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Phenotypic and molecular characterization of *Pasteurella multocida* and *Mannheimia haemolytica* isolated from clinically healthy calves: novel insights from Türkiye

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Abstract

Pasteurella multocida and *Mannheimia haemolytica* are significant bacteria of respiratory tract infections in cattle. Identifying whether these bacteria are commensal or pathogenic is crucial for understanding their role in predisposing calves to respiratory tract infections. This study aimed to determine the prevalence and pathogenic characteristics of *P. multocida* and *M. haemolytica* in nasal swab samples of clinically healthy calves in Konya, Türkiye, and to assess antibiotic resistance profiles. Using conventional culture methods, five samples (1.67%) from four different farms were positive for *P. multocida* and three samples (1.00%) were positive for *M. haemolytica*. The PCR of nasal swab samples yielded *P. multocida* positivity in 17 (5.67%) samples and *M. haemolytica* positivity in 13 (4.34%), including all culture-positive samples. While all *P. multocida* isolates were atypical for the capsule, 17.65% of the PCR-positive samples were L2, 70.59% L3A, and 11.76% L8 serotypes. The *ompA*, *omp87*, *hghA*, and *tbpA* genes were the most prevalent, while the presence of other virulence genes varied. High rates of APX (100%) and S (80.00%) resistance were observed among the isolates. The rates of serotypes A1 and A6 in *M. haemolytica* PCR-positive samples were 53.85 and 46.15%, respectively. The *ssa-1* gene was detected in 33.33% of the isolates. Moderate resistance to SXT, S, and N (66.67%) was recorded. The findings revealed the presence of *P. multocida* and *M. haemolytica* with various virulence characteristics and antimicrobial resistance profiles in the nasal microbiota of healthy calves providing novel insights that could inform improved herd management, disease control, and treatment strategies.

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Introduction

Respiratory tract infections are prevalent among livestock worldwide, resulting in substantial economic losses.[1] The Bovine Respiratory Disease (BRD) is employed to denote ailments of the upper and lower respiratory tracts in cattle, which are frequently the consequence of viral and bacterial co-infections.[2] In respiratory system diseases, which are more important in terms of breeding, significant losses occur in the livestock sector as a result of loss of condition, growth retardation, pneumonia and secondary infections after infection.[3] In Türkiye, the incidence of respiratory system diseases in cattle varies between 22.00 - 59.70%, depending on the region.[4] On a global scale, this rate has been known to reach as high as 67.00%.[5]

A multitude of parameters have been identified as contributing to the etiology of respiratory tract infection including stress, viral, bacterial, environmental, and animal factors. The primary bacterial agents responsible for the respiratory tract infection are *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni,* and *Mycoplasma bovis*.[6] The *M. haemolytica* and *P. multocida*, two members of the *Pasteurellaceae* family, are significant bacterial pathogens of the respiratory system in cattle.[7] It is noteworthy that cattle of all ages and breeds are susceptible (S) to respiratory system infections.[8] The *P. multocida* and *M. haemolytica* are part of the normal flora in the nasal cavity and upper respiratory tract of calves. They can progress to the lower respiratory tract and cause serious infections such as hemorrhagic septicemia and enzootic pneumonia, when the host immune system is suppressed or due to stress factors, including harsh weather, transportation, malnutrition, inadequate host defenses, nasopharyngeal colonization and dehydration.[9,10]

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In clinical cases such as septicemia and pneumonia, sampling of the nasal cavity mucosa and isolation of the causative agent are important. Differentiation of pathogenic species from normal flora by virulence gene analysis provides valuable diagnostic data in animal infections where invasive procedures such as biopsy, are difficult or uncommon. There are various serotypes of *P. multocida* and *M. haemolytica*, which are naturally localized in the upper respiratory tract, especially in the nasal cavity and certain serotypes can be identified as pathogens thanks to the virulence factors they carry.[11,12] The main virulence factors that have been identified as effective in the virulence of infections caused by *P. multocida* are as follows: Outer membrane proteins and porin proteins (omp87, psl, ompH), type 4 fimbriae (ptfA), filamentous hemagglutinin (pfhA), factors related to iron uptake (exbBD-tonB, tbpA, hgbA, hgbB) and dermonecotoxin (toxA).

The *M. haemolytica* possesses a number of virulence factors including capsular polysaccharide, lipo-polysaccharide, adhesin, outer membrane proteins, iron-binding proteins, enzymes, endotoxin and leukotoxin (LKT).[13,14] Among these, the LKT is recognized as the principal virulence factor of *M. haemolytica*, mediating cytotoxic effects on bovine leukocytes and contributing significantly to lung tissue damage and disease progression. The SSA-1 antigen is a significant protein that plays a crucial role in the progression of lung infections.[15]

The use of antibiotics is an important component of the treatment of *P. multocida* and *M. haemolytica* infections in livestock animals. Despite the documented efficacy of antibiotic treatment in managing such infections, the frequent utilization of suboptimal doses or broad-spectrum antibiotics in therapeutic regimens has been identified as a pivotal factor in the emergence of resistance to antibiotics.[16] However, comprehensive data on the nasal carriage, virulence characteristics, and antimicrobial resistance of *P. multocida* and *M. haemolytica* in healthy calves from the Konya region are currently lacking. This study aimed to ascertain the prevalence of nasal carriage of *P. multocida* and *M. haemolytica* in healthy calves raised in Konya province, to determine the pathogenic characteristics of the identified agents, and to evaluate their antibiotic resistance profiles.

