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Molecular Characterization of Virulence Genes among MDR and XDR Avian Pathogenic *E. coli*

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Abstract

One of the most costly diseases is avian colibacillosis. Virulence genes determine *E. coli* pathogenicity. This study was undertaken to explore the existence of some virulence-associated genes and resistant configurations of *Escherichia coli* recovered from broiler chicks. Thirteen *E. coli* isolates were exposed to an investigation of antimicrobial susceptibility profile against 17 antimicrobial agents that exhibited the highest resistance found against amoxicillin, florfenicol, penicillin, amoxicillin clavulanate, tetracycline, meropenem, sulfamethoxazole-trimethoprim, and chloramphenicol in the percentage of 100%, 100%,100%, 92.3%, 76.9%, 69.2%, 61.5%, and 61.5%, respectively while the isolates exhibited highest sensitivity found to fosfomycin, imipenem, azetronam and ciprofloxacine in the percentage of 100%,92.3%,76.9% and 69.2%, respectively. Moreover, the thirteen *E. coli* isolates were exposed to the revealing of some virulence genes (*iss*, *ompT*, *hlyF*, *iroN*, *iutA*, *iucD*, *papC*, *cva*, *astA*, *tsh*, and *irp2*) by polymerase chain reaction (PCR). The results showed that the percentages rates were 84.6, 76.9, 76.9, 76.9, 61.5, 53.8, 38.4, 30.7, 23, 15.3 and 15.3%, respectively. A significant correlation between most antimicrobial-resistant phenotypes and virulence genes in *E. coli* isolates. Antimicrobial use in chickens should be reasonable to prevent antibiotic-resistant microorganisms, according to our findings.

KEYWORDS
Antimicrobial resistance, Broiler Chicks, MDR, *E. coli* , Virulence associated Genes, XDR.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) has a worldwide economic impact. Most broiler chicken illnesses were APEC-caused early and late systemic bacterial infections. It is parted into intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli* (ExPEC) by the infection site (Algammal *et al*., 2022). Avian pathogenic *E. coli* (APEC) strains belonging to the ExPEC group and regarded as the most pathogen-caused morbidity and mortality in chickens including respiratory symptoms, yolk sac infection, enteritis, arthritis, omphalitis, swollen-head syndrome, coli granuloma, salpingitis and oophritis (Mellata, 2013). At the age of 3-6 weeks in broiler chickens, Most colibacillosis forms start as a respiratory illness and progresses systemically (Mellata, 2013).

One of the primary challenges encountered by the poultry sector in Egypt is the occurrence of early embryonic mortality in chicks during the first few days of their life, particularly when attributed to antimicrobial-resistant Gram-negative bacteria (Iraqi *et al*., 2021)

The main uses for antibiotics are treating and preventing microbial infections in both people and animals. However, the frequent antibiotics using may result in the development of antibiotic resistance in humans and animals (Algammal *et al*., 2020). In order to maintain the efficacy of antibiotics and lessen the chance that AMR-bacteria or antibiotic residues will infiltrate the food supply maintain the efficacy of antibiotics and lessen the chance that AMR-bacteria or antibiotic residues will infiltrate the food chain, veterinarians, industry/commodity organizations, and the government collaborate on the practical antibiotics using in food animals (Hayati *et al*., 2019; El-Tarabili *et al*., 2023). Antimicrobials were classified by WHO as being either critically important, highly important, or important due to their significance for human treatment in various parts of the world (WHO, 2012). Antibiotic-resistant infections are challenging to treat, occasionally impossible to cure, and sometimes fatal (Alaali and Thani, 2020).

