Investigation of Important Virulence Genes and Antibiotic Resistance of *Escherichia coli* Isolated from Broiler Chickens

Ilcebaylik, A.[1] and Turkyilmaz, S.[2*]

[1]Health Sciences Institute, Aydin Adnan Menderes University, Aydin, TURKEY.
[2]Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydin, TURKEY.

* **Corresponding author:** Dr. S. Turkyilmaz; Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Isikli, Aydin-Turkey; E-mail: sturkyilmaz@adu.edu.tr

ABSTRACT

Colibacillosis of poultry is caused by pathogenic *Escherichia coli* (APEC) occurring when virulence factors that are encoded by specific genes, which promote adhesion and proliferation of *E. coli* in the host. This study was conducted to investigate the pattern of antibiotic resistance and virulence genes (VG) in the APEC isolates from broiler chickens in the region of western Turkey. All the strains were checked for the presence of 11 virulence genes by polymerase chain reaction (PCR): aerobactin (*iuc*D), iron receptor (*iro*N), iron uptake transport (*iut*A), enteroaggregative toxin (*ast*A), vacuolating autotransporter toxin (*vat*A), hemolysin F (*hly*F), P fimbriae (*papC*), temperature sensitive hemagglutinin (*tsh*), increased serum survival protein (*iss*), outer membrane protein (*omp*T) and structural genes of colicin V operon (*colV*). The resistance status to nine antibiotics in nine antimicrobial families of the isolates was examined by disk diffusion tests. Isolates carrying at least five VG were evaluated as clinical APEC (cAPEC), fewer VG carrying potential APEC (pAPEC), and isolates resistant to at least three or more antimicrobial drug classes as multiple antibiotic resistant (MDR). Chi-Square ($\chi 2$) test was used to compare the frequency of VGs between cAPEC and pAPEC and $P \leq 0.05$ was considered as significant. Hundred and forty (80.1%) APEC were isolated from 173 broilers. Eighty six (61.4%) of the 140 isolates were cAPEC, 54 (38.6%) were pAPEC and these isolates had 47 virulence genotypes. *iss* (78.6%), *iut*A (75.7%), *iro*N (68.6%), *hly*F (61.4%), *omp*T (60.0%) genes were the highest detected genes. Other genes investigated; *iuc*D (40.0%), *tsh* (27.1%), *colV* (25.7%), *pap*C (21.4%), *vat*A (8.6%), *ast*A (4.3%) were detected at lower frequencies. The $\chi 2$ test showed that the distribution of all virulence genes between cAPEC and pAPEC strains was relevant. All isolates were found to be high level resistant to amoxicillin, trimethoprim sulfamethoxazole, amoxicillin clavulanic acid and ciprofloxacin, and 90.0% had MDR. In this study, high levels of MDR were detected in APEC isolates with very different virulence genotypes. It was concluded that PCR screening of virulence genes is more practical than pathogenicity testing, having more than one iron transplantation system playing an important role in the survival of APEC, and the development of VG-based diagnostic methods that characterize virulence genotypes may be useful for future vaccine studies.

Key words: Antibiotic Resistance; Broiler; *Escherichia coli*; Virulence Gene.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) causes avian colibacillosis characterized by multiple organ lesions, septicemia, pericarditis, perihepatitis, peritonitis airsacculitis, and other extra intestinal lesions in poultry species (1). Colibacillosis occurs only in birds infected with pathogenic strains of *E. coli* known as APEC. Recent reports suggest a link between APEC and human disease (2, 3). Thus, improved control of colibacillosis in birds may be beneficial to both animal and human health.

The diagnosis of poultry colibacillosis is based on clinical findings in the affected tissues, typical pathological lesions and *E. coli* culture. Culture methods for isolation and identification of *E. coli* are considered the gold standard. However, culture methods are labor intensive, expensive and time consuming compared to molecular methods. The main drawback of the APEC isolation method is the presence of fecal strains in all birds, and in some cases results in the isolation and characterization of non-pathogenic *E. coli* (4).

The most important virulence factors of *E. coli* are iron collection systems (siderophores), toxins, adhesins, protectins and colicins. These are related to bacterial invasion, colonization and survival of the defense system (5). Iron acquisition genes for example aerobactin (*iuc*D), salmochelin siderophore receptor gene (*iro*N), aerobactin siderophore receptor gene (*iut*A) were significantly associated with virulence of APEC strains (5,6). Toxins like enteroaggregative heat stabile toxin (*ast*A), vacuolating autotransporter toxin (*vat*A), hemolysin F (*hly*F) protect APEC from lysosomes. Adhesins like P fimbriae (*pap*C) and temperature sensitive hemagglutinin (*tsh*) also help to bind APEC to the extra intestinal tract and enter bacteria into tissues. Protectectins like increased serum survival protein (*iss*) and outer membrane protein (*omp*T) inhibit the classical pathway of component activity (3,4). Colicins like colicin V (*col*V) are proteins that prevent bacterial growth (5,6).

The correct identification of virulent strains and virulence genes of *E. coli* is important for the selection of strains that can be used for vaccines. Virulence gene studies are important not only to assist in the characterization of pathogenic strains of *E. coli*, but also in the development of effective vaccines. In previous studies, virulence genes (VG), which can be used as molecular markers in the identification of APEC, have been investigated. First, 9 of the 38 genes examined (*iut*A, *iss*, *omp*T, *iro*N, *tsh*, *cva*C, *sit*A, *fyu*A, *irp*-2) have been shown to be more common in APEC isolates than poultry fecal *E. coli* (AFEC) isolates (3). The authors also reported that *E. coli* strains must have at least 5 virulence genes in order to be evaluated as APEC (6,7). In another study, 46 genes were studied more extensively, and the results showed that five genes (*iut*A, *hly*F, *iss*, *iro*N, and *omp*T) which were associated with virulence were found to be more pathogenic among APEC isolates (8).

Because of inappropriate use of antibiotics, susceptible microorganisms have become multidrug resistant (MDR) over time. There is increasing evidence that APEC isolates are becoming more resistant to antimicrobial agents, so it is important to control MDR in APEC isolates (3,4). MDR isolates are a serious hazard to both humans and other animals as they have the potential for transferring resistance genes to human-specific *E. coli* or other bacteria such as *Staphylococcus aureus* and *Shigella* strains (3). In addition, this pathogen should be considered as a serious public health and food biosecurity problem as it can be transmitted naturally to people through foods consumed daily (9). There is a lack of information regarding the important virulence genes of APEC isolates obtained from broilers with colibacillosis in the region of western Turkey.

Therefore, the aim of this study was to investigate the important virulence genes and antimicrobial resistance patterns of *E. coli* isolates obtained from colibacillosis suspected broilers in the region of western Turkey.

