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Antibiotic resistance of *Gallibacterium anatis* biovar *haemolytica* isolates from chickens

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Abstract

Introduction: *Gallibacterium anatis* is an opportunistic bacteria inducing a range of clinical signs in poultry. *Gallibacterium anatis* strains show multidrug resistance to antibacterial substances. The purpose of this study was to examine the susceptibility of *G. anatis* biovar *haemolytica* isolates collected from the respiratory, reproduction and gastrointestinal tracts of chickens to different antibiotics from various classes. **Material and Methods:** *Gallibacterium anatis* biovar *haemolytica* was identified in tracheal swab and gastrointestinal and reproductive tract tissue samples from Polish layer and broiler chicken flocks. Twenty six isolates with β-haemolysis capability, each from a different flock, obtained from the respiratory (n = 8), reproductive (n = 10) and gastrointestinal (n = 8) tracts were selected and identified by matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry after culturing. A PCR method targeting the 16S genes was used for verification of isolates. The isolates' susceptibility to 20 antimicrobials was evaluated using the disc diffusion method for 8 drugs and the dilution method for the other 12. In addition, they were tested for the presence of the *Gtx*A, *gyr*B and *flf*A virulence genes and *bla*$_{ROB}$, *aph*A, *tet*B and *tet*H antibiotic resistance genes by PCR. **Results:** The most prevalent antibiotic resistance was to tilmicosin, tylosin and quinupristin/dalfopristin (all 100%), erythromycin (96.2%), tetracycline (96.2%), linezolid (92.3%) and teicoplanin (92.3%). Universal susceptibility was to only one antibiotic, chloramphenicol. Statistically significant differences were found between the resistance of gastrointestinal tract strains and that of strains from other tracts to daptomycin, gentamicin, ciprofloxacin and colistin. The *Gtx*A and *gyr*B genes were detected in 100% of isolates and *flf*A in 19.2%. The isolates most frequently contained *tet*B and less frequently *tet*H and *aph*A, and did not contain *bla*$_{ROB}$. **Conclusion:** Most *G. anatis* biovar *haemolytica* isolates were resistant to many classes of antibiotics. Therefore, it is necessary and important to be vigilant for the occurrence of these bacteria and thorough in their diagnosis.

Keywords: *Gallibacterium anatis*, antibiotic resistance, chickens.

Introduction

Infections caused by *Gallibacterium anatis (G. anatis)* can lead to very serious clinical symptoms. Even though this Gram-negative bacterium exists as part of the commensal bacterial flora in birds, with inappropriate environmental factors or co-infections, its pathogenicity can change (19, 31). Mixed bacterial and viral infections are a factor in the development of gallibacteriosis. Bacteria such as *E. coli*, *Avibacterium paragallinarum*, *Mycoplasma gallisepticum* and *M. synoviae* and viruses such as Newcastle disease virus and adenoviruses can intensify symptoms or increase the mortality of birds in a flock infected with *G. anatis* (11, 12, 17, 19, 31, 33). In addition, the influence of other factors such as the strain of bacteria, the age and immune status of the birds or stress can sometimes be decisive in the process of weakening avian health (19, 28). There are two phenotypically different biovars of *G. anatis*. The distinction is made on the basis of their haemolytic properties: the *haemolytica* biovar causes β-haemolysis, and the *anatis* biovar does not. It seems that the *haemolytica* biovar may be more pathogenic (7, 16, 24, 31). *G. anatis* spreads in flocks by the respiratory route through direct contact between birds in the flock as well as by the vertical route through infected eggs. Its spread is also related to its habitat. The *haemolytica* biovar causes a range of symptoms associated with three tracts: oophoritis and salpingitis in the reproductive tract; peritonitis, perihepatitis, liver necrosis and enteritis in the gastrointestinal tract; or air sacculitis and tracheitis in the respiratory tract, in addition to inducing septicaemia in chickens (6, 15, 24, 26, 28, 33). In laying hens, the reproductive organs are chiefly affected, and this bacterium produces lesions including haemorrhagic oophoritis and rupture of ovarian follicles (18, 24, 26).

