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# Multiparametric investigation of Phenotypic Assays, Virulence Genes Detection, and Antimicrobial Susceptibility Testing of *Escherichia coli* Isolated from Ducks

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*Toxin genes: stx1, stx2, hly*
*Other genes: tsh, iss*
*Antimicrobial Susceptibility*
*Congo red*
*Outer membrane proteins: ompA, traT*
*Siderophore: iutA*

**Abstract**

This study investigated 300 duck samples for the isolation of *E. coli*. From 100 cloacal swabs of apparently healthy ducks, 22% were positive. Among 100 diseased ducks, 78% were positive, with the isolates distributed as 28% from the spleen and 50% from the liver. Additionally, *E. coli* isolation rate of bone marrow was 24% of 100 freshly dead ducks. 124 isolates were obtained from 41% of the samples, originating from three governorates: 42% from Qalyubia, 27% from Gharbia, and 31% from Giza. Among the 124 *E. coli* strains analyzed, 12 serogroups were identified , while a large proportion of isolates were untyped . The *E. coli* isolates demonstrated high sensitivity to colistin sulfate (100%), gentamycin (91%), and danofloxacin (83%). Conversely, they exhibited high resistance to ampicillin (100%), erythromycin (91%), and nalidixic acid (66%). All tested strains bound Congo red dye, suggesting pathogenic potential. The study screened for ten virulence genes categorized into five functional groups. Among the adhesion genes, *eaeA* was identified in 33% of isolates, *papC* in 75%, and *tsh* in 33%. Notably, all toxin genes (*stx1, stx2,* and *hly*) were not detected. For serum resistance, the iss gene was located in 58% of isolates, but the iron acquisition gene iutA was found in 100%. Lastly, genes associated with evasion of the host immune system, *ompA* and *traT,* were found in 100% and 91% of isolates, respectively. serotypes O119, O27, O115, and O8 convey six virulence genes but O114 contained only *ompA* and *iutA*. biosecurity measures are crucial to prevent infection and control. The misuse of antibiotics like colistin and preserve their efficacy.

## Introduction

Avian pathogenic *Escherichia coli* (APEC) infections lead to high cost in duck farms and poultry industries **(Hongyan *et al.* (2025)** . *E.* coli is an essential component of the gut microbiota of humans, animals, and fowl. (**Liu *et al* 2019**, **Kaper *et al.*, 2004).** Due to its widespread environmental presence and strong association with fecal matter, *E.* coli

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has long been recognized as a vital warning bacterium for intestinal waste in water sources ( **Ishii and Sadowsky (2008**). Duck is a significant source of meat for human consumption. specific serotypes can acquire virulence genes, enabling them to cause illness in human and duck **(Kim *et al*., 2022)** . These diarrheagenic *E. coli* strains are broken down into six pathotypes centered on their virulence mechanisms: Enteropathogenic *E.coli* (EPEC), the Enterotoxigenic *E. coli (ETEC),* enterohemorrhagic *E. coli* (EHEC) / Shiga toxin-producing E. coli (STEC), enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli (EAEC*), and diffusely adherent *E. coli* (DAEC). (**Ruttler *et al.* 2006; Xia *et al.* 2010**).

Congo red binding effectively reveals bacterial surface properties and hydrophobicity, acting as crucial indicators for virulence and pathogenicity, especially in Avian Pathogenic *E. coli* (APEC) (**Quadri *et al*., 1988**). (**Ahmad *et al*., 2004**).

Reports have exhibited a positive connection among the pathogenicity of *E. coli* and Congo red binding. (**Dubey *et al*., 2000**). Avian Pathogenic *E. coli* infections in chickens are driven by bacterial Virulence factors include binding, invasins, iron uptake systems, protectins, and toxins. Key virulence associated genes **(VAGs)** frequently found in APEC strains linked to colibacillosis include *iutA, hlyF, iss, iroN, ompT, and traT* (**Bhattarai *et al* 2023**). These genes are typically located on pathogenicity islands **(PAIs),** plasmids, or other genetic elements that are mobile, making the exploration of APEC's diversity of genes using these VAGs critical.

This study meticulously investigated the phenotypic characteristics, antibiotic sensitivity, and virulence genes of *E. coli* isolates, utilizing the Congo red binding assay to further elucidate the intricate relationship among these traits and their implications for bacterial pathogenicity.

## Material and Methods

### Sample Collection

The study involved the collection of 300 duck samples.

**Table (1).** Samples from Ducks

| | |
|---|---|
| apparently healthy ducks | 100 cloacal swabs |
| diseased ducks | 100 liver and spleen |
| freshly dead ducks | 100 bone marrow |

### Isolation and Identification of *E. coli*:

*E. coli* was isolated and identified using the methods reported by (**Nolan *et al*. 2013**). Samples were aerobically incubated in peptone-buffered water at 37°C for 24 hours. A loopful of incubated material was cultured on MacConkey's agar (Oxoid, Manchester, UK) and Eosin Methylene Blue agar (Lioflichem, Roseto degli Abruzzi, Italy) before being aerobically incubated at 37°C for 24 hours. The colonies were 1-2 mm in diameter, pink on MacConkey and metallic sheen on Eosin Methylene Blue agar. Suspected *E. coli* colonies received further biochemical tests (indole test, methyl red, Voges Proskauer "VP", citrate utilization, oxidase test, and Triple Sugar Iron "TSI") described by **Bhattarai *et al* 2023** . *E. coli* serotyping was performed at the bacteriology unit of the Animal Health Research Institute, Dokki, Giza, Egypt, using quick diagnostic *E. coli* antisera sets (DENKA SEIKEN CO., Japan), as revealed by **Kok, *et al*. (1996).**

