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Antimicrobial-Resistance and Virulence-Associated Genes of *Pasteurella multocida* and *Mannheimia haemolytica* Isolated from Polish Dairy Calves with Symptoms of Bovine Respiratory Disease

Agnieszka Lachowicz-Wolak [1], Aleksandra Chmielina [2], Iwona Przychodniak [2], Magdalena Karwańska [1], Magdalena Siedlecka [1], Małgorzata Klimowicz-Bodys [1], Kamil Dyba [3] and Krzysztof Rypuła [1,*]

[1] Division of Infectious Diseases of Animals and Veterinary Administration, Department of Epizootiology and Clinic of Birds and Exotic Animals, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, pl. Grunwaldzki 45, 50-366 Wroclaw, Poland; agnieszka.lachowicz-wolak@upwr.edu.pl (A.L.-W.); magdalena.karwanska@upwr.edu.pl (M.K.); magdalena.siedlecka@upwr.edu.pl (M.S.); malgorzata.klimowicz-bodys@upwr.edu.pl (M.K.-B.)

[2] "Epi-Vet" Veterinary Diagnostic Laboratory, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, pl. Grunwaldzki 45, 50-366 Wroclaw, Poland; aleksandra.chmielina@upwr.edu.pl (A.C.); iwona.przychodniak@upwr.edu.pl (I.P.)

[3] Department of Applied Mathematics, Wroclaw University of Environmental and Life Sciences, pl. Grunwaldzki 45, 50-366 Wroclaw, Poland; kamil.dyba@upwr.edu.pl

* Correspondence: krzysztof.rypula@upwr.edu.pl

Abstract: Bovine respiratory disease causes significant economic losses in cattle farming due to mortality, treatment costs, and reduced productivity. It involves viral and bacterial infections, with *Pasteurella multocida* and *Mannheimia haemolytica* key bacterial pathogens. These bacteria contribute to severe pneumonia and are often found together. Poland has one of the highest levels of antimicrobial use in food-producing animals among European Union countries. A total of 70 bacterial strains were analyzed, 48 *P. multocida* and 22 *M. haemolytica*, collected from affected calves' respiratory tracts. The bacterial species were confirmed molecularly using PCR, which was also employed to detect antimicrobial resistance and virulence-associated genes. Antimicrobial susceptibility was determined using the broth microdilution method. Antimicrobial resistance varied between the two bacterial species studied. The highest resistance in *P. multocida* was to chlortetracycline 79.2% (38/48) and oxytetracycline 81.3% (39/48), while *M. haemolytica* showed 63.6% (14/22) resistance to penicillin and tilmicosin. The highest susceptibility was found for fluoroquinolones: *P. multocida* demonstrated 91.7% (44/48) susceptibility to enrofloxacin and 87.5% (42/48) to danofloxacin, while 77.3% (17/22) of *M. haemolytica* were susceptible to both tested fluoroquinolones. The *tetH* and *tetR* genes were observed only in *P. multocida*, at frequencies of 20.8% (10/48) and 16.7% (8/48), respectively. Both species carried the *mphE* and *msrE* genes, though at lower frequencies. All *M. haemolytica* contained the *lkt*, *gs60*, and *gcp* genes. All *P. multocida* carried the *sodA* gene, while the *hgbB* and *ompH* genes were present in 37.5% (18/48) and 20.8% (10/48) of strains, respectively. The highest resistance was observed against the most commonly used antibiotics in the European Union, although the resistance differed between the studied bacterial species and each strain exhibited the presence of at least one virulence gene.

Keywords: bovine pneumonia; shipping fever; MIC; cattle; antimicrobial susceptibility; resistance patterns

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1. Introduction

Bovine respiratory disease (BRD) is a multifactorial disease involving viral and bacterial infections that can be predisposed by environmental factors or stress and can be further aggravated by them [1–15]. This leads to significant economic losses in the cattle industry due to the high costs of treatment, reduced weight gain, and delayed time to first estrus and conception, resulting in later lactation and lower milk production in cows that have recovered from BRD [6–8].

Pasteurella multocida (*P. multocida*) and *Mannheimia haemolytica* (*M. haemolytica*) are two of the most frequently identified bacterial pathogens in calves suffering from BRD [10,16]. These bacteria are often found as coinfections and are generally considered opportunistic pathogens [10,17]. Nevertheless, they possess virulence factors that contribute to enhancing the bacteria's ability to cause pathology and facilitate the development of infections. *Mannheimia haemolytica*, in particular, is linked to BRD outbreaks due to its production of virulence factors, such as leukotoxin, which contribute to clinical symptoms and cause severe pneumonia [18–20]. *Mannheimia haemolytica* harbors additional virulence-associated genes, including *gcp*, which encodes a glycoprotein involved in adhesion, and *gs60*, a heat-shock protein that enhances bacterial survival [20]. In contrast, *P. multocida* possesses *sodA*, which encodes an enzyme that neutralizes reactive oxygen species, aiding in evasion of the host immune response, *hgbB*, which may indicate the bacterium's ability to utilize host hemoglobin, and *ompH*, which facilitates bacterial adhesion to host tissues, further contributing to infection development [15].

In sick calves, discharge from the nasal openings and conjunctivae is visible. It may appear serous, serous–mucous, or purulent. An elevated body temperature, especially in cases of acute disease, may also occur. Affected individuals show an increase in respiratory rate and lethargy and decreased appetite [4,5,11]. Due to the presence of dyspnea, calves adopt a posture with a lowered head and extended neck. In severe cases, they breathe through an open mouth. In the acute form of the disease, respiratory failure and death of the calf may occur within hours after the symptoms are first noticed [11].

BRD is among the most common reasons for antimicrobial use in dairy cattle [21–23]. According to the European Medicines Agency (EMA) [24], Poland has one of the highest levels of antimicrobial use in food-producing animals among European Union countries. Data for 2022 showed that the total sales of antibiotics for food-producing animals in Poland amounted to 838.3 tons, highlighting the significant scale of antimicrobial use in the country. The EU average was 73.9 mg per population correction unit (mg/PCU), while Poland reported 196.0 mg/PCU, which is notably higher. The most commonly sold antibiotics in the EU are penicillins, accounting for 32.7%, followed by tetracyclines at 23.5% [24]. The very frequent administration of these drugs and the irrational use of antimicrobials by veterinarians who do not conduct susceptibility testing contribute to increasing resistance, creating a vicious cycle. Recent research from Poland [22] revealed that over the course of three months on large dairy farms, individual calves received antimicrobial treatment up to two times. Rising antimicrobial resistance is a pressing issue impacting not only the health of calves but also the health of humans, particularly given that *P. multocida* has zoonotic potential [25–29].

To date, there has been a notable absence of published data regarding the antimicrobial susceptibility of *P. multocida* and *M. haemolytica* isolated from Polish calves suffering from BRD. This gap in the literature underscores the urgent need for detailed investigations to better understand the resistance profiles of these pathogens.

The primary aim of this study was to determine the antimicrobial susceptibility of *P. multocida* and *M. haemolytica* strains isolated from dairy calves with BRD. An additional

objective was to assess the frequency of antimicrobial-resistance and virulence-associated genes and among these strains.

