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Gallibacterium anatis: Moleculer Detection of Tetracycline Resistance and Virulence Gene

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ABSTRACT

Gallibacterium anatis causes infections in the reproductive tract of egg-laying hens and it is associated with increased mortality and decreased egg production. For this study we used singeleplex and multiplex PCR with specific primers to assess the presence of tetracycline resistance (Tcr) (*tet* A, B, C, D, E, G, H, K, L, M, O, S, P, Q and X), virulence [cytotoxic (RTX-like toxin, *gtxA*) and fimbrial (*flfA*)] genes and antibiotic resistance in *G. anatis* isolates. Among the 20 isolates tested, the highest antimicrobial resistance patterns were observed in erythromycin, streptomycin, tilmicosin (100%) followed by colistin sulphate (65%), cephalexin and tulathromycin (50%). Among 20 isolates examined, 10 (50%) carried tetracycline resistance genes, 7 (35%) had *tet*(B), 2 (10%) had *tet*(G), and 1 (5%) had *tet*(A), (D), (M) or (L). Of these *G. anatis* isolates were carried out 6 (30%) *gtxA* but none of *flfA* gene. Based on present results, it is concluded that virulence and Tcr genes could contribute to pathogencity of *G. anatis*, which is a major risk to poultry health.

Key words: Antibiotic resistance, G. anatis, Poultry, Virulence genes, Tetracycline resistance genes

INTRODUCTION

Major health problems in the poultry industry can affect egg production. In particular, infectious diseases can reduce egg production and egg quality by directly affecting the reproductive system of hens. Such diseases also can indirectly diminish the overall health status of poultry (Clauer, 2009). Gallibacterium anatis (G. anatis) is a resident of normal microflora of the lower genital and upper respiratory tract in chickens and many other avian species (Bojesen et al., 2004; Rzewuska et al., 2007; Jones et al., 2013; Paudel et al., 2013; Persson and Bojesen 2015; Lawal et al., 2018). Decreased egg production associated with salpingitis, respiratory system problems and mortality in commercial laying hens therefore, G. anatis infections have been the topic of researchers' works in recent years (Bojesen et al., 2011a; Sing, 2016; Chaveza et al., 2017). The knowledge of bacteria-host interactions and antimicrobial susceptibility to G. anatis in laying hens remains limited (Bisgaard et al., 2009; Johnson et al., 2013). Among the most important G. anatis virulence factors involved in colonization and invasion of the epithelium in the trachea, oropharyngeal tissues and oviduct are the IgG destructive protease, RTX-like toxin, *gtxA* and hemagglutinin, which suppress the host immune response (Vaca et al., 2011; Lucio et al., 2012). Bacterial fimbria are also important not only as a virulence factor, but as a target for preventative vaccines (Kudirkiene et al., 2014; Sorour et al., 2015). Tetracycline resistance determinants (Tcrs) are widespread among both Gram negative organisms and *Pasteurellaceae* family and are often found in multi-drug resistant bacterial species (Levy et al., 1989; Roberts, 1996). To better understand *G. anatis* pathogenicity in poultry, this study amied to determine the prevalence of Tcr genes and virulence-specific factor genes in *G. anatis* isolates from laying hens.

MATERIAL AND METHODS

Bacterial Isolates

In the present study, 20 *Gallibacterium anatis* isolates from laying hens obtained from the previous study at the Department of Microbiology, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey were analysed (Yaman and Sahan Yapicier, 2019).

Gallibacterium anatis Strains

G. anatis F149T (non-hemolytic strain, ATCC 43329) and 12656-12 strain (hemolytic strain) was used for analysis in this study.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test was carried out by the agar disk diffusion method on Mueller-Hinton agar (Oxoid Ltd, Hampshire, UK) supplemented with 5% sheep blood according to the guidelines from Clinical and Laboratory Standards Institute (CLSI, 2017). The following antibiotics (spiramycin and tulatromycin, Bioanalyse, Turkey), 18 out of 20 (Oxoid, UK) commonly used in veterinary medicine were selected: ampicillin (10µg; AMP), amoxicillin (10µg; AX), amoxicillin clavulanic acid (30µg; AMC), cephalexin (30µg; CL), ceftiofur (30µg; FUR), ciprofloxacin (5µg; CIP), colistin sulphate (10µg; CT), doxycilin (30µg; DO), enrofloxacin (5µg; ENR), erythromycin (15µg; E), florfenicol(30µg; FFC), gentamicin (10µg; CN), tetracycline (30 µg; T), penicillin (10units; P), spiramycin (100 µg; S), streptomisin (10 µg; S), tilmicosin (15µg; TIL), trimethoprim sulphamethoxazole (25µg; TS), tulathromycin (30µg; TUL), tylosin (30µg; TY). The results were obtained by measuring the diameter of the growth inhibition zone around the antibiotic disc for each isolated bacteria and recorded as sensitive, intermediate and resistant according to the interpretive standards of CLSI and antimicrobials manufacturers' instructions. Isolates displaying resistance to ≥ 3 antimicrobial agents tested were defined as exhibiting multi-drug resistance (MDR) (Tenover et al., 1987; Schwarz et al., 2010). E. coli ATCC 10536 was used as a quality control strain.