Materials and Methods

Study samples. The sample size (n = 384) was initially calculated based on an estimated population size of approximately 130,000 calves in the study area (TÜİK, 2024),[17] assuming a 95.00% confidence level, a 5.00% margin of error. However, due to practical constraints, including the availability of clinically healthy calves and logistical limitations in accessing farms within the study period, a total number of 300 samples were collected and analyzed. Despite being slightly lower than the calculated sample size, 300 samples still provide sufficient statistical power to estimate the prevalence and characterize the isolates reliably, and this number is consistent with sample sizes used in similar studies. The calves were selected using a stratified random sampling approach, taking into account both age groups (1 week to 6 months) and the seven different farms to ensure representative coverage of the study population. The farms included in the study had herd sizes ranging from approximately 50 to 200 animals and were managed under intensive or semi-intensive husbandry systems. The sampled calves represented a mixture of breeds, including Holstein, Swiss Brown and Simmental, with approximately equal distribution of males and females across the age groups. None of the calves sampled had been vaccinated against *P. multocida* or *M. haemolytica*, nor had they been treated with antibiotics. Nasal swap samples were immediately delivered to Necmettin Erbakan University, Faculty of Veterinary Medicine, Department of Microbiology laboratory under cold chain and cultured. Ethical approval of the study was obtained from Selçuk University Faculty of Veterinary Medicine Ethics Committee (2022/94). All procedures subject to the research were carried out within the scope of all guidelines for the scientific use of animals.

Bacterial culture procedure. The nasal swabs were enriched by incubating in 10.00 mL Brain Heart Infusion Broth (Merck, Darmstadt, Germany) under aerobic conditions at 37.00 ˚C for 18 hr. The identification of these bacteria forming suspicious colonies was performed based on their microscopic morphology, motility, ability to grow on MacConkey agar (Neogen, Lansing, USA), and indole and H_2S activities.[18] *Pasteurella multocida* American Type Culture Collection (ATCC) 12945 and *Mannheimia haemolytica* ATCC 43270 were used as positive controls.

DNA extraction. The DNA was extracted from nasal swab samples and culture isolates using a commercial kit (Wizard® Genomic DNA Purification Kit; Promega, Madison, USA) following the manufacturer's instructions. For nasal swab samples, the samples were immediately placed into 1.00 mL of sterile phosphate-buffered saline after collection and vortexed thoroughly to elute the sample material. Approximately 200 µL of the resulting suspension was used for DNA extraction. For culture isolates, 1.00 mL of an overnight culture of the isolates was centrifuged at 13,000 - 16,000 *g* for 2 min to obtain a pellet of the cells. Then, this pellet was used for DNA extraction. For PCR analysis, 10.00 - 40.00 ng μL^{-1} of the extracted DNA was used as the template in each reaction.

Polymerase chain reaction (PCR) analysis. The PCR analysis were performed using 5.00 X PCR master mix according to the manufacturer's instructions (HOT FIREPol; Solis BioDyne, Tartu, Estonia). Detection of *P. multocida* DNA was carried out by a species-specific PCR 1 (hereinafter referred to as tPCR1) targeting the *kmt1* gene,

while *M. haemolytica* DNA was detected by a species-specific PCR 2 (hereinafter referred to as tPCR2) targeting the *mann16s* gene. These PCRs were used both for the confirmation of *P. multocida* and *M. haemolytica* isolates obtained after bacterial culture and for the direct analysis of these agents in the nasal swab samples. Capsule typing of *P. multocida* isolates was conducted using multiplex PCR (hereinafter referred to as kPCR) amplifying the *capA, capB, capD, capE*, and *capF* genes. Serotyping of *P. multocida* isolates was performed by multiplex PCR (hereinafter referred to as sPCR1), which identified serotypes L1 - L8, L3A, and L6A. For *M. haemolytica*, serotyping was conducted by multiplex (hereinafter referred to as sPCR2) and monoplex PCR (hereinafter referred to as sPCR3), targeting serotypes 1, 2, and 6. Virulence-associated genes of *P. multocida* were screened by monospecific PCRs (hereinafter referred to as vPCR1 - vPCR12). These included genes encoding the toxin (*toxA*), adhesins (*ptfA, pfhA, tadD*), iron-acquisition factors (*exbB, exbD, tonB, hgbA*) and protectins (*ompA, ompH, omp87, plpB*). For *M. haemolytica* isolates, virulence analysis targeted the specific antigen 1 (*ssa-1*) and *lkt* genes using monospecific PCRs (hereinafter referred to as vPCR13 and vPCR14). Primer sequences and PCR cycling conditions are summarized in Table 1. All PCR products were analyzed by 1.50% agarose gel electrophoresis and gels were examined under ultraviolet light. Product sizes listed in Table 1 were used to identify the corresponding genes. *Pasteurella multocida* ATCC 12945, NCTC 10323, ATCC 12948, and ATCC 43020 and *Mannheimia haemolytica* ATCC 43270, ATCC 29694 and ATCC 29697 strains were used as positive controls. Negative controls, consisting of nuclease-free water as a no-template control, were included in every PCR run to ensure the absence of contamination.

