JWPR
Journal of World's Poultry Research
2026, Scienceline Publication
J. World Poult. Res. 16(2): 181-194, 2026
Research Paper
DOI: https://dx.doi.org/10.36380/jwpr.2026.17
PII: S2322455X2600017-16
ISSN: 2322-455X

Characterizations of Virulence, Serogroups, and Phylogenetic Groups of Avian Pathogenic *Escherichia coli* in West Java, Indonesia

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Received: March 17, 2026, Revised: April 21, 2026, Accepted: May 25, 2026, Published: June 25, 2026

ABSTRACT

Colibacillosis is an infectious disease in poultry caused by avian pathogenic *Escherichia coli* (APEC), which can invade the host through the intestinal or respiratory tract and subsequently lead to systemic infection mediated by various virulence factors. The current study aimed to characterize the 15 *Escherichia coli* (*E. coli*) isolates obtained from colibacillosis-affected commercial layer farms (n=10) in West Java, Indonesia, between 2016 and 2020. Molecular identification and characterization were performed using Polymerase Chain Reaction (PCR) targeting *usp*A gene, four O-serogroups (O1, O2, O18, and O78), 11 virulence-associated genes, and phylogenetic markers, with appropriate positive and negative controls included in each assay. All isolates were confirmed as *E. coli* by *usp*A amplification. The most prevalent virulence gene was *fimC* in 87% (13/15), which was detected across multiple phylogenetic groups, including B1, B2, A/C, and D/E, followed by *ibeB* and *traT* (73%, 11/15 each), *iucD* and *iroN* (67%, 10/15 each), *papC* and *vat* (33%, 5/15 each), *ibeA* (13%, 2/15), and *iss* (7%, 1/15). Serogroup analysis identified three isolates as O1, two as O18, and one as O78, while the majority of isolates (9/15) were not assignable to the tested serogroups. Phylogenetic analysis classified the isolates into group A (1/15), B1 (2/15), B2 (5/15), F (3/15), A/C (2/15), D/E (1/15), and one unclassified group (1/15), with phylogenetic group B2 harboring the highest number of virulence-associated genes. The findings of the current study demonstrated substantial genetic diversity among APEC isolates circulating in West Java, Indonesia.

Keywords: Colibacillosis, *Escherichia coli*, Phylogenetic group, Virulence gene, Zoonotic disease

INTRODUCTION

Escherichia coli (*E. coli*) is a ubiquitous bacterium that commonly resides in the intestinal tract of humans and animals as part of the commensal microbiota (Ramos et al., 2020). Nevertheless, certain strains of *E. coli* have acquired virulence traits that enable them to cause severe infections (Braz et al., 2020). Avian pathogenic *E. coli* (APEC) constitutes a specialized pathotype responsible for colibacillosis in poultry. The disease is characterized by high morbidity and mortality in commercial flocks, leading to considerable economic losses in the poultry industry worldwide (Joseph et al., 2023). Due to its ability to disseminate systemically beyond the intestinal and respiratory tracts through specific virulence traits, APEC is classified within the extraintestinal pathogenic *E. coli* (ExPEC) group (Kathayat et al., 2021).

The ability of APEC to cause disease is associated with a wide range of virulence determinants, such as adhesins, toxins, iron acquisition mechanisms, and serum resistance factors, which collectively contribute to colonization, evasion of host defenses, and systemic infection (Hu et al., 2022). Key virulence-associated genes reported in previous studies include *fimC* and *papC*, which are involved in adhesion and colonization (Kariyawasam et al., 2006; Wang et al., 2025); *ibeA* and *ibeB*, which facilitate host cell invasion (Germon et al., 2005; Wang et al., 2012); and *iucD* and *iroN*, which play essential roles in

iron acquisition and bacterial survival within the host (Boulbair et al., 2025). In addition, serum resistance and immune evasion are associated with *traT* and *iss* (Johnson et al., 2008; Sonola et al., 2022), while toxin- and plasmid-related genes such as *vat*, *cvaC*, and *tsh* contribute to tissue damage and enhanced pathogenicity (Kostakioti and Stathopoulos, 2004; Díaz et al., 2020). The distribution and combination of virulence genes vary among APEC strains, influencing their pathogenic potential (Nawaz et al., 2024).

Avian pathogenic *E. coli* strains are also classified based on O-serogroups, with serogroups O1, O2, O18, and O78 most frequently associated with avian colibacillosis outbreaks (Kathayat et al., 2021; Mehat et al., 2021). These serogroups play an essential role in host-pathogen interactions and immune recognition (Kathayat et al., 2021). Nevertheless, many APEC isolates do not belong to classical serogroups, suggesting considerable genetic diversity among circulating strains in poultry populations (Mehat et al., 2021). The relationship between serogroup distribution and virulence gene profiles remains an important area of investigation, particularly across different geographic regions (Watts and Wigley, 2024). Previous molecular studies of APEC in the United States and Asia have demonstrated a diverse distribution of serogroups accompanied by considerable variation in virulence gene profiles (Kim et al., 2020; Hossain et al., 2021; Runcharoon et al., 2025). In South Korea, serogroups O78 and O2 were reported as predominant among APEC isolates, along with diverse phylogenetic backgrounds and a high prevalence of virulence-associated genes such as *hlyF* and *iutA* (Kim et al., 2020). Similarly, a study in Bangladesh revealed heterogeneous virulence gene distributions, with *iroN*, *hlyF*, *iss*, and *iutA* detected at varying frequencies among APEC isolates (Hossain et al., 2021). For instance, longitudinal surveillance in Georgia, USA, revealed broad serogroup prevalence and heterogeneous virulence gene patterns among disease-associated APEC isolates (Runcharoon et al., 2025).