2. Materials and Methods

2.1. Sample Collection

The study included samples collected between February 2021 and January 2024. The material consisted of either deep nasal swabs or lung and bronchi swabs from calves that exhibited symptoms of BRD, such as nasal and ocular discharge, fever, cough, increased respiratory rate, enhanced lung field murmur, and respiratory wheezing. Samples from the lower respiratory tract were collected postmortem from calves that had died while showing symptoms of BRD. Swabs were preserved in Amies transport medium with charcoal (Deltalab, S.L., Rubi, Spain) and transported within 48 h to the Epi-Vet veterinary diagnostic laboratory at Wroclaw University of Life Sciences. Samples were collected exclusively from the respiratory tract, with each animal providing a sample from a single location. Due to the commercial nature of the study, samples from several calves were pooled to obtain a single result for a given herd. The tested unit in this study was a bacterial strain isolated from the respiratory tract of a calf suffering from BRD.

2.2. Isolation and Initial Identification

Swabs were initially cultured on Columbia agar with 5% sheep blood (Graso, Stargard Szczeciński, Poland) and on MacConkey agar with crystal violet (Graso, Stargard Szczeciński, Poland). The media were incubated at 37 °C for 16–24 h in an atmosphere containing 5% CO_2. On Columbia agar with 5% sheep blood, round, mucoid colonies with a characteristic "mouse-like" odor were observed, suggesting their affiliation with *P. multocida*, as well as small, gray colonies with β-hemolysis, suggesting their affiliation with *M. haemolytica*. These colonies were checked for cytochrome oxidase production using the Oxidase Test Stick (Liofilchem, Roseto degli Abruzzi, Italy). Colonies that tested positive were streaked onto Columbia agar with 5% sheep blood and MacConkey agar with crystal violet and incubated as previously described. No growth of *P. multocida* was observed on MacConkey agar with crystal violet, whereas *M. haemolytica* grew as small, light-pink colonies. The isolates were biochemically tested according to the manufacturer's instructions using the commercial RaPID NF system (Thermo Fisher Diagnostics B.V., Landsmeer, The Netherlands). The biochemical reactivity patterns were analyzed using dedicated ERIC™ v.1.1 software (Thermo Fisher Diagnostics B.V., Landsmeer, The Netherlands). Selected isolates of *P. multocida* and *M. haemolytica* were cryopreserved in CryoBank (Mast Group Ltd., Reinfeld, Germany) and stored at −80 °C for further studies. Species confirmation and serotyping was subsequently performed molecularly via PCR, as described below.

2.3. PCR

2.3.1. Genomic DNA Extraction

Genomic DNA was extracted using the Genomic Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The quality of the extracted genetic material was assessed using a DS-11 FX spectrophotometer (DeNovix Inc., Wilmington, NC, USA).

2.3.2. Amplification Setup

PCR amplification was performed using the Color Taq DNA polymerase kit (Eurx, Gdańsk, Poland). The reaction mixture contained the following components: 0.625 U of Color Taq DNA polymerase, 1 µL of template DNA, 0.1 µL of each forward and reverse

primer,1 µL of dNTP mixture and 2.5 µL of 10× Buffer B (Eurx, Gdańsk, Poland). The mixture was supplemented with nuclease-free water (A&A Biotechnology, Gdynia, Poland) to a final volume of 25 µL.

2.3.3. Cycling Conditions

PCR was performed in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR protocol began with an initial denaturation step at 95 °C for 5 min. This was followed by 32 amplification cycles, each consisting of denaturation at 94 °C for 30 s, annealing at a temperature specific to the primers used for 30 s and extension at 72 °C for 1 min per kilobase (kb) of the amplified product. After the completion of the cycles, a final elongation step was performed at 72 °C for 10 min. The mixture was then cooled to 4 °C for further analysis.

2.3.4. Primers for Gene Detection

All primers used in this study were synthesized by Genomed S.A. (Warsaw, Poland). For all the genes detected, the primer pair sequences, annealing temperatures, and product sizes are listed in Table 1.

Table 1. Primer sequence, amplicon size, annealing temperature, and gene accession numbers for all the genes detected in this study.

PCR Details for Detected Genes					
PCR Target/Gene	**Primer Name**	**PCR Primer Sequence 5′→3′**	**Amplicon Size (bp)**	**Annealing Temperature (°C)**	**Primer Reference**
Pasteurella multocida					
Bacteria species confirmation, *KMT1*	*KMT1T7* *KMT1SP6*	ATCCGCTATTTACCCAGTGG GCTGTAAACGAACTCGCCAC	460	55	[30]
Capsular type A	*CAPA*	TGCCAAAATCGCAGTCAG TTGCCATCATTGTCAGTG	1044	55	[31]
LPS genotype 3	*BAP7214* *BAP7213*	CAAAGATTGGTTCCAAATCTGAATGGA TGCAGGCGAGAGTTGATAAACCATC	474	52	[32]
Virulence					
B hemoglobin-binding protein	*hgbB*	ACCGCGTTGGAATTATGATTG CATTGAGTACGGCTTGACAT	788	54	[33]
Superoxide dismutase A	*sodA*	TACCAGAATTAGGCTACGC GAAACGGGTTGCTGCCGCT	361	55	[33]
Outer-membrane protein H	*ompH*	CGCGTATGAAGGTTTAGGT TTTAGATTGTGCGTAGTCAAC	438	57	[33]
Mannheimia haemolytica					
Bacteria species confirmation	*MropB*	AACACATAAACGCCGTATCTCG GATATTCGGGCCTTCAGGA	136	55	[34]
Serotype A1	*hyp*	CATTTCCTTAGGTTCAGC CAAGTCATCGTAATGCCT	306	55	[35]
Serotype A2	*Core2*	GGCATATCCTAAAGCCGT AGAATCCACTATTGGGCACC	160	55	[35]
Virulence					
Outer-membrane lipoprotein	*gs60*	GCACATTATATTCTATTGAG AGGCATACTCTAACTTTTGC	429	55	[36]
o-sialoglycoproteinase	*gcp*	CGCCCCTTTTGGTTTTCTAA GTAAATGCCCTTCCATATGG	420	58	[36]
Leukotoxin	*lkt*	GTCCCTGTGTTTTCATTATAAG CACTCGATAATTATTCTAAATTAG	385	58	[35]

Table 1. *Cont.*

PCR Details for Detected Genes					
PCR Target/Gene	**Primer Name**	**PCR Primer Sequence 5′→3′**	**Amplicon Size (bp)**	**Annealing Temperature (°C)**	**Primer Reference**
Antimicrobial resistance phenotype					
CTET, OXY	*tetR*	CGGCTTGGGTTAATAATGGCG ATAACGCGAAAAGCTTCCGC	425	58	[37]
	tetH	ATACTGCTGATCACCGT TCCCAATAAGCGACGCT	1076	60	[37]
TIL, TUL	*msrE*	ACCAGCCACCTTGATCTCAATG GTTCCATTCGATCCAGTTATAGCG	620	60	[37]
	mphE	TCTGTAGCGGGTTTCCAATTGC AATGGTTGCTGCGTATTCCTCG	401	60	[37]

CTET—chlortetracycline, OXY—oxytetracycline, TUL—tulathromycin, TIL—tilmicosin.

