



ISSN: 2456-2912

NAAS Rating (2026): 4.61

VET 2026; 11(4): 112-120

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www.veterinarypaper.com

Received: 06-02-2026

Accepted: 09-03-2026

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Identification of Antibiotic Resistance genes (blaCTX-M, sul1, gyrA, aac(3)-II) and Virulence genes: (tsh, iss, hlyF) of Avian Pathogenic *Escherichia coli* isolated from respiratory and enteric infections of broiler chickens

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DOI: <https://www.doi.org/10.22271/veterinary.2026.v11.i4b.3180>

Abstract

Avian pathogenic *Escherichia coli* (APEC) is the cause of colibacillosis in broiler chickens, resulting in significant economic losses due to high morbidity rates between poultry farms. This study designed to identify the etiology of respiratory and enteric bacterial infection in broiler chickens in Al-Diwaniyah Province, Iraq. By isolation and characterization of multidrug-resistant *E. coli* using bacteriological, histopathological and molecular approaches. Fifty samples were collected from lungs, air sacs and intestines of diseased birds. *E. coli* isolation and identification was performed using conventional culture and confirmed using VITEK system and PCR, while antibiotic sensitivity test revealed high resistance to Ampicillin, Trimethoprim/Sulfamethoxazole, Ciprofloxacin, And Gentamicin. Molecular analyses confirmed the presence of resistance genes (blaCTX-M, sul1, gyrA, aac(3)-II) and virulence genes (tsh, iss, hlyF). Histopathological examination revealed severe lesions including (necrosis, vascular congestion, and inflammatory infiltration), in the intestines, lungs, and air sacs. These findings indicate that APEC infections of broiler chickens are persists in the the area due to antibiotic resistance genes despite continuous antibiotic treatments programs applied to poultry farms also belong to the virulence genes of this bacteria, therefore antibiotic sensitivity test are required during infection to select the ideal antibiotic.

Keywords: APEC, *Escherichia coli*, tsh, iss, hlyF multidrug resistance, blaCTX-M, sul1, gyrA, aac(3)-II

Introduction

Avian colibacillosis, caused by *Escherichia coli*, is a critical bacterial disease affecting poultry worldwide. The disease manifests as septicemia, airsacculitis, pneumonia, enteritis, and systemic organ damage, leading to significant economic losses in the poultry industry (Aworh *et al.*, 2021) ^[9]. The emergence of multidrug-resistant (MDR) strains is a growing concern, particularly strains producing extended-spectrum β -lactamases (ESBLs), which complicate treatment and pose potential public health risks (Bonvegna, 2022) ^[11].

Resistance genes such as blaCTX-M, sul1, gyrA, and aac(3)-II are frequently associated with MDR *E. coli*, while virulence-associated genes, including tsh, iss, and hlyF, enhance bacterial adhesion, serum survival, and pathogenicity (Delicato *et al.*, 2002; Mellata *et al.*, 2009) ^[15, 35]. Molecular typing using Multi-Locus Sequence Typing (MLST) allows characterization of genetic lineages, helping trace epidemiological patterns and potential zoonotic transmission (Vinodhkumar *et al.*, 2024) ^[57].

Given the extensive use of antibiotics in poultry farming, surveillance of antimicrobial resistance, virulence determinants, and genetic diversity in local poultry populations is crucial. This study aims to provide comprehensive data on MDR *E. coli* in broilers in Al-Diwaniyah, Iraq, combining bacteriological, molecular, and histopathological approaches.

Materials and Methods

Bacterial Isolation and Sample Collection

A total Fifty swab samples were aseptically collected from the lungs, intestines, and air sacs of broiler and layer chickens from poultry farms in Al-Diwaniyah Province, Iraq, between September and December 2025. Samples were divided into two groups: Group 1 consisted of tissue specimens fixed in 10% neutral buffered formalin for histopathological examination, while Group 2 included swab samples used for bacteriological and molecular analyses (Figures 1 and 2). Sampling and handling procedures were performed according to standard veterinary microbiological protocols to ensure accurate pathogen recovery (Abd El-Tawab *et al.*, 2020) [1].

Bacteriological identification

Swabs were cultured on nutrient agar, MacConkey agar, and Eosin Methylene Blue (EMB) agar and incubated aerobically at 37°C for 24 hours. Lactose-fermenting colonies showing pink coloration on MacConkey agar and a metallic green sheen on EMB agar were presumptively identified as *Escherichia coli*, consistent with conventional diagnostic criteria (Ibrahim *et al.*, 2019) [23].

Confirmed identification was carried out using the VITEK® 2 automated system (bioMérieux), which has demonstrated high diagnostic accuracy for Enterobacteriaceae in recent evaluations (Moges *et al.*, 2021) [38].

Antibiotic sensitivity testing

Ten confirmed *E. coli* isolates were tested for antimicrobial susceptibility using the VITEK® 2 system against ampicillin/sulbactam, cefotaxime, ciprofloxacin, colistin, gentamicin, piperacillin/tazobactam, and trimethoprim/sulfamethoxazole. Interpretation of resistance profiles followed the standards of the Clinical and Laboratory

Standards Institute (CLSI, 2024), which provides internationally accepted breakpoints for antibiotic testing.

