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Virulence Genes Profile, Biofilm Formation, and Resistance to Disinfectant Agents in Avian Pathogenic *Escherichia coli* (APEC) Isolated from Broiler Farms

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ABSTRACT

Background and Aim: Avian pathogenic *Escherichia coli* (APEC) is a major cause of colibacillosis in poultry, contributing to systemic disease and economic loss. This study investigated virulence genes profile, biofilm formation, and antimicrobial and disinfectant resistance in *Escherichia (E.) coli* isolates from broiler chickens in Qazvin, Iran.

Materials and Methods: Liver samples were collected from 50 broiler chickens diagnosed with colibacillosis. *Escherichia coli* isolates were confirmed biochemically. Antibiotic susceptibility was evaluated using disk diffusion method, and virulence genes were detected via PCR. Biofilm formation was assessed using crystal violet staining. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for formaldehyde, glutaraldehyde, and hydrogen peroxide were determined using broth microdilution.

Results: Among 50 isolates, 82% exhibited multidrug resistance (MDR) and 30% were extended-spectrum beta-lactamase (ESBL) producers. The most frequent and the least common genes were *iss* (66%), and *papC* (4%), respectively. All isolates formed biofilms, with 72% classified as strong producers. Formaldehyde and hydrogen peroxide exhibited the lowest MIC and MBC values (≤ 0.009%), while glutaraldehyde required higher concentrations for inhibitory and bactericidal activity (0.078%).

Conclusion: High rates of MDR and biofilm formation among APEC isolates highlight the need for effective antimicrobial stewardship and disinfection strategies in poultry production systems.

Keywords: Antimicrobial Resistance, Biofilm, Broilers, *Escherichia coli*, Pathogenic Genes

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1. Introduction

Avian pathogenic *Escherichia coli* (APEC) is a subset of extra-intestinal pathogenic *E. coli* (ExPEC) capable of causing colibacillosis, a systemic disease in poultry characterized by septicemia and lesions in multiple organs. Common pathological manifestations include pericarditis, air sacculitis, perihepatitis, and peritonitis **(1)**. Colibacillosis is a leading cause of morbidity and

mortality in poultry, resulting in considerable economic losses, estimated at approximately $40 million annually in the United States alone **(2)**. APEC primarily invades the respiratory and gastrointestinal tracts, particularly under stress-related conditions that compromise epithelial barriers **(3)**. Vertical transmission from infected breeders via contaminated eggs has also been documented as a significant route of spread **(4)**. Importantly, APEC strains share several virulence-associated genes with human ExPEC strains, raising concerns about their potential zoonotic threat **(5)**.

Key virulence genes associated with APEC strains include *vat* (virulence-associated toxin), *papC* (P fimbrial adhesin), *iucD* (a gene involved in the synthesis of the iron-uptake system), *iss* (increased serum survival), *tsh* (temperature-sensitive hemagglutinin), *astA* (heat-stable enterotoxin), and *cvaC* (colicin V production) **(6, 7)**. Previous studies have reported high detection rates of *iss* (60–100%), *iucD* (40–98%), *tsh* (25–70%), and *cvaC* (20–60%) in APEC isolates from poultry, while *papC* and *astA* have been detected less frequently, ranging from 5–30% depending on geographic location and production system **(8-12)**. The presence of these virulence determinants enhances APEC's pathogenic potential, emphasizing the importance of targeted control measures in poultry farming.

Biofilm formation is another key survival mechanism for APEC. Biofilms protect bacteria from environmental stresses and antimicrobial agents, making infections harder to treat and eradicate **(13, 14)**. Moreover, biofilms promote horizontal gene transfer, facilitating the spread of antimicrobial resistance (AMR) and virulence genes among bacterial populations, even across species **(15)**. The extracellular matrix of biofilms offers physical protection, further contributing to persistence against antibiotics, disinfectants, and host immune defense. This resilience complicates the management of biofilm-associated infections in both clinical and agricultural settings **(16)**.

Antibiotic resistance is widespread in poultry operations, with resistant bacteria capable of transmission to humans via contaminated food, water, or direct contact with poultry or waste products **(17)**. Recognizing this threat, international health organizations have identified antimicrobial resistance as a critical global health issue **(18, 19)**. Due to its ubiquity in the gut and adaptability, *E. coli* serves as a useful indicator organism for monitoring resistance trends in livestock. Notably, poultry-derived *E. coli* strains have been identified as potential reservoirs for resistance genes that could be transferred to human pathogens **(20)**.

