Infection and Drug Resistance

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ORIGINAL RESEARCH

Sequence Analysis, Antibiogram Profile, Virulence and Antibiotic Resistance Genes of XDR and MDR *Gallibacterium anatis* Isolated from Layer Chickens in Egypt

Abelazeem M Algammal[1], Marwa E Abo Hashem[1], Khyreyah J Alfifi[2], Amenah S Al-otaibi[2], Marfat Alatawy[2], Reham M ElTarabili[1], Wafaa A Abd El-Ghany[3], Helal F Hetta[4], Ahmed M Hamouda[5], Amira A Elewa[5], Marwa M Azab[6]

[1]Department of Bacteriology, Immunology, and Mycology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt; [2]Biology Department, Faculty of Science, Tabuk University, Tabuk, Saudi Arabia; [3]Poultry Diseases Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt; [4]Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt; [5]Animal Health Research Institute, Zagazig Branch, Zagazig, Egypt; [6]Department of Microbiology and Immunology, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt

Correspondence: Abelazeem M Algammal, Department of Bacteriology, Immunology and Mycology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt, Email abdelazeem.algammal@vet.suez.edu.eg

Background: *Gallibacterium anatis* is incriminated frequently in severe economic losses and mortalities in the poultry industry. This study aimed to detect the prevalence of *G. anatis* in layer chickens, sequence analysis, the antibiogram profiles, and PCR screening of virulence determinants and antibiotic resistance genes.

Methods: Accordingly, 300 samples (tracheal swabs, ovary and oviduct, and lung) were randomly collected from 100 diseased layer chickens from private commercial layer farms at Elsharkia Governorate, Egypt. The bacteriological examination was carried out. The retrieved isolates were tested for *16S rRNA-23S rRNA* gene sequencing, antibiogram profiling, PCR screening of virulence (*gtx*A, *fif*A, and *gyr*B), and antibiotic resistance genes (bla_{ROB}, *aph*A1, *tet*B, and *tet*H).

Results: The prevalence of *G. anatis* was 25% in the examined diseased layer chickens. The sequence analyses emphasized that the tested strains derived from a common ancestor and exhibited a notable genetic similarity with other *G. anatis* strains from USA, China, and Denmark. The isolated *G. anatis* strains were highly resistant to sulfamethoxazole-trimethoprim, oxytetracycline, penicillin, ampicillin, kanamycin, neomycin, and erythromycin. The PCR revealed that the retrieved *G. anatis* strains carried *gtx*A, *gyr*B, and *fif*A virulence genes with a prevalence of 100%, 100%, and 38.3%, respectively. Approximately 30.1% of the retrieved *G. anatis* isolates were XDR to six antimicrobial classes and harbored bla_{ROB}, *aph*A1, and *tet*B resistance genes. Moreover, 20.5% of the isolated *G. anatis* strains were MDR to three different classes and carried bla_{ROB} and *tet*H resistance genes.

Conclusion: Briefly, this study emphasized the existence of XDR and MDR *G. anatis* strains in poultry. Florfenicol and norfloxacin displayed a promising antimicrobial effect against the emerging XDR and MDR *G. anatis* in poultry. The emergence of XDR and MDR *G. anatis* is considered a public health alarm.

Keywords: *G. anatis*, poultry, antibiogram, XDR, sequence analyses, virulence genes, antibiotic resistance genes

Introduction

Globally, there is an increased need for poultry meat and eggs as essential dietary components. Poultry diseases caused by resident microbiota are deleterious diseases that had severe losses in the poultry industry due to the marked decrease in growth and egg production, costs of treatment, and high mortality rates.[1,2]

Based on *16S rRNA* gene sequencing, Genus *Gallibacterium* is categorized as a member of the *Pasteurellaceae* family.[3] *Gallibacterium anatis* (*G. anatis*) is an opportunistic microorganism that normally inhabitant the genital and

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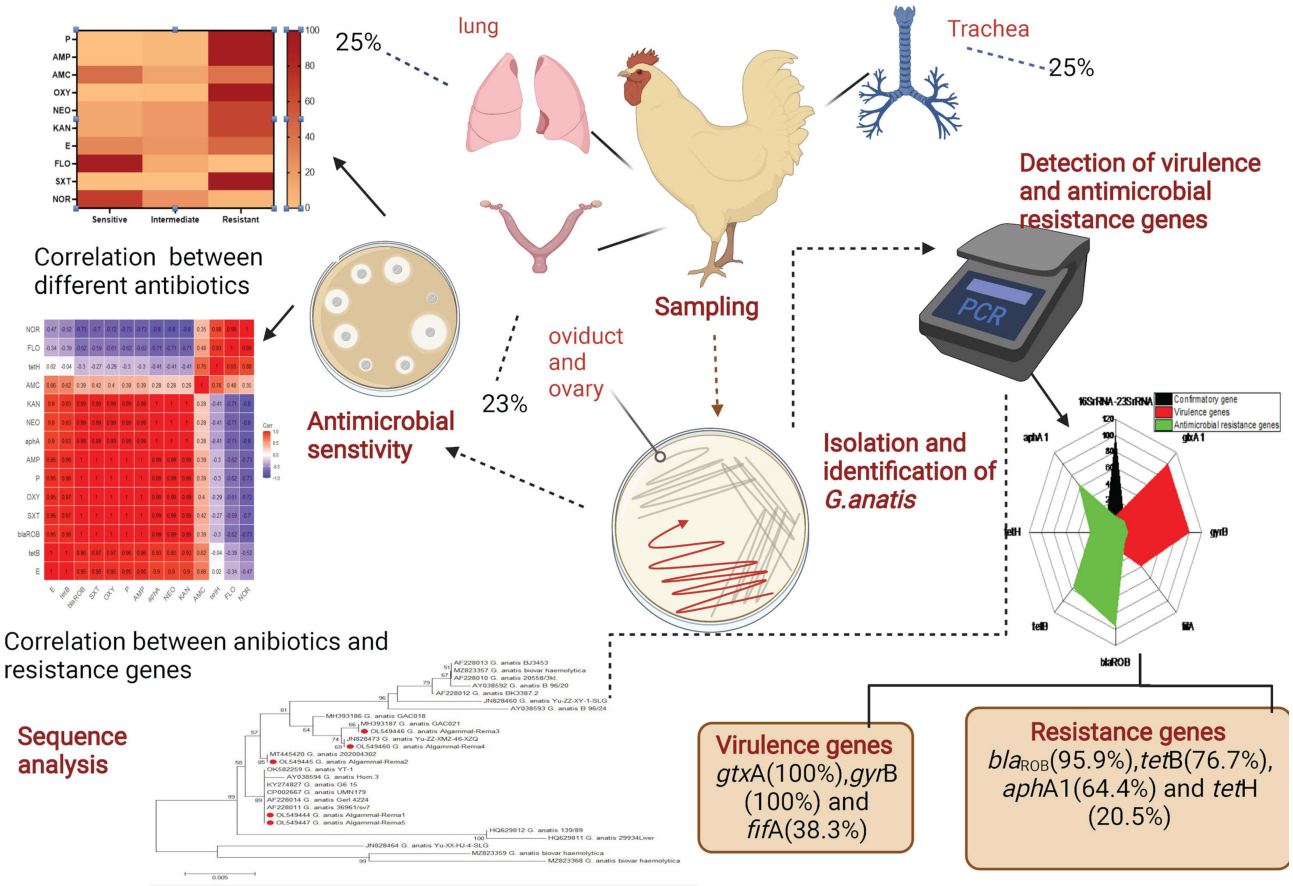
Graphical Abstract



respiratory tracts of intensively reared chickens and other domestic birds. *G. anatis* is a Gram-negative coccobacillus frequently incriminated in mortalities in domestic birds, especially chickens, and sporadic human cases.[4] Phenotypically, *G. anatis* is classified into two different biotypes: *G. anatis* biovar *haemolytica* (hemolytic biotype) and the *G. anatis* biovar *anatis* (non-hemolytic biotype).[5] *G. anatis* is a multidrug-resistant (MDR) pathogen of poultry; causes serious diseases in poultry including salpingitis, decreased egg production, peritonitis, epididymitis, respiratory manifestations, and high mortalities. Moreover, infections caused by *G. anatis* in humans are rarely accompanied by abscessation of the lung, bacteremia, bronchitis, and mortalities.[6]