2.3.5. Electrophoresis of PCR Products

PCR-generated products were detected by electrophoresis in 1.5% agarose gels stained with Midori Green DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany) alongside a DNA Marker 1, 100–1000 bp marker ladder (A&A Biotechnology, Gdynia, Poland) using a PowerPac™ Basic (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 95 V and 400 mA. The electrophoresis results were read using the GelDoc Go imaging system with Image Lab v.6.1 software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.3.6. Controls and Sequencing Validation

As positive controls, *P. multocida* ATCC 12945 was used for the *kmt*, *capA*, *ompH*, *sodA*, and *hgbB* genes, and *M. haemolytica* PCM 2685 from the Polish Academy of Science was used for the *MropB*, *hyp*, *lkt*, *gs60* and *gcp* genes. To confirm the accuracy of the PCR results for all tested *M. haemolytica* genes, gene encoding lipopolysaccharide type 3 and resistance genes, the representative two products were selected and subsequently sent to Genomed S. A. (Warsaw, Poland) for Sanger sequencing with both forward and reverse PCR primers. The obtained sequences were analyzed using BioEdit v5.0.9 software and compared with sequences from the National Center for Biotechnology Information (NCBI) GenBank database, confirming their alignment with those available in the database. Each PCR reaction included a negative control, in which ultrapure water (A&A Biotechnology, Gdynia, Poland) was used instead of the DNA template.

2.4. MIC

2.4.1. MIC Testing Methodology

The minimal inhibitory concentrations (MICs) for *M. haemolytica* and *P. multocida* were assessed using the broth microdilution method with the commercially available test plate Thermo Scientific Sensititre™ BOPO6F (TREK Diagnostic Systems Ltd., East Grinstead, United Kingdom). The antimicrobials included ceftiofur (XNL), tiamulin (TIA), chlortetracycline (CTET), gentamicin (GEN), florfenicol (FFN), oxytetracycline (OXY), penicillin (PEN), ampicillin (AMP), danofloxacin (DANO), sulfadimethoxine (SDM), neomycin (NEO), trimethoprim–sulfamethoxazole (SXT), spectinomycin (SPE), tylosin tartrate (TYLT), tulathromycin (TUL), tilmicosin (TIL), clindamycin (CLI), and enrofloxacin (ENRO).

The testing procedures adhered to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [38]. For optimal growth of the primary cryopreserved

isolates, an inoculum of approximately 5×10^5 cfu/mL was prepared in Thermo Scientific SensititreTM cation-adjusted Mueller–Hinton broth with 5% lysed horse blood (Remel Inc., Lenexa, KS, USA). The inoculated Sensititre plates were incubated in ambient air for 18–24 h at 36 ± 1 °C within a humidified chamber. Quality control is performed on the plates by the manufacturer; however, in addition, we conducted testing on a *P. multocida* ATCC 12945.

2.4.2. Evaluation of MIC Results

The MIC values were recorded using the Sensititre^{TM} Manual MIC Plate Reader (Thermo Fisher, Waltham, MA, USA). The results were evaluated according to the CLSI standards to assess the assessment of the susceptibility of the tested bacterial strains to the antimicrobial agents [38]. Based on these evaluations, each strain was classified as susceptible (S), intermediate (I), or resistant (R) to the respective antimicrobial. In all instances where the term "insusceptible" is used, it refers collectively to both intermediate and resistant classifications. After determining the MIC values for each of the antimicrobials, population analyses were carried out to determine the MIC_{50} (MIC required to inhibit 50% of the organisms) and MIC_{90} (MIC required to inhibit 90% of the organisms) values. The multiple antimicrobial resistance (MAR) index was calculated as the quotient between the number of antimicrobials to which strains were resistant to the number of tested antimicrobials. Multidrug resistance (MDR) was defined as resistance to at least one substance in three or more antimicrobial classes.

2.5. Statistical Analysis

To assess the statistical significance of the difference in resistance to antimicrobials between the two studied bacterial species and to assess the correlation between the presence of virulence genes Pearson's chi-squared tests without Yates's correction were performed. Similarly, to check for correlations between antimicrobial resistance genes and the resistance results demonstrated in the MIC test, cross-tab analyses were performed along with Pearson's chi-squared tests without Yates's correction (χ^2 test) and additionally, Pearson's correlation. The significance level for all tests was set at $\alpha = 0.05$. For binary data, the product of the number of observations (70 in this study) and the square of the sample Pearson correlation coefficient is equal to the chi-squared test statistic of independence computed without Yates's correction. Therefore, to emphasize this relationship, the Yates correction was not applied [39].

Statistical analyses were performed using Statistica 13.3.721.1 (TIBCO Software Inc., Palo Alto, CA, USA). Tables and graphs were prepared using Microsoft Office Excel 2007. Figures were prepared using Adobe Illustrator and VennPainter V.1.2.0 [40].

2.6. Ethics Statement

According to Polish law (Experiments on Animals Act from 15 January 2015, *Journal of Laws of the Republic of Poland* from 2015, item 266), this study did not require ethics committee approval. The samples used in this study were originally from cattle-infection diagnostic material collected by veterinarians treating these herds. Procedures did not cause the animals any pain, suffering, or distress greater than a needle-stick injury. However, the research outline was submitted to the Animal Welfare Advisory Team in Wroclaw, which qualified the study as research that did not require ethics committee approval.

3. Results

3.1. Bacterial Identification and Serotyping

Samples from 42 different herds were tested, all of which were collected from calves exhibiting symptoms of BRD. Three of the isolates were obtained from the lower respiratory tract postmortem, while the remaining isolates were from deep nasal swabs. Based on phenotypic, biochemical, and molecular analyses, a total of 70 bacterial isolates were included for further analysis: 48 *P. multocida* isolates and 22 *M. haemolytica*. Based on the presence of capsule genes, all *P. multocida* isolates were assigned to serogroup A, with 93.8% (45/48) of the isolates genotypically identified as the L3 lipopolysaccharide type, while the remaining three isolates could not be classified in terms of lipopolysaccharide type. Among the *M. haemolytica* isolates, 13.6% (3/22) were classified as serotype A1, while the remaining 86.4% (19/22) were classified as serotype A2. Among the three samples from the lower respiratory tract, two strains of *P. multocida* A:L3 and one strain of *M. haemolytica* serotype A2 were obtained.

3.2. Antimicrobial Susceptibility

The highest susceptibility was observed for fluoroquinolones: *P. multocida* showed 91.7% (44/48) susceptibility to enrofloxacin, while 77.3% (17/22) of *M. haemolytica* strains were susceptible to both enrofloxacin and danofloxacin. The highest percentage of intermediate susceptibility to antimicrobials was observed for *M. haemolytica*, with 50.0% (11/22) of the tested strains exhibiting intermediate susceptibility to FFN and SPE. The *Pasteurella multocida* strains demonstrated the highest resistance to tetracyclines, with 79.2% (38/48) resistant to CTET and 81.3% (39/48) resistant to OXY. In contrast, the *M. haemolytica* strains showed the highest resistance to PEN and TIL, with 63.6% (14/22) resistance. The remaining susceptibility percentages are presented in Table 2.

Table 2. Susceptibility of the tested strains to antimicrobial agents.