Phylogenetic classification provides a broader perspective on the evolutionary relationships of APEC beyond conventional serotyping. *Escherichia coli* is grouped into several principal phylogenetic lineages, including A, B1, B2, D, E, and F. Extraintestinal pathogenic *E. coli* (ExPEC), including APEC, are most often linked to phylogroups B2 and D, which are associated with enhanced virulence (Chakraborty et al., 2015; Nawaz et al., 2024). In contrast, strains belonging to groups A and B1 are generally considered commensal and are associated with lower pathogenic potential (Nawaz et al., 2024). Associations between phylogenetic background and virulence gene distribution have been shown to be informative for predicting the pathogenic potential of APEC isolates and their impact on poultry health (Rybak et al., 2022). Despite extensive studies on APEC in several regions, data on the virulence profiles, serogroups, and phylogenetic backgrounds of APEC circulating in Indonesian poultry farms remain limited. West Java represents one of Indonesia's major commercial layer chicken production areas, underscoring the importance of investigating APEC strains affecting poultry. Improved understanding of the genetic characteristics of local APEC populations is essential for strengthening disease surveillance, guiding control strategies, and supporting the development of locally relevant vaccines.

Recent molecular epidemiological studies have highlighted substantial genetic diversity among APEC populations in regions with intensive poultry production, particularly in East and Southeast Asia (Mehat et al., 2021; Afayibo et al., 2022; Khairullah et al., 2024). These studies report marked variation in virulence gene profiles, phylogenetic lineages, and serogroup distributions, emphasizing the need for region-specific characterization rather than reliance on global reference data (Mehat et al., 2021; Afayibo et al., 2022). Genome-based analyses further demonstrate that APEC pathogenicity is shaped by complex and strain-specific combinations of virulence determinants, with no single marker defining pathogenic potential across populations (Mageiros et al., 2021). In Southeast Asia, APEC isolates from broiler chickens exhibit diverse phylogroups and virulence gene profiles, reflecting their adaptive potential and epidemiological complexity (Yongyod et al., 2026). In Indonesia, where poultry density is high but systematic molecular surveillance remains limited, recent field-based investigations underscore the need for localized APEC profiling to support effective disease prevention and control (Timur et al., 2026). Therefore, the present study aimed to characterize the phylogenetic groups, O-serogroups, and virulence-associated genes of APEC isolates obtained from commercial layer chickens in West Java, Indonesia.

MATERIALS AND METHODS

Ethical approval

All chicken samples and related procedures were ethically approved by the Animal Ethics Committee, School of Veterinary Medicine and Biomedical Sciences,

Bogor, Indonesia, with approval number 231/KEH/SKE/VII/2024.

***Escherichia coli* isolates**

The present study utilized 15 archived *E. coli* isolates originally obtained from commercial layer chicken farms (n = 10) in West Java, Indonesia, between 2016 and 2020, representing a descriptive dataset. The *E. coli* isolates were selected from a laboratory collection using a purposive sampling approach based on their origin from chickens exhibiting clinical signs consistent with colibacillosis. The sampling was designed to ensure that all isolates were clinically relevant to avian colibacillosis while acknowledging the descriptive nature of the study. *Escherichia coli* ATCC 25922, purchased through an authorized distributor of the American Type Culture Collection (ATCC, Manassas, VA, USA), was used as a reference strain. The archived isolates had been previously stored in glycerol deep stocks at −20°C.

For re-identification, isolates were revived and cultured on MacConkey agar (Himedia, India) at 37°C for approximately 18 hours, followed by macroscopic observation of colony morphology. All isolates produced pink-colored colonies with a smooth and circular appearance on MacConkey agar, indicating lactose fermentation. Gram staining was used as an initial step to determine the Gram reaction and cellular morphology, confirming the isolates as Gram-negative rods. The bacterial cultures were subsequently grown on blood agar supplemented with 5% sheep blood at 37°C for 24 hours (Abu-Sini et al., 2023). Distinct colonies were then picked and subcultured for molecular verification.

Phenotypic and biochemical identification

For selective isolation of Gram-negative bacteria, the isolates were streaked onto Eosin Methylene Blue Agar (EMBA, Himedia, India) plates (Bonnet et al., 2020) and incubated at 35°C for 18-24 hours. Colonies with morphology consistent with *E. coli* were then subjected to biochemical confirmation, including assessment of Lauryl Sulfate MUG X-gal (LMX) medium (Himedia, India), followed by incubation at 37°C for 18 hours. Indole production using Kovac's reagent was incubated in tryptose broth at 35°C for 24 ± 2 hours. Catalase activity was determined by applying 3% hydrogen peroxide (H_2O_2) to freshly cultured colonies. The oxidase test was performed by transferring a fresh colony onto a filter paper. Congo Red agar (Himedia, India) was used to assess the invasive potential of the isolates, with cultures incubated at 35°C for 72 hours. Colonies exhibiting a brick-red to dark red coloration were interpreted as Congo Red-positive, indicating potential invasiveness, whereas pale or colorless colonies were considered Congo Red-negative.

DNA extraction and molecular identification

Genomic DNA was extracted using the boiling method as previously described (Junior et al., 2016). Bacterial suspensions were exposed to 95°C for 10 minutes to release cellular DNA, after which the supernatant (100 µL) was collected for further use. Confirmation of *E. coli* was carried out by PCR targeting the *usp*A gene with specific primers *usp*A F (5'-CCGATACGCTGCCAATCAGT-3') and *usp*A R (5'-ACGCAGACCGTAGGCCAGAT-3'), producing an amplicon of 884 bp (Bhargava et al., 2022).

Each PCR reaction was set up in a final volume of 50 µL, comprising KAPA2G Fast HotStart ReadyMix (25 µL; Kapa Biosystems, USA), template DNA (1 µL), forward and reverse primers (3 µL each, 10 µM), and nuclease-free water (19 µL, Himedia, India). Positive control (*E. coli* ATCC 25922) and a negative control without template DNA were included in all runs. The amplification protocol began with an initial denaturation step at 95°C for three minutes, followed by 35 cycles of denaturation (95°C for 30 seconds), annealing at 58°C, extension at 72°C for one minute, with a final extension step at 72°C for five minutes. The amplified DNA fragments were visualized by electrophoresis on 1.5% agarose (Biosharp, China) gel stained with ethidium bromide. Band sizes were estimated using a 100 bp DNA ladder (VC 100 bp Plus DNA Ladder, Vivantis, Malaysia; Kurnia et al., 2018).

