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Molecular Detection of Associated Genes with The Highly Virulent Avian Pathogen *Escherichia Coli* Isolated from Chicken Fecal Samples

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ABSTRACT

Bird disease-causing *E. coli* (Avian colibacillosis), the most common disease-causing morbidity in poultry worldwide, is mainly caused by avian pathogenic *E. coli* (APEC). The present study aimed to detect virulence-associated genes of *E. coli* isolated from chickens of different origins in Basrah Province. 84 isolates were examined by Polymerase Chain Reaction for the existence of the five most related genes defined for avian colibacillosis (*hlyF, ompT, iroN, iutA,* and *iss*). The percent of genes related to the virulence of APEC found in isolates were: *ISS* (75%), *ompT* (60%), *hlyF* (45%), *iroN* (42%), and *iutA* (37%), however, 6% of the isolates had all five genes together. The antimicrobial susceptibility of all isolates to seven antibiotics (Ampicillin, Ciprofloxacin, Colistin, Gentamicin, Chloramphenicol, Cefotaxime, and Imipenem) was verified. The antimicrobial resistance of the isolates (98%) was detected to be resistant to ampicillin, ciprofloxacin (68%), cefotaxime (64%), colistin (61%), gentamicin (59%), chloramphenicol (42%) and imipenem (32%). Regarding the *E. coli* isolates contain all 5 genes linked with the highly virulent APEC, antibiotic susceptibility testing revealed that 5 (100%) of the isolates were resistant to ciprofloxacin and ampicillin, 80% of the isolates were resistant to colistin and gentamicin, and 40% of APEC isolates were resistant to cefotaxime; however, 20% were resistant to imipenem. In conclusion, only a few fecal *E. coli* isolated from chickens had genes associated with virulent APEC, and these isolates have high antibiotic resistance.

Key words: APEC, antimicrobial resistance, virulence genes

INTRODUCTION

The intestinal tract of domestic animals and the environment are the primary and secondary habitats of *Escherichia coli.* In humans and birds, *E. coli* is found in the lower digestive system, where it colonizes within the first 24 hours after hatching or birth (Ballou *et al.*, 2016). *Escherichia coli* that is pathogenic to birds (APEC) is *E. coli* strains that can infect birds of all ages and cause a disease known as colibacillosis, either locally or systemically (Guabiraba & Schouler, 2015). This disease causes great morbidity and mortality, meaning that every aspect of the global poultry industry loses millions of dollars annually (Paixão et al., 2016). In chickens, colibacillosis usually begins as a respiratory infection that affects lungs and air sacs. From there it can spread to other parts of the body and cause septicemia, pericarditis and perihepatitis (Pourbakhsh, et al., 1997). Next, vertical transmission Avian pathogenic E. Coli (APEC) from the breeding flock has the potential to increase early mortality in chicks and cause yolk sac infection (omphalitis) (Giovanardi, et al., 2005).Avian pathogenic *Escherichia coli* (APEC) is a subgroup of extraintestinal pathogenic *Escherichia coli* that is thought to be zoonotic and act as an external reservoir for extraintestinal infections in humans, such as uropathogenic *E. coli* (UPEC). (Jørgensen *et al.*, 2019). ExPEC enters via various routes, particularly the respiratory and genital tracts, and causes various extraintestinal diseases, collectively referred to as colibacillosis (Dissanayake, *et al.*, 2014).Four different evolutionary pathways can lead to the emergence of APEC strains: (1) the extraintestinal dissemination of pathogenic clones possessing particular virulence factors; (2) the selection of highly pathogenic strains through host mediation; (3) the transfer of plasmids between lineages during plasmid-mediated extraintestinal spread; and (4) rearranging virulence factors and plasmids due to horizontal gene transfer, resulting in the generation of new APEC strains (Palmieri, *et al.*, 2023).Five genes were found to be most strongly linked to highly pathogenic APEC strains (Tohmaz *et al.*, 2022;

Palmieri *et al.*, 2023). The gene (*ompT*) encompasses the colicin-cleaving episomal outer membrane protease, the gene of aerobactin (*iutA*) (iron uptake transporter), gene (*iroN*) the iron uptake-related outer membrane siderophore receptor, and the increased serum survival gene (*iss*) enhance resistance to complement, and the novel class of avian hemolysin gene (*hlyF*), which contributes to the synthesis of vesicles with an outer membrane, releasing toxins and enabling the iron absorption (Lounis *et al.*, 2020).The purpose of this present study is to identify the virulence genes of the avian pathogen *Escherichia coli* and the antimicrobial susceptibility of isolates from chicken samples in Basrah Province.