Molecular Detection of Resistance and Virulence Genes

Genomic DNA was extracted using a commercial kit according to the manufacturer's instructions. Conventional PCR was performed to detect resistance genes (*bla*CTX-M, *sul*1, *gyr*A, *aac*(3)-II) and virulence genes (*tsh*, *iss*, *hly*F), which are commonly associated with avian pathogenic *E. coli* (APEC) strains (Paixão *et al.*, 2021) [43]. PCR amplification conditions and gel electrophoresis analysis were performed following established molecular diagnostic protocols (Borges *et al.*, 2019) [12].

Histopathological Examination

Formalin-fixed tissues were processed routinely, embedded in paraffin, sectioned at 4-5 µm, and stained with hematoxylin and eosin (H&E). Microscopic lesions were evaluated according to standard veterinary histopathological criteria (Mesquita *et al.*, 2022) [37].



Fig 1: Swab samples obtained from infected respiratory organs and intestines

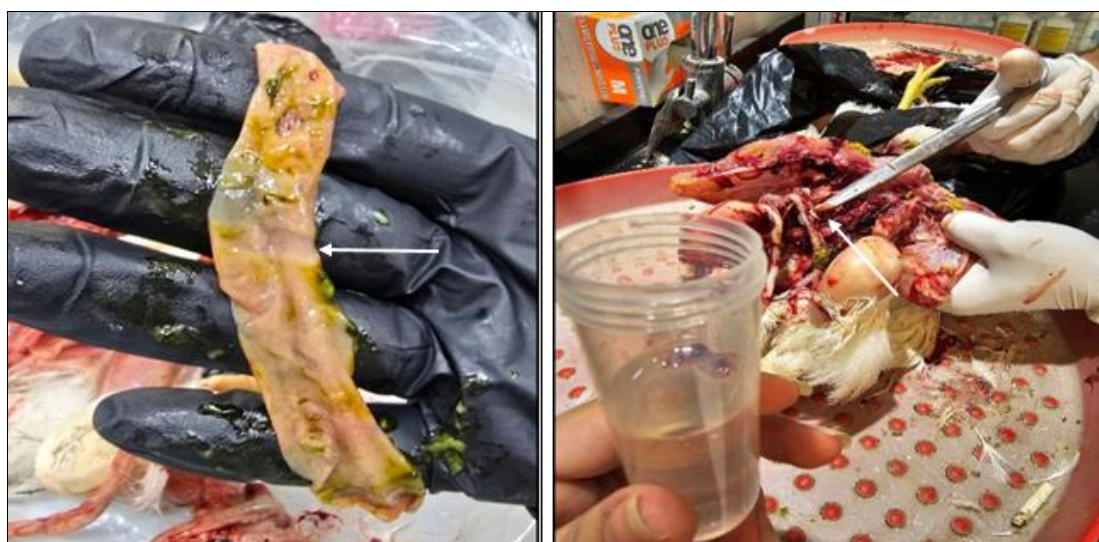


Fig 2: Tissue samples from lungs, air sacs, and jejunum were taken for histological analysis.

Results

Bacteriological Identification of *E. coli*

All lung, intestinal, and air sac swabs were cultured on nutrient agar at 37°C for 24 hours. Suspected colonies were subcultured on MacConkey agar and EMB agar.

All confirmed *E. coli* isolates demonstrated: Green metallic sheen colonies on EMB agar figure 3, Rose-pink colonies on MacConkey agar (figure .4).



Fig 3: *E. coli* growth on Eosin Methylene Blue agar EMB showing green metallic sheen colonies

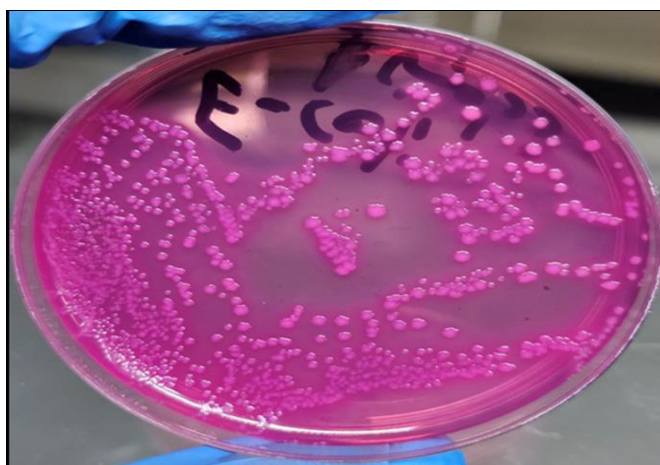


Fig 4: *E. coli* growth on MacConkey agar showing Rose-pink colonies

Antibiotic sensitivity test results

Ten confirmed *E. coli* isolates were tested against seven antimicrobial agents (Fig.5) High resistance was observed to ampicillin/sulbactam and trimethoprim/sulfamethoxazole

(100%). Moderate resistance was observed to gentamicin and ciprofloxacin (80%). Lower resistance was observed to cefotaxime and colistin (40%).