Detection of virulence genes, serogroup, and phylogenetic group

The detection of 12 virulence-associated genes, four O-serogroups (O1, O2, O18, and O78), and phylogenetic groups was performed by PCR using specific primer sets (Table 1). Selected virulence genes represented major functional categories involved in APEC pathogenicity, including adhesion, invasion, iron acquisition, serum resistance, toxin production, and pathogenicity islands, and were chosen based on their frequent use and validation in previous APEC molecular studies.

Phylogenetic grouping of *E. coli* isolates was performed using the updated Clermont PCR-based phylotyping scheme by targeting the *chuA*, *yjaA*, *TspE4.C2*, and *arpA* genes, following the revised framework proposed by Clermont et al. (2015), which

enables classification of *E. coli* into extended phylogenetic groups including A, B1, B2, C, D, E, and F.

PCR reactions were set up in a total volume of 50 µL, consisting of 5 µL of DNA template, 25 µL of MyTaq™ HS Red Mix, 2 µL of each primer, and nuclease-free water. All reactions were conducted in duplicate to confirm reproducibility. Thermal cycling was performed under standardized conditions, with annealing temperatures specific to the primer sets used (Table 1). Amplified fragments were resolved by electrophoresis on 1.5% agarose gels stained with ethidium bromide at 120 V for 35 minutes.

Table 1. Primers used for the detection of virulence genes, O-serogroups, and phylogenetic groups of avian pathogenic *Escherichia coli*

Target gene	Primers (5'-3')	Annealing temperature (ºC)	Amplicon (bp)	References
Virulence gene detection				
fimC	F: GGGTAGAAAATGCCGATGGTG R: CGTCATTTTGGGGGTAAGTGC	59	496	Janßen et al. (2001)
papC	F: TGATATCACGCAGTCAGTAGC R: CCGGCCATATTCACATAA	59	483	Janßen et al. (2001)
ibeA	F: AGGCAGGTGTGCGCCGCGTAC R: TGGTGCTCCGGCAAACCATGC	63	170	Johnson and Stell (2000)
ibeB	F: GTTCTCACTCAGCCAGAACG R: CATCCAGCACTTCCAGATAAC	57	1172	Afayibo et al. (2022)
iucD	F: ACAAAAAGTTCTATCGCTTCC R: CCTGATCCAGATGATGCTC	55	692	Janßen et al. (2001)
iroN	F: CCTCCGACGATGATAATGACG R: GATACCATTATGCGTAATGCC	57	866	Wang et al. (2012)
traT	F: GGTGTGGTGCGATGAGCACAG R: CACGGTTCAGCCATCCCTGAG	63	290	Johnson and Stell (2000)
iss	F: ATGCAGGATAATAAGATGAAA R: CTATTGTGAGCAATATACA	52	270	Ewers et al. (2004)
vat	F: TCCATGCTTCAACGTCTCAGAG R: CTGTTGTCAGTGTCGTGAACG	60	939	Meng et al. (2014)
cvaC	F: CACACACAAACGGGAGCTGTT R: CTTCCCGCAGCATAGTTCCAT	60	680	Johnson and Stell (2000)
tsh	F: ACTATTCTCTGCAGGAAGTC R: CTTCCGATGTTCTGAACGT	55	824	Ewers et al. (2004)
PAI	F: GGACATCCTGTTACAGCGCGCA R: TCGCCACCAATCACAGCCGAAC	65	930	Johnson and Stell (2000)
Serogroup determination				
ECO ECO1 ECO2 ECO18 ECO78	F: GGACATCCTGTTACAGCGCGCA R: CATTAGGTGTCTCTGGCACG R: GATAAGGAATGCACATCGCC R: AGAAGCATTGAGCTGTGGAC R: TAGGTATTCCTGTTGCGGAG	55	 263 355 459 623	Wang et al. (2014)
Quadruplex primers for determining phylogenetic groups				
chuA	F: GACGAACCAACGGTCAGGAT R: TGCCGCCAGTACCAAAGACA	55	279	Clermont et al. (2013)
yjaA	F: TGAAGTGTCAGGAGACGCTG R: ATGGAGAATGCGTTCCTCAAC	55	211	Clermont et al. (2013)
tspE4.C2	F : GAGTAATGTCGGGGCATTCA R: CGCGCCAACAAAGTATTACG	55	152	Clermont et al. (2013)
arpA	F: AACGCTATTCGCCAGCTTGC R: TCTCCCCATACCGTACGCTA	55	400	Clermont et al. (2013)

Data analysis

Results were summarized descriptively in tabular form. Associations among virulence genes and between virulence genes and phylogenetic groups were evaluated using Fisher's exact test. Statistical analyses were performed using R software version 4.4.3 (R Foundation for Statistical Computing, Vienna, Austria). A P-value less than 0.05 was considered statistically significant.

RESULTS

Phenotypic and molecular identification

All 15 isolates grew on MacConkey agar and produced smooth, pink colonies indicative of lactose fermentation (Figure 1A). On blood agar, all isolates exhibited hemolytic activity, with nine isolates showing α-hemolysis and six displaying β-hemolysis. Colonies cultured on Eosin Methylene Blue Agar (EMBA) showed a characteristic metallic green sheen with dark centers (Figure 1B), while growth on Congo Red agar produced red-colored colonies (Figure 1C). All isolates showed positive β-glucuronidase activity on Lauryl Sulfate MUG X-gal (LMX) medium, as indicated by blue-green coloration, and were indole and catalase-positive, evidenced by red ring formation and bubble production, respectively (Figure 1D). In contrast, all isolates were oxidase-negative with no color change. Microscopic examination confirmed that all isolates were Gram-negative, rod-shaped bacteria (Figure 1E). Molecular identification by PCR further confirmed all isolates as *E. coli* based on *usp*A gene amplification (Figure 2).

