH infections; its release by bovine neutrophils during degranulation can exacerbate inflammatory signaling through the induction of both IL-1 and IL-8. Macrophage production of the tissue inhibitor of metalloproteinase 1 (TIMP-1) can be upregulated to offset some of the inflammatory and degradative effects of MMP9, and it is thought that an imbalance of these two proteins may play a role in disease progression (29, 36). Since inflammatory signaling has a profound effect on tissue remodeling processes, we speculated that inhibited IL-17 signaling may alter the balance of MMP production and inhibition.

Relative expression levels of MMP9 (**Figure 6A**) are not significantly different ($p = 0.1755$) between non-treated and UA-treated animals, although the mean expression of MMP9 is higher in control animals (non-treated = 18.44; UA-treated = 9.95). The relative expression of TIMP-1 (**Figure 6B**) did not differ ($p = 0.3223$) between UA-treated animals and non-treated animals. However, only 4/11 control animals have a relative expression value >1, indicating that most of these animals have downregulated TIMP-1 expression relative to healthy lung samples. Meanwhile, 8/11 UA-treated animals have a RE value >1, indicating an upregulation of TIMP-1 compared to healthy lungs. TIMP-1 directly antagonizes the degradative capabilities of MMP9 in a 1:1 ratio (36); therefore, comparing MMP9 expression to TIMP-1 expression provides insight into

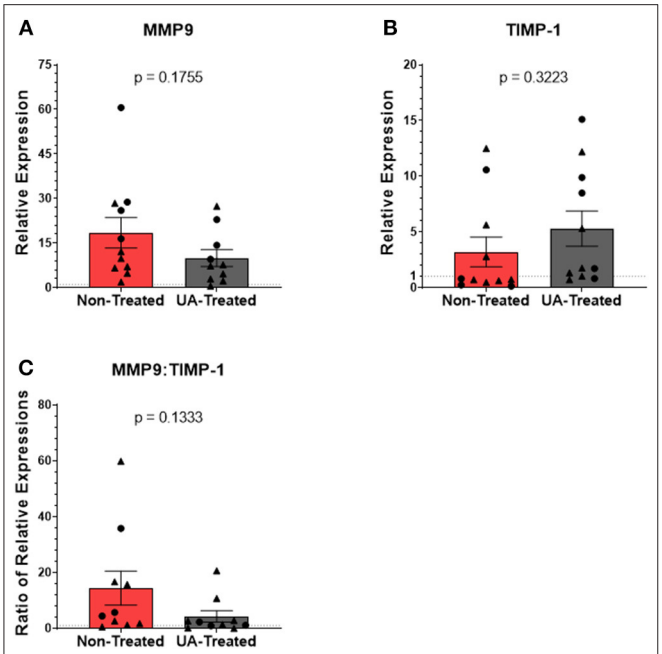



**FIGURE 6 |** Prophylactic UA treatment alters matrix metalloproteinase activity in the lungs following MH infection. Samples of lung tissue were collected at necropsy and stored in RNAlater solution for qPCR analysis of **(A)** MMP9 and **(B)** TIMP-1 mRNA expression; individual animal data from F2019 has been represented as a circle (•), while S2020 animal data has been represented as a triangle (▲). The relative mRNA expression in pneumonic lung tissue from non-treated and UA-treated animals was compared to healthy lung tissue from control animals (F2019 group) for both studies. Graphs depict the average relative expression (RE) ± the SEM for each treatment group across both studies. Outliers in the data sets were first identified using the ROUT method (Prism v9.1.0), and the data were analyzed by standardized unpaired *t*-test (*$p < 0.05$). **(A)** Non-treated $n = 11$, UA-treated $n = 10$; **(B)** non-treated $n = 11$, UA-treated $n = 11$). **(C)** Depicts the ratio of MMP9 to TIMP-1 RE for each individual animal. Data represent mean RE ratios ± the SEM for each treatment group.

the state of tissue repair occurring in the lungs following MH infections. **Figure 6C** shows the ratio of MMP9 to TIMP-1 relative expressions for each animal, and this comparison highlights an interesting tendency ($p = 0.1333$) between UA-treated and non-treated animals, with UA-treated animals having a lower mean ratio and clustering more tightly than non-treated animals. Considering the antagonistic functionality of MMP9 and TIMP-1, these data suggest that our control animals may still be actively remodeling lung tissue to combat infection, while the UA-treated animals with altered IL-17 signaling may be resolving the lung environment to repair functionality.

## DISCUSSION

Inflammatory cytokines, like IL-17A, are thought to contribute to lung congestion by orchestrating the mobilization of neutrophils, inducing the production of ROS and MMPs, and activating the innate defenses from cells lining the respiratory tract. Production of IL-17A, and the subsequent IL-17A-driven inflammation, is regulated by the transcription factor RORγt (12, 13). The small molecule inhibitor, UA, is reported to be a specific and highly effective agonist of RORγt (12). We believe this is the first time the drug has been implemented in a bovine model, and our *in vitro* (**Figure 1B**) and *in vivo* (**Figure 4D**) results underpin UA's inhibitory effects on IL-17A production. Although applications of UA treatment are being investigated clinically, the drug still has limitations in its therapeutic potential. Initial reports have implied that UA treatment is specific for inhibiting IL-17A production, but more recent reports show that treatment effects may not be precise. A recent publication from Zhang et al., has shown that UA is also a potent agonist of peroxisome proliferator-activated receptor γ (PPARγ) in experimental models of multiple sclerosis (37). Other reports have determined that UA treatment suppresses the phosphorylation of STAT3 and JAK2, which further explains the drug's capacity to inhibit Th17 differentiation (13, 38). Despite not having looked at phosphorylation levels, we did see a significant reduction in STAT3 expression in the lungs of UA-treated calves (**Figure 4B**) consistent with these reports. Thus, although UA was a useful tool to impair IL-17 signaling in our studies, its effects may not be specific only to IL-17A production.

There is a significant degree of variability amongst animal trials utilizing experimental MH infection. One study used $4.10 \times 10^7$ CFUs of MH inoculum to induce clinical signs similar to a natural infection (39). Meanwhile, other reports have employed considerably higher doses ($4.4 \times 10^{11}$ CFUs) (36). Considering the variability in disease outcomes and infection doses, we selected an inoculum of $2–4 \times 10^8$ total MH CFUs. While this inoculation strategy did produce clinical MH infection, the resulting disease was severe in some animals (mostly in the F2019 study), which may have limited our ability to discern differences between treatment groups. Although some of these differences may be attributed to natural variations between animals, the F2019 animals also developed a concurrent infection with *Pasteurella multocida* (PM). Subsequently, F2019 animals had higher MH burdens than S2020 animals (**Figure 3A**), and lung cultures from F2019 animals also revealed the presence of PM in both control and UA-treated animals. Importantly, the F2019 animals were evaluated by thoracic ultrasonography the day prior to infection (Porter et al., manuscript under review) and their lungs were clear of consolidation or pleural defects that would indicate a prior or ongoing bacterial infection. This suggests that F2019 animals were either in the early stages of PM infection, or possibly that our intratracheal MH infection created a window of susceptibility for PM infection. We speculate that the increased pathogenesis from PM co-infection may have limited our ability to discern more significant treatment effects, and future studies should consider the balance between inducing pathogenesis and over-challenging beyond the immunomodulatory capabilities of UA.

MH infection is characterized by lung consolidation with infiltrating neutrophils found in the lung exudate (40). There is also a well-established role for IL-17A inflammation in driving the production of neutrophil chemoattractants and activating innate defenses (32, 41). We have previously reported

that MH infection induces IL-17A cytokine production in the lungs (8). Ergo, we hypothesized that altering IL-17A-driven inflammation during MH infection would reduce the presence or activation of infiltrating leukocytes in the lung. Interestingly, we did not observe any changes in the frequency of neutrophils isolated from the BAL fluid of calves treated with the IL-17A inhibitor (**Figure 3B**). Although the UA-treated animals tended to have lower IL-17A expression in their lung tissues (**Figure 4D**), we observed no differences in the expression of IL-8 (**Figure 4E**) (42). It is plausible that neutrophil infiltration was not impacted by altered IL-17 signaling due to IL-17A-independent production of IL-8. To this point, LPS (shed from MH) and damage-associated molecular patterns (induced from ROS and leukotoxins) can directly stimulate alveolar macrophages and epithelial cells to produce IL-8 in the absence of IL-17 signaling (42–44). Others have demonstrated that LPS and flagellin can activate TLR4 and TLR5 signaling cascades in bronchial epithelial cells (through MAPK and NF-κB pathways) to release of large quantities of TNFα, IL-6, and IL-8 (43), which can synergize to recruit and activate neutrophils and macrophages apart from IL-17 signaling (31, 42, 43, 45).

Inflammatory signaling following MH infection is known to induce tissue remodeling by matrix metalloproteinases. MMP9 is an important gelatinase which contributes to lung tissue damage during MH infection (36). Initially, MMP9 production causes degradation of the extracellular matrix, allowing for increased immune cell infiltration. However, prolonged production of MMP9 leads to degradation of the basement membrane of the lung epithelia, which compromises the architecture and barrier functions within the airways, resulting in consolidated lung lesions (36, 46). TIMP-1 is the conjugate inhibitor to MMP9, and its expression is induced to temper the tissue degradation spurred on by inflammatory signaling (36). The inverse ratio between these two proteins is helpful in assessing the state of lung remodeling following acute damage or infections (47, 48). In our study, UA-treated animals had a lower (more inhibitory) expression ratio of MMP9:TIMP-1 (mean expression ratio of 4.287) compared to the non-treated control calves (mean expression ratio of 14.437). Although the treatment difference between the ratios of MMP9:TIMP-1 was not statistically significant, it suggests that lung remodeling and tissue repair may be expedited when inflammatory IL-17 signaling is altered.

## CONCLUSION

Herein, we have shown that inflammatory IL-17 signaling following MH infection plays a dual role in pathogen clearance and immunopathogenesis which may adversely affect disease resolution. We have observed that animals receiving an IL-17A inhibitor tended to have lower lung pathology scores and reduced bacterial colonization in the lungs compared to control animals. Furthermore, blocking the IL-17A signaling pathway resulted in altered expression of inflammatory and innate defense genes in the lungs, and impacted the expression of genes related to tissue remodeling in the lungs following MH infection. Taken together, our results suggest that inflammatory IL-17 signaling plays an important role in MH infection and BRDC pathogenesis, and further investigations into this pathway may offer new therapeutic intervention strategies for respiratory infections.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Iowa State University Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

JS, BC, RB, and JM designed the study. JS and BC collected and analyzed the data. JS and JM wrote the manuscript. All authors reviewed the manuscript.

## FUNDING

Early *in vitro* work was supported through funds from the NIH Grant R21 AI127895. The animals and work detailed in this manuscript were funded by the USDA-NIFA Grant 2018-06904 to JM. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## ACKNOWLEDGMENTS

The authors would also like to acknowledge and thank the ISU Laboratory Animal Resources staff and veterinarians for their help with animal husbandry.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.782872/full#supplementary-material

## REFERENCES

1. Johnson KK, Pendell DL. Market impacts of reducing the prevalence of bovine respiratory disease in United States beef cattle feedlots. *Front Vet Sci.* (2017) 4:189. doi: 10.3389/fvets.2017.00189
2. Dubrovsky SA, Van Eenennaam AL, Karle BM, Rossitto PV, Lehenbauer TW, Aly SS. Bovine respiratory disease (BRD) cause-specific and overall mortality in preweaned calves on California dairies: the BRD 10K study. *J Dairy Sci.* (2019) 102:7320–8. doi: 10.3168/jds.2018-15463
3. Griffin D, Chengappa MM, Kuszak J, McVey DS. Bacterial pathogens of the bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract.* (2010) 26:381–94. doi: 10.1016/j.cvfa.2010.04.004

4. Cozens D, Sutherland E, Lauder M, Taylor G, Berry CC, Davies RL. Pathogenic *Mannheimia haemolytica* invades differentiated bovine airway epithelial cells. *Infect Immun.* (2019) 87. doi: 10.1128/IAI.00078-19
5. Tizard IR. Chapter 16 - Bovine vaccines. In: Tizard IR, editor. *Vaccines for Veterinarians*. St. Louis, MO: Elsevier (2021). p. 193-214.e1.
6. Rice JA, Carrasco-Medina L, Hodgins DC, Shewen PE. *Mannheimia haemolytica* and bovine respiratory disease. *Anim Health Res Rev.* (2007) 8:117–28. doi: 10.1017/S1466252307001375
7. Confer AW, Ayalew S. *Mannheimia haemolytica* in bovine respiratory disease: immunogens, potential immunogens, and vaccines. *Anim Health Res Rev.* (2018) 19:79–99. doi: 10.1017/S1466252318000142
8. McGill JL, Rusk RA, Guerra-Maupome M, Briggs RE, Sacco RE. Bovine gamma delta T cells contribute to exacerbated IL-17 production in response to co-infection with Bovine RSV and *Mannheimia haemolytica*. *PLoS ONE.* (2016) 11:e0151083. doi: 10.1371/journal.pone.0151083
9. McGill JL, Guerra-Maupome M, Schneider S. Prophylactic digoxin treatment reduces IL-17 production *in vivo* in the neonatal calf and moderates RSV-associated disease. *PLoS ONE.* (2019) 14:e0214407. doi: 10.1371/journal.pone.0214407
10. Leite F, O'Brien S, Sylte MJ, Page T, Atapattu D, Czuprynski CJ. Inflammatory cytokines enhance the interaction of *Mannheimia haemolytica* leukotoxin with bovine peripheral blood neutrophils *in vitro*. *Infect Immun.* (2002) 70:4336–43. doi: 10.1128/IAI.70.8.4336-4343.2002
11. Caswell JL, Middleton DM, Gordon JR. The importance of interleukin-8 as a neutrophil chemoattractant in the lungs of cattle with pneumonic pasteurellosis. *Can J Vet Res.* (2001) 65:229–32.
12. Xu T, Wang X, Zhong B, Nurieva RI, Ding S, Dong C. Ursolic acid suppresses interleukin-17 (IL-17) production by selectively antagonizing the function of RORgamma t protein. *J Biol Chem.* (2011) 286:22707–10. doi: 10.1074/jbc.C111.250407
13. Baek SY, Lee J, Lee DG, Park MK, Kwok SK, Cho ML, et al. Ursolic acid ameliorates autoimmune arthritis via suppression of Th17 and B cell differentiation. *Acta Pharmacol Sin.* (2014) 35:1177–87. doi: 10.1038/aps.2014.58
14. McGill JL, Nonnecke BJ, Lippolis JD, Reinhardt TA, Sacco RE. Differential chemokine and cytokine production by neonatal bovine γδ T-cell subsets in response to viral toll-like receptor agonists and *in vivo* respiratory syncytial virus infection. *Immunology.* (2013) 139:227–44. doi: 10.1111/imm.12075
15. Wang Y, Li L, Deng S, Liu F, He Z. Ursolic acid ameliorates inflammation in cerebral ischemia and reperfusion injury possibly. *Front Neurol.* (2018) 9:253. doi: 10.3389/fneur.2018.00253
16. Kim SH, Hong JH, Lee YC. Ursolic acid, a potential PPARγ agonist, suppresses ovalbumin-induced airway inflammation and Penh by down-regulating IL-5, IL-13, and IL-17 in a mouse model of allergic asthma. *Eur J Pharmacol.* (2013) 701:131–43. doi: 10.1016/j.ejphar.2012.11.033
17. West GB, Brown JH. The origin of allometric scaling laws in biology from genomes to ecosystems: towards a quantitative unifying theory of biological structure and organization. *J Exp Biol.* (2005) 208(Pt 9):1575–92. doi: 10.1242/jeb.01589
18. Briggs RE, Tabatabai LB, Tatum FM. Mucosal and parenteral vaccination against pneumonic pasteurellosis in cattle with a modified-live in-frame lktA deletion mutant of *Mannheimia haemolytica*. *Microb Pathog.* (2012) 52:302–9. doi: 10.1016/j.micpath.2012.02.008
19. Frank GH, Wessman GE. Rapid plate agglutination procedure for serotyping Pasteurella haemolytica. *J Clin Microbiol.* (1978) 7:142–5. doi: 10.1128/jcm.7.2.142-145.1978
20. Townsend KM, Boyce JD, Chung JY, Frost AJ, Adler B. Genetic organization of Pasteurella multocida cap Loci and development of a multiplex capsular PCR typing system. *J Clin Microbiol.* (2001) 39:924–9. doi: 10.1128/JCM.39.3.924-929.2001
21. Sacco RE, Nonnecke BJ, Palmer MV, Waters WR, Lippolis JD, Reinhardt TA. Differential expression of cytokines in response to respiratory syncytial virus infection of calves with high or low circulating 25-hydroxyvitamin D3. *PLoS ONE.* (2012) 7:e33074. doi: 10.1371/journal.pone.0033074
22. Cunha P, Vern YL, Gitton C, Germon P, Foucras G, Rainard P. Expansion, isolation and first characterization of bovine Th17 lymphocytes. *Sci Rep.* (2019) 9:16115. doi: 10.1038/s41598-019-52562-2
23. Thacker TC, Palmer MV, Waters WR. T-cell mRNA expression in response to *Mycobacterium bovis* BCG vaccination and *Mycobacterium bovis* infection of white-tailed deer. *Clin Vaccine Immunol.* (2009) 16:1139–45. doi: 10.1128/CVI.00424-08
24. Merriman KE. *Characterizing the Contribution of Vitamin D to Activation of Host Defenses in Dairy Cows*. Gainesville, FL: University of Florida (2015). Available online at: http://ufdc.ufl.edu/UFE0049331/00001
25. Hoorens PR, Rinaldi M, Li RW, Goddeeris B, Claerebout E, Vercruysse J, et al. Genome wide analysis of the bovine mucin genes and their gastrointestinal transcription profile. *BMC Genomics.* (2011) 12:140. doi: 10.1186/1471-2164-12-140
26. Kliem H, Welter H, Kraetzl WD, Steffl M, Meyer HH, Schams D, et al. Expression and localisation of extracellular matrix degrading proteases and their inhibitors during the oestrous cycle and after induced luteolysis in the bovine corpus luteum. *Reproduction.* (2007) 134:535–47. doi: 10.1530/REP-06-0172
27. Gabler C, Killian GJ, Einspanier R. Differential expression of extracellular matrix components in the bovine oviduct during the oestrous cycle. *Reproduction.* (2001) 122:121–30. doi: 10.1530/rep.0.1220121
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* (2001) 25:402–8. doi: 10.1006/meth.2001.1262
29. Kasembeli MM, Bharadwaj U, Robinson P, Tweardy DJ. Contribution of STAT3 to inflammatory and fibrotic diseases and prospects for its targeting for treatment. *Int J Mol Sci.* (2018) 19(8):2299-2328. doi: 10.3390/ijms19082299
30. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer.* (2009) 9:798–809. doi: 10.1038/nrc2734
31. Veldhoen M. Interleukin 17 is a chief orchestrator of immunity. *Nat Immunol.* (2017) 18:612–21. doi: 10.1038/ni.3742
32. Zenobia C, Hajishengallis G. Basic biology and role of interleukin-17 in immunity and inflammation. *Periodontol 2000.* (2015) 69:142–59. doi: 10.1111/prd.12083
33. Kościuczuk EM, Lisowski P, Jarczak J, Strzałkowska N, Józwik A, Horbańczuk J, et al. Cathelicidins: family of antimicrobial peptides. A review. *Mol Biol Rep.* (2012) 39:10957–70. doi: 10.1007/s11033-012-1997-x
34. Kraemer BF, Campbell RA, Schwertz H, Cody MJ, Franks Z, Tolley ND, et al. Novel anti-bacterial activities of β-defensin 1 in human platelets: suppression of pathogen growth and signaling of neutrophil extracellular trap formation. *PLoS Pathog.* (2011) 7:e1002355. doi: 10.1371/journal.ppat.1002355
35. Ackermann MR, Derscheid R, Roth JA. Innate immunology of bovine respiratory disease. *Vet Clin North Am Food Anim Pract.* (2010) 26:215–28. doi: 10.1016/j.cvfa.2010.03.001
36. Starr AE, Dan T, Minhas K, Shewen PE, Coomber BL. Potential involvement of gelatinases and their inhibitors in *Mannheimia haemolytica* pneumonia in cattle. *Infect Immun.* (2004) 72:4393–400. doi: 10.1128/IAI.72.8.4393-4400.2004
37. Zhang Y, Li X, Ciric B, Curtis MT, Chen WJ, Rostami A, et al. A dual effect of ursolic acid to the treatment of multiple sclerosis through both immunomodulation and direct remyelination. *Proc Natl Acad Sci USA.* (2020) 117:9082–93. doi: 10.1073/pnas.2000208117
38. Liu T, Ma H, Shi W, Duan J, Wang Y, Zhang C, et al. Inhibition of STAT3 signaling pathway by ursolic acid suppresses growth of hepatocellular carcinoma. *Int J Oncol.* (2017) 51:555–62. doi: 10.3892/ijo.2017.4035
39. Lhermie G, Ferran AA, Assié S, Cassard H, El Garch F, Schneider M, et al. Impact of timing and dosage of a fluoroquinolone treatment on the microbiological, pathological, and clinical outcomes of calves challenged with *Mannheimia haemolytica*. *Front Microbiol.* (2016) 7:237. doi: 10.3389/fmicb.2016.00237
40. Aulik NA, Hellenbrand KM, Klos H, Czuprynski CJ. *Mannheimia haemolytica* and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. *Infect Immun.* (2010) 78:4454–66. doi: 10.1128/IAI.00840-10
41. Lindén A, Laan M, Anderson GP. Neutrophils, interleukin-17A and lung disease. *Eur Respir J.* (2005) 25:159–72. doi: 10.1183/09031936.04.00032904
42. Malazdrewich C, Thumbikat P, Abrahamsen MS, Maheswaran SK. Pharmacological inhibition of *Mannheimia haemolytica* lipopolysaccharide and leukotoxin-induced cytokine expression in bovine alveolar macrophages. *Microb Pathog.* (2004) 36:159–69. doi: 10.1016/j.micpath.2003.11.002

43. Cai Y, Varasteh S, van Putten JPM, Folkerts G, Braber S. *Mannheimia haemolytica* and lipopolysaccharide induce airway epithelial inflammatory responses in an extensively developed *ex vivo* calf model. *Sci Rep.* (2020) 10:13042. doi: 10.1038/s41598-020-69982-0
44. Ackermann MR, Brogden KA. Response of the ruminant respiratory tract to Mannheimia (Pasteurella) haemolytica. *Microbes Infect.* (2000) 2:1079–88. doi: 10.1016/S1286-4579(00)01262-4
45. Singh K, Ritchey JW, Confer AW. *Mannheimia haemolytica*: bacterial-host interactions in bovine pneumonia. *Vet Pathol.* (2011) 48:338–48. doi: 10.1177/0300985810377182
46. Gorski SA, Hufford MM, Braciale TJ. Recent insights into pulmonary repair following virus-induced inflammation of the respiratory tract. *Curr Opin Virol.* (2012) 2:233–41. doi: 10.1016/j.coviro.2012.04.006
47. Davey A, McAuley DF, O'Kane CM. Matrix metalloproteinases in acute lung injury: mediators of injury and drivers of repair. *Eur Respir J.* (2011) 38:959–70. doi: 10.1183/09031936.00032111
48. Chen G, Ge D, Zhu B, Shi H, Ma Q. Upregulation of matrix metalloproteinase 9 (MMP9)/tissue inhibitor of metalloproteinase 1 (TIMP1) and MMP2/TIMP2 ratios may be involved in lipopolysaccharide-induced acute lung injury. *J Int Med Res.* (2020) 48:300060520919592. doi: 10.1177/0300060520919592

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 08 December 2021
doi: 10.3389/fvets.2021.764909

# Antiviral Effect of Ginsenoside Rb2 and Rb3 Against Bovine Viral Diarrhea Virus and Classical Swine Fever Virus *in vitro*

*Bin Tan[1], Massimo Giangaspero[2], Na Sun[1], Yinping Jin[1], Kexin Liu[1], Qianying Wang[1], Shipeng Cheng[1], Yingping Wang[3] and Shuqin Zhang[1]**

[1] Institute of Special Economic Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, China, [2] Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy, [3] National and Local Joint Engineering Research Center for Ginseng Breeding and Development, Changchun, China

Bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) are members of the genus *Pestivirus* that cause disease in wild and domestic animals and are responsible for extensive economic losses of livestock and biological industry. BVDV is also a significant laboratory contaminant. Currently, no effective antiviral therapeutics are available to control their infection. Ginsenosides, as major pharmacological ingredients in the plants of ginseng, have various biological activities. In the present work, the antiviral activity of 9 ginsenosides and 3 other saponins from *Araliaceae* plants was investigated against *Pestivirus*. Ginsenoside Rb2 and Rb3 showed low cytotoxicity and obvious antiviral effect. They were able to inhibit the replication and proliferation of BVDV and CSFV. In addition, our results suggest that the possible antiviral mechanism of Rb2 might be related to its ability to affect the translation of these viruses. Obtained results suggest that ginsenoside Rb2 and Rb3 have a potential for effective treatment against *Pestivirus* infection.

