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RESEARCH ARTICLE

Molecular and pathomorphological studies of lung lesions in bovine respiratory disease associated with macrolide-resistant bacterial pathogens

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ABSTRACT

This study aimed to evaluate factors influencing the severity of histopathological pneumonia in bovine respiratory disease (BRD) cases, with reference to the complexity of bacterial pathogens and molecular profiles of macrolide resistance genes. A total of 80 formalin-fixed paraffin-embedded (FFPE) lung tissue samples from calves (n=40) and adult cattle (n=40) with fibrinous pneumonia were examined. Histopathological severity was assessed using a modified semi-quantitative scoring system based on inflammation extent, necrotic areas, and vascular damage. The presence of five major bacterial pathogens (*Pasteurella multocida, Mannheimia haemolytica, Histophilus somni, Mycoplasma bovis,* and *Trueperella pyogenes*) and three macrolide resistance genes [*erm(42), msr(E)* and *mph(E)*] were analysed using multiplex real-time PCR. Histopathological evaluations revealed significantly higher severity scores for fibrinonecrotic pneumonia compared to other pneumonia types. Molecular analyses showed multiple bacterial pathogens in 68.8% of cases, significantly correlating with increased histopathological scores. Regarding macrolide resistance genes, at least one resistance gene was detected in 76-95% of positive cases, with the *msr(E) + mph(E)* combination being the most frequent. The combined effect of pathogen number and resistance gene load on pneumonia severity was confirmed by ROC analysis for its ability to predict severe pneumonia. In conclusion, our findings highlight the importance of molecular-based pneumonia severity scoring in guiding rational antimicrobial use and herd management strategies in BRD.

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INTRODUCTION

Bovine respiratory disease complex (BRD) is one of the most important problems of the global cattle industry due to the wide variety of agents, unpredictability in the clinical course, and lack of effective and rapid diagnostic techniques (Pratelli *et al.,* 2021). The disease occurs when opportunistic microorganisms, which are components of the normal microbiota in the upper respiratory tract, invade the lower respiratory tract under the influence of environmental stress factors such as viral infections, transportation, cold weather, and cramped housing (Taylor *et al.,* 2010; Klima *et al.,* 2019; Pratelli *et al.,* 2021). The major bacterial agents that cause BRD are *Mannheimia haemolytica*, *Pasteurella multocida*, *Trueperella pyogenes*, *Histophilus somni*, and *Mycoplasma bovis* (Caswell and Williams, 2016; López and Martinson, 2017). Complex bronchopneumonia caused by these bacterial agents results in serious morbidity and mortality among animals, thereby negatively affecting animal welfare and productivity.

The factors that determine the clinical course, severity and mortality rate of BRD are quite diverse (Dorso *et al.,* 2021). The presence, intensity and duration of stress factors, the capacity of one or more agents to cause infection together and the sensitivity of the agents to antibiotics are the important ones in this regard (Dabo *et al.,* 2007; Klima *et al.,* 2019). Next-generation sequencing studies show that multiple infections in BRD cases increase the severity of the lesion as well as complicate the response to treatment (Buczinski *et al.,* 2024).

It has been reported that the *erm(42)*, *msr(E)* and *mph(E)* genes, which play a role in the development of resistance to macrolide antibiotics and being frequently used in the treatment of BRD, can spread through horizontal gene transfer via mobile genetic elements, and thus resistant bacteria spread rapidly within the herd (Rose

et al. 2012; Beker *et al.,* 2018; Urban-Chmiel *et al.,* 2022). In veterinary medicine, molecular studies on antimicrobial resistance remain limited, and data concerning the distribution of resistance genes against macrolide antibiotics are particularly scarce. The increasing prevalence of these genetic determinants within animal populations, especially in cattle production systems characterized by intensive antimicrobial use, facilitates the natural selection and expansion of resistant strains. Moreover, the environmental dissemination of these resistance factors not only jeopardizes animal health but also contributes to the overall environmental resistance burden, thereby accelerating uncontrolled resistance development across broader ecosystems (Tang *et al.,* 2017; WHO, 2021). Accordingly, molecular surveillance of resistance genes holds strategic importance not only for assessing the current scope of the problem but also for reshaping antimicrobial stewardship policies.

Although studies on the molecular diagnosis of BRD pathogens are available in the literature, investigations that examine the association between these pathogens and histopathological pneumonia severity, particularly in conjunction with antimicrobial resistance gene profiling within the same samples, are extremely limited. In particular, multidisciplinary approaches involving the simultaneous detection of both pathogens and resistance genes via PCR from formalin-fixed paraffin-embedded (FFPE) lung tissues, followed by their correlation with semi-quantitative pneumonia severity scoring, are rarely encountered.

The objective of this study is threefold: firstly; to identify five major bacterial agents associated with BRD losses using multiplex real-time PCR; secondly; to investigate the presence of macrolide resistance genes within the same samples; and thirdly; to correlate all molecular findings with histopathological pneumonia severity. Through this approach, the study seeks to contribute to the identification of potential diagnostic biomarkers, enhance the capacity to predict therapeutic outcomes, and support the development of novel strategies for the prudent use of macrolide antibiotics.

MATERIALS AND METHODS

Animals and Ethical Statement: The study animals consisted of 80 cattle (40 calves <6 months and 40 adults >6 months) diagnosed with fibrinous pneumonia at necropsy and with a clinical history of macrolide antibiotic administration. All animals were obtained from commercial dairy farms located in Konya province and surrounding areas. According to clinical records, the animals had been treated under field conditions with macrolide antibiotics (tulathromycin, tildipirosin, or gamithromycin) following the onset of respiratory symptoms; however, detailed information regarding dosage and duration of treatment was not consistently documented across all cases. Therefore, while the presence of antibiotic use was accepted as a case selection criterion based on clinical history, it was excluded from quantitative analysis. The study protocol was approved by the Animal Experiments Ethics Committee of the Faculty of Veterinary Medicine at Selcuk University (Ethics Approval No: 2021/103).

Histopathological Examination: Lung tissues collected postmortem were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. From each block, three consecutive 5-µm sections were cut (Leica RM 2125RT), stained with hematoxylin and eosin (H&E), and examined microscopically (Olympus BX51). Microscopic lesions were photographed using a digital camera (Olympus EP50, Tokyo, Japan).

