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Antibacterial Resistance and Virulence Potential of Avian Colibacillosis-Causing *Escherichia coli* Isolates

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ABSTRACT

Backgrounds: This study was done to evaluate the distribution of virulence-associated genes and antibiotic resistance in avian colibacillosis-causing *Escherichia coli* (*E. coli*) isolates.

Materials & Methods: In this study, 122 *E. coli* strains isolated from colibacillosis-suspected chickens in commercial broiler poultry farms (Guilan province, Iran) were examined for the presence of 12 virulence genes (*hly*F, *iro*N, *iss, iut*A, *omp*T, *ast*A, *tra, sfa-foc, pap*C, *fim*H, *cvi/cva,* and *Tia*) using polymerase chain reaction (PCR). Antimicrobial susceptibility assessment was performed for the isolates using disc diffusion method against 19 antibiotics.

Findings: The *fim*H, *iut, tra, iss, iro*N, *hly,* and *omp*T genes were detected as the most prevalent genes among colibacillosis-causing isolates (more than 70%), while *sfa-foc* (S fimbriae and F1C fimbriae subunits) had the lowest frequency among colibacillosis-causing *E. coli* isolates (3.28%).

Conclusion: Virulence-associated genes were frequently detected in avian pathogenic *E. coli* strains. These findings could help better understand the pathogenicity potential of *E. coli* in poultry. Preventative measures are necessary to reduce food and environmental contamination with avian *E. coli* strains.

Keywords: *Escherichia coli,* Colibacillosis, Virulence potential, Poultry.

CITATION LINKS

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Introduction

Escherichia coli (*E. coli*) is part of the intestinal flora of healthy broilers, but some strains of this bacterium, designated as avian pathogenic *E. coli* (APEC), are the causative agent of avian colibacillosis. Avian colibacillosis is mainly characterized by airsacculitis and respiratory problems in broiler chickens, leading to systemic infection. This disease is considered as the major cause of production reduction at all ages and economic losses in many poultry farms worldwide [1, 2].

Furthermore, APEC isolates are genetically similar to human extraintestinal pathogenic *E. coli* (ExPEC) strains, including uropathogenic *E. coli* (UPEC). They could potentially be transmitted to humans and cause extraintestinal infections, particularly through the food chain or direct contact with birds and their feces [1, 3, 4]. ExPEC has a complex phylogenetic structure and carries a wide range of virulence factors, including P fimbrial usher *(pap*C, *pap*G), extracellular adhesive fimbriae (*ecp*), salmochelin siderophore receptor *(iro*N, *fyu*A), aerobactin siderophore receptor (*iut*A), episomal outer membrane protein (*omp*T, *ompTp, tsh*), hemolysin (*hly*A), and serum resistance-associated protein (*iss*). Virulence genes of these proteins may be present in pathogenicity islands (PAIs) or plasmids [4, 5]. However, the distribution of these genes could be different in the UPEC and APEC groups of ExPEC. While a set of virulence features including adhesion-related genes (*crl*, *fim*C, and *mat*) are detected in almost all APEC and UPEC strains regardless of their pathotype, other virulence factors such as *hra*, *pap*C, and plasmid-related genes *cvi/cva*, *sit*D ep., *iuc*D, *iss*, *tra*T, and *iro*N are frequently detected in APEC. On the other hand, *iha*, *sfa/foc*, and *afa/dra*B genes are more prevalent in UPEC than in APEC [6].

APEC strains could be a reservoir of virulence genes of ExPEC strains pathogenic to humans. Some human extraintestinal pathogenic *E. coli* strains have the *iss* gene in their genome on the CoIV plasmid, which a huge virulence plasmid typical of avian pathogenic *E. coli* strains. This indicates that the exchange of plasmids and consequently these virulence genes is possible between APEC and UPEC strains [4].

Furthermore, the excessive use of antibiotics in animal husbandry for the treatment and prevention of infectious diseases or as growth promoters contributes to the emergence and dissemination of antibiotic-resistant bacteria [7, 8]. The most common transmission route of multidrug-resistant bacteria to humans is through the food chain [9]. Therefore, commensal and pathogenic *E. coli* strains containing various antimicrobial resistance genes entail a high risk of transmission of drug resistance to human microflora and other pathogenic bacteria [10, 11]. Antibiotic-resistant *E. coli* strains become even more alarming and life threatening if they are pathogenic. In addition, the acquisition of resistance determinants may represent an advantage for the survival of virulent *E. coli* strains. Virulence genetic determinants, if located on the same genetic platform as antimicrobial resistance genes, may be co-transferred under antimicrobial selective pressure, resulting in prolonged illness, greater risk of death, and higher chance of dissemination of resistant microorganisms [12].