In cockerels, the bacterium causes inflammation of the epididymides and leads to reduced semen quality (27). Infection with *G. anatis* decreased the laying rate by 8–10%, lowered productivity, and caused mortality of up to 73% in laying hens subjected to experimental immunosuppression. In young chickens, on the other hand, the lesions were usually systemic (19, 26, 37).

On bovine blood agar, smooth, greyish, opaque, shiny colonies of *G. anatis* biovar *haemolytica* produce a wide haemolytic zone. Responsible for this is the cytotoxin *Gtx*A (*Gallibacterium* toxin A), a two-domained protein with a C- and an N-terminus and one of the virulence factors of *G. anatis* (20, 36). It has the ability to make pores in the plasma membrane of host cells, which can ultimately lead to their necrosis or apoptosis. Cytotoxin *Gtx*A lyses red blood cells in a wide variety of hosts and is also leukotoxic (20, 28, 36). The distribution of *G. anatis* in the world is quite wide. It is found in poultry flocks on different continents, from Europe and Asia to North America and Africa, and has been isolated not only from poultry but also from wild birds, calves and even humans (2, 3, 23, 29, 31, 32, 34).

G. anatis strains show multidrug resistance to antibacterial substances, which is of concern because of their wide distribution. Multidrug resistance of bacteria is a growing problem for the poultry industry as well as for public health (1, 2, 17, 19, 31, 35). Recent studies show common resistance in a large number of *G. anatis* strains to erythromycin and tylosin antibiotics from the macrolide class as well as antibiotics from the tetracycline class (2, 8, 17, 21, 31, 35).

The purpose of this study was to examine the susceptibility of *G. anatis* biovar *haemolytica* isolates collected from the respiratory, reproduction and gastrointestinal tracts of chickens to different antibiotics from various classes.

Material and Methods

Sampling procedures. Twenty six *G. anatis* isolates showing β-haemolysis capability were selected from the sample collection. The isolates were from chicken flocks, collected from the respiratory (n = 8), gastrointestinal (n = 8) and reproductive (n = 10) tracts. Each sample came from a different flock (Table 1).

Table 1. List of *Gallibacterium anatis* isolates obtained from flocks of hens

Isolate	Type of flock	Age of birds (weeks)	Tract of sample origin
GA1	layer	28	respiratory
GA2	layer	28	respiratory
GA3	layer	31	respiratory
GA4	layer	30	reproductive
GA5	layer	28	reproductive
GA6	layer	31	reproductive
GA7	layer	31	reproductive
GA8	layer	28	reproductive
GA9	layer	28	reproductive
GA10	layer	30	reproductive
GA11	layer	25	respiratory
GA12	broiler	4	respiratory
GA13	broiler	4	respiratory
GA14	broiler	3	respiratory
GA15	layer	27	reproductive
GA16	layer	29	reproductive
GA17	layer	28	reproductive
GA18	broiler	4	respiratory
GA19	broiler	4	gastrointestinal
GA20	broiler	3	gastrointestinal
GA21	broiler	4	gastrointestinal
GA22	broiler	4	gastrointestinal
GA23	broiler	3	gastrointestinal
GA24	broiler	4	gastrointestinal
GA25	broiler	4	gastrointestinal
GA26	broiler	4	gastrointestinal

Trachea swab samples were brought to the Department of Poultry Diseases at the National Veterinary Research Institute in Poland as part of a routine diagnostic test and monitoring programme. Tissues from the gastrointestinal and reproductive tracts were aseptically obtained from birds sent for diagnostic purposes. All examined birds were floor reared. Some of the birds had respiratory signs in the form of rales and coughing, and some had swollen heads and poorer laying performance.