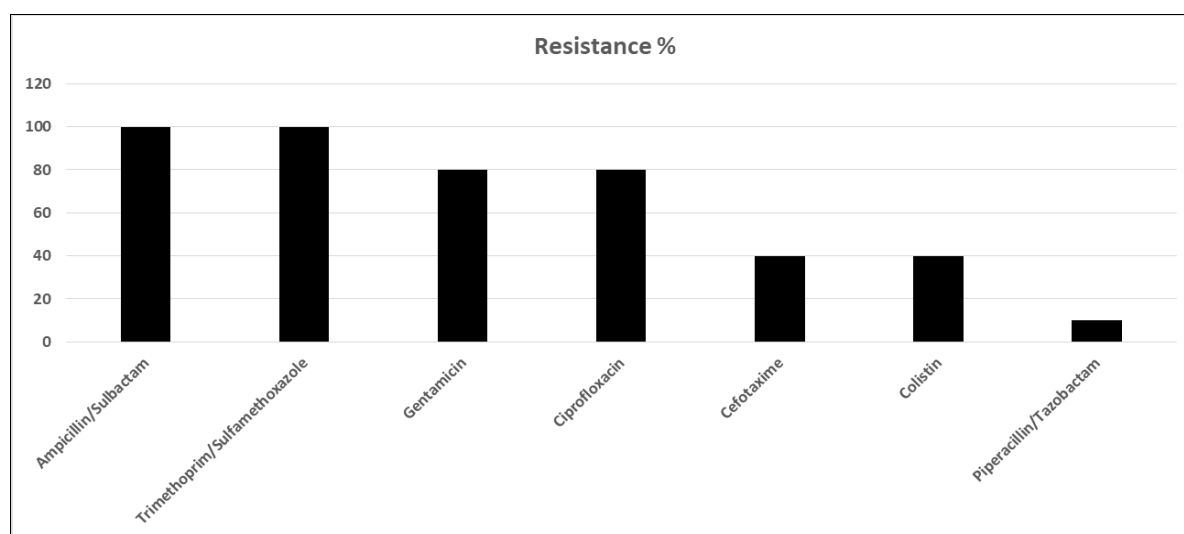


Fig 5: Antibiotic susceptibility profile of *Escherichia coli* isolates showing 100% resistance to ampicillin/sulbactam and trimethoprim/sulfamethoxazole, 80% to gentamicin and ciprofloxacin, and 40% to cefotaxime and colistin

Molecular Detection of Antimicrobial Resistance Genes

The molecular analysis of antimicrobial resistance genes revealed a high prevalence of resistance determinants among the examined *Escherichia coli* isolates. All ten isolates

(100%) demonstrated successful amplification of the blaCTX-M gene at 395 bp, indicating the widespread presence of extended-spectrum β -lactamase (ESBL)-associated resistance (Figure 6A). Similarly, the sul1 gene was detected in all

isolates (100%) at 530 bp (Figure 6B), confirming the genetic basis of sulfonamide resistance observed phenotypically.

Furthermore, the *gyrA* gene was amplified in 8 out of 10 isolates (80%) at 291 bp (Figure 6C), suggesting mutations associated with quinolone resistance. The *aac(3)-II* gene,

responsible for aminoglycoside resistance, was also detected in 8 isolates (80%) with an expected product size of 451 bp (Figure 6D). Collectively, these findings indicate the coexistence of multiple antimicrobial resistance genes among the studied isolates.

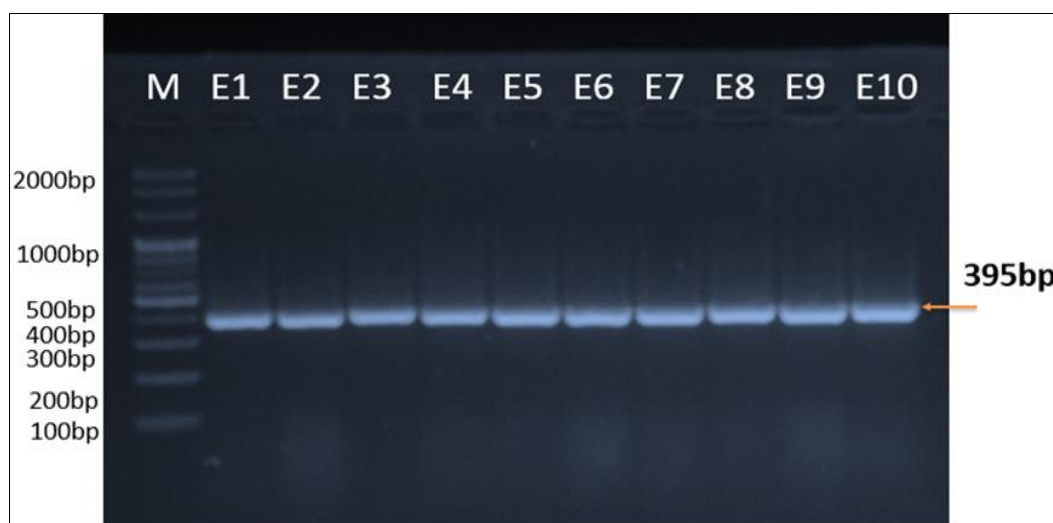


Fig 6A: PCR amplification of antibiotic resistance (Trimethoprim/sul1) gene in *E. coli* isolates using 2% agarose gel electrophoresis. M: Marker ladder (2000-100bp). The positive amplification was showed at 530bp product size