MATERIAL AND METHOD

Material

In this study, a single commercial poultry farm disease diagnostic laboratory investigated samples suspicious of colibacillosis, in 16-41 day old Ross 308 chickens. For this purpose internal organs (heart, lung, and liver) samples were collected aseptically taken during necropsy from 173 broiler chickens. Clinically, the birds had pericarditis, peritonitis, perihepatitis, air sac inflammation and arthritis. For the study, poultry, which had not received antibiotics during the last ten days, were used as material.

Isolation and identification

Isolation of *E. coli* was performed using standard bacteriological methods (10). The surface of the internal organ was branded with flame. Incision with a sterile scalpel was performed on the affected area. The specimen was taken from the incision site with sterile a loopful. A loopful of the sample suspension was streaked onto MacConkey Agar (Merck 1.05465, Germany) and Eosin Methylene Blue (EMB) Agar (Merck 1.01347, Germany) and incubated for 24 h at 37°C aerobically. On the following day the organisms showed lactose fermentation on MacConkey agar and characteristic green metallic sheen on EMB agar. Putative *E. coli* colonies were then transferred on to nutrient agar for further identification using biochemical tests. The identification of

Table 1: Antibiotics, mechanisms of action, evaluation, antibiotic resistance status of all isolates.

Antibiotic	Group	Evaluation		Total (n=140)	cAPEC(n=86)	pAPEC (n=54)
		R	S	R	R	R
AX	Beta Lactam	<14	14≥	96 (68.6)	64 (74.4)	32 (57.1)
AML	Beta Lactamase Inhibitor	<19	≥19	92 (65.7)	62 (72.1)	30 (55.6)
DO	Tetracycline	≤10	≥ 14	9 (64.3)	56 (65.1)	34 (63.0)
SXT	Folate Path Inhibitor	≤11	≥14	88 (62.9)	58 (67.4)	30 (55.6)
CIP	Fluoroquinolone	≤24	≥26	60 (42.9)	34 (39.5)	26 (48.1)
CN	Aminoglycosides	≤14	≥17	52 (37.1)	28 (32.6)	24 (44.4)
FOS	Phosphoric Acid Derivate	<24	≥24	50 (35.7)	32 (37.2)	18 (33.3)
FF	Florfenicol	≤10	≥21	42 (30.0)	26 (30.2)	16 (29.6)
CL	Cephalosporin	<14	≥14	38 (27.1)	24 (27.9)	14 (25.9)

the isolates was performed by Gram staining, oxidase test, EMB agar growth characteristics and indole, methyl red, Voges Proskauer, citrate (IMVIC) tests (10). The isolates were stored in Brain Heart Infusion Broth (Oxoid CM 1135, UK) containing glycerol 20% at -20°C. *E. coli* ATCC25922 (USA) was used as a reference organism.

Antibiotic susceptibility tests

Antimicrobial resistance of the isolates was investigated by the standard disk diffusion method (11). The *E. coli* isolates were characterized for their resistance to 9 antibiotics [amoxicillin (AX, 25 µg), amoxicillin clavulanic acid (AML, 20/10 µg), doxycycline (DO, 30 µg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg), ciprofloxacin (CIP, 5 µg), gentamicin (CN, 10 µg), fosfomycin (FOS, 50 µg), florfenicol (FF, 30 µg), cephalexin (CL, 30 µg)] belonging to nine antibiotic families (Table 1). Zone diameters of susceptibility testing results were categorized as sensitive (S), intermediate (I), or resistant (R) based on the Clinical and Laboratory Standards Institute (CLSI) (11). *E. coli* ATCC 25922 strains were used as quality control for the antibiotic susceptibility tests. Resistance to at least 3 or more unrelated antimicrobial families was defined as MDR (12).

DNA extraction, purity and quantity controls

In this study, DNA extraction was performed by the sonication method as previously reported (13). For this purpose, isolates were passaged from *E. coli* stock cultures to EMB agar and incubated at 37°C for 24 hours. A colony was taken from this bacterial culture and transferred to 5 ml Nutrient Broth (NB) (Merck 1.05443, Germany). Thereafter NB was incubated at 37°C for 18-24 hours. The broth was centrifuged at 13500 rpm for 5 min. The supernatant was discarded. The residue was diluted with 200 µl PBS in an ependorf tube (~10^8/ml). The suspension was held in a sonicator for at 40 Hz 10 minutes. Then centrifuged at 13500 rpm for 5 min. Three microliters of supernatant was as template DNA in each PCR reaction. DNA purity and quantity controls were also performed. The ratio of OD260/OD280 was between 1.6-2.0 indicating that the DNA was pure (14).

Polymerase chain reaction (PCR)

The universal stress protein gene (*usp*A) was used in PCR examination for the verification of the *E. coli* isolates (15). PCR, for each sample was carried out on a volume of 50 µl, final concentration was 10x Taq enzyme buffer solution 1x, 25 mM $MgCl_2$ 2 mM, 10 mM dNTP 0.2 mM, 100 ρmol primer (for each) 0.4 ρmol, 5 U Taq DNA polymerase 1.5 U (Fermentas, USA), 3 µl of each DNA. The prepared tubes were loaded in the thermalcycler (Boeco, Germany).

The DNA was amplified using the following protocol: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (95°C for 10 s), annealing for 15 s [48°C (*iss*), 51°C (*ast*A, *pap*C), 53°C (*iuc*D, *tsh*), 57°C (*usp*A, *vat*A, *colV*), 63°C (*iut*A), 67°C (*iro*N, *hyl*F, *omp*T)] and extension (72°C for 1 min), with a single final extension for 7 min at 72°C. On electrophoresis, a 2% agarose gel stained with Safe View (ABM, Canada) was used and the gel was exposed to 100 volts for 45 min. After electrophoresis, the gel was placed in the chamber of the transilluminator device connected to the computer and photographed under UV light. The primers used in the study are presented in Table 2 (15-22). If the amplified product formed a band of the expected size (Table 2), it was assumed to carry the virulence gene examined.

Table 2: Sequences of primers, product lengths and references.