Isolation and identification of *G. anatis*. Samples were cultured onto Columbia agar with 5% sheep's blood for 24 h at 37 ± 1°C in an atmosphere of 5% CO_2. After incubation, three colonies from each plate with morphology characteristic of β-haemolysis were selected and transferred to nutrient agar and incubated for 24 h at 37 ± 1°C. Identification of colonies was performed using matrix-assisted laser desorption/ ionisation–time-of-flight mass spectrometry (MALDI-TOF) and the MALDI Biotyper system with MBP COMPASS 4.1 software (Bruker Daltonics, Bremen, Germany). Bacterial colonies from the agar plate were transferred to the MALDI target plate and mixed with formic acid and α-cyano-4-hydroxycinnamic acid matrix solution. Strains identified as *G. anatis* by matching them to reference species found in the software's database were preserved and stored at −20°C for further testing.

Antibiotic resistance. The antimicrobial susceptibility of *G. anatis* biovar *haemolytica* was determined by using two methods: disc diffusion and microbroth dilution. With these methods it was possible to test the antibiotic resistance of isolates to 20 antimicrobial substances from 12 different classes of antibiotics. Eight different antibiotics were used in the disc diffusion method (Antimicrobial Susceptibility Testing discs; Oxoid, Basingstoke, UK): florfenicol (30 μg) in the amphenicol class, doxycycline (30 μg) in the tetracycline class, amoxicillin (25 μg) in the β-lactam class, enrofloxacin (5 μg) in the fluoroquinolone class, colistin (50 μg) in the polymyxin class, ceftazidime (30 μg) in the cephalosporin class, and tilmicosin (15 μg) and tylosin (30 μg) in the macrolide class (Table 2). The test applied a bacteria volume of 100 μL at 1.5×10^8 colony-forming units/mL (0.5 McFarland scale) distributed uniformly onto the Columbia agar with 5% sheep's blood. The inhibition zones were interpreted visually.

Determination of minimum inhibitory concentration (MIC) was performed according to the Clinical and Laboratory Standards Institute standard M31-A2(13) using a commercially prepared dehydrated panel for *Enterobacteriaceae* (Sensititre EU Surveillance Enterococcus EUVENC AST plate; Thermo Fisher Scientific, Waltham, MA, USA). The plates were incubated for 20–24 h at 37 ± 1°C under aerobic conditions. Antimicrobial susceptibility testing of *G. anatis* biovar *haemolytica* was performed from a fresh culture on agar and a suspension prepared at 0.5 McFarland density in 0.9% NaCl (bioMérieux, Marcy-'l'Étoile, France), of which 10 μL was transferred to 11 mL of Mueller–Hinton broth (Thermo Fisher Scientific). The suspension was thoroughly vortexed and then 50 μL of it was added to each well of a plate. The plates contained different concentrations of 12 different antibiotics: gentamicin (8–1,024 mg/L) in the aminoglycoside class, ampicillin (0.5–64 mg/L) in the β-lactam class, chloramphenicol (4–128 mg/L) in the amphenicol class, ciprofloxacin (0.12–16 mg/L) in the fluoroquinolone class, teicoplanin (0.5–64 mg/L) and vancomycin (1–128 mg/L) in the glycopeptide class, daptomycin (0.25–32 mg/L) in the lipopeptide class, erythromycin (1–128 mg/L) in the macrolide class, linezolid (0.5–64 mg/L) in the oxazolidinone class, quinupristin/dalfopristin (0.5–64 mg/L) in the streptogramin class, and tetracycline (1–128 mg/L) and tigecycline (0.03–4 mg/L) in the tetracycline class (Table 2). The plates were incubated for 24 h at 37 ± 1°C. The MIC was defined as the lowest concentration preventing visible growth using a plate reader (Sensititre-TREK Vizion Digital MIC Viewing System; Thermo Fisher Scientific). Strains resistant to at least three classes of antimicrobials were identified as multidrug resistant (MDR).