Antimicrobial susceptibility analysis. Antimicrobial susceptibility testing of *P. multocida* and *M. haemolytica* isolates was performed by the Kirby-Bauer disk diffusion method. The evaluation of the results was performed in accordance with the directives of the European Committee on Antimicrobial Susceptibility Testing (Version 15.0; EUCAST, Växjö, Sweden).[25] Ampicillin (AM; 10.00 mg), trimethoprim/sulphamethoxazole (SXT; 1.25/23.75 mg), ceftiofur (FUR; 30.00 mg), penicillin G (P; 10.00 U), ampicillin/cloxacillin (APX; 30.00 mg), cefoperazone (CEP; 75.00 mg), streptomycin (S; 10.00 mg) and neomycin (N; 30.00 mg) disks (Bioanalyse, Ankara, Türkiye) were used to determine the antimicrobial resistance profile. Since no disk diffusion breakpoints are available for *M. haemolytica* in any of the current guidelines, the breakpoint values reported for *P. multocida* (a surrogate microorganism) were used for interpretation. Therefore, breakpoints (susceptible ≥ 17.00 mm and resistant < 17.00 mm) were considered for AM, P and APX; (susceptible ≥ 23.00 mm and resistant < 23.00 mm) for SXT and (susceptible ≥ 26.00 mm and resistant < 26.00 mm for FUR and CEP (based on the values reported for the third-generation cephalosporin cefotaxime). For aminoglycosides (S and N) without defined breakpoints, interpretation was performed according to Kumakawa *et al.*,[26] using the dihydrostreptomycin breakpoints values (susceptible ≥ 8.00 mm for *P. multocida* and susceptible ≥ 10.00 mm for *M. haemolytica*). Multidrug-resistance (MDR) was defined as isolates showing resistance to at least one antimicrobial drug in three or more antimicrobial categories.[27] The antimicrobials tested in this context included AM, P and APX that were in the penicillin group; FUR and CEP in the cephalosporin group; S and N in the aminoglycoside group and SXT in the sulfonamide combined folate synthesis inhibitor group.

Statistical analysis. Descriptive statistical analyses were performed to summarize the data. The prevalence of *P. multocida* and *M. haemolytica* as well as the distribution of virulence genes, serotypes and antimicrobial resistance profiles were expressed as frequencies and percentages. Data were organized and analyzed using Microsoft Excel (version 16.110.2; Microsoft Corp., Redmond, USA). No inferential statistical tests were applied as the study design aimed to provide a descriptive overview rather than to establish statistical associations.

Results

Bacterial culture. Bacterial culture revealed *P. multocida* positivity in five samples (1.67%) from four different farms and *M. haemolytica* positivity in three samples (1.00%) from three different farms. *P. multocida* concurrent culture positivity was detected in all calves from which *M. haemolytica* was isolated.

Identification of *P. multocida* and *M. haemolytica*. All five isolates obtained as a result of cultural analysis and identified as *P. multocida* by phenotypic tests were confirmed as *P. multocida* by species-specific PCR. All three isolates obtained as a result of cultural analysis and identified as *M. haemolytica* by phenotypic tests were confirmed as *M. haemolytica* by species-specific PCR (Fig. 1).

Direct identification from the swab samples. Following direct PCR analysis of the nasal swab samples, *P. multocida* positivity was detected in 17 (5.67%) samples, including five culture-positive isolates, while *M. haemolytica* was detected in 13 (4.34%) samples including three culture-positive isolates (Fig. 1).

PCR-mediated capsule typing. As a result of kPCR, none of the *P. multocida* isolates were found to be *capA*, *capB*, *capD*, *capE*, and *capF* gene positive (data not presented). Thus, all of these isolates were designated as atypical isolates. Although the absence of the capsule genes suggested reduced virulence, these isolates still possessed other virulence genes, as presented below, and could not be definitively classified as non-pathogenic.

PCR-mediated serotyping. Out of the five isolates identified as *P. multocida* by phenotypic tests and PCR,

four (80.00%) were identified as L3A serotype (Fig. 1) and one (20.00%) was L8 serotype. After direct PCR analysis of nasal swab samples, three (17.65%), 12 (70.59%), and two (11.76%) of the *P. multocida* positive samples were identified as L2, L3A, and L8 serotypes, respectively (Table 2). Out of the three isolates identified as *M. haemolytica* by phenotypic tests and PCR, two (66.67%) were identified as Serotype A6 and one (33.33%) was Serotype A1. After direct PCR analysis of nasal swab samples, seven (53.85%) and six (46.15%) of the *M. haemolytica* positive samples were identified as Serotype A1 and Serotype A6, respectively (Table 3).

Table 1. PCRs and conditions used for molecular characterization of *Pasteurella multocida* and *Mannheimia haemolytica*.