Objectives: Surveillance of poultry-isolated *E. coli* is necessary for reducing their health risk and preventing infection. Although several studies have been performed on the virulence potential and antibiotic resistance of *E. coli* bacteria in different parts of Iran, data in Guilan province are very limited. Accordingly, this study aimed to investigate antibacterial resistance and the presence of virulence-associated genes in avian colibacillosis-causing *E. coli* strains in Guilan province, northern Iran.

Materials and Methods

Sampling and *E. coli* isolation: In this study,

122 *E. coli* strains were isolated from heart and liver samples collected from 20 to 45-day-old poultry with characteristic lesions consistent with colibacillosis in commercial broiler poultry farms in Guilan province (northern Iran) during 2020. The collected samples were transferred to MacConkey (MC) and Eosin Methylene Blue (EMB) agar media, and purification was performed according to standard microbiological and biochemical methods such as growth on triple sugar iron agar (TSI) and lysine iron agar (LIA). For oxidative/fermentative degradation of glucose, citrate utilization, urease production, indole production, glucose degradation (methyl red test), and motility tests were used [13].

Antimicrobial susceptibility testing: *E. coli* isolates were subjected to antimicrobial susceptibility testing to ceftriaxone (30 µg), cephalexin (30 µg), cefixime (5 µg), ceftazidime (30 µg), imipenem (10 µg), gentamicin (10 µg), amikacin (30 µg), neomycin sulfate (30 µg), fosbac (200 µg), fosfomycin (200 µg), doxycycline (10 µg), cotrimoxazole (10 µg), Chlortetracycline (10 µg), Ciprofloxacin (5 µg), Enrofloxacin (5 µg), Danofloxacin (10 µg), flumequine (30 µg), florfenicol (30 µg), and linco-spectin (10 µg), purchased from High Media-India, by disc diffusion method on Mueller-Hinton agar (Merck, Germany) according to the CLSI VET01 protocols (2018). *E. coli* ATCC 25922 was included for quality control, and breakpoints were in accordance with CLSI guidelines.

Polymerase chain reaction (PCR) screening for virulence-associated genes: One colony of each bacterial strain was purified, and bacterial genomic DNA was extracted using a GenElute bacterial genomic DNA kit (Sigma-Aldrich, St Louis, MO). The presence of 12 virulence-associated genes, including *hly*F, *iro*N, *iss, iut*A, *omp*T, *ast*A, *tra, sfa-foc, pap*C, *fim*H, *cvi/cva,* and *Tia*, was assessed using PCR as described previously [6, 14-16]. PCR reactions (25 µL) were performed using 2 U of Taq polymerase, 2.5 µL of 10X buffer, 10 mM dNTPs, 20 pmol of each primer, 4 mM $MgCl_2$, and 4 µL of the sample DNA. PCR products were analyzed using agarose gel electrophoresis (1%). Table 1 shows the sequences of oligonucleotide primers used in this study.

Findings

***E. coli* isolation and antimicrobial susceptibility testing:** In total, 122 *E. coli* isolates were obtained from colibacillosis-suspected chickens.

Table 2 shows the results of antibacterial susceptibility testing of *E. coli* isolates to common antibiotics. The highest resistance of colibacillosis-causing *E. coli* strains was related to enrofloxacin, chlortetracycline, and flumequin (more than 90%). No resistance was observed to imipenem and ceftriaxone among the tested isolates, and resistance to gentamicin, amikacin, cephalexin, cefixime, and ceftazidime was low.

Frequency of virulence-associated genes: The frequency of virulence-associated genes and their distribution among colibacillosis-causing *E. coli* isolates are shown in Table 3. Virulence-associated genes *fim*H, *iut, tra, iss, iro*N, *hly,* and *omp*T were identified as the most prevalent genes in colibacillosis-causing isolates (more than 70%). *Sfa-foc* (S fimbriae and F1C fimbriae subunits) had the lowest frequency among colibacillosis-causing *E. coli* isolates (3.28%).