Table 2. List of antibiotics used to determine the antibiotic susceptibility of *Gallibacterium anatis* biovar *haemolytica* isolates

Antibiotic class	Antibiotic
aminoglycoside	gentamicin
β-lactam	amoxicillin
	ampicillin
cephalosporin	ceftazidime
amphenicol	florfenicol
	chloramphenicol
fluoroquinolone	enrofloxacin
	ciprofloxacin
glycopeptide	vancomycin
	teicoplanin
lipopeptide	daptomycin
macrolide	erythromycin
	tylosin
	tilmicosin
oxazolidinone	linezolid
polymyxin	colistin
streptogramin	quinupristin/dalfopristin
tetracycline	tetracycline
	doxycycline
	tigecycline

DNA extraction. DNA was extracted from nutrient agar plate cultures with a Maxwell RSC Cultured Cells DNA Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. The quantity and quality of the DNA was determined using the NanoDrop 1000 system (Thermo Scientific). The tris-ethylenediaminetetraacetic acid used for sample preparation was the negative control. Samples were frozen at −20°C.

PCR. *G. anatis* isolates were identified by the PCR method described earlier by Bojesen *et al.* (9). Specific primers designed to detect the 16–23S ribosomal RNA (rRNA) region were used. The mixture and the conditions described earlier were used for the reaction (21). The PCR amplicons were separated by electrophoresis on a 2% agarose E-gel plate (Invitrogen, Carlsbad, CA, USA) containing ethidium bromide, and were visualised by ultraviolet transillumination.

Virulence and resistance genes. Isolates of *G. anatis* biovar *haemolytica* were tested for presence of the *gyr*B, *Gtx*A and *flf*A virulence genes. All samples were also tested for the presence of the bla_{ROB}, *aph*A, *tet*B and *tet*H antibiotic resistance genes. A PCR method was used for both test steps with the starters described in a previous publication (1).

Statistical analysis. Venn diagrams were constructed showing the number of antibiotics resisted by at least 50% of *G. anatis* isolates from the three chicken anatomical tracts. To determine the statistical significance of antibiotic resistance differences between respiratory, reproductive and gastrointestinal isolates and presence differences of virulence and resistance genes in *G. anatis* isolates, the Mann–Whitney and the one-way ANOVA tests were used. The value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using the Social Science Statistics program (www.socscistatistics.com).

Results

Isolation and identification of *G. anatis* biovar *haemolytica*. Each tested sample showing β-haemolysis on Columbia agar was confirmed by MALDI TOF as *G. anatis*. The bacterium was also identified by PCR by obtaining amplicons of 1,030 base pairs in all samples.

Antibiotic resistance. All isolates showed the highest incidence of resistance to tilmicosin (100%), tylosin (100%) and quinupristin/dalfopristin (100%), followed by resistance to erythromycin (96.2%), tetracycline (96.2%), linezolid (92.3%) and teicoplanin (92.3%). All resistance percentages are given by antibiotic class in Table 3. *G. anatis* isolates showed the greatest susceptibility to chloramphenicol. A full 100% of isolates were susceptible to this antibiotic. Florfenicol and ceftazidime were resisted by only 15% and 19% of isolates, respectively. All isolates were MDR. Five isolates of *G. anatis* biovar *haemolytica* showed multiresistance to 16 antibiotics, and were from the respiratory and reproductive tracts. Resistance to 16, 15, 14 and 13 antibiotics was shown by six, five, six and three *G. anatis* isolates, respectively. Resistance to 12, 11, 10 and 8 antibiotics was shown by two, one, three and one isolates, respectively, noted predominantly (75%) in isolates from the gastrointestinal tract.

Statistically significant differences (P-value < 0.05) were found between the resistance of strains isolated from the gastrointestinal tract and other tracts to the antibiotics: ceftazidime, tylosin, colistin, daptomycin, gentamicin, ciprofloxacin and vancomycin. Nine antibiotics were commonly resisted by at least 50% of the group in the cases of all three anatomical-origin isolate groups (Table 4, Fig. 1). *G. anatis* isolated from the respiratory and reproductive tracts had at least 50% incidence of resistance to four antibiotics. Isolates of *G. anatis* obtained from the gastrointestinal tract showed at least 50% resistance to three antibiotics – colistin, ceftazidime and florfenicol – which belong to the polymyxin, cephalosporin and amphenicol antibiotic classes, respectively (Tables 3 and 4, Fig. 2).

