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CASE REPORT

Isolation and Assessment of Antibiotic Resistance of O142 Avian Pathogenic *Escherichia coli* Causing Black Proventriculus in Broiler Chickens

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Abstract

Avian pathogenic *Escherichia coli* (APEC) serogroup O142 is an uncommon serogroup that causes black proventriculus in poultry. In February 2024, 50 live and dead 7-day-old Arian broiler chickens from a flock of 50,000 were referred to the veterinary hospital of Shahid Bahonar University in Kerman, Iran, with a complaint of a sudden increase in mortality. All chickens were lethargic, and sudden death occurred in all cases. Colisepticemia, mucus hypersecretion, and dark green discoloration of the proventriculus, a dark liver, and pulmonary, and hepatic congestions were observed at postmortem examination. The proventriculus, blood, liver, and spleen from five randomly selected chickens with black proventriculus were sampled for microbiological analysis. In the PCR test, only the liver sample from one bird (1 out of 5) was positive for APEC O142. Conformed APEC isolates were then screened by PCR for detecting the APEC-associated virulence genes *fimC* and *iucD*. The results of the phenotypic antibiotic susceptibility test showed susceptibility to fosfomycin, lincospectin, and sulfamethoxazole-trimethoprim and resistance to florfenicol, colistin, doxycycline, tetracycline, and enrofloxacin. These findings underscore the significance of black proventriculus attributed to *E. coli* O142 and emphasize the importance of targeted antibiotic treatment for affected flocks.

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Introduction

The poultry industry faces many challenges, one of the most significant of which is the management of bacterial infections that can severely impact flock health and productivity. Among these pathogens, *Escherichia coli* (*E. coli*) stands out as a pervasive threat capable of causing various diseases in poultry, including respiratory infections, colibacillosis, and septicemia (1). Black proventriculus is a complication caused mainly by O142 avian pathogenic *E. coli* (APEC). This disease has been reported in young birds, especially those in the early stages of growth. This condition can lead to significant economic losses for poultry producers due to reduced feed conversion, impaired growth rates, and increased mortality rates in affected birds (2,3). Unfortunately, there is not much information about this complication.

In this case report, we present an investigation of a flock of broiler chickens exhibiting clinical signs consistent with black proventriculus, including black discoloration and lesions in the proventriculus. Suspecting the involvement of *E. coli* O142 as a potential causative agent, we conducted a microbiological and molecular (PCR) evaluation to confirm its presence and evaluate its antibiotic resistance profile. By explicitly linking the knowledge gap regarding black proventriculus to the specific questions addressed in our case report, we not only enhance the relevance of our findings but also encourage further research in this underexplored area. Understanding the role of *E. coli* O142 in proventriculus and its associated antibiotic resistance profile is crucial for effective disease management and treatment selection in poultry flocks. Through this case report, we aim to contribute to the existing knowledge base on black proventriculus in poultry and provide valuable insights for veterinarians and poultry producers in managing similar cases effectively.

Case Description

The Animal Ethics Committee of Shahid Bahonar University of Kerman approved all of the stages of the study (Approval No. 1403101). In February 2024, an increase in mortality was observed in 3- to 12-day-old chickens in a 50,000-chicken Arian broiler flock in Kerman, Iran. The death rate increased greatly from day 3. The lowest and highest daily mortality percentages were 0.1% on days 1 and 2, and 3% on day 8. Fifty 7-day-old broilers were referred to the veterinary hospital of Shahid Bahonar University in Kerman, Iran. All of them were lethargic and were euthanized for postmortem examination. Postmortem examination revealed black proventriculus. All birds were kept on litter, and the farm conditions were according to the standard protocol and without any problems.

Sampling and Isolation of *E. coli* Strains

The proventriculus, blood, liver, and spleen of five (a total of 20 organs) chickens with black proventriculus were randomly sampled for microbiological analysis. After homogenizing the inner tissue of samples with sterile PBS, MacConkey agar (Merck, Germany) was used for primary streak culture (37° C for 24 h). Presumptive *E. coli* colonies were examined via biochemical tests, IMViC (Indole, Methyl red, Voges-Proskauer and Citrate) and TSI (Triple Sugar Iron Agar) tests (4). Confirmed *E. coli* isolates (Indole-positive, Methyl red-positive, Voges-Proskauer-negative, Citrate-negative and acid/acid in TSI) were stored for next steps; the confirmed isolates were cultured in Luria-Bertani broth (Merck, Germany) and incubated at 37°C, overnight. Then sterile glycerol was added to achieve 25% concentration, and they were vortexed and immediately stored at -80°C for next steps (5).