To objectively assess pneumonia severity, a modified version of a previously established multiparametric semi-quantitative scoring system (Severity Index) was applied (Booker *et al.,* 2008, Maunsell and Donovan, 2009; Passmore *et al.,* 2018; García-Galán *et al.,* 2021). In this system, each case was scored based on three major histopathological criteria: extent and intensity of inflammation, degree of necrosis and vascular and lymphatic alterations. Each parameter was scored on a scale from 0 (no finding) to 3 (severe), resulting in a total severity score ranging from 0 to 9. In the assessment of inflammation, the intensity of parenchymal infiltration, the degree of red-to-grey hepatization, and pleural surface involvement were considered. For necrosis, the evaluation included sparse necrotic cells at the alveolar level, focal coagulative necrosis, and multifocal or massive necrosis. Vascular evaluation encompassed findings such as congestion, hemorrhage, vasculitis, and thrombosis in blood and lymphatic vessels. All scoring procedures were performed by the same observer using a blinded evaluation method.

Real-Time PCR and primer design: DNA was extracted from paraffin-embedded lung tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen, Cat No: 56404, Hilden, Germany) following the manufacturer's protocol. Three consecutive 5µm sections were obtained from each sample, deparaffinized with xylene, and rehydrated through a graded ethanol series. After proteinase K incubation, the samples were purified through spin columns. DNA concentration and purity were measured by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and only samples with an A260/280 ratio 1.8-2.0 were included in the study.

Molecular analyses were performed using a multiplex Real-Time PCR (LightCycler 96, Roche Diagnostics, Switzerland) method based on TaqMan probe technology. For each sample, a 20µL reaction volume was prepared containing 10µL of 2X Master Mix (Roche), 1µL of each target-specific primer pair (10µM), 1µL of probe (5µM), 5µL of DNA template, and 2µL of nuclease-free water. The amplification protocol included: initial denaturation at 95°C for 10min, followed by 45 cycles of denaturation at 95°C for 15 seconds and extension at 64°C for 45 seconds. A final cooling step was applied at 37°C for 30 seconds.

A total of three separate multiplex PCR panels were designed. The first panel targeted *Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni*; the second panel targeted *Mycoplasma bovis* and *Trueperella pyogenes*. The third panel was designed to detect three macrolide resistance genes: *erm(42)*, *msr(E)*, and *mph(E)*. Primers and probes were selected from previously published studies (Rose *et al.,* 2012; Zhang *et al.,* 2017; Pratelli *et al.,* 2021), synthesized commercially, and labelled with specific fluorophores (FAM, HEX, Texas Red, Cy5), (Table 1-2). Each panel was tested in triplicate, including positive and negative controls. Data analysis was

performed using the LightCycler 96 Software (v1.1.0.1320, Roche Diagnostics, Switzerland).

Statistical Analyses: All statistical analyses in this study were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA), with a significance level set at $P<0.05$. Prior to analysis, the distribution characteristics of pneumonia severity scores, both by pneumonia type and by the number of BRD pathogens identified, were examined using the Shapiro–Wilk test. Since the data did not show normal distribution, comparisons between groups were made with the Kruskal–Wallis test. Then, the Mann-Whitney U test with Bonferroni correction was applied to determine in which groups the statistical difference occurred.

Table 1: Gene Sequences of Major Bacterial Pathogens in BRD

Bacterium	Direction	Primer Sequence (5'–3')
P. multocida	Forward	GGGCTTGTCGGTAGTCTTT
	Reverse	CGGCAAATAACAATAAGCTGAGTA
	Probe	Texas Red-ACGTAGCTAGCTGACTGATCGTACG-BHQ2
M. haemolytica	Forward	ATTAGTGGGTTGTCCTGGTTAG
	Reverse	GCGTGATTTCGGTTCAGTTG
	Probe	HEX-ACGTAGCTAGCTGACTGATCGTACG-BHQ1
H. somni	Forward	AAGGCCTTCGGGTTGTAAAG
	Reverse	CCGGTGCTTCTTCTGTGATTAT
	Probe	FAM-ACGTAGCTAGCTGACTGATCGTACG-BHQ1
M. bovis	Forward	TCAAGGAACCCCACCAGAT
	Reverse	AGGCAAAGTCATTTCTAGGTGCAA
	Probe	FAM-ACGTAGCTAGCTGACTGATCGTACG-BHQ1
T. pyogenes	Forward	CAGTCAAGGGTGAGTCTATT
	Reverse	CTTGAACTCTGTGGAAA
	Probe	HEX-ACGTAGCTAGCTGACTGATCGTACG-BHQ1

Table 2: Macrolide Resistance Gene Sequences

Genes	Direction	Primer Sequence (5'–3')
erm (42)	Forward	TGCACCATCTTACAAGGAGT
	Reverse	CATGCCTGTCTTCAAGGTTT
	Probe	FAM-ACGTAGCTAGCTGACTGATCGTACG-BHQ1
mph(E)	Forward	ATGCCCAGCATATAAATCGC
	Reverse	ATATGGACAAAGATAGCCCG
	Probe	HEX-ACGTAGCTAGCTGACTGATCGTACG-BHQ1
msr (E)	Forward	TATAGCGACTTTAGCGCCAA
	Reverse	GCCGTAGAATATGAGCTGAT
	Probe	Cy5-ACGTAGCTAGCTGACTGATCGTACG-BHQ3

To explore the relationship between pathogen number and pneumonia severity, Spearman's rank correlation was applied. Given the ordinal structure of the severity score, an ordinal logistic regression (OLR) model was also constructed. This model assessed the independent effect of pathogen count while controlling for age group (0–6 months vs. >6 months) and infection complexity (single vs. multiple agents), using the logit link function.

The predictive capacity of both pathogen count and the combined risk score, derived from the sum of bacterial agents and resistance genes, was further evaluated using Receiver Operating Characteristic (ROC) analysis. Cases with a pneumonia severity score of ≥6 were classified as "severe" and analysed as a binary variable (0 = non-severe, 1 = severe). The Area Under the Curve (AUC) values provided a quantitative measure of each model's discriminative performance.

RESULTS

Pathological characterization and pneumonia severity index: Necropsies of 40 calves (<6 months) and 40 adult cattle (>6 months) diagnosed with pneumonia revealed fibrinous bronchopneumonia and characterized by red-brown consolidated areas primarily in the cranial lobes of the lungs. Thickened interlobular septa, pleural fibrinous exudate, and adhesions in the thoracic cavity were also observed. On cut surfaces, purulence (fibrinopurulent bronchopneumonia) was observed in some cases and necrotic areas (fibrinonecrotic pneumonia) in others.