### Antimicrobial susceptibility test

Antibiotic susceptibility testing of bacterial isolates was performed using the standard agar disk diffusion method on Mueller-Hinton agar (Oxoid, UK), in accordance with the guidelines established by the Clinical and Laboratory Standards Institute **(CLSI, 2013).** Pure bacterial colonies were standardized to a 0.5 McFarland turbidity in saline and streaked onto Mueller-Hinton agar plates. The specific antibiotics tested included: Ampicillin (10 µg), Chloramphenicol (30 µg), Colistin sulfate (10 µg), Danofloxacin (5 µg), Doxycycline (30 µg), Erythromycin (15 µg), Gentamicin (10 µg), Nalidixic acid (30 µg), Neomycin (30 µg), Streptomycin (10 µg), Tetracycline (30 µg), Trimethoprim-sulfamethoxazole (1.25/23.75 µg), and Ciprofloxacin (5 µg). Plates were incubated at 37°C for 18-24 hours. The results were interpreted as susceptible (S), intermediate (I), or resistant (R) based on the zone diameter interpretative criteria provided by **(CLSI 2013)** guidelines.

### Pathogenicity test by using congo red

The binding ability of Congo red has been examined utilizing the method given by **Berkhoff and Vinal (1986).** The Congo red medium had been provided with 0.03% Congo

red dye to trypticase soya agar (TSA), the *E. coli* isolates were streaked onto the plates, and the plates were incubated at 37° degrees Celsius for 24 to 72 hours. After 24, 48, and 72 hours of incubation, vibrant orange or brick red colonies appeared, indicating a positive response, whereas pale or white colonies were judged negative.

## Molecular characterization of virulence genes

### DNA extraction

The Polymerase Chain Reaction test was performed on twelve E. coli samples at the PCR lab at the Animal Health Research Institute to identify virulence genes. DNA extraction from samples was conducted out using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications to the manufacturer's instructions. To summarize, 200 µl of the sample suspension was treated with 10 µl of proteinase K and 200µl of lysis buffer at 56°C for 10 minutes. Following incubation, 200µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged according to the manufacturer's instructions. The kit included 100µl of elution buffer, which was used to wash out the nucleic acid.

### Oligonucleotide Primer.

The primers involved were given by Metabion (Germany) and are listed in Table (2).

**Table (2).** Primers sequences for screening *E. coli* virulence-associated genes.

| Gene | Primer Sequence 5'-3' | Amplified product | Reference |
|---|---|---|---|
| ***eaeA*** | ATGCTTAGTGCTGGTTTAGG | 248 bp | **Bisi-Johnson *et al*., (2011)** |
| | GCCTTCATCATTTCGCTTTC | | |
| ***papC*** | TGATATCACGCAGTCAGTAGC | 501 bp | **Wen-jie *et al*., (2008)** |
| | CCGGCCATATTCACATAA | | |
| ***tsh*** | GGT GGT GCA CTG GAG TGG | 620 bp | **Delicato *et al*., (2003)** |
| | AGT CCA GCG TGA TAG TGG | | |
| ***iss*** | ATGTTATTTTCTGCCGCTCTG | 266 bp | **Yaguchi *et al*., (2007)** |
| | CTATTGTGAGCAATATACCC | | |
| ***ompA*** | AGCTATCGCGATTGCAGTG | 919 bp | **Ewers *et al*., (2007)** |
| | GGTGTTGCCAGTAACCGG | | |
| ***traT*** | GATGGCTGAACCGTGGTTATG | 307 bp | **Kaipainen *et al*., (2002)** |
| | CACACGGGTCTGGTATTTATGC | | |
| ***iutA*** | GGCTGGACATGGGAACTGG | 300 bp | **Yaguchi *et al*., (2007)** |
| | CGTCGGGAACGGGTAGAATCG | | |
| ***stx2*** | ACACTGGATGATCTCAGTGG | 779 bp | **Ghanbarpour *et al.,* (2012)** |
| | CTGAATCCCCCTCCATTATG | | |
| ***stx1*** | CCATGACAACGGACAGCAGTT | 614 bp | **Dipineto *et al*., (2006)** |
| | CCTGTCAACTGAGCAGCACTTTG | | |
| ***hly*** | AACAAGGATAAGCAC-TGTTCTGGCT | 1177 bp | **Piva *et al*., (2003)** |
| | ACCATATAA-GCGGTCATTCCCGTCA | | |