Table 3. Antibiotic resistance of *Gallibacterium anatis* biovar *haemolytica* isolates (disc diffusion and minimum inhibitory concentration methods)

Antibiotic class	Antibiotic	Resistance %
aminoglycoside	gentamicin	69.2
β-lactam	amoxicillin	88.0
	ampicillin	73.1
cephalosporin	ceftazidime	19.0
amphenicol	florfenicol	15.0
	chloramphenicol	0.0
fluoroquinolone	enrofloxacin	88.0
	ciprofloxacin	69.2
glycopeptide	vancomycin	88.5
	teicoplanin	92.3
lipopeptide	daptomycin	69.2
macrolide	erythromycin	96.2
	tylosin	100.0
	tilmicosin	100.0
oxazolidinone	linezolid	92.3
polymyxin	colistin	31.0
streptogramin	quinupristin/dalfopristin	100.0
tetracycline	tetracycline	96.2
	doxycycline	46
	tigecycline	57.7

Table 4. Antibiotic resistance of *Gallibacterium anatis* biovar *haemolytica* isolates by anatomical tract of sample origin (disc diffusion method)

Antibiotic	Dose µg	Respiratory (n)	Resistance (%)	Reproductive (n)	Resistance (%)	Gastrointestinal (n)	Resistance (%)
enrofloxacin	5	2	25.0	10	100.0	5	62.5
tilmicosin	15	8	100.0	10	100.0	8	100.0
amoxicillin	25	5	62.5	10	100.0	8	100.0
doxycycline	30	5	62.5	2	20.0	8	100.0
ceftazidime	30	1	12.5	1	10.0	5*	62.5
tylosin	30	8	100.0	10	100.0	3*	37.5
colistin	50	2	25.0	1	10.0	8*	100.0
florfenicol	30	3	37.5	1	10.0	5	62.5

* – statistically significant differences (P-value <0.05) between strains isolated from the gastrointestinal tract and other tracts

MIC Value (mg/L)

Antibiotic	Place of isolation (system)	Resistance (%)	0.032	0.064	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024
Ampicillin	respiratory	50						4						4					
	reproduction	100												10					
	gastrointestinal	75					1		1	1		1		4					
Chloramphenicol	respiratory	0								5			3						
	reproduction	0								7			3						
	gastrointestinal	0								8									
Ciprofloxacin	respiratory	100										8							
	reproduction	100										10							
	gastrointestinal *	0					3	1	4										
Daptomycin	respiratory	87.5								1			7						
	reproduction	80								2			8						
	gastrointestinal *	25				4	1	1					2						
Erythromycin	respiratory	100									5	3							
	reproduction	100									7	2			1				
	gastrointestinal	87.5							1		5				2				
Gentamicin	respiratory	100												8					
	reproduction	100												8	2				
	gastrointestinal *	0									8								
Linezolid	respiratory	100									4	4							
	reproduction	100									2	6		1					
	gastrointestinal	75								2	6								
Quinupristin/ dalfopristin	respiratory	100									3	5							
	reproduction	100									1	9							
	gastrointestinal	100									7	1							
Teicoplanin	respiratory	100												8					
	reproduction	100												10					
	gastrointestinal	75					2			1		2		3					
Tetracycline	respiratory	100											4	4					
	reproduction	100										3	3	3	1				
	gastrointestinal	87.5								1	1	3	2	1					
Tigecycline	respiratory	12,5			1	6	1												
	reproduction	40				6	3	1											
	gastrointestinal	0		2	4	2													
Vancomycin	respiratory	100												1	7				
	reproduction	100												1	9				
	gastrointestinal *	62.5						2		1		1	2	1	1				