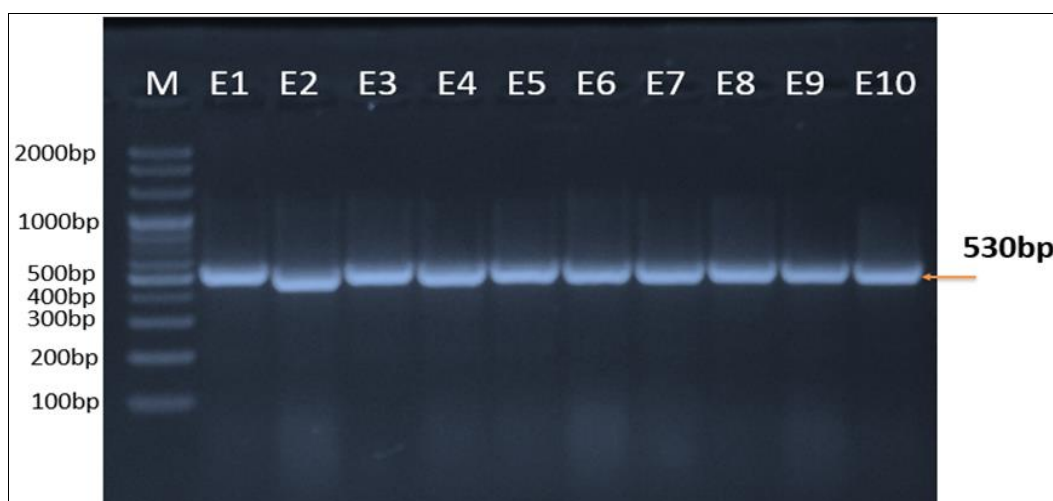


Fig 6B: PCR amplification of antibiotic resistance (Trimethoprim/sul1) gene in *E. coli* isolates using 2% agarose gel electrophoresis. M: Marker ladder (2000-100bp). The positive amplification was showed at 530bp product size

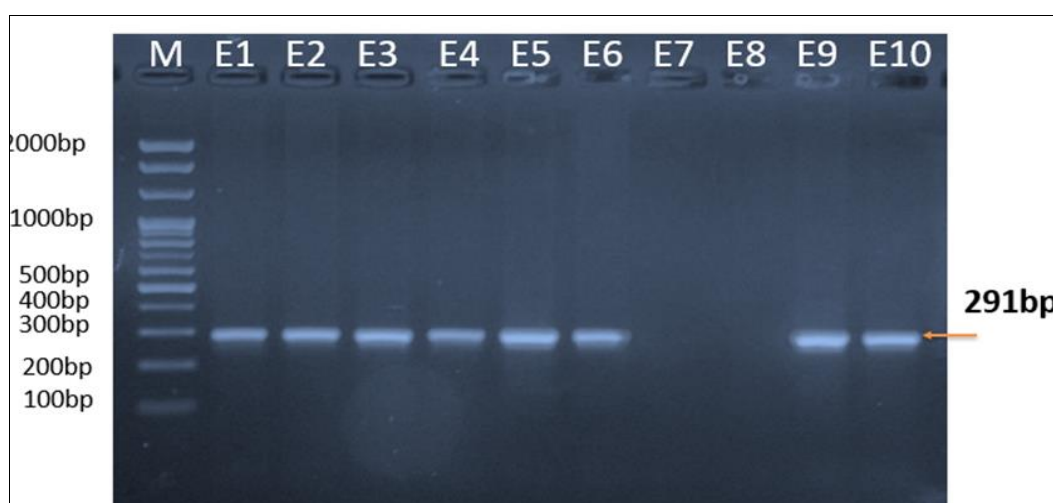


Fig 6C: PCR amplification of antibiotic resistance (Ciprofloxacin/*gyrA*) gene in *E. coli* isolates using 2% agarose gel electrophoresis. M: Marker ladder (2000-100bp). The positive amplification was showed at 291bp product size