Primers	Sequence (5'-3')	Tm (°C)	Product length (bp)	Reference
*usp*F	CCTCCCGCACGATGATC	59.5	884	15
*usp*R	TCCACGCATCGTCAGGC	62.5		
*iut*AF	GGCTGGACATCATGGGAACTGG	65.9	302	16
*iut*AR	CGTCGGGAACGGGTAGAATCG	65.3		
*iro*NF	AATCCGGCAAAGAGACGAACCGCCT	69.1	553	17
*iro*NR	GTTCGGGCAACCCCTGCTTTGACTTT	69.5		
*iuc*DF	ACAAAAAGTTCTATCGCTTCC	55.5	714	18
*iuc*DR	CCTGATCCAGATGATGCTC	57.3		
*hly*FF	GGCCACAGTCGTTTAGGGTGCTTACC	71.0	450	19
*hly*FR	GGCGGTTTAGGCATTCCGATACTCAG	69.5		
*vat*AF	TCCTGGGACATAATGGTCAG	57.3	981	20
*vat*AR	GTGTCAGAACGGAATTGT	63.8		
*ast*AF	TGCCATCAACACAGTATATCC	58.4	116	21
*ast*AR	TCAGGTCGCGAGTGACGGC	51.6		
*tsh*F	ACTATTCTCTGCAG AAGTC	55.3	825	20
*tsh*R	CTTCCGATGTTCTGAACT	54.5		
*pap*CF	TGATATCACGCAGTCAGTAGC	57.9	500	20
*pap*CR	CCGGCCATATTCACATAA	51.4		
*iss*F	ATGCAGGATAATAAGATGAAA	50.1	290	20
*iss*R	CTATTGTGAGCAATATACA	48.0		
*omp*TF	TCATCCCGGAAGCCTCCCTCACTACTAT	71.6	496	22
*omp*TR	TAGCGTTTGCTGCACTGGCTTCTGATAC	70.1		
*col*VF	TGGTAGAATTGTGCCAGAGCAAG	60.6	1180	5
*col*VR	GAGCTGTTTGTAGCGAAGCC	59.4		

APEC evaluation criteria

The term APEC is used for *E. coli* isolates from poultry colibacillosis (4). In this study, the presence of 11 virulence genes of APECs were investigated. Previous studies have reported that virulent APEC strains should carry at least five VG according to accepted genetic criteria for pathogenicity (6,7). Accordingly, isolates carrying at least five or more of the 11 virulence genes examined were considered as virulent and these isolates were named as clinical APEC (cAPEC) and isolates having less than five virulence genes were called potential APEC (pAPEC).

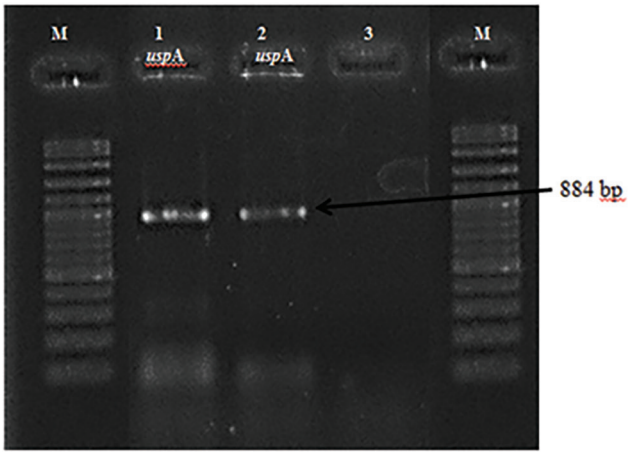

Figure 1. *usp*A PCR: gel electrophoresis images of *E. coli* isolates. 1. *E. coli* ATCC 25922 (Positive control) 2. *E. coli* field isolate 3. Negative Control (*E. faecalis* ATCC 25912 strain) M: Marker (100 bp DNA Ladder).

Statistical analysis

In this study, the frequency of 11 virulence genes was compared between two populations (cAPEC and pAPEC). Chi-square ($\chi 2$) test (95% confidence interval) was carried out using SPSS 22.0 package program for Windows. A $P \leq 0.05$ was considered as significant.

RESULTS

Isolation and identification

In this study, 140 (80.1%) *E. coli* suspicious isolates were obtained from internal organs of 173 broilers. Gram negative rod, oxidase -, indole +, MR +, VP -, citrate - isolates were identified as *E. coli*.

Following PCR with the *usp*A specific primers, 884 bp long product was obtained in all 140 isolates. It was molecularly confirmed that all isolates were *E. coli* (Figure 1). After

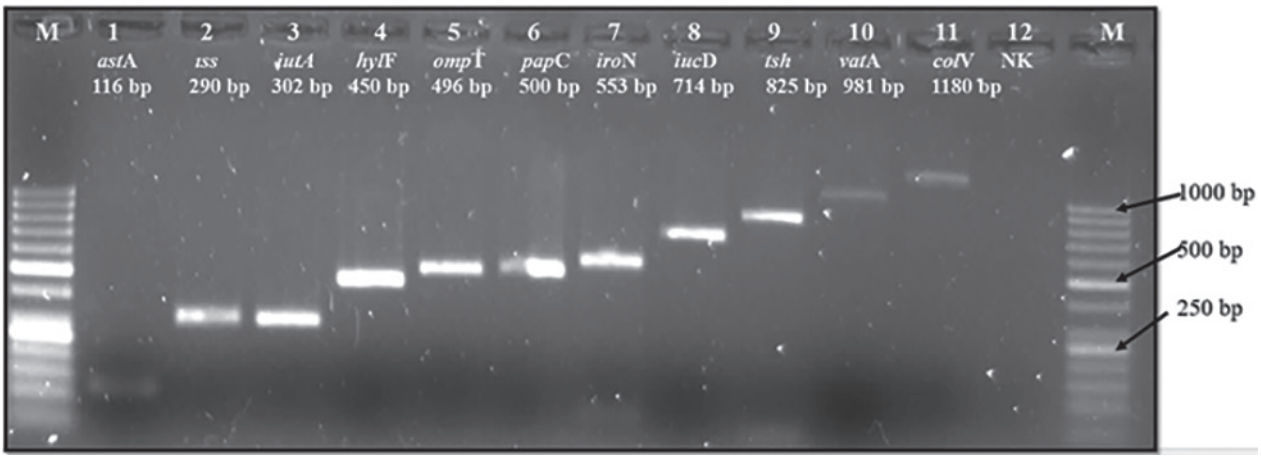

Figure 2: Gel electrophoresis image of APEC virulence genes. **1.** *ast*A (116 bp) **2.** *iss* (290 bp) **3.** *iut*A (302 bp) **4.** *hyl*F (450 bp) **5.** *omp*T (496 bp) **6.** *pap*C (500 bp) **7.** *iro*N (553 bp) **8.** *iuc*D (714 bp) **9**. *tsh* (825 bp) **10.** *vat*A (981 bp) **11.** *col*V (1180 bp) gene positive field isolates **12.** Negative Control (Master mix without DNA) **M:** 50 bp DNA ladder (Fermentas).

Table 3: Number of virulence genes carried of the isolates and evaluation.