Microscopically, the distribution of pneumonia types and diagnoses for each case is summarized in Fig. 1 for both calves and adult cattle. Fibrinous pneumonia was observed most frequently in calves (29 cases) and adult cattle (25 cases), while fibrinonecrotic pneumonia was the second most prevalent type, observed in six and 13 cases, respectively.

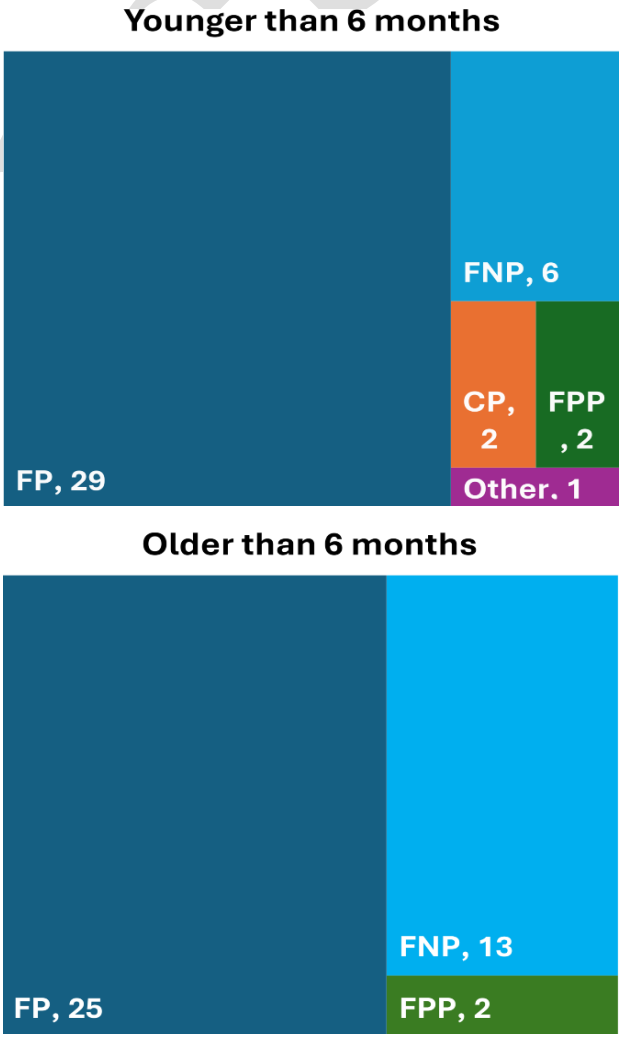

Fig. 1: Distribution of pneumonia types (FP: Fibrinous pneumonia; FNP: Fibrinonecrotic pneumonia; FPP: Fibrinopurulent pneumonia; CP: Catarrhal pneumonia).

In cases of fibrinous pneumonia, initial inflammatory hyperemia was observed, followed by red hepatization, in which the alveoli filled with neutrophils and fibrin, and finally grey hepatization, in which hyperemia decreased, and the alveoli filled with neutrophils, exfoliated epithelial cells, and dense fibrin (Fig. 2A). Additionally, interlobular septa were markedly thickened due to inflammation and fibrinous exudate accumulation (Fig. 2B). In some cases, necrotic foci surrounded by inflammatory infiltrates and characteristic oat cells (elongated nuclei arranged in streaming patterns) were observed (Fig. 2C). Fibrinonecrotic pneumonia was distinguished by extensive areas of coagulative necrosis (Fig. 2D).

In line with histopathological findings, a pneumonia severity score was calculated for each case to reflect the severity associated with pneumonia type. Cases diagnosed as fibrinonecrotic pneumonia exhibited the highest average severity scores compared to all other pneumonia types. Pairwise comparisons of the mean total severity scores among pneumonia types revealed a statistically significant difference particularly between fibrinous and fibrinonecrotic pneumonia ($P<0.0001$). Mean scores, standard deviations, and results of multiple comparisons are presented in Table 3.

Table 3: Comparison of histopathological pneumonia severity index across different pneumonia types (Mean±SD)

Parameter	Comparison	Mean± SD		Bonferroni Adjusted p	Sig.
Pneumonia Severity Index	Fibrinous vs Fibrinonecrotic	5.17±1.26	7.53±1.54	< 0.001	*
	Fibrinous vs Fibrinopurulent	5.17±1.26	6.0±0.82	0.5896	ns
	Fibrinonecrotic vs Fibrinopurulent	7.53±1.54	6.0±0.82	0.1843	ns

* Statistically significant difference was observed between fibrinous and fibrinonecrotic pneumonia types based on Bonferroni-adjusted Mann–Whitney U test following Kruskal–Wallis analysis ($P<0.0001$).

Detection of BRD pathogens and their relationship with pneumonia types: The results of multiplex Real-Time PCR analyses targeting five major bacterial pathogens associated with BRD are summarized by age group and pneumonia type in Fig.s 3A and 3B. In the context of fibrinous pneumonia, the prevalence of major bacterial pathogens was found to be similar in both groups. *M. haemolytica* was the most frequently identified agent in cases of fibrinonecrotic pneumonia. No prominent agent or cluster was identified in the less common cases of fibrinopurulent pneumonia.

The distribution of single and multiple BRD pathogens according to pneumonia type and age group is summarize