It is crucial to understand the pathogenesis and the virulence determinants of *G. anatis* to avoid the adverse effects of this pathogen.[7] The capability of *G. anatis* to adhere and invade the host epithelial cells is believed to induce a remarkable role in the pathogenesis of *G. anatis* infection in poultry.[8] The most prevalent virulence determinants accompanying *G. anatis* are metalloproteases, capsule, fimbriae, hemagglutinin, and innovative elements such as the rtx–like toxin (gtxA). Cell-free filtrates of the hemolytic *G. anatis* strains are highly toxic against the avian-derived macrophage-like cells (HD11). The leukotoxic and hemolytic activities of *G.anatis* are attributed mainly to the gtxA toxin which is encoded by the *gtx*A gene.[9] Moreover, the gtxA toxin has a cytotoxic effect on poultry macrophages in-vivo. Similarly, fimbriae are considered one of the vital virulence determinants of *G. anatis*. The fimbriae of *G. anatis* are categorized in the F17-like family. Mutant strains of *G. anatis* that lack the flfA fimbriae were noticed to be mild pathogenic to the experimentally infected birds. Other virulence determinants of *G. anatis* include the proteolytic proteases and the antiphagocytic capsules that initiate biofilm production.[10]

Multidrug resistance has noticeably increased globally in the last decade, returning as a public health threat. Various modern surveys demonstrated the emergence of XDR and MDR bacterial pathogens from distinct sources such as poultry, fish, animals, food products, and humans.[11–16] Despite that infections induced by *G. anatis* could be treated with antibiotics, certain unresponsive cases were reported.[17,18] Multidrug resistance patterns of *G. anatis* to various antimicrobial classes (such as β-lactam antibiotics, sulfonamides, aminoglycosides, and tetracyclines) are frequently reported by several previous studies.[3,6,19,20]

This study aimed to detect the prevalence of *G. anatis* in layer chickens, sequence analysis, the antibiogram profiles, and PCR screening of virulence determinants (*gtx*A, *fif*A, and *gyr*B) and antibiotic resistance genes (bla_{ROB}, *aph*A1, *tet*B, and *tet*H) among the retrieved *G. anatis* strains.

Methods

Animal Ethics

All procedures were carried out consistent with relevant regulations. All procedures and handling of birds were approved by the Animal Ethics Review Committee, Suez Canal University, Egypt.

Sampling

A total of 300 samples (tracheal swabs, ovary and oviduct, and lung; *n*=100 for each) were randomly collected from 100 diseased layer chickens (3 types of samples from each bird) with an average age of 25–40 weeks from private commercial layer farms at Elsharkia Governorate, Egypt (from February to May 2020). The examined diseased layer chickens were suffering from depression, respiratory signs, occasionally head swelling, and decreased in egg production (5–10%). The post-mortem inspection of sacrificed and/or freshly dead birds displayed oophoritis, peritonitis, salpingitis, and tracheitis. All samples were gathered in sterile plastic bags, placed in an icebox and quickly transferred to the Microbiology laboratory for bacteriological examination.

Isolation and Identification of *G. anatis*

Swabs from the obtained samples (tracheal swabs, ovary and oviduct, and lung) were directly streaked out on 5% Columbia blood agar and MacConkey agar plates (Difco, USA) and incubated aerobically at 37 °C for 24 h. The identification of *G. anatis* was carried out according to Gram's staining, culture characteristics, motility test, and the biochemical characteristics (oxidase, nitrate reduction, catalase, indole production, methyl red, Voges-Proskauer, citrate utilization, urease test, gelatinase, and sugar fermentation tests).[5,21] Moreover, the identification of the retrieved *G. anatis* isolates was ensured using PCR-based detection of the *16S rRNA-23S rRNA* gene according to Bojesen,[22] followed by gene sequencing.

G. anatis 16S rRNA-23S rRNA Gene Sequencing

All the retrieved *G. anatis* isolates displayed harmony in the biochemical and phenotypic features. Consequently, the PCR products of five indiscriminately selected *G. anatis* strains were purified using the QIAquick PCR-Product extraction kit (QIAGEN GmbH, Hilden, D-40724, Germany), then were tested for sequencing in both directions through the Bigdye Terminator V3.1 cycle sequencing kit (Thermo Fisher Scientific GmbH, Dreieich 63,303, Germany). The recovered sequences were placed in the GenBank with accession numbers: OL549444, OL549445, OL549446, OL549447, and OL549460. The BLAST analysis was carried out to investigate the sequence identity. Moreover, the phylogenetic analysis was performed according to the neighbor-joining in MEGA6 as previously described.[23]

Antimicrobial Susceptibility Testing of the Recovered *G. anatis*

The recovered *G. anatis* strains were examined for the susceptibility to several antimicrobial agents using the disc diffusion technique on Muller-Hinton agar (Oxoid, UK) was implemented consistently with the procedures of CLSI, 2018.[24] Ten antimicrobial agents were involved, including; penicillin (PEN, 30μg), ampicillin (AMP, 30μg), amoxicillin-clavulanic acid (AMC, 30 μg), oxytetracycline (OX, 30μg), neomycin (NEO, 10 μg), kanamycin (KAN, 10 μg),

erythromycin (E, 15 µg), florfenicol (FFC, 30µg), sulphamethoxazole-trimethoprim (SXT, 30 µg), and norfloxacin (NOR, 10 µg) (ThermoFisher Scientific, USA). Moreover, *E. coli*-ATCC 25922 was involved as a control strain. The tested *G. anatis* isolates were categorized according to their resistance patterns into MDR (Multidrug-resistant: resistant to ≥ one antimicrobial agent in ≥ 3 classes) and XDR (Extensively drug-resistant: resistant to ≥ one agent in all tested antimicrobial classes except one or two) according to Magiorakos.[25] Moreover, the multiple antibiotic resistance (MAR) index was determined as previously described.[26]

PCR-Based Screening of Virulence and Antimicrobial Resistance Genes in the Isolated *G. anatis*

PCR was used to detect the virulence (*gtx*A: Cytolytic-hemolytic gene, *fif*A: Flageller gene, and *gyr*B: The gyrase subunit B gene) and the antimicrobial resistance genes (bla_{ROB}: β-lactam resistance gene, *aph*A1: aminoglycosides resistance gene, *tet*B and *tet*H: tetracycline resistance gene) in the retrieved *G. anatis* strains. DNA of the tested *G. anatis* strains was extracted following the manufacturer's instructions of the QIAampDNA Mini Kit (Qiagen, GmbH, Germany/Cat. No.56304). The reaction volume was 25-µL, including 5 µL of extracted DNA, 12.5 µL of 2 × Master Mix, 20 pmol of tested primer, and distilled H_2O). Negative control (reactions with no DNA template) and Positive controls (positive strains supplied by A.H.R.I, Egypt) were involved in every reaction. The used primers (Biobasic, Canada) and cycling conditions were demonstrated in Table 1. Lastly, the PCR products were separated using the agar gel electrophoresis (1.5% agarose stained using ethidium bromide 10 mg/mL), and then photographed the gel.

Statistical Analyses

The Chi-square test was used in data analyses (SAS software, 9.4 M6, SAS Institute, Cary, NC, USA) (The significance level was (p-value < 0.05). Besides, the correlation coefficient was estimated via the R-software (version 02.1; http://www.r-project.org/) using "corr" and "corrplot".