Number of virulence genes	Number of isolate (%)	APEC (n=140) (%)	Evaluation
–	2 (1.4)	54 (38.6)	pAPEC
1	14 (10.0)		
2	20 (14.3)		
3	10 (7.2)		
4	8 (5.7)		
5	28 (20.0)	86 (61.4)	cAPEC
6	24 (17.3)		
7	22 (15.7)		
8	4 (2.8)		
9	6 (4.2)		
10	2 (1.4)		

this verification, virulence genes and antibiotic resistance of 140 isolates were examined.

Virulence Genotyping

In this study, the 11 virulence genes [iron collection systems (*iro*N, *iut*A, *iuc*D), toxins (*hly*F, *ast*A, *vat*A), adhesins (*pap*C, *tsh*), colicin (*col*V), serum survival (*iss*, *omp*T)] of 140 *E. coli* isolates were examined by PCR (Figure 2).

According to the evaluation criteria, of 140 APEC isolates 86 (61.4%) were cAPEC and 54 (38.6%) were pAPEC (Table 3). Ten genes detected in 2 isolates (1.4%), 9 genes detected in 6 isolates (4.2%), 8 genes detected in 4 isolates (2.8%), 7 genes detected in 22 isolates (15.7%), 6 genes detected in 24 isolates (17.3%), 5 gene detected in 28 isolates (20.0%), 4 gene detected in 8 isolates (5.7%), 3 gene detected in 10 isolates (7.2%), 2 gene detected in 20 isolates (14.3%), 1 gene detected in 14 isolates (10.0%) whereas no virulence-related gene was found in 2 (1.4%) *E. coli* isolates.

iss (78.6%), *iut*A (75.7%), *iro*N (68.6%), *hly*F (61.4%), *omp*T (60.0%) genes, were the most commonly detected. Other genes investigated; *iuc*D (40.0%), *tsh* (27.1%), *col*V (25.7%), *pap*C (21.4%), *vat*A (8.6%), *ast*A (4.3%) were detected at lower frequencies (Table 4). The detection rates of *iut*A, *iro*N, *iss*, *omp*T, *hyl*F, *iuc*D, *pap*C in cAPEC were relatively higher than the detection rates of genes of pAPEC isolates. On the

Table 4: Virulence gene distributions of APEC, cAPEC and pAPEC isolates

Genes	APEC (n=140) (%)	cAPEC (n=86) (%)	pAPEC (n=54) (%)
*iut*A	106 (75.7)	82 (95.3)	24 (44. 4)
*iro*N	96 (68.6)	82 (95.3)	14 (25.9)
*iuc*D	56 (40.0)	48 (55.8)	8 (14.8)
*hly*F	86 (61.4)	68 (79.1)	18 (33.3)
*vat*A	12 (8.6)	12 (14.0)	0 (00.0)
*ast*A	6 (4.3)	6 (7.0)	0 (00.0)
tsh	38 (27.1)	38 (44.2)	0 (00.0)
*pap*C	30 (21.4)	26 (30.2)	4 (7.4)
iss	110 (78.6)	74 (86.0)	36 (66.7)
*omp*T	84 (60.0)	72 (83.7)	12 (22.2)
*col*V	36 (25.7)	36 (41.9)	0 (00.0)

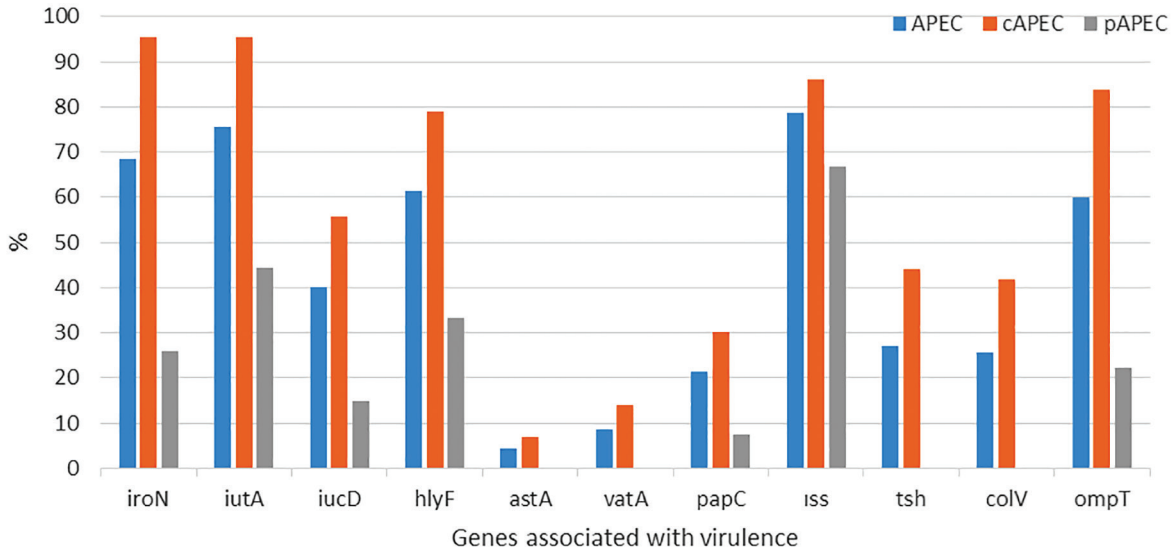

Figure 3: Comparison of virulence gene distributions of APEC, cAPEC and pEPEC isolates.

Table 5: Virulence gene profiles of pAPEC isolates.

Number of Genotypes	Virulence Genotype	Number of virulence genes	Number of isolates
1	–	0	2
2	*iss*	1	10
3	*iut*A	1	4
4	*hyl*F, *iro*N	2	2
5	*hyl*F, *iss*	2	2
6	*omp*T, *iss*	2	2
7	*iro*N, *iss*	2	4
8	*iut*A, *iss*	2	4
9	*hyl*F, *iut*A	2	6
10	*iuc*D, *iut*A, *iss*	3	4
11	*hyl*F, *iro*N, *omp*T	3	2
12	*pap*C, *omp*T, *iss*	3	2
13	*hyl*F, *iut*A, *iss*	3	2
14	*hyl*F, *iro*N, *omp*T, *iss*	4	2
15	*iuc*D, *iut*A, *omp*T, *iss*	4	2
16	*hyl*F, *iuc*D, *iut*A, *iro*N	4	2
17	*pap*C, *iro*N, *omp*T, *iss*	4	2

other hand *tsh*, *colV*, *vat*A and *ast*A genes were not present in the pAPEC isolates (Table 4, Figure 3).