PCR Test Preparation

The DNA of isolates confirmed to be *E. coli* was screened via polymerase chain reaction (PCR). A total of 6, 8, 9, and 7 isolates from liver, blood, spleen, and proventriculus, respectively, were studied. The total genomic DNA of the confirmed *E. coli* strains was extracted by the boiling technique; a single colony was suspended in 300 µL sterile distilled water, heated up to 98–100°C in a heating block (Eppendorf, Germany) for 10–15 min. Then, lysates were centrifuged at 13,000 rpm for 2 min, and the supernatant was moved to a new microtube (6). A NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific) was used to measure template DNA, and the extracted DNA was stored at -20°C.

Molecular Detection of APEC-Associated Virulence Genes

Several genes are known to be associated specifically with APEC. In this study, the *fimC* and *iucD* genes were selected as the molecular markers for the detection of APEC. These genes are among the most commonly detected APEC-associated virulence genes in the majority of the studies focused on APEC (7–9). Confirmed *E. coli* isolates were then screened by PCR for detecting the APEC-associated virulence genes *fimC and iucD*. The primers used for the detection of APEC are listed in Table 2 (10).

Phenotypic Antimicrobial Susceptibility Testing

In compliance with the 2018 standards of the Clinical and Laboratory Standards Institute (CLSI), the disc diffusion method was used to perform antimicrobial susceptibility testing, and the findings were evaluated (Table 1) (11,12). The antibiotics selected for the study were those commonly used in poultry farms; they included florfenicol (FF; 30 µg), colistin (CL; 10 µg), fosfomycin (FOS; 50 µg), ceftriaxone (CRO; 30 µg), doxycycline (D; 30 µg), enrofloxacin (NFX; 5 µg), sulfamethoxazole-trimethoprim (SLT; 23.75 µg and 1.25 µg, respectively), tetracycline (TE; 30 µg), and lincospectin (LS; 2 µg). The diameters of growth inhibition zones were measured, and according to CLSI breakpoints, the *E. coli* isolates were determined to be resistant, intermediate, or susceptible.

Molecular Assay to Identify O142 APEC

The primer sequences and PCR protocol information are provided in Table 2 (10,13). PCR volume was 25 µl containing 1 µl DNA extract, 0.5 µl of each primer (20 µM), 10 µl 2× ready Taq DNA Polymerase Master Mix (ParsTous, Iran), and sterile distilled water up to reaction volume. A positive control (from the Microbiology Bank of Shahid Bahonar University, Kerman) and a negative control (distilled water) were used during the PCR test. The PCR products were electrophoresed on a 1.3% agarose gel for 45 minutes at 110 V, and GelDoc 1000 was used for imaging with UV (Vilber Lourmat, France).

Table 1. Zone diameter interpretive criteria (mm) by disk diffusion method for *E. coli* (11,12)

Class	Antibacterial Agent	Disk Content (µg)	Susceptible	Intermediate	Resistant
Phenicols	Florfenicol (FF)	30 µg	≥ 19	15–18	≤ 14
Polymyxin	Colistin (CL)	10 µg	≥ 11	-	≤ 10
Phosphonic acid	Fosfomycin (FOS)	200 µg	≥ 16	13–15	≤ 12
Cephalosporins	Ceftriaxone (CRO)	30 µg	≥ 23	20–22	≤ 19
Tetracyclines	Doxycycline (D)	30 µg	≥ 14	11–13	≤ 10
	Tetracycline (TE)	30 µg	≥ 23	18-22	≤ 17
Fluoroquinolones	Enrofloxacin (NFX)	5 µg	≥ 23	17–22	≤ 16
Aminoglycoside	Lincospectin (LS)	2 µg	≥ 20	17-19	≤ 16
Folate Pathway Inhibitors	Sulfamethoxazole + Trimethoprim (SLT)	23.75 + 1.25 µg	≥ 16	11–15	≤ 10

Table 2. Primer sequences and PCR condition to identify O142 APEC

Primer	Sequence (5'–3')	PCR condition	Product size (bps)	Reference
***iucD* F**	ACAAAAAGTTCTATCGCTTCC	95°C (10 m), 40 cycles: 95°C (15 s), 55°C (60 s), 72°C (60 s), 72°C (10 m)	692	(10)
***iucD* R**	CCTGATCCAGCTGATGCTC			
***fimC* F**	GGGTAGAAAATGCCGATGGTG	95°C (10 m), 40 cycles: 95°C (15 s), 59°C (60 s), 72°C (60 s), 72°C (10 m)	496	(10)
***fimC* R**	CGTCATTTTGGGGGTAAGTGC			
O142 F wzx	TCTCCATCCCCGTTTATTTG	95°C (10 m), 40 cycles: 95°C (15 s), 60°C (60 s), 72°C (60 s), 72°C (10 m)	285	(13)
O142 R wzx	CCCCAAACATTAGCATTCGT			

Results

At postmortem examination, mucus hypersecretion, dark green discoloration of the proventriculus, a dark liver, and pulmonary and hepatic congestions were observed (Figures 1 and 2).