Table 1 Primers Sequences and PCR Cycling Conditions

Genes	Oligonucleotides Sequences	Amplicon size (bp)	(35 Cycles)			References
			Den.	Annealing	Ext.	
***16SrRNA* (1133fgal)-*23SrRNA* (114r)**	F: TATTCTTTGTTACCARCGG R: GGTTTCCCCATTCGG	1032	94°C 30 sec	55°C 50 sec	72°C 1 min	[22]
***gtx*A** **(Cytolytic-hemolytic gene)**	**F**: CAAACCTAATTCAATCGGATG **R**: TGCTTCAATAATTTTCCATTTTC	1257	94°C 30 sec	51°C 40 sec	72°C 1 min	[1]
***fif*A** **(Flageller gene)**	**F**: CACCATGGGTGCATTTGCGGATGATCC **R**: TATTCGTATGCGATAGTATAGTTC	538	94°C 30 sec	55°C 40 sec	72°C 45 sec	[27]
***gyr*B** **(The gyrase subunit B gene)**	**F**: TGTGCGTTTCTGGCCAAGTC **R**: CGCTCACCAACTGCAGATTC	561	94°C 30 sec	55°C 40 sec	72°C 45 sec	[28]
bla_{ROB} **(Beta lactam resistance gene)**	**F**: AATAACCCTTGCCCCAATTC **R**: TCGCTTATCAGGTGTGCTTG	685	94°C 30 sec	60°C 40 sec	72°C 45 sec	[29]
***aph*A1** **(Aminoglycosides resistance gene)**	**F**: TTATGCCTCTTCCGACCATC **R**: GAGAAAACTCACCGAGGCAG	489	94°C 30 sec	54°C 40 sec	72°C 40 sec	
tetH **(Tetracycline resistance gene)**	**F**: ATACTGCTGATCACCGT **R**: TCCCAATAAGCGACGCT	1076	94°C 30 sec	60°C 40 sec	72°C 1 min	
tetB **(Tetracycline resistance gene)**	**F**: CCT TAT CATGCC AGT CTTGC **R**: ACT GCCGTT TTT TTCGCC	774	94°C 30 sec	50°C 40 sec	72°C 1 min	[30]

Table 2 The Prevalence of *G. anatis* Isolated from Examined Diseased Layer Chickens (*n*=73)

No. of Examined Diseased Birds	Types of Organs	No. of Organs	No. of Positive Samples	Percentage of Positive Samples
100	Ovary and oviduct	100	23	23
	Tracheal swab	100	25	25
	Lung	100	25	25
Total		300	73	24.3
Chi square ***p* value**			0.10959 0.9467^{NS}	

Abbreviation: NS, Non-significant.

Results

Phenotypic Characteristics of the Isolated *G. anatis* from Diseased Layers

The recovered *G. anatis* colonies were bright translucent, low convex, fine, circular, smooth-edged with greyish color, and mostly showed β-hemolysis on blood agar. Moreover, the colonies were small (pin point-like) and pink (lactose fermenter) on MacConkey agar. Gram's staining displayed Gram-negative coccoid to small pleomorphic bacilli. The retrieved *G. anatis* isolates were positive for oxidase, catalase, nitrate reduction, sucrose, and mannitol fermentation tests. Conversely, the recovered isolates were negative for citrate utilization, indole, urease, gelatinase, motility, methyl red, and Voges-Proskauer tests.

Prevalence of *G. anatis* in the Examined Samples of Diseased Layer Chickens

A total of 73 *G. anatis* isolates were recovered from 300 bacteriologically examined samples obtained from 100 diseased layer chickens. Twenty-five isolates (25%) were obtained from tracheal swabs, 25 isolates (25%) from lung samples, and 23 isolates (23%) from ovary and oviduct samples. From the twenty-five infected birds with *G. anatis*, the pathogen was isolated from tracheal swab, lung, and ovary and oviduct samples of the same bird in 23 infected birds. Moreover, *G. anatis* was recovered from the lung and tracheal swabs of the same bird in two infected birds. There is no significant difference in the dissemination of *G. anatis* among the examined organs obtained from the diseased layer chickens ($p > 0.05\%$), as illustrated in Table 2 and Figure 1. The prevalence of *G. anatis* was 25% (25/100) in the examined diseased layer chickens.

Phylogenetic Analyses of *G. anatis 16S rRNA-23S rRNA* Gene

Phylogenetic and sequence analyses of the *16S rRNA-23S rRNA* gene emphasized that the tested *G. anatis* strains (*n*=5) derived from a common ancestor (Accession Numbers: OL549444, OL549445, OL549446, OL549447, and OL549460). In the present study, the tested *G. anatis* strains exhibited a notable genetic similarity (96.7–100%) with other *G. anatis* strains from various origins. For example, *G. anatis* strain G6_15 (99–100%) was isolated from a tracheal swab of

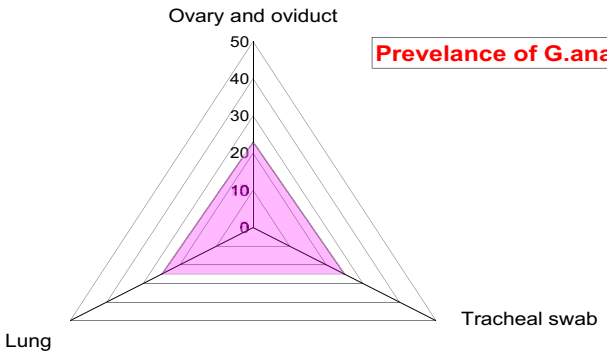


Figure 1 Prevalence of *G. anatis* among different examined samples obtained from diseased layers.

diseased layer chicken in Egypt (Accession No. KY274827). *G. anatis* strain UMN179 (99–100%) was isolated from an Iowa laying hen with peritonitis in the USA (Accession No. CP002667). *G. anatis* biovar hemolytica (98.3–98.7%) was isolated from poultry in Pennsylvania, USA (Accession No. MZ823357). *G. anatis* strain 202,004,302 (99.7–100%) was isolated from layer chicken in China (Accession No. MT445420), *G. anatis* strain GAC021 (99–100%) was isolated from poultry in China (Accession No. MH393187), *G. anatis* strain Yu-ZZ-XMZ-46-XZQ (99–100%) isolated from a cloacal sample in China (Accession No. JN828473), *G. anatis* strain YT-1 (99–100%) isolated from oviduct in China (Accession No. OK582259), *G. anatis* strain 36,961/sv7 (99.4–99.7%) in Denmark (Accession No. AF228011), and *G. anatis* strain Gerl.4224 (99–100%) in Denmark (Accession No. AF228014) as shown in Figures 2 and 3.

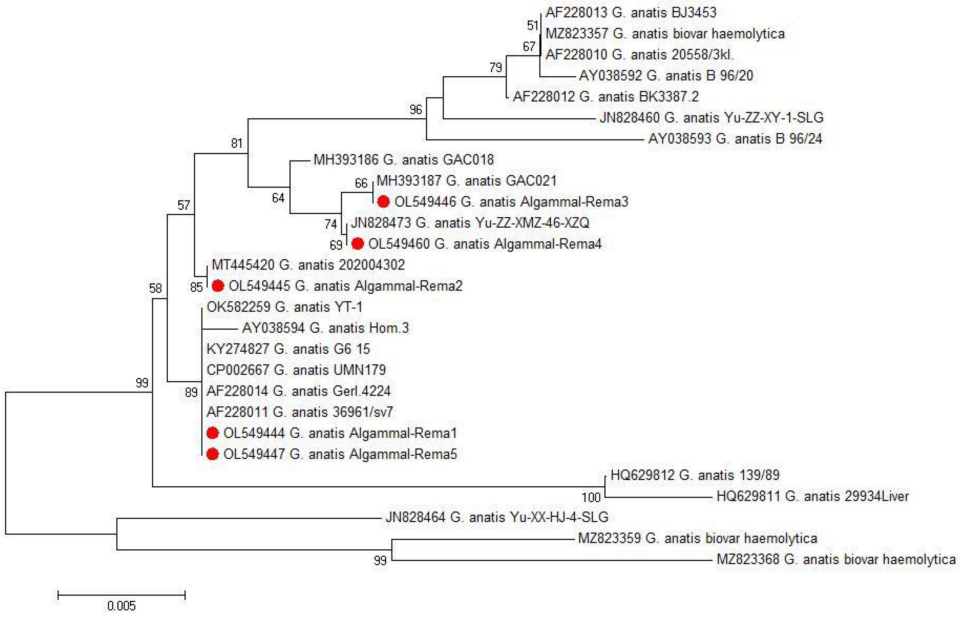


Figure 2 The phylogenetic analysis was carried out according to the *16SrRNA-23SrRNA* gene sequencing. The phylogenetic tree clarifies the genetic relatedness between the recovered *G. anatis* strains and other strains deposited in the GenBank. The retrieved *G. anatis* strains in the present study were emphasized with red circles.