Effective hygiene practices and proper use of disinfectants are essential components of infection control. Broad-spectrum disinfectants play a crucial role in reducing environmental bacterial loads and preventing re-infection of flocks and workers **(21)**. However, misapplication, such as using incorrect concentrations, expired products, or failing to clean surfaces before disinfection, can undermine their efficacy **(22)**. Given these challenges, the present study aims to isolate *E. coli* strains from broiler chickens diagnosed with colibacillosis in Qazvin, Iran, and to characterize their virulence genes profile, biofilm-forming ability, and resistance to both antimicrobial drugs and common disinfectants. The findings will provide valuable insights to inform clinical treatment strategies and preventive measures, contributing to improved poultry health and reduced economic impact on the industry.

2. Materials and Methods

2.1 Sampling and Bacterial Isolation

Between May 2023 and March 2024, liver samples were collected from 50 broiler chickens diagnosed with colibacillosis. Birds were selected using a convenience sampling method from commercial broiler farms in Qazvin Province, Iran, based on the presence of clinical signs and postmortem lesions consistent with colibacillosis. All procedures involving animals were reviewed and approved by the Ethics Committee of Qazvin University of Medical Sciences (Approval code: IR.QUMS.REC.1402.185). Sample collection was conducted following institutional and national guidelines for animal care and use. The samples were transferred to the Microbiology Research Center of Qazvin University of Medical Sciences for further processing under sterile conditions. Aseptic swabs from the liver samples were streaked onto MacConkey agar (HiMedia, M081) and incubated aerobically at 37°C for 24 hr. Colonies were then sub-cultured onto eosin methylene blue (EMB) agar (HiMedia, M317) and incubated at 37°C overnight. Colonies exhibiting a characteristic green metallic sheen, as well as other typical *E. coli* morphologies, were selected as suspected *E. coli*. These isolates were then confirmed by colony morphology, Gram staining, and a series of biochemical tests (indole, methyl red, Voges-Proskauer, citrate utilization, catalase, oxidase, and motility indole ornithine tests).

2.2 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was conducted using disk diffusion method under the Clinical and Laboratory Standards Institute (CLSI 2024) guidelines. CLSI M100-34 (2024) was used for antibiotics with

human breakpoints, and CLSI VET01-S2 was used for veterinary-specific agents. The following antibiotics were evaluated: cefoxitin (FOX), ceftazidime (CAZ), ceftazidime-clavulanate (CZA), cefotaxime (CTX), cefotaxime-clavulanate (CTC), cefepime (FEP), chloramphenicol (C), meropenem (MEM), gentamicin (GM), ciprofloxacin (CP), trimethoprim-sulfamethoxazole (SXT), nitrofurantoin (F), doxycycline (D), enrofloxacin (NFX), and florfenicol (FF). In addition, the minimum inhibitory concentration (MIC) of colistin was determined using broth microdilution method. Quality control for the antibiotic disks was performed using *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas (P.) aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 as standards for the Mueller-Hinton agar culture medium. Extended-spectrum beta-lactamase (ESBL)-producing isolates were detected using phenotypic combination disk diffusion techniques as described previously **(19)**. Multidrug resistance (MDR) refers to the acquired inability to respond to at least one drug from three or more different categories of antimicrobials. On the other hand, extensively drug resistance (XDR) is characterized by the lack of susceptibility to at least one drug in all but two or fewer categories of antimicrobials **(23)**.

2.3 Detection of virulence genes

Seven virulence genes associated with APEC isolates were investigated using Polymerase Chain Reaction (PCR) with specific primer sequences outlined in Table 1. A positive control strain was included for each gene. DNA extraction was performed using the boiling method **(24)**. Briefly, bacterial suspensions were adjusted to 0.5 McFarland standard in 1X TE buffer, vortexed, boiled for 10 min, centrifuged, and the supernatant was stored at -20°C. PCR amplification involved an initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 sec at 94°C (denaturation), 30 sec at 54°C (annealing), and 2 min at 72°C (extension), concluding with a final extension at 72°C for 10 min. PCR products were visualized on 2% agarose gel using transillumination.