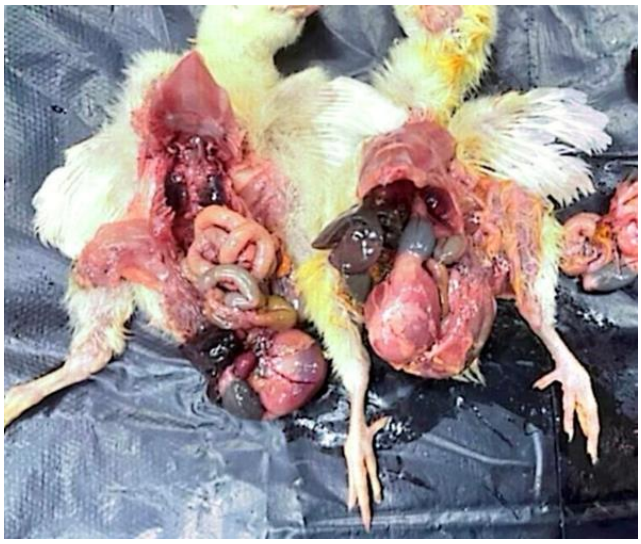

Figure 1. Black proventriculus and dark liver in chickens.

Figure 2. Black proventriculus in a broiler chickens.

A total of 30 distinct smooth, pink colonies on MacConkey were seen as suspected *E. coli* colonies, all of which were confirmed by IMViC tests (indole, methyl red, Voges-Proskauer, and citrate). In the PCR test, only the liver sample from one bird (1 out of 5) was positive for O142 *E. coli* (Figure 3). Also, APEC O142 DNA was confirmed by the presence of *fimC* and *iucD* virulence genes via PCR (Figures 4 and 5). The antibiotic resistance results were determined based on the diameter of the growth-inhibiting zones of the positive isolate in the PCR test as resistant (R), intermediate (I), and susceptible (S) (Table 3 and Figure 6).

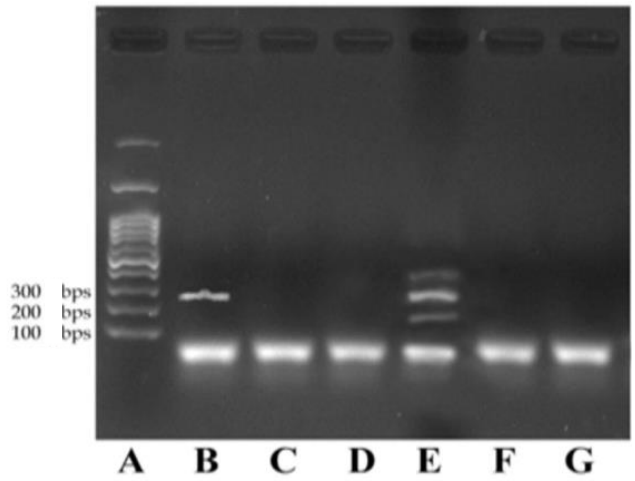

Figure 3. Agarose gel electrophoresis analysis of the PCR method to determine the presence of *E. coli* O142 DNA in proventriculus, blood, liver, and spleen samples; Column A: molecular weight index (DNA ladder 100 bp); Column B: positive control; Column E: liver sample from bird 2; Column F: negative control; other columns: a number of examined samples.