The genes encoding virulence factors that distinguish APEC from normal flora were horizontally transferred into APEC, along with other pathogenic *E. coli*. Pathogenicity islands that are placed on chromosomes or plasmids may include these virulence genes (Navarro-Garcia *et al*., 2019). The virulence factors, which include poisons, iron acquisition mechanisms (siderophores), and protectins, have an impact on invasion, colonization, adhesion, and survival against host defenses (Ghunaim *et al*., 2014). The (*iucD*) gene regulates the pathogenicity of APEC and the synthesis of aerobactin (Ngeleka *et al*., 1996). The Yersinia iron-acquisition proteins, *irp2*, present in human *E. coli* strains. (Jeong *et al*., 2012). The *iroN* gene, which is an outer membrane siderophore receptor, enhances APEC pathogenicity (Dozois *et al*., 2003).

High serum survival *iss* gene is linked to APEC complement resistance (Pfaff-McDonough *et al*., 2000). *E. coli* strains from broilers were found to have hemolysin, or *hlyF*, which affects APEC pathogenicity (Johnson *et al*., 2006). *OmpT* cleaves protamine and plasminogen was detected in APEC (Johnson *et al*., 2006). Although many virulence genes in APEC strains have been

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identified, it is unclear what frequencies of combination genes and patterns of gene exist. This limits our understanding of how APEC evolution influences pathogenicity (Dziva and Stevens, 2008).

The available data revealed the possible steps in virulence gene acquisition based on gene combinations, We examined the frequency of the genes responsible for virulence as *tsh*, *hlyF*, *papC*, *iroN*, *iucD*, *irp2*, *ompT*, *iutA*, *iss*, *astA*, and *cva*. We also investigated the correlation between phenotypic resistance and APEC pathogenicity.

MATERIALS AND METHODS

Statement of Ethical Considerations

In the current study followed ARRIVE guidelines. The Animal Ethics Review Committee at Suez Canal University (AERC-SCU), Egypt, endorsed all broiler chicks handling and experiments.

Bacterial Strains

Thirteen fully defined strains of *E. coli* were obtained from broiler farms from freshly dead birds from yolk sac and viscera (brain,heart,kidney and liver). The collected samples were inoculated into MacConkey broth (Difco, USA) and subsequently incubated at a temperature of 37°C for 24 hours. A loopful of the incubated broth was streaked onto MacConkey agar and Eosin Methylene Blue agar (EMB) plates (Difco, USA) and thereafter incubated at a temperature of 37°C for 24 hours. The identification of the purified colonies was conducted based on their cultural characteristics, Gram staining,and biochemical tests(MacFaddin, 1985). Their antibiotic resistance pattern and virulence gene molecular typing were investigated.

Antibiogram

Using a Kirby-Bauer disk diffusion assay, thirteen strains were tested for antimicrobial susceptibility technique according to the standards and interpretive criteria described by CLSI (2020). Seventeen antimicrobial substances were used: amoxicillin (AML, 10ug), penicillin (P,10ug), aztreonam (ATM, 30ug), amoxicillin-clavulanate (AMC, 20/10µg), cefepime (FEB, 30µg); gentamicin (GEN 10ug), kanamycin (K, 5µg), fosfomycin (FOS, 50ug); imipenem (IMP, 10µg), meropenem (MEM,10µg); ciprofloxacin (CIP,5 µg); doxycycline (DO, 30 µg), tetracycline (TE,30 µg); sulfamethoxazole-trimethoprim (SXT, 23.7/1.25µg); chloramphenicol (C, 30 µg), florfenicol (OFX, 30µg); tigecycline (TGC, 15µg). According to Magiorakos *et al.* (2012), the *E. coli* strains were MDR(Multidrug resistant) and XDR(Extensively drug resistant). Furthermore, the MAR index was evaluated using (Krumperman, 1983).

Virulence Genotyping

Thirteen strains were examined for the existence of the elven *E. coli* virulence genes in this study, were *ompT* (outer membrane proteins T), *tsh* (temperature-sensitive hemagglutinin), *iss* (serum survival gene), The Yersinia high-pathogenicity island (*irp2*), catechol siderophore receptor (*iroN*), aerobactin systems (*iutA*), cytotoxic necrotizing factors (*cva*), heat-stable toxin (*astA*), and hemolysin (*hlyF*), Iron uptake chelate gene D (*iucD*), gene required formation of galactoside-binding Pap pili (*papC*) by PCR assay.