Table 1. Primer sequences of *E. coli* virulence genes.

Genes	Primer sequences (5′➔3′)	Size (bp)	Hybridization Temperatures
vat	F-GTATATGGGGGGCAACATAC R-GTGTCAGAACGGAATTGTCG	708	58
papC	F-GTGGCAGTATGAATGACCGTTA R-ATATCCTTTCTGCAGGGATGCAATA	205	60
iucD	F-ACAAAAAGTTCTATCGCTTCC R-CCTGATCCAGATGATGCTC	693	58
iss	F-CAGCAACCCGAACCACTTGATG R-AGCATTGCCAGAGCGGCAGAA	323	60
tsh	F-GGGAAATGACCTGAATGCTGG R-CCGCTCATCAGTCAGTACCAC	420	54
astA	F-TGCCATCAACACAGTATATCC R-TAGGATCCTCAGGTCGCGAGTGA	111	50
cvaC	F-TGGTAGAATGTGCCAGAGCAAG R-GACTGTTTGTAGCGAAGCC	1181	60

2.4 Biofilm Formation

The biofilm formation ability of the isolates was assessed using crystal violet staining method **(25)**. Confirmed *E. coli* isolates were streaked on tryptic soy agar (TSA) and incubated at 37°C for 24 hr. Fresh colonies were then inoculated into 5 mL of sterile tryptic soy broth (TSB) and incubated at 37°C for 18–24 hr with shaking at 120 rpm. The optical density (OD) was measured at 600 nm after standardizing the bacterial suspension to 0.5 McFarland standard (1.5×10^8 CFU/mL). Each bacterial suspension (200 µL) was placed in a sterile 96-well microplate, alongside a positive control (strong biofilm producer; *P. aeruginosa*) and a negative control (TSB without bacteria), and incubated for 24 hr at 37°C. After incubation, the media were removed, and the wells were washed three times with PBS (pH 7.2). To fix the

biofilm, 200 µL of 95% methanol was added for 15 min at room temperature. Wells were then stained with 200 µL of 1% crystal violet for 15 min and washed again with PBS. Finally, 200 µL of 33% acetic acid was added and shaken for 15 min, and biofilm formation was quantified by measuring the absorbance at 570 nm (OD_{570}) using a microplate reader (BioTek, Epoch, USA). Each assay was performed using two technical replicates per isolate, and the experiment was repeated independently three times on separate days to ensure consistency and reproducibility of results. The average OD of the negative control wells (OD_C) was 0.112 ± 0.03. Based on this value, the isolates were categorized as weak (OD_C< OD ≤2×OD_C), moderate (2×OD_C < OD ≤4×OD_C), and strong biofilm producers (OD >4×OD_C) **(26)**.

2.5 Disinfectant Susceptibility

Formaldehyde, glutaraldehyde, and hydrogen peroxide (H_2O_2) were selected for testing as they are routinely used disinfectants in poultry farms in Iran and worldwide, known for their broad-spectrum antimicrobial activity and widespread application in farm sanitation protocols. The MIC for three disinfectants against APEC strains, was determined using broth microdilution assay. The initial stock concentrations were 37% for formaldehyde, 25% for glutaraldehyde, and 35% for hydrogen peroxide. To prepare working solutions, 10% concentrations of each were made, and subsequently, two-fold serial dilutions were performed on these 10% solutions. The assay was performed in a 96-well plate, with each well containing 100 µL of TSB medium and serial dilutions of disinfectants. A 100 µL suspension of bacteria (1.5×10^6 cfu/mL) was added to each well, and the plate was incubated at 37°C for 24 hr. MIC was defined as the lowest disinfectant concentration without visible turbidity compared to control (ATCC strain 25922) wells. For minimum bactericidal concentration (MBC), 100 µL from wells at or above the MIC was plated on Mueller-Hinton agar. After 24-hr incubation at 37°C, if fewer than 15 colonies formed, that dilution was considered MBC. If more than 15 colonies appeared in the MIC well, the previous dilution was identified as MBC. If the MIC well had 15 or fewer colonies, it was classified as MIC and MBC both.

2.6 Statistical Analysis

The data analysis was performed using Chi-square and Fisher's exact tests, with a significance level set at 5%, using SPSS version 16 software.