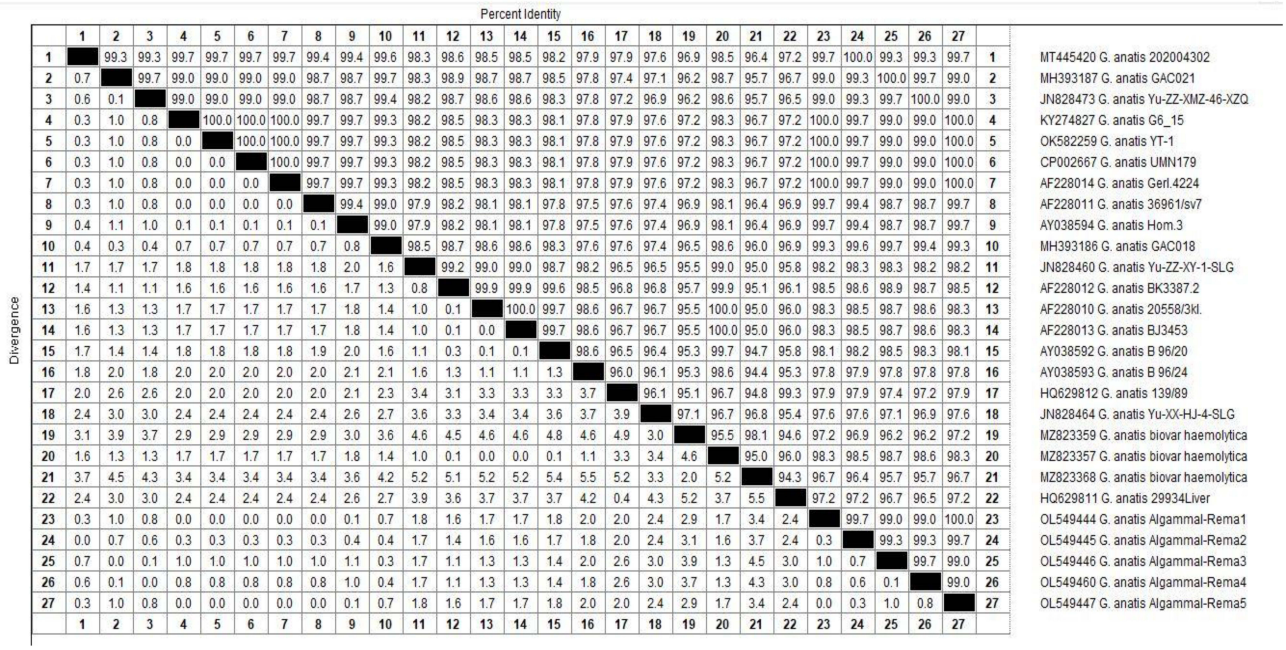


Figure 3 Illustrates the percentage of *G. anatis 16SrRNA-23SrRNA* nucleotides sequences identity.

Table 3 The in-vitro Antimicrobial Susceptibility Testing of the Isolated *G. anatis* (*n*=73)

Antimicrobial Class	Antimicrobial Agents	Sensitive		Intermediate		Resistant	
		n	%	*n*	%	*n*	%
Penicillin	**Penicillin**	0	0	3	4.1	70	95.9
	Ampicillin	0	0	3	4.1	70	95.9
β-lactam-β Lactamase inhibitor combination	**Amoxicillin -clavulanic acid**	32	43.8	11	15.1	30	41.1
Tetracyclines	**Oxytetracycline**	0	0	2	2.7	71	97.3
Aminoglycosides	**Neomycin**	10	13.7	16	21.9	47	64.4
	Kanamycin	10	13.7	16	21.9	47	64.4
Macrolides	**Erythromycin**	22	30.1	17	23.3	34	46.6
Phenicols	**Florfenicol**	65	89	8	11	0	0
Sulfonamides	**Sulfamethoxazole trimethoprim**	0	0	0	0	73	100
Fluroquinolones	**Norfloxacin**	55	68.5	18	24.6	5	6.8
Chi-square ***p* value**		267.75 0.0001		47.702 0.0001		149.62 0.0001	

The in-vitro Antimicrobial Susceptibility of *G. anatis*

The antimicrobial susceptibility of the retrieved *G. anatis* isolates showed that the tested strains were resistant to sulfamethoxazole-trimethoprim (100%), oxytetracycline (97.3%), penicillin and ampicillin (95.9%), kanamycin and neomycin (64.4%), and erythromycin (46.6%). Moreover, the recovered isolates were sensitive to florfenicol (89%), norfloxacin (68.5%), and amoxicillin-clavulanic acid (43.8%) (As shown in Table 3 and Figure 4). Statistically, the recovered *G. anatis* strains displayed a significant difference in their susceptibility patterns to different tested antimicrobial classes ($p < 0.05$). Besides, the correlation coefficient was detected among the involved antimicrobial agents. Accordingly, strong positive correlations were detected between: KAN, NEO, P, AMP, OXY, and SXT (r=0.99); FLO and NOR(r=0.99); E, P, AMP, OXY, and SXT (r=0.95); E, KAN, and NEO (r=0.90). Moreover, moderate positive correlations were noticed between AMC and E (r=0.66); AMC and FLO (r=0.48); AMC and SXT (r=0.42); AMC and OXY (r=0.40); AMC, P, and AMP (r=0.39) (Figure 5).

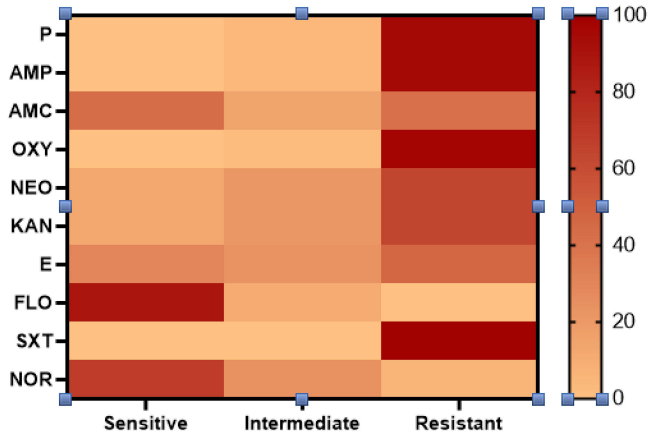


Figure 4 Illustrates the antimicrobial susceptibility of the recovered *G. anatis* from diseased layer chickens.