PCR	Genes	Primary	Primer Sequence (5'—3')	Cycling conditions	Product (bp)	References
PCR type						
tPCR1	*kmt1*	kmt1 F	ATCCGCTATTTACCCAGTGG	95.00 ˚C 5 min, 30 cycle (95.00 ˚C 30 sec, 55.00 ˚C 30 sec, 72.00 ˚C 30 sec), 72.00 ˚C 5 min	460	19
		kmt1 R	GCTGTAAACGAACTCGCCAC			
tPCR2	*mann16S*	mann16s F	TGGGCAATACGAACTACTCGGG	95.00 ˚C 5 min, 35 cycle (95.00 ˚C 1 min, 53.00 ˚C 1 min, 72.00 ˚C 1 min), 72.00 ˚C 15 min	227	20
		mann16s R	CTTTAATCGTATTCGCAG			
P. multocida capsule typing						
kPCR	*capA*	capA F	TGCCAAAATCGCAGTCAG	95.00 ˚C 5 min, 30 cycle (95.00 ˚C 30 sec, 55.00 ˚C 30 sec, 72.00 ˚C 30 sec), 72.00 ˚C 5 min	1,044	19
		capA R	TTGCCATCATTGTCAGTG			
	capB	capB F	CATTTATCCAAGCTCCACC		760	
		capB R	GCCCGAGAGTTTCAATC			
	capD	capD F	TTACAAAAGAAAGACTAGGAGCCC		657	
		capD R	CATCTACCCACTCAACCATATCAG			
	capE	capE F	TCCGCAGAAAATTATTGACTC		511	
		capE R	GCTTGCTGCTTGATTTTGTC			
	capF	capF F	AATCGGAGAACGCAGAAATCAG		851	
		capF R	TTCCGCCGTCAATTACTCTG			
P. multocida serotyping						
sPCR1	L1	L1 F	ACATTCCAGATAATACACCCG	96.00 ˚C 5 min, 30 cycle (96.00 ˚C 30 sec, 52.00 ˚C 30 sec, 72.00 ˚C 90 sec), 72.00 ˚C 5 min	1307	21
		L1 R	ATTGGAGCACCTAGTAACCC			
	L2	L2 F	CTTAAAGTAACACTCGCTATTGC		810	
		L2 R	3'TTTGATTTCCCTTGGGATAGC			
	L3	L3 F	TGCAGGCGAGAGTTGATAAACCATC		474	
		L3 R	CAAAGATTGGTTCCAAATCTGAATGGA			
	L4	L4 F	CTTTATTTGGTCTTTATATATACC		550	
		L4 R	AGATTGCATGGCGAAATGGC			
	L5	L5 F	CAATCCTCGTAAGACCCCC		1175	
		L5 R	TCTTTATAATTATACTCTCCCAAG			
	L6	L6 F	AATGAAGGTTTAAAAGAGATAGCTGGAG		668	
		L6 R	CCTATATTTATATCTCCTCCCC			
	L7	L7 F	CTAATATATAAACCATCCAACGC		931	
		L7 R	CTAATATATAAACCATCCAACGC			
	L8	L8 F	GAGAGTTACAAAAATGATCGGC		255	
		L8 R	TCCTGGTTCATATATAGGTAGG			
	L3A	L3A F	TCCTTATCTGACATTGAAATCG		415	
		L3A R	CTAGACATCTGGTGGTTGCG			
	L6A	L6A F	AATATCTTTATAATTATACTCTCCC		668	
		L6A R	AATGAAGGTTTAAAAGAGATAGC			
M. haemolytica serotyping						
sPCR2	Serotype 1	Serotype 1 F	CATTTCCTTAGGTCAGC	95.00 ˚C 5 min, 35 cycle (94.00 ˚C 1 min, 55.00 ˚C 1 min, 72.00 ˚C 1 min), 72.00 ˚C 10 min	306	12
		Serotype 1 R	CAAGTCATCGTAATGCCT			
	Serotype 2	Serotype 2 F	GGGCATATCCTAAAGCCGT		160	
		Serotype 2 R	AGAATCCACTATTGGGCACC			
sPCR3	Serotype 6	mannsero 6 F	GCGTGACCCTGAATA	94.00 ˚C 5 min, 35 cycle (94.00 ˚C 1 min, 56.00 ˚C 1 min, 72.00 ˚C 2 min), 72.00 ˚C 10 min	357	
		mannsero 6 R	CGAACGGGAAAACCCCAAAC			

Table 1. Continued.

PCR	Genes	Primary	Primer Sequence (5'—3')	Cycling conditions	Product (bp)	References
P. multocida virulence genes						
vPCR1	*toxA*	toxA F	CTTAGATGAGCGACAAGG	94.00 ˚C 5 min, 40 cycle (94.00 ˚C 30 sec, 55.00 ˚C 30 sec, 72.00 ˚C 30 sec), 72.00 ˚C 10 min	846	
		toxA R	GAATGCCACACCTCTATAG			
vPCR2	*ptfA*	ptfA F	TGTGGAATTCAGCATTTTAGTGTGTC	94.00 ˚C 5 min, 35 cycle (94.00 ˚C 60 sec, 68.00 ˚C 60 sec, 72.00 ˚C 60 sec), 72.00 ˚C 10 min	488	
		ptfA R	TCATGAATTCTTATGCGCAAAATCCTGCTGG			
vPCR3	*pfhA*	pfhA F	TTCAGAGGGATCAATCTTCG	94.00 ˚C 5 min, 35 cycle (94.00 ˚C 60 sec, 68.00 ˚C 60 sec, 72.00 ˚C 60 sec), 72.00 ˚C 10 min	286	
		pfhA R	AACTCCAGT TGGTTTGTCG			
vPCR4	*tadD*	tadD F	TCTACCCATTCTCAGCAAGGC	94.00 ˚C 5 min, 35 cycle (94.00 ˚C 60 sec, 68.00 ˚C 60 sec, 72.00 ˚C 60 sec), 72.00 ˚C 10 min	416	
		tadD R	ATCATTTCGGGCATTCACC			
vPCR5	*ompA*	OmpA F	CGCATAGCACTCAAGTTTCTCC	94.00 ˚C 5 min, 35 cycle (94.00 ˚C 30 sec, 55.00 ˚C 30 sec, 72.00 ˚C 60 sec), 72.00 ˚C 5 min	201	22
		OmpA R	CATAAACAGATTGACCGAAACG			
vPCR6	*ompH*	OmpH F	CGCGTATGAAGGTTTAGGT	94.00 ˚C 5 min, 35 cycle (94.00 ˚C 30 sec, 55.00 ˚C 30 sec, 72.00 ˚C 60 sec), 72.00 ˚C 5 min	438	
		OmpH R	TTTAGATTGTGCGTAGTCAAC			
vPCR7	*omp87*	Omp87 F	GGCAGCGAGCAACAGATAACG	94.00 ˚C 5 min, 35 cycle (94.00 ˚C 30 sec, 55.00 ˚C 30 sec, 72.00 ˚C 60 sec), 72.00 ˚C 5 min	838	
		Omp87 R	TGTTCGTCAAATGTCGGGTGA			
vPCR8	*plpB*	plpB F	TTTGGTGGTGCGTATGTCTTCT	94.00 ˚C 5 min, 35 cycle (94.00 ˚C 30 sec, 55.00 ˚C 30 sec, 72.00 ˚C 60 sec), 72.00 ˚C 5 min	282	
		plpB R	AGTCACTTTAGATTGTGCGTAG			
vPCR9	*exbB tonB*	ExbB F	GGTGGTGATATTGATGCGGC	95.00 ˚C 5 min 35 cycle (94.00 ˚C 30 sec, 53.00 ˚C 30 sec, 72.00 ˚C 45 sec), 72.00 ˚C 10 min	1144	
		tonB R	GCATCATGCGTGCACGGTT			
vPCR10	*hgbA*	hgbA F	TGGCGGATAGTCATCAAG	95.00 ˚C 5 min, 35 cycle (94.00 ˚C 30 sec, 53.00 ˚C 30 sec, 72.00 ˚C 45 sec), 72.00 ˚C 10 min	419	
		hgbA R	CCAAAGAACCACTACCCA			
vPCR11	*hgbB*	hgbB F	ACCGCGTTGGAATTATGATTG	95.00 ˚C 5 min, 35 cycle (94.00 ˚C 30 sec, 54.00 ˚C 30 sec, 72.00 ˚C 45 sec), 72.00 ˚C 10 min	788	
		hgbB R	CATTGAGTACGGCTTGACAT			
vPCR12	*tbpA*	tbpA F	TTGGTTGGAAACGGTAAAGC	95.00 ˚C min, 35 cycle (94.00 ˚C 30 sec, 53.00 ˚C 30 sec, 72 45 sec), 72.00 ˚C 10 min	728	
		tbpA R	TAACGTGTACGGAAAAGCCC			
M. haemolytica virulence genes						
vPCR13	*ssa-1*	ssa-1 F	TTCACATCTTCATCCTC	94.00 ˚C 15 min, 40 cycle (95.00 ˚C 1 min, 48.00 ˚C 1 min, 72.00 ˚C 2 min), 72.00 ˚C 15 min	327 bp	23
		ssa-1 R	TTTTCATCCTCTTCGTC			
vPCR14	*lkt*	lkt-A F	CACTCGATAATTATTCTAAATTAG	94.00 ˚C 10 min, 35 cycle (94.00 ˚C 1 min, 58.00 ˚C 1 min, 72.00 ˚C 1 min), 72.00 ˚C 15 min	3,000 bp	24
		lkt-A R	GTCCCTGTGTTTTCATTATAA G			
		parC R	GCCATTCCCACCGCAATCC			

