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Virulence factor genes analysis of extended spectrum β-Lactamase producing *Escherichia coli* isolated from broiler chickens in Indonesia

Nofita NURBIYANTI[1,2], Chaerul BASRI[3], Puji RAHAYU[4], Imron SUANDY[5], Hadri LATIF[3]*

[1]Study Program of Animal Biomedical Science, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia
[2]Center for Indonesian Veterinary Analytical Studies (CIVAS), Bogor, Indonesia
[3]Division of Veterinary Public Health and Epidemiology Division, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor, Indonesia
[4]National Quality Control and Certification for Animal Products Laboratory (BPMSPH), Ministry of Agriculture, Bogor, Indonesia
[5]Directorate General of Livestock and Animal Health Services, Ministry of Agriculture, Jakarta, Indonesia

ABSTRACT. Extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) is recognized as an opportunistic pathogen with zoonotic potential, posing a threat to public health. This study aimed to identify virulence factor genes (VFGs), determine ESBL-Ec pathotypes and pathovars, and analyze their distribution in isolates from broiler cecum across eight Disease Investigation Center (DIC) areas in Indonesia. A total of 120 ESBL-Ec isolates were proportionally selected based on regional prevalence. The VFGs were detected using Oxford Nanopore Technologies (ONT) MinION sequencing and analyzed with bioinformatics pipelines. Of the total isolates, 113 met the quality threshold. A total of 4,269 non-redundant orthologs and alleles related to VFGs were detected, representing 99 VFG types grouped into 25 clusters, indicating substantial genetic diversity. The *fim* gene cluster was the most frequently detected (20.1%), whereas the *esp* cluster exhibited the highest allele diversity (18.2%). The VFGs encoding the ExPEC were predominant (66.7%), while the remaining VFGs encoded the InPEC (33.3%). Each isolate potentially encodes at least two pathovars, with nearly 80% encoding three or more. The diversity of VFGs and pathovar combinations highlights the disease potential and zoonotic risk of ESBL-Ec.

KEYWORDS: broiler chicken, extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec), pathotypes, virulence factor genes (VFGs), whole genome sequencing (WGS)

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INTRODUCTION

Extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) is a major public health concern because it can spread between species and cause serious infections that are difficult to treat. These bacteria produce extended spectrum β-lactamase enzymes that confer resistance to many antibiotics, including penicillins, third- and fourth-generation cephalosporins, and monobactams [37]. This resistance makes infections difficult to treat and can lead to severe complications or even death [23].

The World Health Organization recommends ESBL-Ec as a critical indicator for monitoring antimicrobial resistance (AMR) worldwide, using the One Health approach [39]. This approach recognizes that humans, animals, and the environment are closely interconnected in the spread of resistant bacteria. In poultry, ESBL-Ec commonly colonizes the intestinal tract but can also cause diseases such as colibacillosis, salpingitis, and omphalitis [14, 38]. These infections often lead to indiscriminate use of antibiotics in day-old chicks (DOCs), accelerating resistance development [34].

The pathogenic potential of ESBL-Ec largely depends on *virulence factor genes* (VFGs). These genes encode proteins that help the bacteria attach to host cells, evade the immune system, and survive inside the body [25]. Based on the site of infection and virulence characteristics, *E. coli* is classified into two major pathotypes: intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC). Each pathotype comprises several pathovars, such as enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*

*Correspondence to: Latif H: hadrilatif@apps.ipb.ac.id, Veterinary Public Health and Epidemiology Division, School of Veterinary Medicine and Biomedical Science, IPB University, Bogor 16680, Indonesia
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(EHEC), and enteroaggregative *E. coli* (EAEC) within the InPEC group, whereas ExPEC includes pathovars such as uropathogenic *E. coli* (UPEC) and neonatal meningitis-associated *E. coli* (NMEC) [7].

The spread of pathotypes and pathovars has significant public health implications. The ExPEC pathotypes, particularly UPEC, are responsible for more than 90% of urinary tract infections (UTIs) in humans [31]. In contrast, InPEC pathotypes such as EHEC are the leading cause of bloody diarrhea and hemolytic uraemic syndrome (HUS) due to Shiga toxin production [27]. Several studies have shown that ExPEC strains isolated from broiler chickens are genetically similar to those found in human clinical cases, suggesting a zoonotic risk through potential exposure to contaminated poultry meat or its products [17, 38].

Despite its major public health impact, research on the distribution of VFGs and pathotypes of ESBL-Ec in poultry in Indonesia remains limited and lacks a comprehensive investigation. Most studies have focused only on detecting specific genes, such as *fimA and papC*, using Polymerase Chain Reaction (PCR) [38]. This method is insufficient to capture the broader genetic diversity and its potential risks to human, animal, and environmental health.

Therefore, to address this information gap, this study was conducted to provide a comprehensive overview of zoonotic risk by identifying virulence factor genes, pathotypes, and pathovars of ESBL-Ec isolates obtained from the cecum of broiler chickens in eight Disease Investigation Center (DIC) regions across Indonesia. Through this approach, this study aims to elucidate the distribution pattern of virulence genes associated with transmission potential and to provide insights for developing zoonotic disease control strategies, strengthening AMR surveillance programs, and formulating more targeted and risk-based veterinary public health policies.