Using a Genomic DNA Purification kit, crude DNA extracts were obtained. The finished DNA template stored at 20°C for further usage. The company does not publish the primers used for PCR; they are internally created. Electrophoresis on a horizontal gel in 2% agarose was performed on all samples.

Statistical Analysis

RStudio (4.1.2) assessed correlation and Chi-square test. Investigating AR phenotype-virulence gene relationships. An association was significantly measured if $p < 0.05$.

RESULTS

Antibigram testing

The antimicrobial resistance results of the retrieved E.coli isolates exhibited that the recovered strains were resistant against Amoxicillin (100%), Florfenicol (100%), Penicillin (100%), amoxicil-

Table 1. The antibiogram testing of the isolated *E. coli*.

Antimicrobial class	Antimicrobial agent	Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
B lactamase	Amoxicillin	0	0	0	0	13	100
	Penicillin	0	0	0	0	13	100
	Aztreonam	10	77	0	0	3	23
	Amoxicillin-Clavulanate	0	0	1	7.7	12	92.3
	Cefepime	1	7.7	10	77	2	15.4
Aminoglycosides	Gentamicin	3	23	4	30.7	6	46.1
	Kanamycin	0	0	11	84.6	2	15.4
Phosphonic	Fosfomycin	13	100	0	0	0	0
Carbapenems	Imipenem	12	92.3	1	7.7	0	0
	Meropenem	0	0	4	30.7	9	69.2
Fluoroquinolones	Ciprofloxacin	9	69.2	1	7.7	3	23
Tetracycline	Doxycycline	9	69.2	0	0	4	30.7
	Tetracycline	3	23	0	0	10	77
Sulfonamides	Sulfamethoxazole-trimethoprim	4	30.7	1	77	8	61.5
Amphenicols	Chloramphenicol	5	38.5	0	0	8	61.5
	Florfenicol	0	0	0	0	13	100
Glycylcycline	Tigecycline	6	46.1	6	46.1	1	7.7

lin-clavulanate (92.3%), Tetracycline (76.9%), Meropenem (69.2%), Sulfamethoxazole-Trimethoprim (61.5%), and Chloramphenicol (61.5%). Moreover, all recovered isolates were sensitive to fosfomycin (100%) and imipenem (92.3%) followed by aztroname (77%), and ciprofloxacin (69.2%), as shown in Table 1.

Virulence genotyping

The *iss* gene showed the highest percentage 84.6% (n=11) followed by both *ompT*, *iroN* and *hlyF* genes with 76.9% percentage. The lowest proportion 15.3% was exhibited for the genes *tsh* and *irp2* (n=2 for each gene) (Table 2). Out of 13 *E. coli* isolates, 9 (69.2%) isolates exhibited 5 virulence genes, and 4 (30.7%) isolates showed 5 virulence genes, making them virulent strains (Table 3). Non-significant differences among virulence genes of recovered isolates.

Table 2. percentage of APEC virulence genes (VAGs) in *E. coli* isolates (n=13).

Gene	No. of isolates harbour genes	Percentage
Iss	11	84.60%
omp-T	10	76.90%
hlyF	10	76.90%
iroN	10	76.90%
iutA	8	61.50%
iucD	7	53.80%
papC	5	38.40%
cva	4	30.70%
astA	3	23.00%
tsh	2	15.30%
2 irp	2	15.30%
Chi-square	18444	
P value	0.04791^{NS}	