**Table (3).** Cycling conditions for the different primers during cPCR were as follows

| Gene | Primary denaturation | Secondary denaturation | Annealing | Extension | cycles | Final extension | Reference |
|---|---|---|---|---|---|---|---|
| *eaeA* | 94˚C 5 min. | 94˚C 30 sec. | 51˚C 30 sec. | 72˚C 30 sec. | 35 | 72˚C 7 min. | **Bisi-Johnson *et al.*, (2011)** |
| *papC* | 94˚C 5 min. | 94˚C 45 sec. | 59˚C 45 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. | **Wen-jie *et al.*, (2008)** |
| *Tsh* | 94˚C 5 min. | 94˚C 45 sec. | 54˚C 45 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. | **Delicato *et al.*, (2003)** |
| *iss* | 94˚C 5 min. | 94˚C 30 sec. | 54˚C 30 sec. | 72˚C 30 sec. | 35 | 72˚C 7 min. | **Yaguchi *et al.*, (2007)** |
| *ompA* | 94˚C 10 min. | 94˚C 1 min. | 58˚C 1 min. | 72˚C 1 min. | 35 | 72˚C 10 min. | **Ewers *et al.*, (2007)** |
| *traT* | 95˚C 5 min. | 94˚C 1 min. | 55˚C 1 min. | 72˚C 1 min. | 35 | 72˚C 7 min. | **Kaipainen *et al.*, (2002)** |
| *iutA* | 94˚C 5 min. | 94˚C 30 sec. | 63˚C 30 sec. | 72˚C 30 sec. | 35 | 72˚C 7 min. | **Yaguchi *et al.*, (2007)** |
| *Stx1* | 94˚C 10 min. | 94˚C 1 min. | 58˚C 1 min. | 72˚C 1 min. | 35 | 72˚C 10 min. | **Dipineto *et al.* (2006)** |
| *Stx2* | 94˚C 10 min. | 94˚C 1 min. | 58˚C 1 min. | 72˚C 1 min. | 35 | 72˚C 10 min. | **Ghanbarpour *et al.* (2012 )** |
| *hly* | 94˚C 15 min. | 94˚C 1 min. | 60˚C 1 min. | 72˚C 1.5 min | 35 | 72˚C 12 min. | **Pivaet *al.*, (2003)** |

**PCR amplification.**

In a 25-µl reaction, add 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer (20 pmol concentration), 5.5 µl of water, and 5 µl of DNA template. The reaction was transported out in a T3 Biometra thermal cycler.

**Analysis of the PCR Products.**

PCR products were filtered by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE (Tris-Borate-EDTA) buffer at room temperature with a 5V/cm gradient. Gel analysis involved loading 20 µl of products into each slot. Fragment sizes were determined using a gene ruler 100 bp ladder. A gel documentation system (Alpha Innotech, Biometra) captured an image of the gel and analyzed the data using computer software.

## Result

124 *E.coli* bacteria were identified from 300 duck samples from various governorates. *E. coli* was isolated. The isolation rate was (22 isolates )22% from cloacal swabs from apparently healthy ducks. Among diseased ducks suffering from colisepticemia, *E. coli* was found in (78 isolate) 78%, with isolation from (50 isolates) 50% the liver and(28 isolates) 28% in the spleen. For freshly dead ducks, the isolation rate from bone marrow was(24 isolate) 24%. 124 *E. coli* isolates were collected from various governorates, with the following distribution: 42% from Qalyubia, 27% from Gharbia, and 31% from Giza. The *E. coli* isolates produced characteristic rose-pink colonies on MacConkey agar and displayed a green metallic sheen on Eosin Methylene Blue (EMB) agar. Of the 124 *E. coli* isolates that were styped and different serotypes were identified were identified. 124 *E. coli* strains were serotyped as depicted in Table (4).

**Table (4).** Serotypes of *E. coli* isolates from organs and cloacal swabs

| **Serotypes of *E.coli* isolates recoverd from organ (liver , spleen and bone marrow)** | | **Serotypes of *E.coli* isolates recovered from cloacal swabs** | |
|---|---|---|---|
| **Serotypes** | **Number of isolates** | **Serotypes** | **Number of isolates** |
| **O26** | 34 | **O27** | 2 |
| **O119** | 2 | **O158** | 2 |
| **O157** | 1 | **O86a** | 4 |
| **O115** | 3 | **O164** | 4 |
| **O27** | 28 | **O44** | 4 |
| **O167** | 10 | **O114** | 3 |
| **O8** | 12 | **Untypable** | 3 |
| **Untypable** | 12 | | |
| **Total** | 102 | **Total** | 22 |

Twelve *E. coli* strains of different serotypes were screened against 13 antibiotics. All strains (100%) were resistant to Ampicillin and 100% sensitive to both colistin as shown in figure (1) and table (5).

**Table (5).** Antibiotic susceptibility test against *E.coli* isolates

| **Antimicrobial agent** | **Resistant** | | **Intermediates** | | **Senstive** | |
|---|---|---|---|---|---|---|
| | **Num-ber** | **Percent %** | **Number** | **Percent %** | **Number** | **Percent %** |
| **Ampicillin (10 µg)** | 12 | 100 | 0 | 0 | 0 | 0 |
| **Chloramphenicol (30 µg)** | 5 | 41 | 2 | 16 | 5 | 41 |
| **Colistin Sulphate (10 µg)** | 0 | 0 | 0 | 0 | 12 | 100 |
| **Danofloxacine (5 µg)** | 1 | 8 | 1 | 8 | 10 | 83 |
| **Doxycycline (30 µg)** | 4 | 33 | 3 | 25 | 5 | 41 |
| **ERYTHROmycin (15 µg)** | 11 | 91 | 1 | 8 | 0 | 0 |
| **Gentamycin (10 µg)** | 1 | 8 | 0 | 0 | 11 | 91 |
| **Naldixic Acid (30 µg)** | 8 | 66 | 1 | 8 | 3 | 25 |
| **Neomycin (30 µg)** | 2 | 16 | 8 | 66 | 2 | 16 |
| **Streptomycin (10 µg)** | 9 | 75 | 3 | 25 | 0 | 0 |
| **Tetracycline (30 µg)** | 0 | 0 | 0 | 0 | 2 | 16 |
| **Trimethoprim-sulphamethoxezole (1.25/23.75 µg)** | 2 | 16 | 0 | 0 | 6 | 50 |
| **Ciprofloxacin (5 µg)** | 5 | 41 | 2 | 16 | 5 | 41 |