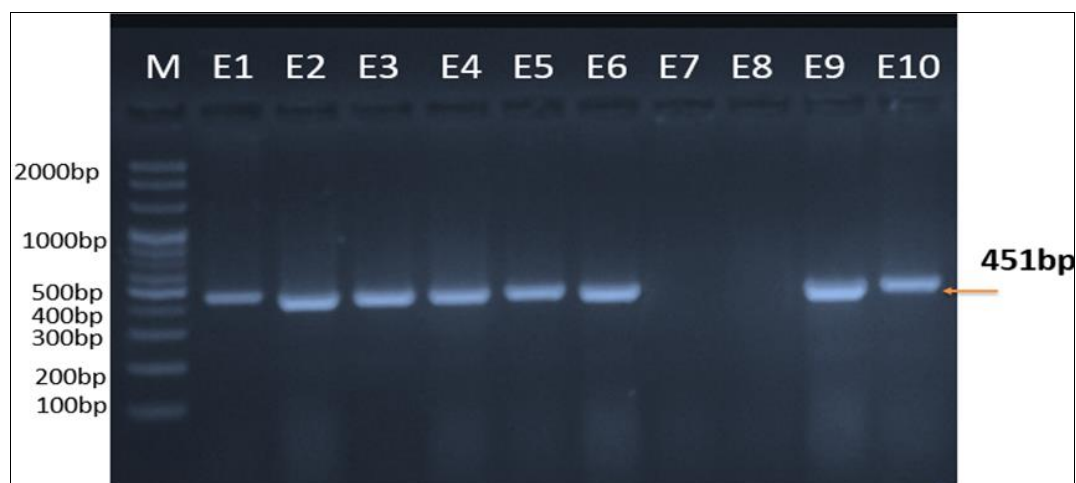


Fig 6D: PCR amplification of antibiotic resistance (Gentamicin/aac(3) II) gene in *E. coli* isolates using 2% agarose gel electrophoresis. M: Marker ladder (2000-100bp). The positive amplification was showed at 451bp product size

Molecular Detection of Virulence Genes

The distribution of virulence-associated genes demonstrated a high pathogenic potential among the isolates. The *tsh* gene was detected in all isolates (100%) with an amplicon size of 420 bp (Figure 7A). Likewise, all isolates (100%) were positive for the *iss* gene at 323 bp (Figure 7B), highlighting their enhanced serum survival capability.

In addition, the *hlyF* gene was identified in 9 out of 10 isolates (90%) at 599 bp (Figure 7C), indicating the presence of hemolysin-associated virulence factors. The high prevalence of these virulence determinants supports the classification of the isolates as avian pathogenic *E. coli* (APEC) strains.



Fig 7A: PCR amplification of virulence factor (*tsh*) gene in *E. coli* isolates using 2% agarose gel electrophoresis. M: Marker ladder (2000-100bp). The positive amplification was showed at 420bp product size

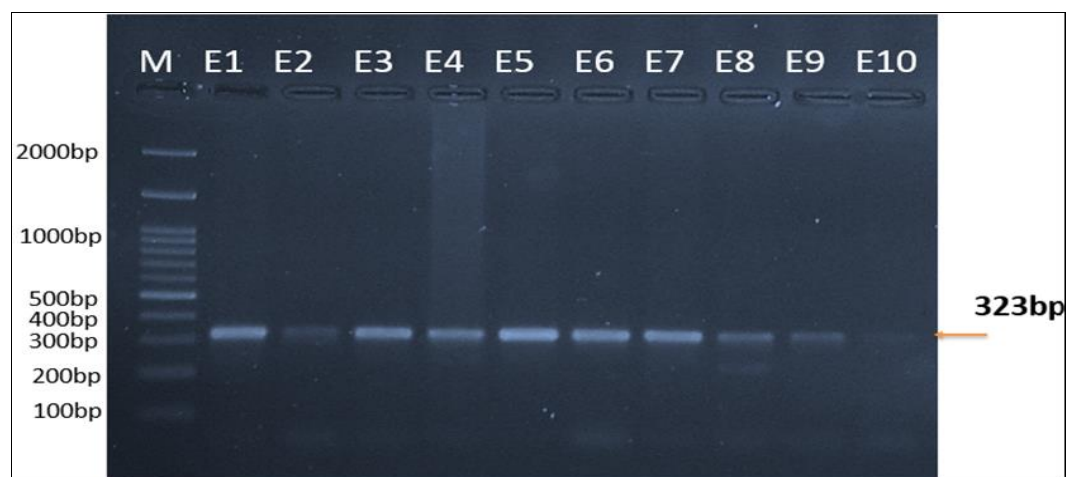


Fig 7B: PCR amplification of virulence factor (*iss*) gene in *E. coli* isolates using 2% agarose gel electrophoresis. M: Marker ladder (2000-100bp). The positive amplification was showed at 323bp product size