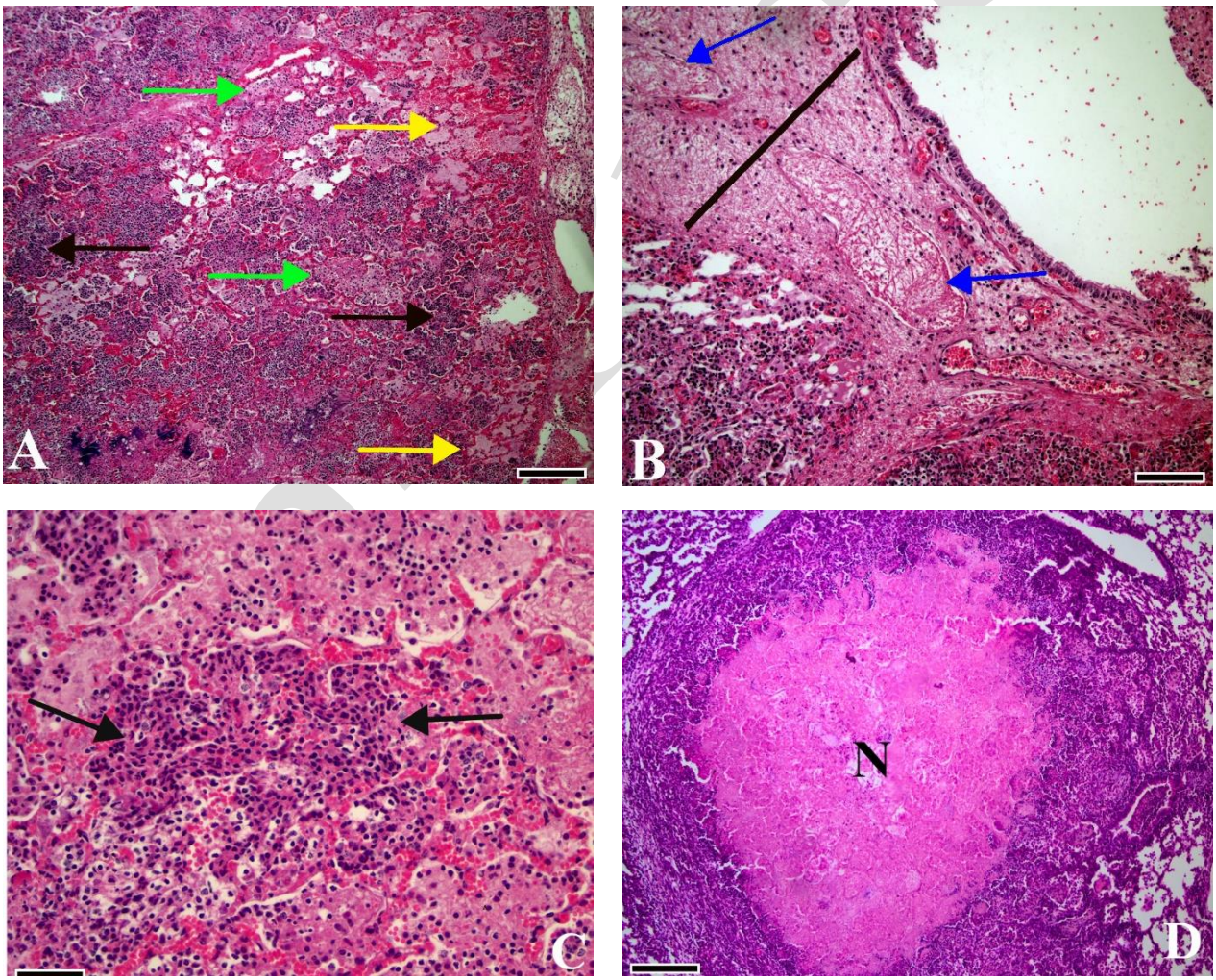

Fig. 2: Histopathological microphotographs. A: Fibrinous pneumonia. Inflammatory hypremia (yellow arrow), red hepatization (green arrow), gray hepatization (black arrow), HE, Scale bar: 200 µm; B: Enlargement of interlobular septa due to inflammation and fibrinous exudate (black line) and fibrinous thrombus in lymph vessels (blue arrow), HE, Scale bar: 100µm; C: Oat cell formations (black arrows), HE, Scale bar: 50 µm; D: Fibrinonecrotic pneumonia, extensive area of necrosis (N), HE, Scale bar: 100 µm.

in Fig. 4. As clearly illustrated in the Fig., a substantial proportion of cases involved more than one bacterial agent contributing simultaneously to the infectious process. Multiple infections with two or more agents were detected in approximately 70% of cases in the 0–6-month age group (28/40 cases) and the adult group (27/40 cases).

The results of the pathogen combination analysis detailed the distribution of concurrently detected BRD agents by pneumonia type and age group (Fig. 5). The data indicate that multi-agent infections are predominant in BRD cases and that specific combinations vary by pneumonia type and age group. A broader spectrum of pathogen diversity and combination patterns was observed in fibrinous pneumonia cases within the 0–6-month age group, whereas in older animals, the combinations were fewer but more frequently clustered around specific pathogen sets.

Comparison of mean pneumonia severity index by age group and pathogen complexity: For each individual within the same age group and pathogen complexity category, histopathological pneumonia severity scores ranging from 0 to 9 were summed to calculate a total pneumonia score. This total was then divided by the number of cases in each group to determine the mean severity index. The results of this analysis, based on age group (0–6 months / >6 months) and pathogen complexity (single / multiple), are presented in Table 4.

As demonstrated in Table 4, infections involving multiple pathogens exhibited higher mean severity scores compared to single-agent cases, both in the 0–6-month group and in animals older than 6 months. This trend remained consistent across all age groups, and the presence of multiple pathogens was statistically associated with significantly increased pneumonia severity ($P<0.05$).

Effect of agent count on pneumonia severity: The Kruskal–Wallis test revealed statistically significant differences in pneumonia severity scores among groups defined by different pathogen counts ($P<0.0001$). Further analyses showed that cases with five detected pathogens had significantly higher pneumonia scores compared to all other groups. Additionally, the presence of three or more pathogens was associated with a marked increase in severity scores (Fig. 6A). Spearman's rank correlation analysis demonstrated a strong positive correlation between the number of pathogens and pneumonia severity scores ($\rho=0.76$, $P<0.0001$) (Fig. 6B). Ordinal logistic regression analysis confirmed that the number of pathogens had an independent and statistically significant effect on pneumonia severity ($\beta=1.56$, $P<0.001$) (Fig. 6C). Finally, ROC analysis indicated that pathogen count had high predictive performance for identifying severe pneumonia cases (defined as a score ≥ 6), with an AUC value of 0.90 (Fig. 6D).

Table 4: Pneumonia severity index and statistical significance by age group and agent complexity

Age Group	Pathogen Complexity	Number of Cases	Total Score	Mean Score	p-value	Significance
0–6 months	Single	10	47	4.7	<0.05	*
	Multiple	28	171	6.11	<0.05	*
>6 months	Single	11	48	4.36	<0.001	**
	Multiple	27	183	6.78	<0.001	**
All Ages	Single	21	95	4.53	<0.0001	***
	Multiple	55	354	6.44	<0.0001	***

*Significance levels are indicated by asterisks based on Bonferroni-adjusted Mann–Whitney U test: $P < 0.05$ (*), $P < 0.001$ (**), $P<0.0001$ (***).

Resistance gene profiles by pathogen and pneumonia type: Among cases in which at least one BRD pathogen was detected, macrolide resistance gene analysis by multiplex Real-Time PCR revealed a very high rate of positivity (76-94%) in both age groups.