MATERIALS AND METHODS

Samples collection

Chicken samples were obtained by taking fresh fecal and cloacal swabs from various locations within Basrah province. Between October 18, 2023 and January 13, 2024, 204 samples were collected from backyard chickens, poultry fields, and chicken shops (Table 1).

Table (1): Number and sources of samples used in this study

Source of samples	Type of samples		
	Cloacal swabs	Droppings swabs	Total No.
Backyard chicken	67	35	102
Poultry fields	48	0	48
Chicken shop (markets)	38	16	54
Total	153	51	204

Microbiological techniques

Escherichia coli was isolated from fecal and cloacal swabs and immediately brought to the central research unit in the College of Veterinary Medicine in a cool box under completely aseptic conditions. In the laboratory, the samples were cultured in peptone water at 37 ℃ for 24 hours (Baccus-Taylor *et al.*, 2015). Isolates were subcultured on MacConkey agar overnight at 37℃ (Bravata-Alcantara *et al.*, 2019). Separate streaks of positive colonies on MacConkey agar were placed on eosin-methylene blue agar and incubated for a full day at 37°C (Mohammad *et al.*, 2018). Isolates with characteristic features were identified by biochemical tests using Gram stains, and IMVIC (indole, methyl red, Voges Proskauer, and citrate) (Mustika *et al.*, 2024).

Molecular techniques

The suspected *E. coli* isolates were further identified using a polymerase chain reaction. It involves several steps including DNA extraction, PCR, and gel electrophoresis.

DNA Extraction

The boiling method was utilized to extract genomic DNA from all presumed isolates according to (Junior *et al.*, 2016).

Molecular confirmation of *E. coli*

The *uidA* gene was amplified to verify the suspected *E. coli* isolates identified by conventional microbiological methods and thermal cycling conditions according to (Farhan & Al-Iedani, 2019) have a product size of 203 bp using the PCR technique.

The mixture volumes used for amplification of the *uidA* gene were a total of 25 µl PCR reaction, 12.5 µl master mix (Promega/USA), 1 µl of each individual primer,7.5 µl nuclease-free water, and 3 µl DNA template.

Molecular detection of APEC genes

By using PCR, the presence of virulence-associated genes was examined in each *E. coli* isolate. To explore the potential targets, this study created the gene-specific primers listed in Table 2 using software tools and then compared them to an appropriate nucleotide sequence database using tools such as BLAST.

Table (2): Primers sequences of virulence associated genes of APEC

Name of primers	Primer sequences (5- 3)	length	Size of product	GenBank accession no	Reference	Manufacturer
hlyF	F: 5′-AGGGTGCTTACCTTCAACCA-3′	20bp	228bp	KP657527.1	This study	Promega / USA
	R: 5′-CGGGCTACAGCTTCGTCAAT-3′					
iroN	F: 5′-ACCAATCGCCTGAGTTCCTG-3′	20bp	146bp	KP657537.1	This study	Promega/ USA
	R: 5′-CTGTCATTCACCGTCAGGCT-3′					

iss	F: 5′-GTTATTTTCTGCCGCTCTGG -3′ R: 5′- AACCGAGCAATCCATTTACG -3′	20bp	227bp	FJ8248853.1	This study	Promega/ USA
ompT	F:5′- CGGAGATTGATTTTGGCACT -3′ R: 5′- CCTCCACGACCAGCTAATGT -3′	20bp	196bp	NC_000913.3	This study	Promega/ USA
iutA	F: 5′-ATGCCGTACTGGTCGGTATC -3′ R:5′-AGCAGCACACCATACGTCAG -3″	20bp	230bp	OP081602.1	This study	Promega/ USA

The volumes of the mixtures used to amplify the virulence genes hlyF and iroN by duplex PCR were a total of 25 µL, 5 µL of nuclease-free water, 3.5 µL of DNA template, 1 µL of every primer, and 12.5 µL of the master mix (Promega, USA). For (iss, ompT, iutA) genes, the mixture volumes used for amplification by uniplex PCR were a total of 20 µl PCR reaction, 10 µl master mix (Promega/USA), 2 µl DNA template, 6 µl nuclease-free water, and 1 µl of each primer.