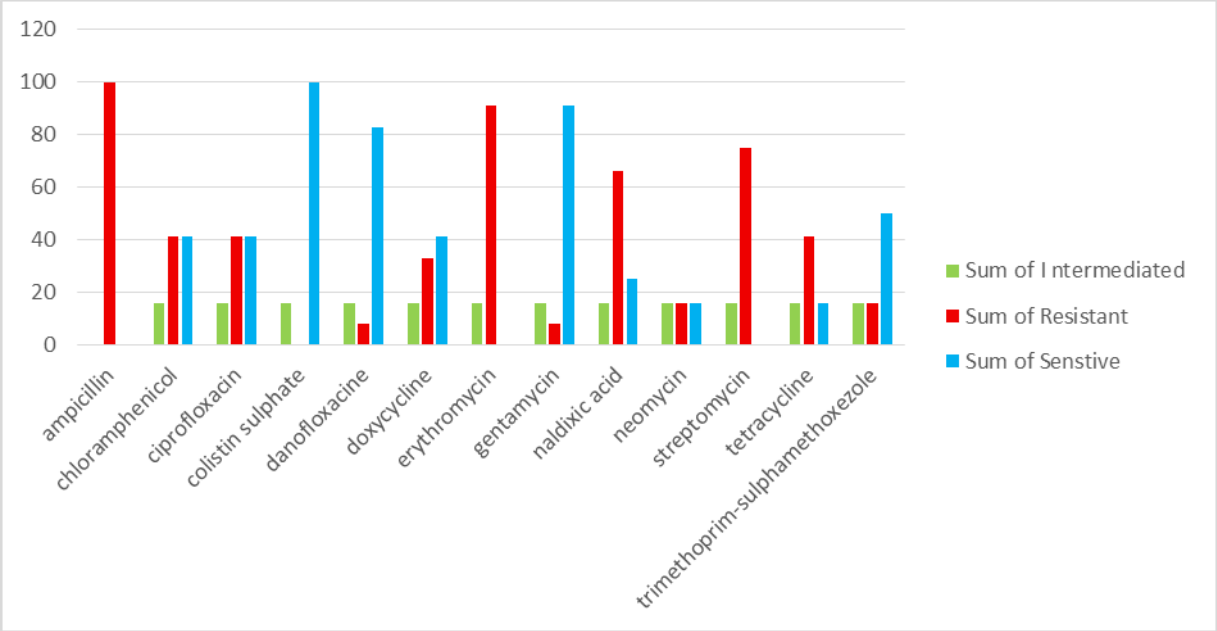


**Figure (1).** Antibiotic susceptibility profiles of *E. coli* isolates, showing the distribution of sensitive, intermediate, and resistant

A pathogenicity test of twelve *E. coli* serotypes using Congo red dye revealed varying binding affinities. Serotype O8 demonstrated high binding with Congo red, while O157, O158, and O144 exhibited less binding. The results of this research were put forward in table (2) and figure (2).

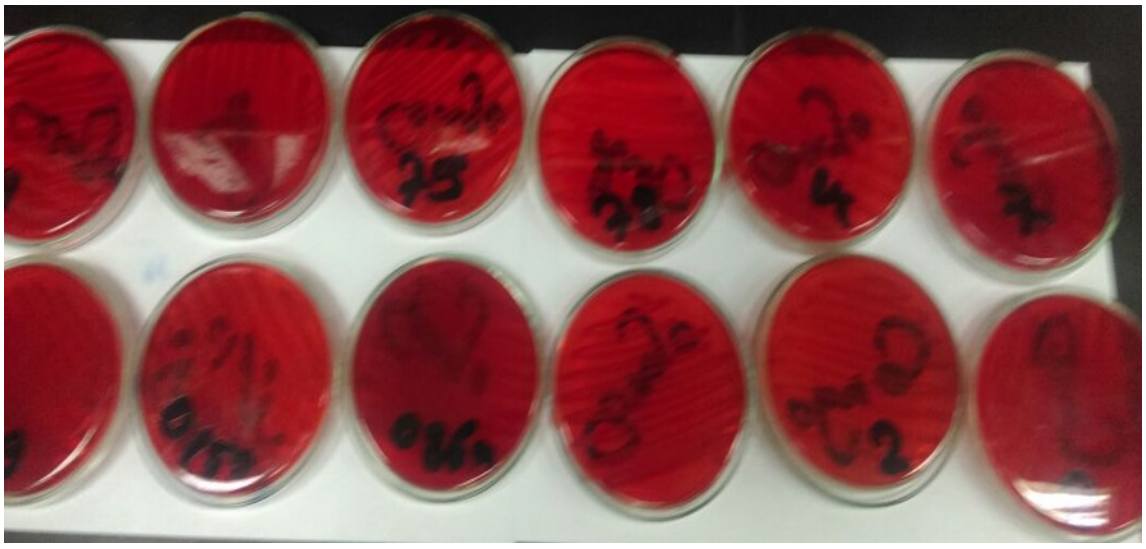

**Figure (2).** Congo Red binding by twelve *E. coli* strains on agar plates, indicating varying levels of binding.

**Table (6).** Congo Red binding by twelve *E. coli* strains on agar plates, indicating varying levels of binding.

| Serotypes | Degree of Congo red binding | Serotypes | Degree of Congo red binding |
|---|---|---|---|
| **O167** | ++ | **O8** | ++ |
| **O26** | ++ | **O158** | + |
| **O119** | ++ | **O86a** | ++ |
| **O27** | ++ | **O164** | ++ |
| **O157** | + | **O44** | ++ |
| **O115** | ++ | **O114** | + |