3. Results

3.1 Bacterial Isolation and Antimicrobial Susceptibility

A total of 50 *E. coli* isolates were collected from 50 broiler chickens' specimens with colibacillosis and confirmed biochemically. The antibiogram test results indicated that the highest levels of sensitivity were recorded for nitrofurantoin (96%), meropenem (94%), cefoxitin (94%), gentamicin (92%), cefepime (92%), and colistin (90%). In contrast, the isolates showed the greatest resistance to tetracycline (86%), trimethoprim-sulfamethoxazole (82%), chloramphenicol (80%), florfenicol (72%), doxycycline (68%), and levofloxacin (66%) (Figure 1). Results indicated that 41 (82%) isolates displayed multidrug resistance (MDR). Notably, none of the strains showed characteristics of extensively drug-resistant (XDR) or pan-drug-resistant (PDR) profile. Furthermore, 15 (30%) of the isolates were identified as producers of ESBL using combination disk diffusion method.

3.2 Detection of Virulence Genes

The PCR investigation of the seven virulence-associated genes (*iss, tsh, papC, iucD, vat, cva,* and *astA*) revealed varying frequencies among the isolates (Figure 2). The most prevalent gene was *iss*, found in 33 isolates (66%), while the least common gene was *papC*, which was detected in only 2 isolates (4%). Notably, 28% of the samples did not carry any virulence genes. Additionally, 8% of the samples harbored six genes simultaneously, 4% contained five genes, 14% had four genes, 10% had three genes, 16% had two genes, and 20% had at least one gene.

3.3 Biofilm Formation

The quantitative results of biofilm formation in *E. coli* isolates indicated that all tested isolates (100%) were capable of forming biofilms. Among them, approximately 72% of *E. coli* strains demonstrated high biofilm formation ability, 24% exhibited moderate biofilm formation, and about 4% showed weak biofilm formation (Figure 3). Additionally, statistical analysis revealed significant correlation between MDR phenotype and biofilm strength (P=0.046), suggesting that all MDR isolates were more likely to exhibit strong biofilm-forming ability. However, when the number of virulence genes was compared across different biofilm categories (weak, moderate, strong), no significant association (P=0.144) was found, implying that virulence gene load does not predict biofilm strength.

3.4 MIC and MBC Determination Results for Disinfectants

The MIC and MBC of 50 APEC isolates were evaluated against three commonly used disinfectants; formaldehyde, hydrogen peroxide (H_2O_2), and glutaraldehyde, using broth microdilution method. For formaldehyde and hydrogen peroxide, all isolates exhibited MIC and MBC values at 1/512 dilution, which corresponds to a concentration of ≤ 0.009%. For

glutaraldehyde, the MIC and MBC were observed at 1/64 dilution (equivalent to 0.078%) for the tested isolates. These results are summarized in Table 2.

Table 2. MIC and MBC values of *E. coli* isolates (n = 50) against selected disinfectants.

Disinfectant	MIC Dilution	MIC value	MBC Dilution	MBC value
Formaldehyde	1/512	≤ 0.009	1/512	≤ 0.009
Hydrogen Peroxide	1/512	≤ 0.009	1/512	≤ 0.009
Glutaraldehyde	1/64	0.078	1/64	0.078