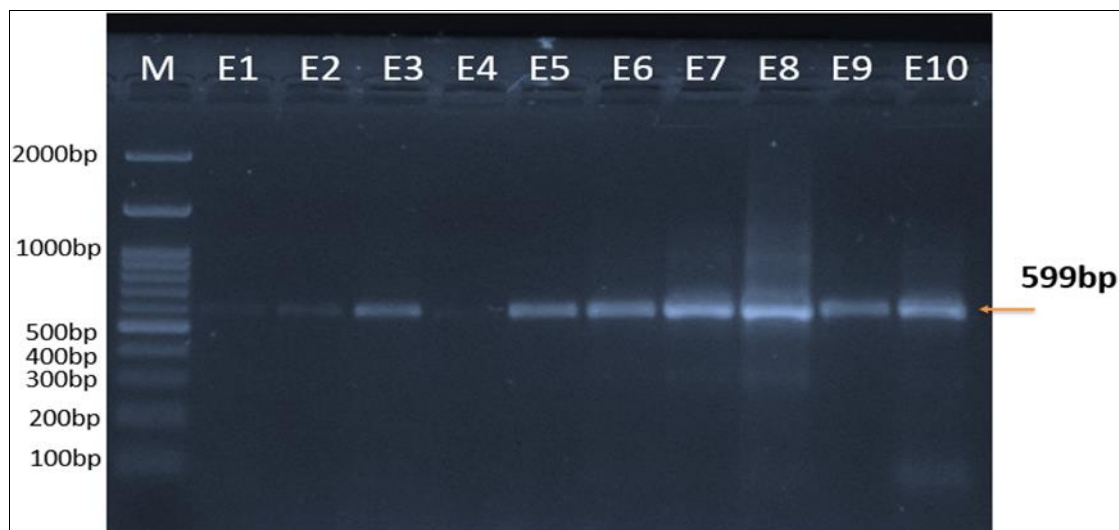


Fig 7C: PCR amplification of virulence factor (*hlyF*) gene in *E. coli* isolates using 2% agarose gel electrophoresis. M: Marker ladder (2000-100bp). The positive amplification was showed at 599bp product size

Results of Histopathological Changes of Affected Organs Intestine

Microscopic examination of intestinal sections from infected chickens revealed severe pathological alterations consistent with hemorrhagic enteritis. The mucosal layer showed marked erosion and focal areas of necrosis, accompanied by extensive inflammatory cell infiltration extending into the submucosa. The inflammatory infiltrate consisted predominantly of lymphocytes and macrophages, indicating an active immune response. Additionally, pronounced vascular dilation and congestion were observed within the lamina propria and submucosal blood vessels. These lesions collectively reflect significant intestinal tissue damage associated with *E. coli* infection (Figure8).

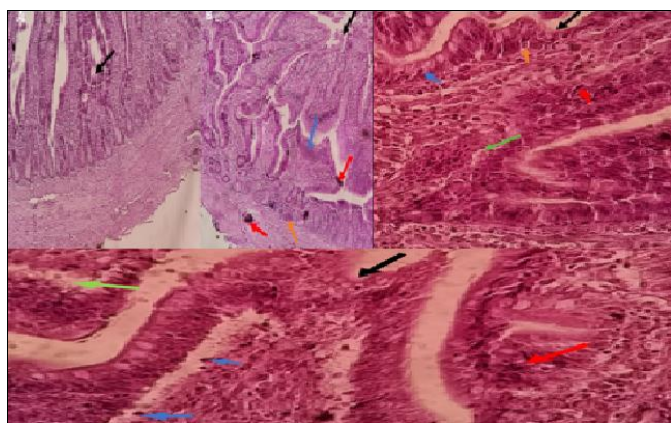


Fig 8: Histopathological changes in the intestine of chickens infected with *E. coli*.

- (A) Longitudinal section showing mucosal erosion and inflammatory infiltration (H&E, 100×).
 (B) Cross section demonstrating necrosis and submucosal inflammatory cell infiltration (H&E, 400×).
 (C) High magnification illustrating lymphocytes (blue arrows), macrophages (orange arrows), mucosal erosion (black arrows), necrosis (green arrows), and vascular congestion (red arrows) (H&E, 400×).

Lung

Histopathological evaluation of lung tissues revealed lesions compatible with serous to purulent pneumonia. There was extensive inflammatory infiltration, mainly composed of

lymphocytes, surrounding and infiltrating the alveolar structures. Marked alveolar necrosis and structural disruption were evident, along with vascular dilation and congestion within pulmonary vessels. In some areas, fibrosis was observed, suggesting chronic inflammatory progression. These findings indicate severe pulmonary involvement in response to *E. coli* infection (Figure9).

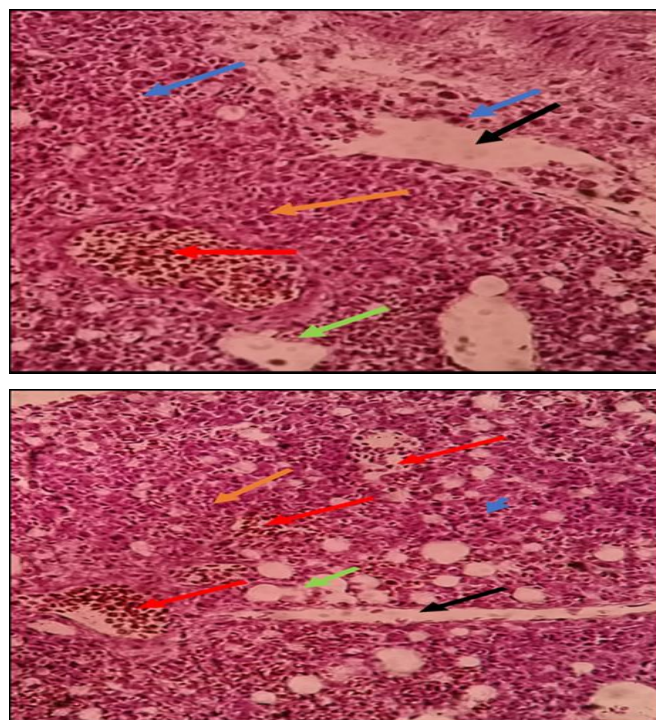


Fig 9: Histopathological changes in the lung of chickens infected with *E. coli*

- (A) Longitudinal section showing inflammatory infiltration and vascular congestion (H&E, 400×).
 (B) Cross section demonstrating alveolar necrosis, fibrosis, lymphocytic infiltration (blue arrows), erosion (black arrows), necrosis (green arrows), vascular dilation and congestion (red arrows), and fibrosis (orange arrows) (H&E, 400×).