In total, 140 APEC isolates had 47 virulence genotypes. Fifty-four pAPEC isolates had 17 (Table 5) genotypes while 86 cAPEC isolates had 30 virulence genotypes (Table 6).

Statistical analysis

Statistical analysis revealed that the distribution of all virulence genes between cAPEC and pAPEC strains was significantly different ($P \leq 0.05$). The high frequency (*iss*, *omp*T, *hly*F, *iro*N and iucD) of the five virulence genes was detected among the cAPEC isolates compared to pAPECs (Table 7).

Antibiotic resistance

Resistance of 140 *E. coli* strains to 9 antimicrobial drugs was investigated using agar disc diffusion method. The results showed that the isolates were at a high-level resistance to four antimicrobial drugs: 96 (68.6%) were resistant to amoxicillin, 92 (65.7%) were resistant to trimethoprim sulfamethoxazole, 90 (64.3%) were resistant to amoxicillin clavulanic acid, 88

Table 6: Virulence gene profiles of cAPEC isolates.

Number of Genotypes	Virulence Genotype	Number of virulence genes	Number of isolates
1	*hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T	5	2
2	*hyl*F, *iuc*D, *iut*A, *iro*N, *iss*	5	2
3	*iuc*D, *iut*A, *iro*N, *omp*T, *iss*	5	2
4	*ast*A, *hyl*F, *iro*N, *omp*T, *iss*	5	2
5	*hyl*F, *iut*A, *iro*N, *omp*T, *iss*	5	4
6	*tsh*, *iut*A, *iro*N, *omp*T, *iss*	5	2
7	*tsh*, *hyl*F, *iut*A, *omp*T, *iss*	5	2
8	*tsh*, *iut*A, *iro*N, *omp*T, *iss*	5	2
9	*pap*C, *hyl*F, *iut*A, *omp*T, *iss*	5	2
10	*papC*, *vat*A, *hyl*F, *iroN*, *iss*	5	2
11	*col*V, *iut*A, *iro*N, *omp*T, *iss*	5	6
12	*col*V, *hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T	6	4
13	*tsh*, *hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T	6	2
14	*hyl*F, iucD, *iut*A, *iro*N, *omp*T, *iss*	6	4
15	*pap*C, *hyl*F, *iuc*D, *iut*A, iroN, *iss*	6	2
16	*tsh*, *hyl*F, *iuc*D, *iut*A, *iro*N, *iss*	6	2
17	*ast*A, *hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T	6	2
18	*col*V, *ast*A, *iut*A, *iro*N, *omp*T, *iss*	6	2
19	*col*V, *hyl*F, *iut*A, *iro*N, *omp*T, *iss*	6	4
20	*tsh*, *pap*C, *hyl*F, *iut*A, *iro*N, *omp*T	6	2
21	*pap*C, *hyl*F, *iuc*D, *iut*A, *iro*N, ompT, *iss*	7	2
22	*col*V, *hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T, *iss*	7	4
23	*vat*A, *hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T, *iss*	7	2
24	*tsh*, *hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T, *iss*	7	6
25	*tsh*, *papC*, *hyl*F, *iuc*D, *iut*A, *iro*N, *iss*	7	4
26	*tsh*, *pap*C, *col*V, *iut*A, *iro*N, ompT, *iss*	7	4
27	*tsh*, *col*V, *hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T, *iss*	8	4
28	*tsh*, *pap*C, *col*V, vatA, *hyl*F, *iuc*D, *iut*A, *iro*N, *iss*	9	2
29	*tsh*, *pap*C, *col*V, *vat*A, *hyl*F, *iut*A, *iro*N, *omp*T, *iss*	9	4
30	*tsh*, *pap*C, *col*V, *vat*A, *hyl*F, *iuc*D, *iut*A, *iro*N, *omp*T, *iss*	10	2

(62.9%) were resistant to ciprofloxacin. Moderate level resistance to the remaining five drugs was detected: 60 (42.9%) were resistant to doxycycline, 52 (37.1%) were resistant to gentamicin, 50 (35.7%) were resistant to cephalexin, 42 (30.0%) were resistant to fosfomycin, 38 (27.1%) were resistant to florfenicol (Table 2, Figure 4).

Of the total 140 APEC isolates, 4 (2.98%) were one, 10 (7.1%) were two, 24 (17.1%) were three, 32 (22.9%) were four, 38 (27.1%) were five, 32 (22.9%) were resistant to six antibiotics; 126 (90.0%) of the isolates had multiple antibiotic resistance (Table 8, Figure 5).

DISCUSSION

Modern poultry production practices provide favorable conditions for the emergence and spread of infectious diseases. Colibacillosis is a localized or systemic infection caused by *E. coli* and is frequently reported in the country of the authors, Turkey (23) and in the world's leading poultry breeding countries (24, 25, 26, 27,28). In this study, 82.9% isolation of *E. coli* from the internal organs of broiler chickens suspected of colibacillosis showed that *E. coli* played an important role in the epidemiology of respiratory system diseases in broiler farms. In addition, 98.6% of *E. coli* isolates carried at least one virulence gene, from *E. coli* suggesting that the isolates used could be APEC. Furthermore, 17.5% of the samples VG could not be isolated for two possible reasons: First, the poultry from which these samples were taken may have used antibiotics that could inhibit the isolation of *E. coli* immediately after the onset of the disease, or the chickens examined may be suffering from a viral respiratory disease

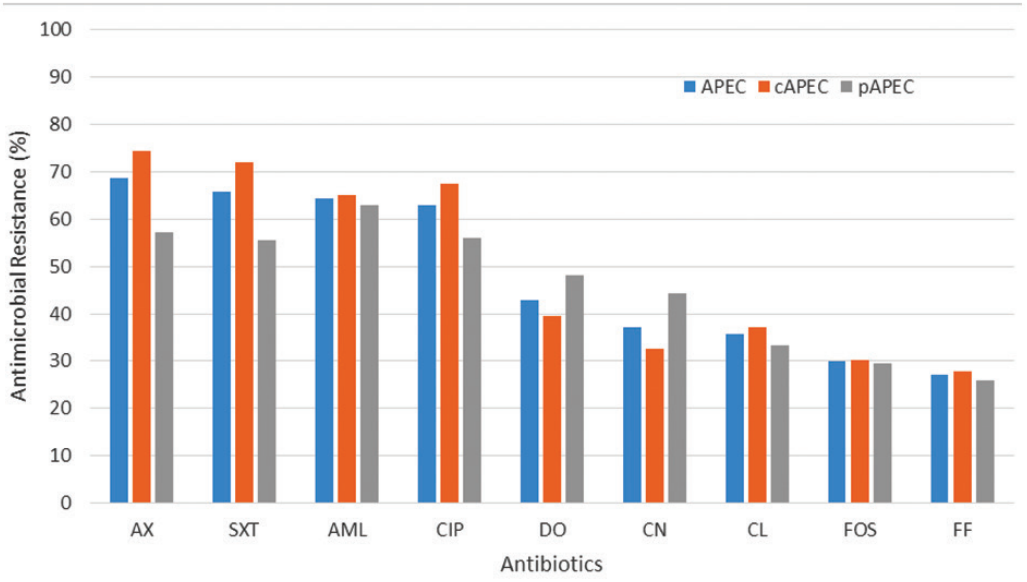

Figure 4: Antibiotic resistance rates of APEC, cAPEC and pAPEC isolates.