Phenotypic patterns of resistance and virulence genes of the E.coli strains

Alarmingly, 23.1% (3/13) of the *E. coli* strains were XDR to seven different classes, while only one strain (0.7%) was XDR to eight different classes. In contrast, 61.5% (8/13) of the isolated *E. coli* strains were multiresistant, with 15.4% (2/13) being MDR to six classes, 23.1% (3/13) being MDR to five classes, and 23.1% (3/13) being MDR to four classes, as shown in Table 3. The multiple antibiotic resistance (MAR) index values ranged from 0.35-0.65 indicating high-risk contamination (Table 3). Moreover, Between the tested antimicrobial drugs and the virulence genes, the correlation coefficient (r) was calculated. Antibiotic resistance and the presence of virulence genes were shown to be positively correlated, as shown in Figure 1.

DISCUSSION

In the existing study, Thirteen *E. coli* isolates were subjected to investigation of antimicrobial susceptibility profile against 17 antimicrobial agents that exhibited the highest resistance found against amoxicillin, florfenicol, penicillin, amoxicillin-clavulanate, tetracycline, meropenem, sulfamethoxazole-trimethoprim, and chloramphenicol in the percentage of 100%, 100%,100%, 92.3%, 76.9%, 69.2%, 61.5%, and 61.5%, respectively while the isolates exhibited highest sensitivity found to fosfomycin, imipenem, azetronam and ciproflxacin in the percentage of 100%,92.3%, 76.9% and 69.2%, respectively. The results agree with Ashraf *et al*. (2016) recorded resistance to amoxicillin, but the isolates were very susceptible to gentamycin. Jude *et al*. (2022) exhibited resistance by penicillinase likewise; prolonged noticing resistance to amoxicillin followed by resistance to ceftriaxone or cefotaxime. Abu Daud *et al*. (2014) revealed *E. coli* isolates showed resistance to amoxicillin, clindamycin, streptomycin, and trimethoprim moreover, they were intermediate to oxytetracycline and sensitive to gentamicin.

Contrarily, high resistances to all other antimicrobial sub-

Table 3. Patterns of resistant and virulence genes of the obtained *E. coli* (n = 13).

Isolate	ID	Type	Patterns of resistant	Virulence genes	MARI
1	789	MDR	7 Antimicrobial agents/ 4 Classes: AML, P, ATM, AMC, TE, SXT, and OFX	10 genes *iutA, papC, iss, iucD, tsh, ompT, hlyF, cva, astA, iroN*	0.41
2	798	MDR	6 Antimicrobial agents/ 4 Classes: AML, P, AMC, MEM, SXT, and OFX	8 genes *iutA, papC, iss, iucD, tsh, ompT, hlyF, iroN*	0.35
3	806	XDR	10 Antimicrobial agents/ 7 Classes: AML, P, AMC, MEM, SXT, TE, DO,C,CN and OFX	7 genes *iutA, iss, iucD, ompT, hlyF, cva, iroN*	0.59
4	807	Resistant	3 Antimicrobial agents/ 2 Classes: AML, P and OFX	5 genes *papC, iss, ompT, hlyF, iroN*	0.18
5	823	MDR	7 Antimicrobial agents/ 5 Classes: AML, P, AMC, MEM, C, TE and OFX	6 genes *iutA, iss, ompT, hlyF, astA, iroN*	0.41
6	843	MDR	11 Antimicrobial agents/ 6 Classes: AML, P, AMC, FEB, ATM, SXT, CIP, C, TE,DO and OFX	8 genes *iutA, papC,iss,iucD,irp2,ompT,hlyF, iroN*	0.65
7	849	XDR	11 Antimicrobial agents/ 7 Classes: AML, P, AMC, FEB, ATM, MEM, CIP, C,TE,CN and OFX	6 genes *iutA, iss, iucD, ompT, hlyF, iroN*	0.65
8	853	MDR	6 Antimicrobial agents/ 4 Classes: AML, P, AMC, C, MEM and OFX	0 genes	0.35
9	854	MDR	7 Antimicrobial agents/ 5 Classes: AML, P, AMC, C, MEM, TE and OFX	9 genes *iutA, iss, iucD, irp2, ompT,hlyF,cva, iroN*	0.41
10	874	XDR	9 Antimicrobial agents/ 7 Classes: AML, P, AMC, CIP, MEM, SXT, TE, K and OFX	5 genes *iutA, iss, ompT, hlyF, iroN*	0.53
11	886	MDR	9 Antimicrobial agents/ 6 Classes: AML, P, AMC, C, MEM, TE, DO, SXT and OFX	3 genes *iss, iucD, cva*	0.53
12	887	MDR	7 Antimicrobial agents/ 5 Classes: AML, P, AMC, TE, SXT, K and OFX	4 genes *iss, ompT, hlyF, iroN*	0.41
13	889	XDR	11 Antimicrobial agents/ 8 Classes: AML,P,AMC,C,TE,DO,TCG,SXT,CN,MEM and OFX	2 genes *papC, astA*	0.65