Antimicrobial	Antimicrobial Susceptibility											
	Pasteurella multocida						*Mannheimia haemolytica*					
	S		I		R		S		I		R	
	n/N	%	*n/N*	%	*n/N*	%	*n/N*	%	*n/N*	%	*n/N*	%
CTET	9/48	18.8%	1/48	2.1%	38/48	79.2%	10/22	45.5%	2/22	9.1%	10/22	45.5%
OXY	9/48	18.8%	0/48	0.0%	39/48	81.3%	11/22	50.0%	1/22	4.5%	10/22	45.5%
TUL	37/48	77.1%	0/48	0.0%	11/48	22.9%	9/22	40.9%	1/22	4.5%	12/22	54.5%
TIL	34/48	70.8%	2/48	4.2%	12/48	25.0%	7/22	31.8%	1/22	4.5%	14/22	63.6%
PEN	36/48	75.0%	3/48	6.3%	9/48	18.8%	3/22	13.6%	5/22	22.7%	14/22	63.6%
ENRO	44/48	91.7%	3/48	6.3%	1/48	2.1%	17/22	77.3%	4/22	18.2%	1/22	4.5%
DANO	42/48	87.5%	4/48	8.3%	2/48	4.2%	17/22	77.3%	2/22	9.1%	3/22	13.6%
XNL	36/48	75.0%	5/48	10.4%	7/48	14.6%	14/22	63.6%	1/22	4.5%	7/22	31.8%
FFN	38/48	79.2%	9/48	18.8%	1/48	2.1%	9/22	40.9%	11/22	50.0%	2/22	9.1%
SPE	29/48	60.4%	7/48	14.6%	12/48	25.0%	10/22	45.5%	11/22	50.0%	1/22	4.5%

S—susceptible, I—intermediate, R—resistant, CTET—chlortetracycline, OXY—oxytetracycline, TUL—tulathromycin, TIL—tilmicosin, PEN—penicillin, ENRO—enrofloxacin, DANO—danofloxacin, XNL—ceftiofur, FFN—florfenicol, SPE—spectinomycin.

Statistically significant differences in resistance to some of the tested antimicrobial agents were observed between the *P. multocida* isolates and the *M. haemolytica* isolates: PEN χ^2 = 13.778; TUL χ^2 = 9.646; OXY χ^2 = 9.205; CTE χ^2 = 7.956; and TIL χ^2 = 6.841, with *p*-values < 0.05. The mentioned antimicrobials are marked with an asterisk in the following graph depicting the percentage of insusceptible strains (Figure 1).

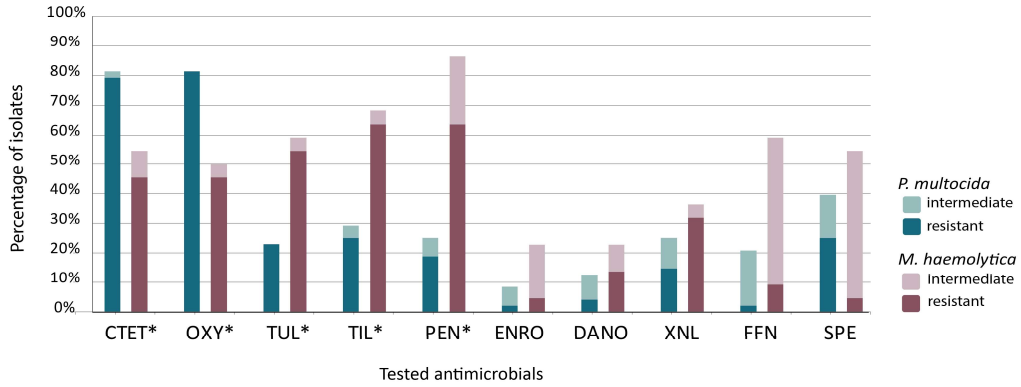


Figure 1. Percentages of insusceptibility to antimicrobials among the tested strains of *Pasteurella multocida* (*P. multocida*) and *Mannheimia haemolytica* (*M. haemolytica*). * Statistically significant difference in resistance to the given antimicrobial agent between bacterial species ($p < 0.05$). CTET—chlortetracycline, OXY—oxytetracycline, TUL—tulathromycin, TIL—tilmicosin, PEN—penicillin, ENRO—enrofloxacin, DANO—danofloxacin, XNL—ceftiofur, FFN—florfenicol, SPE—spectinomycin.

3.3. MIC Values

The effectiveness of individual antimicrobials was determined using the MIC_{50} and MIC_{90} (Figure 2). Due to the lack of bacterial growth inhibition in the presence of certain antimicrobials, it was not possible to identify the MIC_{50} for 22.2% (4/18) of the antimicrobials tested against *P. multocida* or 22.2% (4/18) of the antimicrobials tested against *M. haemolytica*. The MIC_{90} could not be determined for 55.6% (10/18) of the antimicrobials for *P. multocida* and 61.1% (11/18) for *M. haemolytica*.

Antimicrobial	Species	No. of all isolates	Minimal inhibitory concentration µg/mL												Non-inhibitory effect	MIC_{50} µg/mL	MIC_{90} µg/mL
			0.12	0.25	0.5	1	2	4	8	16	32	64	2/38	256			
CTET	Pm	48			4		5	1	7						31	>8	>8
	Mh	22			4	3	3	2	1						9	4	>8
OXY	Pm	48			6	3									39	>8	>8
	Mh	22			6	4	1	1							10	2	>8
TUL	Pm	48				6	22	9				1			10	2	>64
	Mh	22					1	6	1	1	1				12	>64	>64
TIL	Pm	48						31	3	2		1			11	4	>64
	Mh	22						2	5	1					14	>64	>64
PEN	Pm	48	17	19	3	1	2	2	2						2	0.25	4
	Mh	22	1	2	5	4	2	1	2						5	1	>8
AMP	Pm	48		29	9	1	2	1	2						4	0.25	8
	Mh	22		13	1		2	1	3	1					1	0.25	8
ENRO	Pm	48	37	7	1	2									1	0.12	0.25
	Mh	22	8	9	3	1									1	0.25	0.5
DANO	Pm	48	36	6	4	1									1	0.12	0.5
	Mh	22	9	8	2	1									2	0.25	1
XNL	Pm	48		29	1	3	3	5	6						1	0.25	8
	Mh	22		8		2	4	1	1						6	2	>8
FFN	Pm	48		8	22	6	2	9	1							0.5	4
	Mh	22			1	8		11	1						1	4	4
SPE	Pm	48							1	5	23	7			12	32	>64
	Mh	22								3	7	11			1	64	64
CLI	Pm	48							1						47	>16	>16
	Mh	22							2	7					13	>16	>16
GEN	Pm	48				6	23	13	5						1	2	8
	Mh	22				12	8			1					1	1	2
NEO	Pm	48						28	9	1					10	4	>32
	Mh	22						20	1	1					4	4	
SDM	Pm	48												3	45	>256	>256
	Mh	22												17	5	256	>256
TIA	Pm	48							2	16	17				13	32	>32
	Mh	22			5		1		2	6	1				7	16	>32
SXT	Pm	48											44		4	2/38	2/38
	Mh	22											14		8	2/38	>2/38
TYLT	Pm	48								2	31				15	32	>32
	Mh	22									2				20	>32	>32

Figure 2. Number of bacterial strains of *P. multocida* (Pm) and *M. haemolytica* (Mh) showing growth inhibition at the specified concentrations of the tested antimicrobial agents. The range of tested concentrations

is indicated by the frames. The colored fields indicate the concentrations at which bacterial growth was inhibited. Darker shades represent a higher number of inhibited isolates at a given concentration. Green corresponds to *P. multocida*, while red represents *M. haemolytica*. The number of strains not inhibited at the tested concentrations was also included. MIC_{50} and MIC_{90} values marked with ">" indicate that bacterial growth inhibition was not achieved at the tested concentrations. CTET—chlortetracycline, OXY—oxytetracycline, TUL—tulathromycin, TIL—tilmicosin, PEN—penicillin, AMP—ampicillin, ENRO—enrofloxacin, DANO—danofloxacin, XNL—ceftiofur, FFN—florfenicol, SPE—spectinomycin, CLI—clindamycin, GEN—gentamicin, NEO—neomycin, SDM—sulfadimethoxine, TIA—tiamulin, SXT—trimethoprim–sulfamethoxazole, TYLT—tylosin tartrate.