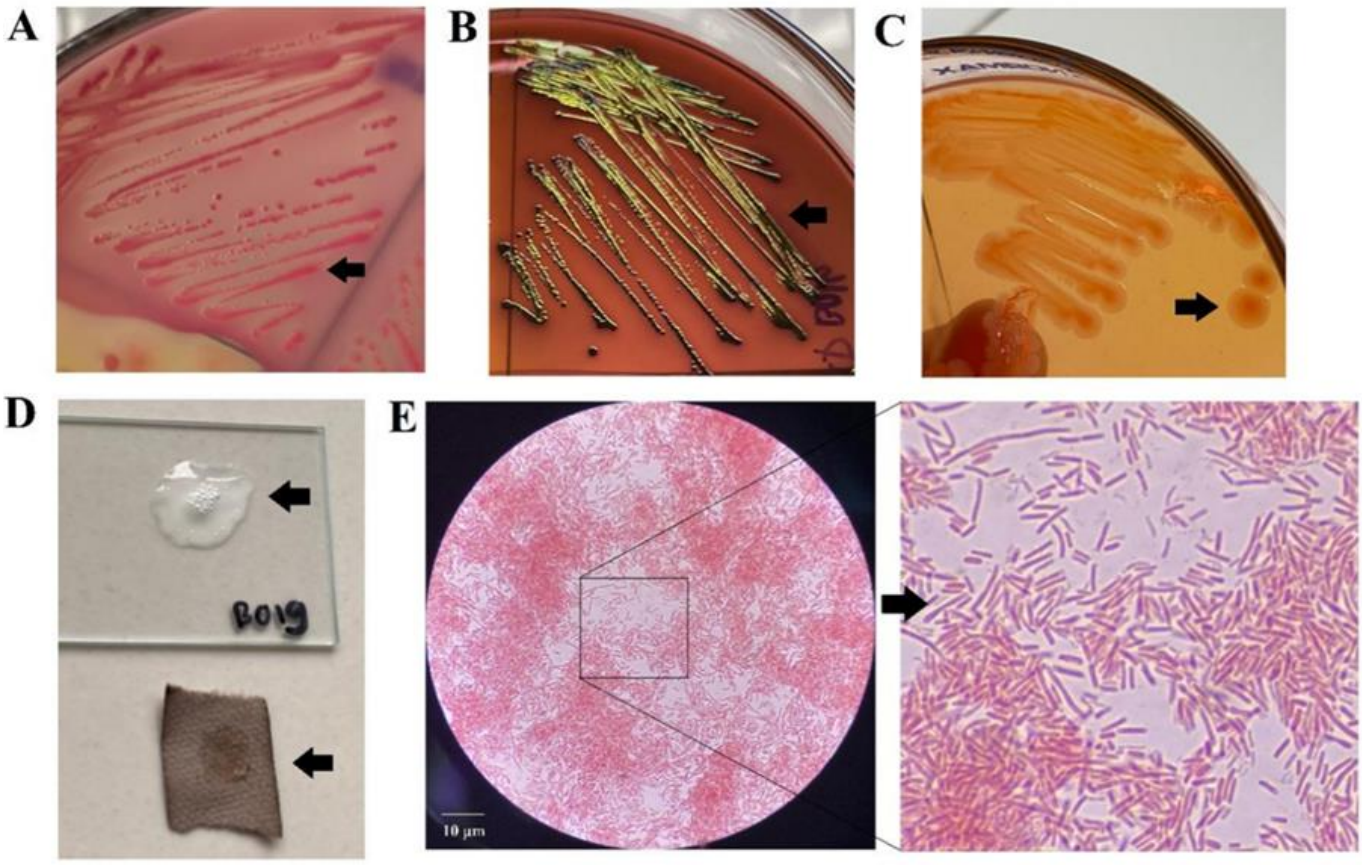

Figure 1. Identification of *Escherichia coli* isolates recovered from commercial layer farms in West Java, Indonesia, between 2016 and 2020. **A**: Pink colonies on MacConkey agar indicating lactose fermentation. **B**: Colonies with a black center and metallic green sheen on Eosin Methylene Blue Agar (EMBA). **C**: Red-colored colonies on Congo Red agar. **D**: Catalase-positive reaction indicated by immediate bubble formation after the addition of 3% H_2O_2, and oxidase-negative reaction indicated by the absence of color change on filter paper. **E**: Microscopic appearance of Gram-negative, rod-shaped bacterial cells after Gram staining at 1000x magnification.