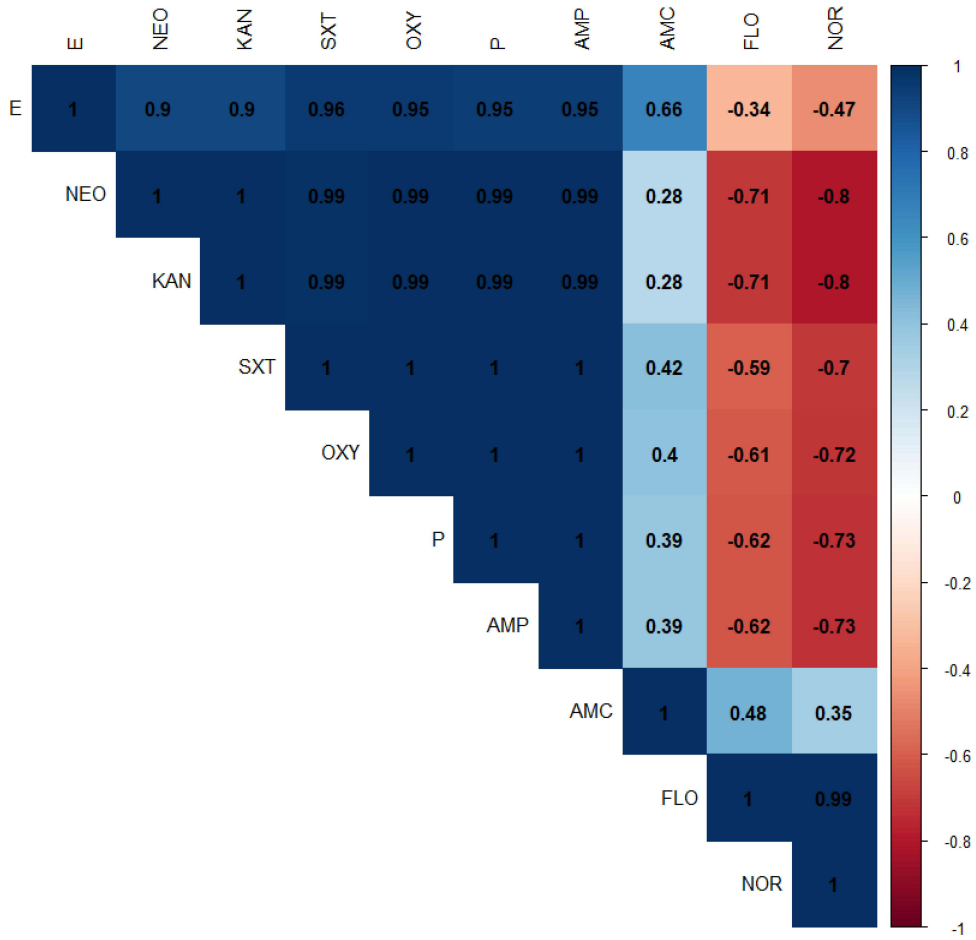


Figure 5 The heat map demonstrates the correlation coefficient (r) between different tested antimicrobial involved in the current study.

Virulence Determinant and Antibiotic Resistance Genes of the Isolated *G. anatis* Strains

Using PCR, all the retrieved *G. anatis* isolates (100%) were positive for the confirmatory gene (*16SrRNA-23SrRNA*). Moreover, the recovered *G. anatis* strains commonly harbored the virulence genes *gtx*A1 (cytolytic-hemolytic gene) (100%) and *gyr*B (gyrase subunit B gene) (100%), followed by *fif*A gene (fimbrial gene) (38.3%). Furthermore, the tested *G. anatis* strains harbored the bla_{ROB} (β-lactam resistance), *tet*B (tetracycline resistance), *aph*A (aminoglycosides resistance gene), and *tet*H (tetracycline resistance) resistance genes with a prevalence of 95.9%%, 76.7%, 64.4%, and 20.5%, respectively (Table 4 and Figure 6). The statistical analysis showed a significant difference ($p < 0.05$) in the distribution of antibiotic resistance and virulence genes in the isolated *G. anatis* strains.

Multidrug Resistance Patterns of the Retrieved *G. anatis* Strains

Our results evidenced that 30.1% (22/73) of the retrieved *G. anatis* isolates were XDR to six antimicrobial classes (Penicillins: PEN and AMP, β-lactam-β-Lactamase-inhibitor combination: AMC, Tetracyclines: OX, Aminoglycosides: NEO and KAN, Macrolides: E, and Sulfonamides: SXT) and harbored bla_{ROB}, *aph*A1, and *tet*B resistance genes. Moreover, 20.5% (15/73) of the isolated *G. anatis* strains were MDR to three different classes (Penicillins: PEN and AMP, Tetracyclines: OX, and Sulfonamides: SXT) and carried bla_{ROB} and *tet*H resistance genes. Furthermore, 17.8% (13/73) of the retrieved *G. anatis* strains were MDR to four different classes (Penicillins: PEN and AMP, Tetracyclines: OX, Aminoglycosides: NEO and KAN, and Sulfonamides: SXT) and possessed bla_{ROB}, *aph*A1, and *tet*B resistance genes. In addition, 10.9% (8/73) of the isolated *G. anatis* strains were MDR to

Table 4 Prevalence of Virulence and Antimicrobial Resistance Genes Among the Recovered *G. anatis* (*n*=73)

Types of Genes		*n*	%	Chi-Square *p* value
Confirmation gene	*16SrRNA-23SrRNA*	73	100	
Virulence-determinant genes	*gtx*A1	73	100	23.276 0.001
	*gyr*B	73	100	
	*fif*A	28	38.3	
Antimicrobial resistance genes	bla_{ROB}	70	95.9	34.766 0.0001
	*tet*B	56	76.7	
	*tet*H	15	20.5	
	*aph*A1	47	64.4	

four different classes (Penicillins: PEN and AMP, Tetracyclines: OX, β-lactam-β-Lactamase-inhibitor combination: AMC, and Sulfonamides: SXT) and carried bla_{ROB} and *tet*B resistance genes. Besides, 9.6% (7/73) of the isolated *G. anatis* strains were MDR to five different classes (Penicillins: PEN and AMP, Tetracyclines: OX, Aminoglycosides: NEO and KAN, Macrolides: E, and Sulfonamides: SXT) and carried bla_{ROB}, *aph*A1, and *tet*B resistance genes as described in Table 5 and Figure 7. In the present study, the MAR index values (0.3–0.6) revealed multiple resistance patterns suggesting that the recovered *G. anatis* strains are derived from high-risk contamination. The correlation coefficient (r) was calculated among the demonstrated antimicrobial resistance genes in the recovered *G. anatis* strains and different involved antimicrobial agents. Our findings revealed positive correlations between: bla_{ROB} gene, P, and AMP (r=1); a*ph*A1 gene, NEO, and KAN (r=1); *tet*B and OXY (r=0.97) as illustrated in Figure 8.

Discussion

This study aimed to investigate the prevalence of *G. anatis* in layer chickens, sequence analysis, the antibiogram profiles, and PCR screening of *gtx*A, *fif*A, and *gyr*B virulence genes and bla_{ROB}, *aph*A1, *tet*B, and *tet*H antibiotic resistance genes among the retrieved *G. anatis* strains.

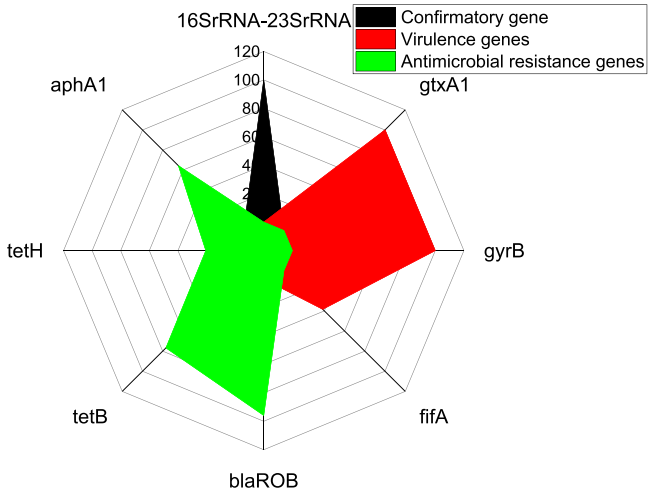


Figure 6 The distribution of antimicrobial resistance and virulence determinant genes in the retrieved *G. anatis* strains from diseased layer chickens.