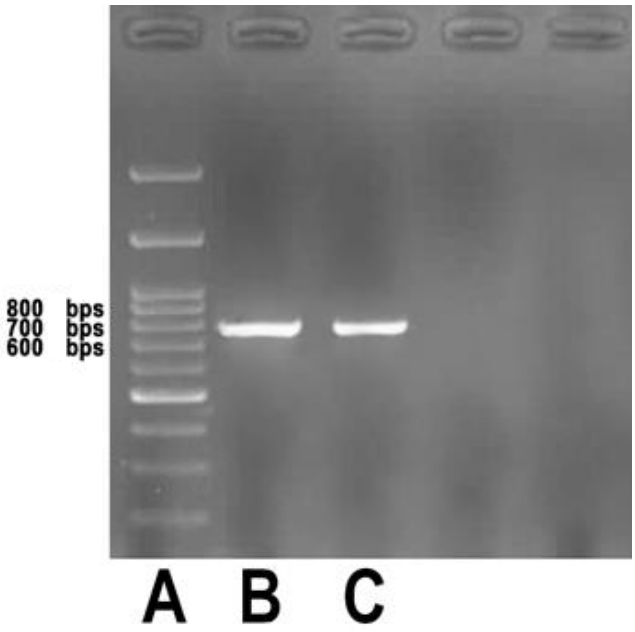

Figure 4. Agarose gel electrophoresis analysis of the PCR method to determine the presence of the *iucD* gene to confirm APEC O142 DNA.

Discussion

E. coli serogroup O142 is an uncommon serogroup that causes black proventriculus in poultry (2,3). *E. coli* O142 has been reported in gastroenteritis in premature human

babies and diarrhea in calves and lambs (14,15). This serogroup was not found in the studies conducted on APEC isolated from chickens in China, the United States, or Germany in 1999, 2005, and 2007, respectively (16–18). *E. coli* O142 in poultry was first found in broiler breeders (Arbor Acres) in China in 2015 (3). It was also reported in 2020 in broiler breeders (Cobb 500) in the northwest of Iran (2). This study found this pathogen in broiler chickens (Arian) in southeastern Iran. This pathogen seems to be circulating in industrial broiler breeders and broiler flocks. The emergence of *E. coli* O142 poses a significant threat to broiler chickens, highlighting the need for increased awareness and monitoring within the poultry industry. Our findings underscore the importance of addressing this pathogen to safeguard animal health and ensure food safety. Tissues affected by colisepticemia develop a green discoloration on exposure to air, possibly due to bacterial indole production. These observations suggest that black proventriculus is mainly observed during postmortem examinations rather than in live chicks, probably due to this phenomenon (3). Furthermore, in a study conducted by Wang et al. (2015), the *feoB* gene (which plays a role in ferrous iron transport) was seen only in the O142 APEC isolate isolated from the dark green proventriculus, which probably indicates its involvement in the color change of the proventriculus (2,3,19). In our research, similar to previous reports, mortality was not observed in the first three days. In previous studies, the chickens had no clinical symptoms, but in this study, the chickens were lethargic (2,3). Also, similar to Wang et al. (2015), mucus hypersecretion was observed in addition to the color change in the proventriculus (3).

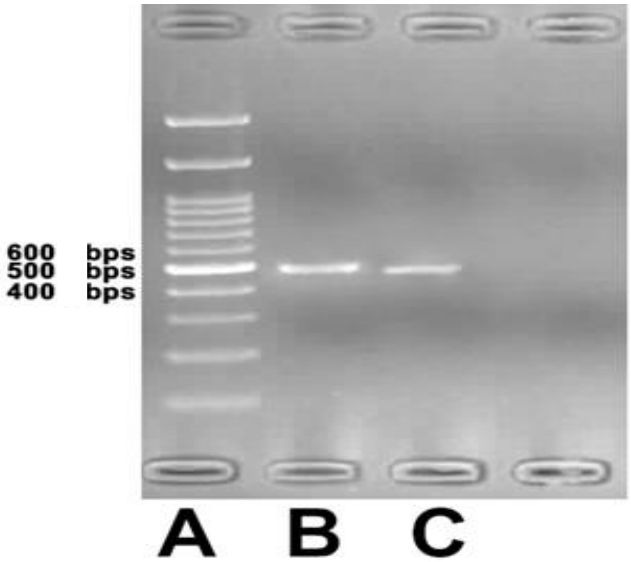

Figure 5. Agarose gel electrophoresis analysis of the PCR method to determine the presence of the *fimC* gene to confirm APEC O142 DNA.

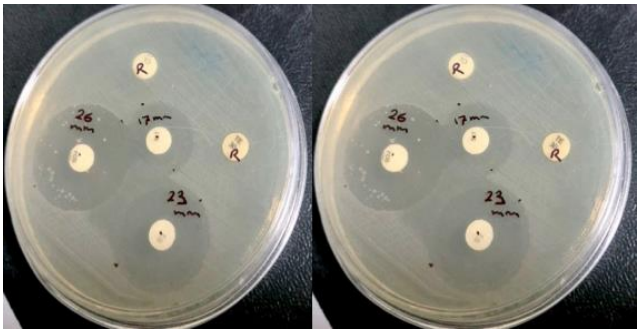

Figure 6. Disk diffusion method for positive *E. coli* O142 isolate of liver.