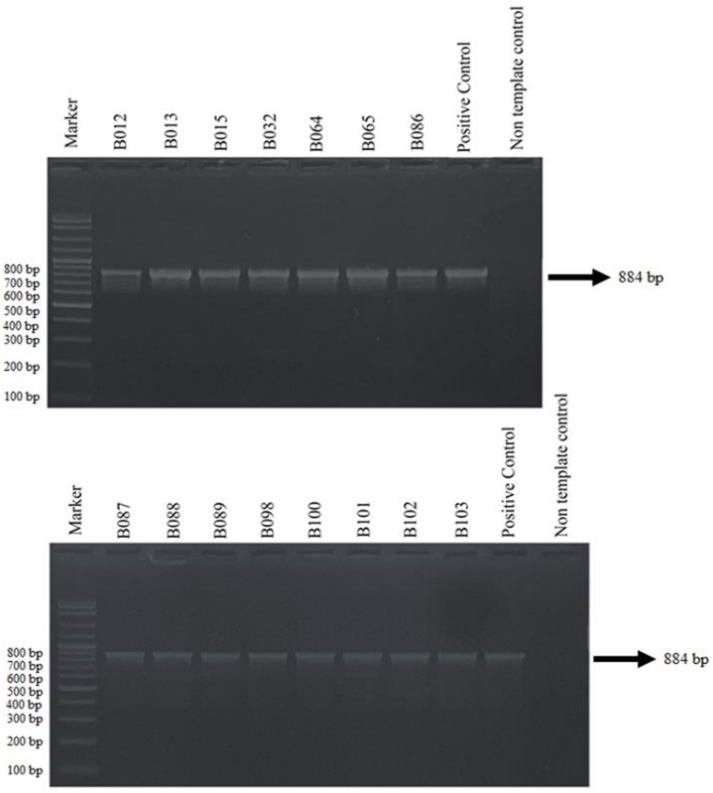

Figure 2. PCR amplification of the *usp*A gene (884 bp) was performed on *Escherichia coli* isolates obtained from APEC isolates obtained from commercial layer chickens in Bogor, West Java, Indonesia. All *E. coli* isolates showed positive amplification of the *usp*A gene. Marker: 100 bp DNA ladder, PC: Positive control (*E. coli* ATCC 25922).

Serogroup distribution and phylogenetic classification

Serogroup analysis showed that three isolates (3/15; 20%) were assigned to serogroup O1, two (2/15; 13.3%) to O18, and one (1/15; 6.7%) to O78, whereas the majority of isolates (9/15; 60%) were not assignable to the tested serogroups. Phylogenetic analysis classified the isolates into groups A (1/15; 6.7%), B1 (2/15; 13.3%), B2 (5/15; 33.3%), F (3/15; 20%), A/C (2/15; 13.3%), and D/E (1/15; 6.7%), with one isolate (1/15; 6.7%) remaining unclassified (Table 2). Using the revised Clermont phylogenetic scheme, several isolates were assigned to combined phylogenetic categories, such as A/C and D/E, while one isolate (1/15; 6.7%) remained unclassified, reflecting the limitations of PCR-based phylotyping in resolving closely related *E. coli* lineages.

Distribution and prevalence of virulence-associated genes

The most prevalent virulence-associated genes among the *E. coli* isolates were *fimC* (87%, 13/15), which encodes an adhesin. In contrast, the *iss* gene, associated with serum resistance, showed the lowest prevalence (7%, 1/15). Among adhesin-related genes, *fimC* was more frequently detected than *papC* (33%, 5/15). For invasion-associated genes, *ibeB* (73%, 11/15) was more prevalent than *ibeA* (13%, 2/15). Iron acquisition–related genes *iucD* and *iroN* were detected at equal frequencies (67%, 10/15). In terms of serum resistance, *traT* (73%, 11/15) was more common than *cvaC* (47%, 7/15) and *iss*. Among toxin-associated genes, *vat* (33%, 5/15) was detected more frequently than *tsh* (27%, 4/15). In addition, the pathogenicity island (*PAI*) gene was identified in 40% (6/15). The distribution of virulence-associated genes across phylogenetic groups is summarized in Table 3.

Associations among virulence genes and phylogenetic groups

Patterns of associations among several virulence genes were observed (Table 4), with cautious interpretation due to the limited sample size. Associations between the presence of individual virulence genes were observed, including *vat* and *papC* (OR = 19.0, p = 0.017), *cvaC* and *iucD* (OR = 2.27, p = 0.026), and *PAI* and *iroN*

(OR = 10.20, p = 0.044), indicating that these genes co-occur more frequently than expected by chance. Such associations suggest possible functional linkage or co-localization, including plasmid or pathogenicity island–associated gene clustering, rather than direct causal relationships. Additional gene pairs showed moderate associations, such as *fimC* and *iroN* (p = 0.095), reflecting partial overlap in virulence gene distribution among isolates. In addition to gene–gene associations, analysis of virulence genes in relation to phylogenetic background indicated associations between phylogenetic group B2 and the virulence genes *papC* (OR = 231, p = 0.000333) and *vat* (OR = 19, p = 0.017).

Table 2. Phenotypic characteristics, serogroups, phylogenetic groups, and virulence gene profiles of APEC isolates recovered from commercial layer farms in West Java, Indonesia, between 2016 and 2020

Number	Isolate number	Phylogenetic groups	Number of isolates	Type of hemolysis	O serotype	Number of virulence genes
1	B012	A	1	α	TT	1
2	B065	B1	2	α	O18	3
3	B103			β	O18	8
4	B032	B2	5	α	TT	3
5	B086			α	O1	8
6	B088			α	O1	7
7	B089			α	O1	8
8	B098			α	TT	9
9	B064	F	3	α	TT	3
10	B101			β	TT	6
11	B102			β	O78	7
12	B013	A/C	2	β	TT	3
13	B100			β	TT	4
14	B015	D/E	1	β	TT	2
15	B087	Unknown	1	α	TT	7

APEC: Avian pathogenic *Escherichia coli*

Table 3. Distribution of virulence-associated genes among phylogenetic groups of APEC isolates recovered from commercial layer farms in West Java, Indonesia, between 2016 and 2020

Phylogenetic groups / Virulence genes	A (n = 1)	B1 (n = 2)	B2 (n = 5)	F (n = 3)	A/C (n = 2)	D/E (n = 1)	Unknown (n = 1)
Adhesion-associated genes							
fimC	0	100% (1/1)	100% (5/5)	66.6% (2/3)	100% (2/2)	100% (1/1)	100% (1/1)
papC	0	0	100% (5/5)	0	0	0	0
Invasion-associated genes							
ibeA	0	0	0	0	50% (1/2)	0	0
ibeB	100% (1/1)	0	80% (4/5)	66.6% (2/3)	100% (2/2)	0	100% (1/1)
Iron uptake–associated genes							
iucD	0	0	80% (4/5)	100% (3/3)	50% (1/2)	0	100% (1/1)
iroN	0	100% (1/1)	100% (5/5)	66.6% (2/3)	50% (1/2)	0	0
Serum resistance–associated genes							
traT	0	100% (1/1)	80% (4/5)	100% (3/3)	0	100% (1/1)	100% (1/1)
iss	0	0	0	0	0	0	100% (1/1)
cvaC	0	0	60% (3/5)	66.6% (2/3)	0	0	100% (1/1)
Toxin-associated genes							
vat	0	0	80% (4/5)	0	0	0	100% (1/1)
tsh	0	0	20% (1/5)	66.6% (2/3)	0	0	0
Pathogenicity island							
PAI	0	0	80% (4/5)	66.6% (2/3)	0	0	0