Primers

A primer pair specific for 14 tetracycline resistance genes and *G. anatis* virulence genes were listed in Tables 1 and 2 (Ng et al. 2001; Bager et al. 2013; Paudel et al. 2013).

DNA Extraction

DNA extraction from *G. anatis* isolates were performed according to the instructions of the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA). DNAs were stored for use as template DNA at -20°C until amplification.

PCR Conditions

Singleeplex PCR assay was carried out for virulence genes. 25 μ l reaction volumes containing 3 μ l MgCl (25

mM), 0.5 μ l dNTP (10 mM), 10 pmols of primers and 0.2 μ l Taq polymerase (5U/ μ l). The following cycling conditions were used: 3 min at 94°C, followed by 30 cycles of 1 min at 94°C (denaturation) and 1 min at 54°C (primer annealing), 1 min at 72°C (extension), and 7 min at 72°C (final extension). Multiplex PCR was performed for tetracycline resistant genes and these genes grouped (Group I: *tet*(B), *tet*(C) and tet(D); Group II: *tet*(A), *tet*(E) and tet(G); Group III: *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(S); Group IV: *tet*A(P), *tet*(Q) and *tet*(X)) described by Ng et al. (2001). Each multiplexed group's PCR reaction mix concentration and amplification conditions were carried out following the previous research (Zhao and Aoki, 1992).

 Table 1. Tetracyline resistance specific primers

Tcrs	primer sequence (5'-3')	Amplicon size (bp)	
tet(A)	GCT ACA TCC TGC TTG CCT TC	210	
	CAT AGA TCG CCG TGA AGA GG		
tet(B)	TTG GTT AGG GGC AAG TTT TG	659	
	GTA ATG GGC CAA TAA CAC CG		
<i>tet</i> (C)	CTT GAG AGC CTT CAA CCC AG	418	
	ATG GTC GTC ATC TAC CTG CC		
<i>tet</i> (D)	AAA CCA TTA CGG CAT TCT GC	787	
	GAC CGG ATA CAC CAT CCA TC		
<i>tet</i> (E)	AAA CCA CAT CCT CCA TAC GC	278	
101(12)	AAA TAG GCC ACA ACC GTC AG		
tet(G)	GCT CGG TGG TAT CTC TGC TC	468	
. ,	AGC AAC AGA ATC GGG AAC AC		
tet(G)	CAG CTT TCG GAT TCT TAC GG	844	
(-)	GAT TGG TGA GGC TCG TTA GC		
tet(K)	TCG ATA GGA ACA GCA GTA CAG	169	
	CAG ATC CTA CTC CTT		
tet(L)	TCG TTA GCG TGC TGT CAT TC	267	
(_)	GTA TCC CAC CAA TGT AGC CG		
tet(M)	GTG GAC AAA GGT ACA ACG AG	406	
	CGG TAA AGT TCG TCA CAC AC		
$tet(\mathbf{O})$	AAC TTA GGC ATT CTG GCT CAC	515	
lel(0)	TCC CAC TGT TCC ATA TCG TCA	515	
tot(S)	CAT AGA CAA GCC GTT GAC C	667	
101(3)	ATG TTT TTG GAA CGC CAG AG	00/	
4 a4(D)	CTT GGA TTG CGG AAG AAG AG	676	
iei(1)	ATA TGC CCA TTT AAC CAC GC	0/0	
tet(Q)	TTA TAC TTC CTC CGG CAT CG	004	
	ATC GGT TCG AGA ATG TCC AC	204	
tet(X)	CAA TAA TTG GTG GTG GAC CC	169	
	TTC TTA CCT TGG ACA TCC CG	400	

Virulence genes	Primer sequence (5'-3')	Amplicon size (bp)
GalNtx	TGCGCAAGTGCTAAATGAAG GGATAATCGTTGCGCTTTG	925
flfA	CACCATGGGTGCATTTGCGGATGATC C TATTCGTATGCGATAGTATAGTTC	538

Table 2. Spesific primers for virulence genes of G. anatis

Ethical Approval

This study was approved by Animal Research Ethics Committee of Burdur Mehmet Akif Ersoy University, Burdur, Turkey (Protocol No. MAKU-HADYEK/ 2017-314).