Amplification of *hlyF and iroN* genes was achieved with the initial denaturation of 95°C for 5 minutes, ensued by 30 amplification cycles of 94°C for 1 minute, 56°C for 30 seconds, and 72℃ for 2 minutes. This step was followed by a final extension of 72 ℃ for 5 minutes.

Amplification of *iss, ompT, and iutA* genes was achieved with the initial denaturation of 95°C for 1 min, 30 cycles of 45s at 95°C, 45 s at 59°C, and 2 min at 72℃, with a last extension step of 72 °C for 5 min.

Determination of antimicrobial susceptibility

Antimicrobial susceptibility was tested on *E. coli* isolates utilizing the Kirby-Bauer method. Mueller-Hinton agar (Himedia/India) was prepared according to the manufacturer's instructions; A broth culture of E. coli isolates was standardized by dilution to 0.5 McFarland standard. A sterile swab was inserted into the suspension and dried on the insides of the tube to remove any additional inoculum load. The entire surface of the MHA plate was coated with the dipping swab. Various antibiotic discs with known concentrations for E. coli, including cefotaxime (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), colistin (10 µg), gentamicin (10 µg), imipenem (10 µg), and chloramphenicol (30 µg), were fixed on the MHA plate with sterile forceps. The inoculated MHA was incubated at 37°C for 18-24 hours. (Widodo *et al*., 2023).

RESULTS

Isolation of *E. coli*

The study used 204 samples in total (153 cloacal and 51 fecal swabs), of which 109 (53.4%) tested positive for *E. coli* according to biochemical testing and morphological culture features. The *E. coli* isolate was identified using eosin-methylene blue agar (EMB) medium with metallic green color, gram-negative rod-shaped bacteria, and pink colonies on MacConkey agar. Indole and MR positive tests, VP, and citrate negative tests (Figure 1).

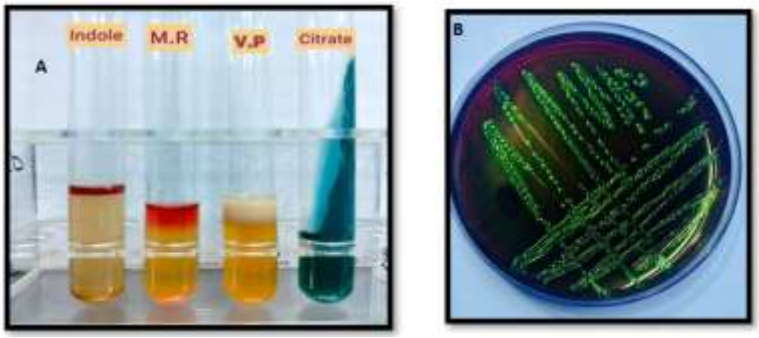

Figure (1): Morphological and biochemical characteristics of isolates
A: IMVIC test (+ + - -). **B**: Growth of *E. coli* on EMB (green metallic sheen)

Molecular identification of *E. coli* by PCR

PCR amplification of the *uidA* gene was used with a product size of 203 bp to confirm the suspected isolates as *Escherichia coli.* Of 109 isolates suspected by conventional bacteriological testing, 84 (77%) were confirmed to be *E. coli* (Table 3; Figure 2).

Table (3): Results of *E. coli* identification using conventional microbiological techniques and PCR

	Conventional microbiological Techniques	Detection by *uidA*

Source	Total No.				
		No.	%	No.	%
Backyard chicken	102	39	38.2	34	87.1
Poultry fields	48	44	91.6	36	81.8
Chicken shop (Market)	54	26	48.1	14	53.8
Total	204	109	53.4	84	77

Figure (2): Product of PCR of uidA gene on 1.5 percent agarose gel, using DNA ladder (100-bp).

Presence of the APEC virulence-associated genes

This study targeted five virulence genes typically associated with APEC. 84 isolates of *E. coli* were examined, some isolates contained one virulence gene, others contained five. These combinations of the five virulence genes varied, each isolate had at least one virulence-associated factor, according to PCR analysis. Specifically, of the 84 isolates, 63 (75%) were positive for *iss*, 50 (60%) for *ompT*, 40 (48%) for *hlyF*, 35 (42%) for *iroN*, and 31 (37%) for *iutA*. The presence of five isolates containing the five virulence genes was determined. Figures (3-5) and Table (4) show the presence of APEC virulence-associated genes.