Air Sacs

Microscopic examination of air sac tissues showed characteristic lesions of airsacculitis. The sections revealed

marked inflammatory cell infiltration, predominantly lymphocytes, along with hyperplasia of the epithelial lining. Areas of necrosis were observed in association with fibrinous exudate deposition. Prominent vascular dilation, congestion,

and early fibrotic changes were also evident, reflecting both acute and chronic inflammatory processes. These pathological findings confirm severe air sac involvement in infected chickens (Figure10).

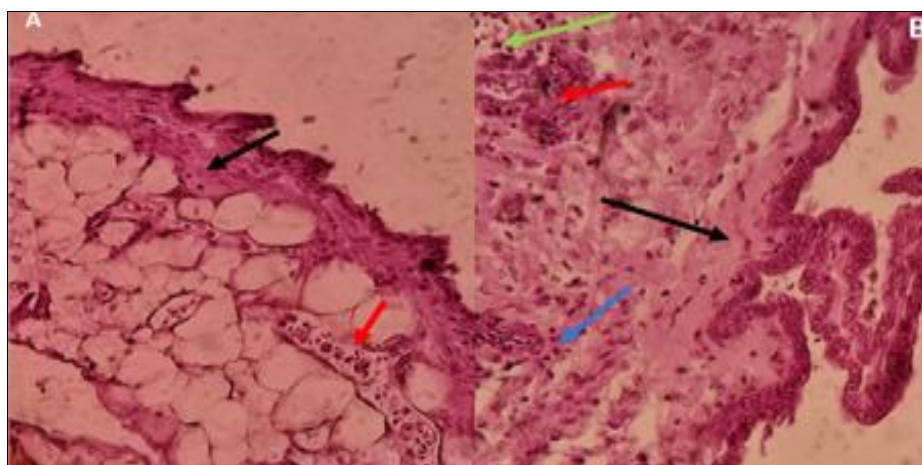


Fig 10: Histopathological changes in the air sacs of chickens infected with *E. coli*

(A) Cross section showing hyperplasia and inflammatory infiltration (H&E, 400×).

(B) Longitudinal section demonstrating lymphocytic infiltration (blue arrows), hyperplasia (black arrow), necrosis (green arrow), and vascular congestion (red arrow) (H&E, 400×).

Discussion

The present study revealed a high prevalence of multidrug-resistant (MDR) *Escherichia coli* isolates recovered from poultry farms in Al-Diwaniyah Province. The observed 100% resistance to ampicillin/sulbactam and trimethoprim/sulfamethoxazole is consistent with previous global findings reporting elevated resistance rates to β -lactams and sulfonamides in poultry-associated *E. coli* isolates (Xexaki *et al.*, 2023; Soodan, 2022; Nasrollahian *et al.*, 2024) [40]. The extensive use of these antimicrobial agents in poultry production, either for therapeutic or prophylactic purposes, is considered a major selective pressure driving this resistance (Islam *et al.*, 2023) [24].

The 80% resistance observed to gentamicin and ciprofloxacin further supports the presence of MDR phenotypes in poultry environments. Similar patterns of increasing resistance to fluoroquinolones and aminoglycosides have been documented in poultry isolates worldwide (Tigabie *et al.*, 2023; Shariati *et al.*, 2022; Veloo *et al.*, 2025) [48, 56]. Although lower resistance rates were detected for cefotaxime and colistin (40%), the emergence of resistance to colistin is particularly alarming because it is considered a last-resort antibiotic in human medicine (Amegayibor *et al.*, 2025) [8]. The spread of mobile colistin resistance genes such as *mcr* has been widely reported and represents a serious One Health concern (Mousavi *et al.*, 2025).

Molecular analysis demonstrated that all isolates (100%) harbored the *bla*CTX-M gene. This finding indicates widespread dissemination of ESBL-producing strains in poultry farms, similar to reports from Indonesia (Faridah *et al.*, 2023), Senegal (Cissé *et al.*, 2024) [13], Pakistan (Saeed *et al.*, 2023) [20], and Iran (Moradzadeh *et al.*, 2025) [39]. The dominance of CTX-M-type ESBL genes in animal production systems has been widely documented (Bonvegna, 2022) [11], confirming their global expansion across different hosts and ecological niches.

Similarly, the *sul1* gene was detected in 100% of isolates. This high prevalence aligns with local Iraqi findings reported by (Hussein *et al.* 2025), as well as international data describing frequent detection of *sul* genes in poultry-associated *E. coli* (Elmorsy *et al.*, 2025; Zahoor *et al.*, 2024) [19, 61]. Sulfonamide resistance genes are commonly associated with integrons and plasmids, facilitating both vertical and horizontal transmission (Ben *et al.*, 2017; Rehman *et al.*, 2017). The ability of mobile genetic elements to enhance dissemination of *sul* genes has been well described (Sköld, 2001; Perreten & Boerlin, 2003; Sagor *et al.*, 2022) [44, 45, 49].