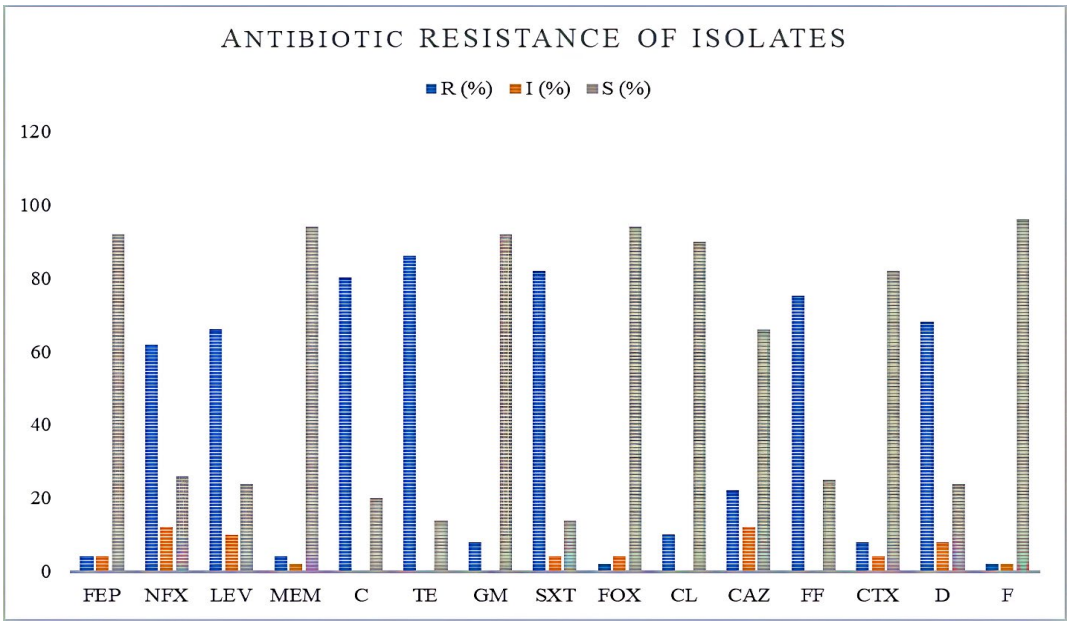

Figure 1. Antibiotic resistance percentages of *E. coli* isolates (n = 50) recovered from broiler chickens with colibacillosis. FOX = cefoxitin; CAZ = cftazidime; CZA = ceftazidime–clavulanate; CTX = cefotaxime; CTC = cefotaxime–clavulanate; FEP = cefepime; MEM = meropenem; GM = gentamicin; CP = ciprofloxacin; SXT = trimethoprim–sulfamethoxazole; F = nitrofurantoin; LEV = levofloxacin; CL = colistin; D = doxycycline; NFX = enrofloxacin; C = chloramphenicol; TE = tetracycline; FF = florfenicol. **(Designed by Authors, 2025)**.

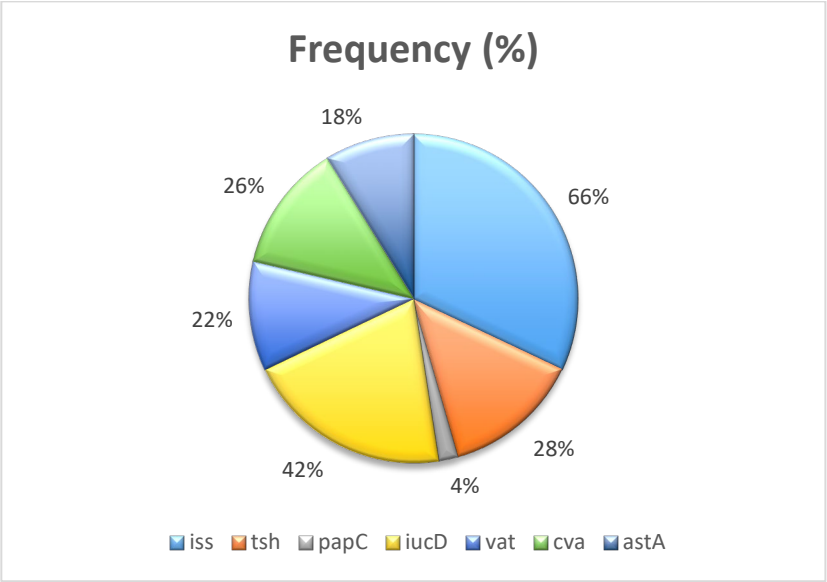

Figure 2. Frequency of virulence genes in *E. coli* isolates **(Designed by Authors, 2025)**.