Fig. 1. Antibiotic resistance of *Gallibacterium anatis* biovar *haemolytica* isolates by the anatomical tracts from which they were isolated (minimum inhibitory concentration (MIC) method)
red line – dilution at which resistance started; * – statistically significant differences (P-value < 0.05) between strains isolated from the gastrointestinal tract and other tracts

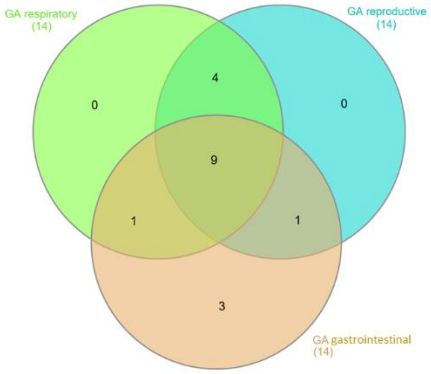

Fig. 2. Venn diagram of the numbers of antibiotics resisted by at least 50% of isolates from the chicken respiratory, reproductive and gastrointestinal tracts
GA – *Gallibacterium anatis*

Presence of virulence and resistance genes. The *Gtx*A and *gyr*B virulence genes were present in 100% of isolates. In contrast, the *flf*A gene was present in only 19.2% of isolates. There was no statistically significant difference (P-value >0.05) between the presence of the *Gtx*A gene and the presence of the *gyr*B gene. However, statistically significant differences (P-value <0.05) were found between the presence of the *Gtx*A and *gyr*B virulence genes and that of the *flf*A gene (Fig. 3a).

The *tet*B and *tet*H tetracycline resistance genes constituted the largest group of genes isolated from *G. anatis* biovar *haemolytica* strains. The *tet*B gene was detected in all tested isolates, while *tet*H was noted in 34.6% of isolates, mostly isolated from the gastrointestinal (23%) and the respiratory tract (12%). The *tet*H gene was not found in *G. anatis* isolates from the reproductive tract. A low percentage of *G. anatis* isolates were detected with the *aph*A gene (3.8%), and