The correlation between the presence of virulence factors and antibiotic resistance: Most virulence-associated genes were predominant in antibacterial-resistant strains. The *hly-f* gene was the most prevalent virulence gene among antibiotic-resistant isolates. Most *fim*H and *pap*C-positive isolates were antibiotic-resistant. The *sfa-foc* gene was observed only in fluoroquinolone- and sulfonamide-resistant groups. Among the 12 virulence genes investigated, ten genes were detected in cephalexin- and cefixime-resistant isolates, and five genes including

Table 1) Nucleotide sequences of primers used in this study, amplicon size (bp), and specific PCR program for amplification of each gene

Target Gene	Function	Primer Sequence (5′-3′)	Cycles Temp / Time (Seconds) 35-40 Cycles					Amplicon Size (bp)	References
			Initial Denaturation °c/min	Denaturation	Annealing	Extension	Final Extension °c/min		
iroN-F	Salmochelin siderophore receptor gene	AATCCGGCAAAGAGACGAACCGCCT	94/4min	94/30S	63/30S	72/45S	72/10min	553	15
iroN-R		TTCGGGCAACCCCTGCTTTGACTTT							
Hly-f-F	Putative avian hemolysin	GGCCACAGTCGTTTAGGGTGCTTACC	94/4min	94/30S	63/30S	72/45S	72/10min	450	15
Hly-f-R		GGCGGTTTAGGCATTCCGATACTCAG							
iss-F	Episomal increased serum survival gene	CAGCAACCCGAACCACTTGATG	94/4min	94/30S	63/30S	72/45S	72/10min	323	15
iss-R		AGCATTGCCAGAGCGGCAGAA							
Ompt-F	Episomal outer membrane protease gene	TCATCCCGGAAGCCTCCCTCACTACTAT	94/4min	94/30S	63/30S	72/45S	72/10min	496	15
Ompt-R		TAGCGTTTGCTGCACTGGCTTCTGATAC							
ast A -F	EAST1 (heat-stable cytotoxin associated with enteroaggregative E. coli)	TGCCATCAACACAGTATATCC	94/4min	94/30S	55/30S	72/40S	72/10min	116	16
ast A -R		TAGGATCCTCAGGTCGCGAGTGACGGC							
Tra-F	Transfer protein	GTGGTGCGATGAGCACAG	94/4min	94/30S	58/30S	72/40S	72/10min	290	6
Tra-R		TAGTTCACATCTTCCACCATCG							
Sfa-foc-F	S fimbriae (sialic acid-specific) and F1C fimbriae	CTCCGGAGAACTGGGTGCAT CTTAC	94/4min	94/30S	63.5/30S	72/45S	72/10min	410	14
Sfa-foc-R		CGGAGGAGTAATTACAAACCTGGCA							
Pap C-F	Pilus associated with pyelonephritis	GTGGCAGTATGAGTAATGACCGTTA	94/4min	94/30S	63.5/30S	72/40S	72/10min	200	14
Pap C-R		ATATCCTTTCTGCAGGGATGCAATA							
Fim H-F	Type 1 fimbriae (D-mannose-specific adhesin)	TGCAGAACGGATAAGCCGTG	94/4min	94/30S	63.5/30S	72/45S	72/10min	508	14
Fim H-R		GCAGTCACCTGCCCTCCGGT							
CVI/Cva-F	Structural genes of colicin V operon (microcin ColV)	TCCAAGCGGACCCCTTATAG	95/4min	94/30S	58/30S	72/50S	72/10min	679	6
CVI/Cva-R		CGCAGCATAGTTCCATGCT							
Iut-A-F	Aerobactin siderophore receptor gene	*GGCTGGACATCATGGGAACTGG*	94/4min	94/30S	63/30S	72/40S	72/10min	302	15
Iut-A-R		*5′- 5′- GGCTGGACATCATGGGAACTGG*							
Tia-F	Toxigenic invasion locus in ETEC strains	5′- 5AGCGCTTCCGTCAGGACTT	94/4min	94/30S	58/30S	72/45S	72/10min	512	6
Tia-R		ACCAGCATCCAGATAGCGAT							