tPCR 1/2: species-specific PCR 1/2, kPCR: capsule typing PCR, sPCR: serotyping PCR, vPCR: virulence gene specific PCR.

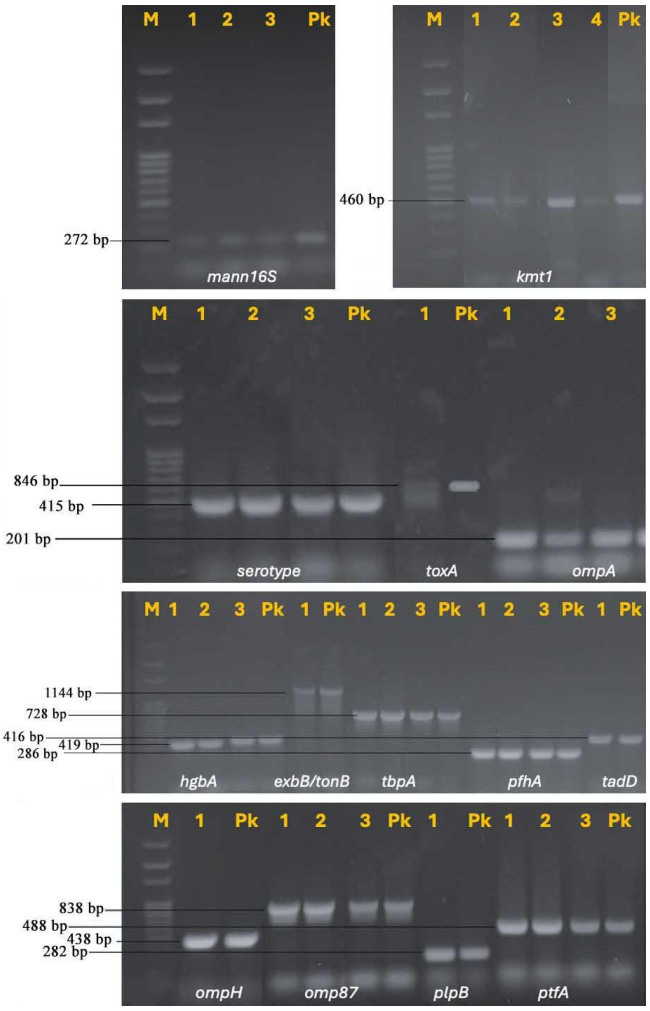


Fig. 1. Electrophoresis image of PCR products. M: 100 bp DNA ladder, 1 -3: field isolates, Pk: positive control.

Virulence gene analysis. All five isolates identified as *P. multocida* by phenotypic tests and PCR were positive for the *ompA*, *omp87*, *hghA*, and *tbpA* virulence genes, while the presence of other virulence genes (*toxA*, *ptfA*, *pfhA*, *tadD*, *ompH*, *plpB*, and *exbB-tonB*) varied, ranging from 20.00 to 80.00%. Following direct PCR analysis of nasal swab samples, the prevalence of virulence genes among the *P. multocida*-positive samples ranged from 23.53 to 94.12%. The most frequently detected genes were *hghA*, *ptfA*, and *tbpA*, respectively. The *hgbB* gene was not detected in any of the *P. multocida* isolates or PCR-positive samples (Table 2 and Fig. 1). The *ssa-1* gene was detected in one (33.33%) of the three *M. haemolytica* isolates identified by phenotypic tests and PCR, and in four (30.77%) of the *M. haemolytica*-positive nasal swab samples analyzed by direct PCR (Table 3).