MATERIALS AND METHODS

Ethics of animal research

This study did not require animal ethics approval because live animals were not used. All samples originated from the national surveillance archive, previously collected by official government agencies.

Selection of samples and isolates

Samples used in this study were obtained from the national archive of the AMR surveillance program coordinated by the Indonesian government. All isolates were ESBL-Ec obtained from broiler cecum samples collected at poultry slaughterhouses within the working areas of eight DICs. The DICs are technical units under the Directorate General of Livestock and Animal Health Services, Ministry of Agriculture, responsible for collecting samples as part of AMR surveillance in accordance with the Indonesian AMR Surveillance Guidelines [22].

Of the 835 confirmed *E. coli* isolates, 259 were identified as ESBL-Ec through active surveillance conducted by the eight DICs in Indonesia in 2023 and subsequently archived at the National Quality Control and Certification for Animal Products Laboratory (BPMSPH) [33]. Based on ESBL-Ec prevalence data, a representative subset of isolates were selected for this study using an expected prevalence of 50%, an acceptable error rate of 7%, and a 95% confidence level. The sample size was calculated using WinEpi software (http://www.winepi.net/), resulting in 120 isolates as the representative sample. The number of isolates assigned to each DIC was determined proportionally to the regional prevalence of ESBL-Ec reported in the national surveillance program, and isolates were selected using simple random sampling [28, 33].

Only isolates that met the quality criteria based on whole genome sequencing (WGS) results were included in the analysis, with genome completeness of ≥90% and contamination rate of ≤5% [4]. The distribution of isolates across the DIC working areas is shown in Fig. 1 and Table 1.

Identification and confirmation of ESBL-Ec

The isolates were initially identified using standard bacteriological culture techniques on MacConkey agar (Oxoid, Hampshire, UK), and presumptive *E. coli* colonies were confirmed by indole testing, biochemical assay, and Matrix-Assisted Laser Desorption/Ionization–Time of Flight (MALDI–TOF) Mass Spectrometry (Bruker Daltonics, Bremen, Germany). The ESBL production was subsequently confirmed phenotypically using the double-disk diffusion test (Kirby–Bauer disk diffusion method) on Mueller–Hinton agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), in accordance with Clinical and Laboratory Standards Institute (CLSI, Wayne, PA, USA) guidelines [39].

DNA extraction and quality assessment

Deoxyribonucleic acid (DNA) was extracted from 120 ESBL-Ec isolates using the DNeasy® PowerWater® Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The DNA concentration was measured using a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and samples with at least 1 ng/μL were selected for library preparation [30]. The purified DNA was subsequently end-repaired and ligated with sequencing adapters to generate libraries compatible with the Oxford Nanopore platform (Oxford Nanopore Technologies/ONT, Oxford, UK).

Whole genome sequencing

All DNA isolates that passed the selection were analyzed using MinION™ Sequencer (ONT), together with the Rapid Sequencing gDNA Barcoding Kit (SQK-RBK110.96; Oxford Nanopore Technologies). The prepared DNA library was loaded into a Flow Cell (FLO-MIN106D R9.4.1; Oxford Nanopore Technologies) for sequencing. Concerning library preparation, high molecular weight genomic DNA was used without mechanical shearing to preserve long fragments. The library was prepared following the manufacturer's