**Keywords: antivirus activity, ginsenoside Rb2/Rb3, BVDV, CSFV, *in vitro***

OPEN ACCESS

***Edited by:***
*Annamaria Pratelli, University of Bari Aldo Moro, Italy*

***Reviewed by:***
*Mohsan Ullah Goraya, University of Agriculture, Faisalabad, Pakistan*
*Lauro Velazquez-Salinas, United States Department of Agriculture (USDA), United States*

****Correspondence:***
*Shuqin Zhang*
*shuqinzhang2008@163.com*

***Specialty section:***
*This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science*

***Received:*** *26 August 2021*
***Accepted:*** *02 November 2021*
***Published:*** *08 December 2021*

***Citation:***
*Tan B, Giangaspero M, Sun N, Jin Y, Liu K, Wang Q, Cheng S, Wang Y and Zhang S (2021) Antiviral Effect of Ginsenoside Rb2 and Rb3 Against Bovine Viral Diarrhea Virus and Classical Swine Fever Virus in vitro. Front. Vet. Sci. 8:764909. doi: 10.3389/fvets.2021.764909*

## INTRODUCTION

Bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) are members of the genus *Pestivirus*, family *Flaviviridae*. They are viral pathogens affecting wild fauna and domestic livestock, causing extensive economic losses worldwide (1). Their genomes are single stranded positive polarity RNA, which consist of a single large open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTR). The ORF encodes a polyprotein of ∼3,900 aa and cleaved by viral and cellular proteinases into the individual viral proteins (2). The highly conserved 5′ UTR of genome binds to the host ribosome using the internal ribosome entry site (IRES) mechanism that facilitates translation of *Pestivirus* proteins (3).

CSFV infection mainly causes high fever, multiple hemorrhages, and leukopenia, leading to high morbidity and mortality, the severity of which might be due to the host species and the virulence of viral strains (4). Infections with BVDV are related to various clinical conditions such as reproductive failure, subclinical infection, persistent infections, severe acute disease with or without hemorrhagic diathesis, and even fatal mucosal disease (5, 6). BVDV is not only an important clinical pathogen, but also a significant laboratory contaminant. It has been reported that bovine serum,

canine, bovine and feline cell lines, and vaccine commercially available have been found to be contaminated with BVDV (7–9).

Panax ginseng Meyer, a perennial herb of the *Araliaceae* family, has been used as a preventive and/or therapeutic herbal medicine in China, Japan, and Korea to strengthen holistic health for thousands of years (10). Ginsenosides are considered the major components of ginseng, they have a variety of biological activities, such as anti-aging, anti-oxidative, anti-cancer, and other actions improving the health (11). Currently, numerous studies have reported the beneficial effects on infections with pathogenic viruses. 20(R)-ginsenoside Rh2 was shown to suppress the replication of gamma herpesviruses in mouse and humans (12), 20(S)-protopanaxtriol possessed potent *in vitro* antiviral effect on the CVB3 (13). In particular, Re, Rf, and Rg2 could protect the host from the infections of rhinovirus 3 and coxsackievirus. In addition, ginsenoside Rg2 has also been found to have significant anti-EV71 activity (14). Ginsenoside Rg3 presented antiviral activity on Hepatitis C virus (HCV), inhibiting HCV-induced abnormal mitochondrial fission and mitophagy (supporting mechanisms for the establishment of persistent viral infection) (15). Rb2 showed the ability to reduce the virus titers and protect against infection of rotavirus and Sendai virus in mice (16, 17). However, to date, little is known about antiviral activity of the ginsenosides against BVDV. Currently, no effective antiviral therapeutics are available to control the BVDV infection. Thus, we have evaluated the antiviral activities of ginsenosides and other saponins for *in vitro* toxicity and activity against BVDV, also for a possible application to other closely genetically related pathogens as HCV.

## MATERIALS AND METHODS

### Cells and Viruses

Madin Darby Bovine Kidney (MDBK) cells and swine testicle (ST) cells were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM), which was added with 8% fetal bovine serum (FBS). The FBS was confirmed to be free of BVDV antibody with commercial BVDV antibody test kit (IDEXX Laboratories, Inc.). All cells and FBS were tested negative for the presence of *Pestivirus* antigen by RT-PCR (18). The cpBVDV C24V and CSFV Shimen strains were obtained from the China Institute of Veterinary Drug Control, and propagated in MDBK and ST cells, respectively.

### Ginsenosides

Ginsenosides Rb1, Rb2, Rb3, Rc, Re, Rf, Rg1, Rh1, 20(S)-Rh2, pseudo ginsenoside Rh2 (PRh2), pseudo ginsenoside F11 (PF11), and NotoginsenosideR1 (NR1), screened in this study, were obtained from the Jilin University. The purity of these ginsenosides was above 98%, as estimated by high performance liquid chromatography. Stock solutions (40 mg/mL) of the compounds were dissolved in dimethyl sulfoxide (DMSO) or ethanol and were subsequently diluted in culture medium. As a negative control, DMSO was also added to all no-drug control samples.

### Cytotoxicity Assays

Cytotoxicity assays were carried out as described previously (19). In short, MDBK cells, exponentially grown in the 96-well plates (approximately $5 \times 10^3$ cells/well), were incubated in 5% $CO_2$ at 37°C for 24 h. Then, the medium was removed and serial dilutions of compounds were added. DMSO was used as a negative control. The cells were proliferated at 37°C for 3 days, and their overall metabolic activity was determined by the method of MTS/PMS (Promega, USA). The 50% cytotoxic concentration ($CC_{50}$) was calculated using a non-linear regression fitting of the data as the compound concentration necessary to reduce 50% cell viability compared to control non-treated cells.

### Antiviral Screening Using CPE Inhibition Assay

The activity of compounds against BVDV was achieved by inhibiting the cpBVDV C24V strain induced cytopathogenicity in MDBK cells. Cells were seeded in 96-well plates and incubated overnight in growth medium in 5% $CO_2$ at 37°C. Then, the cells were infected with virus at $10^3$ $TCID_{50}$/mL, using a multiplicity of infection (MOI) of 0.05. After 2 h, the virus was removed and maintenance medium (DMEM supplemented with 2% FBS) with or without serial dilutions of compounds was added. Concentrations of compounds were from 6.25 to 200 μg/mL. DMSO was used as a control sample. The cells were incubated at 37°C for 3 days. Subsequently, the antiviral activity was evaluated through the viral titration and the cytopathic effect (CPE) inhibition, by MTS/PMS (Promega, USA). The 50% effective concentration ($EC_{50}$) was calculated using a non-linear regression fitting of the data as the compound concentration necessary to reduce 50% cytopathic effect on MDBK cells compared to DMSO treated control cells.

### Antiviral Activity of Ginsenoside Rb2 and Rb3 Against cpBVDV Strain

MDBK cells were seeded in 6-well plates and cultured overnight, then inoculated with cpBVDV strain C24V with 0.05 MOI and Rb2 or Rb3 (200 μg/mL) mixture. Uninfected cells, added with DMSO, were used as controls. The plates were incubated for 72 h at 37°C to be further submitted for BVDV RNA and E2 protein detection by real-time PCR and Western blot.

### Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted, retrotranscribed, and quantified by the real-time PCR. Following manufacturer's instructions, TRIzol reagent was used to extract RNA (Invitrogen, China) and the M-MLV Reverse Transcriptase was used to conduct the reverse transcription reaction (Promega, USA). The specific primer of BVDV, as described previously, was used to conduct the real-time PCR (20). In brief, a 135-bp fragment from

**Abbreviations:** BVDV, bovine viral diarrhea virus; CSFV, classical swine fever virus; HCV, hepatitis C virus; ORF, open reading frame; UTR, untranslated regions; IRES, internal ribosome entry site; FBS, fetal bovine serum; PRh2, pseudo ginsenoside Rh2; PF11, pseudo ginsenoside F11; NR1, notoginsenoside R1; DMSO, dimethyl sulfoxide; CPE, cytopathic effect; EC50, 50% effective concentration.

the 5′UTR of BVDV was amplified: 5′UTR forward, 5′-GGTAGCAACAGTGGTGAGTTC-3′, and 5′UTR reverse, 5′-CTCAGGTTAAGATGTGCTGTG-3′. The qPCR reactions were performed with the GoTaq qPCR master mix (Promega, USA) in the Bio-Rad iQ5 Real-Time PCR System (Bio-Rad Laboratories, USA). A 91-bp fragment of bovine β-actin mRNA in each sample was amplified and used as the endogenous control (BBA forward, 5′-CCCACACGGTGCCCATCTAT-3′, and BBA reverse, 5′-CCACGCTCCGTGAGGATCTTC-3′), in order to normalize the target amplification data (21). The 2–ΔΔCT method was used to perform the relative quantification of RNA of treated cells compared with that of untreated and infected cells. Each sample was tested in triplicate, including the positive and negative controls.

## Western Blot Analysis

Treatment cells were washed using the icy PBS, and total protein samples were extracted using the RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, USA). According to the instructions of the manufacturer, after being centrifuged at 13,000 rpm for 20 min, concentration of proteins in supernatants was detected using a BCA Protein Assay Kit. Proteins were equally separated on 12–15% SDA-PAGE and then moved onto the PVDF membrane (Merck Millipore, USA). After being blocked in 5% skimmed milk for 2 h, the membrane was incubated with the anti-BVDV E2 antibodies (VMRD, USA) at 4°C overnight and washed, and then incubated with the HRP-conjugated goat anti-mouse IgG (Sigma, USA) at room temperature for 1 h. Signals were developed with an ECL Detection Kit (Thermo Fisher Scientific, USA).

## Rb2 and Rb3 Effect on Viral Replication

To expound the stage of viral replication at which ginsenoside Rb2 and Rb3 exerts their activity, three different assays were performed as described previously (22, 23) with some modifications. MDBK cells were treated with Rb2 and Rb3 before virus infection (pre-treatment group), after infection (post-treatment group), or virucidal group (pre-treatment and post-treatment group) (**Figure 6A**). Rb2 and Rb3 were always used at the non-cytotoxic concentration (200 μg/mL) and the cpBVDV strain C24V titration was always used at 0.05 MOI.

The virucidal assay was conducted with equal amounts of BVDV and ginsenoside. The mixtures were incubated in the microcentrifuge tubes at 37°C for 2 h, and then they were added into the MDBK cell monolayers. The pre-treatment and post-treatment groups were conducted in the procedures that MDBK cell monolayers were incubated with ginsenoside or BVDV virus at 37°C for 2 h. After removal of the liquid, the BVDV or ginsenoside was added. Virus and DMSO were also included on each plate as controls. The plates were incubated at 37°C for 72 h, followed by fixation and staining with BVDV specific antibody labeled by FITC (VMRD, USA).

## Time of Addition Effect of Rb2: Effect on Viral RNA Synthesis

MDBK cells were seeded in 12-well plates and cultured overnight, then infected with cpBVDV strain C24V at a MOI of 0.05, then Rb2 (200 μg/mL) was added at different time points after infection and the cells were further incubated to complete 24 h of infection. All samples were processed for viral RNA analysis.

## Rb2 Effect on CSFV

Using the FA-based virus inhibition assay, the antiviral efficacy of Rb2 against CSFV was investigated.

CSFV Shimen strain with 0.05 MOI was separated and added into the ST cell monolayers and allowed to inoculation for 2 h at 37°C. After inoculation, cells were washed with PBS and serial dilutions of ginsenoside Rb2 was added. Concentrations of compounds were from 25 to 200 μg/mL. Uninfected cell and virus treated with DMSO were used as controls. The plates were incubated at the temperature of 37°C for 72 h, cells were fixed with icy stationary liquid (acetone:methanol = 3:1) at 4°C for 20 min. The cells were further incubated with mouse anti-CSFV E2 mAb WH303 at 37°C for 1 h, washed three times with PBS and incubated with FITC-conjugated rabbit anti-mouse IgG (Sigma, USA) at 37°C for 30 min. After three washes, immunofluorescence was observed using a fluorescence microscope.

## Luciferase Reporter Constructs

The BVDV and CSFV IRES were cloned into the firefly luciferase plasmids pGL4.20 (Promega, USA), respectively. The specific primer for CSFV was described previously (24). The BVDV primer sequences were: F: 5′CCGAGCTCGTATA CGAGAATTT GCCTAGGAC-3′; R: 5′ CCC AAGC TTGGCATAAACAGGTTCTTCCACCC3′. Similar 470 bp fragments of the BVDV were amplified and subcloned into the pGL4.20 using restriction enzymes with *Hind*III and *Sac*I. All reconstructed plasmids were sequenced at Invitrogen Co., Ltd.

## DNA Transfection

The 293T cells were grown in 96-well plates until 80% confluence, reconstructed plasmids and Renilla luciferases plasmids pGL4.74 (Promega, USA) were co-transfected with the X-treme GENETM HP DNA transfection reagent (Roche, Philadelphia, PA), using standard operation, respectively. The parental vector pGL4.20 and pGL4.74 co-transfected group served as mock controls. The cells were allowed to inoculate for 4 h at 37°C. After inoculation, cells were washed with PBS, added with the ginsenoside Rb2 (200 μg/mL) and incubated at 37°C for 48 h. The luciferase activity was determined using the dual luciferase reporter assay system (Promega, USA).

## Statistical Analysis

Student's *t*-test was conducted to examine the statistical differences among ginsenoside treatment groups, virus control, DMSO control, and mock groups. Expression of results was in the form of mean ± standard deviation, representing three independent experiments. Statistical analyses were conducted with GraphPad Prism 6 (GraphPad Software, San Diego, CA). In the case of a value of $p < 0.05$, it was regarded statistically significant.

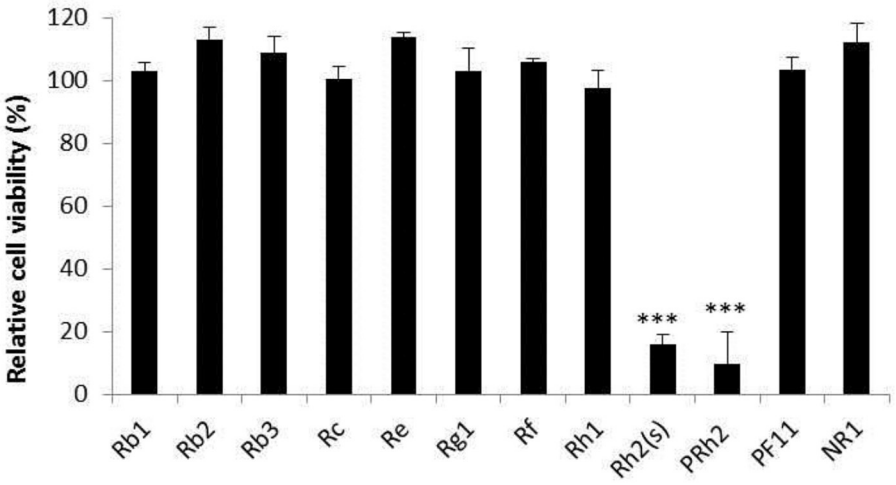



**FIGURE 1 |** Cytotoxicity of compounds. The result of cell viability is expressed as the percentage of control. The data are expressed as the mean of three experiments. ***$p < 0.001$. PRh2, pseudoginsenoside Rh2; PF11, pseudoginsenoside F11; NR1, notoginsenosideR1 (NR1).

# RESULTS

## Cytotoxicity of Compounds

Most compounds had no effect on the cell viability at 200 μg/mL, while ginsenoside 20(S)-Rh2 and pseudo ginsenoside Rh2 reduced the viability of MDBK cells significantly (**Figure 1**). Therefore, ginsenoside 20(S)-Rh2 and pseudo ginsenoside Rh2 were not included in further experiments.

## Cytopathic Effect Inhibition by the Compounds

Concerning the antiviral tests undertaken on the 10 compounds, ginsenoside Rb2 and Rb3 significantly inhibited BVDV induced CPE. CPE inhibition was 94.5 and 91.2% at the 200 μg/mL concentration. All other compounds demonstrated a limited or no inhibitory effect on CPE (**Figure 2**). To further determine the antiviral effect of ginsenoside Rb2 and Rb3, and calculate the $EC_{50}$, different concentrations of Rb2 and Rb3 (6.25–200 μg/mL) were added into the BVDV infected MDBK cells. The viral titer and CPE inhibition were inversely proportional to the ginsenoside Rb2 and Rb3 concentrations (**Figures 3**, **4**). The $EC_{50}$ of Rb2 was 57.76 μg/ mL, that of Rb3 was 60.25 μg/ mL (**Table 1**).

## Antiviral Activity of Ginsenoside Rb2 and Rb3 Against BVDV

BVDV replication was significantly inhibited by Rb2 or Rb3, as shown by reduction of viral RNA levels when compared to DMSO (**Figure 5A**). Similar results were obtained on BVDV E2 protein with Western blot (**Figure 5B**). Compared with the virus control and DMSO treated group, there is almost nothing or slight bands in the Rb2 and Rb3 treated groups.

## Rb2 and Rb3 Effect on Viral Replication

The different assays performed to elucidate the mechanism of action of the ginsenoside Rb2 and Rb3, applied at different periods, showed that Rb2 and Rb3 completely suppressed BVDV infection with virucidal assay and post-treatment of cells. Pre-treatment of cells assay also showed effects on BVDV infection, but the inhibitory effect was weaker than other assays (**Figures 6B,C**).

## Time of Addition Effect of Rb2: Effect on Viral RNA Synthesis

In order to give a better perspective about the time of action of Rb2, time of drug addition experiments were carried out. A concentration of Rb2 of 200 μg/mL was added at different points of time after infection, and then viral replication was evaluated as the yield of viral RNA synthesis. Viral RNA was markedly reduced when Rb2 was added during the first 8 h, after which a gradual reduction in drug inhibition was observed. The antiviral effect of Rb2 can be exerted within in 14 h p.i. (**Figure 7**).

## Rb2 Effect on CSFV

To further understand the ginsenoside Rb2 antiviral activity for other pestiviruses, CSFV Shimen was investigated. Using the FA-based virus inhibition assay, ginsenoside Rb2 demonstrated to have a similar inhibition ability on CSFV to that observed on the BVDV reference strain CV24V (**Figure 8**).

## Effect of Ginsenoside Rb2 on the *Pestivirus* IRES

Direct antiviral activity of ginsenoside Rb2 at the level of *Pestivirus* IRES mediated translation in 293T cell culture was investigated. The translation efficiency in 293T cells was analyzed by a reporter RNA encoding Flufly under the *Pestivirus* IRES control, in the presence or absence of the Rb2. As shown in **Figure 9**, the luciferase activity (Flufly/Renilla) in the presence of Rb2 was significantly reduced than that of the control in absence of Rb2. But the luciferase activity of vector was not changed in the presence or absence of Rb2. These results suggest that

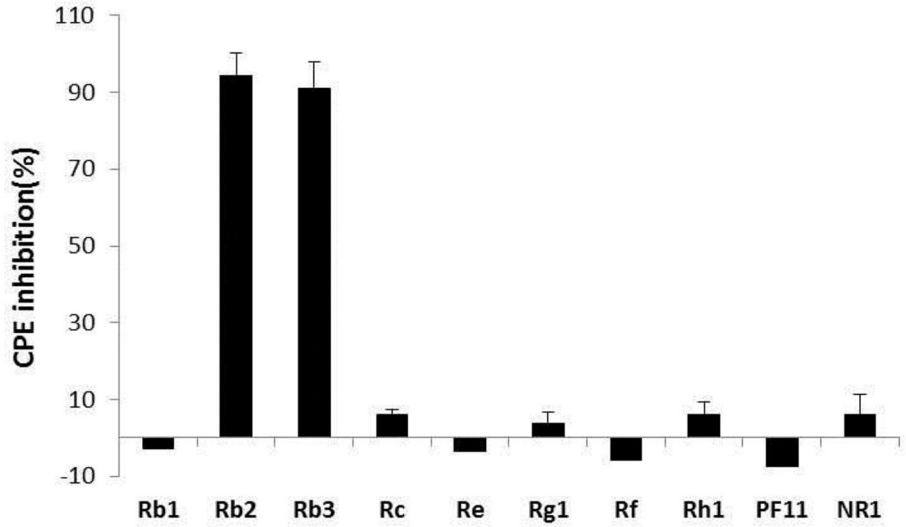



**FIGURE 2 |** Antiviral activity of compounds. All compound concentrations were used with 200 μg/ml, and uninfected and virus infected cells were used as control. The data are expressed as the mean of three experiments.

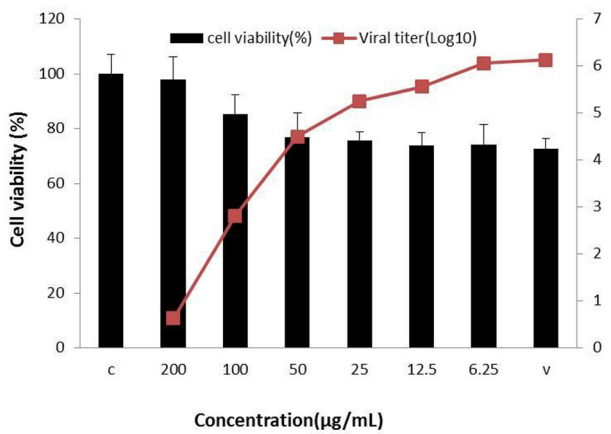



**FIGURE 3 |** Ginsenoside Rb2 antiviral activity against bovine viral diarrhea virus (BVDV). All serial dilutions Rb2 concentrations were used and uninfected and virus infected cells were controls.

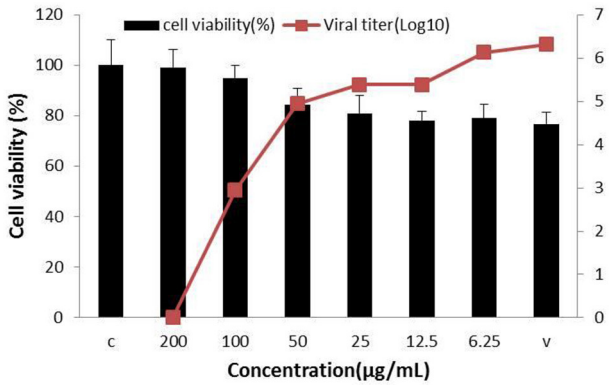


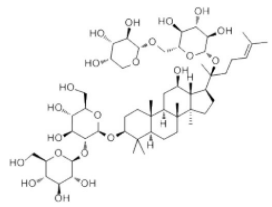
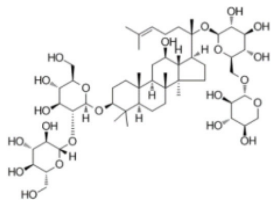

**FIGURE 4 |** Ginsenoside Rb3 antiviral activity against bovine viral diarrhea virus (BVDV). All serial dilutions Rb3 concentrations were used and uninfected and virus infected cells were controls.

ginsenoside Rb2 can inhibit the translation process mediated by the *Pestivirus* IRES.

## DISCUSSION

Ginseng (Panax ginseng) is often used as herbal tonic to increase immunity and reduce stress. These effects are attributed to ginsenosides, major pharmacological components. Although the antiviral activities of ginsenosides have also been reported in several previous studies, the mechanisms underlying the antiviral activity have not yet been elucidated. Several studies about the antiviral mechanism of ginsenosides focused on the immunomodulatory action. The Kang Laboratory (Georgia State University, GA) has published several studies on the immunomodulatory and antiviral effects of KRG extract (RGE) on RSV. Their results indicated that RGE possesses an immunomodulatory effect by balancing Th1 and Th2 immune responses, and protects the host from severe pulmonary inflammation upon FI-RSV immunization and RSV infection (25). The Kang et al. report showed that ginsenoside Rb1 is an immune-stimulatory agent with antiviral activity against enterovirus71. They demonstrated the effective antiviral activities of Rb1 against EV71 *in vitro* and *in vivo*. Furthermore, Rb1 treatment could induce high cellular and humoral immune responses *in vivo*. Meanwhile, Rb1 contributed to the enhanced type I IFN responses and IFN-β knockdown reversed the antiviral activity of Rb1 *in vitro* (26).

**TABLE 1 |** The structure and antiviral activity of Rb2 and Rb3.

| Compound | Structure | MDBK $CC_{50}$ (μg/mL) | BVDV $EC_{50}$ (μg/mL) | SI($CC_{50}/EC_{50}$) |
|---|---|---|---|---|
| Rb2 |  | >200 | 57.76 | >3.46 |
| Rb3 |  | >200 | 60.25 | >3.32 |

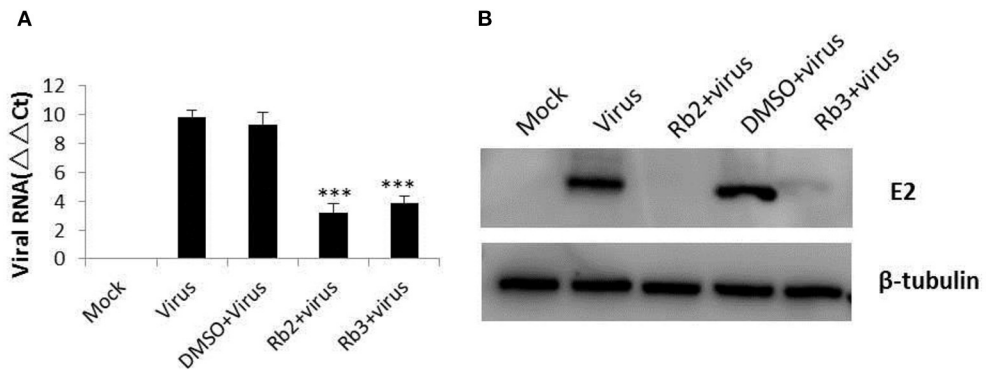



**FIGURE 5 |** Effect of ginsenoside Rb2 and Rb3 on BVDV replication. **(A)** Real-time PCR analysis of the viral RNA. **(B)** Western blot analysis of BVDV E2 protein expression. Uninfected cells and DMSO-treated cells were used as control. ***$p < 0.001$.