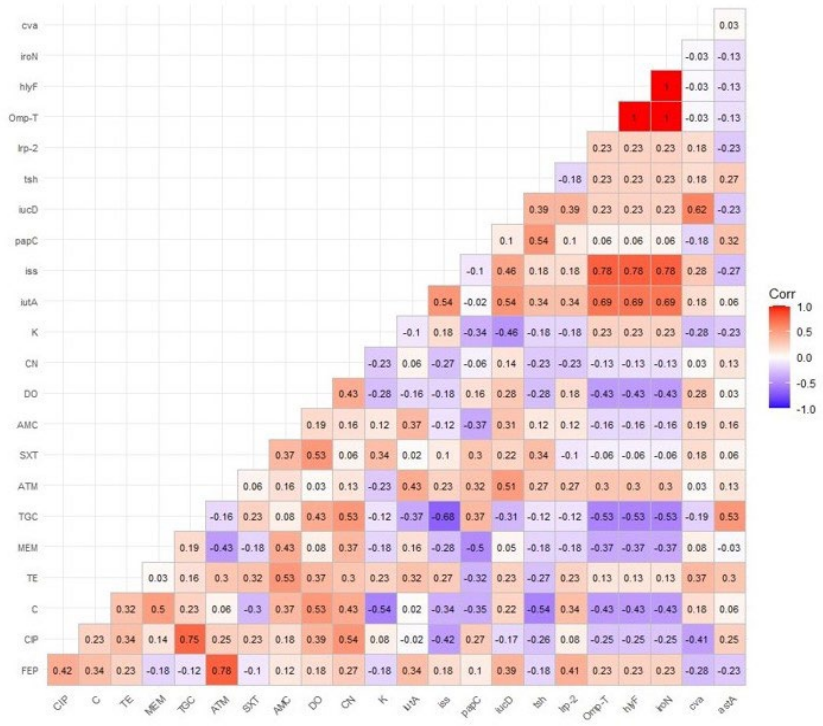

Figure 1. Relation between phenotypic resistant and virulence genes of the obtained *E. coli*

stances were noticed, including cefotaxime sodium and florfenicol (96.6% for each), apramycin, ciprofloxacin, and gentamicin (91.4% for each), enrofloxacin and lincomycin (91.4% for each), streptomycin (89.7%), sulphamethoxazole-trimethoprim, doxycycline HCl (77.6% for each) and spiramycin (75.9%); Iraqi *et al.* (2021) revealed 80% resistant to ampicillin and colistine sulfate. Ghada *et al* (2021) stated *E. coli* strains showed resistance to streptomycin. Amer *et al.* (2018) stated the result of isolated *E. coli* resistance to (kanamycin and oxytetracycline), 80% to (clindamycin, ampicillin and streptomycin), 75% to enrofloxacin, 65% to chloramphenicol, 55% to (gentamicin and cefotaxime), 45% to trimethoprim+sulfamethoxazole, 35% to erythromycin and 30% to oxacillin.