3.4. Multidrug-Resistance and Phenotypic Resistance Patterns

MDR was detected in 31.4% (22/70) of all the tested strains. Among the *P. multocida* strains, the rate was 27.1% (13/48), while among the *M. haemolytica* strains, it was 40.9% (9/22). The MAR index ranged from 0.275 for *P. multocida* to 0.336 for *M. haemolytica*.

The most frequently noted phenotypic resistance pattern was "CTET, OXY," which was observed exclusively among *P. multocida* strains at 37.5% (18/48). Among the *M. haemolytica* strains, the most common resistance pattern was the cooccurrence of resistance to "XNL, CTET, OXY, PEN, TIL, TUL," which was present in 18.2% (4/22) of the tested strains. All the recorded resistance patterns are presented in Table 3.

Table 3. Resistance patterns detected among the tested strains of *P. multocida* and *M. haemolytica* along with their frequencies.

Phenotypic Resistance Pattern							
No. of Antimicrobial in Pattern	**Antimicrobial Names**	***P. multocida***		***M. haemolytica***		**All**	
		n*/*N	**%**	***n*/*N***	**%**	***n*/*N***	**%**
1	PEN	1/48	2.1%	3/22	13.6%	4/70	5.7%
	SPE	1/48	2.1%	0/22	0.0%	1/70	1.4%
2	XNL, PEN	1/48	2.1%	0/22	0.0%	1/70	1.4%
	PEN, TIL	0/48	0.0%	1/22	4.5%	1/70	1.4%
	OXY, SPE	1/48	2.1%	0/22	0.0%	1/70	1.4%
	CTET, OXY	18/48	37.5%	0/22	0.0%	18/70	25.7%
3	PEN, TIL, TUL	0/48	0.0%	1/22	4.5%	1/70	1.4%
	XNL, CTET, OXY	1/48	2.1%	0/22	0.0%	1/70	1.4%
	OXY, PEN, TIL *	0/48	0.0%	1/22	4.5%	1/70	1.4%
	CTET, TIL, TUL	0/48	0.0%	1/22	4.5%	1/70	1.4%
	CTET, OXY, SPE	5/48	10.4%	0/22	0.0%	5/70	7.1%
4	XNL, CTET, OXY, SPE *	1/48	2.1%	0/22	0.0%	1/70	1.4%
	XNL, PEN, TIL, TUL *	0/48	0.0%	1/22	4.5%	1/70	1.4%
	CTET, OXY, PEN, SPE *	1/48	2.1%	0/22	0.0%	1/70	1.4%
	CTET, OXY, TIL, TUL	1/48	2.1%	2/22	9.1%	3/70	4.3%
5	XNL, CTET, OXY, TIL, ENRO *	1/48	2.1%	0/22	0.0%	1/70	1.4%
	XNL, CTET, OXY, TIL, TUL *	1/48	2.1%	0/22	0.0%	1/70	1.4%
	CTET, OXY, PEN, TIL, TUL *	4/48	8.3%	0/22	0.0%	4/70	5.7%
	CTET, OXY, SPE, TIL, TUL *	2/48	4.2%	0/22	0.0%	2/70	2.9%
6	CTET, OXY, PEN, TIL, TUL, DANO *	0/48	0.0%	1/22	4.5%	1/70	1.4%
	XNL, CTET, OXY, PEN, TIL, TUL *	1/48	2.1%	4/22	18.2%	5/70	7.1%
7	CTET, OXY, FFN, PEN, TIL, TUL, DANO *	1/48	2.1%	0/22	0.0%	1/70	1.4%
	XNL, CTET, OXY, TIL, TUL, DANO, SPE *	1/48	2.1%	0/22	0.0%	1/70	1.4%
8	XNL, CTET, OXY, FFN, PEN, TIL, TUL, DANO *	0/48	0.0%	1/22	4.5%	1/70	1.4%
9	not detected	-		-		-	
10	XNL, CTET, OXY, FFN, PEN, TIL, TUL, DANO, ENRO, SPE *	0/48	0.0%	1/22	4.5%	1/70	1.4%

* Configurations indicating multidrug resistance. CTET—chlortetracycline, OXY—oxytetracycline, TUL—tulathromycin, TIL—tilmicosin, PEN—penicillin, NRO—enrofloxacin, DANO—danofloxacin, XNL—ceftiofur, FFN—florfenicol, SPE—spectinomycin,.

3.5. Frequency of Virulence-Associated Genes (VAGs)

Among the tested strains, 100% of the *P. multocida* strains possessed the *sodA* gene, while the other VAGs, namely, *hgbB* and *ompH*, were found in 37.5% (18/48) and 20.8% (10/48) of the strains, respectively. All three genes together were detected in 8.3% (4/48) of the strains. The *M. haemolytica* strains contained the *lkt*, *gs60*, and *gcp* genes in 100% (22/22) of the isolates.

3.6. Frequency of Resistance Genes

The genes *tetH* and *tetR* were observed exclusively among *P. multocida* strains, at 20.8% (10/48) and 16.7% (8/48), respectively. The genes *mphE* and *msrE* were detected in *P. multocida* at 6.3% (3/48) and 14.6% (7/48), respectively, and in *M. haemolytica* at 9.1% (2/22) each.

3.7. Associations

No statistically significant correlations were found between the pair *hgbB* and *ompH*. Moreover, the 100% presence of the remaining VAGs and the low frequency of antimicrobial-resistance genes rendered inferences based on the statistical correlation tests potentially unreliable. Regardless, these tests did not show any significant correlation at $p < 0.05$. However, the cooccurrence of the *tetR* or *tetH* genes was always consistent with phenotypic tetracycline resistance in MIC assays.

Therefore, we decided to present the results in the form of sets showing cooccurrences, as illustrated by the Venn diagrams (Figures 3 and 4).

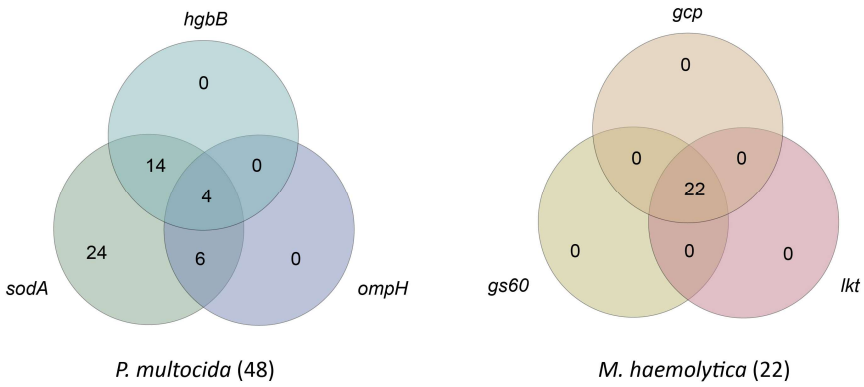


Figure 3. Venn diagrams showing the cooccurrence of virulence-associated genes in the studied strains of *P. multocida* and *M. haemolytica*. The common parts indicate the types of configurations, while the numbers in the respective fields represent the frequency of each configuration.