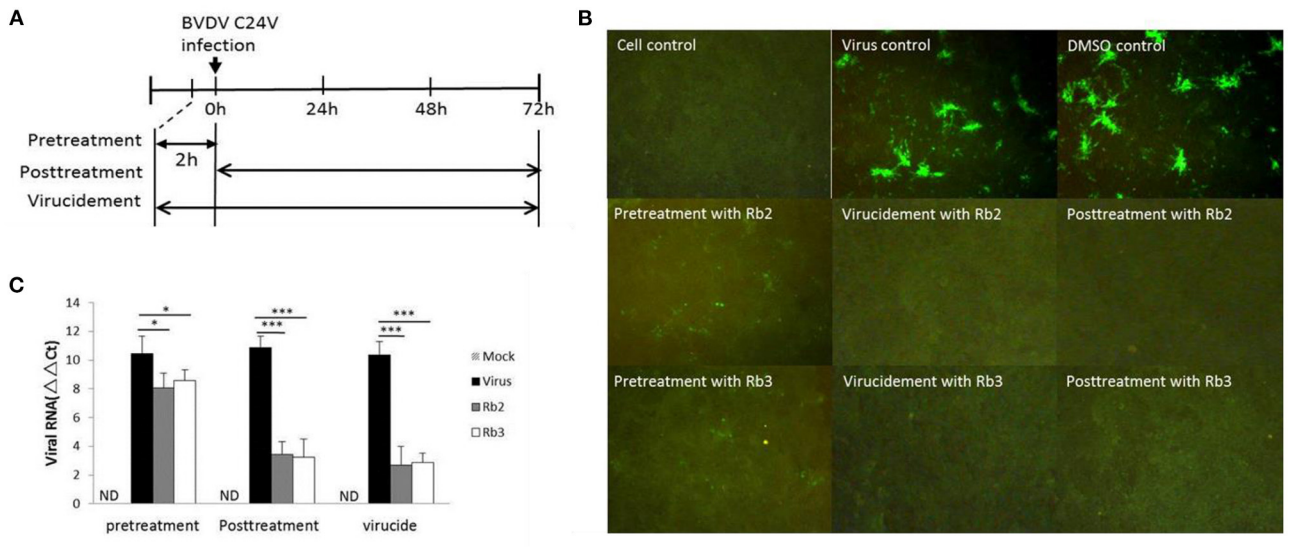



**FIGURE 6 |** Stage of antiviral activity against cpBVDV C24V by ginsenoside Rb2 and Rb3. **(A)** A schematic diagram describing the experimental scheme for measuring the stage at which ginsenoside exerts its effects. **(B)** The FA result of plaque reduction assays. **(C)** Changes in viral RNA level. *$p < 0.05$; ***$p < 0.001$.

The antiviral mechanism of ginsenoside was scarcely studied, and focused on the quantification of inhibitory effect on virus growth, thus indirectly estimating the inhibition of the virus translation process. To investigate if ginsenosides have the same inhibitory effect on virus translation as other synthetic antiviral drugs, we studied the antiviral effect of Rb2 on *Pestivirus* IRES. It is known that the 5′ UTR of *Pestivirus* and HCV RNA genome used for IRES mediated translation, many drugs and antibodies were designed and synthesized according to it (27, 28). The effect of Rb2 on the *Pestivirus* IRES was evaluated through luciferase reporter assay. In the presence of Rb2, the luciferase activity was significantly reduced than that of the control in the absence of Rb2. Compared to CSFV IRES, the luciferase activity in BVDV IRES was lower because the luciferase reporter plasmid of BVDV IRES deleted the 5′ boundary, reducing the IRES function (29). But it did not affect the inhibitory effect of Ginsenoside Rb2. These results suggest that ginsenoside Rb2 could inhibit the translation process mediated by the *Pestivirus* IRES. However, further research is needed to be carried out.

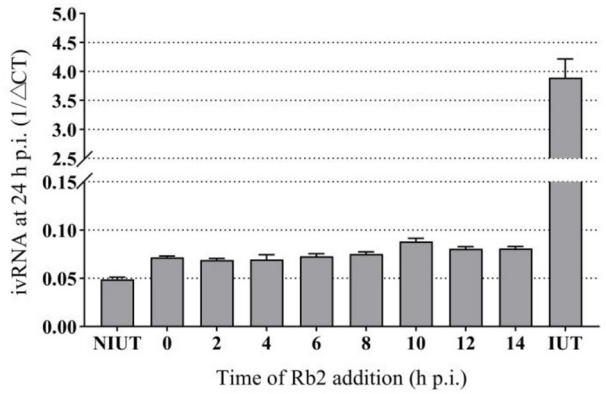



**FIGURE 7 |** Effect of Rb2 addition on viral RNA synthesis.

The interest of the observations reported in this study is not necessarily directly related to the control or the prevention of the diseases caused in animals by the pathogens used as model (CSFV and BVDV). While for pestiviruses, prophylactic means are available, no curative compounds can be applied to infected animals. Nevertheless, the current strategy is focused on the eradication of these pathogens to progressively gain an officially free status. In addition, for BVDV, measures rely on the identification and elimination of persistently infected

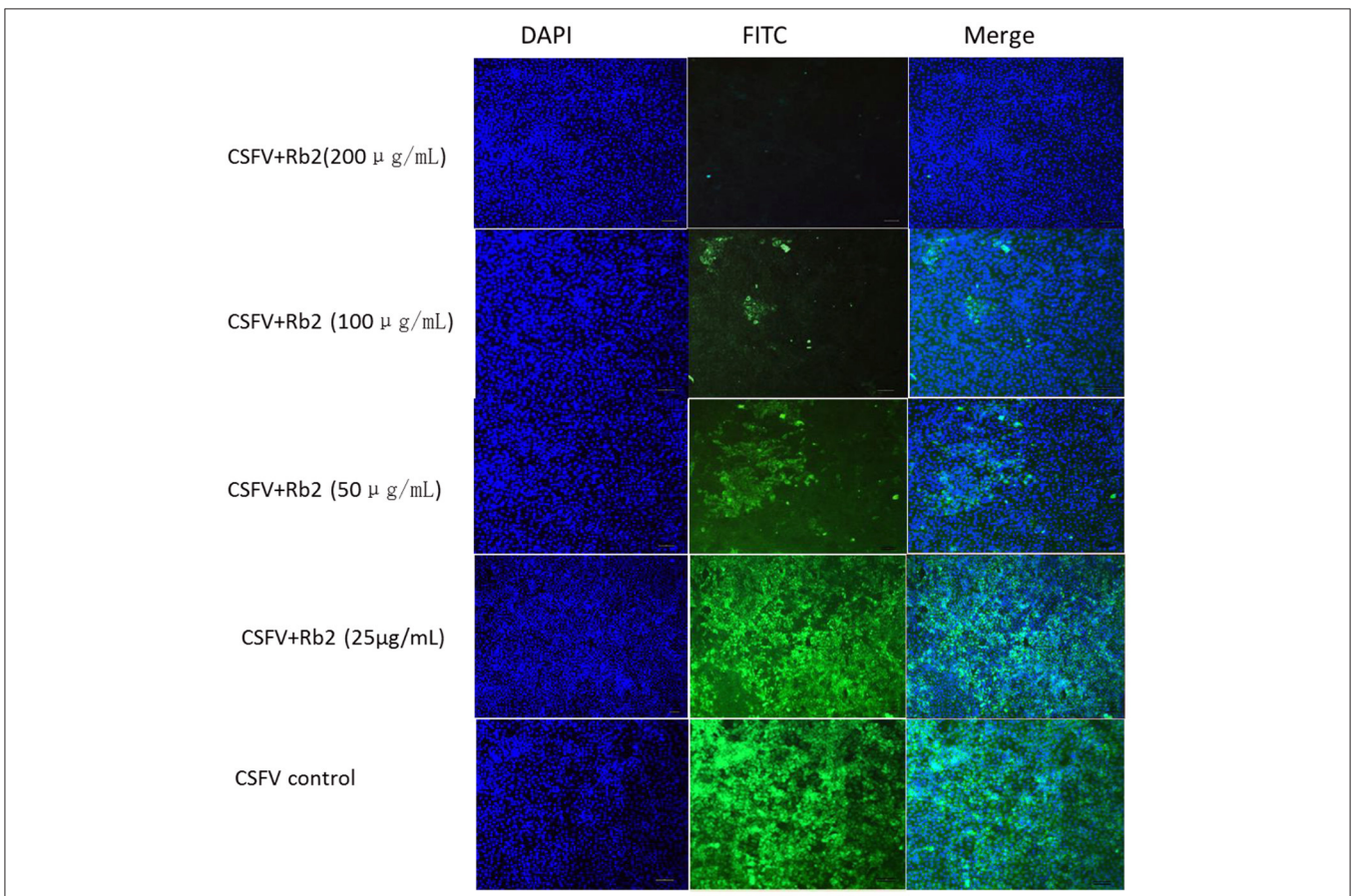



**FIGURE 8 |** The effect of Rb2 on CSFV Shimen with IFA.

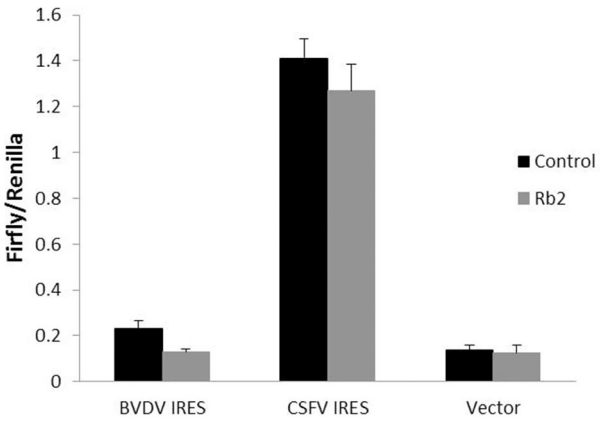



**FIGURE 9 |** The effect of Rb2 on the activity of IRES of BVDV and CSFV with dual-Luciferase assay. $^{**}p < 0.01$.

immunotolerant animals, and for CSFV, when the disease is eradicated in a country or zone, stamping out policy is strictly applied.

Bovine-derived biological materials are important to medicine and other biological applications. But contamination is frequently reported with BVD-associated viruses. Contamination of FBS leads to contamination of biological products that use FBS for their production, including cell cultures and vaccines. BVDV contamination can affect the outcome of cell-culture-based research and diagnostic procedures, resulting in misinterpretation of research data or an incorrect diagnosis. Vaccine contamination may not only influence the results of vaccination but may also lead to new infections, causing serious BVD outbreaks (30). With concern to biological product contamination by BVDV, antiviral properties of ginsenosides might be taken into account with a possible use in the framework of laboratory testing and biological products manufacturing activities to counter occurrence of adventitious pestiviruses. However, the problem will be resolved through the application of stringent laboratory safety practices and the compulsory use of biologicals such as bovine fetal serum derived only from officially BVDV-free animals, guaranteed by relevant health competent authorities, and not based on claims from manufacturers. Official free status is contemplated by the recent EU regulations 429/2016 and 689/2020. Possibly, in veterinary medicine, the use of antivirals for pestiviruses might be restricted to highly valuable animals, in order to reduce the impact of the infection on their health and welfare.

HCV is the main causative agent of chronic hepatitis in humans, responsible for more than 185 million infections worldwide. Not only are vaccines absent, but the treatment of hepatitis C has improved only over the past decade. Initially, with interferons and ribavirin, cure rates did not exceed 60% (31, 32), and almost all of them had side effects so severe that some people abandoned the treatment. Highly effective, well-tolerated therapies are now available. However, the therapeutic constraint is represented by very high costs (up to about 97,000 USD for a 12-week treatment). BVDV is genetically similar to HCV in structure and, in general, both of them can produce chronic infections in their hosts, respectively. Due to the limitations in the discovery and development of HCV drugs, compounds with the antiviral activity against BVDV are regarded as a surrogate model system for efforts to discover drugs effective against HCV (33). Therefore, the search for antiviral properties of ginseng using animal models may offer alternatives for the cure and possibly also the prevention of the disease in humans.

In this study, we demonstrated that ginsenoside Rb2 and Rb3 have antiviral effects on inhibition BVDV replicated in MDBK cell. Furthermore, ginsenoside Rb2 and Rb3 showed antiviral potency, thus, representing potentially useful antiviral compounds for controlling contamination in biological products.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

BT, NS, YJ, KL, and QW participated in conducting experiments and analysis of biophysical data. MG participated in editing the manuscript. SC and YW participated in the design of the study. SZ proposed the original idea, designed and supervised the experiments, and edited the manuscript. All authors read and approved the final manuscript.

## FUNDING

This work is funded by National Natural Science Foundation of China (31602093), National Public Welfare of China for Agriculture Special Purpose (CAAS-ASTIP-ISAPS-2021038), and the basic scientific research funding of the Chinese Academy of Agricultural Sciences (0032014016).

## REFERENCES

1. Newcomer BW, Givens MD. Givens, Approved and experimental countermeasures against pestiviral diseases: bovine viral diarrhea, classical swine fever and border disease. *Antiviral Res.* (2013) 100:133–50. doi: 10.1016/j.antiviral.2013.07.015
2. Callens N, Brügger B, Bonnafous P, Drobecq H, Gerl MJ, Krey T. Morphology and molecular composition of purified bovine viral diarrhea virus envelope. *PLoS Pathog.* (2016) 12:e1005476. doi: 10.1371/journal.ppat.1005476
3. Friis MB, Rasmussen TB, Belsham GJ. Modulation of translation initiation efficiency in classical swine fever virus. *J Virol.* (2012) 86:8681–92. doi: 10.1128/JVI.00346-12

4. Tong W, Zheng H, Li GX, Gao F, Shan TL, Zhou YJ, et al. Recombinant pseudorabies virus expressing E2 of classical swine fever virus (CSFV) protects against both virulent pseudorabies virus and CSFV. *Antiviral Res.* (2020) 173:104652. doi: 10.1016/j.antiviral.2019.104652
5. Evans CA, Pinior B, Larska M, Graham D, Schweizer M, Guidarini C, et al. Global knowledge gaps in the prevention and control of bovine viral diarrhea (BVD) virus. *Transbound Emerg Dis.* (2019) 66:640–52. doi: 10.1111/tbed.13068
6. Małaczewska J, Kaczorek-Łukowska E, Wójcik R, Siwicki AK. Antiviral effects of nisin, lysozyme, lactoferrin and their mixtures against bovine viral diarrhoea virus. *BMC Vet Res.* (2019) 15:318. doi: 10.1186/s12917-019-2067-6
7. Newcomer BW, Neill JD, Marley MS, Ridpath JF, Givens MD. Givens. mutations induced in the NS5B gene of bovine viral diarrhea virus by antiviral treatment convey resistance to the compound. *Virus Res.* (2013) 174:95–100. doi: 10.1016/j.virusres.2013.03.007
8. Giangaspero M, Vacirca G, Harasawa R, Büttner M, Panuccio A, De Giuli Morghen C, et al. Genotypes of pestivirus RNA detected in live virus vaccines for human use. *J Vet Med Sci.* (2001)63:723-733. doi: 10.1292/jvms.63.723
9. Pecora A, Aguirreburualde SP, Ridpath JH, Santos MJ. Molecular characterization of pestiviruses in fetal bovine sera originating from argentina: evidence of circulation of HoBi-like viruses. *Front Vet Sci.* (2019) 6:359. doi: 10.3389/fvets.2019.00359
10. Jung K, An JM, Eom DW, Kang KS, Kim SN. Preventive effect of fermented black ginseng against cisplatin-induced nephrotoxicity in rats. *J Ginseng Res.* (2017) 41:188–94. doi: 10.1016/j.jgr.2016.03.001
11. Hong Y, Lin Y, Yang L, Dong W, Gu X, Ginsenoside Rb2 alleviates obesity by activation of brown fat and induction of browning of white fat. *Front Endocrinol.* (2019) 10:153. doi: 10.3389/fendo.2019.00153
12. Kang S, Im K, Kim G, Min H. Antiviral activity of 20(R)-ginsenoside Rh2 against murine gammaherpesvirus. *J Ginseng Res.* (2017) 41:496–502. doi: 10.1016/j.jgr.2016.08.010
13. Wang X, Wang Y, Ren Z, Qian C, Li Y, Wang Q, et al. Protective effects of 20(s)-protopanaxtriol on viral myocarditis infected by coxsackievirus B3. *Pathobiology*. (2012) 79:285–9. doi: 10.1159/000331229
14. Lee MH, Lee BH, Jung JY, Cheon DS, Kim KT, Choi C. Antiviral effect of Korean red ginseng extract and ginsenosides on murine norovirus and feline calicivirus as surrogates for human norovirus. *J Ginseng Res.* (2011) 35:29–35. doi: 10.5142/jgr.2011.35.4.429
15. Kim S, Jang JY, Kim E, Cho EY, Ahn D, Kim C, et al. Ginsenoside Rg3 restores hepatitis C virus-induced aberrant mitochondrial dynamics and inhibits virus propagation. *Hepatology.* (2017) 66:758–71. doi: 10.1002/hep.29177
16. Yang H, Oh K, Kim H, Cho Y, Yoo Y. Ginsenoside-Rb2 and 20(S)-Ginsenoside-Rg3 from Korean red ginseng prevent rotavirus infection in newborn mice. *J Microbiol Biotecnol.* (2018) 28:391–6. doi: 10.4014/jmb.1801.01006
17. Yoo Y, Lee J, Park S, Nam K, Cho Y, Choi J. Protective effect of ginsenoside-Rb2 from Korean red ginseng on the lethal infection of haemagglutinating virus of Japan in mice. *J Ginseng Res.* (2013) 37:80–86. doi: 10.5142/jgr.2013.37.80
18. Ridpath JF, Bolin SR. Differentiation of types 1a, 1b and 2 bovine viral diarrhoea virus (BVDV) by PCR. *Mol Cell Prob.* (1998) 12:101–6. doi: 10.1006/mcpr.1998.0158
19. Musiu S, Pürstinger G, Stallinger S, Vrancken R, Haegeman A, Koenen F, et al. Substituted 2,6-bis(benzimidazol-2-yl)pyridines: a novel chemical class of pestivirus inhibitors that targets a hot spot for inhibition of pestivirus replication in the RNA-dependent RNA polymerase. *Antiviral Res.* (2014) 106:71–9. doi: 10.1016/j.antiviral.2014.03.010
20. Zhang SQ, Tan B, Li P, Wang FX, Guo L, Yang Y, et al. Comparison of conventional RT-PCR, reverse-transcription loop-mediated isothermal amplification, and SYBR green I-based real-time RT-PCR in the rapid detection of bovine viral diarrhea virus nucleotide in contaminated commercial bovine sera batches. *J Virol Methods.* (2014) 207:204–9. doi: 10.1016/j.jviromet.2014.05.020
21. Castro EF, Fabian LE, Caputto ME, Gagey D, Finkielsztein LM, Moltrasio GY, et al. Inhibition of bovine viral diarrhea virus RNA synthesis by thiosemicarbazone derived from 5,6-dimethoxy-1-indanone. *J Virol.* (2011) 85:5436–45. doi: 10.1128/JVI.00859-10
22. Kubica TF, Alves SH, Weiblen R, Lovato LT. *In vitro* inhibition of the bovine viral diarrhea virus by the essential oil of *Ocimum basilicum* and momoterpenes. *Braz J Micro.* (2014) 45:209–14. doi: 10.1590/S1517-83822014005000030
23. Astani A, Reichling J, Schnitzler P. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. *Phytother Res.* (2010) 24:673–79. doi: 10.1002/ptr.2955
24. Luo QH, Jia JJ, Zhang BK Mi SJ, Zhang L, Xie JX. Liu, Y, Tu CC. Effects of the Nproprotein of classical swine fever virus and cellular stresses on the CSFV internal ribosome entrysite-dependent translation. *Chinese J Pre Vet Med.* (2016) 38:844–8. doi: 10.3969/j.issn.1008-0425
25. Im K, Kim J, Min H. Ginseng. The natural effectual antiviral: protective effects of Korean Red Ginseng against viral infection. *J Ginseng Res.* (2016) 40:309–14. doi: 10.1016/j.jgr.2015.09.002
26. Kang N, Gao H, He L, Liu Y, Fan H, Xu Q, et al. Ginsenoside Rb1 is an immune-stimulatory agent with antiviral activity against enterovirus 71. *J Ethnopharmacol.* (2021) 266:113401. doi: 10.1016/j.jep.2020.113401
27. Panigrahi R, Hazari S, Chandra S, Chandra PK, Datta S, Kurt R, et al. Interferon and ribavirin combination treatment synergistically inhibit HCV internal ribosome entry site mediated translation at the level of polyribosome formation. *PLoS ONE.* (2013) 8: e72791. doi: 10.1371/journal.pone.0072791
28. Deepak K, Lewicka A, Koldobskaya Y, Huang H, Piccirilli J A. Synthetic antibody binding to a preorganized RNA domain of hepatitis C virus internal ribosome entry site inhibits translation. *ACS Chem Biol.* (2020) 15:205–216. doi: 10.1021/acschembio.9b00785
29. Seung K, Daniel R, Ruben O. Genetic analysis of the internal ribosome entry segment of bovine viral diarrhea virus. *Virology.* (1998) 251:370–82. doi: 10.1006/viro.1998.9425
30. Gómez-Romero N, Velazquez-Salinas L, Ridpath J, Verdugo-Rodríguez A, Basurto-Alcántara F. Detection and genotyping of bovine viral diarrhea virus found contaminating commercial veterinary vaccines, cell lines, and fetal bovine serum lots originating in Mexico. *Arch Virol.* (2021) 166:1999–2003. doi: 10.1007/s00705-021-05089-9
31. Kohli A, Shaffer A, Sherman A, Kottilil S. Treatment of hepatitis C: a systematic review. *JAMA.* (2014) 312:631–40. doi: 10.1001/jama.2014.7085
32. Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut.* (2006) 55:1350–9. doi: 10.1136/gut.2005.076646
33. Buckwold VE, Beer BE, Donis RO. Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. *Antiviral Res.* (2013) 60:1–15. doi: 10.1016/S0166-3542(03)00174-8


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 14 December 2021
doi: 10.3389/fvets.2021.763972

# Use of Thoracic Ultrasonography to Improve Disease Detection in Experimental BRD Infection

*Madison M. Porter, Paiton O. McDonald, Jamison R. Slate, Amanda J. Kreuder and Jodi L. McGill**

*Department of Veterinary Microbiology and Preventative Medicine, Iowa State University, Ames, IA, United States*

Bovine respiratory disease (BRD) is caused by complex interactions between viral and bacterial pathogens, host immune status, and environmental stressors. In both clinical and research settings, current methods for detecting BRD in calves commonly focus on visual indicators such as attitude, nasal discharge, and cough, in addition to vital signs such as rectal temperature and respiration rate. Recently, thoracic ultrasonography (TUS) has become more commonly used in clinical settings, in addition to physical examination to diagnose BRD. To assess the value of performing TUS during experimental BRD infection, 32 calves were challenged with bovine respiratory syncytial virus, to mimic a viral infection, and 30 calves were infected with *Mannheimia haemolytica*, to mimic a bacterial infection. TUS was performed at regular intervals using a standardized method and scoring system in addition to daily clinical scoring. Although overall correlations between clinical scores and TUS scores were generally weak (maximum $R^2 = 0.3212$), TUS identified calves with abnormal lung pathology that would have otherwise been misclassified on the basis of clinical scoring alone, both on arrival and throughout the studies. In addition, TUS had an increased correlation with gross lung pathology on necropsy (maximum $R^2 = 0.5903$), as compared to clinical scoring (maximum $R^2 = 0.3352$). Our results suggest that TUS can provide additional information on calf health at enrollment and throughout a study and may provide an alternative to terminal studies, due to the high correlation with lung pathology at necropsy.

**Keywords: bovine respiratory disease, thoracic ultrasonography, calves, clinical scoring, pneumonia**

OPEN ACCESS

***Edited by:***
*Barbara Padalino, University of Bologna, Italy*

***Reviewed by:***
*Tara G. McDaneld, U.S. Meat Animal Research Center, United States*
*Laurel J. Gershwin, University of California, Davis, United States*
*Philip John Griebel, University of Saskatchewan, Canada*

****Correspondence:***
*Jodi L. McGill*
*jlmcgill@iastate.edu*

***Specialty section:***
*This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science*

***Received:*** *16 September 2021*
***Accepted:*** *04 November 2021*
***Published:*** *14 December 2021*

***Citation:***
*Porter MM, McDonald PO, Slate JR, Kreuder AJ and McGill JL (2021) Use of Thoracic Ultrasonography to Improve Disease Detection in Experimental BRD Infection. Front. Vet. Sci. 8:763972. doi: 10.3389/fvets.2021.763972*

## INTRODUCTION

Bovine respiratory disease (BRD) is a leading cause of morbidity and mortality among pre-weaned dairy calves and is a major health concern among feedlot cattle (1, 2). In addition to the cost of treatment, BRD causes economic losses through reduced growth, reduced lifetime milk production, and increased mortality (3, 4). BRD results from a complex interaction between viral and bacterial pathogens, host immune status, and environmental stressors that commonly presents as a primary viral infection followed by a secondary bacterial infection. Early identification is crucial to successful treatment but difficult with current approaches to disease detection. Current detection methods primarily focus on clinical scoring, which relies on indicators such as rectal temperature, difficulty breathing, increased respiration, ear position, and nasal discharge (5). However, these indicators have a low sensitivity and specificity for lesions within the lung (6). In addition, many of

these clinical signs such as temperature and depression can be caused by environmental conditions and are nonspecific disease indicators (7). Some clinical scoring methods also fail to identify calves with subclinical pneumonia if they rely on external, visual indicators associated with more obvious clinical disease. Lung auscultation performed by an experienced veterinarian can improve the accuracy of clinical scoring models; however, studies have suggested that this method may lack sensitivity for diagnosing BRD (6), and it is difficult to assign a quantitative score. In the laboratory, research of potential BRD interventions is often performed with controlled infections using clinical scoring as the main method of determining the severity of disease progression. With the current detection methods, many markers of disease severity in these intervention studies can only be observed post-mortem, limiting the ability to analyze disease progression longitudinally.