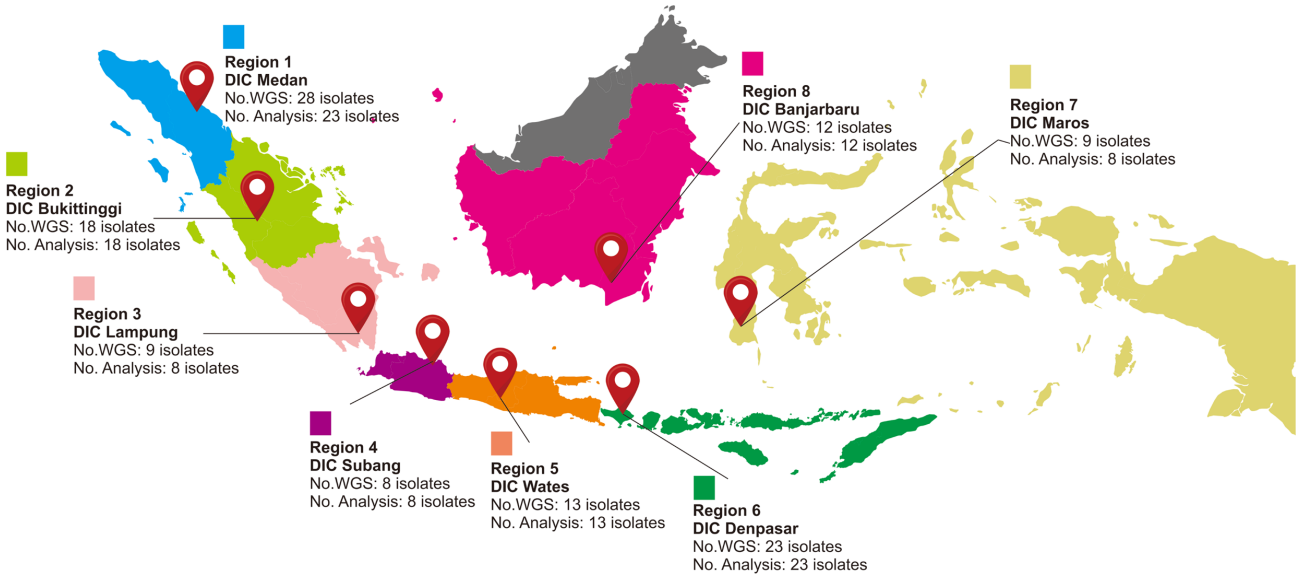

Fig. 1. Sample distribution map of extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) isolates from broiler cecum collected at eight Disease Investigation Centers (DICs) in Indonesia in 2023. Number of whole genome sequencing (WGS) are samples tested by whole genome sequencing and those included in the analysis.

protocol, which included DNA repair and end-preparation steps, adaptor ligation, and clean-up procedures before sequencing. These steps ensured optimal read length and sequencing quality. During sequencing, DNA molecules pass through nanopores, altering the ionic current, to generate long-read sequence data for downstream bioinformatic analysis. The long-read sequencing approach allows more comprehensive and accurate genome analysis, particularly for detecting complex virulence factors in bacteria [32].

Bioinformatic analysis

Sequencing data in FASTQ format were analyzed using Ubuntu operating system (Canonical Ltd., London, UK) and bioinformatics software such as NanoStat (Oxford Nanopore Technologies) for raw data quality checking, Filtlong (Ryan Wick, Melbourne, Australia) for isolate filtering based on completeness ≥90% and contamination ≤5%, Flye (Kolmogorov Laboratory, University of California, San Diego, CA, USA) for *de novo* assembly, Quast (St. Petersburg State University, St. Petersburg, Russia) and CheckM (Australian Centre for Ecogenomics, Brisbane, Australia) for quality evaluation of genome assembly results, Multi-Locus Sequence Typing (MLST; Torsten Seemann, Melbourne, Australia), and Platon (University of Würzburg, Würzburg, Germany) for genomic feature identification, and ABRicate (Torsten Seemann, Melbourne, Australia) with the VFDB database (Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China) for virulence factor gene detection.

All VFG detection results were confirmed using a suitability standard of at least 95% for both coverage and identity [4]. Pathotypes and pathovars were identified using the VFDB platform (http://www.mgc.ac.cn/VFs/), which contains over 4,300 virulence factor genes based on experimental data. The collected data were then analyzed descriptively by counting how often each unique allele gene appeared, using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

RESULTS

DNA and genome quality of whole genome sequencing results

Of the 120 ESBL-Ec isolates, only 113 met the quality criteria and were subsequently included in bioinformatics analysis [4]. The genome quality of ESBL-Ec isolates obtained from broiler chicken cecum samples in Indonesia is summarized in Table 2. The genomic data supporting these findings of this study are publicly available in the European Nucleotide Archive (ENA) under accession number PRJEB97707.

Virulence factor genes

The identification of VFGs in 113 ESBL-Ec isolates revealed 99 gene types grouped into 25 main gene clusters based on 4,269 non-redundant orthologs and allele occurrences (Table 3). The *fim* cluster had the highest gene frequency, reaching 20.1%. High gene frequencies were also observed in the *ent* cluster (16.2%) and the *yag/ecp* cluster (14.3%). Although the *esp* cluster contained the greatest diversity of gene types (18.2%), its occurrence frequency was only 9.3%, indicating that not all genes within a cluster are equally present in all isolates. Other clusters with high gene diversity include the *fim* cluster (9.1%) and the *pap* cluster (8.1%).

Conversely, clusters like *sfa*, *vat*, and *pic* were found with very low frequencies of <0.01%, indicating that these genes are only present in a small proportion of isolates with a low frequency of occurrence (Table 3). The *nle* gene cluster was divided into two

subgroups, *nle** and *nle***. The *nle** subgroup potentially encodes EHEC pathovars, while *nle*** (*nleA* and *nleD*) is associated with EPEC pathovars [2, 3, 24] (Tables 3 and 4).

Cluster profile of virulence factor genes that potentially encode pathotypes and pathovars

An analysis of gene occurrence frequency in 113 ESBL-Ec isolates showed that most VFGs were strongly linked to the ExPEC pathotype, accounting for 66.7%. Among these, the majority were from gene clusters likely encoding UPEC pathovars (63.8%), while the rest (2.9%) were associated with NMEC pathovars (Table 4).

The remaining VFGs were strongly associated with InPEC pathotypes, making up 33.3%. Most of these were from gene clusters likely encoding EHEC pathovars (30.3%), while the rest were associated with EAEC (2%) and EPEC (1%) pathovars (Table 4).