Antimicrobial susceptibility analysis. As a result of antimicrobial susceptibility analysis performed by disk diffusion, all *P. multocida* isolates were found susceptible to SXT; AM and P susceptibility was 80.00% (4/5) and FUR,

Table 2. Characteristics of the *Pasteurella multocida* isolates and positive samples.

No.	Farm No.	Culture	Direct PCR	Serotypes	Virulance genes									Antimicrobial analyses						
					toxA	*ptfA*	*pfhA*	*tadD*	*ompA*	*ompH*	*omp87*	*plpB*	*exbB-tonB*	*hghA*	*hghB*	*tbpA*	AM	SXT	FUR	P	APX	CEP	S	N
10	1	-	+	L3A	-	+	-	-	+	+	+	+	+	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
25	1	-	+	L2	-	+	-	+	-	-	-	+	+	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND
32	1	*P. multocida*	+	L3A	-	+	+	+	+	-	+	+	+	+	-	+	S	S	S	S	**R**	S	**R**	**R**
53	1	*P. multocida*	+	L8	-	+	-	-	+	+	+	+	+	+	-	+	S	S	S	S	**R**	**R**	**R**	S
73	2	-	+	L3A	+	+	-	-	-	-	+	-	+	-	-	+	ND	ND	ND	ND	ND	ND	ND	ND
82	2	-	+	L2	-	+	-	+	+	+	-	+	+	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
84	2	-	+	L3A	-	+	-	-	-	+	+	-	+	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
106	2	-	+	L3A	-	+	-	-	+	-	+	+	+	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
121	2	-	+	L8	-	+	-	+	+	-	+	+	+	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
157	3	*P. multocida*	+	L3A	+	-	+	-	+	-	+	-	-	+	-	+	**R**	S	I	**R**	**R**	**R**	**R**	**R**
178	3	-	+	L2	-	-	-	-	+	-	-	+	-	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND
203	4	*P. multocida*	+	L3A	-	+	+	-	+	+	+	+	+	+	-	+	S	S	S	S	**R**	S	S	S
210	4	-	+	L3A	+	+	-	-	+	-	+	-	-	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
265	6	-	+	L3A	-	+	+	-	+	-	+	+	+	+	-	-	ND	ND	ND	ND	ND	ND	ND	ND
286	7	-	+	L3A	-	+	-	-	+	-	+	+	+	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
288	7	-	+	L3A	+	+	-	-	-	+	-	+	+	+	-	+	ND	ND	ND	ND	ND	ND	ND	ND
295	7	*P. multocida*	+	L3A	-	+	-	-	+	-	+	+	+	+	-	+	S	S	I	S	**R**	S	**R**	S

ND: not detected S: susceptible, I: intermediate susceptible, R: resistant, AM: ampicillin, SXT: trimethoprim/sulfamethoxazole, FUR: ceftiofur, P: penicillin G, APX: ampicillin/cloxacillin, CEP: cefoperazone, S: streptomycin, N: neomycin.
Red rectangle indicates: multidrug-resistance.

CEP, and N susceptibility was 60.00% (3/5). High APX (100%) and S (80.00%) resistance was detected among the isolates (Table 2). While all *M. haemolytica* isolates were susceptible to P, AM, FUR and CEP susceptibility was 66.67%, and SXT, S and N resistance was 66.67% (Table 3).

The MDR was detected in 2 (40.00%) *P. multocida* isolates and 2 (66.67%) *M. haemolytica* isolates and the most common resistance in MDR combinations was detected against APX and S. It was noteworthy that one *M. haemolytica* isolate (#295) with serotype 6 but without *ssa-1* and *lkt* virulence genes had multiple resistance to other antimicrobials tested, except P (Tables 2 and 3).

Discussion

It is imperative to comprehend the temporal dynamics of the nasal microbiome. This understanding helps to determine the conditions under which pathobionts, microbes that can cause respiratory infections, exhibit either a commensal or a pathogenic phenotype. In our study, nasal swab samples from healthy calves were examined for the presence of *P. multocida* and *M. haemolytica* by cultural methods and PCR. The results demonstrated the presence of both bacteria at low rates in the nasal flora of healthy calves (1.67% *P. multocida* and 1.00% *M. haemolytica* positivity by cultural analysis; 5.67% *P. multocida* and 4.34% *M. haemolytica* positivity by direct PCR). The fact that all strains isolated by cultural analysis were confirmed by PCR demonstrates that the phenotypic and molecular diagnostic methods used are complementary. A plethora of studies have been conducted that focus on the analysis of the nasal microbiome to determine the susceptibility of calves to develop respiratory tract infections and the role of the *Pasteurellaceae* family members. When compared to other studies, the isolation rates obtained were similar to Centeno-Martinez *et al.*,[28] (reported to be below 1.00%). However, Seker *et al.*,[29] and Myrenås *et al.*,[30] reported higher positivity rates of 11.40 and 37.40% for *P. multocida* and 0.50 and 14.30% for *M. haemolytica*. It is thought that these differences might be due to various variables such as the sensitivity of the methods used, sampling periods, environmental factors (*e.g.*, temperature and humidity fluctuations, housing conditions, animal density hygiene) age, and immune status of the animals. On the other hand, studies in calves with pneumonia reveal that the prevalence of these pathogens in the nasal flora is significantly higher. For example, Akalu *et al.*,[31] reported *P. multocida* positivity of 17.00% and *M. haemolytica* positivity of 54.40%, while Jamali *et al.*,[32] found *P. multocida* positivity in calves with respiratory tract infections to be as high as 83.40%. These data indicated that colonization of the nasal cavity by *P. multocida* and *M. haemolytica* was increased in the presence of respiratory tract infections. As a result, it appears that these pathogens may be present in the nasal flora of healthy calves and may constitute an important reservoir for transmission. Molecular methods were once again shown to be more sensitive than cultural analysis.