Thoracic ultrasonography (TUS) has been identified as a rapid, on-farm, validated predictor of lung lesions in pre-weaned dairy calves (8). TUS can identify calves with both clinical and subclinical pneumonia with increased sensitivity and specificity (6, 8). Limited research has been done on the detection of subclinical pneumonia, but calves that suffer from BRD early in life are known to have lower production later in life, such as decreased average daily gain, decreased carcass value, and decreased survival of heifers to first calving (2, 4). These findings suggest that the effects of lung damage persist even after clinical signs return to normal (9). Many studies of TUS and its ability to accurately detect disease within the lungs have taken place in field experiments where the exact infectious agent is unknown. The BRD monitored in these studies is likely complex with coinfections by multiple bacterial or viral agents. Thus, in uncontrolled field settings, there is little opportunity to assess the differences in changes in lung appearance as observed by TUS between bacterial or viral pneumonia and the different ways that these diseases progress within the lung.

The objective of this study was to compare TUS with clinical scoring, gross pathology, and pathogen burden in controlled infection of bovine respiratory syncytial virus (BRSV), to represent the primary viral infection, or *Mannheimia haemolytica*, to represent the secondary bacterial infection, in pre-weaned dairy calves. Our secondary objective was to track the progression of lung lesion development as observed by TUS throughout a controlled infection trial.

# MATERIALS AND METHODS

## Study Population

This study includes calves from three different trial populations. All animal procedures were conducted in strict accordance with federal and institutional guidelines and were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC protocols 18-058 and 19-081). Trial 1 consisted of 32 4-week-old, mixed gender Holstein calves that were challenged with aerosolized BRSV. Calves were colostrum-replete with BRSV titers ranging from 32 to 256 (mean 116). Calves were confirmed negative for BVDV before enrollment. Trial 2, also referred to as the Fall 2019 cohort, consisted of 16 4-week-old, mixed gender Holstein calves that were challenged with *Mannheimia haemolytica*. Trial 3, also referred to as the Summer 2020 cohort, consisted of 14 4-week-old, mixed gender Holstein calves that were challenged with *Mannheimia haemolytica*. Antibody titers to *M. haemolytica* ranged from 32 to 128 and did not differ between trials. The animals from all three trials were purchased from the same, single source. The animals were transported approximately 3 h from a farm in Eastern Iowa. Calves were housed individually at the farm but were comingled and house in groups of n = 4 upon arrival at the research facility. Calves were confirmed negative for BVDV before enrollment. Animals from all three trials were housed under AgBSL2 conditions at the Iowa State University Livestock Infectious Disease Isolation Facility. Calves were allowed to acclimate for 4 days before beginning the studies. Animals were fed milk replacer twice daily and had *ad libitum* access to food and water.

## BRSV and *M. haemolytica* Challenge

BRSV inoculum was prepared and administered as previously described (10). Briefly, BRSV strain 375 was prepared from virus stock re-isolated from the lung of an infected animal and passaged on bovine turbinate cells less than four times. The inoculum was determined to be uncontaminated with BVDV by PCR. Calves received the inoculum *via* an aerosolized challenge with $\sim 10^4$ $TCID_{50}$/ml of BRSV strain 375.

*Mannheimia haemolytica* was prepared and administered as previously described (11). Briefly, *M. haemolytica* strains NADC-D153 and NADC-D174 were grown to log phase in Columbia broth for approximately 2.5 h. Growth was then diluted 50-fold for challenge. Calves were challenged intratracheally with 25 ml of *M. haemolytica* in Earle's Balanced Salt Solution (EBSS) at $2 \times 10^7$ colony-forming units (CFU)/ml. All preparations were kept on ice before inoculation.

## Clinical Scoring

Calves were scored for clinical illness daily using a modified University of Wisconsin calf health respiratory scoring chart (5). The original scoring chart assigns a number (0–3) to each calf for various clinical signs including fever, ear position, eye discharge, nasal discharge, and cough. Additional scoring categories that were added to the chart include expiration effort (0–3) and lung auscultation (0 = abnormal lungs sound absent, 1 = abnormal lung sounds present) (6). The scores for each day are then totaled, and a total score of $\geq$5 was used to indicate clinical disease. Clinical scoring was performed by one, blinded individual for the duration of the study. The humane endpoint defined by our IACUC protocol included prolonged increases in body temperature (>40.5° C for more than 48 h), inappetence for more than two feeding periods, and respiratory effort scores of 3 for more than 48 h.

## Thoracic Ultrasonography

TUS was performed on days 0, 3, 7, 10, and 14 with the BRSV-challenged calves and days 0, 1, 2, 3, and 4 with the *M. haemolytica*–challenged calves. TUS was performed with an IBEX® EVO® (E.I. Medical Imaging, Loveland, CO) using the

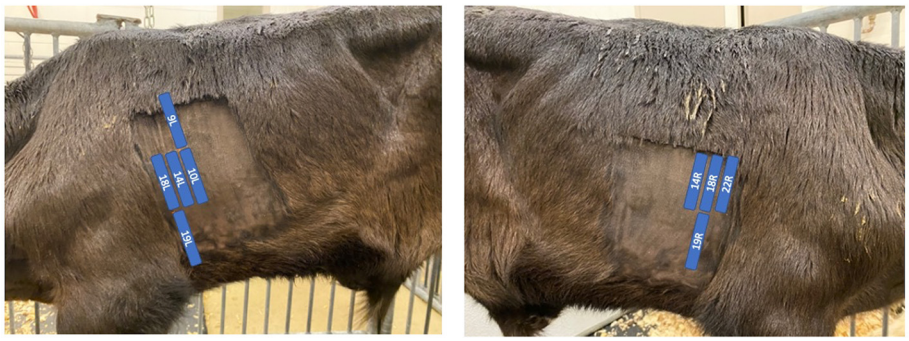



**FIGURE 1 |** Position of ultrasound probe and description of locations. The labeled blue locations in **(A)** and **(B)** indicate the positioning of the ultrasound probe within each ICS on the left and right sides of the calf, respectively. Location labels are derived from Rademacher et al. (12).

**TABLE 1 |** Anatomical landmarks associated with each ultrasound location.

| Location name | Anatomical landmarks |
|---|---|
| 19L | ICS immediately caudal to shoulder muscle, bottom of probe even with point of elbow |
| 18L | One probe length dorsal to 19L, middle of probe even with point of shoulder |
| 14L | One ICS caudal from 18L, middle of probe even with point of shoulder |
| 10L | One ICS caudal from 14L, middle of probe even with point of shoulder |
| 9L | One probe length dorsal to 10L, bottom of probe even with point of shoulder |
| 22R | ICS immediately caudal to shoulder muscle, middle of probe even with point of shoulder |
| 18R | One ICS caudal from 22R, middle of probe even with point of shoulder |
| 19R | One probe length ventral to 18R, bottom of probe even with point of elbow |
| 14R | One ICS caudal to 18R, middle of probe even with point of shoulder |

*L in the location name denotes the location was on the left side of the calf and R in the location name denotes a location on the right side of the calf. Ultrasounds were always taken in the order listed.*

L7HD linear transducer probe (5–9 MHz) set to a depth of 8.7 cm for all scans with 70% isopropyl alcohol applied to the areas of interest. The thoraxes of all calves were clipped within 1 week before the start of challenge to improve image quality. Nine locations on the calf were identified for ultrasonography with five locations on the left side of the calf and four locations on the right side of the calf. Ultrasound locations were based on previously described locations of interest (12) and are shown in **Figures 1A,B**. Locations were observed in the same order each time beginning with the most cranial and ventral location on the left side and moving caudally and dorsally on the left side and then repeated on the right side. The first ultrasound location on the left side of the calf was identified by placing the probe so that the bottom of the probe was even with the point of the elbow and in the intercostal space (ICS) immediately caudal to the shoulder muscle. The remaining four locations on the left side of the calf were placed, as described in **Table 1**. On the right side of the calf, the first ultrasound location was found by again placing the probe in the ICS that was immediately caudal to the shoulder muscle with the first location on the right side even with the point of the shoulder. The remaining three locations on the right side of the calf were placed, as described in **Table 1**. At each location, a four second clip was recorded and stored for later image review. Images were captured by study personnel without previous ultrasound experience (MP and PM) but who were trained on ultrasound image collection by an experienced ultrasonographer [AK; diplomate, American College of Veterinary Internal Medicine (Large Animal)] at the beginning of trial 1.

## Image Review

Images were scored by a reviewer without previous ultrasound experience (MP) who was trained on ultrasound interpretation by an experienced ultrasonographer (AK). Initial training consisted of review of images concurrently for identification of lung consolidation, pleural defects including comet tail artifacts and B-lines, pleural irregularity, pulmonary abscessation, and pleural fluid presence. A subset of images was then scored independently by the trained ultrasonographer and the trainee, and results were compared and confirmed to be consistent between reviewers. All ultrasound scoring utilized in this study was thereafter performed by the trainee; the final review of any substantially discordant results between necropsy scoring and TUS was also performed on as needed basis by the experienced ultrasonographer. Ultrasound clips for each location were scored for presence of pleural defects and depth of consolidation. Pleural defects were assigned a score of 0–3, 0 = no comet tails/B-lines observed; 1 = one to two comet tails/B-lines observed; 2 = three to five comet tails/B-lines observed; 3 = six and above comet tails/B-lines observed. Depth of consolidation was also assigned a score of 0–3 based on the maximum depth of consolidation seen at each location: 0 = no consolidation, 1 = <2 cm of consolidation, 2 = >2 cm but <4 cm of consolidation, and 3 = >4 cm of consolidation. Once all nine locations for a day were scored, the scores for presence of pleural defects were combined

for a final pleural defect score and the maximum consolidation depth seen in any of the nine locations on each day was assigned as the final depth of consolidation score. Calves were assigned a final ultrasound score for the day on a scale of 0–4: 0 = no consolidation present, final pleural defects score of <5; 1 = no consolidation present, final pleural defects score >5; 2 = final depth of consolidation score of 1; 3 = final depth of consolidation score of 2; 4 = final depth of consolidation score of 3.

## Necropsy

BRSV-challenged calves were humanely euthanized on day 14 after infection or as clinical symptoms deemed necessary. *M. haemolytica*–challenged calves were euthanized on day 4 after infection or as clinical symptoms deemed necessary. All were euthanized by barbiturate overdose using sodium pentobarbital administered at 100 mg/kg. On necropsy, the lungs were removed, and 11 lobes (left cranial, right cranial, middle, upper right caudal, middle right caudal, lower right caudal, lower left caudal, middle left caudal, upper left caudal, accessory, and left cranial caudal lobes), as shown in **Supplementary Figure 1**, were assessed for the presence of lesioned lung on a percent basis. Lung tissue was assessed for any visual changes to the surface of the lung and palpated for any changes to lung texture. Necropsy data were not included from BRSV-challenged calves that were euthanized before day 14. To identify the lung lobes that were captured on ultrasound, the calf was placed on its side, the ultrasound locations were identified, as shown in **Supplementary Figures 2A,B**, and Trypan blue dye was injected into the lung tissue. Once the lungs were removed from the calf, the blue injection sites were identified and marked, as shown in **Supplementary Figures 2C,D**. Five lobes of interest were identified: left cranial, left cranial caudal, upper left caudal, right cranial, and middle.

## Real-Time PCR and Quantitative Culture

Viral load was determined in lung tissue and nasal swab samples by quantification of BRSV NS2 copy number, as previously described (13).

Briefly, randomly selected samples, ∼0.5 g each, lesioned and non-lesioned lung tissues were collected from two separate lung lobes each calf and stored in RNAlater (ThermoFisher). RNA was then isolated and pooled from the separate locations (lesioned samples pooled together, non-lesioned lung tissues pooled together) using Trizol Reagent (Life Technologies) and then cleaned up using a Qiagen RNeasy isolation column, as described (14). Nasal swabs samples were collected from the upper nasal cavity of BRSV infected calves on days 0, 3, 7, 10, and 14 after infection. Viral RNA was isolated from nasal swab samples using the MagMax Viral Isolation Kit (Applied Biosystems, Life Technologies). cDNA synthesis and quantitative rtPCR reactions were carried out with the TaqMan RNA-to-CT 1-step-kit (Applied Biosystems) according to the instructions of the manufacturer. Primers and probes for the BRSV NS2 gene and the bovine RPS9 gene have been published (13). Reactions were performed using a ThermoFisher QuantStudio 3 Real-Time PCR machine under previously described cycling conditions (10). Standard curves for NS2 and RPS9 genes were run in parallel with test samples, and all were run in duplicate. Viral NS2 copy numbers were calculated using standard curves and normalized to RPS9 to account for differences in input materials.

Quantitative culture for *M. haemolytica* was performed, as previously described (11). Briefly, lung tissue samples were ground in EBSS to produce a homogenized suspension that was then diluted 10-fold in EBSS. The dilutions were then spread on blood agar base plates containing 5% defibrinated bovine blood and incubated overnight at 37°C. Colonies with typical *M. haemolytica* morphology were enumerated, and representative colonies were selected for plate agglutination. Cotton-tipped applicators were rolled on half of a fresh blood agar plate then a sterile loop was used to semi-quantitatively streak for isolation on the remaining two quarters.

## Statistical Analysis

Statistical analysis was performed using Prism v9.0.1 (GraphPad Software, Inc.). Data were analyzed using linear regression and R values were calculated using the Pearson method.

# RESULTS

## Infection Models

In the first trial, 32 4-week-old, mixed gender Holstein calves were infected with BRSV strain 375 *via* aerosol inoculation. Calves were monitored daily and assigned a clinical disease score on the basis of an adapted University of Wisconsin calf health scoring model. TUS images were collected on days 0, 3, 7, 10, and 14 after infection. Because of elevated clinical disease scores, two calves were euthanized on day 6, and four calves were euthanized on day 7. The remaining 26 calves were euthanized on day 14 after infection. As shown in **Figure 2A**, both clinical disease and TUS scores peaked on day 7 after infection. Clinical scores gradually decreased through day 14; however, ultrasound scores remained elevated for the remainder of the study.

In the second and third trials, a total of 30 4-week-old, mixed gender Holstein calves were infected *via* intratracheal inoculation with $10^7$ CFU *M. haemolytica* strain NADC-D153. Calves were monitored and assigned a clinical disease score daily with TUS images collected daily on days 0–4 after infection. In trial 2, six calves were euthanized on day 1, one calf was euthanized on day 2, and the remaining nine calves were euthanized on day 4 after infection. In trial 3, all 14 calves were euthanized on day 4 after infection. The Fall 2019 cohort of *M. haemolytica*–infected calves had peak clinical and ultrasound score on day 1 after infection, and the Summer 2020 cohort had clinical scores and TUS scores that peaked on days 3 and 4, respectively, as shown in **Figure 2B**.

## Disease Progression

All calves had TUS performed before inoculation with either BRSV or *M. haemolytica*. In trial 1, seven of the 32 BRSV-infected calves had a TUS score ≥2 on arrival indicating the presence of previous clinical disease or current subclinical pneumonia. In trials 2 and 3, none of the 30 *M. haemolytica*–infected calves had a TUS ≥ 2 on arrival.

After experimental infection, consistent trends, unique to each disease process, were observed in the development of pleural

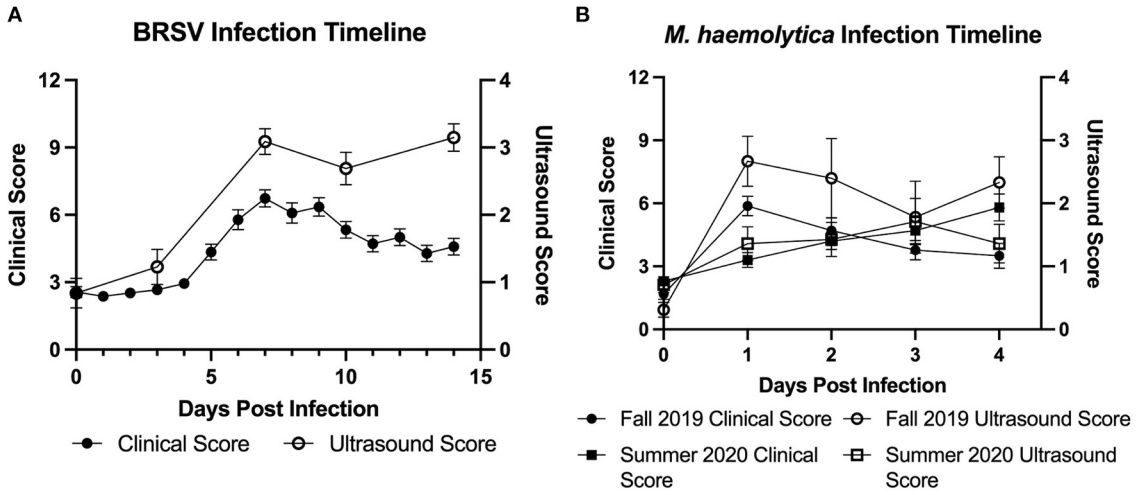



**FIGURE 2 |** Timeline of experimental BRSV and *M. haemolytica* infection. Calves were challenged with either BRSV **(A)** or *M. haemolytica* **(B)**. Unless clinical signs necessitated earlier euthanasia, calves challenged with BRSV were euthanized on day 14 after infection, whereas calves challenged with *M. haemolytica* were euthanized on day 4 after infection. All calves were assigned a clinical score each day by a trained observer based on fever, eye and nose discharge, severity of lung sounds, and ear position. All calves were assigned an ultrasound score on selected days (days 0, 3, 7, 10, and 14 in BRSV-challenged calves; all days in *M. haemolytica*–challenged calves) by a trained reviewer based on presence and severity of consolidated lung tissue and pleural defects such as B-lines and comet tail artifacts. Data represent mean ± SEM.

defects and consolidation that were detected by TUS as shown in **Figure 3**. In *M. haemolytica*–infected calves, a majority of calves (12/16) in the Fall 2019 cohort (**Figure 3A**) developed lesions consistent with consolidation (TUS score $\geq$ 2) on TUS within 24 h of infection. After the peak observed on day 1 after infection, the average ultrasound score steadily decreased until the final observation on day 4 after infection. Notably, however, six of the most severe calves were euthanized 24 h after infection, and an additional two were euthanized 48 h after infection, including the calf depicted in **Figure 3A**, which likely contributed to the decrease in ultrasound score as the study progressed. In the Summer 2020 cohort (**Figure 3B**), one third of the calves (5/15) developed lesions consistent with consolidation on TUS within 24 h of infection and one additional calf developed lesions consistent with consolidation by day 4 of the study. No calves were euthanized before necropsy on day 4 after infection. In general, the consolidation lesions observed on ultrasound in the *M. haemolytica*–infected calves had well-defined borders; this was consistent with the appearance of localized, firm lesions observed in the lung tissue on necropsy.

In the calves that had no prior consolidation, lesions consistent with lung consolidation (TUS score $\geq$ 2) were commonly first identified *via* TUS on day 7 after BRSV infection and remained detectable until necropsy (**Figure 3C**). The calves with positive TUS scores before infection were no more likely to develop positive clinical scores or elevated TUS scores; however, these elevated scores were detected sooner. In addition, pleural defect scores peaked on day 10 after infection and were not always associated with the presence of consolidation. Lesions consistent with consolidation observed on ultrasound in BRSV infection had less defined borders than those of the *M. haemolytica*–infected calves; this was also consistent with the appearance of more diffuse, regionalized lesions within the lung tissue on necropsy.

## Clinical Score and Ultrasound Score

Diagnosing BRD through clinical disease scoring relies primarily on visual cues and an elevated body temperature. A number of field studies have demonstrated the poor sensitivity of clinical disease scoring systems, which fail to detect a significant population of calves with subclinical pneumonia (6). Few studies have compared the relationship between clinical disease scoring progression with TUS scoring progression in controlled infection settings. Therefore, we compared clinical disease score and TUS score from all calves from all days of the study. In trial 1, 131 clinical disease score and TUS score pairs from all days that TUS images were collected were compared (**Figure 4A**). In trials 2 and 3, 59 and 69 clinical disease score and TUS score pairs from all days of the study were compared (**Figures 4B,C**). In the BRSV infection model, there was a weak to mild positive correlation between clinical disease score and TUS score ($R^2 = 0.3212$). Given the differences in disease progression, the *M. haemolytica*–infected calves from trials 2 and 3 were treated as two separate cohorts. The correlation between clinical disease score and TUS score was lower after *M. haemolytica* infection, regardless of cohort ($R^2 = 0.2866$ in the Fall 2019 cohort and $R^2 = 0.0677$ in the Summer 2020 cohort).

To further explore the relationship between clinical disease score and TUS score, the scatterplots were then divided into four quadrants on the basis of a positive clinical score (CS$\geq$5) and the presence of consolidation (TUS score$\geq$ 2). The bottom left quadrant represents calves with negative clinical scores and no

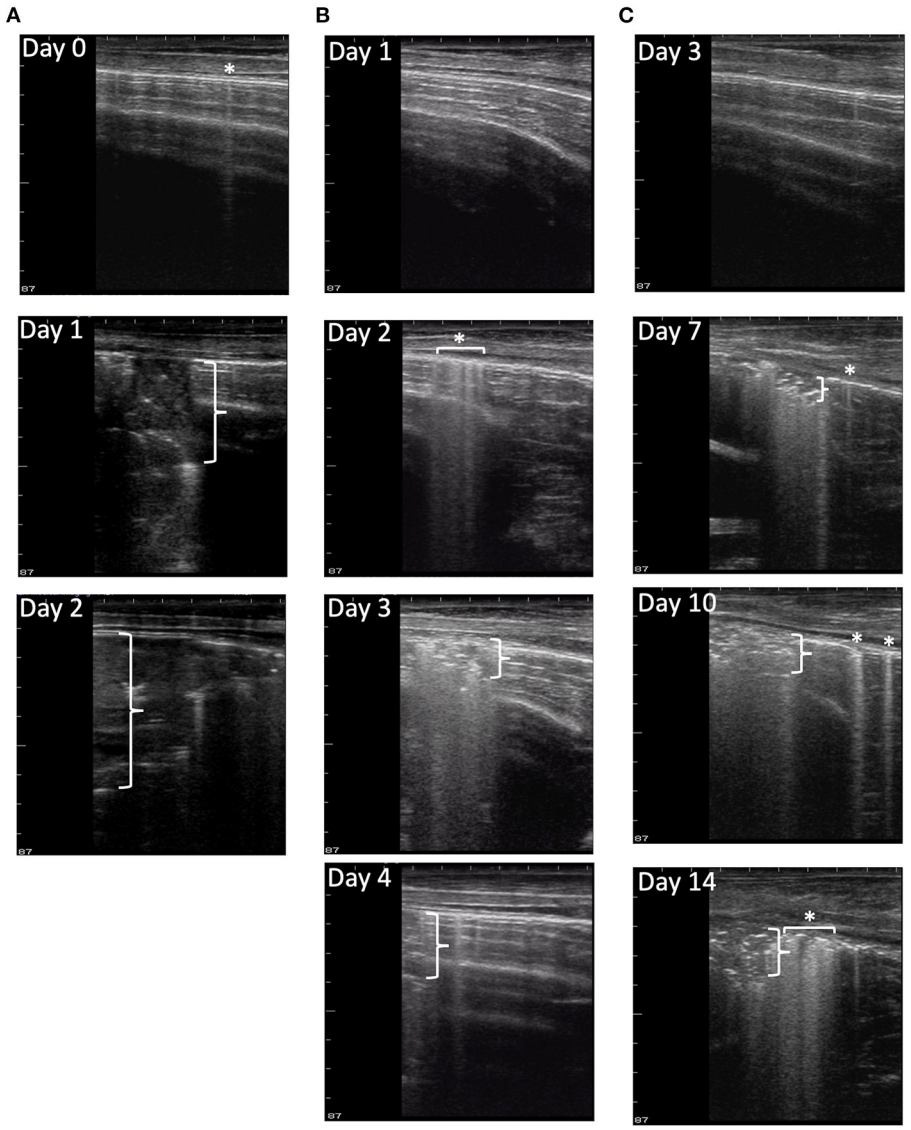



**FIGURE 3 |** Progression of disease in ultrasound images. Ultrasound images were collected from nine locations each day for each calf in the study. **(A)** depicts ultrasound image progression from location 14R on days 0, 1, and 2 of a *M. haemolytica*–infected calf from the Fall 2019 cohort. The final TUS scores of the calf were 0, 4, and 4, and its clinical scores were 2, 7, and 9, respectively. The calf was euthanized on day 2 after infection because of the severity of disease progression. **(B)** depicts the ultrasound image progression from location 18L on days 1, 2, 3, and 4 of a *M. haemolytica*–infected calf from the Summer 2020 cohort. The final TUS scores of the calf were 1, 1, 4, and 4, and its clinical scores were 4, 4, 7, and 2, respectively. The calf was euthanized on day 4 after infection. **(C)** depicts the ultrasound image progression from a location 18L on days 3, 7, 10, and 14 of a representative BRSV-infected calf. The final TUS scores of calf were 0, 3, 3, and 3, and its clinical scores were 1, 9, 6, and 2, respectively. The calf was euthanized on day 14 after infection. Asterisks with or without horizontal brackets indicate pleural defects and vertical brackets indicate maximum depth of consolidation.

consolidation present; these calves make up 41% (107 of 259) of all the data points collected. The top left quadrant represents calves with a positive clinical score but with no consolidation present; these calves make up 10% (27 of 259) of the data points collected. The top right quadrant represents calves with a positive clinical score and consolidation present; these calves have clinical pneumonia and make up 31% (81 of 259) of the data points collected. The bottom right quadrant represents calves with a negative clinical score but consolidation present; these calves have subclinical pneumonia that is undetectable by other methods of disease progression monitoring and make up 17% (44 of 259) of the data points collected.