Prevalence of virulence factor gene clusters

Analysis of 113 ESBL-Ec isolates revealed varying prevalence levels among the detected VFG clusters, representing the proportion of isolates carrying each gene. This differs from gene frequencies, which represent the total number of specific genes detected across all isolates. Of the 25 identified gene clusters, 16 were classified as virulence genes linked to the ExPEC pathotype, while the remaining nine clusters potentially encode the InPEC pathotype. Notably, the *esp* and *yag/ecp* clusters exhibited high prevalence rates of 96.5% and 91.2%, respectively (Fig. 2). These results indicate a widespread presence of both ExPEC and InPEC virulence genes in the analyzed isolate population. The comprehensive distribution and prevalence of each gene cluster for both pathotype groups are shown in Fig. 2.

Distribution of virulence factor gene clusters in eight regions of DICs in Indonesia

The distribution of VFGs in ESBL-Ec exhibited varying patterns across DIC regions in Indonesia. Based on the frequency of VFG clusters in each DIC, the highest proportion was found in DIC Region 6 (Denpasar) at 20%, followed by Region 1 (Medan) at 17.6%, and Region 2 (Bukittinggi) at 16.3%. In contrast, the lowest proportion was recorded in Region 4 (Subang), with only 7.1% (Table 5).

Overall, most regions exhibited a prevalence of the *fim* cluster, a significant gene cluster potentially encoding the UPEC pathovar (ExPEC pathotype). The highest frequency of the *fim* gene cluster was found in Region 1 (Medan) at 22.6%, followed by Region 4 (Subang), Region 6 (Denpasar), and Region 7 (Maros) at 21.6%, 21%, and 20.3%, respectively. However, an exception exists in Region 3 (Lampung) where the most dominant gene cluster is not *fim* but *ent*, accounting for 16.2% (Table 5).

Table 1. Distribution of extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) isolates and representative samples across Disease Investigation Center (DIC) regions

DIC regions	Confirmed ESBL-Ec isolates (N)	Representative isolates for WGS*	Isolates included in bioinformatics analysis**
1 Medan	61	28	23
2 Bukittinggi	39	18	18
3 Lampung	20	9	8
4 Subang	17	8	8
5 Wates	27	13	13
6 Denpasar	49	23	23
7 Maros	19	9	8
8 Banjarbaru	27	12	12
Total	259	120	113

Whole genome sequencing (WGS). *Representative samples proportionally allocated from confirmed ESBL-Ec isolates across DIC regions. **Isolates that met the quality criteria and were eligible for bioinformatics analysis.

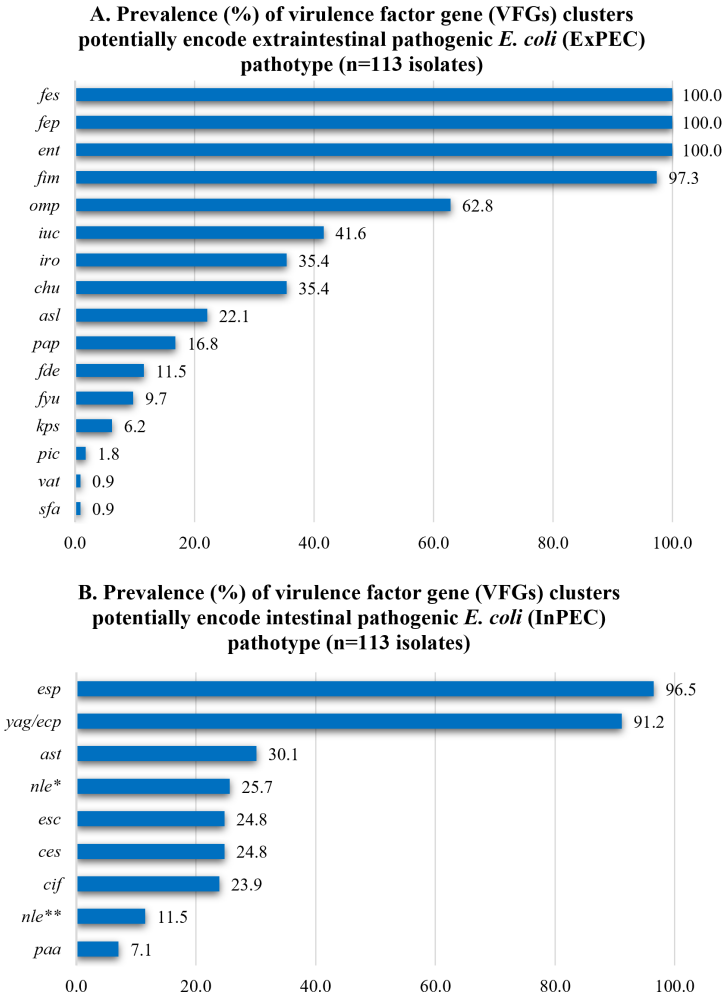

Fig. 2. Prevalence of virulence factor gene (VFG) clusters in extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) from broiler cecum (n=113); (A) Potentially encoding extraintestinal pathogenic *E. coli* (ExPEC), and (B) Potentially encoding intestinal pathogenic *E. coli* (InPEC).