The distribution of macrolide resistance gene combinations by age group-pathogen positivity, and age group-pneumonia type is presented in Fig. 7. The most frequently observed resistance gene combination was *msr(E)* + *mph(E)*, whereas *erm(42)* alone or in combination was detected in only a few cases. Notably, dual and triple resistance gene combinations were markedly clustered in calves aged 0–6 months who tested positive for *M. haemolytica* and *P. multocida* (Fig. 7A). According to the type of pneumonia, fibrinous and fibrinonecrotic pneumonias stood out in terms of both positivity rates and the diversity of resistance gene combinations. Triple gene profiles were predominant in these pneumonia types, and *msr(E)* + *mph(E)* combination was also observed in these groups. In contrast, resistance gene profiles were limited in cases of fibrinopurulent pneumonia (Fig. 7B).

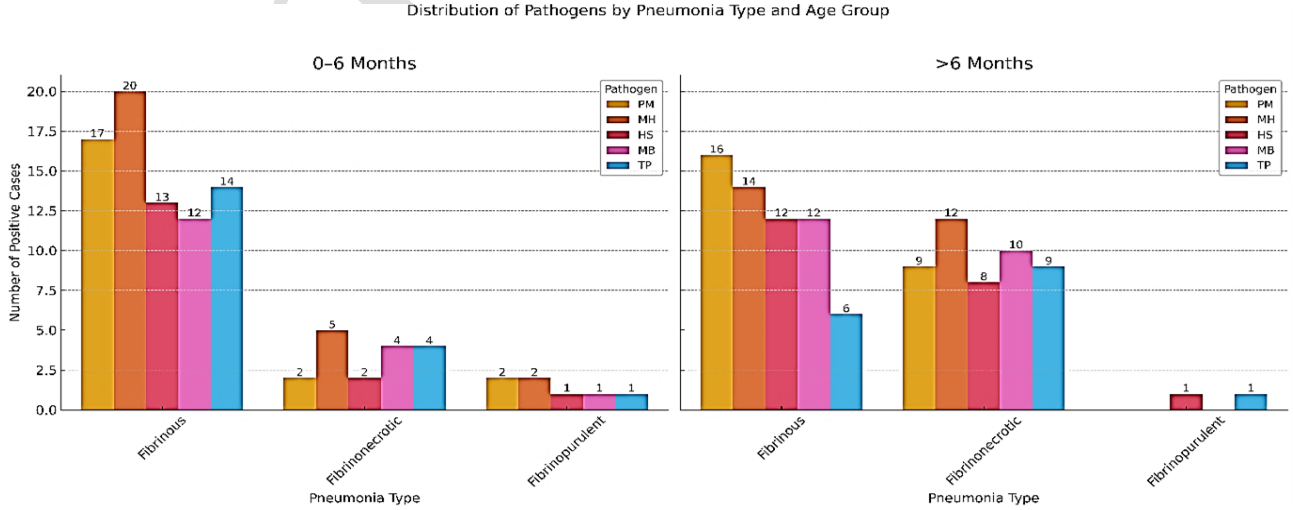

Fig. 3: Distribution of BRD pathogens by pneumonia type and age group (PM: *Pasteurella multocida*, MH: *Mannheimia haemolytica*, HS: *Histophilus somni*, MB: *Mycoplasma bovis*, TP: *Trueperella pyogenes*).

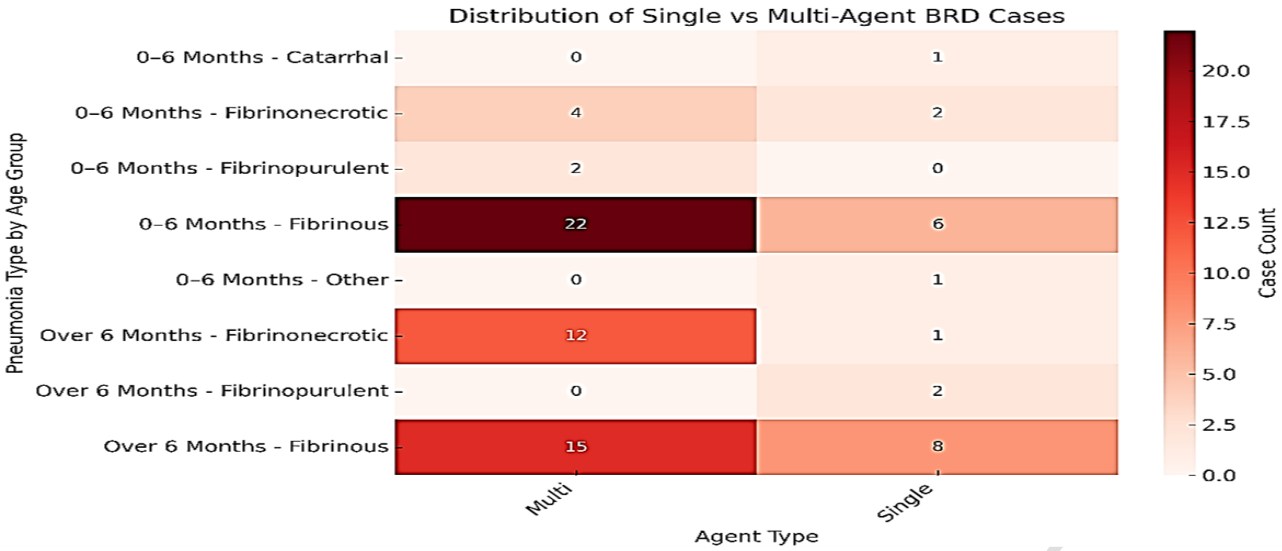

Fig. 4: Distribution of single and multi-agent BRD cases by pneumonia type and age group.