Of the 32 BRSV-infected calves, 19 were classified as having subclinical pneumonia on at least one day that TUS was performed. The highest incidence of subclinical pneumonia, 11 of the 26 remaining calves, occurred on day 14 after infection (**Figure 5A**). On day 0 of the study, 24 calves had negative clinical and TUS scores. By day 7 after infection, 24 calves were in the

**TABLE 2 |** TUS and clinical score progression of calves across BRSV and *M. haemolytica* infection.

| **BRSV cohort** | | **Day 0** | **Day 3** | **Day 7** | **Day 10** | **Day 14** |
|---|---|---|---|---|---|---|
| | CS(–)/TUS(–)[a] | 24 | 16 | 1 | 3 | 2 |
| | CS(+)/TUS(–) | 1 | 0 | 2 | 2 | 1 |
| | CS(–)/TUS(+) | 6 | 5 | 3 | 1 | 11 |
| | CS(+)/TUS(+) | 1 | 1 | 24 | 15 | 12 |
| ***M. haemolytica* Fall 2019 cohort** | | **Day 0** | **Day 1** | **Day 2** | **Day 3** | **Day 4** |
| | CS(–)/TUS(–) | 16 | 0 | 1 | 3 | 3 |
| | CS(+)/TUS(–) | 0 | 4 | 2 | 2 | 0 |
| | CS(–)/TUS(+) | 0 | 3 | 3 | 3 | 3 |
| | CS(+)/TUS(+) | 0 | 8 | 4 | 1 | 2 |
| ***M. haemolytica* Summer 2020 cohort** | | **Day 0** | **Day 1** | **Day 2** | **Day 3** | **Day 4** |
| | CS(–)/TUS(–) | 14 | 8 | 7 | 7 | 2 |
| | CS(+)/TUS(–) | 0 | 1 | 2 | 3 | 7 |
| | CS(–)/TUS(+) | 0 | 3 | 1 | 1 | 1 |
| | CS(+)/TUS(+) | 0 | 2 | 4 | 3 | 4 |

[a]*CS(–)/TUS(+) is also referred to as subclinical pneumonia. CS(+)/TUS(+) is also referred to as clinical pneumonia.*

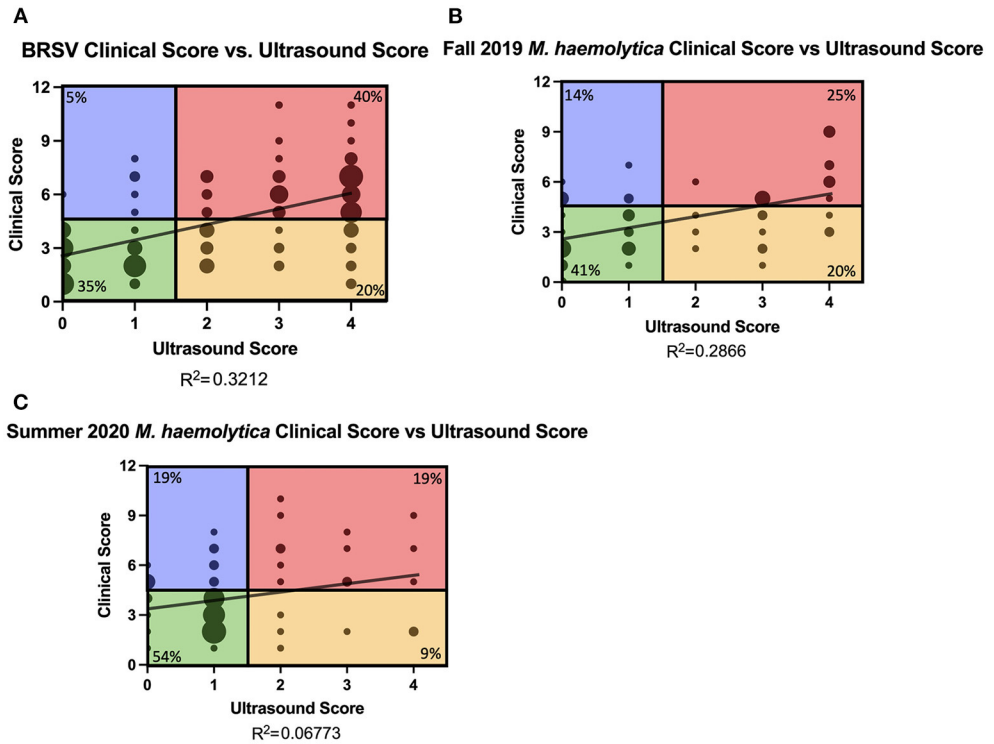



**FIGURE 4 |** Relationship of clinical score and TUS score and classification of calves. Daily clinical scores were compared to daily ultrasound scores from BRSV-infected calves **(A)** and *M. haemolytica*–infected calves **(B,C)**. The graphs include all data points from days 0, 3, 7, 10, and 14 in BRSV-infected calves and from days 0 to 4 in *M. haemolytica*–infected calves. The size of the point represents the number of calves represented in each location: **(A)** the maximum dot size represents 10 calves; **(B)** the maximum size dot represents seven calves; **(C)** the maximum sized dot represents 12 calves. The plots were divided into four quadrants according to positive clinical score (≥5) and positive ultrasound score (≥2). Green quadrant represents calves that are negative for clinical score and ultrasound score. Blue quadrant represents calves with positive clinical scores but negative ultrasound scores. Red quadrant represents calves with elevated clinical and ultrasound scores (clinical). Yellow quadrant represents calves who had non-elevated clinical scores but had positive ultrasound scores (subclinical). The percentage in each quadrant represents the frequency of points from that study represented in each quadrant.

clinical pneumonia classification with positive clinical and TUS scores. Six of these calves were euthanized before day 10 because of severe clinical disease symptoms, so on day 10 after infection, 15 calves remained in the clinical pneumonia classification. By day 14, the number of clinical pneumonia calves was reduced to 12, and there were 11 calves classified with subclinical pneumonia

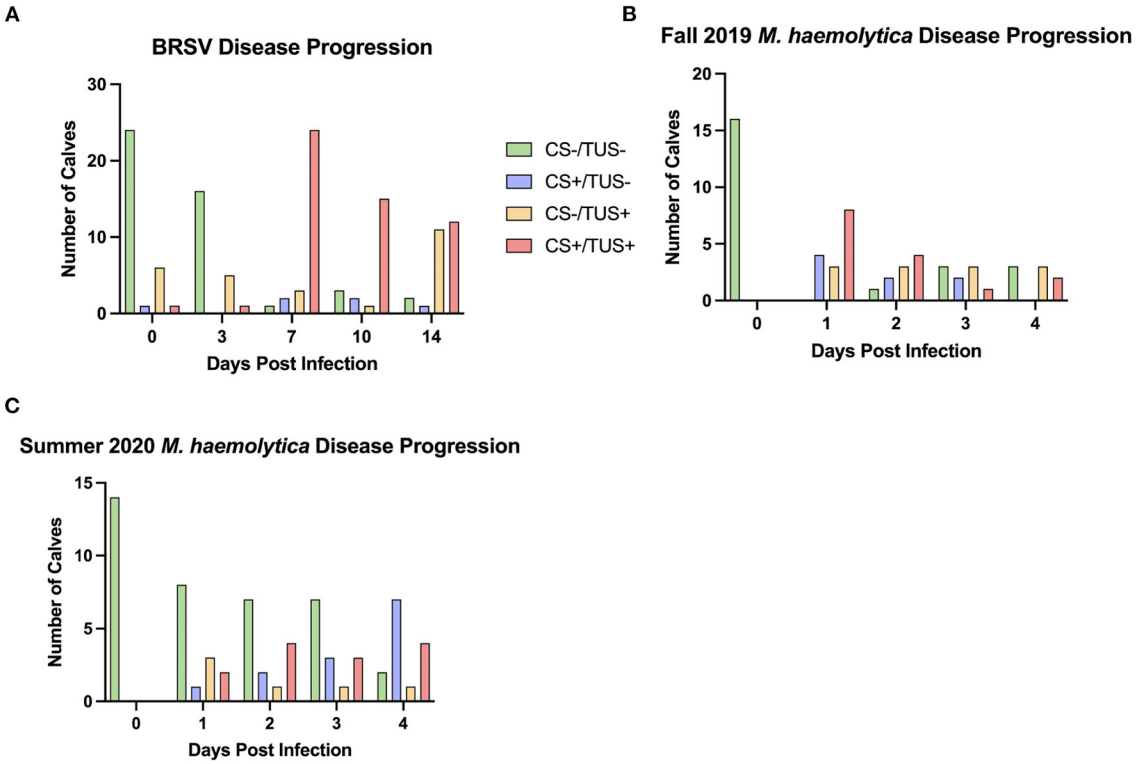



**FIGURE 5 |** The distribution of calves represented in each of the four quadrants over the course of BRSV **(A)** and *M. haemolytica* **(B,C)** infection. For the BRSV-infected calves **(A)**, TUS was performed on days 0, 3, 7, 10, and 14 after infection. For the *M. haemolytica*–infected calves **(B,C)**, TUS was performed on days 0, 1, 2, 3, and 4 after infection.

(**Table 2**). On average, the calves had negative clinical disease and TUS scores on days 0 and 3 after infection and then entered the clinical pneumonia classification on day 7 after infection. The calves that survived to day 10 generally continued to present with clinical pneumonia scores and then moved into the subclinical pneumonia classification by day 14 after infection.

In the two trials with *M. haemolytica*–infected calves, seven of the 16 calves in the Fall 2019 cohort and four of the 14 calves in the Summer 2020 cohort were classified as having subclinical pneumonia on at least one day that TUS was performed (**Figures 5B,C**). In these two trials, the highest incidence of subclinical pneumonia was on day 1 after infection, with six of the 30 calves in this classification. In trial 2, all 16 calves began the study with negative clinical and TUS scores. On day 1 after infection, half the calves were classified as having clinical pneumonia and four calves had positive clinical scores with negative TUS scores. Six of these animals were euthanized because of severe clinical disease symptoms. By day 2 after infection, calves were mostly distributed in the subclinical and clinical pneumonia classifications. An additional calf was euthanized because of severe symptoms. On days 3 and 4 after infection, approximately half the calves had negative clinical and TUS scores, and the remainder was classified with subclinical pneumonia. In trial 3, all 14 calves entered the study with negative clinical and TUS scores. On day 1 after infection, eight calves remained in this classification and the remaining six were distributed among the other three classifications. On day 2 after infection, seven calves were still negative in both scores, but four calves had entered the clinical pneumonia classification, and similar numbers were seen on day 3 after infection. By day 4 after infection, only two calves remained negative for both clinical and TUS scores, seven calves had positive clinical scores and negative TUS scores, and four calves were in the clinical pneumonia classification. By using TUS, calves that lacked clinical symptoms but still developed lung consolidation after experimental infection were frequently identified.

## Pathogen Burden and Ultrasound Score

Although it is known that a portion of the lung damage arising during BRD is a result of immunopathology, there is little information available regarding the relationship between the development and progression of lung lesions and pathogen load. Therefore, nasal swabs samples were collected from BRSV-infected calves for quantification of viral shedding in Trial 1, and representative lesioned and non-lesioned lung tissues were collected for evaluation of pathogen burden in Trials 2 and 3. In **Figure 6A**, quantitative Real-Time (qRT)-PCR was performed for the BRSV NS2 gene on nasal swabs collected from the BRSV-infected animals on day 7 after infection, the peak of virus shedding in this model (15). Virus quantity, described as copy

numbers of the NS2 gene, was then plotted against the ultrasound scores. There was no association between ultrasound scores and peak virus shedding on day 7 after infection ($R^2 = 0.01324$). Samples of lesioned and non-lesioned lung tissue were also collected from the BRSV-infected calves on day 14 after infection. As seen in **Supplementary Figure 3**, few BRSV NS2 gene copies were detected at this timepoint, and there was no association between TUS score and lung viral load at day 14 after infection ($R^2 = 0.03588$ in non-lesioned lung tissue and $R^2 = 0.02543$ in lesioned lung tissue). Lung tissue samples were collected from *M. haemolytica*–infected calves at necropsy, and lung bacterial loads were determined by quantitative culture. As shown in **Figure 6B**, lung bacterial loads ranged from undetectable to as high as $7 \times 10^7$ CFU/g of lung tissue. The lung bacterial loads depicted in **Figure 6B** include calves that were euthanized at humane endpoint and thus include samples that were collected on days 1 (six calves), 2 (one calf), and 4 (23 calves) after infection. There was no association between *M. haemolytica* lung burden at necropsy and ultrasound score ($R^2 = 0.08831$). Thus, there is no discernible relationship between pathogen load and TUS score at the peak of virus shedding (day 7 after infection) for BRSV-infected calves or at the day of necropsy for *M. haemolytica*–infected calves.

## Pathology and Clinical Score

Lung tissue damage can have long-term effects on calf health and performance. Because clinical disease scoring is a widely used disease detection model, its correlation to gross lung pathology can provide a useful basis for determining whether TUS is more or less accurate in detecting the presence of lesions in the lung tissue. All calves that had necropsy data collected from all three studies showed visible lung lesions on necropsy. In **Figure 7**, the area of pneumonic lung, expressed as a percent, was compared to clinical disease score on the day of necropsy. There was a weak to mild positive correlation among BRSV-infected calves ($R^2 = 0.1961$). In the *M. haemolytica*–infected calves, the relationship between clinical disease score and gross lung pathology score was stronger ($R^2 = 0.3352$) but still failed to show a strong relation between the two parameters. This result is not unexpected and underlines the point that clinical disease score is not a strong predictor of the disease severity in the lung. Therefore, for researchers who want to determine the efficacy of a BRD intervention, clinical disease scoring on its own only provides limited information related to disease progression and severity.

## Pathology and Ultrasound Score

To further explore the accuracy and sensitivity of TUS scores for predicting gross lung damage, the correlation between ultrasound score on necropsy and gross lung pathology score was determined in **Figures 8A,B**. The nine ultrasound locations that were evaluated were focused in the cranial and ventral regions of the lung field because consolidation is more likely to develop in these lobes. One caveat of the ultrasound system is the restricted depth of penetration if normal lung tissue is present. This limits the ability of the ultrasound to detect lesions that are deep to normal aerated lung and certain regions of the lung. Therefore, in addition to comparing the total percentage of lung affected, the percentage of pneumonic lung within the five cranial and ventral lobes of interest that were directly examined using ultrasound (as indicated by the location highlights in **Figure 8C**) was also calculated and compared to the respective TUS score. A moderate correlation between the gross lung pathology score encompassing the entire lung and TUS score on necropsy was observed for calves infected with BRSV ($R^2 = 0.5137$). This correlation increased when the gross lung pathology score of only the five lobes of interest was considered ($R^2 = 0.5903$). For the *M. haemolytica*–infected calves, there was also a moderate correlation when the pathology score of the entire lung was considered ($R^2 = 0.4324$) and the correlation increased when TUS score was compared to the five lobes of interest ($R^2 = 0.4899$). In both infection models, there was a greater correlation between gross lung pathology score and ultrasound score than between gross lung pathology score and clinical score. Thus, TUS provides a more accurate representation of gross lung pathology than clinical scoring and can be obtained at any point in the disease process.

To further investigate the relationship between clinical score, TUS score, and gross lung pathology score, the three variables were compared in **Figure 9**. The location of each point on the graph is based on the clinical score and ultrasound score on the day of necropsy, and the focused gross lung pathology score (denoted in **Figure 8C**) is represented by the color of each data point. For the BRSV-infected calves, a higher gross lung pathology score was associated with a higher TUS score, regardless of clinical score. For the *M. haemolytica*–infected calves, this trend was still present but less pronounced. The differences seen between these two models may be associated with the types of lesions associated with the disease. In BRSV infection, more diffuse and regionalized lesions were observed within the tissue after necropsy. This leads to a higher likelihood of identifying lesions on ultrasound of only a select number of locations. In *M. haemolytica* infection, more localized lesions were observed on necropsy, leading to a decreased likelihood of identifying lesions on ultrasound when only observing select locations. However, there is a trend for both viral and bacterial infections, where, as both clinical and TUS scores increase, the percentage of lung affected increases as well.

# DISCUSSION

There have been many studies on the detrimental effects, both in short and long terms, and the costs of BRD (1, 2, 4). Early detection remains a key factor in mitigating the negative effects of the disease. Previous studies on BRD detection have focused on clinical scoring models that rely on visual indicators, such as nasal discharge, cough, attitude, and physical assessment such as rectal temperature or expiration rate (5). Some of these markers, such as attitude and rectal temperature, are not BRD specific and can be caused by a wide variety of illnesses. In addition, not all calves with BRD will present with the same symptoms or with symptoms that are severe enough to be considered clinically ill. Thus, calves with active, but subclinical, BRD are not identified.

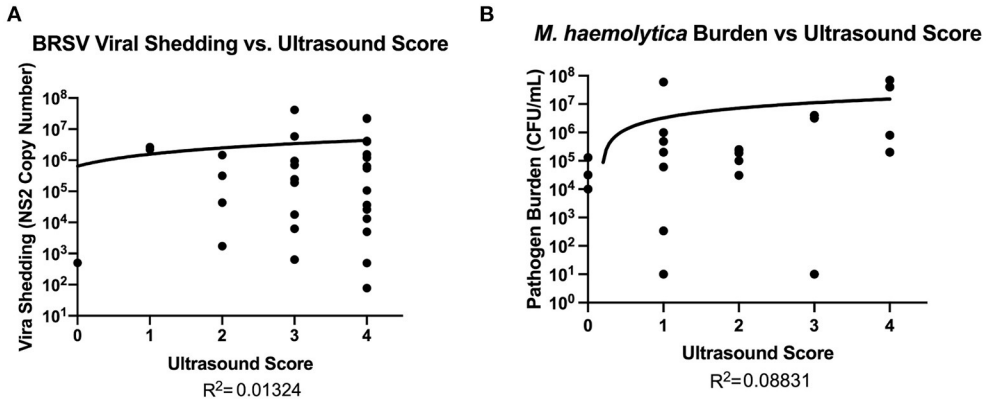



**FIGURE 6 |** Relationship between pathogen burden and TUS score. For BRSV–infected calves **(A)**, qRT-PCR was performed on nasal swabs for the BRSV NS2 gene to quantify the amount of virus being shed in nasal secretions on day 7. For *M. haemolytica*–infected calves **(B)**, quantitative culture was performed using samples of lung tissue to estimate bacterial lung burden.

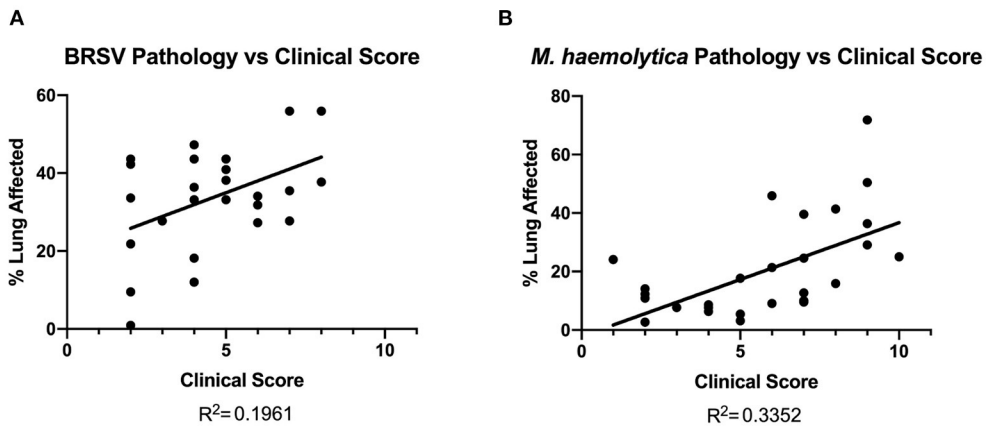



**FIGURE 7 |** Gross lung pathology on necropsy plotted against clinical score on day of necropsy. In both BRSV-infected **(A)** and *M. haemolytica*–infected calves **(B)**, the lungs were divided into 11 lobes and scored based on percentage of lung affected by gross lesions. There was a weak correlation between clinical score and gross lung pathology in both models.

In recent studies comparing on-farm detection of clinical disease, TUS has been shown to have increased sensitivity and specificity over clinical scoring, allowing it to capture even subclinical pneumonia (9, 16). Therefore, the goal of our study was to determine whether TUS could provide the same improvements to disease monitoring during experimental BRD trials.

In experimental models of BRD, lung pathology is typically utilized as the gold standard for detection of disease (17–19). For our studies, we compared TUS to lung pathology as the gold standard, and our results show an improved relationship between TUS and lung pathology compared to traditional clinical disease scoring. Thus, TUS was shown to be a more accurate predictor of disease presence within the lungs than clinical scoring. Although the $R^2$ value between TUS score and gross lung pathology was only moderate, the TUS data in our study were collected from a small portion of the lung, and using TUS, lesions that are deep to aerated lung cannot be observed. When considering these limitations, the increased correlation between TUS and lung pathology as compared to clinical scoring is increasingly significant. Furthermore, although lung pathology is considered the gold standard, it is highly invasive and requires the animal to be euthanized. In contrast, TUS is non-invasive and can provide results that are well correlated with gross lung pathology, especially if more of the lung field is included in the scan (20), thus providing researchers with an alternative to euthanasia.

The two experimental infection models used in the present study were selected to mimic a viral infection and a bacterial infection due to the known variations in disease presentation and progression in these two types of infection. In most of the parameters, we examined that there was a decreased correlation seen in the *M. haemolytica*–infected calves compared with that in the BRSV-infected calves. This decreased correlation may be related to the increased variation of the disease process itself. Progression of disease in *M. haemolytica* infection models

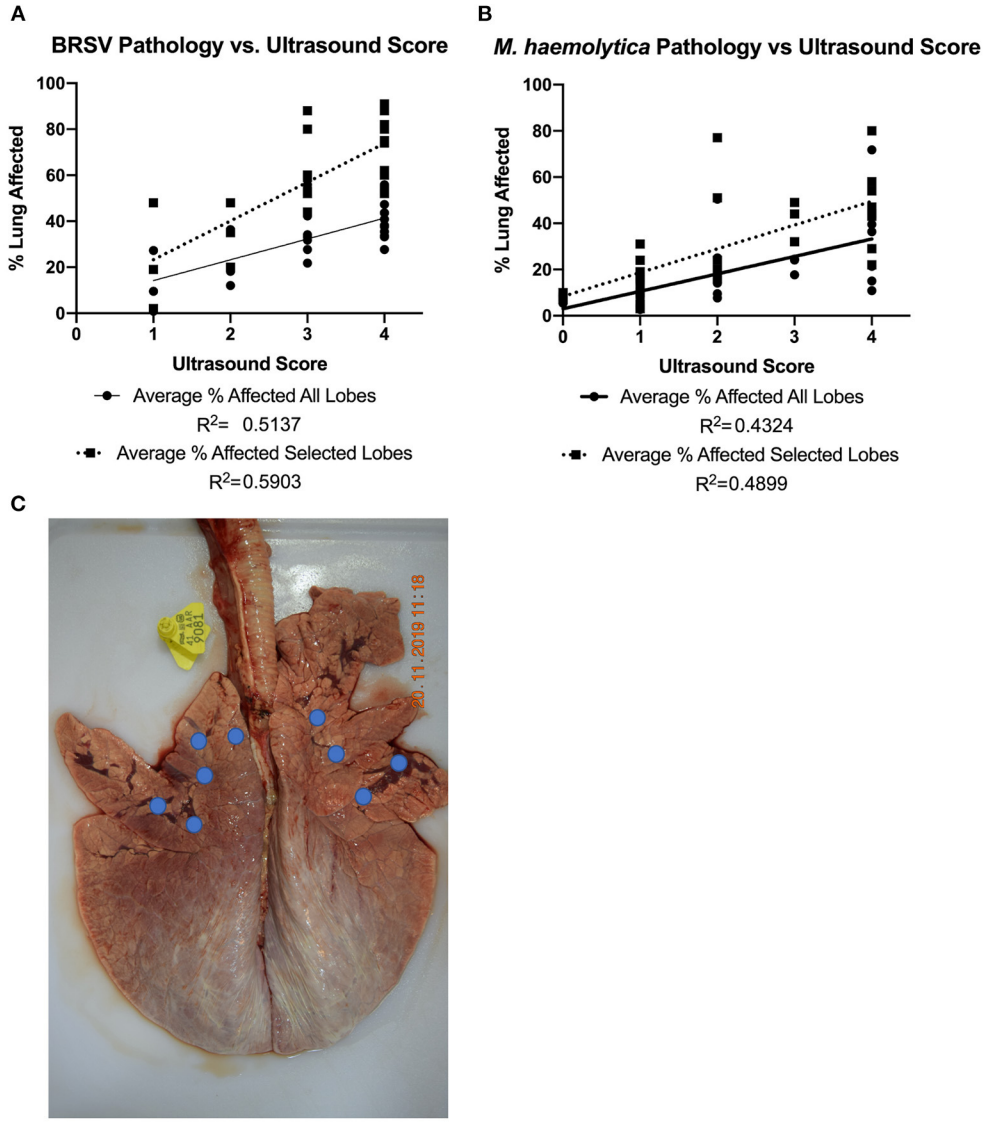



**FIGURE 8 |** Gross lung pathology on necropsy plotted against ultrasound score on necropsy. In both BRSV-infected **(A)** and *M. haemolytica*–infected calves **(B)**, the lungs were divided into 11 lobes and scored based on percentage of lung affected by gross lesions. The average percent affected across all lobes was plotted in addition to an average across the five lobes imaged *via* ultrasound. The approximate ultrasound locations for both the left and right sides of the lung are shown **(C)**. Because the entire lung field was examined *via* TUS, the average of the lobes that were imaged was plotted separately. In both disease models, there was a correlation between gross lung pathology on necropsy and ultrasound score and the correlation increased when only the lobes with the TUS locations were used.

is frequently inconsistent due to variations in endotoxin and leukotoxin production between strains and challenge dose. Previous studies have used doses ranging from $10^6$ to $10^9$ CFU/ml in their inoculation and experienced a range of disease severity even with the same concentration of bacteria (7, 17, 21). Generally, BRSV has a much more consistent pattern of disease (22–24). Within the present study, there was an increased variation in clinical score and TUS score in *M. haemolytica*–infected calves compared with that in BRSV-infected calves. During the Fall 2019 cohort, there was 50% mortality by 48 h after infection, whereas in the Summer 2020 cohort, all infected calves survived to necropsy. When considered together the high variation in clinical score, TUS score and mortality confirm previous observations of the increased variability in disease progression seen in *M. haemolytica* infection. Despite this variation, it is important to note the stronger correlation between TUS score and gross lung pathology compared with the correlation between clinical score and gross lung pathology in *M. haemolytica*–infected calves. Thus, although there was higher variation and somewhat decreased relationships, TUS was still able to accurately capture the differences in disease progression and severity.