Table 2. Summary of genome quality of extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) isolates from broiler cecum in Indonesia (n=113 isolates)

Variable	Mean	SEMean	StDev	Minimum	Maximum
Completeness (%)	98.28	0.14	1.44	90.52	99.51
Contamination (%)	1.41	0.07	0.76	0.36	4.59
Genome length fastq (bp)	418,124,977	19,909,803	211,644,106	51,598,774	924,360,929
Median read length (bp)	4,329	111	1,183	1,222	6,387
Mean read length (bp)	7,375	154	1,639	2,815	10,330
Genome size (bp)	5,508,883	31,005	329,588	4,757,790	6,613,543
N50 (bp)	4,597,676	119,936	1,274,938	305,168	5,670,062

Base pair (bp); standard error of the mean (SEMean); standard deviation (StDev); genome length refers to the total length of all genome reads; genome size refers to the length of a single genome, reflecting species variation; N50 represents the contig length at which 50% of the genome is contained, with values ≥100 kilobase pairs (kbp) indicating high-quality assembly.

Table 3. Frequency and virulence factor genes (VFGs) cluster of extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) isolates in broiler cecum in Indonesia (n=113)

No	VFG Types	VFGs clusters (n=25)	Variation clusters		Gene occurrence frequency	
			Number (n)	Proportion (%)	Number (n)	Proportion (%)
1	*fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI*	*fim*	9	9.1	857	20.1
2	*entA, entB, entC, entD, entE, entF, entS*	*ent*	7	7.1	690	16.2
3	*yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*	*yag/ecp*	6	6.1	607	14.3
4	*fepA, fepB, fepC, fepD, fepG*	*fep*	5	5.1	462	10.8
5	*espFu/tccP, espJ, espK, espL1, espL4, espM1, espM2, espR1, espR3, espW, espX1, espX2, espX4, espX5, espY1, espY2, espY3, espY4*	*esp*	18	18.2	398	9.3
6	*iroB, iroC, iroD, iroE, iroN*	*iro*	5	5.1	208	4.9
7	*escD, escF, escO, escR, escS, escU, escV*	*esc*	7	7.1	193	4.5
8	*chuS, chuT, chuU, chuV,chuW,chuX,chuY*	*chu*	7	7.1	184	4.3
9	*iucB, iucC, iucD*	*iuc*	3	3.0	147	3.4
10	*fes*	*fes*	1	1.0	113	2.6
11	*astA*	*ast*	1	1.0	85	2
12	*ompA*	*omp*	1	1.0	71	1.7
13	*nleA/espI, nleB2, nleD, nleF, nleG7, nleH1, nleH2*	*nle**	7	7.1	51	1.2
14	*papB, papC, papD, papF, papG, papI, papJ, papK*	*pap*	8	8.1	48	1.1
15	*cesD2, cesT*	*ces*	2	2.0	40	0.9
16	*aslA*	*asl*	1	1.0	25	0.6
17	*cif*	*cif*	1	1.0	27	0.6
18	*fyuA*	*fyu*	1	1.0	11	0.3
19	*fdeC*	*fde*	1	1.0	14	0.3
20	*kpsD, kpsM*	*kps*	2	2.0	12	0.3
21	*nleA, nleD*	*nle***	2	2.0	14	0.3
22	*paa*	*paa*	1	1.0	8	0.2
23	*pic*	*pic*	1	1.0	2	0.05
24	*sfaX*	*sfa*	1	1.0	1	0.02
25	*vat*	*vat*	1	1.0	1	0.02
	Total		99	100	4,269	100

The *nle* gene cluster was divided into two subgroups: *nle**, potentially encoding enterohemorrhagic *E. coli* (EHEC) pathovars, and *nle*** (*nleA* and *nleD*), associated with enteropathogenic *E. coli* (EPEC) pathovars.

Cluster distribution pattern of potential virulence factor genes encoding pathovars of each isolate

Based on the analysis of 113 ESBL-Ec isolates, each isolate was found to potentially carry at least two types of pathovars encoded by VFG clusters. Interestingly, the majority of isolates—around 80%—had the potential to carry three or more pathovars. The distribution of the number of pathovars per isolate varied across regions, with the highest proportion observed in isolates carrying three pathovar types (46.9% of the total), followed by four pathovar types (27.4%), two pathovar types (20.4%), and five pathovar types (5.3%) (Table 6).

Table 4. Cluster of virulence factor genes (VFGs) potentially encoding pathovars and pathotypes of extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) from broiler cecum in Indonesia (n=113)

No.	VFGs cluster	Potential pathovar		Potential pathotype	
		Type	Proportion (%)	Type	Proportion (%)
1	*fim, ent, fep, iro, chu, iuc, fes, pap, fyu, pic, sfa, vat*	UPEC	63.8	ExPEC	66.7
2	*omp, asl, fde, kps*	NMEC	2.9		
3	*yag/ecp, esp, esc, nle*, ces, paa*	EHEC	30.3		
4	*ast*	EAEC	2	InPEC	33.3
5	*cif, nle***	EPEC	1		

Extra-intestinal pathogenic *E. coli* (ExPEC); intestinal pathogenic *E. coli* (InPEC); uropathogenic *E. coli* (UPEC); neonatal meningitis-associated *E. coli* (NMEC); enterohemorrhagic *E. coli* (EHEC); enteroaggregative *E. coli* (EAEC); enteropathogenic *E. coli* (EPEC).