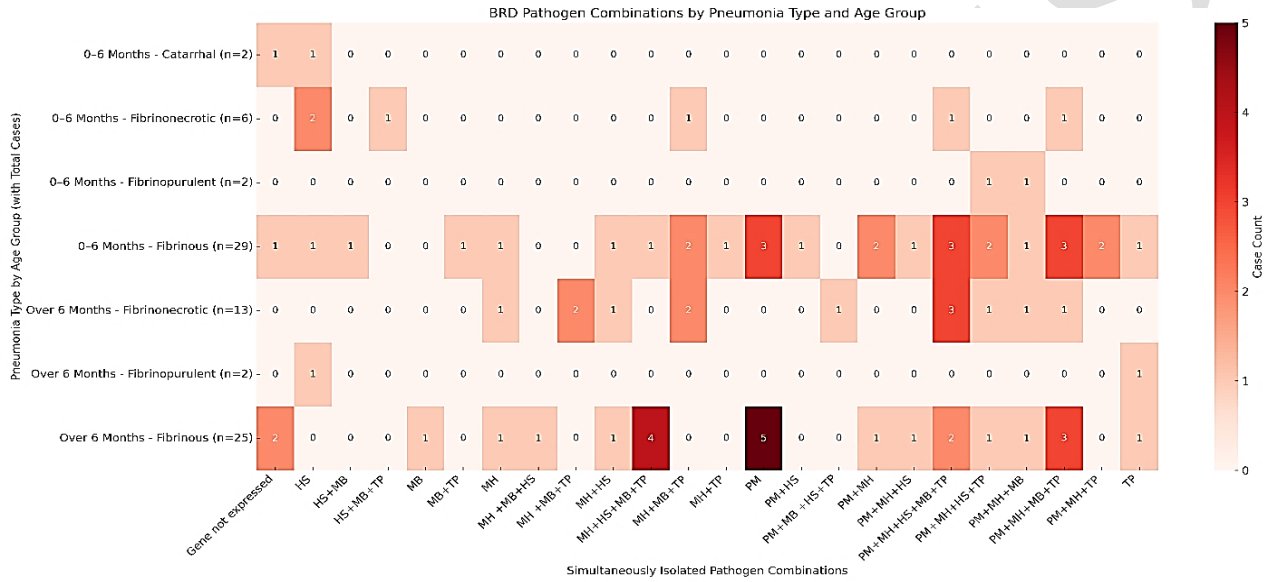

Fig. 5: Distribution of BRD pathogen combinations by pneumonia type and age group.

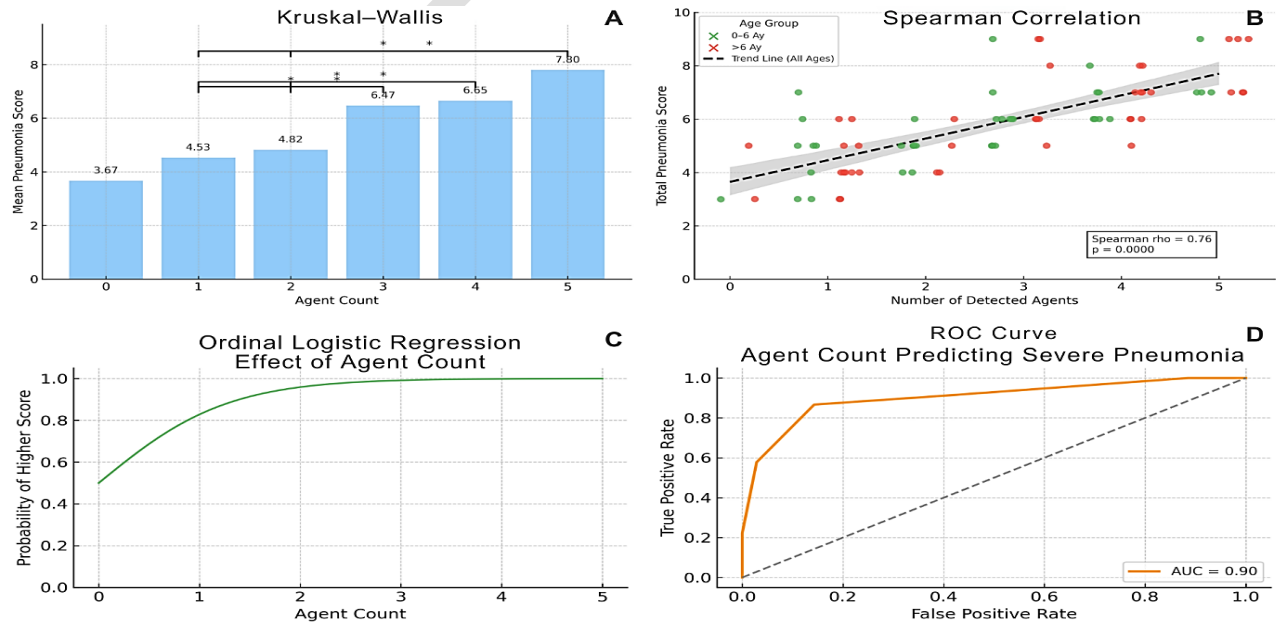

Fig. 6: Multidimensional analyses of the effect of agent count on pneumonia severity.