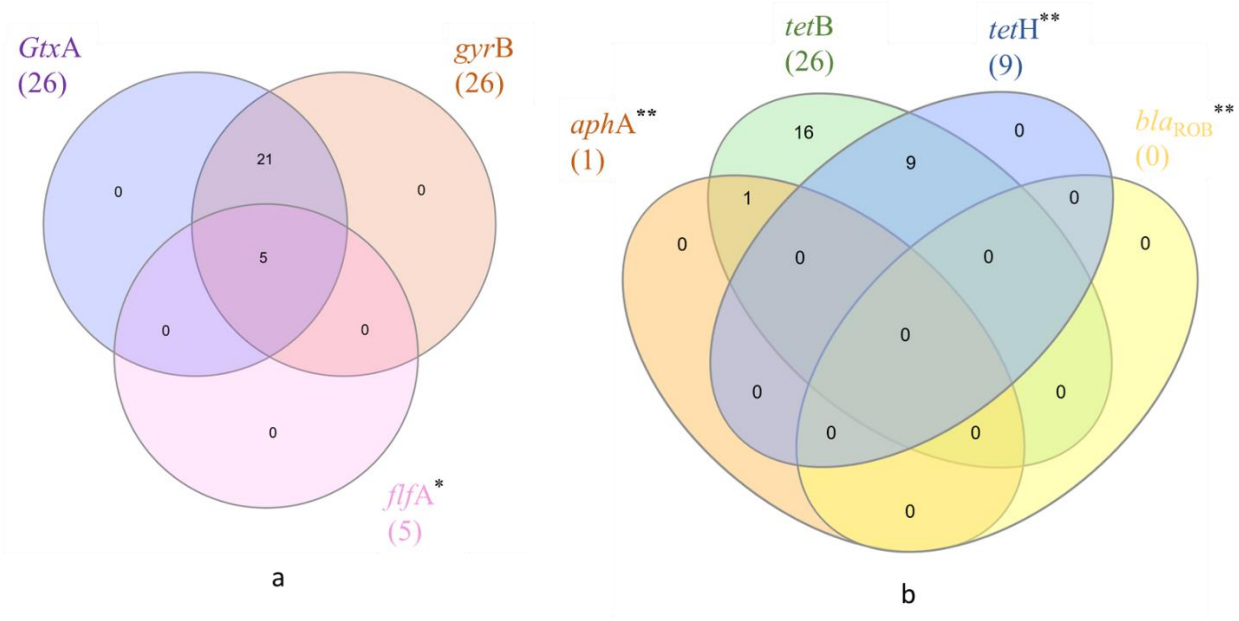

Fig. 3. Venn diagram of a) virulence genes and b) resistance genes in *Gallibacterium anatis* biovar *haemolytica*
* – statistically significant differences (P-value < 0.05) between the presence of the *Gtx*A and *gyr*B virulence genes and presence of the *flf*A gene
** – statistically significant differences associated with the presence of virulence genes *Gtx*A and *gyr*B, and the presence of resistance genes *tet*H, *aph*A and bla_{ROB} (P-value < 0.05)

none were identified with the bla_{ROB} gene. Five isolates (19.2%) had the *Gtx*A and *gyr*B genes plus the *flf*A gene. Only in one isolate (3.8%) originating from the gastrointestinal tract was the presence of the *aph*A gene also found besides *tet*B and *tet*H (Fig. 3b). There were significant differences associated with the presence of virulence genes *Gtx*A and *gyr*B, and the presence of resistance genes *tet*H, *aph*A and bla_{ROB} (P-value < 0.05).

Discussion

Gallibacterium anatis is one of the major poultry pathogens. The haemolytic biovar can be responsible for multiple clinical signs caused by either single infections or mixed infections with other pathogens, leading to serious economic losses in the poultry industry (15, 24, 31). The present study aimed to investigate the antibiotic resistance of isolates of *G. anatis* biovar *haemolytica* obtained from chicken respiratory, reproductive and gastrointestinal tracts. We also examined the presence of resistance genes to three groups of antibiotics and the presence of virulence genes.

This bacterium can be isolated from the trachea as well as the cloaca of healthy commercial chickens because it forms part of the normal chicken microflora (19, 24). Infections can be associated with a variety of clinical signs occurring together in mixed infections. There are also reports that describe single *G. anatis* infections causing disorders in the reproductive tract of chickens (24, 26). In addition to type of strain, route of infection and involvement of secondary factors influencing the progress of *G. anatis* infection, there are many other factors involved in the development of disease. The age of the bird, its subjection to stress or the action of particular hormones are all host-related factors. Environmental factors that can affect the development of infection are seasonal changes or cold stress (10, 22, 25). Many reports inform of an increase of antibiotic resistance among *G. anatis* isolates (8, 17, 21, 35). In our study, we used *G. anatis* biovar *haemolytica* strains isolated from three chicken anatomical tracts. Antibiotic resistance of respiratory, reproductive and gastrointestinal isolates was tested against 20 different antibiotics from 12 different classes using two methods. The majority of *G. anatis* isolates showed high resistance to antibiotics of the macrolide class: resistance to erythromycin, tylosin and tilmicosin was found in 96.2%, 100% and 100%, respectively. Our results are in agreement with the results of other authors (1, 14, 29). Isolates from our collection also showed high resistance to tetracycline (96.2%), tigecycline (57.7%) and doxycycline (46%), proving similar to resistance noted in previous studies (1, 14, 15, 21). In addition, high resistance was evident to gentamicin (69.2%), an antibiotic of the aminoglycoside class. Resistance to antibiotics from these three classes is common as the MDR described on animal farms, and is escalating. This is related to the frequent use of these substances in animal production. Tetracycline resistance was not only very common among our isolates but also in other studies (5, 8, 35). High tetracycline resistance has also been linked to the presence of resistance genes. In our study, two different tetracycline resistance genes were found. The *tet*B gene was detected in all tested isolates, while *tet*H was in 34.6% of isolates, which is comparable to the results obtained by other authors (1). Most *G. anatis* isolates having the *tet*H gene were from the gastrointestinal tract (23%) and the respiratory tract (12%). The presence of *tet* genes has been reported in *G. anatis* isolates in other studies, where it appeared to be frequent in isolates implicated in all types of infections (1, 8, 35). The *aph*A gene, which encodes a protein