APEC: Avian pathogenic *Escherichia coli*

Table 4. Pairwise associations among virulence genes detected in APEC isolates recovered from commercial layer farms in West Java, Indonesia, between 2016 and 2020

Virulence genes	*fimC*	*papC*	*ibeA*	*ibeB*	*iucD*	*iroN*	*traT*	*iss*	*vat*	*cvaC*	*tsh*	*PAI*
fimC	NA											
papC	0.524	NA										
ibeA	1.0	(0.524)	NA									
ibeB	(1.0)	1.0	1.0	NA								
iucD	1.0	0.600	(1.0)	0.077*	NA							
iroN	0.095*	0.1009	(1.0)	(1.0)	0.251	NA						
traT	0.476	1.0	(0.476)	(1.0)	0.077*	0.560	NA					
iss	1.0	(1.0)	(1.0)	(1.0)	1.0	(0.333)	1.0	NA				
vat	0.524	0.017**	(0.524)	0.231	0.101	0.600	0.231	0.333	NA			
cvaC	0.467	0.608	1.0	0.569	0.026**	0.282	0.077*	0.467	0.119	NA		
tsh	1.0	(1.0)	0.476	1.0	0.231	0.231	0.516	(1.0)	(1.0)	0.026**	NA	
PAI	0.486	0.089	(0.486)	(1.0)	0.580	0.044*	0.604	(1.0)	0.329	0.315	1.0	NA

Pairwise associations between virulence genes were evaluated using Fisher's exact test. Values shown represent p-values. Statistically significant associations are indicated by asterisks ($p \leq 0.05$ **; $0.05 < p \leq 0.10$ *). Values shown in parentheses indicate negative associations based on the contingency table distribution, whereas values without parentheses indicate positive associations. These tendencies are descriptive and do not represent calculated odds ratios. "NA" indicates comparisons that were not applicable. APEC: Avian pathogenic *Escherichia coli*

DISCUSSION

The isolates analyzed in this study were obtained from laying hens showing clinical signs of colibacillosis and were confirmed as *E. coli* through selective culture and PCR-based identification. Hemolysin production is recognized as an important virulence factor in extraintestinal pathogenic *E. coli*, contributing to host cell damage and the progression of systemic infections such as pyelonephritis and peritonitis (Sora et al., 2021; Ranabhat et al., 2024). In addition, Congo Red binding is widely used as a phenotypic indicator of invasiveness in pathogenic *E. coli* strains, reflecting the ability of bacteria to express virulence-associated traits (Indrawati and Kurnia, 2019).

The *usp*A gene encodes the universal stress protein A, which plays a key role in bacterial survival, growth, adhesion, and motility. The expression of the *usp*A gene is induced under stressful environmental conditions, including heat stress, nutrient limitation, and osmotic pressure, highlighting its importance in bacterial persistence during infection (Mishra et al., 2017). The role of stress-response and virulence-associated genes in enhancing bacterial fitness and persistence has been widely reported, particularly in pathogenic *E. coli*, where multiple virulence determinants act synergistically to support infection and environmental adaptation (Rezatofighi et al., 2021). The consistent detection of *usp*A across all isolates further supports its reliability as a molecular marker for pathogenic *E. coli*.

The predominance of adhesion and serum resistance–associated genes observed in the current study is consistent with virulence patterns commonly reported in APEC, where adhesins and serum survival factors play key roles in host colonization and systemic persistence (Rezatofighi et al., 2021). The high prevalence of *fimC* (87%, 13/15), encoding type 1 fimbriae, aligns with large-scale studies identifying the *fimC* gene as one of the most conserved APEC virulence determinants, underscoring its central role in host colonization and initial attachment (Nawaz et al., 2024). In contrast, the low detection of *iss* (7%, 1/15) differs from reports describing its frequent occurrence among highly virulent, systemically invasive APEC strains, suggesting variability in serum resistance strategies across geographic regions and strain backgrounds (Johnson et al., 2008; Hossain et al., 2021). Although both *iss* and *traT* genes contribute to complement resistance, their unequal distribution, reflected by the markedly higher prevalence of *traT* (73%, 11/15), indicates functional redundancy and highlights the importance of plasmid-mediated mechanisms in local APEC survival strategies (Johnson et al., 2008).

Collectively, findings of the current study support the concept that APEC pathogenicity is driven by specific combinations of virulence factors rather than by individual genes alone, with gene prevalence shaped by ecological context and genetic background.

Comparable patterns of virulence gene prevalence have been observed in APEC isolates from neighboring Southeast Asian regions. For example, a study in Bangladesh reported high frequencies of APEC-associated *fimC*, *iucD*, and *papC* in layer farms, similar to results of the present study (Ievy et al., 2020). In Thailand, variations in virulence gene profiles and genetic diversity between broiler APEC populations have also been documented, demonstrating geographic heterogeneity within the region (Thomrongsuwannakij et al., 2020; Laopiem et al., 2025). Evidence from Bangladesh also supports the heterogeneous distribution of virulence genes in APEC, with *iroN* and *iss* frequently detected among poultry isolates (Hossain et al., 2021). Consistent with the results of the current study, a recent field-based study from Indonesia also reported substantial genetic diversity among APEC isolates circulating in commercial poultry farms, with heterogeneous virulence gene profiles and phylogenetic backgrounds highlighting the importance of region-specific molecular surveillance (Timur et al., 2026).