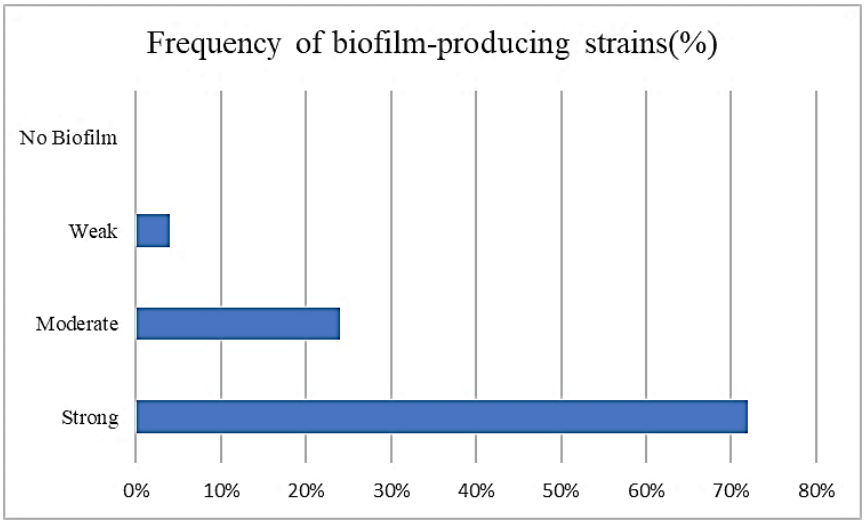

Figure 3. Frequency of biofilm-producing strains **(Designed by Authors, 2025)**.

4. Discussion

Avian colibacillosis caused by APEC remains a significant concern for poultry production and public health, particularly in regions with expanding poultry industries and limited antimicrobial regulation **(27-29)**. The emergence, dissemination, and persistence of antibiotic-resistant *E. coli* strains are especially problematic in low and middle-income countries (LMICs), where antimicrobial stewardship practices may be suboptimal **(30)**. It is projected that global antimicrobial use in food animal production will increase by 67% by 2030, with 129% rise expected in the Asia-Pacific region for chicken production alone **(31)**. In Iran, reliable data on prevalence of resistance among APEC strains to various antimicrobial agents and disinfectants is limited. Thus, this research aimed to examine the patterns of antibiotic and disinfectant resistance, assess biofilm-forming capabilities, and identify virulence genes in *E. coli* isolated from broiler chickens.

In this work, high levels of resistance were observed for commonly used antibiotics, notably tetracycline (86%), trimethoprim-sulfamethoxazole (82%), and chloramphenicol (80%). These rates exceed those reported in the United States **(32)** and align closely with findings from recent studies in Iran **(33)** and Egypt **(34)**. Dolejská et al **(35)** also documented elevated tetracycline resistance among *E. coli* isolates from black-tailed chickens; however, their isolates remained fully susceptible to the third-generation cephalosporins like ceftazidime, unlike our study **(35)**. Tetracycline has been approved for use in the United States, China, Poland, the United Kingdom, France,

Brazil, and Spain for therapeutic, prophylactic, and growth promotion purposes for more than 50 years **(36)**. Additionally, our study revealed that 82% of APEC isolates exhibited MDR, and 30% were ESBL producers that is higher than a similar previous study in Iran **(37)**. In China, a longitudinal study of *E. coli* isolates from sick chickens showed a substantial increase in antimicrobial resistance from 1993 to 2013, with MDR rates approaching 90% by the end of the study period **(38)**. Similarly, ESBL prevalence among APEC isolates from broilers in eastern Thailand was significantly higher than our 30% rate, indicating regional variation in ESBL dissemination **(39)**. Notably, Dutch surveillance studies have reported ESBL-producing *E. coli* on 100% of broiler farms, with strong genetic similarity between poultry, meat products, and clinical isolates, emphasizing the zoonotic risk and interconnectivity underscored by the One Health approach **(40, 41)**. In our study, we also found that 10% of *E. coli* isolates exhibited resistance to colistin. This is particularly alarming as colistin is considered a last-resort antibiotic for treating bacterial infections in humans **(42)**.

The APEC-associated virulence gene profiling revealed a diverse array of virulence factors among the *E. coli* isolates. Our study found different frequencies in *iss* (66%), *iucD* (42%), *tsh* (28%), cvi/cva (26%), *astA* (18%), and *papC* (4%) genes. A similar order of prevalence of *iss* (100%)> *iucD* (97.8%)> *tsh* (62.2%)> cvi/cva (57.8%) was reported in Nepal **(28)**. However, the prevalence of *papC* gene (4%) in our study was lower than that in previous studies developed in Turkey **(43)**, and Germany **(44)**. Interestingly, 28% of the isolates in our study lacked any targeted virulence genes. Several possible explanations may account for this finding. First, these isolates may harbor other untested virulence genes or possess novel or less-characterized genetic determinants contributing to their pathogenicity **(7)**. Second, since the isolates were obtained from diseased birds based on clinical diagnosis and macroscopic lesions, some may represent opportunistic strains that proliferated in compromised hosts rather than the infection with a specific pathotype **(45)**. Future studies using whole-genome sequencing (WGS) or expanded PCR panels are warranted to provide a more comprehensive virulence profile and confirm APEC classification.