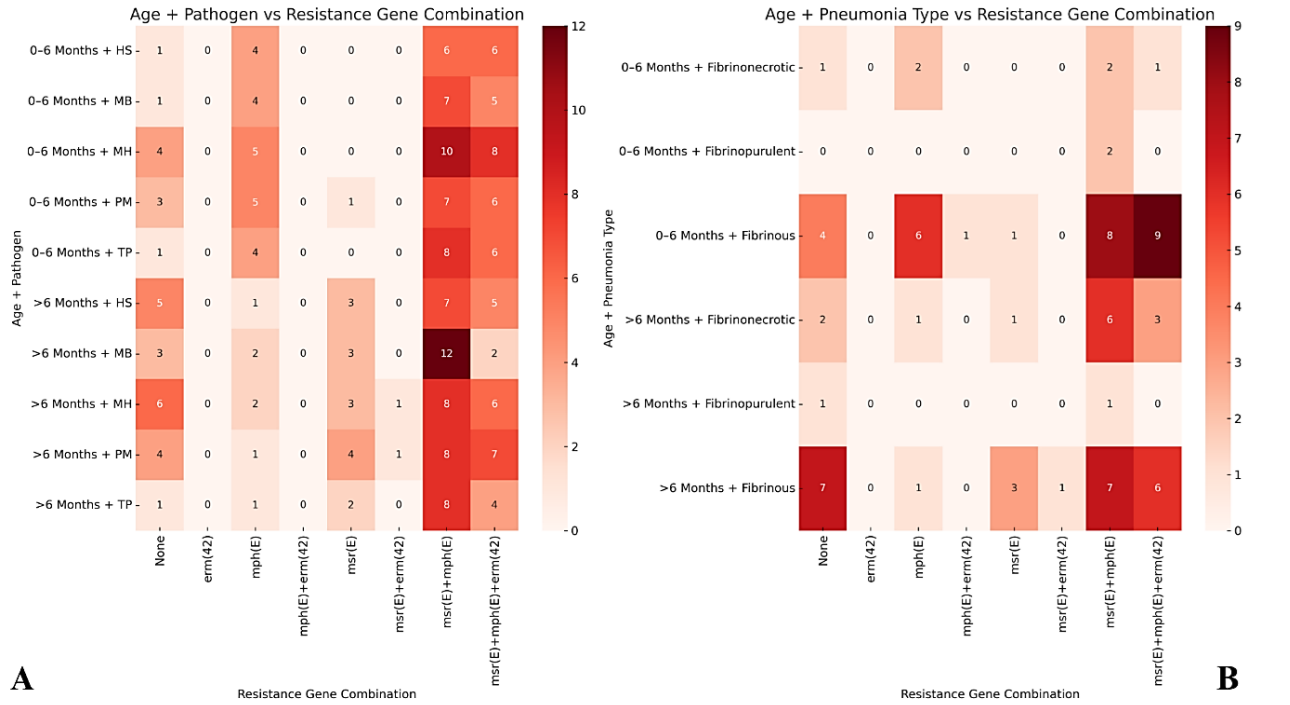

Fig. 7: Distribution of macrolide resistance gene combinations by age group, pathogen, and pneumonia type. **A)** Resistance gene profiles by age group and BRD pathogen positivity. B) Resistance gene profiles by age group and histopathological pneumonia type.

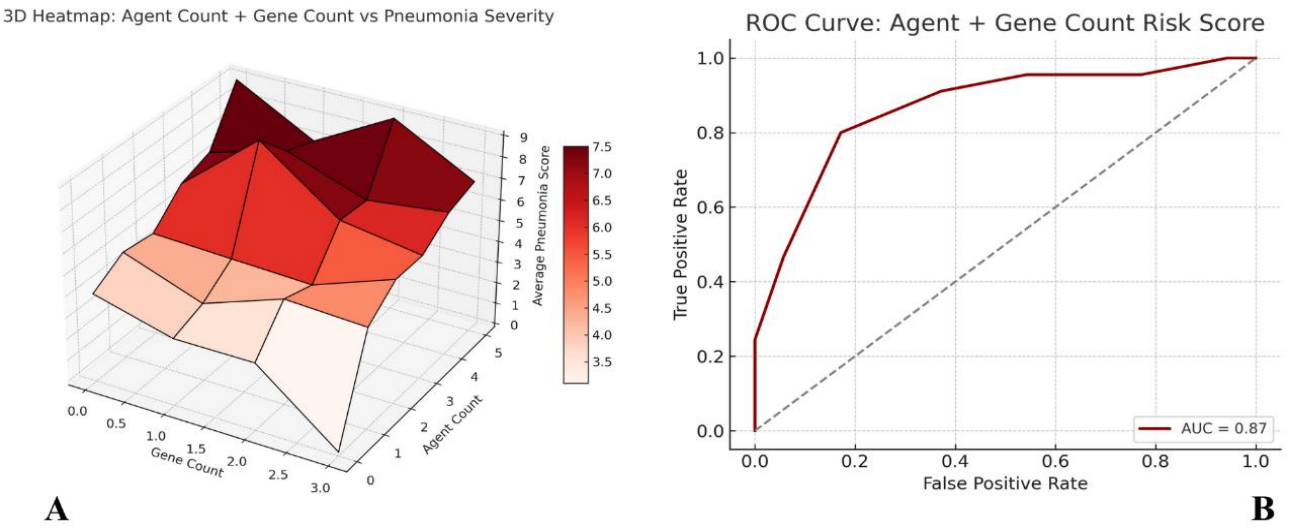

Fig. 8: Combined effect of agent count and resistance gene burden on pneumonia severity. A) 3D surface plot showing average pneumonia cores by agent and gene count. B) ROC curve for combined risk score predicting severe pneumonia (AUC=0.87).

The combined analysis of pathogen count and resistance gene load demonstrated a parallel increase in pneumonia severity (Fig. 8A). Specifically, in cases infected with two or more pathogens and carrying multiple resistance genes simultaneously, the mean pneumonia severity score approached 6, with some combinations exceeding a score of 7. ROC analysis demonstrated that this combined risk score had high predictive power for severe pneumonia (score ≥6; AUC = 0.87; Fig. 8B).

DISCUSSION

Bovine respiratory disease complex (BRD) causes serious economic losses due to its high lethality in calves and adult cattle (Deepak *et al.,* 2021; Chai *et al.,* 2022). This study aimed to assess the histopathological severity of pneumonia and its association with major bacterial pathogens and macrolide resistance genes in BRD cases with a history of macrolide use.

Histopathological examinations revealed that fibrinous and especially fibrinonecrotic pneumonias were dominant in both age groups. According to the histopathological severity scoring, the fibrinonecrotic form exhibited higher scores compared to all other types. In most of these cases, *Mannheimia haemolytica* was identified, which aligns with its known ability to produce leukotoxins that damage endothelial cells and contribute to necrosis (Klima *et al.,* 2019; Hatipoglu *et al.,* 2024). Dorso *et al.* (2021) reported that fibrinonecrotic bronchopneumonia lesions were clearly associated with *M. haemolytica*; Booker *et al.* (2008) reported that the fibrinonecrotic pattern was dominant in fatal BRD cases; Singh *et al.*

(2011) showed that this pattern correlated with the progressive and systemic course of the infection. Similarly, Caswell and Williams (2016) emphasized that fibrinonecrotic bronchopneumonia was linked to rapidly progressing forms of pneumonia and poor prognosis. Accordingly, widespread vascular damage and necrosis observed in BRD deaths may be a prognostically significant and reliable marker in predicting pneumonia severity.

Although pathological pneumonia diagnoses generally determine the disease type, it is crucial to consider that severity and prevalence can vary even within the same type. It is clear that important factors such as the clinical course of the disease, its spread within the herd and the severity of the lesion, which affect mortality, need to be evaluated more sensitively. Therefore, we propose that the histopathological severity score applied in our study should be integrated into academic research and veterinary field applications for more effective pneumonia management.

The multiplex qPCR results for major BRD pathogens were remarkable. Multiple agents were detected simultaneously in the majority of cases (55 multi-agent vs. 21 single-agent), a tendency consistent across both age groups. Crucially, pneumonia severity increased significantly as the number of agents increased. This finding aligns with the known multifactorial nature of BRD and also supports the view that a higher number of agents may increase tissue damage (Lachowicz-Wolak *et al.,* 2022; Hatipoglu *et al.,* 2024; Kamel *et al.,* 2024).