associated with aminoglycoside resistance, was found in one isolate from our collection. The low percentage prevalence of the gene detected was not, however, to any extent proportionate to the frequency of resistance of the isolates to the aminoglycoside antibiotic gentamicin, which was high at 69.2%. High antibiotic resistance was also found to quinupristin/dalfopristin in the streptogramin class (100%), teicoplanin (92.3%) and vancomycin in the glycopeptide class (88.5%), amoxicillin in the β-lactam class (88%) and linezolid in the oxazolidinone class (92.3%). Resistance to these antibiotics may be a grave problem because they are used in human medicine. The elevated rate of colistin resistance among the isolates (31%) is also alarming. A finding which contrasted with the instances of resistance noted was that all isolates were susceptible to chloramphenicol. Multidrug resistance is a huge problem in veterinary and human medicine. Recent antibiotic susceptibility studies of field strains of *G. anatis* have shown a high number of MDR strains. Our research shows that a significant proportion of isolates showed resistance to at least eight different antibiotics. Five isolates were resistant to 16 antibiotics, which is very alarming.

Virulence factors are involved in many aspects of host–pathogen relationships, including colonisation, nutrient acquisition, immune evasion and immunosuppression (1, 28, 35). They include toxins, enzymes and adhesion molecules and are very important virulence factors that confer the ability to cause disease and thus determine the pathogenicity of microorganisms. The main feature of *G. anatis* biovar *haemolytica* isolates is the ability to form a wide β-haemolytic zone around the colony on plates (15, 24, 35). This trait was observed in all isolates used in this study. The protein responsible is the secreted toxin *Gtx*A, which exhibits haemolytic activity against erythrocytes (20). The toxin is a specific virulence factor of *G. anatis*. Its presence gives *G. anatis* a means of adhering to cells and changes cell permeability and expression of inflammatory factors, resulting in cell damage and apoptosis (20, 30, 36). Kristensen *et al.* (20) in their research concluded that *Gtx*A may represent a new form of RTX-like toxin with immune evasion function. In this study, this virulence gene was detected in all tested isolates. In addition to *Gtx*A toxin production, *G. anatis* is known to produce a variety of other virulence factors. One of them, the *gyr*B gene encoding for the B subunit of the DNA gyrase, was also present in all of our isolates (1, 35). *G. anatis* biovar *haemolytica* has the ability to adhere to chicken epithelial cells because of its fimbriae-like structures involved in adhesion to host cells. One of the genes encoding F17-like fimbriae that bind receptors containing N-acetyl-D-glucosamine (Glc-NAc) on the host cell surface is *flf*A (4, 28, 35). Among the isolates tested in this study, the presence of this gene was found in five of them.

Considering the clinical signs of the hens from which the test material was obtained, it can be concluded that biovar *haemolytica* may have had an effect on the onset of laying-related symptoms. The hens from which *G. anatis* isolates were obtained from the gastrointestinal tract did not show any clinical signs. This may also explain the differences in these strains' resistance to that of isolates from the other two tracts.

Conclusion

Our results imply that the high prevalence of MDR isolates is an alarming threat, requiring immediate action to prevent the spread of *G. anatis* isolates resistant to antibiotics used in human medicine such as vancomycin and colistin. In light of our results as well as reports from other poultry researchers around the world, it seems very important to prevent *G. anatis* infection more effectively.

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