Holland *et al.* (2000) and Chui *et al.* (2010) noted that virulence genes are needed to identify specific microorganisms, and In the absence of the species-specific gene, the same bacteria may exist. The genes in this study were chosen because they are frequent in APEC which is identified by PCR. Investigation of some virulence-associated genes was performed by PCR on thirteen E.coli isolates including *iss* (11/13), omp-T (10/13), *hlyF* (10/13), *iroN* (10/13), *iutA* (8/13), *iucD* (7/13), *papC* (5/13), *cva* (4/13), *astA* (3/13), *tsh* (2/13) and *irp2* (2/13)with percentage rates (84.6, 76.9, 76.9, 76.9, 61.5, 53.8, 38.4, 30.7, 23, 15.3 and 15.3%), respectively.

The rates of *iutA*, *hlyF*, *iucD*, *iss*, *iroN*, and *ompT* were greater in the current study than those of *irp2*, *tsh*, and *astA*. Distribution of *hlyF*, *iucD*, *iss*, and *irp2* suggest co-transmission, although no report exists (Johnson *et al.*, 2006). APEC strains have redundant genes (*iroN*, *iucD*, and *irp2*) associated with iron uptake, however, virulence genes differ depending on the host and site. Although the main roles of these redundant genes are unknown, they might change depending on the niche. Repetitive proteins related to iron uptake, which is essential for APEC pathogenicity, may aid in evading humoral defense (Jeong *et al.*, 2012).

The obtained results agreed with Yaguchi *et al.* (2007) who detected *cva* C, *iss*, *iutA*, papA, *tsh*, and usp Virulence genes of *E. coli* recovered from sick chickens; Manita *et al.* (2018) distinguished eleven virulence genes (*iutA*, *iss*, *papC*, *iucD*, *tsh*, irp-2, *ompT*, *hlyF*, *iroN*, *cva/cvi*, and *astA*) related to colibacillosis collected from suspected broiler chickens and reported the majority of the APEC genes were *iss*, *iucD*, *hlyF*, *ompT*, *iroN*, and *iutA*, although, to a lesser range *irp2*, *papC*, *cva/cvi*, and *tsh* genes demonstrated the crucial function for virulence of APEC strains.

Three virulence genes which were (*iutA*, *iss* and *tsh*) with percentage rates 70, 60 and 30 %, respectively. The detected results showed that 90% of the isolates had at least 4 virulence genes, while only 10% had none (Walid *et al.*, 2020). On the other hand, Ashraf *et al.* (2014) conducted Multiplex PCR on all *E. coli* serovars recovered from 44 commercial broiler chicken farms aged 20-30 days old in Egypt and *papC* virulence gene was detected only in O55 serotype. Stated the virulence associated Genes (sitA, *iss*, *iucD*, iucC, *astA*, *tsh*, *cvi*, and *irp2*) were revealed a rate of descending order was 97.4, 93.3, 75, 74, 71, 46.5, 39 and 34%, respectively (Rekaz *et al.*, 2019). eaeA, traT, and vat virulence genes of *E. coli* strains and arranged in descending order all eighteen *E. coli* isolates were positive for vat genes, 16 were for traT genes and 5 were positive for eaeA gene positive (100%, 88.8 and 27.7%), respectively (Hassan *et al.*, 2020).

CONCLUSION

This study showed high resistance against different common antimicrobials used in poultry medicine including; beta-lactams, tetracyclines, and trimethoprim. In addition, the isolates have a significant number of APEC virulence genes (VAGs). These isolates, according to our findings, are possible sources of antibiotic resistance in APEC, offering major public health implications to individuals who are directly or indirectly exposed to them. To overcome these problems, in vivo, challenge studies are needed to investigate the efficacy of antibiotic treatment against highly resistant APEC isolates.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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