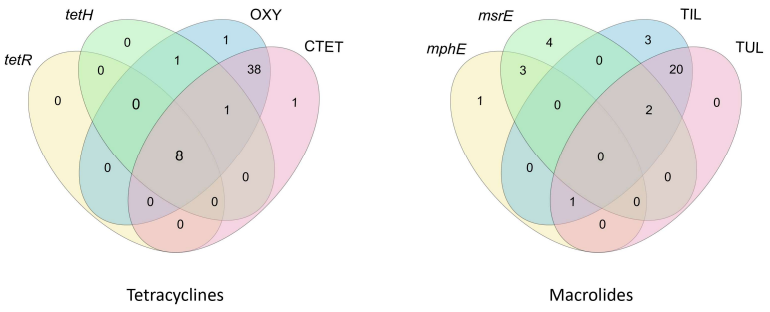


Figure 4. Venn diagrams showing the cooccurrence of antimicrobial resistance genes and phenotypic resistance in the studied strains of *P. multocida* and *M. haemolytica*. The common parts indicate the types of configurations, while the numbers in the respective fields represent the frequency of each configuration. CTET—chlortetracycline, OXY—oxytetracycline, TUL—tulathromycin, TIL—tilmicosin.

4. Discussion

Bovine respiratory disease (BRD) is the leading reason for antimicrobial use in calves [21–23]. The increasing resistance to antimicrobials represents a significant concern, not only for the health of calves but also for human health [24–29]. Recent molecular studies conducted by our team have revealed that *P. multocida* and *M. haemolytica* are the most frequently identified pathogens among Polish calves exhibiting symptoms of BRD [10].

According to an EMA report [24], the most commonly sold antimicrobials in the EU are penicillins (mainly extended-spectrum penicillins in Poland) and tetracyclines, which together account for 56.2% of the aggregated sales for food-producing animals by antimicrobial class in European countries in 2022. Compared with other countries in the European Union, Poland has a relatively high antimicrobial consumption. Of concern is the upward trend in antibiotic sales in Poland over the last decade, as presented in the EMA report [24]. Most of the other European countries included in this report exhibited a downward trend in antibiotic sales. In 2022, the overall antibiotic sales for use in the animal production sector in Poland were more than 2.5 times higher than the European average [24].

To the best of the authors' knowledge, there is a lack of published studies on the antimicrobial susceptibility of *P. multocida* and *M. haemolytica* isolated from Polish calves with BRD. This gap in the literature highlights the urgent need for comprehensive studies to better understand the resistance profiles of these pathogens. All studies cited below refer exclusively to bacteria isolated from the cattle respiratory tract, as our research focuses solely on bacteria derived from this animal species.

4.1. Serotype Detection and Virulence-Associated Genes

The study we conducted on *P. multocida* was designed in accordance with the methodology outlined by Townsend et al. (1998) [30]. It was intended to detect the specific *kmt1* gene and identify all capsular genotypes.

However, our results revealed the presence of only genotype A. Based on the available literature [41] and preliminary (unpublished) studies, we determined that lipopolysaccharide 3 is the most prevalent. This finding was confirmed in our study, as we detected it in 93.8% (45/48) of *P. multocida* isolates. Studies conducted on symptomatic Spanish cattle [42] also revealed that the majority of isolates belonged to serotype A:L3, accounting for 97.6% of all isolates (166/170). Similar results from other studies show that A:L3 was commonly detected in *P. multocida* isolates associated with BRD [15,18,43–46].

All *P. multocida* strains examined in our study exhibited the presence of the *sodA* gene, which helps bacteria evade the host immune response. In studies from Spain [42], Germany [33], and Japan [45], this gene was also detected at a rate of 100% in *P. multocida* serotype A strains. In a study from Iran [47], this gene was less frequently observed, but it was in other serotypes of *P. multocida* strains (B, E), occurring at a rate of 63.6%. A study from Egypt [29], where other serotypes in addition to A were also present, demonstrated a complete absence of this gene.

Ewers et al. (2006) [33] demonstrated a correlation between the presence of the *hgbB* gene and the manifestation of clinical symptoms in cattle. Moreover, this gene was found in 57.7% (104/180) of isolates from cattle. The *hgbB* gene, which may indicate the bacterium's ability to utilize host hemoglobin, was found in 37.5% (18/48) of the strains investigated in our study. A high prevalence of this gene, at 74.0% (176/238), was reported by Katsuda et al. (2013) [45] among strains from diseased calves. Conversely, in the study by Calderón Bernal et al. (2022) [42], the prevalence was 0.6% (1/170).

The *ompH* gene encodes a protein that aids bacteria in adhering to host tissues, thereby facilitating colonization. In our study, it was the least frequently detected virulence gene in *P. multocida*, present in only 20.8% (10/48) of the strains. Similar results to ours were obtained by Calderón Bernal et al. (2022) [42], who reported a prevalence of 14.1% (24/170), whereas Ewers et al. (2006) [33] and Katsuda et al. (2013) [45] reported 100% presence of the gene. Interestingly, Gülaydin et al. (2020) [15] determined that if this gene is present alone (without any other VAGs cooccurring), it is not associated with the manifestation of the disease. In our study, however, it was consistently found in conjunction with other VAGs that we investigated. In our study, only sick calves were included, and the mentioned gene was detected. The relationship between the presence of this gene and the occurrence or absence of the disease requires further investigation.

We did not find a statistically significant correlation between the *ompH* and *hgbB* genes. Similarly, Ewers et al. (2006) [33] did not report such a correlation and also found no correlation between the other VAGs included in our study.

In our study, 86.4% (19/22) of the *M. haemolytica* isolates were genotypically classified as serotype A2, while the remaining 13.6% (3/22) were classified as serotype A1. No serotype A6 was detected, despite conducting the analysis according to the protocol outlined by Klima et al. (2017, 2014) [35,37], which included primers for all three genes determining serotypes.

Mannheimia haemolytica serotype A2 was considered nonpathogenic to cattle and was regarded as a commensal of the normal respiratory flora [35,48]. Current research provides updated insights into the role of this serotype. It is also recognized as an opportunistic pathogen capable of causing disease in calves. It was clearly associated with fatal acute lung disorder in calves in the Netherlands, with all of these *M. haemolytica* isolates (n = 49) belonging to serotype A2 [19]. Moreover, a study by het Lam et al. (2023) [19] reported that 96.1% (49/51) of the isolates from symptomatic calves were serotype A2, while all 45 isolates from cows were classified as serotypes A1 and A6.

In a study by Mason et al. (2022) [49], *M. haemolytica* serotype A2 was identified in 29.8% (31/104) of the *M. haemolytica* isolates tested, which were obtained from bovine clinical pathology and postmortem samples associated with pneumonia cases in Great Britain.

A study conducted in Denmark by Kudirkiene et al. (2021) [18] showed that serotype A1 was detected equally in healthy 52.4% (11/21) and diseased calves 47.6% (10/21), whereas serotype A2 was more frequently isolated from diseased calves 70.0% (7/10). In addition, similarly to our study, they did not detect serotype A6 [18].

Abed et al. (2020) [50], reported that 60% (9/15) of *M. haemolytica* isolates were classified as serotype A2, whereas 40% (6/15) were identified as serotype A1. All of these strains were obtained from pneumonic calves presenting with respiratory symptoms. Similarly, in the present study focusing on diseased calves, the majority of *M. haemolytica* isolates were identified as A2, including one sample obtained from lung tissue, whereas serotype A1 was considerably less common in our study.

Interestingly, the study by het Lam et al. (2023) [19] demonstrated that two serotype A2 strains were genetically closer to serotypes A1 and A6 than to other serotype A2 strains. Such results underscore the need for further research to elucidate the role of these serotypes in the pathogenesis of BRD in calves.