The virulence gene content varied markedly among the APEC isolates examined in this study, underscoring the heterogeneous nature of APEC pathogenicity. In the present study, isolate B098, belonging to phylogenetic group B2, harbored the highest number of virulence-associated genes, whereas isolate B012 carried only a single detected virulence gene (*ibeA*) yet was associated with colibacillosis. Recent genome-based and molecular epidemiological studies have demonstrated that APEC pathogenicity is not defined by a fixed number of virulence genes but rather by diverse and strain-specific combinations shaped by genetic background and host–pathogen interactions (Kravik et al., 2023; Li et al., 2025). Whole-genome sequencing analyses of APEC isolates from chickens further indicated that several strains associated with clinical disease may lack subsets of classical virulence genes, reinforcing that pathogenic potential is context-dependent rather than determined solely by gene count, as previously assumed in earlier virulence paradigms (Kravik et al., 2023).

In the present study, a positive association was identified between phylogenetic group B2 and two virulence genes, namely *papC* and *vat*. The *papC* gene functions as an adhesin and plays a role in the formation of colonization factors involved in extraintestinal infections (Sora et al., 2021). In contrast, *vat* encodes a proteolytic toxin that induces vacuolization in host cells (Díaz et al., 2020). These findings are consistent with previous studies indicating that phylogenetic group B2 is commonly associated with higher virulence and specific virulence gene profiles. The co-occurrence of virulence genes, including *cvaC*, *iucD*, and *tsh*, likely reflects their co-localization on mobile genetic elements such as plasmids, which play a key role in coordinating virulence traits in APEC (Table 4). One of the plasmid markers commonly used to identify *Colicin V* (*ColV*) plasmids is *cvaC* (Reid et al., 2022). *ColV* plasmids are frequently detected in *E. coli* strains and are often associated with invasive properties and enhanced pathogenicity, as plasmids can carry multiple virulence genes that facilitate bacterial survival and replication within the host (Johnson et al., 2022; Reid et al., 2022). The *iucD* gene is involved in iron uptake through the synthesis and regulation of the aerobactin system, while *tsh* functions as an autotransporter protein involved in bacterial localization and tissue infection (Kostakioti and Stathopoulos, 2004; Johnson et al., 2022). According to Johnson et al. (2023), both *iucD* and *tsh* can be located on *ColV* plasmids, supporting the findings of the present study. The observed associations among virulence genes may reflect non-random combinations of virulence determinants that could synergistically contribute to the pathogenic potential of APEC. Such patterns may indicate potential co-localization of virulence genes on mobile genetic elements, including plasmids or pathogenicity islands.

Similarity among virulence genes may reflect their association with mobile genetic elements, including plasmids or pathogenicity islands (Shoaib et al., 2025). The detection of *ibeA* in isolate B012, belonging to phylogenetic group A, highlights the complex relationship between phylogenetic background and pathogenic potential in APEC. Previous studies have shown that *ibeA* plays a key role in the invasion of brain microvascular endothelial cells and contributes to systemic dissemination in animal infection models (Wang et al., 2011). Although phylogenetic group A is predominantly associated with commensal *E. coli*, increasing evidence indicates that strains from this group may acquire virulence-associated traits and cause disease under certain conditions, such as host immunosuppression, environmental stress, or the acquisition of mobile genetic elements carrying virulence genes (Dale and Woodford, 2015). Comparative genomic analyses have further shown that virulence determinants in *E. coli* are frequently acquired through horizontal gene

transfer, often via pathogenicity islands and other mobile genetic elements, enabling commensal lineages to transition toward pathogenic phenotypes (Lo et al., 2015; Desvaux et al., 2020). Previous studies have also reported virulence-associated genes such as *iucD*, *iss*, and *papC* in commensal *E. coli* isolates from healthy chickens, underscoring the fluid boundary between commensal and pathogenic strains (Al-Kandari and Woodward, 2019). Taken together, these findings support the results of the current study that isolate B012, despite belonging to a phylogroup commonly regarded as commensal, may retain pathogenic potential through the acquisition of specific virulence determinants, reinforcing the notion that APEC virulence is shaped by gene content and genetic context rather than phylogenetic affiliation alone (Desvaux et al., 2020).

The pathogenicity of isolate B012 in colibacillosis may be partly attributed to its alpha-hemolytic activity on blood agar. This interpretation is supported by the observation that all isolates belonging to phylogenetic group B2 also exhibited alpha-hemolysis. Alpha-hemolysin is a well-recognized virulence-associated toxin of *E. coli* that contributes to host cell damage by disrupting membrane integrity, inducing ATP depletion, and altering potassium homeostasis, ultimately leading to cell death (Verma et al., 2020; Cané et al., 2024). A previous report on extraintestinal pathogenic *E. coli* emphasized that alpha-hemolysin primarily functions as a mediator of tissue damage and inflammatory responses rather than as a direct indicator of overall virulence gene burden (Sora et al., 2021). In the same line, several alpha-hemolytic isolates in the present study harbored relatively few virulence-associated genes, suggesting that hemolytic activity may contribute to pathogenicity independently or synergistically with other virulence determinants rather than serving as a direct proxy for virulence gene content.

Pathogenicity islands (PAIs) were detected in 40% of the isolates and were exclusively associated with phylogenetic groups B2 and F. Pathogenicity islands are chromosomally encoded regions that harbor one or more virulence-associated genes and are known to enhance pathogenic potential (Sora et al., 2021). However, APEC virulence is not solely dependent on chromosomal determinants, as key virulence genes may also be plasmid-borne (Ovi et al., 2023). The dual contribution of chromosomal and mobile genetic elements suggests that pathogenicity arises from integrated genetic architectures rather than from a single virulence locus, as no individual gene has been found to be uniquely associated with pathogenic strains and virulence is instead driven by the interplay of multiple genetic pathways (Palmieri et al., 2023). Accordingly, isolates lacking PAIs may still exhibit pathogenic behavior through alternative genetic and regulatory mechanisms that support host-associated survival, reflecting the overall genomic complexity and multifactorial nature of APEC pathogenicity (Palmieri et al., 2023; Kamal et al., 2025).