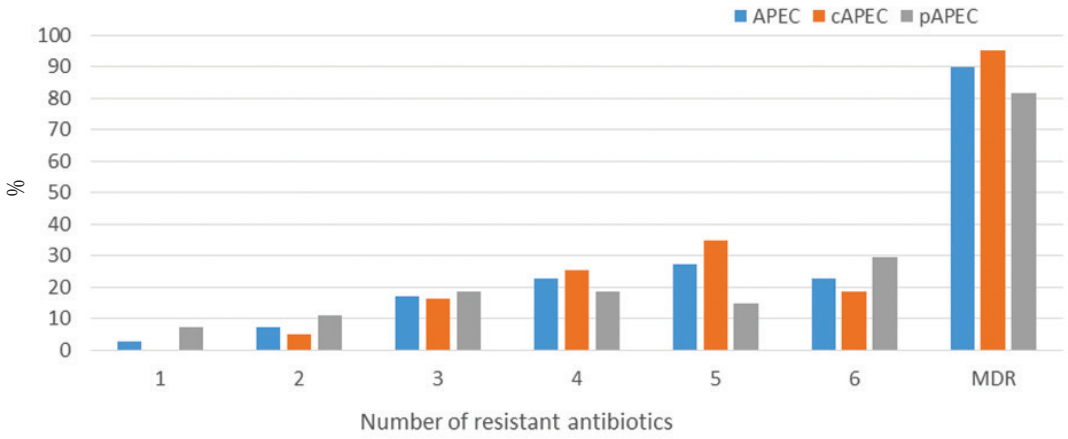

Figure 5: Comparison of antibiotic resistance rates of APEC, cAPEC and pAPEC isolates.

such as infectious bronchitis virus and Newcastle disease virus.

Bacterial iron collection systems have many functions under different conditions, and different iron transport systems can be expressed at different times in different host tissues (3,4). Iron collection systems, especially in bacteria that cause septicemia, are known to be associated with bacterial virulence (5,6,8). Moreover, iron recovery protects bacteria from host humoral immunity and the accumulation of these genes is a potential risk factor for APEC infection (29). In this study, 75.7% of the isolates had *iut*A, 68.8% *iro*N and 40% *iuc*D. This result shows the importance of iron collection systems for the bacterial pathogenesis. In fact, bacteria are dependent on iron collection systems to survive in the host (5,6,8). For these reasons, the presence of multiple iron-related genes may allow APEC to survive under different conditions.

In this study, remarkably, the *iut*A gene belonging to the aerobactin iron uptake system was identified as the main factor for regulating iron uptake in *E. coli*. The *iut*A gene is one of the five genes of the aerobactin operon. It encodes an outer membrane protein that provides high affinity binding of Fe^{3-} aerobactin, can be found on the plasmid (17) or chromosome (30) in some APEC strains. The aerobactin system

Table 7: Comparison of virulence gene distribution in cAPEC and pAPEC isolates.

Gene	APEC (n=140)		cAPEC (n=86)		pAPEC (n=54)		P	χ2
	+	–	+	–	+	–		
*iro*N	96	44	82	4	14	40	0.000***	74.183
*iut*A	106	34	82	4	24	30	0.000***	46.746
*iuc*D	56	84	48	38	8	46	0.000***	23.233
*hly*F	86	54	68	18	18	36	0.000***	29.285
*ast*A	6	134	6	80	0	54	0.047*	3.936
*vat*A	12	128	12	74	0	54	0.004***	8.241
*pap*C	30	110	26	60	4	50	0.001***	10.264
tsh	38	102	38	48	0	54	0.000***	32.75
*col*V	36	104	36	50	0	54	0.000***	30.429
iss	110	30	74	12	36	18	0.007**	7.400
*omp*T	84	56	72	14	12	42	0.000***	52.274

*P<0.05; **P<0.01; ***P<0.001.

Table 8: Antibiotic resistance status of APEC, cAPEC and pAPEC isolates.

Number of Antimicrobial Families	APEC (n=140) (%)	cAPEC (n=86) (%)	pAPEC (n=54) (%)
1	4 (2.9)	–	4 (7.4)
2	10 (7.1)	4 (4.9)	6 (11.1)
3	24 (17.1)	14 (16.3)	10 (18.5)
4	32 (22.9)	22 (25.5)	10 (18.5)
5	38 (27.1)	30 (34.8)	8 (14.8)
6	32 32 (22.9)	16 (18.5)	16 (29.7)
Number of MDR isolate	126 (90.0)	82 (95.1)	44 (81.5)

plays a role in the persistence and formation of lesions in APEC infected chickens. Current findings are consistent with other studies that found a high prevalence of the iutA gene in APEC isolates (3,8,28,29).

Secretory toxins play a key role in the activation of host biological processes by pathogenic *E. coli* (31). The *vat*A gene, which has been shown to induce cytotoxic effects in host cells, was present in 8.6% of all APEC isolates and in 14.0% of cAPEC isolates. Although this finding was not lower than in other studies, the low prevalence rate is similar to those reported (25,28,32). In this study, *ast*A gene was detected in 4.3% of all APEC isolates and in 7% of cAPEC isolates. The *ast*A gene has been shown to be present among avian fecal *E. coli* strains as compared to APEC. Therefore, it has been suggested that this gene is not related to *E. coli* virulence characteristics (24).

Adhesion of bacteria to lung cells is very important in the occurrence of colibacillosis in poultry. Pilus is the most important virulence factor in the binding of pathogenic *E. coli* to host cells. *pap*C is the main functional gene of P pilus (4,5). In this study, the *pap*C gene was detected in 21.8% of all APEC isolates and 30.2% of cAPEC isolates. This ratio is lower than those found by some researchers (26) and higher than those found by others (33). The *tsh* gene is another factor associated with adhesion. Subedi *et al.* (2018) reported that they detected this gene in 65.2% of APEC isolates. In this study, although the *tsh* gene was detected in 27.1% of all APEC isolates and 44.2% of cAPEC isolates, this gene was not detected in pAPEC isolates. This may indicate that the presence of the *tsh* gene is necessary to increase the level of pathogenicity of cAPEC. It is interesting to note that 72.9% of the isolates do not have the *tsh* gene and 78.6% do not have the *pap*C gene. This may not signify that the isolates do not contain any genes encoding the adhesion factor. In fact, there are other genes associated with adhesion which were not studied in this work. Thus, it is possible that such isolates contain some of the adhesion factors that have not been studied.