Twelve *E. coli* isolates, representing different serotypes, were tested for the presence of ten specific virulence genes via PCR. The outer membrane protein A gene (***ompA***) and the ferric enterobactin receptor gene (***iutA***) were ubiquitously present, with positive amplification observed in all Twelve tested strains as cleared in figure (2.3). Similarly, the plasmid-encoded protein ***traT*** gene was noticed in all strains except O114 is negative as illustrated in figure (4). In contrast, all 12 strains consistently tested negative for an existence of the Shiga toxin 1 gene (*stx1*), the Shiga toxin 2 gene (*stx2*), and the hemolysin gene (*hly*) as displayed in figure (5). The intimin gene (*eaeA*) was detected in isolates belonging to serogroups O167, O26, O119, and O27. The P fimbrial structural subunit gene (*papC*) was only negative in O167, O157, and O114. The temperature-sensitive hemagglutinin gene (*tsh*) was present in *E. coli* isolates from O119, O157, O115, and O8 serogroups. The increased serum surviving gene (*iss)* was amplified in isolates of serogroups O167, O27, O115, O8, O86a, O164, and O44.

**Table (7).** Prevalence of genes related to virulence among selected E. coli strains utilizing PCR.

| Genes | *eae*A | *pap*C | tsh | iss | *omp*A | *iut*A | *tra*T | *stx*1 | *stx*2 | *hly* |
|---|---|---|---|---|---|---|---|---|---|---|
| **O114** | - | - | - | - | + | + | - | - | - | - |
| **O44** | - | + | - | + | + | + | + | - | - | - |
| **O164** | - | + | - | + | + | + | + | - | - | - |
| **O86a** | - | + | - | + | + | + | + | - | - | - |
| **O158** | - | + | - | - | + | + | + | - | - | - |
| **O8** | - | + | + | + | + | + | + | - | - | - |
| **O115** | - | + | + | + | + | + | + | - | - | - |
| **O157** | - | - | + | - | + | + | + | - | - | - |
| **O27** | + | + | - | + | + | + | + | - | - | - |
| **O119** | + | + | + | - | + | + | + | - | - | - |
| **O26** | + | + | - | - | + | + | + | - | - | - |
| **O167** | + | - | - | + | + | + | + | - | - | - |
| **Total Positive** | **4** | **9** | **4** | **7** | **12** | **12** | **11** | **0** | **0** | **0** |

(- Negative virulence genes , + positive virulence genes )