A substantial positive correlation was observed between the *vat* and *papC* genes, suggesting coordinated roles in APEC pathogenicity. The *vat* gene encodes a proteolytic toxin that induces host cell vacuolization (Díaz et al., 2020), whereas *papC* functions as an adhesin facilitating extraintestinal colonization (Sora et al., 2021). In addition, the presence of *cvaC*, which encodes colicin V and is associated with ColV plasmids, supports the involvement of plasmid-associated virulence factors in enhancing bacterial survival and host interaction (Reid et al., 2022).

Classical APEC serotypes associated with colibacillosis include O1, O2, O18, and O78 (Kathayat et al., 2021). In the present study, three isolates belonged to serogroup O1, two to O18, and one to O78, whereas the majority of isolates were not assignable to the tested serogroups. Phylogenetic analysis classified the isolates A (1/15), B1 (2/15), B2 (5/15), F (3/15), A/C (2/15), D/E (1/15), and one unclassified group. Consistent with the previous report, phylogenetic group B2 was predominantly associated with extraintestinal pathogenic potential, while groups A and B1 are more commonly linked to commensal strains (Chakraborty et al., 2015).

Previous studies have demonstrated substantial O-serogroup diversity among APEC isolates beyond the classical serotypes, indicating the involvement of additional and potentially emerging serogroups (Newman et al., 2021). A limitation of this study is that serogroup analysis was restricted to O1, O2, O18, and O78; therefore, other serogroups, such as O24, O25, O86, and the emerging O145, may be present among the untypeable isolates.

Although the revised Clermont PCR method improves the specificity of phylogenetic classification compared to the original triplex approach, it remains a targeted method that examines only a limited portion of the bacterial genome. Previous studies have highlighted limitations in resolving closely related phylogenetic groups and potential overlaps between lineages, particularly among groups such as A/C and D/E (Clermont et al., 2015; Odoki et al., 2020). The observed pattern is consistent with genome-based population studies demonstrating that *E. coli* exhibits a highly complex and continuous population

structure, in which clear phylogenetic boundaries are not always sharply defined, potentially leading to ambiguous assignments when PCR-based typing methods are applied (Clermont et al., 2019). Genome-based approaches, including multilocus sequence typing and whole-genome sequencing, are currently regarded as the gold standard for *E. coli* phylogenetic resolution (Beghain et al., 2018). Nevertheless, PCR-based Clermont typing remains a practical and widely accepted tool for epidemiological investigations, especially in large-scale surveillance studies and in settings where genome sequencing is not routinely available (Clermont et al., 2015).

In line with findings from Brazil, where phylogenetic group G has been reported among APEC isolates (Barbosa et al., 2023), the absence of this group in the present study may be related to the exclusion of the *ybgD* marker from the phylogenetic typing scheme in this study. Overall, these findings underscore that APEC pathogenicity reflects a multifactorial and context-dependent process shaped by genetic composition, mobile elements, and host interactions, reinforcing the importance of integrated molecular and genomic approaches in future epidemiological investigations.

CONCLUSION

This study provides valuable insight into the virulence gene profiles, serogroup distribution, and phylogenetic characteristics of APEC isolates from commercial layer farms in West Java, Indonesia, revealing considerable genetic diversity among isolates. Phylogenetic group B2 harbored more virulence-associated genes, supporting its potential role in pathogenicity, while observed associations between *papC* and *vat*, as well as among *cvaC*, *iucD*, *tsh*, and *vat*, suggest possible clustering of plasmid- and pathogenicity-related determinants. Although *fimC* was the most prevalent gene, the presence of additional factors such as *ibeB*, *traT*, and *iroN* highlights the multifactorial nature of APEC pathogenic mechanisms. The detection of O1, O18, and O78 serogroups, together with unclassified isolates, indicates the circulation of diverse APEC populations in the studied region. However, the relatively limited sample size (n=15), restricted geographic coverage, and reliance on PCR-based characterization may constrain broader generalization and affect the robustness of the statistical analysis; therefore, the findings should be interpreted with caution and require validation in larger-scale studies. Future studies incorporating larger and more diverse populations, expanded serogroup panels, genome-based approaches, and improved phylogenetic typing methods, including the quadruplex scheme and additional markers such as *ybgD*, are required to enhance classification accuracy and more comprehensively capture the full diversity of APEC, ultimately supporting more effective surveillance and control strategies.

DECLARATIONS

Acknowledgments

The authors gratefully acknowledge Lusianawati Widjaja for her valuable assistance in enhancing the clarity of the manuscript and for her constructive input during the response-to-reviewers process.

Authors' contributions

Difa Widasari, Ryan Septa Kurnia, Muhammad Ade Putra, Christian Marco Hadi Nugroho, Agustin Indrawati, and Surachmi Setiyaningsih wrote the original text, contributed to the experimental design, carried out the experiments, and conducted the statistical analysis. Difa Widyasari, Christian Marco Hadi Nugroho, Agustin Indrawati, and Surachmi Setiyaningsih analyzed and reviewed the results and then composed the final essay. All authors have reviewed and approved the final edition of the manuscript before publication in the present journal.

Availability of data and materials

All data from the current study are available upon reasonable requests from the authors.

Competing interests

The authors declared no conflicts of interest.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. The authors have reviewed additional ethical concerns, including research misconduct, data fabrication, and redundancy. The authors declare that artificial intelligence (AI) tools, specifically Grammarly (Grammarly Inc., version 1.2.255.1882), were used only for language refinement and grammar checking. All scientific content, data interpretation, and conclusions were developed entirely by the authors.

Funding

The present study was financially supported by the Ministry of Higher Education, Science, and Technology (Kemdiktisaintek), Indonesia, through the RIKUB grant

under the main contract number of 012/C3/DT.05.00/RIKUB/2025 and sub-contract number of PKS-686/UN2.RST/HKP.05.00/2025.

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