In this study, the *iss* gene was positive in 67.7% of pAPEC isolates and 86% of cAPEC isolates. In a previous study, it was reported that *iss* were significantly associated with APEC strains compared to non-pathogenic strains and that the strain could be an indicator of the potential for causing disease (34). Three alleles of the *iss* gene are known; two are chromosomal and one is found in ColV virulence plasmids (34). Thus, the *iss* gene may play a vital role in the pathogenicity of *E. coli* and may be a potential target for developing disease prevention strategies.

The *omp*T gene encodes an episomal outer membrane protease that cleaves colicins (35). In this study, 83.7% of *omp*T gene cAPEC isolates were detected. Other studies on APEC have shown higher detection rates of this gene (3, 8, 28).

Mobile genetic elements such as islands of pathogenicity and some plasmids harboring certain genes contribute to

bacterial virulence. In this study, the *ColV* plasmid gene was detected in 41.9% of cAPEC isolates. In previous studies, it has been reported in rates ranging from 27.8% to 57.8% (24, 26).

Antibiotic resistance varies between countries and even regions, because the different use of antimicrobial drugs in each geographical region. Penicillin, folate pathway inhibitors, tetracycline, quinolones, sulfonamides are frequently used in the treatment of *E. coli* infections in broilers (36). In some studies, it has been reported that antibiotic resistant bacteria isolated from animals can be transmitted to humans through direct contact, meat or contamination (37). The general concern is that antibiotics administered in animals will affect conditions for therapeutic benefit in humans. In this study, none of the nine antibiotics examined showed a 100% effect against APEC isolates. APEC isolates were found to be resistant to amoxicillin (highest) (68.6%) and (27.1%) florfenicol (lowest) . The resistance of APEC isolates studied was high against trimethoprim sulfamethoxazole, amoxicillin clavulanic acid and ciprofloxacin whereas to doxycycline, gentamicin, cephalexin, phosphomycin and florfenicol were moderate. These antibiotic resistance rates of *E. coli* strains are similar, although slightly lower than previous reported (23, 26).

MDR pathogens, which cause treatment problems in both animal and human diseases are, a major public health problem. In this study, 90.0% of APEC isolates (95.1% of cAPEC isolates and 81.5% of pAPEC isolates) were MDR. The prevalence of MDR in APEC has also been reported in studies in Bangladesh (38) and Nepal (26). This is a strong indicator of non-discriminatory and poor antibiotic administration for prophylaxis or infection. It is important to control MDR in *E. coli* because these strains are a potential source for transferring multidrug resistant genes to human specific *E. coli* or other bacteria such as *Staphylococcus aureus* and *Shigella* strains. Therefore, they pose a serious danger to both humans and other animals (21). Since this pathogen can be taken up with food consumed on a daily basis, it should also be considered as a serious public health and food biosecurity problem.

In this study, it has been proposed that having multiple iron transport systems can play an important role in the activity of APEC and the presence of virulence genes based diagnostic methods in which virulence genotypes are identified may be beneficial for future vaccination studies. The importance of virulence genes in *E. coli* isolates can be better understood by examining the expression of these genes *in vivo* and *in vitro*. In particular, regular examination of the virulence genes of multiple antibiotic resistant APEC strains is necessary to implement a conservation program to reduce the risk of colibacillosis.

ACKNOWLEDGEMENT

This manuscript was compiled from the first author's Master Thesis, supported by Aydin Adnan Menderes University Scientific Research Projects Unit (Project Number: VTF-19006) and the authors would like to thank to Prof. Dr. Bulent Bozdogan (Aydin Adnan Menderes University, Medical Faculty, Department of Medical Microbiology, Aydin, Turkey) and Merve Engin KURT (Ege University, Faculty of Letters, Department of Translation and Interpreting Studies, Izmir, Turkey) for help and support.

REFERENCES

1. Dziva, F. and Stevens, M.P.: Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol. 37:355-366, 2008.
2. Ewers, C., Li, G., Wilking, H., Kiessling, S., Alt, K., Antao, E.M., Laturnus, C., Diehl, I., Glodde, S., Homeier, T., Bohnke, U., Steinruck, H., Philipp, H.C. and Wieler., L.H.: Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? Int. J. Med. Microbiol. 297:163-176, 2007.
3. Rodriguez-Siek, K.E., Giddings, C.W., Doetkott, C., Johnson, T.J. and Nolan, L.K.: Characterizing the APEC pathotype. Vet. Res. 36: 241-256, 2005.
4. Nolan, L.K., Barnes, H., Jean Pirre, V., Abdul-Aziz, T. and Louge, C.M.: Colibacillosis. In Diseases of Poultry. D. Swayne, E. ed. John Wiley and Sons, Ames, Iowa, pp. 751-785, 2013.
5. Janben, T., Schwarz, C., Preikschat, P., Voss, M., Philipp, H.C. and Wieler, L.H.: Virulence-assocated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. Int. J. Med. Microbiol. 291:371-378, 2001.
6. Ewers, C., Janssen, T., Kiessling, S., Philipp, H.C. and Wieler, L.H.: Rapid detection of virulence-associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. Avian Dis. 49: 269-273, 2005.
7. De Carli, S., Ikuta, N., Lehmann, F.K., da Silveira, V.P., de Melo Pedrebon, G., Fonseca, A.S. and Lunge, V.R.: Virulence gene content in *Escherichia coli* isolates from poultry flocks with clinical signs of colibacillosis in Brazil. Poult. Sci. 94: 2635-2640, 2015.
8. Johnson, T.J., Wannemuehler, Y., Johnson, S.J., Stell, A.L., Doetkott, C., Johnson, J.R., Kim, K.S., Spanjaard, L. and Nolan, L.K.: Comparison of extraintestinal pathogenic *Escherichia coli* strains from human and avian sources reveals a mixed subset representing potential zoonotic pathogens. Appl. Environ. Microbiol. 74:7043-7050, 2008.