VAGs are essential for the pathogenicity of *M. haemolytica*, influencing its ability to cause disease and interact with the host's immune system. One of the most important virulence factors is considered to be the bacterium's ability to produce leukotoxin, which has cytotoxic potential and damages host leukocytes [20].

Abed et al. (2020) [50] demonstrated the presence of the *lkt* and *gcp* genes in 80% (4/5) of strains obtained from diseased calves. The *gcp* gene encodes a glycoprotein involved in

bacterial adhesion to host tissues, facilitating colonization and infection [20]. In a separate study by Dokmak et al. (2015) [51], both the *gcp* and *gs60* genes were detected in all strains from cattle exhibiting symptoms of BRD. The *gs60* gene encodes a heat-shock protein that enables the bacterium to withstand hostile environmental conditions, enhancing its survival [20].

All the *M. haemolytica* strains examined in our study exhibited the presence of the VAGs investigated, suggesting that these strains have high pathogenic potential, despite a significant proportion belonging to serotype A2, which was previously considered nonpathogenic.

4.2. Antimicrobial Susceptibility

According to the EMA report [24], tetracyclines account for 23.5% of the total sales of antibiotics used in food-producing animals in the European Union. In Poland, they are used at a rate of 39.0 mg/PCU, whereas the average in the EU is 17.4 mg/PCU [24]. This group of antibiotics is classified under Category D in the EMA classification [52], indicating that they should constitute the first line of treatment. In our study, the highest resistance rates were among *P. multocida* strains, with resistance levels ranging from 79.2% to 81.3%. Similarly, among the *M. haemolytica* strains analyzed, half of them exhibited insusceptibility to tetracyclines. The elevated resistance to tetracyclines observed among the strains we studied may likely be contributed to by this high usage rate in Poland. The resistance of these bacterial species to tetracyclines has been reported in numerous studies worldwide. It is quite common for resistance rates to exceed 80%, reaching up to 100%, particularly among *P. multocida* strains [4,21,29,53–56]. Compared to those obtained in our study, lower resistance rates were reported by Dutta et al. (2021) [57] from Nebraska, USA (4.2–40.8%).

Mannheimia haemolytica strains presented the highest resistance to PEN and TIL, with rates of 63.6%, whereas *P. multocida* exhibited resistance rates of 18.8% and 25.5% for these antimicrobials, respectively.

Considering insusceptibility as a combination of resistant and intermediate strains, it is noteworthy that the highest level of insusceptibility recorded in our study was to PEN, with an observed rate of 86.4% among *M. haemolytica* strains. Furthermore, this antimicrobial showed the greatest difference in resistance levels between the studied bacterial species, and this difference was also statistically significant. Penicillins account for 32.7% of the total sales of antibiotics used in food-producing animals in the European Union, while macrolides account for 8.5%. In Poland, penicillins are used at a rate of 69.1 mg/PCU compared to the EU average of 24.2 mg/PCU. The usage of macrolides in Poland is 28.8 mg/PCU, while the European average is 6.3 mg/PCU. These are the highest rates among EU countries [24]. Penicillins, like tetracyclines, are classified under Category D in the EMA classification, while macrolides are classified under Category C, referred to as "caution" [52]. Notably, in studies of cattle from Denmark [18], where the usage of penicillins is 9.7 mg/PCU and macrolides is 5.0 mg/PCU [24], all the examined strains of *P. multocida* and *M. haemolytica* were found to be susceptible to PEN and TIL. This may suggest that high antibiotic usage contributes to the development of resistance among bacterial strains.

In the European Union, fluoroquinolones account for only 2.8% of the total sales of antimicrobials. Compared to other EU countries, fluoroquinolone usage in Poland is high, reaching 11.8 mg/PCU, while the European average is 2.1 mg/PCU [24]. However, in comparison to the other groups of antibacterial agents discussed in our study, fluoroquinolones are used significantly less frequently in Poland. This is most likely reflected in the high susceptibility levels of the bacterial strains we studied to this group of antimicrobials. In our study, the highest susceptibility was observed for fluoroquinolones: *P.*

multocida presented a 91.7% (44/48) susceptibility rate to enrofloxacin, while 77.3% (17/22) of *M. haemolytica* strains were susceptible to both ENRO and DANO. Our results differ from those of studies conducted outside the European Union, which reported high percentages of resistant strains for both bacterial species with respect to the aforementioned fluoroquinolones [4,21,29,53,54,56,58]. However, our findings are consistent with those among Canadian cattle reported by Klima et al. (2020) [55], who also observed low levels of resistance to fluoroquinolones. It is beneficial that we observe low resistance rates to fluoroquinolones, as ENRO, DANO and SPE, which ranked next in terms of susceptibility, fall under Category B in the EMA classification [52]. This classification signifies that these agents are categorized as "restricted" and emphasizes the ongoing need for careful stewardship in their application. Therefore, low resistance to fluoroquinolones is a positive finding, as it helps preserve their effectiveness, which is especially important in the context of treating human infections.

We present and discuss antimicrobials for which breakpoints have been established by the CLSI in the context of susceptibility categories (S, I, R). On the other hand, for antimicrobials for which breakpoints are not defined by the the CLSI, we discuss the data in terms of MIC_{50} and MIC_{90} values.

For the bacteria we studied, which were sourced from the respiratory tracts of cattle, the breakpoint for resistance to AMP was established by the CLSI at 0.25 μg/mL [38]. The concentration range on the plates we used starts precisely at this level. Consequently, it was not possible to assess the susceptibility of the examined strains to this antimicrobial using CLSI guidelines. Nonetheless, for both bacterial species, the MIC_{50} was 0.25 μg/mL, with the MIC_{90} reaching 8 μg/mL (Figure 2). In a study from Japan by Katsuda et al. (2013) [45], a narrower disparity between these metrics was observed: the MIC_{50} for *P. multocida* was 1 μg/mL, while the MIC_{90} was 4 μg/mL. In contrast, for *M. haemolytica*, this disparity was more pronounced, with the MIC_{50} at 2 μg/mL and the MIC_{90} reaching as high as 128 μg/mL [59].

Moreover, for both bacterial species in our study, the MIC_{50} for NEO was the same as the lowest concentration tested, i.e., 4 μg/mL. Additionally, for *M. haemolytica*, this concentration also corresponds to the MIC_{90}.

In contrast, the situation is different for TYLT and CLI. Most of the tested strains did not exhibit growth inhibition or did so only at the highest concentrations tested. However, this may be attributed to the intrinsic resistance of these bacterial species to these substances.

Elevated MIC values for TYLT and CLI were also reported by Depenbrock et al. (2021) [53] who studied bacteria obtained from California cattle; however, their study differs from ours in the concentrations of NEO, where the MIC_{50} and MIC_{90} values were notably higher than those observed in our study, exceeding 32 μg/mL. Similarly, high concentrations of 32 μg/mL and above for NEO and TYLT were reported by Andres-Lasheras et al. (2021) [4], who conducted studies on Canadian cattle.

Another significant issue is the simultaneous occurrence of resistance to various classes of antimicrobial agents. In our study, resistance to at least one substance in three or more antimicrobial classes was 27.1% (13/48) for *P. multocida*, while among *M. haemolytica* strains, resistance was notably higher at 40.9% (9/22). The resistance rate for *P. multocida* in our study was relatively low compared with that reported in other studies, whereas the resistance level for *M. haemolytica* fell within the moderate range.