Table 5 The Prevalence of Multidrug-Resistance Profiles and the Resistance Genes Among the Recovered *G. anatis* Strains (*n* =73)

No. of Strains	%	Type of Resistance	Multidrug Resistance Profiles	Antimicrobial-Resistance Genes	MAR-Index
22	30.1	XDR	**- Six classes**: PEN and AMP AMC OX NEO and KAN E SXT	bla_{ROB}, *aph*A1,and *tet*B	0.6
15	20.5	MDR	**- Three classes**: PEN and AMP OX SXT	bla_{ROB} and *tet*H	0.3
13	17.8	MDR	**- Four classes**: PEN and AMP OX NEO and KAN SXT	bla_{ROB}, *aph*A1, and *tet*B	0.4
8	10.9	MDR	**- Four classes**: PEN and AMP AMC OX SXT	bla_{ROB}, and *tet*B	0.4
7	9.6	MDR	**- Five classes**: PEN and AMP OX NEO and KAN E SXT	bla_{ROB}, *aph*A1, and *tet*B	0.5
5	6.8	XDR	**-Six classes**: PEN and AMP OX NEO and KAN E SXT NOR	bla_{ROB}, *aph*A1, and *tet*B	0.6

In the current work, *G. anatis* was identified in layer chickens that suffered from respiratory manifestations and a drop in egg production. Besides, the PM inspection displayed oophoritis, peritonitis, salpingitis, and tracheitis. In the last decade, *G. anatis* was recovered from diseased chickens with tracheitis and salpingitis in different geographical areas all over the world. *G. anatis* is associated mainly with severe economic losses in the poultry industry.[20,31,32] *G. anatis* is frequently retrieved from the genital and respiratory tracts of diseased poultry suffering from severe pathological lesions.[33]

In the present study, the bacteriological examination proved that the prevalence of *G. anatis* was 25% in the examined diseased layer chickens, where the lung and trachea are the most predominant infected organs. Moreover, there is no inconsistency in the biochemical and phenotypic features of the isolated *G. anatis* strains that showed an obvious harmony. The recovered *G. anatis* colonies were circular, smooth-edged with greyish color, and mostly β-hemolytic on

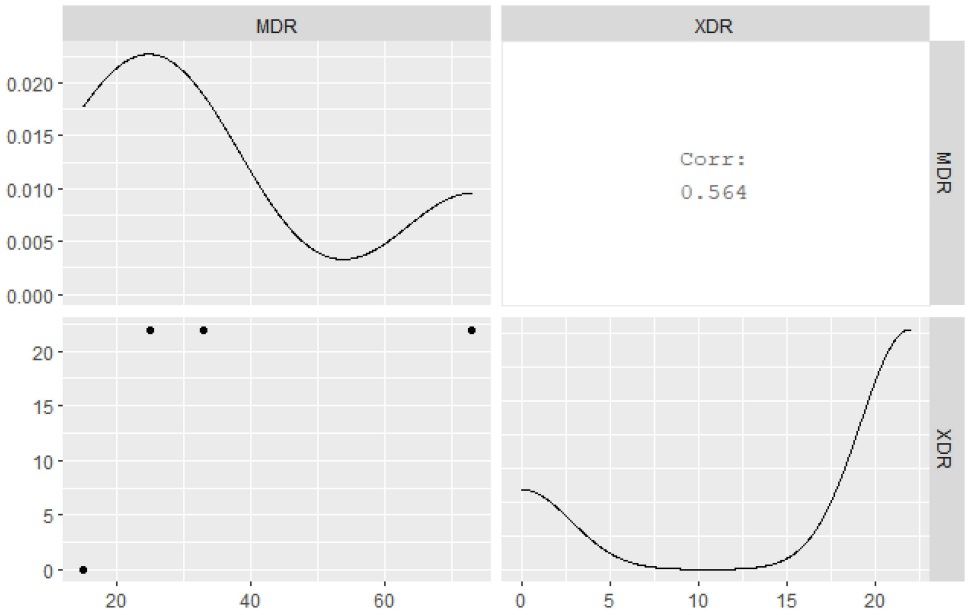


Figure 7 The emergence of XDR and MDR patterns between among the recovered *G. anatis* strains from diseased layer chickens.

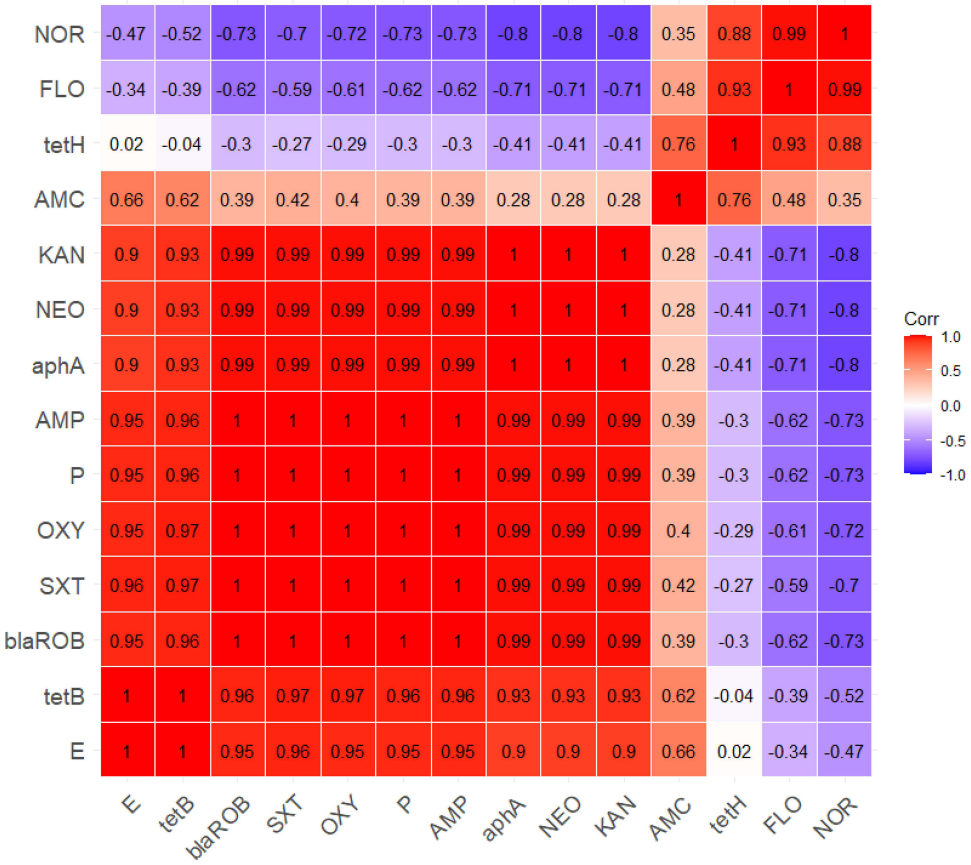


Figure 8 The heat map illustrates the correlation coefficient (r) between the demonstrated antimicrobial resistance genes in the retrieved *G. anatis* strains and different involved antimicrobial agents.

blood agar. Moreover, the colonies were small (poor growth) and pink on MacConkey agar. Furthermore, the retrieved *G. anatis* isolates were positive for oxidase, catalase, nitrate reduction, sucrose, and mannitol fermentation tests. On the other hand, the recovered isolates were negative for citrate utilization, indole, urease, gelatinase, and Voges-Proskauer tests. Our findings are consistent with those reported by Johnson[34] and Van Driessche.[4]

In the current study, the *16S rRNA-23S rRNA* phylogenetic analyses highlighted that the tested *G. anatis* strains have a common ancestor (Accession Numbers: OL549444, OL549445, OL549446, OL549447, and OL549460). Moreover, the tested *G. anatis* strains showed high genetic similarity with other *G. anatis* strains isolated from diseased poultry in different regions, such as *G. anatis* strain G6_15 isolated by Elbestawy[20] from a tracheal swab of diseased layer chicken in Egypt, *G. anatis* strain UMN179 isolated from an Iowa laying hen with peritonitis in USA.[34] *G. anatis* strain Gerl.4224 was isolated from poultry in Denmark,[5] and *G. anatis* strain GAC021 was isolated from silky chicken in China.[35] Our findings disagreed with the results of Paudel,[1] who reported that strains of *G. anatis* are divergent and usually exhibit genetic differences. Besides, these findings illustrated the epidemiological map and ensured the public health importance of *G. anatis*.