9. Aarestrup, F.M.: Monitoring of antimicrobial resistance among food animals: principles and limitations. J. Vet. Med. B Infect. Dis. Vet. Public Health 51:380-388, 2004.
10. Quinn, P.J., Markey, B.K., Leonard, F.C., FitzPatrick, E.S., Fanning, S. and Hartigan, P.J.: Veterinary Microbiology and Microbial Disease. Second Edition, Blackwell Science Ltd., Oxford, UK, 2011.
11. Clinical and Laboratory Standards Institute, 2012. Supplement M100-S22, 32, 3.
12. Bywater, R., Silley, P. and Simjee, S.: Antimicrobial breakpoints – definitions and conflicting requirements. Vet. Microbiol. 118: 158-159, 2006.
13. Maniatis, T. and Sambrook, J.: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, USA, 1989.
14. Aggarwal, S.: Techniques in Molecular Biology. Lucknow: International Book Distributing CO. Short tandem repeat genotyping; pp. 127-34, 2008.
15. Chen, J. and Griffiths, N.W.: PCR differentiation of *Escherichia coli* from other Gram-negative bacteria using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein. Lett. Appl. Microbiol. 27: 369-371, 1998.
16. Johnson, T.J., Wannemuehler, Y., Doetkott, C., Johnson, S.J., Rosenberger, S.C. and Nolan, L.K.: Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46: 3987-3996, 2008.
17. Johnson, T.J., Sie, K.E., Johnson, S.J. and Nolan, L.K.: Complete DNA sequence of a ColBM plasmid from avian pathogenic *Escherichia coli* suggests that it evolved from closely related ColV virulence plasmids. J. Bacteriol. 188, 5975-5983, 2006.
18. Janssen, T., Schwarz, C., Preikschat, P., Voss, M., Philipp, H.C. and Wieler, L.H.: Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. Int. J. Med. Microbiol. 291: 371-378, 2001.
19. Morales, C., Lee, M.D., Hofacre, C. and Maurer, J.J.: Detection of a novel virulence gene and a *Salmonella* virulence homologue among *Escherichia coli* isolated from broiler chickens. Foodborne Pathol. Dis. 1: 160-165, 2004.
20. Ewers, C., Janssen, T., Kiessling, S., Philipp, H.C. and Wieler, L.H.: Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticemia in poultry. Vet. Microbiol. 104: 91-101, 2004.
21. Ewers, C., T. Janssen, S. Kiessling, H. C. Philipp, and L. H. Wieler.: Rapid detection of virulence-associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. Avian Dis. 49:269-273, 2005.
22. Johnson, T.J., Siek, K.E., Johnson, S.J. and Nolan, L.K.: Complete DNA sequence of a ColBM plasmid from avian pathogenic *Escherichia coli* suggests that it evolved from closely related ColV virulence plasmids. J. Bacteriol. 188, 5975-5983, 2006.
23. Ocak, F., Çöven, F., Ertunç, E., Eğilmez, T. and Türkyılmaz, S.: Determination of the most significant serotypes and antimicrobial susceptibilities of avian pathogenic *Escherichia coli* isolates in Turkey. EPS. 82, 1-12, 2018.
24. Mohamed, M.A., Shehata, M.A. and Rafeek, E.: Virulence genes content and antimicrobial resistance in *Escherichia coli* from broiler chickens. Vet. Med. Int. 1–6, 2014.
25. Vhm, L., Serrano, I.Q., Delgado, P.D.P.M., Lev, R., Olague-Marchan, M., Shs, R., Mal, L., Af, D.T. and Rmr, S.: Genes of virulence and phylogenetic group in isolates of avian pathogenic *Escherichia coli*. Archives of Medicine. 9, 1-5, 2017.
26. Subedi, M., Luitel, H., Devkota, B., Bhattarai, R.K., Phuyal, S. and Panthi, P.: Antibiotic resistance pattern and virulence genes content in avian pathogenic *Escherichia coli* (APEC) from broiler chickens in Chitwan, Nepal. BMC Vet. Res. 14: 113, 2018.
27. Azam, M., Mohsin, M., Rahman, S. and Saleemi, M.K.: Virulence-associated genes and antimicrobial resistance among avian pathogenic *Escherichia coli* from colibacillosis affected broilers in Pakistan. Trop. Anim. Health Pro. 51; 1259-1265, 2019.
28. Mbanga, J. and Nyararai, Y.O.: Virulence gene profiles of avian pathogenic *Escherichia coli* isolated from chickens with colibacillosis in Bulawayo, Zimbabwe. Onderstepoort J. Vet. Res. 82:1-8, 2015.
29. Paauw, A., Leverstein-van Hall, M. A., van Kessel, K. P., Verhoef, J. and Fluit, A. C.: 2009. Yersiniabactin reduces the respiratory oxidative stress response of innate immune cells. PLoS ONE. 4:1–9
30. Schouler, C., Schaeffer, B., Brée, A., Mora, A., Dahbi, G., Biet, F., Oswald, E, Mainil, J., Blanco, J. and Moulin-Schouleur, M.: Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. J. Clin. Microbiol. 50, 1673–1678, 2012,
31. Qadri, F., Svennerholm, A.M., Faruque, A.S. and Sack, R.B. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin. Microbiol. Rev. 18, 465-83, 2005.
32. Kwon, S.G., Cha, S.Y., Choi, E.J., Kim, B., Song, E. and Jang, HK: Epidemiological prevalence of avian pathogenic *Escherichia coli* differentiated by multiplex PCR from commercial chickens and hatchery in Korea. J. Bacteriol. Virol. 38: 179-188, 2008.
33. Roseliza, R., Khairani-Bejo, S., Zunita, Z., Ramlan, M. and Khoo, E.: Phylogenetic grouping and virulence gene profiles of *Escherichia coli* isolated from chicken. MJVR. 8, 65-74: 2017.
34. Johnson, T.J., Wannemuehler, Y.M. and Nolan, L.K.: Evolution of the *iss* gene in *Escherichia coli*. Appl. Environ. Microbiol. 74: 2360-2369, 2008.
35. Cavard, D. and Lazdunski, C.: Colicin cleavage by *omp*T protease during both entry into and release from *Escherichia coli* cells, J. Bacteriol. 172: 648-652, 1990.
36. Zhao, S., Maurer, J.J., Hubert, S., De Villena, J.F., McDermott, P.F., Meng, J., Ayers, S., English, L. and White, D.G.: Antimicrobial susceptibility and molecular characterization of avian pathogenic *Escherichia coli* isolates. Vet. Microbiol. 107: 215-224, 2005.
37. Woolhouse, M. and Farrar, J.: An intergovernmental panel on antimicrobial resistance. Nature. 509: 555-557, 2014.
38. Matin, M.A., Islam, M.A. and Khatun, M.M.: Prevalence of colibacillosis in chickens in greater Mymensingh district of Bangladesh. Vet. World. 10: 29-33, 2017.