Table 5. Distribution of virulence factor genes (VFGs) of extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) from broiler cecum in eight regions Disease Investigation Centers (DICs) in Indonesia

Potential pathotype	Potential pathovar	VFGs clusters	Regions (%)							
			1 (n=753)	2 (n=696)	3 (n=315)	4 (n=301)	5 (n=513)	6 (n=854)	7 (n=315)	8 (n=522)
ExPEC	UPEC	*fim*	22.6	19.4	15.2	21.6	19.5	21	20.3	18.4
		ent	18.9	16.4	16.2	15.9	15	16	13	15.3
		fep	12.2	10.6	10.2	11.3	10.3	10.7	11.1	9.8
		iro	2	3.6	12.7	5	2.7	2.9	7.9	9.4
		chu	2.7	4	4.8	7.6	4.3	2.6	6	6.7
		iuc	1.6	1.6	6.3	7	2.9	2.3	6.7	5.2
		fes	3.1	2.6	2.5	2.7	2.5	2.7	2.5	2.3
		pap	1.1	0	0.6	0.3	2.9	1.3	0.3	1.9
		fyu	0	0.1	0.6	0.3	0.2	0.1	0.6	0.6
		pic	0	0	0	0	0.2	0	0	0.2
		sfa	0.1	0	0	0	0	0	0	0
		vat	0	0	0	0	0	0	0	0.2
	Sub-subtotal UPEC		64.3	58.3	69.1	71.7	60.5	59.6	68.4	70
	NMEC	*omp*	1.3	1.6	1.6	2	1.8	1.4	1.9	2.3
		asl	0.4	0.4	0.6	1	0.8	0.2	1	1
		fde	0.1	0	0.3	1	0.4	0.1	1	0.6
		kps	0.4	0	0	0	0.2	0.2	1.3	0.4
	Sub-subtotal NMEC		2.2	2	2.5	4	3.2	1.9	5.2	4.3
Subtotal ExPEC			66.5	60.3	71.6	75.7	63.7	61.5	73.6	74.3
InPEC	EHEC	*yag/ecp*	15.7	13.6	13.3	13.6	15.2	15.3	15.2	10.3
		esp	9.2	10.6	7	9	9	10	10.2	8.2
		esc	3.7	7.5	4.4	0	6.8	5	0	4
		*nle**	0.7	2.3	1.3	0	1.6	1.4	0	1.1
		ces	0.7	1.3	1.3	0	1.2	1.3	0	1
		paa	0	0.7	0	0	0	0.2	0	0.2
	Sub-subtotal EHEC		30.0	36.0	27.3	22.6	33.8	33.2	25.4	24.8
	EAEC	*ast*	2.8	2.2	0.3	1.7	0.6	4.1	1	0.4
	Sub-subtotal EAEC		2.8	2.2	0.3	1.7	0.6	4.1	1	0.4
	EPEC	*cif*	0.5	1	0.6	0	1.2	0.7	0	0.4
		*nle***	0.4	0.4	0	0	0.8	0.4	0	0.2
	Sub-subtotal EPEC		0.9	1.4	0.6	0	2.0	1.1	0	0.6
Subtotal InPEC			33.7	39.6	28.2	24.3	36.4	38.4	26.4	25.8
Total percentage (%)			17.6	16.3	7.4	7.1	12	20	7.4	12.2

This data is presented as a proportion based on the frequency of genes detected in each DIC; extra-intestinal pathogenic *E. coli* (ExPEC); intestinal pathogenic *E. coli* (InPEC); uropathogenic *E. coli* (UPEC); neonatal meningitis-associated *E. coli* (NMEC); enterohemorrhagic *E. coli* (EHEC); enteroaggregative *E. coli* (EAEC); enteropathogenic *E. coli* (EPEC).

Table 6. Distribution patterns of the number of potential pathovars based on virulence factor genes (VFGs) clusters present in each extended spectrum β-lactamase producing *Escherichia coli* (ESBL-Ec) isolate from broiler cecum in eight regions Disease Investigation Centers (DICs) in Indonesia

DIC regions	Number of isolates	Proportion of pathovars (%)				
		1	2	3	4	5
1 Medan	23	0.0	34.8	39.1	21.7	4.3
2 Bukittinggi	18	0.0	16.7	33.3	33.3	16.7
3 Lampung	8	0.0	37.5	37.5	12.5	12.5
4 Subang	8	0.0	12.5	62.5	25.0	0.0
5 Wates	13	0.0	0.0	69.2	30.8	0.0
6 Denpasar	23	0.0	26.1	43.5	26.1	4.3
7 Maros	8	0.0	25.0	37.5	37.5	0.0
8 Banjarbaru	12	0.0	0.0	66.7	33.3	0.0
Average of total isolates (%)	113	0.0	20.4	46.9	27.4	5.3

Each isolate was found to potentially contain at least two pathovars, and the dominant consisted of three pathovars.