Table (4): Distribution of genes associated with the highly virulent APEC in *E. coli* isolates

Gene	Percent	Number of isolates
ISS	75%	63
ompT	60%	50
hlyF	48%	40
iroN	42%	35
iutA	37%	31
APEC	6%	5

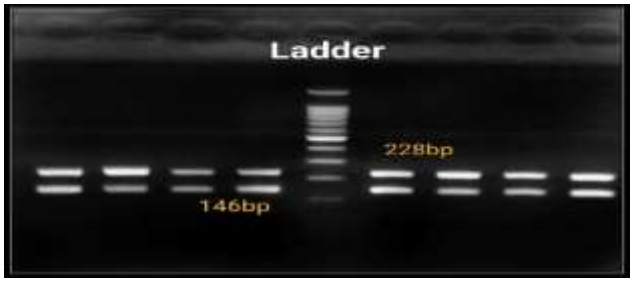

Figure 3: An agarose image showing the *hlyF* (228 bp) and *iroN* (146 bp) genes amplified from *E. coli*.

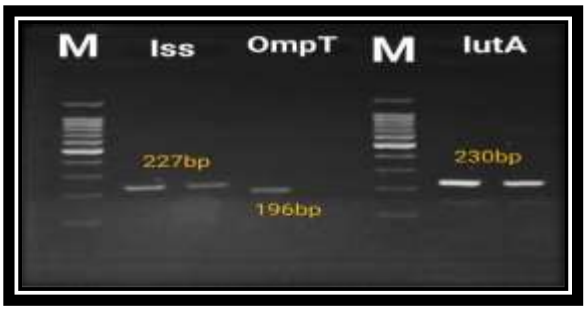

Figure 4: An agarose image showing *iss* (227bp), *ompT* (196bp), and *iutA* (230bP) genes amplified from *E. coli*.

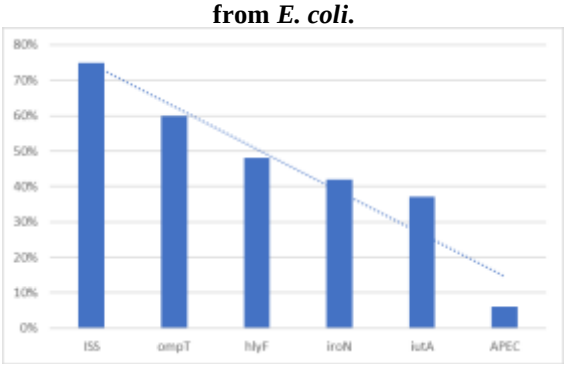

Figure 5: Distribution of virulence-associated genes in *E. coli* isolates

Antibiotic susceptibility test

The susceptibility of each isolate to veterinary-relevant antibiotics was examined. The isolates showed the highest resistance rates to ampicillin (98%), ciprofloxacin (68%), cefotaxime (64%), colistin (61%), gentamicin (59%), and chloramphenicol (42%). Imipenem (32%) appeared to be the most effective antibiotic against these isolates, according to CLSI (2020). This is shown in Table 5 and Figure 6. For the *E. coli* isolates, which contained all five (VAGs) of the highly virulent APEC, antimicrobial susceptibility testing exhibited that 5 (100%) of the isolates were resistant against ampicillin and ciprofloxacin, (80%) colistin, gentamicin, and chloramphenicol; (40%) Cefotaxime. In comparison, (20%) of isolates were resistant to imipenem, as shown in Table 6.

Table (5): Antimicrobial susceptibility test of *E. coli* isolates.