Table 2) Antimicrobial resistance properties in AFEC isolates

Antibiotic	Sensitive Isolates (APEC Group) N=122 N(%)	Intermediate Isolates (APEC Group) N=122 N(%)	Resistant Isolates (APEC Group) N=122 N(%)
Amikacin	100 (82)	22 (18.03)	0(0)
Gentamicin	118(96.72)	3 (2.46)	1(0.82)
Neomycin Sulphate	37 (30.33)	50 (40.98)	35 (28.68)
Ciprofloxacin	18(14.75)	62 (50.82)	42(34.43)
Flumequin	2 (1.64)	7 (5.74)	113 (92.62)
Enrofloxacin	0 (0)	14 (11.47)	118 (96.72)
Danofloxacin	13 (10.66)	21 (17.21)	88 (72.13)
Co-trimaxazol	8 (6.58)	8 (6.58)	106 (86.88)
Chlortetracycline	2 (1.64)	3 (2.46)	117 (95.90)
Doxicyclin	26 (21.31)	12 (9.84)	84 (68.85)
Florphenicol	13 (10.66)	11 (9.02)	98 (80.33)
Lincospectin	103(84.43)	11 (9.1)	8 (6.56)
Imipenem	120(98.4)	2 (1.64)	0(0)
Cephalexine	89(73)	26 (21.31)	7(5.73)
Cefixime	21(17.21)	95 (78)	6(4.92)
Ceftazidime	119(97.54)	1 (0.82)	2(1.64)
Ceftriaxon	117 (96)	5 (4.1)	0(0)
Fosbac	98(80.32)	6 (4.92)	18(14.75)
Fosfomycin	80(65.57)	6 (4.92)	36(29.51)

hly-f, *iss*, *pap*C, *cvi/cva*, and *iut*A were detected in ceftazidime-resistant isolates.

Table 3) Distribution of virulence associated genes in APEC strains

Gene	Frequency, N=122 N(%)
iroN	96(78.69)
Hly-f	72.95))89
iss	97 (79.51)
ompT	88 (72.13)
astA	63 (51.64)
tra	103 (84.43)
Sfa-foc	4(3.28)
papC	60(49.18)
fimH	122(100.00)
CVI/Cva	76 (62.29)
Iut-A	105 (86.06)
Tia	71 (58.2)

Discussion

APEC causes a variety of diseases and significant economic losses in the poultry industry worldwide. The expression of several virulence factors is responsible for the pathogenicity of APEC strains. In this study, the prevalence of 12 different virulence-associated genes was investigated in *E. coli* strains isolated from colibacillosis-suspected samples. Virulence-associated genes *fim*H, *iut, tra, iss, iut, iro*N, *hly,* and *omp*T were identified as the most prevalent genes in these isolates (more than 70%). Previous studies have found significant associations between these virulence genes and APEC strains [3, 15, 17]. In the present study, the prevalence rates of *iut*A, *hly*F, *iro*N, *omp*T, and *iss* virulence genes among *E. coli* isolates were much higher than those reported in a

previous study on *E. coli* isolates collected from colibacillosis cases in broiler poultry farms in Alborz, Tehran, and Golestan provinces in Iran [18].

Studies have shown that the expression of surface adhesion factors and close contact between bacteria and the host cell wall increase the virulence of *E. coli* and help bacteria colonize infected host cells [4]. In the present study, the *fim*H gene had the highest frequency among the tested *E. coli* isolates. Consistent with this finding, Stromberg et al. (2017) identified the *fim*H gene in 90% of ExPEC isolates [19]. Another study showed that *fim*H was present in 61.7% of ExPEC isolates collected from chickens in southwestern Iran [20]. The prevalence of type 1 fimbrial adhesion gene (*fim*H) in APEC isolates in a study in southeastern Iran was 66.66%, and the *pap* gene was detected in 63.85% of these isolates [21].