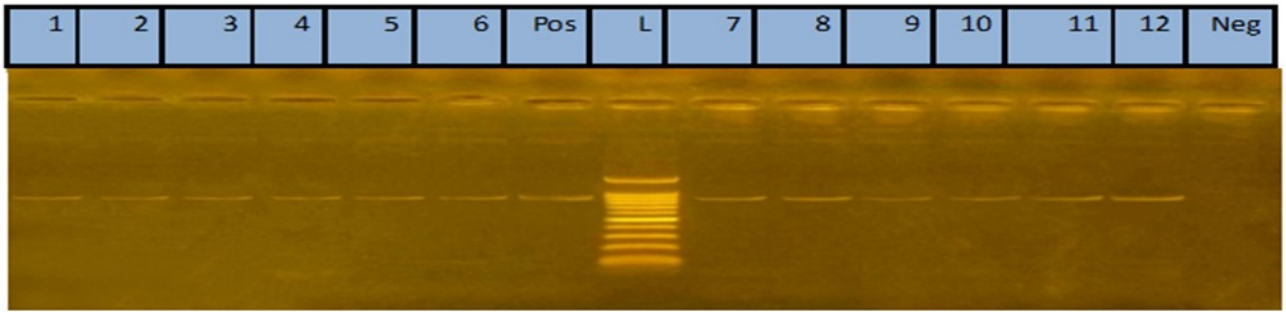


**Figure (3).** Agarose electrophoresis on gel exhibited *ompA* gene (919 bp) in selected *E. coli* isolates from ducks

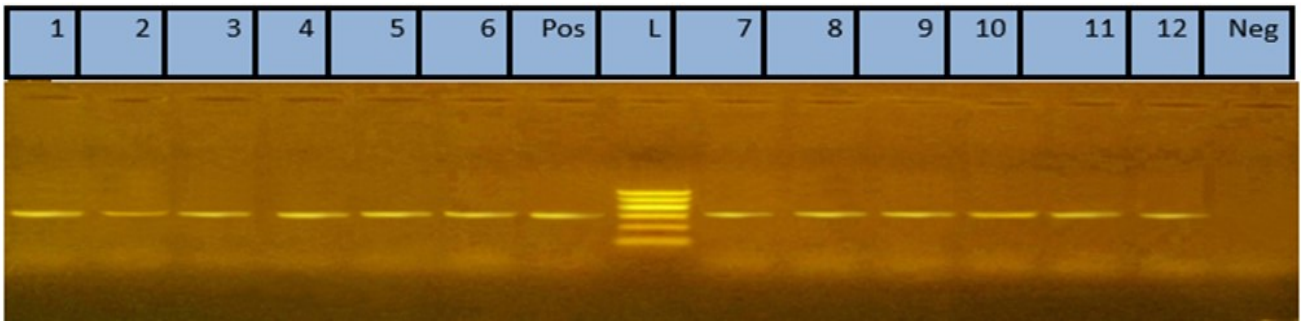


**Figure (4).** Agarose electrophoresis on gel exhibited *iutA* gene (300 bp) in selected *E. coli* isolates from ducks.

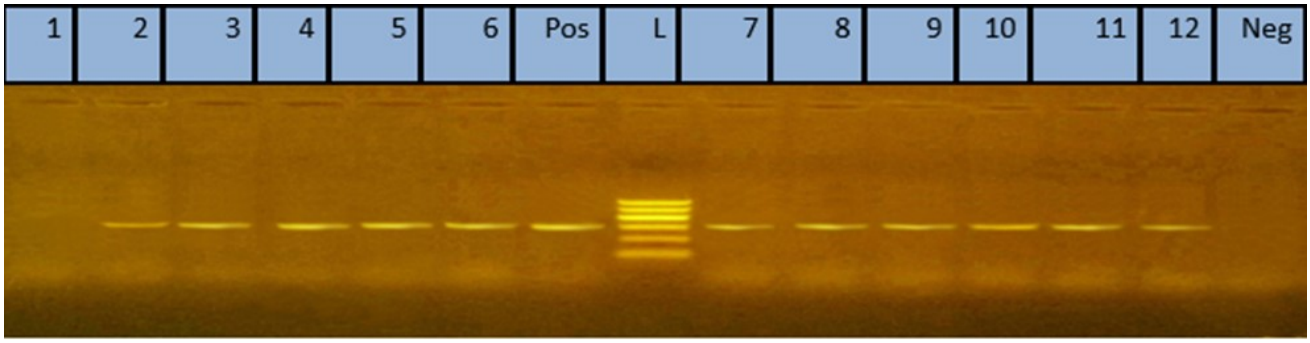


**Figure (5).** Agarose electrophoresis on gel exhibited *traT* gene (307 bp) in selected *E. coli* isolates from ducks

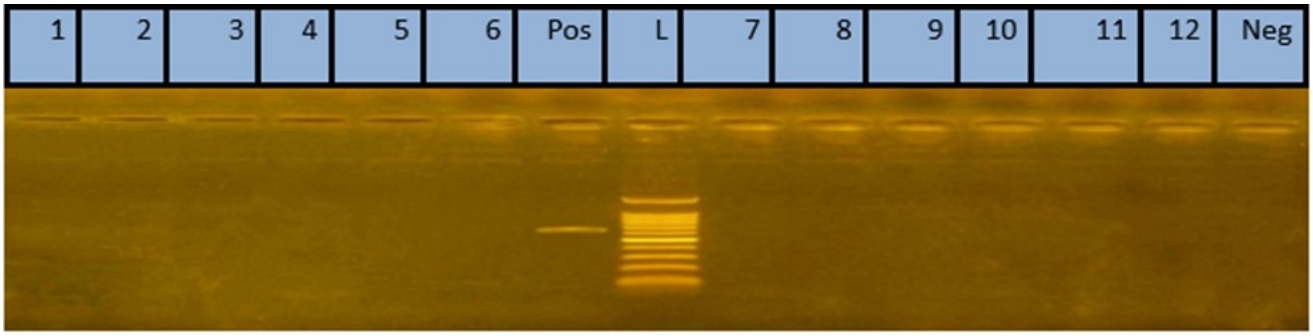


**Figure (6).** Agarose electrophoresis on gel exhibited *stx1* gene (614 bp) in selected *E. coli* isolates from ducks.

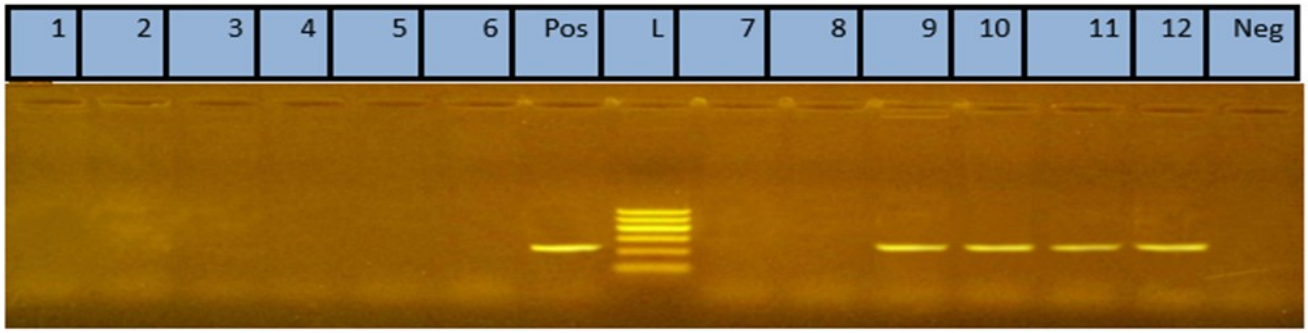


**Figure (7).** Agarose electrophoresis on gel exhibited *eaeA* gene ( (248 bp) in selected *E. coli* isolates from ducks.