The *gyrA* gene was detected in 80% of isolates, confirming the molecular basis of ciprofloxacin resistance. Mutations in the quinolone resistance-determining region (QRDR) of *gyrA* are widely recognized as key mechanisms of fluoroquinolone resistance (Song *et al.*, 2020) [51]. High prevalence of ciprofloxacin-resistant *E. coli* in broiler farms has been reported in Bangladesh (Das *et al.*, 2023), although regional variations have been observed (Das *et al.*, 2020; Masood *et al.*, 2019). Local Iraqi studies have also documented *gyrA*-mediated resistance among clinical isolates (Al-Fayyadh *et al.*, 2017) [6].

The *aac*(3)-II gene, detected in 80% of isolates, explains the phenotypic resistance to gentamicin. Aminoglycoside-modifying enzymes such as AAC (3)-II reduce antibiotic binding to ribosomes, thereby impairing protein synthesis inhibition (Cirit *et al.*, 2019). The co-occurrence of aminoglycoside resistance genes with other antimicrobial resistance determinants in poultry isolates has been reported in genomic studies (Aworh *et al.*, 2021; Mak *et al.*, 2022) [9, 33].

Regarding virulence factors, the universal detection of *tsh* and *iss* genes (100%) and the high prevalence of *hlyF* (90%) indicate that these isolates possess significant pathogenic potential. The *tsh* gene plays an important role in adhesion and colonization of the avian respiratory tract (Dozois *et al.*, 2000; Delicato *et al.*, 2002) [15, 17]. High prevalence of *tsh* among APEC isolates has been reported in recent studies (Abdelhady *et al.*, 2025; Hasanet *et al.*, 2023; Shah *et al.*, 2025) [2, 22, 47].

The *iss* gene enhances serum survival and resistance to complement-mediated killing (Subedi *et al.*, 2018) [52], and its high prevalence in poultry isolates has been documented in

several countries including Italy, Chile, Ireland, and Bangladesh (Gambi *et al.*, 2022; Delicato *et al.*, 2003; McPeake *et al.*, 2005; Hossain *et al.*, 2021) [16, 34].

Detection of hlyF in 90% of isolates suggests the presence of ColV-like virulence plasmids, which frequently carry multiple virulence determinants (Mellata *et al.*, 2009; Zhuge *et al.*, 2018) [35, 63]. High prevalence of hlyF in poultry isolates has been reported in Brazil and South Korea (Jeong *et al.*, 2021; Lúcio *et al.*, 2025) [26], and its dissemination may reflect selective pressure in intensive poultry systems (Alanazi, 2016) [5].

Histopathological findings in intestines, lungs, and air sacs confirmed severe inflammatory and necrotic changes. Hemorrhagic enteritis and mucosal erosion observed in the present study are consistent with intestinal barrier disruption mechanisms described previously (Olkowski *et al.*, 2005; Koynarski *et al.*, 2010) [41]. Inflammatory cell infiltration reflects activation of host immune defense mechanisms (Kogut *et al.*, 2018) [29], while tissue necrosis represents advanced pathological progression (Kapakin *et al.*, 2015).

Pulmonary lesions including serous/purulent pneumonia and alveolar necrosis correspond with previously described pathological stages of bacterial pneumonia (Song *et al.*, 2022; Li *et al.*, 2023; Abdelhamid *et al.*, 2024; Jahan, 2023) [3, 25, 50]. Air sac lesions characterized by congestion, fibrinous exudate, and fibrosis are typical manifestations of colibacillosis in poultry (Dutta *et al.*, 2013; Srinivasan *et al.*, 2014; Govindhasamy *et al.*, 2023; Zaki *et al.*, 2025) [18, 21, 62].

From an immunological perspective, bacterial virulence factors interact with host immune regulatory mechanisms, potentially affecting disease severity. Recent research highlights the importance of enzymatic immune regulators in poultry antiviral and antibacterial responses, including the role of amylase-mediated pathways in immune modulation (Al-Ibadi, *et al.*, 2025) [7]. Such interactions may influence the progression and systemic impact of bacterial infections.

Conclusion

The study revealed that *Escherichia coli* which have been isolated from broiler chickens in Al-Diwaniyah strong antibiotic resistance, including ampicillin and trimethoprim/sulfamethoxazole, along with the presence of multiple resistance genes (blaCTX-M, sul1, gyrA, aac(3)-II). Virulence-associated genes (tsh, iss, hlyF) were also frequently detected, indicating a high pathogenic potential. These findings underscore the importance of continuous monitoring of antibiotic use in poultry production and the implementation of biosecurity and infection control measures to limit the spread of resistant and pathogenic bacteria and protect both animal and human health.

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How to Cite This Article

Alameer DH, Al-Ibadi INA. Identification of Antibiotic Resistance genes (blaCTX-M, sul1, gyrA, aac(3)-II) and Virulence genes: (tsh, iss, hlyF) of Avian Pathogenic *Escherichia coli* isolated from respiratory and enteric infections of broiler chickens. *International Journal of Veterinary Sciences and Animal Husbandry*. 2026; 11(4): 112-120.

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