The isolation of *E. coli* O142 as the causative agent in this study corroborates previous findings and underscores the importance of targeted microbial surveillance (2,3). Among the liver, proventriculus, spleen, and blood samples from five birds with black proventriculus, only the liver of one bird tested positive for *E. coli* O142 by PCR. This limited detection can be attributed to several factors. First, the heterogeneous distribution of pathogens within different tissues may result in variable bacterial loads, affecting detection rates. For instance, certain organs may harbor higher concentrations of bacteria due to tissue-specific immune responses. Additionally, variations in host immune responses can influence both clinical signs and pathogen distribution. Technical factors also play a role; inhibitors present in biological samples can interfere with PCR amplification, leading to false negatives. Furthermore, differences in DNA extraction methods can impact test sensitivity. Overall, the discrepancy between clinical signs of black proventriculus and PCR results highlights the complexity of pathogen detection and underscores the need for further investigation into these factors to improve diagnostic accuracy in avian medicine (3,20).

Table 3. Phenotype antimicrobial resistance of a liver-positive *E. coli* O142 isolate.

Antibacterial Agent	Zone inhibition (mm)	Antibiotic-resistant group
Florfenicol (FF)	0	R
Colistin (CL)	0	R
Fosfomycin (FOS)	26	S
Ceftriaxone (CRO)	20	I
Doxycycline (D)	0	R
Tetracycline (TE)	0	R
Enrofloxacin (NFX)	9	R
Lincospectin (LS)	23	S
Sulfamethoxazole-trimethoprim (SLT)	17	S

Multidrug resistance (MDR) is a serious concern in both human medicine and the poultry industry. According to the MDR definition, these bacteria are resistant to at least one antimicrobial agent from three or more antibiotic categories (1). Understanding antibiotic resistance can help poultry producers and veterinarians tailor management and disease prevention strategies accordingly. Antibiotic susceptibility testing revealed alarming levels of resistance to antibiotics commonly used in veterinary medicine, as well as to colistin-a critically important human antibiotic and last-line defense against many Gram-negative bacilli (21). The transfer of antimicrobial agents to humans via animal products harboring antibiotic residues can potentially lead to the emergence of antibiotic-resistant bacteria in humans (22). This situation exemplifies the One Health concept, which emphasizes the interconnectedness of human, animal, and environmental health. Furthermore, the increasing frequency of this disease in poultry raises significant public health concerns, particularly through the consumption of contaminated chicken. Improper disposal of infected carcasses can pose additional risks by potentially transmitting pathogens to wildlife, perpetuating a cycle of infection that affects both animal populations and human health. The results of this study indicated resistance to florfenicol, colistin, doxycycline, tetracycline, and enrofloxacin. Similar to the results of previous studies, a good susceptibility to fosfomycin was observed (2,3). Since florfenicol is the only phenicol approved for treating bacterial infections in food-producing animals, the resistance levels to this drug are concerning (23). These findings emphasize the urgent need for a collaborative One Health approach to explore new strategies and alternatives for effectively combating antibiotic resistance.

Conclusion

In conclusion, black proventriculus represents a novel pathological manifestation of *E. coli* infection in chickens, necessitating comprehensive surveillance, diagnostic refinement, and targeted therapeutic interventions. This study's findings revealed MDR in O142 APEC and underscored the importance of using an antibiogram during disease treatment. Advancing our understanding of its pathophysiology and optimizing treatment and management strategies will help mitigate the impact of black proventriculus on poultry health and welfare. Furthermore, these findings highlight the need for ongoing research into the mechanisms of antibiotic resistance and the development of alternative strategies to combat this emerging threat, ultimately contributing to improved health outcomes for both poultry and humans within the framework of the One Health approach.

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Authors' Contributions

Hemad Shafie: Conceptualization, Methodology, Writing-original draft, and Writing-review& editing; **Maziar Jajarmi and Reza Molaei:** Methodology**; Pouneh Hajipour:** Methodology and Writing-original draft.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Ethical Approval

All procedures were conducted with the approval of the Animal Ethics Committee of Shahid Bahonar University of Kerman (Approval No. 1403101).

Conflict of Interest

The author reports no conflicts of interest.

Consent for Publication

Not applicable.

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