Among dairy heifers in California [53], the MDR rates were 76% (110/145) for *P. multocida* and 70% (83/119) for *M. haemolytica*. In contrast, among Canadian dairy cattle [4], the MDR rates were 67.8% (164/242) for *P. multocida* and 25.4% (53/209) for *M. haemolytica*.

Klima et al. (2020) [55] reported varying levels of MDR, ranging from 50% to 90% for *P. multocida* and 55% to 81.8% for *M. haemolytica* across different years. Additionally,

the majority of serotype A2 strains from calves with fatal acute lung disorders (95.6%) harbored multiple antimicrobial-resistance genes, which contributed to three to five antimicrobial classes, including phenicols, sulfonamides, tetracyclines, aminoglycosides, and beta-lactams [19].

MDR rates of antimicrobial agents in our study are lower than those reported in other parts of the world. This may suggest that in Poland, only a few groups of antibiotics are primarily used, but they are administered very frequently.

Klima et al. (2020) [55] identified 104 unique multidrug-resistance patterns involving 16 different antimicrobials. In our study, we observed 25 configurations, although the number of antimicrobials considered was 11. Similarly, Depenbrock et al. (2021) [53] reported the same number of configurations as our study, while examining the same number of antimicrobials.

In our study, the most frequently observed phenotypic resistance pattern was "CTET, OXY." However, this pattern was exclusively observed among *P. multocida* strains, occurring in 37.5% (18/48) of the strains. Furthermore, these antimicrobials agents belong to the same class.

In contrast, among the *M. haemolytica* strains, resistance to "XNL, CTET, OXY, PEN, TIL, TUL" was the most frequently observed configuration and was present in 18.2% (4/22) of the tested strains. Although this configuration involves six antimicrobials, they belong to four distinct classes. Depenbrock et al. (2021) [53] found that *P. multocida* was most commonly resistant to four classes, while *M. haemolytica* was resistant to three classes, which contrasts with our findings.

Furthermore, their study revealed that resistance to fluoroquinolones was present in the most common configurations for both *P. multocida* and *M. haemolytica*, which also distinguishes their findings from ours [53]. In contrast, in our study, configurations containing ENRO and/or DANO were observed in only 8.6% (6/70) of the results.

They also noted a similarity in the resistance patterns, suggesting that this might be due to both bacterial species being isolated from the nasal cavity [53]. In our study, however, these patterns appeared to differ, and we have demonstrated statistically significant differences in antimicrobial resistance between these two bacterial species. Therefore, it seems that the previous use of antimicrobials in the studied populations is more likely to have influenced the emergence of resistance patterns than the fact that they were isolated from a single location.

4.3. Resistance Genes

In our study, the presence of the *tetH* and *tetR* genes was consistently associated with phenotypic to tetracycline resistance, but the genes *mphE* and *msrE* were detected not only in strains exhibiting phenotypic resistance to macrolides but also in those that did not show such resistance (Figure 4). Similar findings were reported by Klima et al. (2020) [55]; however, in their study, the presence of the *tetH* gene was not associated with phenotypic resistance to chlortetracycline in more than 70% of *P. multocida* and *M. haemolytica* strains. It implies that the presence of resistance genes does not always correlate with phenotypic resistance, as gene expression can be influenced by various regulatory mechanisms. The *tetR* gene was always found in cooccurrence with the *tetH* gene in the examined strains (n = 8). Each of these strains exhibited phenotypic resistance to both tested tetracyclines (CTET and OXY). Among all the strains in which the *tetH* gene was detected, phenotypic resistance to OXY was observed in 10 out of 10 cases, while one of these strains did not show resistance to CTET (1/10). Cooccurring resistance to both CTET and OXY in the presence of the *tetH* gene was noted in 9 out of 10 strains. These findings are consistent with

those of the study by Klima et al. (2020) [55], which demonstrated a statistically significant association between the presence of *tetH* and resistance to OXY.

In our study, resistance genes for antimicrobials such as *tetR*, *tetH*, *mphE*, and *msrE* were relatively rare. Therefore, we were not able to make inferences based on correlation tests. However, phenotypic resistance to tetracyclines and macrolides was observed at a high level (Figure 1). The observed antimicrobial resistance may result from the presence of various genes as well as other factors, such as biofilm production or mutations [60,61]. More than 40 genes responsible for tetracycline resistance are known. For example, among cattle, the *tetG* gene was found in Germany [61,62], *tetL* in Belgium [63], and *tetB* in France, [60], while *tetH* emerged as the most frequently detected gene in North America [55,64].

Understanding the mechanisms underlying resistance in the strains studied would require more extensive research.

4.4. Limitations

This study used only clinical samples submitted to a single Polish laboratory, which primarily receives specimens from the southwestern region of the country. Due to financial constraints, we were unable to perform LPS typing for three *P. multocida* strains that were negative on the confirmation test for LPS type three. Additionally, the small number of *M. haemolytica* serotype A1 strains (3/22) precluded the possibility of conducting reliable comparative analyses between serotypes.

4.5. Future Directions

In this study, the MIC_{90} values for more than half of the tested antimicrobials fell outside the range of the concentrations examined. Furthermore, the statistically significant differences observed in the susceptibility of the bacterial species to antimicrobial agents complicate the formulation of recommendations for field veterinarians regarding the most appropriate first-line antimicrobials to use for BRD in calves. Despite the high susceptibility of the bacteria to fluoroquinolones, it is important to note that these drugs are highly classified by the EMA and should not be used indiscriminately. Therefore, we believe that microbiological testing, including determining the susceptibility of isolated bacterial strains, should precede any decision to use antimicrobial agents in calves in Poland.

The levels of average antimicrobial resistance among *M. haemolytica* strains are relatively high, raising some concerns. It is intriguing to consider whether over the next few years, these values may evolve into full resistance and what factors might contribute to this trend. Such dynamics in resistance could significantly impact treatment efficacy and necessitate the development of new therapeutic strategies. It will be important to monitor these trends in the future to better understand the mechanisms of resistance evolution and to implement appropriate preventive measures.

The potential role of *M. haemolytica* serotype A2 as a pathogen, which was previously considered exclusively nonpathogenic in cattle, makes it an intriguing subject for further research. Investigating its role in respiratory diseases could provide valuable insights to aid in the development of prevention strategies.

5. Conclusions

The results of this study expand the knowledge on the pathogenicity and antimicrobial resistance of *P. multocida* and *M. haemolytica* in Polish cattle. All the tested strains of both bacterial species possessed at least one virulence-associated gene, while all *M. haemolytica* strains carried each of the investigated virulence genes, clearly indicating their potential to cause disease. *Pasteurella multocida* exhibited the highest resistance to tetracyclines, while *M. haemolytica* demonstrated the greatest lack of sensitivity to penicillin. The simultaneous

high usage of these antibiotics in the EU, especially in Poland, indicates a potential impact of their widespread use on the development of resistance. A significant difference in antimicrobial resistance between the bacterial species studied complicates effective BRD therapy based solely on empirically chosen antibacterial drugs. Given the above, it is crucial for clinicians to perform microbiological tests with antimicrobial-susceptibility assessments as frequently as possible before applying them to sick calves.

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Institutional Review Board Statement: Approval Waived according to Polish law (the Experiments on Animals Act from 15 January 2015, Journal of Laws of the Republic of Poland from 2015, item. 266), this study did not require the Ethics Committee's approval. The samples used in this study were originally from cattle infection diagnostic material collected by veterinarians treating these herds.

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