The two infection models vary in many ways, but, in addition to the disease mechanisms, there was also variation in the

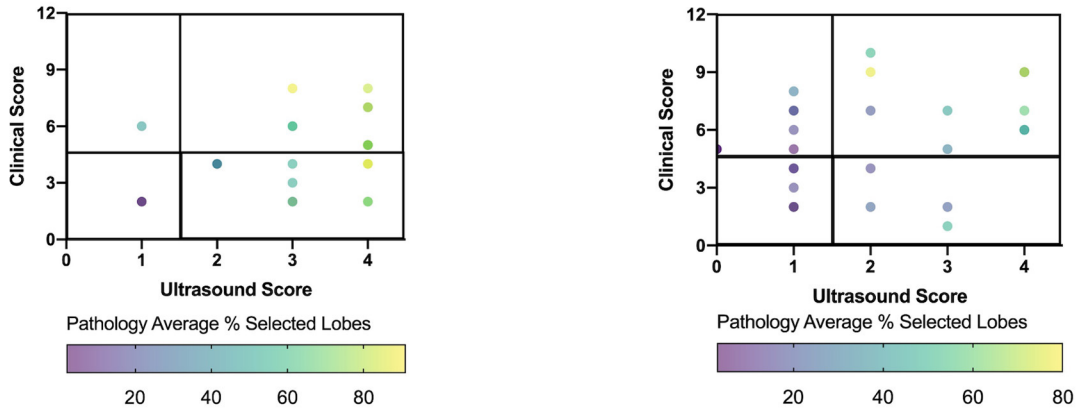



**FIGURE 9 |** Gross lung pathology plotted against clinical score and ultrasound score on day of necropsy for BRSV-infected **(A)** and *M. haemolytica*–infected calves **(B)**. Clinical score for each calf was plotted against the final ultrasound score with the color of dot representing the percentage of lung affected with gross lesions. The plot is divided into four quadrants, similar to **Figure 5**.

calf populations. In the BRSV infection trial, consolidation was observed before experimental infection in seven of the 32 calves that were entered in the trial. This suggests that these calves had previous respiratory infections that may not have been fully resolved at the time of enrollment in the study. TUS was able to capture the disease in these calves that would have otherwise been missed by clinical scoring alone. The presence of pre-inoculation lesions has the potential to influence all subsequent data that were collected on these calves, in particular TUS and necropsy results, because lesions had already developed before study initiation. Previous studies have also noted suspected pre-enrollment respiratory illness and its effect on the subsequent data that were collected (18, 25). In our study, calves that entered the study with consolidation already present were more likely to remain in the clinical and subclinical pneumonia quadrants. BRD trials with experimental infections are attempting to simplify a complicated disease to study a more specific part of the infection process. Calves that enter experimental trials with undiagnosed or unresolved subclinical respiratory infections can significantly affect study outcomes. Therefore, the results of our study strongly suggest that TUS can be a powerful tool for screening calves for existing disease before they are entered into a trial.

By plotting the relationship between clinical score and TUS score, we identified four different subgroups of calves. Previous papers have identified these calves as having upper respiratory tract infections (positive clinical score and negative TUS score), subclinical pneumonia (negative clinical score and positive TUS score), and clinical pneumonia (positive clinical and TUS score) (8). Because of the nature of our experimental infections, where pathogens were introduced directly into the lungs, the term upper respiratory tract was not used for animals with a positive clinical score that lacked ultrasonographic changes. In our study population, this subset of animals most likely included animals with mild pathology of the lungs or pathology that was either deep to normal aerated lung or in a location that was not visualized using our TUS system. However, we did utilize the "subclinical" and "clinical" terminology for calves that had negative clinical score and positive TUS score (subclinical) and positive clinical and TUS score (clinical). Utilization of these classifications highlights the advantages of using TUS in conjunction with clinical scoring. The calves in the subclinical group represent calves that would have been considered within normal limits if TUS was not used in the study but clearly had changes to their lung tissue that were confirmed on necropsy. It is also important to note that some of the calves within the subclinical pneumonia group may also represent calves who are in the process of disease resolution. Changes in lung tissue that are brought on by pathogens may persist even after the pathogens have been cleared, therefore creating a population of healthy calves with significant abnormalities in lung tissue appearance. The lesions persisting in the lungs of the BRSV-infected calves on day 14 after infection, after the virus has been eliminated from the lungs, are one example of this occurrence. The disease resolution process and its timeline are not well understood, but the presence of long-term effects suggests that the resolution process is likely prolonged compared to the duration of active infection. Disease resolution is an important area for future study of BRD pathogenesis, because it may have long-term implications for animal performance.

The weakest relationship observed was between viral shedding or pathogen burden and TUS score. Our measurements of viral shedding quantified the amount of specific BRSV genes present in nasal secretions on a given day. The peak of BRSV shedding usually occurs around day 6 after infection (23, 26). By day 14 after infection, when the calves in the present study were necropsied, the amount of virus that remains in the tissue is very low or has been completely cleared, as previous studies have shown (23, 26). Therefore, to more accurately reflect pathogen

load in the BRSV study, we used the amount of virus shed in nasal secretions on day 7 after infection as our indicator of pathogen burden. Despite selecting the peak shedding day, our dataset still did not detect a correlation with viral shedding. This is not surprising given that viral shedding is not always well correlated with lung damage, which can instead be attributed to the host immune response and subsequent immunopathology (27, 28). In the *M. haemolytica* trials, we used quantitative culture of lung tissue to determine pathogen burden. The presence alone of pathogenic bacteria in the lungs is also not necessarily related to disease severity because some level of *M. haemolytica* presence, which is a normal commensal of the upper respiratory tract, can be considered within normal limits (29, 30). As with viral infection, much of the lung damage resulting from *M. haemolytica* infection can be attributed to immunopathology rather than pathogen burden. Limited work has been done on the relationship between markers of immune function and classifications of BRD as determined by TUS, and all work that has been performed previously has been under field conditions (9, 31).

Most previous research of TUS has focused on its value as a tool to help in clinical diagnosis and treatment of BRD (6, 8, 12). These studies have focused on using TUS in natural BRD infections to confirm its sensitvity and specificity and have primarily taken place in the field where pathogen identification is limited. There are few studies that have used TUS in an experimental infection (7). Our data clearly demonstrate that TUS can provide researchers with additional data on the respiratory health of the animals enrolled in their studies. It is important to note that TUS scanning for clinical diagnosis is typically performed using a different scanning pattern with inclusion of more of the lung surface. In this study, the TUS images were collected by previously untrained individuals; training was able to be completed in a few sessions, and the difficulty of training for image collection was comparable to training for blood collection. It is also important to note that the images were collected as research data to be analyzed and not used to make any clinical diagnosis of disease. When utilized in this manner, TUS can serve as another tool for researchers to use for measuring disease progression, similar to the way that clinical scoring has been integrated in BRD research. In this context, TUS could be especially beneficial in interventional studies that do not call for a final necropsy, such as in the feedlot setting. In these studies, researchers rely on clinical scoring and various blood tests to determine severity of disease (32, 33), and the addition of TUS could provide further information on the health of the lung itself even without necropsy of the animals. Using TUS as a determinant of disease severity could help researchers increase the size of their data pools while decreasing the number of calves that must be euthanized.

## CONCLUSION

When performed in conjunction with clinical disease scoring, in both controlled infections and field studies, TUS can provide a non-invasive method of assessing disease progression within the lung tissue and provide researchers with a more complete image of calf health. TUS can also identify additional diseased calves that clinical scoring alone cannot capture and provide valuable information on calf lung health at the time of study enrollment.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Iowa State University Institutional Care and Use Committee.

## AUTHOR CONTRIBUTIONS

MP, PM, and JM designed the study. MP and PM collected and analyzed the data. AK provided training and oversight of all TUS-related data collection and interpretation. MP, AK, and JM wrote the manuscript. All authors reviewed the manuscript.

## FUNDING

This work was funded by NIH grant R21 AI127895 and USDA-NIFA grant 2018-06904 to JM. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## ACKNOWLEDGMENTS

The authors wish to thank Dr. Robert Briggs and Bradley Chriswell for their assistance and expertise with preparation of the *M. haemolytica* inoculum. The authors also wish to thank the ISU Laboratory Animal Resources staff and clinical veterinarians for their attentive care of the animals.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.763972/full#supplementary-material

## REFERENCES

1. USDA. Heifer Calf Health and Management Practices on U.S. Dairy Operations. In: Fort Collins, CO: United States Department of Agriculture, National Veterinary Services Laboratories (2007). doi: 10.3168/jds.2018-15550
2. Schneider MJ, Tait RG, Busby WD, Reece JM. An evaluation of bovine respiratory disease complex in feedlot cattle: Impact on performance and carcass traits using treatment records and lung lesion scores. *J Anim Sci.* (2009) 87:1220–8. doi: 10.2527/jas.2008-1283
3. Cramer CM, Ollivett TL. Growth of preweaned, group-housed dairy calves diagnosed with respiratory disease using clinical respiratory scoring

and thoracic ultrasound-A cohort study. *J Dairy Sci.* (2019) 102:4322–31. doi: 10.3168/jds.2018-15420
4. Stanton AL, Kelton DF, LeBlanc SJ. Wormuth, J, Leslie KE. The effect of respiratory disease and a preventative antibiotic treatment on growth, survival, age at first calving, and milk production of dairy heifers. *J Dairy Sci.* (2012) 95:4950–60. doi: 10.3168/jds.2011-5067
5. McGuirk SM, Peek SF. Timely diagnosis of dairy calf respiratory disease using a standardized scoring system. *Anim Health Res Rev.* (2014) 15:145–7. doi: 10.1017/S1466252314000267
6. Buczinski S, Forté G, Francoz D, Bélanger AM. Comparison of thoracic auscultation, clinical score, and ultrasonography as indicators of bovine respiratory disease in preweaned dairy calves. *J Vet Intern Med.* (2014) 28:234–42. doi: 10.1111/jvim.12251
7. Baruch J, Cernicchiaro N, Cull CA, Lechtenberg KF, Nickell JS, Renter DC. Performance of multiple diagnostic methods in assessing the progression of bovine respiratory disease in calves challenged with infectious bovine rhinotracheitis virus and Mannheimia haemolytica. *J Anim Sci.* (2019) 97:2357–67. doi: 10.1093/jas/skz107
8. Ollivett TL, Buczinski S. On-farm use of ultrasonography for bovine respiratory disease. *Vet Clin North Am Food Anim Pract.* (2016) 32:19–35. doi: 10.1016/j.cvfa.2015.09.001
9. Ollivett TL, Caswell JL, Nydam DV, Duffield T, Leslie KE, Hewson J, et al. Thoracic ultrasonography and bronchoalveolar lavage fluid analysis in holstein calves with subclinical lung lesions. *J Vet Intern Med.* (2015) 29:1728–34. doi: 10.1111/jvim.13605
10. McGill JL, Guerra-Maupome M, Schneider S. Prophylactic digoxin treatment reduces IL-17 production in vivo in the neonatal calf and moderates RSV-associated disease. *PLoS ONE.* (2019) 14:e0214407. doi: 10.1371/journal.pone.0214407
11. Briggs RE, Tabatabai LB, Tatum FM. Mucosal and parenteral vaccination against pneumonic pasteurellosis in cattle with a modified-live in-frame lktA deletion mutant of Mannheimia haemolytica. *Microb Pathog.* (2012) 52:302–9. doi: 10.1016/j.micpath.2012.02.008
12. Rademacher RD, Buczinski S, Tripp HM, Edmonds MD, Johnson EG. Systematic thoracic ultrasonography in acute bovine respiratory disease of feedlot steers: impact of lung consolidation on diagnosis and prognosis in a case-control study. *Bov Pract.* (2014) 48:1–10. doi: 10.21423/bovine-vol48no1 p1-10
13. McGill JL, Kelly SM, Kumar P, Speckhart S, Haughney SL, Henningson J, et al. Efficacy of mucosal polyanhydride nanovaccine against respiratory syncytial virus infection in the neonatal calf. *Sci Rep.* (2018) 8:3021. doi: 10.1038/s41598-018-21292-2
14. Diaz FE, Guerra-Maupome M, McDonald PO, Rivera-Pérez D, Kalergis AM, McGill JL, et al. Recombinant BCG vaccine is safe and immunogenic in neonatal calves and reduces the clinical disease caused by the respiratory syncytial virus. *Front Immunol.* (2021) 12:664212. doi: 10.3389/fimmu.2021.664212
15. McGill JL, Rusk RA, Guerra-Maupome M, Briggs RE, Sacco RE. Bovine gamma delta T cells contribute to exacerbated IL-17 production in response to co-infection with bovine RSV and Mannheimia haemolytica. *PLoS ONE.* (2016) 11:e0151083. doi: 10.1371/journal.pone.0151083
16. Berman J, Francoz D, Dufour S, Buczinski S. Bayesian estimation of sensitivity and specificity of systematic thoracic ultrasound exam for diagnosis of bovine respiratory disease in pre-weaned calves. *Prev Vet Med.* (2019) 162:38–45. doi: 10.1016/j.prevetmed.2018.10.025
17. Amrine DE, White BJ, Larson RL, Mosier DA. Pulmonary lesions and clinical disease response to Mannheimia haemolytica challenge 10 days following administration of tildipirosin or tulathromycin. *J Anim Sci.* (2014) 92:311–9. doi: 10.2527/jas.2013-6577
18. Corrigan ME, Drouillard JS, Spire MF, Mosier DA, Minton JE, Higgins JJ, et al. Effects of melengestrol acetate on the inflammatory response in heifers challenged with Mannheimia haemolytica. *J Anim Sci.* (2007) 85:1770–9. doi: 10.2527/jas.2006-396
19. Amrine DE, White BJ, Larson R, Anderson DE, Mosier DA, Cernicchiaro N. Precision and accuracy of clinical illness scores, compared with pulmonary consolidation scores, in Holstein calves with experimentally induced Mycoplasma bovis pneumonia. *J Am Vet Med Assoc.* (2013) 310–5. doi: 10.2460/ajvr.74.2.310
20. Pravettoni D, Buczinski S, Sala G, Ferrulli F, Bianchi F, Boccardo A. Short communication: Diagnostic accuracy of focused lung ultrasonography as a rapid method for the diagnosis of respiratory disease in dairy calves. *J Dairy Sci.* (2021) 104:4929–35. doi: 10.3168/jds.2020-19377
21. Fajt VR, Apley MD, Roth JA, Frank DE, Brogden KA, Skogerboe TL, et al. The effects of danofloxacin and tilmicosin on neutrophil function and lung consolidation in beef heifer calves with induced Pasteurella (Mannheimia) haemolytica pneumonia. *J Vet Pharmacol Ther.* (2003) 26:173–9. doi: 10.1046/j.1365-2885.2003.00477.x
22. Salt JS, Thevasagayam SJ, Wiseman A, Peters AR. Efficacy of a quadrivalent vaccine against respiratory diseases caused by BHV-1, PI3V, BVDV and BRSV in experimentally infected calves. *Vet J.* (2007) 174:612–26. doi: 10.1016/j.tvjl.2006.10.007
23. Grissett GP, White BJ, Larson RL. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. *J Vet Intern Med.* (2015) 29:770–80. doi: 10.1111/jvim.12597
24. Vangeel I, Antonis AF, Fluess M, Riegler L, Peters AR, Harmeyer SS. Efficacy of a modified live intranasal bovine respiratory syncytial virus vaccine in 3-week-old calves experimentally challenged with BRSV. *Vet J.* (2007) 174:627–35. doi: 10.1016/j.tvjl.2006.10.013
25. Hanthorn CJ, Dewell RD, Cooper VL, Frana TS, Plummer PJ, Wang C, et al. Randomized clinical trial to evaluate the pathogenicity of Bibersteinia trehalosi in respiratory disease among calves. *BMC Vet Res.* (2014) 10:1–8. doi: 10.1186/1746-6148-10-89
26. Bem RA, Domachowske JD, Rosenberg HF. Animal models of human respiratory syncytial virus disease. *Am J Physiol Lung Cell Mol Physiol.* (2011) 301:L148–56. doi: 10.1152/ajplung.00065.2011
27. Leite F, Kuckleburg C, Atapattu D, Schultz R, Czuprynski CJ. BHV-1 infection and inflammatory cytokines amplify the interaction of Mannheimia haemolytica leukotoxin with bovine peripheral blood mononuclear cells in vitro. *Vet Immunol Immunopathol.* (2004) 99:193–202. doi: 10.1016/j.vetimm.2004.02.004
28. N'jai AU, Rivera J, Atapattu DN, Owusu-Ofori K, Czuprynski CJ. Gene expression profiling of bovine bronchial epithelial cells exposed in vitro to bovine herpesvirus 1 and Mannheimia haemolytica. *Vet Immunol Immunopathol.* (2013) 155:182–9. doi: 10.1016/j.vetimm.2013.06.012
29. Klompmaker AF, Brydensholt M, Michelsen AM, Denwood MJ, Kirkeby CT, Larsen LE, et al. Estimating clinically relevant cut-off values for a high-throughput quantitative real-time pcr detecting bacterial respiratory pathogens in cattle. *Front Vet Sci.* (2021) 8:674771. doi: 10.3389/fvets.2021.674771
30. Thomas AC, Bailey M, Lee MRF, Mead A, Morales-Aza B, Reynolds R, et al. Insights into Pasteurellaceae carriage dynamics in the nasal passages of healthy beef calves. *Sci Rep.* (2019) 9:1–14. doi: 10.1038/s41598-019-48007-5
31. Cuevas-Gómez I, McGee M, McCabe M, Cormican P, O'Riordan E, McDaneld T, et al. Growth performance and hematological changes of weaned beef calves diagnosed with respiratory disease using respiratory scoring and thoracic ultrasonography. *J Anim Sci.* (2020) 98:skaa345 doi: 10.1093/jas/skaa345
32. Word AB, Broadway PR, Burdick Sanchez NS, Roberts SL, Richeson JT, Liang YL, et al. Immune and metabolic responses of beef heifers supplemented

with Saccharomyces cerevisiae to a combined viral-bacterial respiratory disease challenge. *Transl Anim Sci.* (2018) 3:135–48. doi: 10.1093/tas/txy117

33. Kayser WC, Carstens GE, Washburn KE, Welsh TH, Lawhon SD, Reddy SM, et al. Effects of combined viral-bacterial challenge with or without supplementation of Saccharomyces cerevisiae boulardii strain CNCM I-1079 on immune upregulation and DMI in beef heifers. *J Anim Sci.* (2019) 97:1171–1. doi: 10.1093/jas/sky483

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 26 January 2022
doi: 10.3389/fvets.2022.825257

# Development of a One-Step Multiplex Real-Time PCR Assay for the Detection of Viral Pathogens Associated With the Bovine Respiratory Disease Complex

*Juan Zhang, Wan Wang, Mujiao Yang, Jun Lin, Fei Xue*, Yuanmao Zhu* and Xin Yin**

*State Key Laboratory of Veterinary Biotechnology, Division of Livestock Infectious Diseases, Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences, Harbin, China*

OPEN ACCESS

**Edited by:**
*Annamaria Pratelli, University of Bari Aldo Moro, Italy*

**Reviewed by:**
*Wojciech Socha, National Veterinary Research Institute (NVRI), Poland*
*Ivan Toplak, University of Ljubljana, Slovenia*

***Correspondence:**
*Xin Yin*
*yinxin@caas.cn*
*Yuanmao Zhu*
*zhuyuanmao@caas.cn*
*Fei Xue*
*xuefei@caas.cn*

**Specialty section:**
*This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science*

**Received:** *30 November 2021*
**Accepted:** *04 January 2022*
**Published:** *26 January 2022*

**Citation:**
*Zhang J, Wang W, Yang M, Lin J, Xue F, Zhu Y and Yin X (2022) Development of a One-Step Multiplex Real-Time PCR Assay for the Detection of Viral Pathogens Associated With the Bovine Respiratory Disease Complex. Front. Vet. Sci. 9:825257. doi: 10.3389/fvets.2022.825257*

Bovine respiratory disease complex (BRDC) occurs widely in cattle farms. The main viral pathogens include bovine viral diarrhea virus (BVDV), Bovine herpesvirus 1 (BoHV-1), bovine parainfluenza virus type 3 (BPIV3), and bovine respiratory syncytial virus (BRSV), and the newly emerged influenza D virus (IDV). In this study, we have developed a one-step multiplex real-time Polymerase Chain Reaction (PCR) capable of simultaneously detecting these five viral pathogens causing BRDC. The established assay could specifically detect targeted viruses without cross-reaction with others. The detection limit was ∼10 copies/reaction for single real-time PCR and 100 copies/ reaction for multiplex real-time PCR assay. A total of 213 nasal samples from cattle with signs of respiratory tract disease were then collected for performance evaluation of the established platform, proving that the method has good specificity and sensitivity. The surveillance data suggested that BVDV and BoHV-1 infections are the dominant cause of BRDC in the herd, whereas the detection rate of IDV, BIPV3, and BRSV is relatively lower. In summary, the established assay provides technical support for rapid clinical detection of BRDC associated viral pathogens to guide the formulation of BRDC prevention and control measures.

**Keywords: BRDC, IDV, BVDV, BoHV-1, BRSV, BPIV3, quantitative PCR**

## INTRODUCTION

Bovine respiratory disease complex (BRDC) has posed a great threat to the dairy and beef industries throughout the world (1). The disease is usually resulted from stress, primary viral infection and secondary bacterial infection, leading to high mortality and morbidity in cattle (2, 3). It's considered that single or combinational infections by viral pathogens are the prerequisites to developing BRDC (4). So far, the viral pathogens including Bovine Viral Diarrhea Virus (BVDV), Bovine herpesvirus 1 (BoHV-1), Bovine Parainfluenza Virus Type 3 (BPIV3), Bovine Respiratory Syncytial Virus (BRSV) and Influenza D Virus (IDV) have been proposed to be directly associated with BRDC (5). Moreover, secondary bacterial infections such as *Mannheimia haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus* during or after viral infections potentially cause fatal complication of viral infections (6, 7). Thus, early diagnosis of viral pathogens associated with each outbreak could be beneficial to timely managing and controlling BRDC (1).