Regarding the antimicrobial resistance patterns, the isolated *G. anatis* strains showed remarkable resistance patterns to sulfonamides, tetracyclines, β-Lactam antibiotics, aminoglycosides, and macrolides. These findings nearly agreed with Bojesen[17] and Elbestawy.[36] The extensive uncontrolled use of antibiotics in the poultry farms, as well as the ability of *G. anatis* to gain antibiotic resistance genes from other resistant bacteria, are the main factors that favor of the existence of these superbugs.[18]

In the present study, PCR evidenced that the tested *G. anatis* strains are commonly harbored *gtx*A and *gyr*B, virulence genes, followed by the *fif*A gene. Our results are nearly agreed with those reported by Krishnegowda,[2] Sorour,[37] and Nassik.[38] The *rtx*-like toxin (*gtx*A) is one of the main virulence determinants of *G. anatis* encoded by the *gtx*A gene.[27] The *gtx*A toxin was demonstrated for the first time in a hemolytic *G. anatis* strain isolated from diseased chicken in 2010 in Denmark. The toxin is accountable for hemolytic and leukotoxic activities of *G. anatis*. Lacking the *gtx*A gene indicates a reduced bacterial virulence.[9] Besides, the *gyrB* gene encodes the ATPase domain of DNA gyrase which is necessary for the replication of DNA in *G. anatis*. The *gyrB* gene is used frequently to identify *G. anatis* infection in poultry.[39] Fimbriae are a common virulence determinant of *G. anatis* that plays a significant role in bacterial adhesion to the glycoprotein receptors in the host mucous membranes. Different F17-like fimbrial genes were recently determined in *G. anatis* from different origins, where the *flf*A gene is the most predominant one.[27,28]

Regarding the multidrug resistance patterns in the isolated *G. anatis* strains, a high proportion of the retrieved *G. anatis* isolates were XDR to 6 antimicrobial classes and harbored bla_{ROB}, *aph*A1, and *tet*B resistance genes. Moreover, a high percentage of the recovered *G. anatis* strains were MDR to 3–5 different classes and carried bla_{ROB}, *aph*A, and *tet*B or *tet*H resistance genes. Antimicrobial resistance is considered one of the main threats to public health globally. It remains occurred due to the abuse of antibiotics in both veterinary and health practices and the bacterial acquirement of antibiotic resistance genes through mobile genetic components.[40,41] The resistance to β-lactam antibiotics (such as amoxicillin, penicillin, and ampicillin) is mainly mediated by the bla_{ROB-1} gene. Stimulatingly, the bla_{ROB-1} gene is the most prevalent β-lactamase gene, frequently demonstrated in the members of *Pasteurellaceae*.[42] The *tet*B gene is the most prevalent tetracycline resistance gene in the present study, followed by the *tet*H gene. These findings were supported by Bojesen, who reported the determination of the *tet*B gene in 27 (27/49) tetracycline-resistant *G. anatis* strains.[17] Moreover, the acquired resistance to aminoglycosides antibiotics is mainly attributed to the enzymatic alteration mechanism, resulting in the inactivation of aminoglycosides in several bacterial pathogens, especially *G. anatis*. The aminoglycosides phosphotransferases, encoded by the *aph*A gene, are the most common aminoglycosides modifying enzymes.[4,43]

Briefly, from what we know, this is the first report that emphasized the existence of XDR and MDR *G. anatis* strains in diseased layers in Egypt, with particular reference to the sequence analyses and multidrug resistance profiles. The retrieved *G. anatis* strains commonly harbor the *gtx*A and *gyr*B virulence genes, followed by the *fif*A gene. The obtained *G. anatis* strains are XDR or MDR to several antimicrobial classes (such as sulfonamides, tetracyclines, β-Lactam antibiotics, aminoglycosides, and macrolides) commonly harbored bla_{ROB}, *aph*A, and *tet*B or *tet*H resistance genes. Florfenicol and norfloxacin displayed a promising antimicrobial effect against the emerging

XDR and MDR *G. anatis* strains. Combining the conventional and molecular techniques is a consistent epidemiological tool used to diagnose *G. anatis* infection in poultry. Alarmingly, the emergence of XDR and MDR *G. anatis* strains establishes a public threat that specifies a lousy prognosis of diseases induced by these superbugs. Besides, it has an adverse impact on the poultry production. Consequently, it encourages the routine application of antimicrobial susceptibility testing along with the appropriate use of antimicrobial agents in the health sector and the veterinary practice.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflict of interest.

References

1. Paudel S, Alispahic M, Liebhart D, Hess M, Hess C. Assessing pathogenicity of *Gallibacterium anatis* in a natural infection model: the respiratory and reproductive tracts of chickens are targets for bacterial colonization. *Avian Pathol*. 2013;42(6):527–535. doi:10.1080/03079457.2013.843160
2. Krishnegowda DN, Dhama K, Mariappan AK, et al. Etiology, epidemiology, pathology, and advances in diagnosis, vaccine development, and treatment of *Gallibacterium anatis* infection in poultry: a review. *Vet Q*. 2020;40(1):16. doi:10.1080/01652176.2020.1712495
3. Singh SV, Singh B, Sinha D, et al. *Gallibacterium anatis*: an emerging pathogen of poultry birds and domiciled birds. *J Vet Sci Technol*. 2016;7(3):324.
4. Van Driessche L, Vanneste K, Bogaerts B, et al. Isolation of drug-resistant *Gallibacterium anatis* from calves with unresponsive bronchopneumonia, Belgium. *Emerg Infect Dis*. 2020;26(4):721. doi:10.3201/eid2604.190962
5. Christensen H, Bisgaard M, Bojesen AM, Mutters R, Olsen JE. Genetic relationships among avian isolates classified as *Pasteurella haemolytica*,'Actinobacillus salpingitidis' or *Pasteurella anatis* with proposal of *Gallibacterium anatis* gen. nov., comb. nov. and description of additional genomospecies within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol*. 2003;53(1):275–287. doi:10.1099/ijs.0.02330-0
6. El-Adawy H, Bocklisch H, Neubauer H, Hafez HM, Hotzel H. Identification, differentiation and antibiotic susceptibility of *Gallibacterium* isolates from diseased poultry. *Ir Vet J*. 2018;71(1):1–10. doi:10.1186/s13620-018-0116-2
7. Bager RJ, Persson G, Nesta B, et al. Outer membrane vesicles reflect environmental cues in *Gallibacterium anatis*. *Vet Microbiol*. 2013;167(3–4):565–572. doi:10.1016/j.vetmic.2013.09.005
8. Zhang X-P, Lu C-J, Li Y-T, et al. In vitro adherence and invasion of primary chicken oviduct epithelial cells by *Gallibacterium anatis*. *Vet Microbiol*. 2017;203:136–142. doi:10.1016/j.vetmic.2017.02.009
9. Kristensen BM, Frees D, Bojesen AM. GtxA from *Gallibacterium anatis*, a cytolytic RTX-toxin with a novel domain organisation. *Vet Res*. 2010;41(3):25. doi:10.1051/vetres/2009073
10. Persson G, Bojesen AM. Bacterial determinants of importance in the virulence of *Gallibacterium anatis* in poultry. *Vet Res*. 2015;46(1):1–11. doi:10.1186/s13567-015-0206-z
11. Algammal AM, Hashem HR, Alfifi KJ, et al. atpD gene sequencing, multidrug resistance traits, virulence-determinants, and antimicrobial resistance genes of emerging XDR and MDR-Proteus mirabilis. *Sci Rep*. 2021;11(1):1–15.
12. Algammal AM, Hashem HR, Al-Otaibi AS, et al. Emerging MDR-*Mycobacterium avium* subsp. *avium* in house-reared domestic birds as the first report in Egypt. *BMC Microbiol*. 2021;21(1):1–11. doi:10.1186/s12866-021-02287-y
13. Hetta HF, Al-Kadmy I, Khazaal SS, et al. Antibiofilm and antivirulence potential of silver nanoparticles against multidrug-resistant *Acinetobacter baumannii*. *Sci Rep*. 2021;11(1):1–11.
14. Algammal AM, Alfifi KJ, Mabrok M, et al. Newly emerging MDR *B. cereus* in *Mugil seheli* as the first report commonly harbor nhe, hbl, cytK, and pc-plc virulence genes and bla1, bla2, tetA, and ermA resistance genes. *Infect Drug Resist*. 2022;15:2167–2185. doi:10.2147/IDR.S365254
15. Algammal AM, Mabrok M, Ezzat M, et al. Prevalence, antimicrobial resistance (AMR) pattern, virulence determinant and AMR genes of emerging multi-drug resistant *Edwardsiella tarda* in Nile tilapia and African catfish. *Aquaculture*. 2022;548:737643. doi:10.1016/j.aquaculture.2021.737643
16. Kareem SM, Al-Kadmy IMS, Kazaal SS, et al. Detection of gyrA and parC Mutations and Prevalence of Plasmid-Mediated Quinolone Resistance Genes in *Klebsiella pneumoniae*. *Infect Drug Resist*. 2021; 12;14:555-563. doi: 10.2147/IDR.S275852
17. Bojesen AM, Vazquez ME, Bager RJ, Ifrah D, Gonzalez C, Aarestrup FM. Antimicrobial susceptibility and tetracycline resistance determinant genotyping of *Gallibacterium anatis*. *Vet Microbiol*. 2011;148(1):105–110. doi:10.1016/j.vetmic.2010.08.011
18. Hess C, Grafl B, Bagheri S, Kaesbohrer A, Zloch A, Hess M. Antimicrobial resistance profiling of *Gallibacterium anatis* from layers reveals high number of multiresistant strains and substantial variability even between isolates from the same organ. *Microbial Drug Resist*. 2020;26(2):169–177. doi:10.1089/mdr.2019.0056
19. Jones K, Thornton J, Zhang Y, Mauel M. A 5-year retrospective report of Gallibacterium anatis and *Pasteurella multocida* isolates from chickens in Mississippi. *Poult Sci*. 2013;92(12):3166–3171. doi:10.3382/ps.2013-03321