In support of this interpretation, our correlation and regression analyses confirmed a significant effect of agent number on pneumonia severity. In addition, ROC analysis was performed to evaluate the ability of agent count to predict severe pneumonia (score≥6; AUC: 0.90). These results provide strong evidence that multi-agent combinations increase the severity of morphological pneumonia in BRD. However, additional studies with larger populations are needed to translate the findings to clinical practice and develop early warning systems.

Our study revealed that the most frequent agent combination was *Mannheimia haemolytica*, *Pasteurella multocida*, and *Mycoplasma bovis*. The synergistic harmful effects of these agents on the alveolar epithelium, bronchioles, and vessels likely play a significant role in the pathogenesis of fatal BRD (Caswell and Williams 2016; Buczinski *et al.,* 2024). The concentration of these multi-agent combinations in the fibrinous and fibrinonecrotic pneumonia types exacerbates the complex morphological course, increasing the risk of severe clinical pictures. This suggests that the presence of these specific multi-agent combinations is a critical determinant of disease severity and outcome in BRD.

The polymicrobial nature of BRD is influenced by major viral pathogens, including bovine respiratory syncytial virus (BRSV), parainfluenza virus type 3 (PI-3), bovine herpesvirus-1 (BoHV-1), and bovine coronavirus (BcoV) (Gaudino *et al.,* 2022; Buczinski *et al.,* 2024). According to Caswell *et al.* (2016), these viral agents are known to harm the immune system and the respiratory tract epithelium, which promotes secondary bacterial colonization and intensifies symptoms.

Epidemiological studies have revealed the presence of viral-bacterial co-infection in more than 50% of BRD cases (Murray *et al.,* 2017; Saegerman *et al.,* 2022,). Although viral agents were not screened in our study, the fatal clinical course and severe fibrinous-necrotic lesions in the studied cases are likely a consequence of this synergistic interaction. This is also consistent with metagenomic studies that have detected significantly higher viral loads in sick animals in BRD cases compared to healthy animals (Murray *et al.,* 2017; Zhang *et al.,* 2019).

In the current study, at least one macrolide resistance gene was found in 76-95% of BRD pathogen-positive cases. Of these resistance genes, *msr(E)* encodes an efflux pump that transports the antibiotic molecule out of the cell, and the *mph(E)* gene encodes an enzyme called macrolide phosphotransferase that inactivates the drug through phosphorylation (Rose *et al.,* 2012). The *erm(42)* gene, which is less frequent than the others, prevents ribosomal binding of antibiotics through 23S rRNA methylation, thus changing the target region and causing the drugs to become inactive (Leclercq, 2002). In our study, the *msr(E)* + *mph(E)* combination was the most prevalent. This finding aligns with other studies as Rose *et al.* (2012) and Urban-Chmiel *et al.* (2022) reported that the prevalence of macrolide resistance genes in the *Pasteurellaceae* family was over 70%. The higher prevalence in the present study is likely attributable to the inclusion of only fatal, treatment-non-responsive cases.

For years, antibiotic resistance has been determined by measuring minimal inhibitory concentrations (MICs) (Nagaraja, 2022). Studies have reported that genes such as *erm(42)*, *msr(E)*, and *mph(E)* are responsible for resistance to macrolides and can increase MIC values by up to 1024-fold, especially when present in triple combination (Rose *et al.,* 2012; Desmolaize *et al.,* 2011; Woolums *et al.,* 2018). This suggests that treatment failures in BRD may be due to the number and diversity of pathogens and due to resistance gene load. This phenomenon is supported by our finding that cases with multi-agent infections and multiple resistance genes had higher histopathological severity scores.

Although the clinical history of prevalent macrolide uses in the field guided our focus towards the macrolide resistance genes in our study, it should be noted that this represents merely a segment of the extensive array of antimicrobial resistance in BRD. The most frequently observed resistance profile among the major pathogens of BRD is that of tetracycline resistance, which is encoded by the *tet(H)* gene (Urban-Chmiel *et al.,* 2022) while blaOXA-2-mediated beta-lactam resistance has recently exhibited an increasing trend, aminoglycoside resistance, encoded by the *ant(2")-Ia, aph(3")-Ia,* and *aadA25* genes, and fluoroquinolone resistance due to *qnr* gene and *gyrA/parC* mutations, have been reported sporadically (Stanford *et al.,* 2020; Urban-Chmiel *et al.,* 2022). Consequently, resistance genes that limit the effectiveness of antibiotics other than macrolides also play a critical role in the management of BRD.

In multi-etiological infections, multiple resistance mechanisms can aggravate the disease course, beyond just rendering treatment ineffective. This may lead to prolonged inflammation, exacerbated tissue damage, and significantly delayed recovery (Jahan *et al.,* 2022; Fan *et al.,* 2025). Therefore, new approaches that require the evaluation of antibiotic resistance gene load together with pathogen load,

which go beyond traditional diagnostic methods, are needed. In this respect, our study contributes to literature in terms of both the identification of molecular biomarkers for BRD prognosis and the management of antibiotic strategies on a herd basis.

Necropsy, which is considered the gold standard in the diagnosis of BRD, is a data source that can be obtained after death as a traditional diagnostic method (Buczinski *et al.,* 2021; Kamel *et al.,* 2024). As in the present study, the combined evaluation of the number of pathogens and resistance gene load determined by modern molecular methods with the findings obtained with traditional diagnostic models is a rarely addressed topic in the literature. In this respect, the present study presents a biomarker model that connects diagnosis, prognosis and antimicrobial decision-making in the management of BRD. It is also very critical to identify risky animals in advance and to monitor the development of antibiotic resistance in BRD management. Therefore, the obtained combined scoring system is thought to have the potential for the development of future pneumonia prognosis prediction models and epidemiological monitoring systems.

Nevertheless, the study is not without its limitations. First, our analysis was limited to macrolide resistance genes. Additionally, the study cohort included only severe clinical cases, excluding mild or subclinical ones. The absence of viral diagnostics is a limitation, particularly given the known impact of viral-bacterial co-infections in BRD. Future studies using larger pathogen panels including viral agents and metagenomic approaches will provide a more holistic approach to the molecular epidemiology of BRD (Freeman *et al.,* 2022).

Conclusions: This study shows that the severity of pneumonia in cases of BRD is strongly affected by the pathogen combination and the number of resistance genes against macrolide antibiotics. By combining molecular diagnostics with scoring lung lesions, the study introduces a novel approach to better predict disease outcomes. These results highlight the value of using molecular and pathology-based tools together in veterinary diagnostics and point to the need for broader monitoring that looks at both bacterial and viral co-infections.

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