RESULTS

Antimicrobial Susceptibility Test

The highest antimicrobial resistance patterns in 20 isolates tested were observed for erythromycin, streptomycin, tilmicosin (100%) followed by colistin sulphate (65%), cephalexin and tulathromycin (50%) which are shown in table 3. 100% of the *G. anatis* isolates exhibited sensitivity to doxycilline while 15% and 85%, respectively, showed intermediate resistance to tetracycline.

Molecular Detection of *tet* Genes and Virulence Genes

Twenty isolates of *G. anatis* contained 10 (50%) tetracycline resistance, 7 (35%) tet(B), 2(10%) tet(G), 1(5%) tet(A), (D), (M) and (L). 2(%10) were found to carry both tet(B) and tet(G); 1(5%) were carried both tet(B), (D) and (A) genes (Figure 1). The amplification of

genes by PCR showed that 6 (30%) strains contains gtxA and no *flfA* genes encoded. Based on the correlation of isolates; one of the two isolates carried both tet(B), (D) and gtx gene and the other carried tet (B) and gtx genes.

Table 3. Antimicrobial resistance of 20 G. anatis isolates

Items	S (n%)	I (n%)	R (n%)
AMP	20(100)	0	0
AMC	20(100)	0	0
AX	20(100)	0	0
CIP	15(75)	0	5(25)
CL	12(50)	0	12(50)
CN	16(80)	0	4(20)
СТ	7(35)	0	13(65)
DO	20(100)	0	0
Е	0	0	20(100)
ENR	20(100)	0	0
FFC	20(100)	0	0
FUR	20(100)	0	0
Р	20(100)	0	0
SP	15(75)	0	5(25)
S	0	0	20(100)
TE	0	17(85)	3(15)
TIL	0	0	20(100)
TS	19(95)	0	1(5)
TUL	10(50)	0	10(50)
TY	15(75)	0	5(25)

S: Sensitive, I: Intermediate, R: Resistant, AMP: Ampicillin, AMC: Amoxicillin clavulanic acid, AX: Amoxicillin, CIP: Ciprofloxacin, CL: Cephalexin, CN: Gentamicin, CT: Colistin sulphate, DO: Doxycillin, E: Erythromycin, ENR: Enrofloxacin, FFC: Florfenicol, FUR: Ceftiofur, P: Penicillin: SP: Spiramycin, S: Streptomisin, TE: Tetracycline, TIL: Tilmicosin, TS: Trimethoprim sulphamethoxazole, TUL: Tulathromycin, TY: Tylosin



Figures 1. Multiplex PCR assay was performed using Group I-II-III Tcr primers respectively. M: 100bp marker; 1, 2, 4, 5: *tet* (B), 6: *tet* (B) and (D); 2: *tet* (A), 3, 4, 6: *tet* (G); 4: *tet*(M), 7: *tet* (L)

DISCUSSION

G. anatis is commonly found among normal flora of both the upper respiratory tract and lower genital tract of chickens and other avian species, and can therefore be regarded as an opportunistic pathogen. The pathogenesis of G. anatis is not well-characterized, particularly at the molecular level, and little is known about which antibiotic resistace, genes and mechanisms are associated with the ability of G. anatis to cause disease. The current investigation is the first study of the antimicrobial resistance, tet and virulence genes of G. anatis in Turkey. Among the 20 isolates tested, the highest antimicrobial resistance patterns were observed for erythromycin, streptomycin, tilmicosin (100%) followed by colistin sulphate (65%), cephalexin and tulathromycin (50%) which are shown in table 3 The majority of the isolates were exhibited susceptibility against to amoxicillin clavulanic acid, ceftiofur, enrofloxacin, florfenicol. gentamicin, trimethoprim sulphamethoxazole which is in agreement with the other studies (Jones et al., 2013; El-Bastawy, 2014; El-Adawy et al., 2018; Lawal et al., 2018). About 100% of the G. anatis isolates exhibited sensitivity to doxycilline while 15% and 85%, respectively, showed intermediate resistance to tetracycline. Especially high level of tetracycline resistance was similar with the previous researches (Bojesen et al., 2011b; Jones et al., 2013; Abd El-Hamid et al., 2016; Lawal et al., 2018). In contrast to these findings, Lin et al. (2001) also reported moderate sensitivity to tetracycline. Multi-drug resistance reveals that 13 isolates representing large percentage (65%) resistance against three or more antibiotics. Especially, MDR patterns in this study were similar to those observed in previous study (Bojesen et al., 2011b). In this study, singleplex and multiplex PCR were used to detect Tcr and virulence genes in G. anatis isolates from laying hens. This study can be one of the first tries to examine the prevalence of these genes in G. anatis isolates in Turkey and also to test for the presence of tet (P), (Q), (S), and (X) in addition to the previously studied tet (A), (B), (C), (D), (E), (G), (H), (K), (L), (M) and (O) genes (Hansen et al., 1993; Bojesen et al., 2011b). Four multiplex PCR groups were used in this study to detect 14 tetracycline resistance genes and singleplex PCR to target virulence-associated gtxA and flfA genes. Twenty isolates of G. anatis contained 10 (50%) carried genes for tetracycline resistance, 7 (35%) had tet(B), 2 (10%) had tet(G), and 1(5%) had tet(A), (D), (M) or (L). Another 2 (10%) carried both tet(B) and tet(G) while 1 (5%) had