BVDV is single-stranded, positive-sense RNA viruses that belongs to genus *Pestivirus*, family *Flaviviridae* (8). BVDV are genetically divided into genotype 1 (BVDV-1), genotype 2 (BVDV-2) and genotype 3 (BVDV-3) (9). Previous studies have concluded that BVDV aggravates the respiratory disease syndrome by causing immune tolerance and persistent infection (10). BoHV-1 harbors a linear double-stranded DNA genome and causes infectious bovine rhinotracheitis (IBR), a highly contagious infectious respiratory disease (11, 12). The World Organization for Animal Health (OIE) lists IBR as a Category B animal disease. As reported, BoHV-1 preferentially infects the basal epithelium of injured monolayers and/or genital tracts to induce mucosal barrier injury (13, 14). Serological survey of BoHV-1 infection in China suggested that the seropositive rate was approximately 40% (11). Unlike BoHV-1, BPIV3 is restricted to replicate in the apical ciliated epithelium causing tissue damage and immunosuppression which often progresses to bronchial pneumonia in calves and adult cattle (15). BRSV infection is the major cause of BRDC in calves during their first year (16). It infects both ciliated bronchial epithelia and type II pneumocytes, and causes minimal to extreme respiratory diseases (17). In addition to these well-known viral pathogens, IDV, a new type of influenza virus with cattle as a primary reservoir, can infect and cause influenza diseases in cattle (18). Recently, IDV was reported as a crucial viral agent that drives the occurrences of bovine respiratory disease syndrome (18). However, the contribution of IDV to the bovine respiratory disease development as well as the co-infection between IDV and other respiratory viral pathogens remains unknow. Due to the similarity of clinical signs and co-infections involved in BRDC, it's nearly impossible to rapidly and accurately identify the causative agents solely based on the clinical signs and conventional methods such as pathogens isolation. Therefore, single and multiplex real-time PCR assays have been developed for the rapid detection of pathogens associated with BRDC in the past years (13, 17, 19–21). However, the single target real-time PCR requires separate amplification by consuming excess resources along with inefficient processing. Direct diagnostic methods assay for the presence of the viruses associated with BRDC are still lacking. In addition, the relative contribution of these viruses in BRDC development has not been defined yet, especially in China (22). In this study, we developed a multiplex real-time PCR assay for synchronously detecting five viral pathogens associated BRDC. We showed that the optimized assay displayed excellent performance. The detection limits for all these five viral pathogens were 100 copies/reaction with the multiplex real-time PCR assay. More importantly, our established assay can effectively detect BRDC related pathogens from the nasal swabs collected from the cattle with signs of respiratory tract disease.

# MATERIALS AND METHODS

## Viruses and Cell Cultures

BVDV-1a NADL strain (ATCC VR-534), Bovine rotavirus (BRV) strain NCDV (ATCC VR-452) *Pasteurella multocida* P-1059 (capsular serogroup A) were obtained from China Veterinary Culture Collection Center (CVCC). BoHV-1 isolate HLJ07, BRSV field isolate HLJ01, Bovine coronavirus (BCoV) isolate HM and *Mannheimia haemolytica* SH1801 (Serotype 1), and BPIV-3 (HQ530153) were isolated by our laboratory (23). BVDV-2 HLJ-10 strain was provided by Dr. Mingchun Gao at Northeast Agricultural University (24). The Nucleic Acid of CSFV vaccine strain (C-strain) was provided by Dr. HuaJi Qiu at Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences (25). BVDV, BoHV-1, BPIV3 were propagated on Madin-Darby bovine kidney (MDBK) cells. BRSV was propagated on bovine Turbinate (BT) cells cultured in Minimum Essential Medium (MEM) (Thermo Fisher, Inc.; USA) supplemented with 2% bovine serum (GE, Inc.; USA). IDV strain D/bovine/Mississippi/C00046N/2014 was obtained via reverse genetics system and propagated on Madin-Darby Canine Kidney (MDCK) cells (26).

## Primer and Probe Design

All available sequences of BVDV, BoHV-1, BRSV, BPIV3 and IDV were retrieved from GenBank for alignment via MEGA X. To obtain the specific primer/probe set for the assay development, we selected the highly conserved regions BVDV 5'UTR region, BoHV-1 glycoprotein E (*gE*) gene, BRSV nucleocapsid (*N*) gene, BPIV3 matrix (*M*) gene, IDV *PB1* gene for primer/probe design using the Oligo7 and Primer Express 3.0.1 software. The designed primer/probe sets were then subjected to Primer-BLAST for specificity validation. The primer/probe sets with excellent specific properties were finally chosen for the assay development. All oligonucleotides listed in **Table 1** were synthesized by RuiBiotech (Beijing, China).

## Nucleic Acid Extraction

DNA/RNA was extracted from cell cultures or clinical samples following the instruction of Axygen Body Fluid Viral DNA/RNA Miniprep Kit. Briefly, 2 mL of DMEM was added into the nasal swabs and mixed well. After centrifugation at 5,000 rpm for 5 min, the clarified supernatants were then collected and used for nucleic acid extraction. To obtain the DNA/RNA from either clinical samples or infected culture fluids, 200 μL of the supernatants were mixed with equal amount of lysis buffer. After incubation at room temperature for 5 min, the mixture was mixed with 75 μL of V-N buffer. The supernatants were then collected after centrifugation for RNA extraction. Finally, the resulting RNA was eluted using 30 μL elution buffer and stored at −80 °C for further use.

## Virus Titration on Cell Cultures

BVDV, BoHV-1, and BPIV3 were propagated in MDBK cells. Briefly, the monolayer MDBK cells were inoculated with the indicated virus stock at MOI of 0.02. After one hour adsorption, the cultures were rinsed and washed three times with PBS. DMEM supplemented with 2% horse serum was then added for maintenance. The cell cultures are collected once 100% cytopathic effect (CPE) is observed. The $TCID_{50}$ values were determined using standard methods after incubation for 48–120 h.

**TABLE 1 |** Primer and probes used in this study.

| Pathogens | Primer or probes | Sequence (5'-3') | Position | Length |
|---|---|---|---|---|
| BVDV | BVDV-F228 | TCGAGATGCCACGTGGAC | 228–245 | 162 bp |
| | BVDV-UTR | ATGTGCCATGTACAGCAGA | 371–390 | |
| | BVDV-Pro | CY5-ACCCTATCAGGCTGT-MGB | 322–337 | |
| BOHV-1BoHV-1 | gE-85bp-F | CCGCCAATAACAGCGTAGA | 122735–122753 | 85 bp |
| | gE-85bp-R | CCGTTGTACTGCAGCACAA | 122801–122819 | |
| | BOHV-1-gE-Pro | FAM-CCTCCGGGCTTTAC-MGB | 122783–122796 | |
| BRSV | BRSV-N-141bp-F | ATACAAAGGACTCATCCCGAAAG | 155–173 | 75 bp |
| | BRSV-N-141bp-R | AAGATTCCTTCTACCCTACTACCTCC | 201–220 | |
| | BRSV-N-Probe | NED-AGTATTTGAAAAGTACCCTC-MGB | 178–192 | |
| BPIV3 | BPIV3M-113bp-F | CAGGAACTCCTACAAGCCGC | 164–183 | 113 bp |
| | BPIV3M-113bp-R | CATGGGTACAGTTCAGGTTTAATG | 317–338 | |
| | BPIV3-MTPro | VIC-CTATCATCTCCGTGGC-MGB | 218–237 | |
| IDV | IDV-F (19) | AATTCTGTGCCAATGAAGCTG | 320–340 | 104 bp |
| | IDV-R (19) | TGGCATATTTCTTTCACTTGTCC | 401–423 | |
| | IDV-Pr | ROX-CATAAGTTTGYCTTCCTTCAGTG-MGB | 375–397 | |

## Sensitivity Test

To determine the lowest detection limit of primers and probes used in this study, the specificity tests of single real-time PCR and multiple real-time PCR were conducted in single tube. Liner positive control with the copy number ranging from $10^7$ to $10^1$ was used as standards.

## Real-Time PCR

The fixed reaction conditions for the multiplex reaction system were as follow: 10 μL of 2 × one step RT-PCR buffer III (Takara), 0.4 μL of Ex Taq HS, 0.4 μL of PrimeScript RT Enzyme Mix II, 0.1 μL of each primer set (10 μmol), 0.2 μL of Probe (10 μmol), 2 μL of nucleic acid template, and enzyme-free water 5.7 μL. Amplification was carried out using the following program: 42°C, 5 min, 95°C, 10 s, 45 cycles of 95°C 5 s, 60°C 30 s.

## Clinical Samples

A total of 213 nasal swab samples were collected from the cattle with signs of respiratory tract disease in the northeastern part of China. Among them, 30 nasal swabs were collected from dairy herds in 2019, 38 nasal swabs were collected from dairy herds in 2020, while the remaining 145 nasal swabs were collected from dairy herds in 2021. Most these sampled animals had either one or several of the following symptoms: fever >39 °C, cough, serious nasal and/or lacrimal discharge, and breath sounds. The nasal samples were stored frozen at −80°C until use.

# RESULTS

## Analytical Specificity of the Primer/Probe Sets Used in the One-Step Real-Time RT-PCR Assay

To experimentally evaluate the specificity of the selected primer/probe sets, we isolated the viral genomes from the cultured viruses including BVDV NADL strain, BoHV-1 isolate HLJ07, BPIV3 strain HQ510351, BRSV field isolate HLJ01, IDV strain D/bovine/Mississippi/C00046N/2014, BCoV isolate HM and BRV strain NCDV as well as the bacterial genomes from *Pasteurella multocida* and *Mannheimia haemolytica* for testing. We found that the designed primer/probe set could successfully recognize and amplify the gene fragment derived from the corresponding virus. No amplification and fluorescent signals were observed for non-related pathogens (**Figures 1A–E**). These results proved that each set of primers and probes exhibited good specificity which can be applied for the assay development.

## Establishment of the Standard Curve for the Multiplex Real-Time PCR

To further verify the sensitivity of the multiplex detection method, we generated the recombinant plasmids containing the five target fragments amplified from IBPV3 [*M* gene (4039-4194)], BoHV-1 [*gE* gene (120290-123438)], BVDV-1b [*5'-UTR* (64-494)], BRSV [*N* gene (1342-2289)], and IDV [*PB1*gene (129-488)] and cloned into pLVX-IRES-zsGreenI vector created by Clontech Laboratories Inc. The primers used for amplification were shown in **Table 2**, respectively. The standard curves for the five detected viruses were then determined using a range of 10-fold diluted recombinant plasmids with concentrations from $10^7$ to $10^1$ copies/μL. The qPCR amplifications of each standard plasmid DNA showed amplification plots corresponding to mean Ct values of 16.272–35.966 for BVDV, 15.697–35.694 for BoHV-1, 17.563–36.402 for IBPV3, 20.394–39.084 for BRSV, 17.385–36.634 for IDV (**Figures 2A–E** left panel). The slope of the standard curve, correlation coefficient ($R^2$), and amplification efficiency (Eff%) were calculated as follows: −3.125, 0.999, and 108.905 % for BVDV; −3.312, 0.992, and 100.424% for BoHV-1, −3.206, 0.999, and 105.053% for BPIV3, −3.322, 0.995, and 100.104% for BRSV, −3.165, 0.990, and 107.012% for IDV (**Figures 2A–E** right panel), showing an excellent amplification efficiency and linear equation required for RNA quantification.

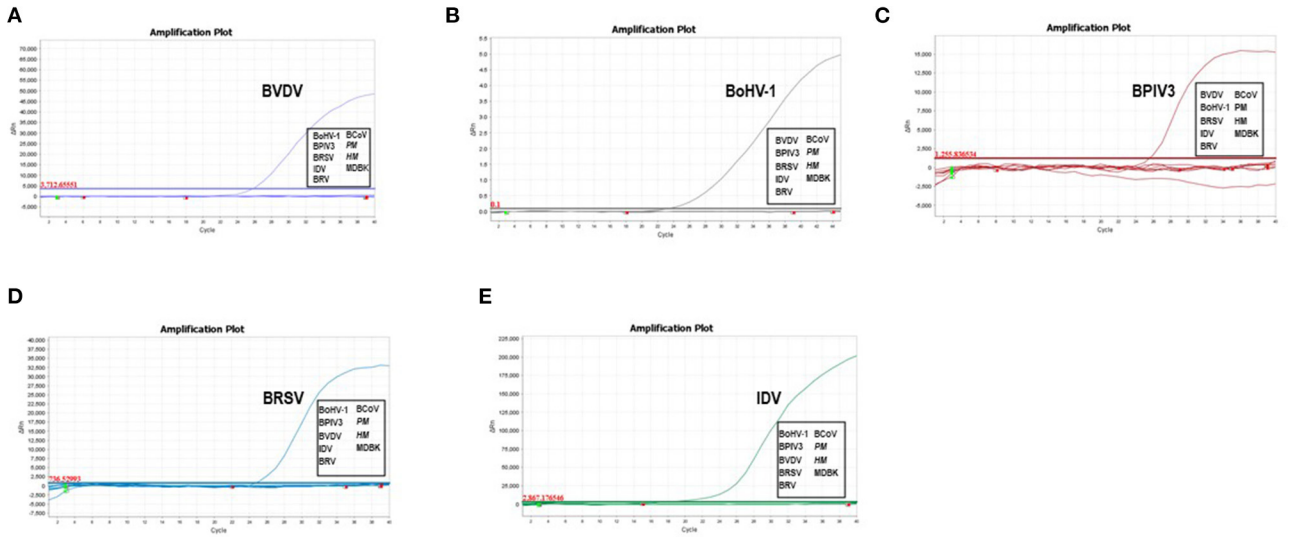



**FIGURE 1 |** Analytical specificity of the primer/probe sets used in the one-step real-time RT-PCR assay: Amplification curves represent samples positive for BVDV, BoHV-1, BPIV3, BRSV and IDV detected by the real-time qRT-PCR assay. Negative samples include BCoV, BRV, *Pasteurella multocida* and *Mannheimia haemolytica*. Equal amount of isolated viral/bacterial genome were mixed for the assay. The non-detected samples were highlighted in the box.

**TABLE 2 |** Primers used for standard constructions.

| Pathogens | Primer or probes | Sequence (5'-3') | Position | Length |
|---|---|---|---|---|
| BVDV | BVDV-F54 | CCGGAATTCCGGGACAAATCCTCCTTAGCGAA(EcoRI) | 64–85 | 450 bp |
| | BVDV-2F54 | CGC ctcgag TCTCCTCTCTCGCCAAACA(Xhol) | 476–494 | |
| BOHV-1 | BOHV-1-gI-gE-L | gttaTCTAGAgtgatggtgatggtgatgGCGGAGGATGGACTTGAGTCG(xba I) | 120290–120309 | 3188 bp |
| | BOHV-1-g3E-U | GATC gactagt CGGTGCCTGTTGCTCTGGAT(Spel I) | 123418–123438 | |
| BRSV | BRSV-NF2 | GATCGACTAGTC GCCACCATG ATGTTATATGCTATGTCCCGAT (Spel I) | 200–221(N) | 980 bp |
| | BRSV-NR2 | GTTA CTCGAG CCAATTGGTTCTTGATTGCCTC (Xhol) | 1127–1147(N) | |
| BPIV3 | BPIV3 MF1 | CTAG TCTAGAAACGAACAAAGGAAGGCAAT(Xbal) | 3834-3853 | 881 bp |
| | BPIV3 MR1 | CGC GGATCCATGATGCCCATATAACCAGA (BamHI) | 4676–4695 | |
| IDV | IDV-F | GATCGACTAGTCGCCACCATG acgtcaatgatatcattgaca(Spel I) | 68–88 | 2135 bp |
| | IDV-R | GTTACTCGAGC ttcaattgcctctcccatcga (Xhol) | 2150–2170 | |

## Optimization of the Multiplex Reaction System

To improve the performance of the multiplex reaction system and reduce the interference among the fluorophores, the parameters including primer concentration, probe concentration, annealing temperatures, and cycling conditions were selected for optimization. The optimized multiple real-time PCR reactions were further evaluated by using the nucleic acids isolated from related pathogens including BVDV, BoHV-1, BPIV3, IDV, and BRSV. Amplification was carried out on Applied Biosystems QuantStudio 5 (Thermo Fisher Scientific). We found that a good amplification effect can be achieved when the total reaction system is 10 μL. Consistently, the optimized multiplex reaction system could efficiently recognize the specific pathogen with the corresponding primer/probe set (**Figures 3A–F**). By using the 10-fold serial diluted plasmid standards, we further validated the amplification efficiency and detection limits, and found that the sample with the concentrations as low as $1 \times 10^1$ copies/μL are detectable. Unfortunately, this lowest number of nucleic acid copies per unit volume ($1 \times 10^1$ copies/μL) was not able to be detected in 95% of BVDV and IDV detection. Therefore, the lowest acceptance limit was defined as $1 \times 10^2$ copies/μL for BVDV and IDV. The cutoff Ct value for BVDV positivity was defined at 36, which means the sample with a Ct value less than or equal to 36 ($\leq 36$) was considered as positive, but higher than 36($>36$) are negative. The cutoff Ct value for BoHV-1 and BPIV3 positivity was defined at 36, and the cutoff Ct value for IDV positivity was defined at 37 (**Figures 4A–E**).

## Repeatability of the Multiplex Real-Time PCR Assay

To assess the repeatability of the multiplex real-time PCR assay, four dilutions of the plasmid standards ranging from $10^3$ to $10^6$

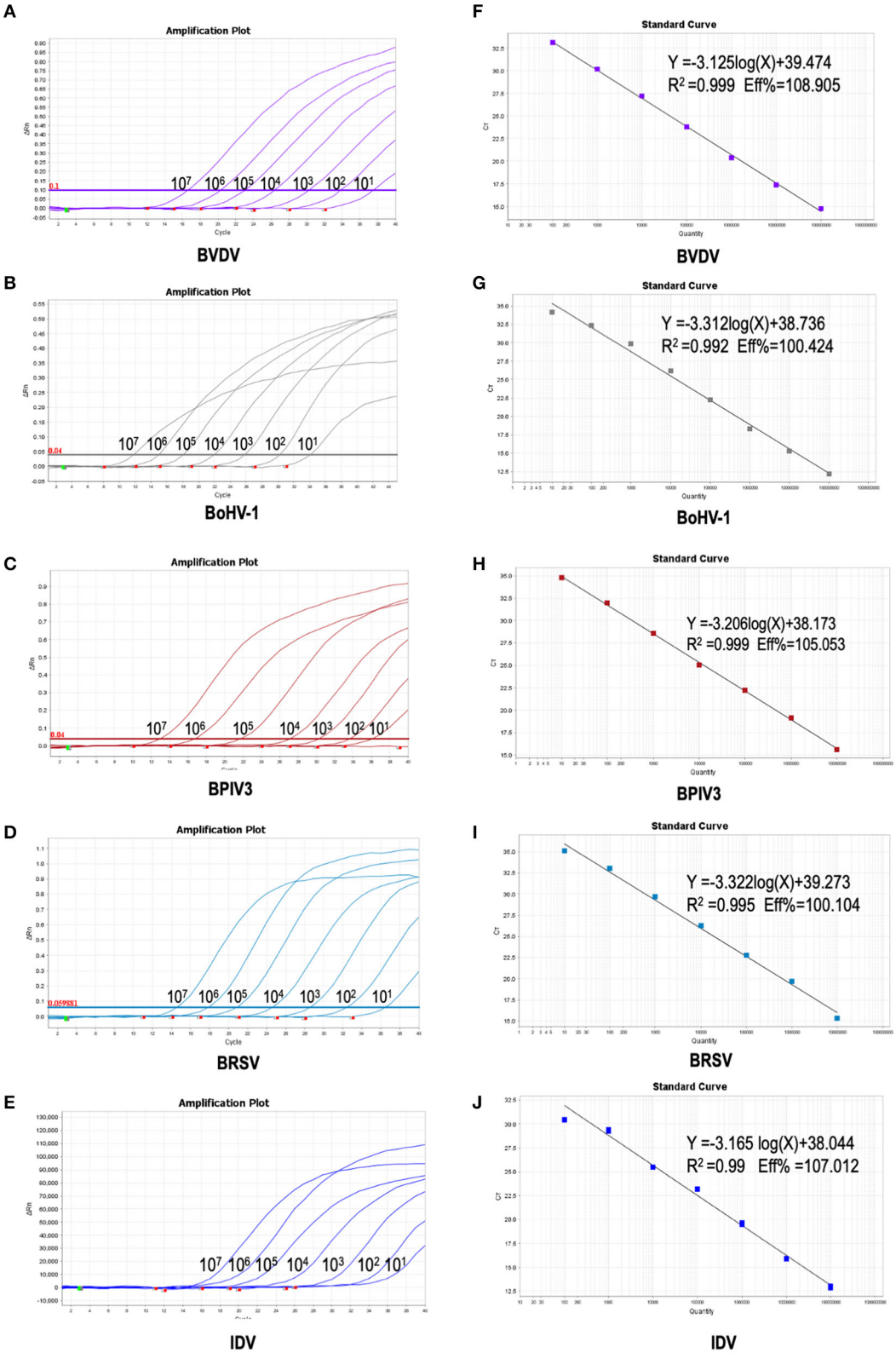



**FIGURE 2 |** Preparation of plasmid standards: **(A–E)**: amplification curves (X-axis: Cycle, Y-axis: ΔRn) of BVDV, BPIV3, BRSV, BoHV-1 and IDV for each plasmid standard with concentrations ranging from $1 \times 10^7$ copies/μL to $1 \times 10^1$ copies/μL; **(F–H)**: standard curves of plasmid standards of BVDV, BPIV3, BRSV, BoHV-1and IDV. **(F)**: BVDV: $Y = -3.125\log(X)+39.474$ $R^2 = 0.999$ Eff% = 108.905; **(G)**: BoHV-1: $Y = -3.312\log(X)+38.736$ $R^2 = 0.992$ Eff% = 100.424; **(H)**: BPIV3: $Y = -3.206\log(X)+38.173$ $R^2 = 0.999$ Eff% = 105.053; I: BRSV: $Y = -3.322\log(X)+39.273$ $R^2 = 0.995$ Eff% = 100. 104; **(J)**: IDV: $Y = -3.165$ log(X)+38.044 $R^2 = 0.99$ Eff% =107.012.

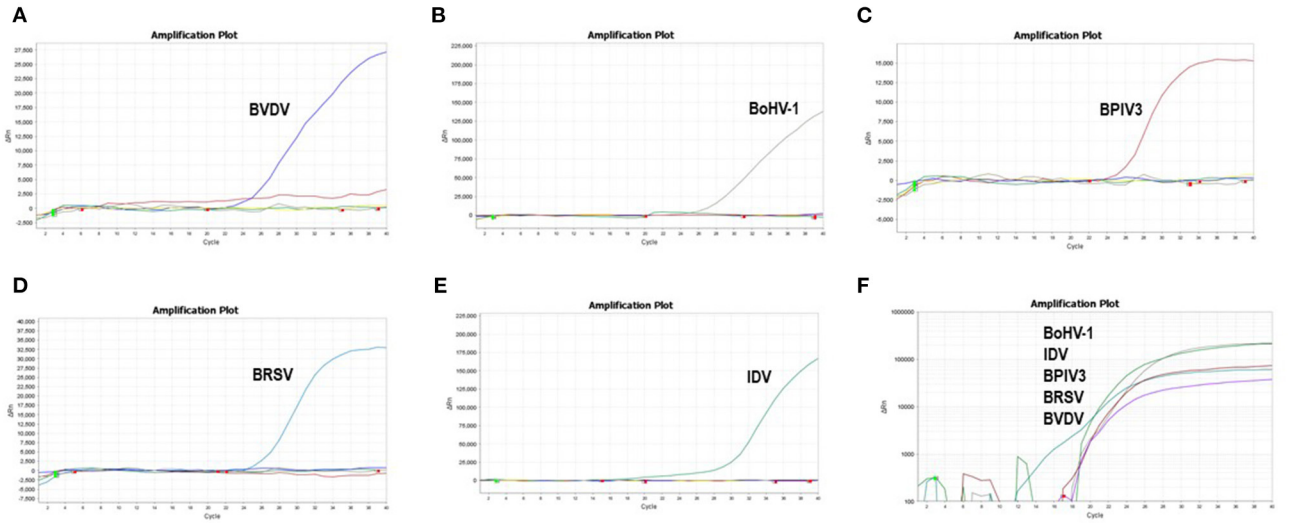



**FIGURE 3 |** Specificity of the multiplex real-time PCR assay: The data were represented with amplification curves (X-axis: Cycle, Y-axis: ΔRn) **(A–E)** Amplification curves represent samples positive for BVDV, BoHV-1, BPIV3, BRSV and IDV detected by the multiplex real-time PCR assay. **(F)** Co-infection simulation experiments with nucleic acids of five pathogens extracted from cell culture The viruses can be detected by our multiplex real-time PCR.

are used for intra-batch and inter-batch repetitions. The standard deviation and coefficient of variation were calculated based on the Ct values obtained from three replicates. As shown in **Table 3**, the coefficient of variation is <3%, indicating the method has good repeatability.

## Clinical Sample Detection

To further verify its clinical applicability for the differential diagnoses of BRDC associated viral pathogens, 213 clinical samples collected from the cattle with clinical signs of BRDC were tested. Among them, 63 (29.577 %) were detected as positive for BVDV, 55 (25.822 %) were detected as positive for BoHV-1, 32 (15.023 %) were detected as positive for BPIV3, 6 (2.817 %) were detected as positive for BRSV. Interestingly, 15 (7.042 %) IDV positive sample was also detected (**Figure 5**). Among all samples, 52 (24.413 %) clinical specimens were co-infected with two or more than two pathogens. Of note, 26(12.207 %) clinical samples were co-infected with BoHV-1and BPIV3, among which the co-infections of BPIV3 and BoHV-1 were the most serious (**Table 4**), indicating that BVDV and BoHV-1 is widespread in the dairy herds in China and exist serious co-infections.