Antimicrobial	Resistant (%)	Intermediate (%)	Susceptible (%)
Ampicillin	82 (98%)	2 (2%)	0 (0)
Cefotaxime	54 (64%)	6 (7%)	24 (29%)
Chloramphenicol	35 (42%)	19 (22%)	30 (36%)
Ciprofloxacin	57 (68%)	9 (11%)	18 (21%)
colistin	51 (61%)	21 (25%)	12 (14%)
Gentamicin	49 (59%)	2 (2%)	33 (39%)
Imipenem	27 (32%)	27 (32%)	30 (36%)

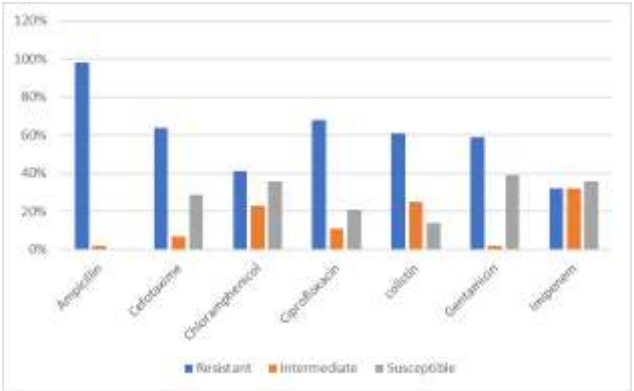

Figure 6: susceptibility of *E. coli* isolates to antimicrobial agents.

Table (6): Antimicrobial resistance of APEC isolates screened using the disk diffusion method.

Antimicrobial	Resistance %
Ampicillin	100
Ciprofloxacin	100
Colistin	80
Gentamicin	80
Chloramphenicol	80
Cefotaxime	40
Imipenem	20

DISCUSSION

The avian pathogenic *Escherichia coli* (APEC) infection known as colibacillosis is a significant bacterial infection that causes significant losses for the poultry industry globally (Tohmaz *et al.*, 2022).

In the present study, 84 isolates were examined for 5 genes associated with APEC virulence. 100% of isolates carried a minimum of one gene. The genes *ompT, iroN, hlyF, iutA, and iss* form the most widespread profile that characterizes the APEC pathotype. This result is consistent with previous studies that reported high frequencies of 98% (Mohamed *et al.*, 2018) and 100% (Lounis *et al*., 2020). However, this contradicts a previous study showing a lower percent of 47% (Li *et al*., 2015).

The *iss* (enhanced serum survival) gene typically encodes a protein involved in serum survival and contributes to the increased virulence of *Escherichia coli* in day-old chicks. The *iss* gene was the most abundant gene in this study at 75%. Similar observations have also been reported (Varga *et al*., 2018). All these data suggest that *iss* may play an essential role in the pathophysiology of colibacillosis in poultry.

This research also identified a high frequency of 60% of the *ompT* gene, which encoded an outer episomal membrane protein. *ompT* may play a role in outer membrane protease secretion and colicin resistance (Johnson *et al.*, 2006). The findings of our study are consistent with other reports indicating that this gene is highly prevalent (Mohamed *et al*., 2018). Moreover, they reported a lower frequency (Mbanga & Nyararai, 2015).

The *hlyF* and *iroN* genes are involved in iron absorption, which was noted with a high frequency (48% and 42%, respectively) in this study. This frequency is generally lower than that described in a publication by (Pilati, *et al*., 2024).

The *iutA* gene participates in the regulation and synthesis of the aerobactin uptake system (Ling *et al.*, 2013). The frequency among the isolates was 37%. This is considered a lower rate compared to the higher detection rates described by (Akbari & Asadpour, 2022).

Only 6% of the isolates in this study show positive results for the combination of the five genes *iss, ompT, hlyF, iutA and iroN*.

Antimicrobial resistance is a very serious difficulty due to excessive use of antibiotics, In addition to expensive costs and a number of side effects. Because chickens could contribute to the transmission and spread of *E. coli*, which is resistant to antibiotics. It is important to understand the level of antibiotic resistance in chicken isolates to avoid the Utilize of antibiotics to treat or prevent diseases in these birds that could damage humans.

Antibiotic susceptibility and the presence of virulence genes were assessed in APEC isolates. Our data illustrated high rates of resistance to ampicillin and ciprofloxacin (100%) and colistin (80%). These results are congruent with those of recent studies in Egypt (Awad *et al.*, 2020) and Qatar (Johar *et al.*, 2021). Gentamicin and chloramphenicol 80%, cefotaxime 40%, and imipenem 20%.

CONCLUSION

In conclusion, while only a few fecal E. coli isolates from chickens possessed genes linked to virulent APEC, these isolates exhibited high levels of antibiotic resistance. The potential association between virulence genes and elevated antibiotic resistance may pose a significant risk factor.

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