The *omp*T gene promotes the formation of bacterial communities during APEC infection, and more than 80% of APEC isolates carry this gene, suggesting the essential role of this gene in APEC infection [22]. In this study, the prevalence of *omp*T was relatively high among the tested bacteria (72.13%). The *omp*T gene encodes an outer membrane protease (OmpT) that activates plasminogen to plasmin and plays an important role in protein degradation [23]. The *hly*F gene is often found in APEC isolates and expressed during extraintestinal infection. This gene induces the formation of outer membrane vesicles (OMV) which are involved in the transmission of bacterial virulence factors and promote the pathological process in the infected host [24]. In this study, the *hyl*F gene was identified in 72.95% of APEC isolates. The frequency of *hly*F in this study was lower than that previously reported by Li and colleagues (2015) [3]. However, the present study findings corroborated the results of other studies, which identified the *hly*F gene in most APEC strains and suggested it as a virulence marker associated with mammalian species and considered its presence in birds as an indicator of interspecies barrier transposition [24, 25].

The other virulence genes investigated, including *iro*N and *iut*A, encode outer membrane receptors for iron and siderophore aerobactin, respectively. In this study, these genes had high frequencies of 78.69 and 86.06%, respectively. Previous studies have shown that the prevalence of these genes is higher in APEC isolates compared with commensal *E. coli* [15, 26, 27]. The Tia protein contributes to invasive phenotypes and adhesion, and the *ast* gene encodes EAST1 protein, which is often compared with *E. coli* STa enterotoxin [28]. These genes were also detected in more than 50% of APEC strains in this study. Furthermore, in the present study, *sfa-foc* was identified in 3.28% of APEC isolates. S fimbria encoded by *sfa* operon is a common virulence factor among APEC and ExPEC strains. *E. coli sfa+* strains are very important since S fimbriae is associated with the pathogenesis of urinary infections, meningitis, and septicemia in human patients [25]. The low prevalence of *sfa-foc* in the present study was consistent with the findings of another study on *E. coli* isolates collected from pathological conditions of broiler chickens in poultry slaughterhouses in southeastern Iran [21].

Also, *cvi/cva,* as structural genes of colicin V operon (microcin ColV), was detected in more than 62.29% of APEC isolates. The frequency of this virulence gene was comparable to the literature. In a recently published study in Italy, the *cvi/ cva* gene was detected in 16 (31%) out of 51 *E. coli* isolates [29]; also, Subedi et al. (2018) reported a prevalence of 57.8% for the *cvi/ cva* virulence gene in APEC isolates collected from broiler chickens in Nepal [30].

Based on the results of antimicrobial susceptibility testing in the present study, *E. coli* isolates showed high drug resistance to at least two categories (quinolones and tetracycline) of antimicrobial agents. Among the 19 antimicrobial agents tested, APEC isolates showed the highest resistance to quinolones, such as flumequine (92.62%) and enrofloxacin (96.72%), as well as chlortetracycline (95.90%). Quinolones and tetracyclines are widely used to fight poultry pathogens in Iran [31, 32]. Flumequine is a broad-spectrum veterinary antibiotic that is widely used for prevention and treatment worldwide. Therefore, there are several reports of bacterial resistance to these antibacterial agents from all over the world [32-35]. Also, the high resistance rate to tetracyclines could be explained by the common use of oxytetracycline as a feed additive in commercial broiler rearing. On the other hand, less common antibiotics such as third-generation cephalosporins were found to be highly effective against *E. coli* isolates. These findings confirm that the incidence of antimicrobial resistance in *E. coli* isolates depends on the frequency of antibiotic use as feed additives or for the treatment of diseases in poultry.

In this study, APEC isolates carried many virulence factors. Some virulence-related genes were predominantly associated with antibiotic resistance, which could cause a serious public health problem. The *hly-f* gene was found to be the most prevalent virulence gene among antibiotic-resistant isolates. Yazdanpour and co-workers (2020) also reported *hly*A as the most prevalent virulence gene among highly resistant community-acquired uropathogenic *E. coli* isolates [36].

Also, adhesin-encoding genes *fim*H and *pap*C were abundantly detected in antibiotic-resistant isolates in this study. In accordance with this result, some studies have shown that the *pap*C gene is a predictor of antibiotic resistance in uropathogenic *E. coli* isolates [36, 37]. Co-spreading of virulence factors and resistance genes indicates the presence of both groups of genes on the same transferable elements and horizontal gene transfer between bacteria.

Conclusion

The prevalence of virulence-associated genes and antibacterial resistance was high in colibacillosis-causing *E. coli* strains. Preventative measures are necessary to reduce food and environmental contamination with avian *E. coli* strains.

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Conflicts of interests: The authors declare that there is no conflict of interest.

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