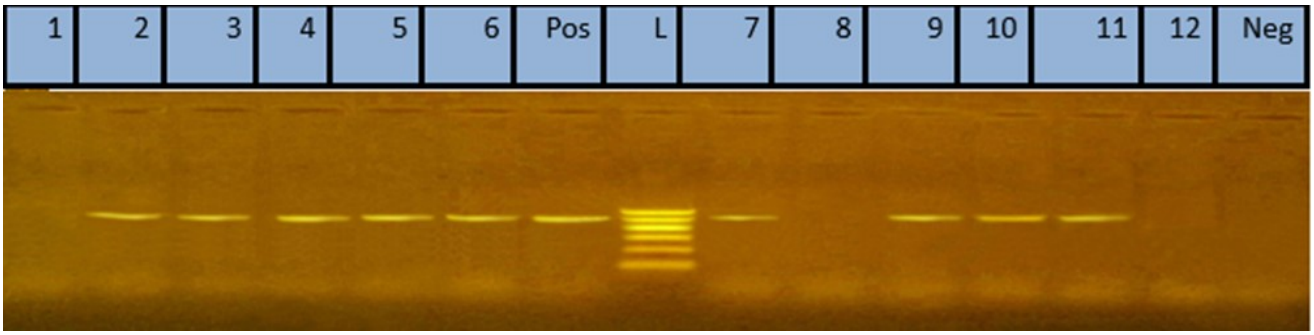


**Figure (8).** Agarose electrophoresis on gel exhibited *papC* gene (501 bp) in selected *E. coli* isolates from ducks.

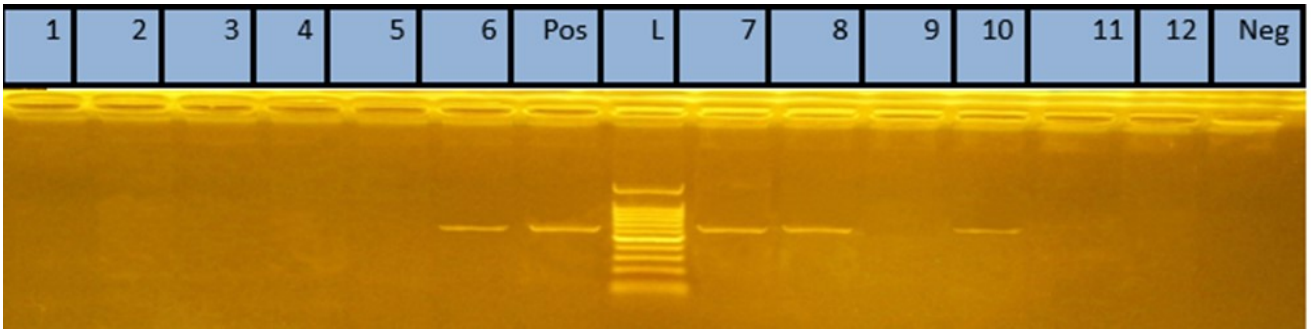


**Figure (9).** Agarose electrophoresis on gel exhibited *tsh* gene (620 bp) in selected *E. coli* isolates from ducks.

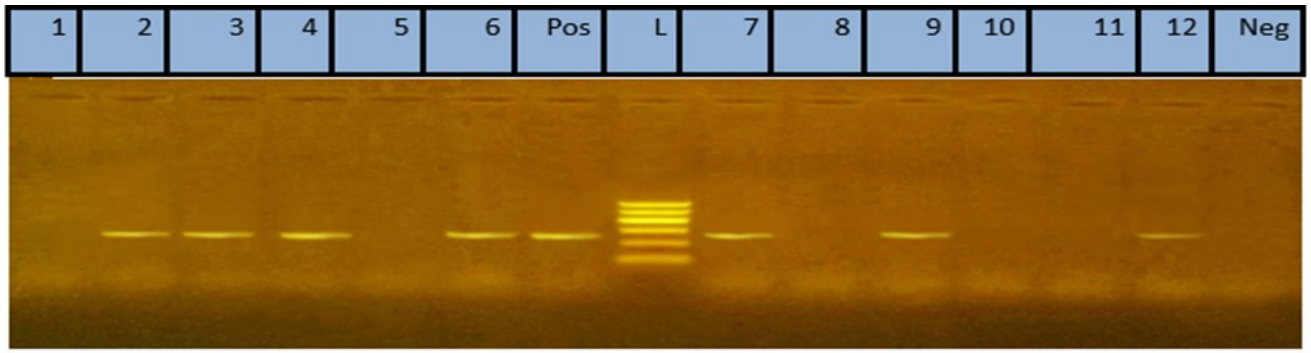


**Figure (10).** Agarose electrophoresis on gel exhibited *iss* gene (266 bp) in selected *E. coli* isolates from ducks.

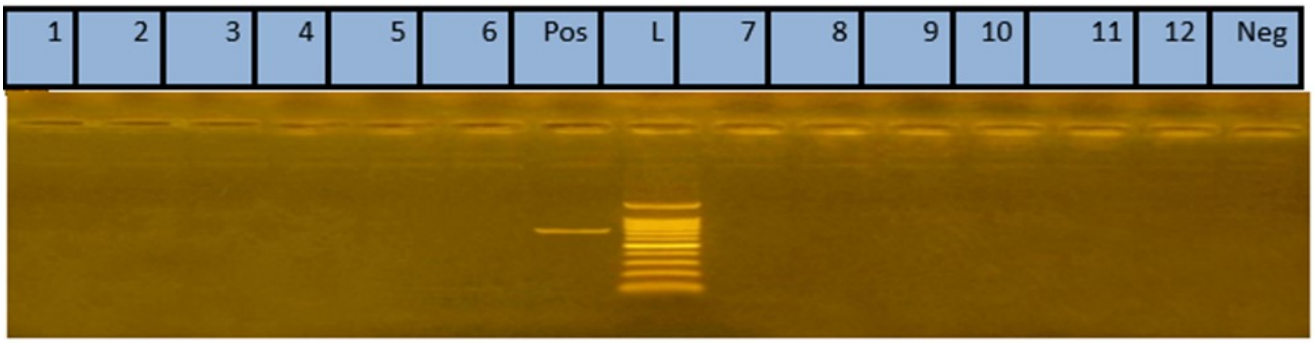


**Figure (11).** Agarose electrophoresis on gel exhibited *stx2* gene (799 bp) in selected *E. coli* isolates from ducks.