In the literature, it has been reported that BRD caused by *P. multocida* are mostly associated with serovar A:3 (L3A).[33,34] On the other hand, L2 (14.00%) and L8 (1.50%) serotypes were also found, albeit at low rates.[33] In our study, the majority of *P. multocida* isolates obtained from healthy calves (80.00%) and those detected by direct PCR (70.59%) belonged to the L3A serotype, while L8 (11.76 – 20.00%) and a small number of L2 (17.65%) serotypes were also detected in samples analyzed by direct PCR. These findings indicated the presence of different serotypes exhibiting heterogeneous colonization as well as dominant pathogenic serotypes associated with the respiratory system in healthy calves. They also highlighted the importance of considering local serotype distribution when devising disease control strategies.

Table 3. Characteristics of the *Mannheimia haemolytica* isolates and positive samples.

No.	Farm No.	Culture	Direct PCR	Serotype	Virulence gene		Antimicrobial analyses							
					ssa-1	*lkt*	AM	SXT	FUR	P	APX	CEP	S	N
10	1	-	+	A6	-	-	ND	ND	ND	ND	ND	ND	ND	ND
32	1	*M. haemolytica*	+	A1	+	-	S	S	S	S	**R**	S	S	S
53	1	-	+	A1	-	-	ND	ND	ND	ND	ND	ND	ND	ND
82	2	-	+	A6	-	-	ND	ND	ND	ND	ND	ND	ND	ND
84	2	-	+	A1	+	-	ND	ND	ND	ND	ND	ND	ND	ND
113	2	-	+	A1	-	-	ND	ND	ND	ND	ND	ND	ND	ND
117	2	-	+	A1	-	-	ND	ND	ND	ND	ND	ND	ND	ND
203	4	*M. haemolytica*	+	A6	-	-	S	**R**	S	S	**R**	S	**R**	**R**
206	4	-	+	A1	-	-	ND	ND	ND	ND	ND	ND	ND	ND
244	5	-	+	A6	+	-	ND	ND	ND	ND	ND	ND	ND	ND
265	6	-	+	A6	-	-	ND	ND	ND	ND	ND	ND	ND	ND
288	7	-	+	A1	+	-	ND	ND	ND	ND	ND	ND	ND	ND
295	7	*M. haemolytica*	+	A6	-	-	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**

ND: not detected, S: susceptible, I: intermediate susceptible, R: resistant, AM: ampicillin, SXT: trimethoprim/sulfamethoxazole, FUR: ceftiofur, P: penicillin G, APX: ampicillin/cloxacillin, CEP: cefoperazone, S: streptomycin, N: neomycin.
Red rectangle indicates: multidrug-resistance.

It has been reported that *M. haemolytica* serotype A2 is more commonly isolated from healthy animals as a commensal, whereas, serotypes A1 and A6 are the primary agents of BRD.[34,35] Mason *et al.*,[35] reported serotype distribution in cattle with pneumonia as A1 (44.20%), A2 (29.80%), and A6 (17.30%), while Lachowicz-Wolak *et al.*,[34] found that the majority of isolates from BRD-affected cattle were serotype A2. In our study, 66.67% of *M. haemolytica* isolates from healthy calves were identified as serotype A6 and 33.33% as serotype A1, and among PCR-positive samples, 53.85 and 46.15% were serotype A1 and A6, respectively. The paradoxical carriage of BRD-associated serotypes in asymptomatic calves suggested that serotype alone did not determine pathogenicity; rather, strain-specific virulence attributes were likely decisive. In particular, the absence of the *lkt* gene in these A1 carriers underscored that despite serotype A1 known disease-association, virulence might depend on the presence of key genes. This aligned with findings showing that A1 but not A2 strains invade bovine airway epithelial cells and cause damage.[36] Together, these findings underscored the importance of supplementing serotyping with molecular virulence profiling, as healthy calves can asymptomatically harbor BRD-associated serotypes and thus contribute to silent transmission within herds.

The capsule structure of *P. multocida* is a virulence factor that plays a significant role in the pathogenicity of the pathogen. In particular, Type A capsule and its A:3 subtype are the variants most commonly associated with respiratory tract infections in cattle.[37] A plethora of studies have been conducted in the extant literature on the determination of *P. multocida* capsule types. Gülaydın and Gürtürk,[38] found that all *P. multocida* isolates obtained from healthy and diseased cattle were *capA* type, while Katsuda *et al.*,[39] reported *capA* and *capD* positivity in calves with pneumonia at rates of 93.70 and 6.30%, respectively. Furthermore, rarer capsule types such as *capB*, *capD*, and *capE*, have been identified in *P. multocida* isolates of bovine origin.[40] In our study, the *capA*, *capB*, *capD*, *capE*, and *capF* genes were absent in all *P. multocida* strains isolated. All isolates were classified as atypical. This finding indicated the presence of new or modified variants that could not be included in the classical capsule classification system similar to previously reported atypical isolates in the literature.[41] It is imperative to elucidate the epidemiological role and virulence potential of these atypical isolates to facilitate the development of effective control strategies. In our study, the *capA*, *capB*, *capD*, *capE*, and *capF* genes were absent in all *P. multocida* isolates which were therefore classified as atypical. The identification of atypical *P. multocida* isolates represented a novel insight with important implications. These strains might reflect novel capsular types or mutations in capsule biosynthesis loci, potentially affecting virulence, immune evasion and vaccine coverage. Similar atypical isolates have been reported previously, highlighting their role as emerging variants that could complicate epidemiological surveillance and control strategies.[41,42] Further genomic and functional analyses are needed to fully understand their epidemiological and pathogenic significance.