The high percentage of strong biofilm producers suggests that biofilm-associated infections could be a persistent challenge in poultry farming **(46)**. A study conducted by Pavlickova et al **(47)** in 2017 found that 68% of *E. coli* isolates obtained from chicken meat and wild poultry were capable of forming biofilms **(47)**. However, in the present study, 100% of the isolates exhibited some form of biofilm formation. Bacteria that form biofilms pose a threat to public health because they can attach to different surfaces. When these biofilm structures are disrupted, they can release pathogenic microorganisms and lead to the contamination of products **(48)**. Given that the adhesion of these bacterial cells can affect the colonization process on non-living surfaces. Although we did not directly assess environmental persistence, the high proportion of strong biofilm-producing isolates observed in this study supports potential for increased survival and resistance to cleaning agents, as biofilms are well-documented to protect bacteria from environmental stressors **(49)**.

GLU, FOR, and H_2O_2 are the primary active components found in disinfectants utilized for cleaning and disinfection processes on poultry farms **(50)**. The disinfectant MIC and MBC results showed that all isolates were inhibited and killed at concentrations ≤ 0.009% for FOR and H_2O_2, and 0.078% for GLU. However, the higher MIC of GLU could be due to the interference of media components with GLU, resulting in fewer active compounds available to interact with bacteria **(51, 52)**. While no established resistance breakpoints exist for disinfectants, these values are substantially lower than recommended in-use concentrations, indicating effective *in vitro* activity. Our findings suggest no reduced susceptibility to these disinfectants under the test conditions. These results are consistent with studies by Oosterik et al **(53)**. There is limited information regarding the basis of *E. coli* resistance to disinfectants, with only the resistance mechanisms against quaternary ammonium compounds (QAC) having been documented **(54, 55)**. Elevated concentrations of these disinfectants pose significant health risks to the healthcare workers and patients. They are potent biocidal agents but are also associated with respiratory irritation, skin sensitization, and mucous membrane damage. Formaldehyde is recognized as a human carcinogen, and its vapor can cause severe irritation and long-term health issues **(56)**. Using these disinfectants, adhering to recommended concentrations, safety protocols, and integrating comprehensive infection control measures is essential to maximize benefits while minimizing risks. Further studies could explore the long-term effects of these disinfectants, their potential resistance development, and environmental impacts to ensure sustainable practices in poultry farming.

5. Conclusion

This study highlights significant public health and biosecurity concerns associated with APEC in broiler farms in Qazvin, Iran, evidenced by high rates of multidrug resistance (82%) and ESBL production (30%).

Frequent detection of virulence genes, particularly *iss* (66%), combined with widespread biofilm formation, indicates a strong potential for environmental persistence and zoonotic transmission. Based on the demonstrated *in vitro* efficacy of commonly used disinfectants such as formaldehyde and hydrogen peroxide, we recommend routine rotation of disinfectants to prevent reduced effectiveness due to microbial adaptation. Additionally, the prudent use of antimicrobials, including limiting colistin use in veterinary practice, is essential to preserve the efficacy of critically important antibiotics. To mitigate the spread of resistant and virulent APEC strains, enhanced surveillance, strengthened biosecurity, and implementation of antibiotic stewardship programs are urgently needed in poultry production systems.

6. Declarations

6.1 Acknowledgment

The authors are thankful to the Medical Microbiology Research Center, Qazvin University of Medical Science for their support and cooperation.

6.2 Ethical Considerations

This study was conducted in full compliance with ethical standards (Ethical Number: IR.QUMS.REC.1402.185) and approved by the Ethics Committee guidelines of Qazvin University of Medical Science.

6.3 Authors' Contributions

All authors contributed to the study conception and design. N.A.: Conceptualization, writing original draft. M.K.: Data curation, methodology, writing original draft. S.S.: Writing, reviewing, and editing. F.F.: Writing original draft. A.A.S.: Writing, editing. S.S.: Software, validation. F.N.: Investigation, visualization, supervision. All authors have read and approved the final manuscript.

6.4 Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6.5 Financial Support and Sponsorship

This research project was conducted without any financial support from external sources.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors did not utilize AI Tools.

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