DISCUSSION

This study represents one of the first comprehensive efforts in Indonesia to identify VFGs in ESBL-Ec isolates obtained from broiler cecum. The findings revealed considerable diversity in cluster types, gene frequencies, prevalence, and the number of pathovars within a single isolate. This diversity suggests the potential for the pathogens to cause disease, expand their host range, enhance adaptability, and facilitate transmission, and the combination of these factors may contribute to the emergence of hybrid pathogens with enhanced virulence [1, 40].

In particular, it was found that the *esp* cluster was twice as abundant as the *fim* cluster, but *fim* showed the highest frequency of occurrence, being twice that of *esp* (Table 3). The presence of the *esp* cluster is not necessarily indicative of pathogenic potential, as it may also be found in commensal or environmental *E. coli* strains [8, 25]. The observed genetic diversity is likely driven by mechanisms such as point mutations, genetic recombination, horizontal gene transfer (HGT) via plasmids, transposons, or bacteriophages, as well as gene duplication and deletion [7]. Within the One Health framework, the variation of virulence gene clusters reflects the adaptive flexibility of bacteria when responding to diverse pressures, including antibiotic exposure, host immune defenses, and microbiota shifts [6]. This adaptability facilitates the spread of pathogens across species and geographical boundaries, thereby increasing the risk of foodborne transmission from animal-derived products and complicating effective pathogen control [17].

In contrast, high-frequency genes such as *fim*, which encode proteins involved in *fim*brial structure and host cell adhesion, play a critical role in the early stages of bacterial colonization [16]. The high prevalence of *fim* suggests an enhanced capacity for adhesion to the host epithelium, thereby potentially facilitating rapid and efficient colonization. However, the presence of this gene does not necessarily lead to its phenotypic expression. This mechanism can promote intensive infection within a short time frame and enable pathogen dissemination before detection in the early stages of infection [10, 12]. Therefore, the resulting infections are likely to be acute and progressive, with the potential to cause systemic complications such as HUS and UTIs [9, 16].

The *ent*, *fep*, and *fes* gene clusters were highly prevalent across all isolates, while *fim*, *esp*, and *yag/ecp* were found in almost all isolates (Fig. 2). Functionally, these gene clusters contribute to diverse virulence mechanisms, including iron uptake, adhesion, the type III secretion system (T3SS) activity, and biofilm formation—all essential for bacterial survival, colonization, and establishment of infection [7, 21, 25]. In Enterobacteriaceae, the most common virulence-associated clusters are those linked to iron uptake, which enable bacteria to adapt and compete successfully within the gastrointestinal tract and host tissues [21, 31]. At the population level, the high prevalence of clusters can drive widespread infections, reduce the effectiveness of treatments, and support the spread of pathogens in the environment, such as in food-processing areas and wastewater systems [17, 39].

The *fep*, *iro*, *esc*, and *chu* gene clusters also exhibited moderate diversity and prevalence among the isolates (Table 3). These gene clusters contribute to iron acquisition and protein secretion, which can enhance the bacterium's ability to establish infections in various tissues [27, 31]. Although less frequently observed, their presence remains important because they may contribute to diarrhea and other gastrointestinal issues, particularly in immunocompromised or otherwise vulnerable individuals [27]. Other gene clusters with potential invasive function include *asl*, *omp*, *kps*, and toxins such as *ast* [10, 31]. The *asl* and *omp* clusters may facilitate translocation across host cell barriers, especially during systemic infection like meningitis [10]. The *kps* gene product mediates capsule biosynthesis, helping the bacteria evade host immune defenses, including phagocytosis and complement-mediated killing [19]. Meanwhile, *ast* was the only gene cluster found that may be linked to the EAEC pathovar; it encodes a heat-stable enterotoxin 1 (EAST1), which can trigger fluid secretion in the intestines, leading to diarrhea [15]. These variations in virulence profiles among isolates highlight the need to consider specific genomic traits when assessing infection risk and designing genome-informed control strategies.

Notably, this study demonstrates that each ESBL-Ec isolate may harbor multiple pathovars, with most carrying three or more (Table 6). This means that different types of pathovars can combine in a single isolate, possibly leading to the formation of hybrid pathovars. The presence of hybrid pathogens that may use complex virulence strategies, expand their host range, and become more difficult to control [1]. Furthermore, ESBL production confers resistance to β-lactam antibiotics, compounding infection risks and

making treatment more difficult in both animals and humans [39]. These findings are important for improving early detection and monitoring of possible hybrid pathogens through national surveillance systems, as well as developing genomic-based treatment guidelines to improve the management of severe infections.

Geographically, the distribution of virulence genes varied across regions. The highest proportion was observed in isolates from Denpasar DIC, whereas the lowest was from Subang DIC (Table 4).In most regions, *fim* genes predominated, except in Lampung DIC, where *ent* genes were more frequent. These variations in VFG distribution among *Escherichia coli* isolates may be influenced by differences in environmental selective pressures, such as climate, temperature, humidity, rainfall, or chemical exposure, as well as farm and slaughterhouse management practices, including waste management [6, 17, 26, 36].