Numerous virulence factors involved in the pathogenesis of *P. multocida* and *M. haemolytica* have been identified.[11-14,43] The presence and prevalence of these factors, which facilitate bacterial adhesion to the host, evasion of the immune system, access to nutrient sources and tissue invasion, differ between clinically healthy and respiratory-infected animals. Katsuda *et al.*,[39] reported that the *ptfA, tonB, hgbA, omp87*, and *ompH* genes were detected at high rates (94.70 - 100%) in isolates obtained from healthy and diseased calves, while the *pfhA*, *tbpA*, and *hgbB* genes were found at variable frequencies (52.40 - 76.20%). Sakmanoğlu *et al.*,[33] on the other hand, frequently detected genes such as *ptfA* (80.00%), *tadD* (76.00%) and *ompA* (73.00%) in *P. multocida* isolates, while genes such as *ompH* (4.50%) and *toxA* (3.00%) were detected at lower frequencies. In our study, the presence of the *ompA, omp87, hgbA*, and *tbpA* genes was detected in all *P. multocida* isolates. The presence of other virulence genes (*toxA, ptfA, pfhA, tadD, ompH, plpB*, and *exbB-tonB*) was detected at rates ranging from 20.00 to 80.00%. In particular, the *hgbA*, *ptfA*, and *tbpA* genes were among the most commonly detected virulence factors in both isolates and PCR-positive samples. In contrast, the *hgbB* gene was not detected in any of the samples. The differences in virulence gene positivity suggested genetic variation between isolates or variability in the virulence profiles of strains obtained from different geographical regions. Leukotoxin, an important virulence factor for *M. haemolytica* was not detected in any isolates or PCR-positive samples in this study. However, Balevi *et al.*,[44] reported the presence of *lkt* in 33.84% of *M. haemolytica* isolates while Lachowicz-Wolak *et al.*,[34] reported the presence of *lkt* in 100% of their isolates. The absence of the *lkt* gene in our study suggested that the isolated strains might be low-virulence variants. The lower prevalence of *ssa-1* positivity (30.77 - 33.33%) in our study compared to that reported by Abed *et al.*,[9] (60.00%), reflected the lower prevalence of *ssa-1* positivity (30.77 - 33.33%) variation across different regions and animal populations.

It is widely accepted that *P. multocida* and *M. haemolytica* are generally susceptible to a range of antibiotics, including beta-lactams, fluoroquinolones and tetracyclines. Nevertheless, the indiscriminate and pervasive utilization of antibiotics has precipitated the emergence of resistance among these pathogens.[41] Numerous studies have evaluated the antibiotic susceptibility of these bacteria isolated from the bovine respiratory system. For example, Jamali *et al.*,[32] reported a 30.50% resistance rate to P in *P. multocida* from calves with respiratory infection. Schönecker *et al.*,[45] described

27.00% P resistance and 2.00% FUR resistance in *P. multocida* isolates. In a study of calf pneumonia pathogens, Algammall *et al.*,[46] found high levels of resistance in *P. multocida* and Zareh *et al.*,[47] similarly reported resistance to AM among these isolates. Another study documented a 77.70% streptomycin-resistance rate in *P. multocida* isolates.[48] In our study, all *P. multocida* isolates were susceptible to SXT, 80.00% were susceptible to AM and P and 60.00% were susceptible to FUR, CEP, and N, however, resistance reached 100% for APX and 80.00% for S. In the case of *M. haemolytica*, Lee *et al.*,[49] reported about 80.00 % susceptibility to AM, P and SXT. Meanwhile, Schönecker *et al.*,[45] found that all isolates were susceptible to FUR and 26.00% showed resistance to P. In a recent Turkish study, complete resistance to AM and SXT was observed in both M. *haemolytica* and *P. multocida* isolates, whereas FUR susceptibility was 100% for *P. multocida* and 88.90% for *M. haemolytica.*[50] In our study, all *M. haemolytica* isolates were found to be susceptible to penicillin, while resistance rates of 66.67% were observed for SXT, S, and N. These findings highlighted significant diversity in the antimicrobial-susceptibility profiles of *P. multocida* and *M. haemolytica* strains and indicated that resistance rates to commonly used antimicrobials might increase regionally or over time. Of particular concern is the high resistance to APX, S and N, drugs frequently used as first-line therapies for BRD in Türkiye, which suggests that current treatment protocols may no longer be optimally effective and warrant urgent re-evaluation, especially given the alarming prevalence (40.00 to 66.67%) of MDR in our isolates.

In conclusion, the data obtained in our study reveal that *P. multocida* and *M. haemolytica* could be found in healthy calves at certain prevalence rates indicating that clinically healthy calves may contribute to the maintenance of these bacteria populations, which harbor diverse pathogenic potentials. The identification of variants associated with BRD underscores the epidemiological significance of this carriage. Moreover, the marked diversity in antimicrobial-susceptibility profiles among the isolates emphasized the need to monitor resistance trends to commonly used antimicrobials such as AM, S and SXT and to update treatment protocols accordingly. This study had some limitations that should be acknowledged. First, the number of culture-positive isolates was relatively small, which could limit the strength of inferences about isolate-based characteristics, and no inferential statistical tests were applied. Thus result interpretation remained descriptive and should be viewed with caution. Second, sampling was restricted to nasal swabs from the upper respiratory tract, which might not fully reflect lower-lung pathogen populations in BRD cases. Finally, the study was geographically confined to a single province, which could limit the generalizability of the findings to other regions or production systems.

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Conflicts of interest

The authors declare no conflict of interest.

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