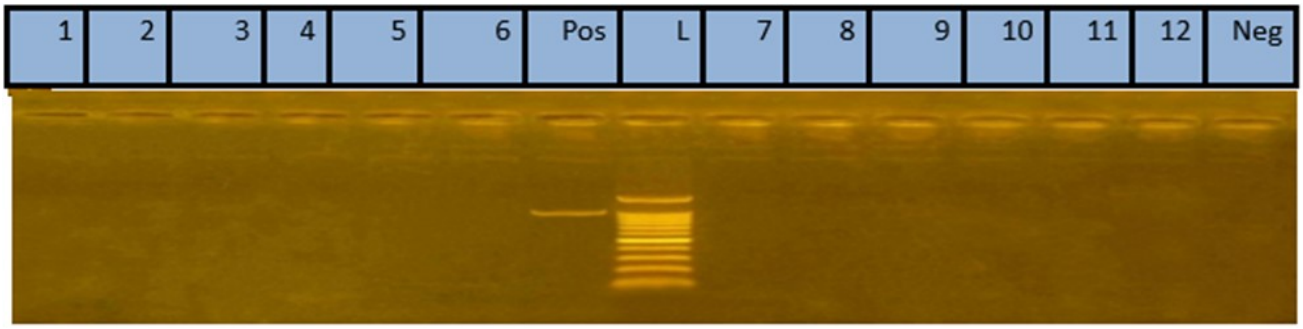


**Figure (12).** Agarose electrophoresis on gel exhibited *hly* gene (1177 bp) in selected *E. coli* isolates from ducks.

## Discussion

Initial infections in ducks typically occur via the respiratory tract, often secondary to other viral or mycoplasmal infections. While acute cases may present with generalized congestion, chronic infections are characterized by severe lesions such as pericarditis, perihepatitis, liver enlargement, air sacculitis, and pneumonia (**Johnston *et al.* 2007**).

The recovery rate of *E. coli* from cloacal swabs of apparently healthy ducks was 22%, whereas **Olawale *et al.* (2023) and El-Shall *et al.* (2020)** reported different percentages (60% and 75%) in similar contexts. Furthermore, the frequency of *E. coli* recovered from the liver and spleen of diseased ducks was consistent with the findings of **Nguyen *et al.* (2021).** A high rate of isolation from the liver and spleen indicated colisepticemia caused by invasive strains that owned iss, iutA, and tsh genes **(Kumar *et al.*, 2023).**

The presence of untypeable isolates highlights a challenge for vaccine development, as serotyping alone may not be sufficient (**Asawy *et al.*, 2010**). While some serogroups like O44, O158, and O114 are **commonly associated** with colibacillosis (**El-Jakee, *et al.* (2018).),** the isolation of O26 from internal organs disagrees with studies suggesting its rarity in chickens and ducks (**Marwah *et al.*, 2010; Amin *et al.*, 2013**). Furthermore, the isolation of O157, O119, and O114 is concerning, as these serogroups are often associated with enterohemorrhagic *E. coli* (EHEC) in humans (**Alexandre *et al.*, 2001**). All examined *E. coli* strains in this research were Congo red positive, aligning with some reports (**Hassan, 2009**) but disagreeing with others (**El-ashker, 2006**), indicating their potential pathogenicity, as Congo red binding is linked to virulence (**Bearkhoff and Vinal, 1986**).

Antibiotic susceptibility testing exhibited high levels of resistance between the *E. coli* isolates, with 100% resistance to ampicillin, 91% to erythromycin, 75% to streptomycin, 66% to naldixic acid, 41% to ciprofloxacin, and 33% to doxycycline. Conversely, all 12 isolates were 100% sensitive to colistin sulphate. These high antibiotic resistance was consistent with findings from (**Eljaaly *et al.*, 2019 Makhol *et al.* (2011)** and underscores the challenge in controlling colibacillosis due to limited effective antimicrobial options (**Aaron *et al.*, 2012**). The study also investigated a total of ten path-

ogenicity genes, revealed that *ompA* and *iutA* were present in all tested strains. Other genes *traT, papC, iss, eaeA,* and *tsh* were found in various combinations across different serotypes, with stx1, stx2, and *hly* being absent. The absence of Shiga toxin genes (*stx1, stx2*) and *hly* in all strains aligns with some studies (**Shimaa, 2013; Wen Jie *et al*., 2008**) but contradicted others (**Yao *et al*., 2011**).

## Conclusion

This study provides a vital foundation, encouraging deeper research into *APEC* virulence genes and antimicrobial resistance evolution. Its findings contribute to developing effective disease control strategies in poultry, reducing economic losses. This also supports prudent antibiotic use to preserve efficacy for both animal and human health, thereby enhancing poultry safety for human consumption, reducing zoonotic risks, and improving public health in the future.

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