# DISCUSSION

BRDC is an important disease that plagues the cattle industry and causes significant economic losses (1). Since BRDC is a muti-factorial disease, involving both viral and bacterial pathogens (26). The early diagnosis and detection of BRDC is of great economic and clinical significance (27). At present, the commonly used detection assays are classified into antigen detection and antibody detection (28). Conventional RT-PCR and PCR have been widely applied for pathogen detection. However, this method is only suitable for infection by a single pathogen, and time-consuming (29, 30). As bovine respiratory disease syndrome is mainly caused by co-infection of multiple pathogens, it is urgent to develop a rapid and convenient detection method to simultaneously the pathogens causing BRDC. So far, a variety of detection methods have been developed for the BRDC diagnosis (3, 21). For instance, Liu et al. developed a nanoparticle-assisted PCR assay for detection of BRSV, BoHV-1, BVDV, and BPIV3 with a detection limit of 1.43 $\times 10^2$ copies of recombinant plasmids per reaction (31). Leenadevi et al. developed a one-step multiplex real time RT-PCR for the detection of BRSV, BoHV-1 and BPIV3. The assay was rapid, highly repeatable, specific and had a sensitivity of 97% in detecting $10^2$ copies of BRSV, BoHV-1 and BPIV3 (22). Mari et al. developed a multiplex real-time RT-PCR assay for BVDV-1, BVDV-2 and HoBi-like pestivirus. The assay was found to be sensitive, specific and repeatable, ensuring detection of as few as $10^0$-$10^1$ viral RNA copies (32). Although these methods have good specificity and sensitivity, the detected pathogens were only limited to BVDV, BoHV-1, or BPIV3. The accumulating studies have found that IDV and BRSV are also highly associated with bovine respiratory disease syndrome (19, 27). Therefore, the assays that are capable of detecting all these five pathogens are needed. In this study, primers and probes that could specifically recognize the dedicated pathogens were designed in the highly conserved regions. To improve the amplification efficiency of different primers and probes, the annealing temperature of the primers was set at 57°C, the annealing temperature of the probe was set at 67°C. Notably, in this developed assay, the primer/probe set of BVDV showed the lowest amplification. One possible explanation is that the fluorescence of Cy5 was the weakest and very susceptible to interference from other fluorophores due to its own physical

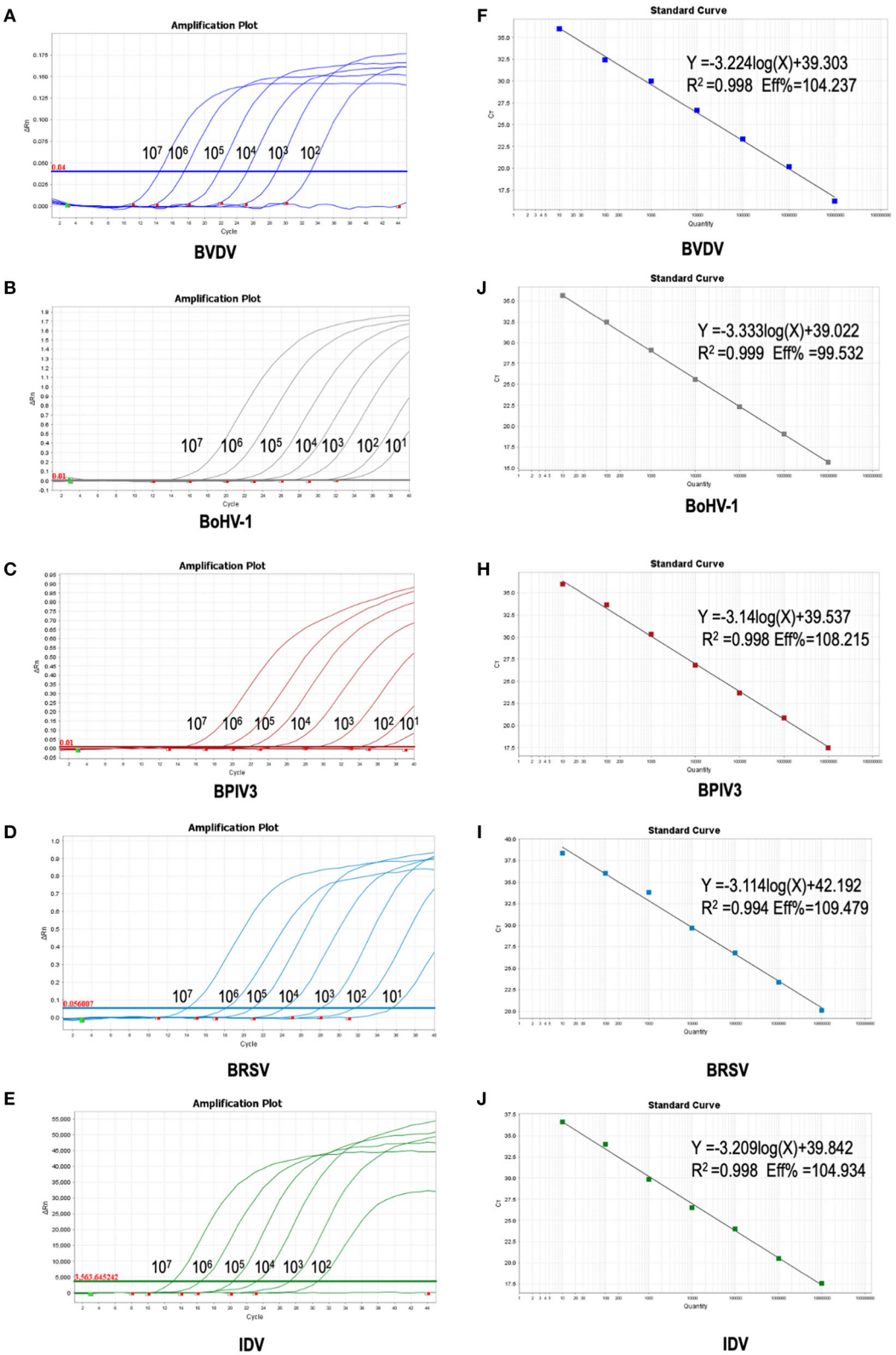



**FIGURE 4 |** Sensitivity of the multiplex real-time PCR assay: **(A–E)** amplification curves (X-axis: Cycle, Y-axis: ΔRn) of plasmid standards of BVDV, BoHV-1,BPIV3,BRSV and IDV detected by multiplex real-time PCR. **(F–J)** standard curves of plasmid standards of BVDV, BoHV-1, BPIV3, BRSV and IDV. F: BVDV: Y = −3.224log(X)+39.303 $R^2$ = 0.998 Eff% = 104.237; **(G)**: BoHV-1: Y = −3.333log(X)+39.022 $R^2$ = 0.999 Eff% = 99.532; **(H)**: BPIV3: Y = −3.14log(X)+39.537 $R^2$ = 0.998 Eff% = 108.215; **(I)**: BRSV: Y = −3.114log(X)+42.192 $R^2$ = 0.994 Eff% = 109.479; **(J)**: IDV: Y = −3.209 log(X)+39.842 $R^2$ = 0.998 Eff% = 104.934.

properties (30). Despite the optimization such as primer/probe concentration and annealing temperature, the improvement effect was not significant. Among all these viruses, BoHV-1exhibited the highest amplification efficiency probably due to its double-stranded DNA structure which enables the amplification more efficient.

In the clinical samples tested, the highest prevalence was observed for BoHV-1 and BVDV, while the infection rate of BRSV is relatively lower. Our observations were consistent with the previous reports showing the prevalence of BoHV-1 and BVDV in China was ∼40 and 50.10%, respectively (11, 33). As reported previously, the infection rate of BPIV3 and BRSV is relatively lower (34). However, a survey in Slovenia showed that the most prevalent virus was BRSV instead, followed by BCoV, BPIV3, and BVDV, while BoHV-1 was less frequently detected (35). The observed differences of BRSV and BoHV-1 prevalence indicated that the major causes of BRDC in different country/regions need to be determined by conducting routine surveillance. In addition, we found that the positive rate of IDV is more than 7.042%, suggesting that a certain prevalence of IDV

**TABLE 3 |** Repeatability of the multiplex real-time PCR assay.

| Virus | Concentration of template (copies/μL) | Intra-coefficient of variation | | Inter-coefficient of variation | |
|---|---|---|---|---|---|
| | | X ± SD | CV(%) | X ± SD | CV(%) |
| BVDV | $10^6$ | 19.323 ± 0.134 | 0.70 | 19.319 ± 0.227 | 1.17 |
| | $10^5$ | 23.188 ± 0.016 | 0.07 | 23.140 ± 0.153 | 0.66 |
| | $10^4$ | 26.157 ± 0.044 | 0.17 | 26.411 ± 0.366 | 1.39 |
| | $10^3$ | 29.904 ± 0.123 | 0.41 | 29.598 ± 0.281 | 0.95 |
| BOHV-1 | $10^6$ | 18.807 ± 0.034 | 0.18 | 18.874 ± 0.136 | 0.72 |
| | $10^5$ | 22.414 ± 0.038 | 0.17 | 22.428 ± 0.042 | 0.19 |
| | $10^4$ | 26.785 ± 0.089 | 0.33 | 26.727 ± 0.137 | 0.51 |
| | $10^3$ | 29.617 ± 0.179 | 0.60 | 29.428 ± 0.315 | 1.07 |
| BRSV | $10^6$ | 21.283 ± 0.038 | 1.70 | 21.759 ± 0.465 | 1.45 |
| | $10^5$ | 25.067 ± 0.032 | 0.12 | 25.177 ± 0.084 | 0.33 |
| | $10^4$ | 28.681 ± 0.023 | 0.80 | 28.852 ± 0.45 | 1.56 |
| | $10^3$ | 32.118 ± 0.125 | 0.39 | 33.081 ± 1.14 | 2.14 |
| BPIV3 | $10^6$ | 20.815 ± 0.064 | 0.31 | 20.694 ± 0.362 | 1.75 |
| | $10^5$ | 24.435 ± 0.012 | 0.53 | 24.348 ± 0.559 | 2.29 |
| | $10^4$ | 27.510 ± 0.438 | 0.25 | 27.400 ± 0.285 | 1.04 |
| | $10^3$ | 30.747 ± 0.028 | 0.09 | 30.640 ± 0.288 | 0.94 |
| IDV | $10^6$ | 19.278 ± 0.042 | 0.21 | 19.425 ± 0.55 | 2.80 |
| | $10^5$ | 22.185 ± 0.349 | 1.57 | 22.650 ± 0.104 | 0.45 |
| | $10^4$ | 25.070 ± 0.074 | 0.30 | 25.608 ± 0.389 | 1.50 |
| | $10^3$ | 28.230 ± 0.099 | 0.35 | 28.664 ± 0.243 | 0.85 |

**TABLE 4 |** The number of clinical specimens with co-infection.

| Co-infection | Number |
|---|---|
| BoHV-1, BPIV3, IDV | 3 |
| BVDV, BoHV-1, BPIV3 | 5 |
| BVDV, BoHV-1, BPIV3 | 5 |
| BoHV-1, BPIV3 | 15 |
| BPIV3, IDV | 1 |
| BoHV-1, IDV | 1 |
| BVDV, BoHV-1 | 11 |
| BPIV3, BRSV | 1 |
| BVDV, IDV | 4 |
| BPIV3, BRSV, IDV | 1 |
| BoHV-1, BRSV | 1 |
| BVDV, BPIV3 | 1 |
| BoHV-1, BPIV3, BRSV | 1 |

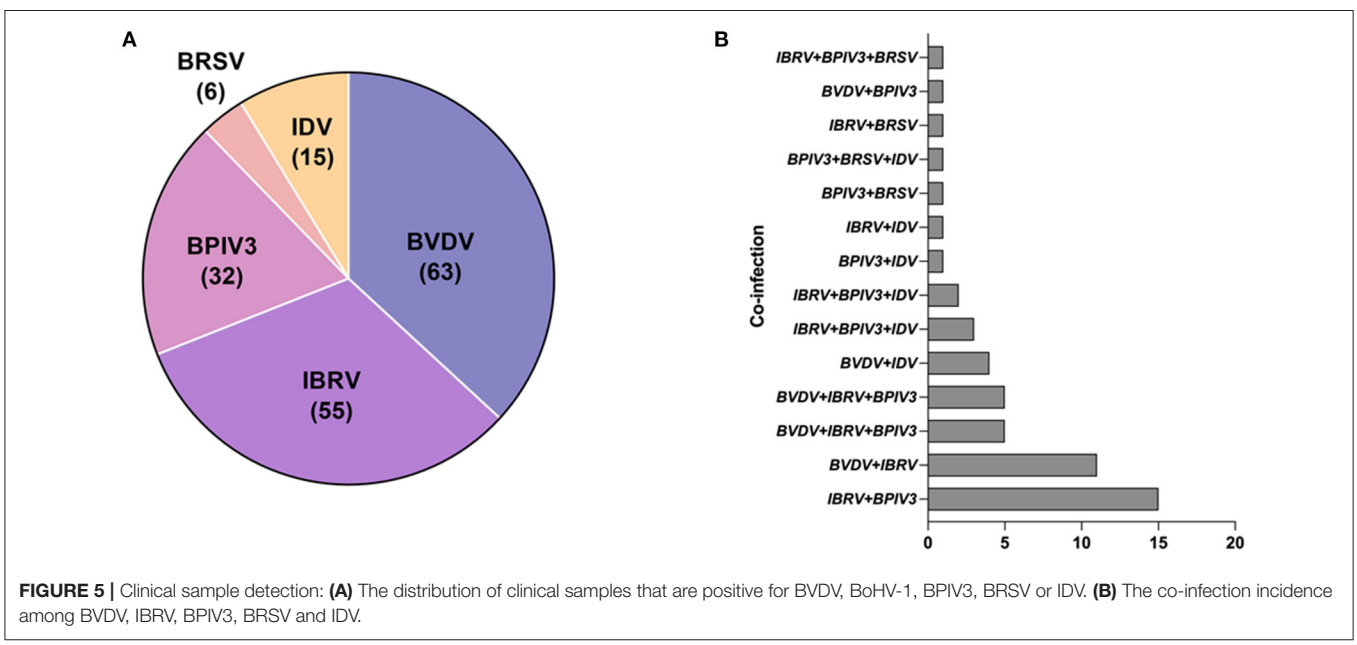



**FIGURE 5 |** Clinical sample detection: **(A)** The distribution of clinical samples that are positive for BVDV, BoHV-1, BPIV3, BRSV or IDV. **(B)** The co-infection incidence among BVDV, IBRV, BPIV3, BRSV and IDV.

occurs in the northeast part of China. The surveillance of IDV in cattle population in China needs to be performed.

Due to the sampling season and sampling method, the positive rate may be affected. As reported, the high incidence of respiratory diseases mainly occurs in spring (4). while the clinical samples used in this study were collected from the northeast part of China during 2019–2021. Notably, we found that the dual infections (BoHV-1 and BPIV3, BVDV and BoHV-1) were identified most often in the cattle with BRD symptoms. Due to the limited sample size, further investigation is required to verify the observation. In conclusion, this study established efficient multiple real-time PCR detection method which can detect the five major viral pathogens causing BVDV, BoHV-1, BRSV, BPIV3, and IDV, and provided support for the clinical surveillances.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

XY, YMZ, FX, and JZ designed the study, reviewed, and edited the manuscript. ZJ, WW, MJY, and JL collected the samples. ZJ performed the experiments. ZJ and XY wrote and edited the manuscript. All the authors read and approved the final manuscript.

## FUNDING

This work was supported by the Natural Science Foundation of Heilongjiang Province JQ2021C005 (XY). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## ACKNOWLEDGMENTS

The authors would like to thank the members from Yin Laboratory for their critical suggestions.

## REFERENCES

1. Bell RL, Turkington HL, Cosby SL, The bacterial and viral agents of BRDC: immune evasion and vaccine developments. *Vaccines (Basel).* (2021) 9. doi: 10.3390/vaccines9040337
2. Liu Z, Li J, Liu Z, Li J, Li Z, Wang C, et al. Development of a nanoparticle-assisted PCR assay for detection of bovine respiratory syncytial virus. *BMC Vet Res.* (2019) 15:110. doi: 10.1186/s12917-019-1858-0
3. Thanthrige-Don N, Lung O, Furukawa-Stoffer T, Buchanan C, Joseph T, Godson DL, et al. A novel multiplex PCR-electronic microarray assay for rapid and simultaneous detection of bovine respiratory and enteric pathogens. *J Virol Methods.* (2018) 261:51–62. doi: 10.1016/j.jviromet.2018.08.010
4. Guo T, Zhang J, Chen X, Wei X, Wu C, Cui Q, et al. Investigation of viral pathogens in cattle with bovine respiratory disease complex in Inner Mongolia, China. *Microb Pathog.* (2021) 153:104594. doi: 10.1016/j.micpath.2020.104594
5. Mehinagic K, Pilo P, Vidondo B, Stokar-Regenscheit N. Coinfection of Swiss cattle with bovine parainfluenza virus 3 and Mycoplasma bovis at acute and chronic stages of bovine respiratory disease complex. *J Vet Diagn Invest.* (2019) 31:674–80. doi: 10.1177/1040638719861686
6. Gershwin LJ, Van Eenennaam AL, Anderson ML, McEligot HA, Shao MX, Toaff-Rosenstein R, et al. Single Pathogen Challenge with Agents of the Bovine Respiratory Disease Complex. *PLoS ONE.* (2015) 10:e0142479. doi: 10.1371/journal.pone.0142479
7. Walz PH, Chamorro MF, Falkenberg SM, Passeler T, van der Meer F, Woolums AR. A bovine viral diarrhea virus: An updated American College of Veterinary Internal Medicine consensus statement with focus on virus biology, hosts, immunosuppression, and vaccination. *J Vet Intern Med.* (2020) 34:1690–706. doi: 10.1111/jvim.15816
8. Wenzhi Xue HCM. Identification of bovine viral diarrhea virus receptor in different cell types. *Vet Microbiol.* (1996) 49:67–79. doi: 10.1016/0378-1135(95)00167-0
9. Peddireddi L, Foster KA, Poulsen EG, An B, Hoang QH, O'Connell C, et al. Molecular detection and characterization of transient bovine viral diarrhea virus (BVDV) infections in cattle commingled with ten BVDV persistently infected cattle. *J Vet Diagn Invest.* (2018) 30:413–22. doi: 10.1177/1040638717753962
10. Baxi M, McRae D, Baxi S, Greiser-Wilke I, Vilcek S, Amoako K, et al. A one-step multiplex real-time RT-PCR for detection and typing of bovine viral diarrhea viruses. *Vet Microbiol.* (2006) 116:37–44. doi: 10.1016/j.vetmic.2006.03.026
11. Chen X, Wang X, Qi Y, Wen X, Li C, Liu X, et al. Meta-analysis of prevalence of bovine herpes virus 1 in cattle in Mainland China. *Acta Trop.* (2018) 187:37–43. doi: 10.1016/j.actatropica.2018.07.024
12. Xu J, Zhang X, Zhou S, Shen J, Yang D, Wu J, et al. A DNA aptamer efficiently inhibits the infectivity of Bovine herpesvirus 1 by blocking viral entry. *Sci Rep.* (2017) 7:11796. doi: 10.1038/s41598-017-10070-1
13. Hou P, Wang H, Zhao G, He C, He H. Rapid detection of infectious bovine Rhinotracheitis virus using recombinase polymerase amplification assays. *BMC Vet Res.* (2017) 13:386. doi: 10.1186/s12917-017-1284-0
14. Muylkens B, Thiry J, Kirten P, Schynts F, Thiry E. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Vet Res.* (2007) 38:181–209. doi: 10.1051/vetres:2006059
15. Ellis JA. Bovine Parainfluenza-3 Virus. *Vet Clin North Am Food Anim Pract.* (2010) 26:575–93. doi: 10.1016/j.cvfa.2010.08.002
16. Sarmiento-Silva RE, Nakamura-Lopez Y, Vaughan G. Epidemiology, molecular epidemiology and evolution of bovine respiratory syncytial virus. *Viruses.* (2012) 4:3452–67. doi: 10.3390/v4123452
17. Valarcher JF, Taylor G. Bovine respiratory syncytial virus infection. *Vet Res.* (2007) 38:153–80. doi: 10.1051/vetres:2006053
18. Su S, Fu X, Li G, Kerlin F, Veit M. Novel Influenza D virus: Epidemiology, pathology, evolution and biological characteristics. *Virulence.* (2017) 8:1580–91. doi: 10.1080/21505594.2017.1365216
19. Zhang X, Outlaw C, Olivier AK, Woolums A, Epperson W, Wan XF. Pathogenesis of co-infections of influenza D virus and Mannheimia haemolytica in cattle. *Vet Microbiol.* (2019) 231:246–53. doi: 10.1016/j.vetmic.2019.03.027
20. Zoccola R, Mazzei M, Carrozza ML, Ricci E, Forzan M, Pizzurro F, et al. A newly developed BVDV-1 RT-qPCR Taqman assay based on Italian isolates: evaluation as a diagnostic tool. *Folia Microbiol (Praha).* (2017) 62:279–86. doi: 10.1007/s12223-017-0497-8
21. Kishimoto M, Tsuchiaka S, Rahpaya SS, Hasebe A, Otsu K, Sugimura S. et al. Development of a one-run real-time PCR detection system for pathogens associated with bovine respiratory disease complex. *J Vet Med Sci.* (2017) 79:517–23. doi: 10.1292/jvms.16-0489
22. L. Thonur. One-step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine herpesvirus 1 and bovine parainfluenza virus 3. *BMC Vet Res.* (2012) 8:1746–6148. doi: 10.1186/1746-6148-8-37
23. Zhu YM, Shi HF, Gao YR, Xin JQ, Liu NH, Xiang WH, et al. Isolation and genetic characterization of bovine parainfluenza virus type 3 from cattle in China. *Vet Microbiol.* (2011) 149:446–51. doi: 10.1016/j.vetmic.2010.11.011

24. Liu H, Li Y, Gao M, Wen K, Jia Y, Liu X, et al. Complete genome sequence of a bovine viral diarrhea virus 2 from commercial fetal bovine serum. *J Virol.* (2012) 86:10233. doi: 10.1128/JVI.01581-12
25. Wang C, Li S, Jia H, Chen G, Fang Y, Zeng S, et al. Monoclonal and oligoclonal TCR AV and BV gene usage in CD4(+) T cells from pigs immunised with C-strain CSFV vaccine. *Sci Rep.* (2018) 8:1655. doi: 10.1038/s41598-018-19974-y
26. Oguejiofor CF, Thomas C, Cheng Z, Wathes DC. Mechanisms linking bovine viral diarrhea virus (BVDV) infection with infertility in cattle. *Animal Health Research Reviews.* (2019) 20:72–85. doi: 10.1017/S1466252319000057
27. Sheridan MP, Regev-Shoshani G, Martins J, Vimalanathan S, Miller C. Nitric oxide modulates the immunological response of bovine PBMCs in an in vitro BRDc infection model. *Res Vet Sci.* (2016) 109:21–8. doi: 10.1016/j.rvsc.2016.09.004
28. Parreno V, Romera SA, Makek L, Rodriguez D, Malacari D, Maidana S, et al. Validation of an indirect ELISA to detect antibodies against BoHV-1 in bovine and guinea-pig serum samples using ISO/IEC 17025 standards. *J Virol Methods.* (2010) 169:143–53. doi: 10.1016/j.jviromet.2010.07.014
29. I.M. Mackay. Real-time PCR in the microbiology laboratory. *Clin Microbiol Infect.* (2004) 10:190–212. doi: 10.1111/j.1198-743X.2004.00722.x
30. Pan Z, Lu J, Wang N, He WT, Zhang L, Zhao W, et al. Development of a TaqMan-probe-based multiplex real-time PCR for the simultaneous detection of emerging and reemerging swine coronaviruses. *Virulence.* (2020) 11:707–18. doi: 10.1080/21505594.2020.1771980
31. Liu C, Liu Y, Liang L, Cui S, Zhang Y. RNA-Seq based transcriptome analysis during bovine viral diarrhoea virus (BVDV) infection. *BMC Genomics.* (2019) 20:774. doi: 10.1186/s12864-019-6120-4
32. Tsuchiaka S, Masuda T, Sugimura S, Kobayashi S, Komatsu N, Nagai M. et al. Development of a novel detection system for microbes from bovine diarrhea by real-time PCR. *J Vet Med Sci.* (2016) 78:383–9. doi: 10.1292/jvms.15-0552
33. Sun WW, Meng QF, Cong W, Shan XF, Wang CF, Qian AD. Herd-level prevalence and associated risk factors for Toxoplasma gondii, Neospora caninum, Chlamydia abortus and bovine viral diarrhoea virus in commercial dairy and beef cattle in eastern, northern and northeastern China. *Parasitol Res.* (2015) 114:4211–8. doi: 10.1007/s00436-015-4655-0
34. Shen Y, Liu J, Zhang Y, Ma X, Yue H, Tang C. Prevalence and characteristics of a novel bovine adenovirus type 3 with a natural deletion fiber gene. *Infect Genet Evol.* (2020) 83:104348. doi: 10.1016/j.meegid.2020.104348
35. Tomislav Paller PH, Pogačnik M, Toplak I. The prevalence of ten pathogens detected by real-time PCR methods in nasal swabs samples collected from live cattle with respiratory disease. *Slov Vet Res.* (2017) 54.

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

# Advantages of publishing in Frontiers

**OPEN ACCESS**
Articles are free to read for greatest visibility and readership

**FAST PUBLICATION**
Around 90 days from submission to decision

**HIGH QUALITY PEER-REVIEW**
Rigorous, collaborative, and constructive peer-review

**TRANSPARENT PEER-REVIEW**
Editors and reviewers acknowledged by name on published articles

**Frontiers**
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

**Visit us:** www.frontiersin.org
**Contact us:** frontiersin.org/about/contact

**REPRODUCIBILITY OF RESEARCH**
Support open data and methods to enhance research reproducibility

**DIGITAL PUBLISHING**
Articles designed for optimal readership across devices

**FOLLOW US**
@frontiersin

**IMPACT METRICS**
Advanced article metrics track visibility across digital media

**EXTENSIVE PROMOTION**
Marketing and promotion of impactful research

**LOOP RESEARCH NETWORK**
Our network increases your article's readership