tet(B), (D) and (A) genes. None of the other resistance genes were detected. Together, tet(A), (B), (D), (G), (M) and (L) genes, which are associated with efflux and/or ribosomal protection mechanisms of G. anatis were detected (Ng et al., 2001; Michalova et al., 2004). Unsuprisingly, presence of these genes was explained according to the previous studies (Kehrenberg et al., 2001; Kehrenberg et al., 2006; Bojesen et al., 2011b). It is indicated that group I tet(B) genes had the most numbers among the 20 isolates, which is consistent with a report by Bojesen et al. (2011b). The tet(B) gene compared to the others. represented especially among Enterobacteriaceae (Roberts, 1996; 1998; Levy, Kehrenberg et al., 2006) and reported to be widely distributed among Pasteurellacea (Vaca et al., 2011; Lucio et al., 2012; Bager et al., 2013; Kudirkiene et al., 2014; Persson and Bojesen 2015; Zhang et al., 2017). The pathogenicity of G. anatis is influenced by various factors encoded by different virulence genes that play important roles in different pathogenic activities such as adhesion. invasion, intracellular survival, systemic infection, and toxin production (Kristensen et al., 2011; Persson and Bojesen, 2015; Sorour et al., 2015; Sing et al., 2016). In particular, the gtx toxin is responsible for the hemolytic and leukotoxic affects of G. anatis (Bager et al., 2013; Kudirkiene et al., 2014; Persson and Bojesen, 2015). The flfA gene is also implicated in G. anatis virulence and is a target for prevention of diseases caused by G. anatis in laying hens (Bager et al., 2013; Kudirkiene et al., 2014; Persson and Bojesen 2015). PCR amplification of these genes (gtxA and flfA) in this study showed that 6 (30%) of the tested strains carried gtxA, but none had flfA. All of the isolates in this study displayed hemolytic characteristics, which is consistent with the expectations about the value of detecting gtx for determination of pathogenic activity. A previous study that focused on hemolytic strains of G. anatis found that gtx was present in 7/12 (58%) and 5/13 (38.4%) samples from chickens and ducks, respectively (Sorour et al., 2015). Meanwhile, a study by Kristensen et al. (2011) revealed that gtx is associated with nonhemolytic G. anatis strains. The other studies found high incidences (50-75%) of flfA gene (Kudirkiene et al., 2014; Sorour et al., 2015), whereas none of the isolates in present study had *flf*A. Moreover, the absence of fimbria in the isolates that examined could have contributed to the lower pathogenicity of these G. anatis strains. The findings of this study indicated no correlation between the presence of Tcr genes and genes associated with virulence in the isolates tested. The virulence mechanisms

associated with the ability of *G. anatis*, which is typically a non-pathogenic component of the normal respiratory microflora of animals, to induce opportunistic respiratory tract infections under conditions that compromise immune responses or those that cause stress, such as inadequate nutritional intake (Bojesen et al., 2003), require further investigation.

CONCLUSION

The present study detected the genes associated with virulence and tetracycline resistance of *Gallibacterium anatis* that isolated from laying hens in Turkey for the first time and presented the first evidence to support the use of specific primers for *tet* P, Q, S and X genes in this breed. The findings of this study can increase the knowledge of *Gallibacterium anatis* pathogenicity in poultry.

DECLARATIONS

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

OSY planned and designed the study. OSY performed the experiments, SY and OSY contributed to the analysis and interpretation of data. OSY drafted the manuscript. All authors read and approved the final manuscript.

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