20. Elbestawy AR, Ellakany HF, Abd El-Hamid HS, et al. Isolation, characterization, and antibiotic sensitivity assessment of *Gallibacterium anatis* biovar haemolytica, from diseased Egyptian chicken flocks during the years 2013 and 2015. *Poult Sci*. 2018;97(5):1519–1525. doi:10.3382/ps/pey007
21. Kleven S, Dufour-Zavala L, Swayne D, et al. *A Laboratory Manual for the Isolation, Identification, and Characterization of Avian Pathogens*. American Association of Avian Pathologists. 2008.
22. Bojesen AM, Vazquez ME, Robles F, et al. Specific identification of *Gallibacterium* by a PCR using primers targeting the 16S rRNA and 23S rRNA genes. *Vet Microbiol*. 2007;123(1–3):262–268. doi:10.1016/j.vetmic.2007.02.013
23. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*. 2013;30(12):2725–2729. doi:10.1093/molbev/mst197
24. CLSI. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals*. 4th ed. CLSI supplement VET08. Wayne: Clinical Laboratory Standards Institute; 2018
25. Magiorakos A-P, Srinivasan A, Carey R, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect*. 2012;18(3):268–281. doi:10.1111/j.1469-0691.2011.03570.x
26. Krumperman PH. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl Environ Microbiol*. 1983;46(1):165–170. doi:10.1128/aem.46.1.165-170.1983
27. Bager RJ, Nesta B, Pors SE, et al. The fimbrial protein FlfA from *Gallibacterium anatis* is a virulence factor and vaccine candidate. *Infect Immun*. 2013;81(6):1964–1973. doi:10.1128/IAI.00059-13
28. Johnson TJ, Danzeisen JL, Trampel D, et al. Genome analysis and phylogenetic relatedness of *Gallibacterium anatis* strains from poultry. *PLoS One*. 2013;8(1):e54844. doi:10.1371/journal.pone.0054844
29. Klima CL, Alexander TW, Hendrick S, McAllister TA. Characterization of *Mannheimia haemolytica* isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. *Can J Vet Res*. 2014;78(1):38–45.
30. Petrocchi-Rilo M, Gutiérrez-Martín C-B, Pérez-Fernández E, Vilaró A, Fraile L, Martínez-Martínez SJA. Antimicrobial resistance genes in porcine *Pasteurella multocida* are not associated with its antimicrobial susceptibility pattern. *Antibiotics*. 2020;9(9):614. doi:10.3390/antibiotics9090614
31. Yaman S, Sahan Yapicier O. Diagnosis of *Gallibacterium anatis* in layers: first report in Turkey. *Braz J Poultry Sci*. 2019:21. doi:10.1590/1806-9061-2019-1019
32. Lozica L, Kazazić SP, Gottstein Ž. High phylogenetic diversity of *Gallibacterium anatis* is correlated with low biosecurity measures and management practices on poultry farms. *Avian Pathol*. 2020;49(5):467–475. doi:10.1080/03079457.2020.1765970
33. Antenucci F, Arak H, Gao J, Allahgadry T, Thøfner I, Bojesen AM. Hydrostatic filtration enables large-scale production of outer membrane vesicles that effectively protect chickens against *Gallibacterium anatis*. *Vaccines*. 2020;8(1):40. doi:10.3390/vaccines8010040
34. Johnson TJ, Fernandez-Alarcon C, Bojesen AM, Nolan LK, Trampel DW, Seemann T. Complete genome sequence of *Gallibacterium anatis* strain UMN179, isolated from a laying hen with peritonitis. *J Bacteriol*. 2011;193(14):3676–3677. doi:10.1128/JB.05177-11
35. Huangfu H, Dong Q, Wang H, et al.Isolation, identification and phylogenetic analysis of *Gallibacterium anatis* from silky population in Henan Province. *Acta Vet*. 2018;49(11):2451–2460.
36. Elbestawy A Studies on *Gallibacterium anatis* infection in chickens. *PhD Fac Vet Med*, Alexandria University, Egypt. 2014.
37. Sorour HK, Atfeehy NMA, Shalaby AG. *Gallibacterium anatis* infection in chickens and ducks. *Assiut Vet Med J*. 2015;61(147):80–86.
38. Nassik S, Tallouzt S, Karbach N, et al. First report of isolation of *Gallibacterium anatis* from layer chickens in Morocco with decrease in laying performance. *Avian Dis*. 2019;63(4):727–730. doi:10.1637/aviandiseases-D-19-00119
39. Wang C, Robles F, Ramirez S, Riber AB, Bojesen AM. Culture-independent identification and quantification of *Gallibacterium anatis* (*G. anatis*) by real-time quantitative PCR. *Avian Pathol*. 2016;45(5):538–544. doi:10.1080/03079457.2016.1184743
40. Soler N, Forterre P. Vesiduction: the fourth way of HGT. *Environ Microbiol*. 2020;22(7):2457–2460. doi:10.1111/1462-2920.15056
41. Rodríguez-Beltrán J, DelaFuente J, Leon-Sampedro R, MacLean RC, San Millan A. Beyond horizontal gene transfer: the role of plasmids in bacterial evolution. *Nat Rev Microbiol*. 2021;19(6):347–359. doi:10.1038/s41579-020-00497-1
42. Michael GB, Bossé JT, Schwarz S. Antimicrobial resistance in *Pasteurellaceae* of veterinary origin. *Microbiol Spectr*. 2018;6(3). doi:10.1128/microbiolspec.ARBA-0022-2017
43. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. *Drug Resist Updat*. 2010;13(6):151–171. doi:10.1016/j.drup.2010.08.003