Environmental factors can affect bacterial survival and proliferation, shaping microbial communities [6, 17] and influencing the presence or expression of virulence genes. Moreover, differences in sanitation and waste management practices at farms and slaughterhouses can create selective pressures that either promote or limit the spread of pathogenic bacteria [36, 42]. In practical terms, this indicates that each region has its own microbiological characteristics, and therefore, control efforts should be adjusted to fit the local context. Collectively, these data reveal that ESBL-Ec from broiler cecum in Indonesia are not only antibiotic resistant but also possess diverse and multifaceted virulence profiles.

From a public health perspective, the predominance of ExPEC pathotypes—approximately twice as frequent as InPEC (Table 4), represents a significant risk of severe extraintestinal infections, including UTIs, pyelonephritis, sepsis, and meningitis. These finding highlights that the threat extends beyond the digestive system to systemic disease. Accordingly, effective control strategies should follow a One Health approach that integrates human, animal, and environmental health. Most gene clusters identified were associated with UPEC pathovars, which encode *fim*briae that promote adhesion to the urogenital tract, initiate colonization, and lead to urinary tract infections [31]. These findings support the concern that broiler chickens may serve as a reservoir for human transmission. Previous studies have also confirmed that UPEC strains from chickens are genetically similar to those found in human infections [13]. Additionally, genes related to NMEC pathovars were also detected, implying the potential to cross the blood–brain barrier (BBB) and cause meningitis, especially in newborns [5].

In contrast, genes that encode InPEC pathotypes, primarily from EHEC pathovars, were implicated in biofilm formation and adherence to the intestinal epithelium. These functions are important for colonization and the establishment of gastrointestinal disease [25]. Although their frequency was relatively low (≤2%), the detection of EAEC and EPEC pathovars remains clinically important, as both are recognized as major contributors to diarrheal diseases in vulnerable populations, particularly young children [27]. Certain virulence gene clusters, such as *esp, nle,* and *ces,* were even found to be linked with bloody diarrhea, colitis, and serious complications like HUS [11, 18]. The major outbreak in Germany in 2011, caused by EHEC-EAEC hybrid strains, suggests that the coexistence of multiple pathovars within a single isolate may have contributed to the observed severity of the outbreak [1].

Overall, this study demonstrates that ESBL-Ec isolated from broiler farms in Indonesia are antimicrobial resistant and simultaneously harbor multiple virulence traits associated with pathogenicity. Importantly, the mere presence of virulence genes does not necessarily result in infection, as their expression is influenced by host condition (the organism being infect), environmental factors (where the pathogen resides), and epigenetic regulation (the regulation of gene expression at the epigenetic level). For example, the *fim*brial expression can occur in the ON (*fim*briated) and OFF (non-*fim*briated) phases, regulated by the *fimS*, *fimB*, and *fimE* genes [12]. In addition, the host immune response, imbalance in gut microbiota, temperature, pH, nutrient levels, and other environmental stresses also affect the bacteria's ability to cause disease [6]. These findings indicate a relatively high level of virulence complexity among the isolates.

From a veterinary epidemiology perspective, these findings are critical because broiler chickens may serve as reservoirs for zoonotic transmission [17, 38]. The ESBL-Ec can spread to humans through several pathways, including consumption of undercooked poultry products, direct contact with animals or contaminated environments, and exposure to farm wastewater [38]. The likelihood of transmission increases when hygiene and sanitation measures are inadequate [17].

Since 2020, the rising prevalence of ESBL-Ec in Indonesian poultry farms has mirrored global trends. Both the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have underscored the importance of integrated monitoring systems for antimicrobial resistance [39]. The Indonesian government has also responded through cross-sector regulations that highlight the importance of AMR surveillance [28]. However, systematic monitoring of virulence factor genes remains limited, despite evidence that the coexistence of resistance and virulence determinants enhances bacterial survival and dissemination in the environment [41]. The reported incidence of urinary tract infections is approximately 90 to 100 cases per 100,000 people annually [20], which serves as a reminder that the presence of UPEC-associated genes from poultry sources should not be ignored [35]. Without effective control measures, the circulation of pathogenic bacteria through poultry products represents a significant zoonotic risk and may contribute to the rising burden of infectious diseases in the community.

Therefore, this study highlights the genetic complexity and zoonotic potential of ESBL-Ec isolates from broilers in Indonesia. Considering the public health risks, practical One Health measures are recommended to limit pathogen transmission. These include improving hygiene and sanitation practices in slaughterhouses, implementing prudent and regulated use of antibiotics in poultry production, and enhancing biosecurity measures on farms to prevent the spread of antimicrobial-resistant and virulent strains. Collectively, these strategies can help mitigate the potential transmission of pathogenic ESBL-Ec to humans and the environment.

Conclusions

The ESBL-producing *Escherichia coli* from broiler cecum in Indonesia exhibited high genetic diversity, with VFGs predominantly associated with ExPEC. All isolates harbored multiple VFG combinations spanning several pathovars, suggesting the potential for

hybrid pathogens. These findings highlight that broiler chickens in Indonesia can act as reservoirs of antimicrobial-resistant and virulent *E. coli*, posing a significant threat to public health.

CONFLICT OF INTEREST. We declare that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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