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Prevalence and molecular characterization of ESBL-producing *Escherichia coli* isolated from broiler chicken and their respective farms environment in Malaysia

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Abstract

Background Extended spectrum beta-lactamase-producing *Escherichia coli* (ESBL-EC) is an increasing public health threat. This study aimed to determine the prevalence and characterization of ESBL-producing *Escherichia coli* (*E. coli*) isolated from broiler chicken and their farm environment, in Kelantan Malaysia.

Methods *Escherichia coli* was isolated from 453 collected samples, including 210 cloacal swabs and 243 environmental samples. The antimicrobial susceptibility profile of the *E. coli* isolates was assessed for sixteen antibiotics using the disc diffusion method. The *E. coli* isolates were evaluated for phenotypic ESBL production using modified double disc synergy. After extraction of genomic DNA, ESBL resistance genes, phylogenetic group, and virulence genes were detected by PCR using appropriate primers. ESBL genes were further confirmed by sequencing. The molecular typing of *E. coli* strains was determined by Multilocus Sequence Typing (MLST).

Results A total of 93.8% (425/453) *E. coli* were isolated from the collected samples. Out of 334 *E. coli* isolates screened, 14.7% (49/334) were phenotypically ESBL producers. All the ESBL-EC were resistant to tetracycline, ciprofloxacin, and ampicillin. Thus, 100% of the ESBL-EC were multidrug resistant. Of the ESBL-EC 81.6% were positive for at least one ESBL encoding gene. The most prevalent ESBL gene detected was bla_{TEM} (77.6%; 38/49) followed by bla_{CTX-M} (32.7%; 16/49) and bla_{SHV} (18.4%; 9/49). The majority of ESBL-EC belonged to phylogenic groups A followed by B1 accounting for 44.9% and 12.2%, respectively. The most frequently identified sequence types were ST10 ($n=3$) and ST206 ($n=3$). The most detected virulence genes in the *E. coli* isolates were *astA* (33.3%; 22/66) followed by *iss* (15.2%; 10/66).

Conclusions Our results show both broiler chicken and their respective farms environment were reservoirs of multi-drug resistant ESBL-producing *E. coli* and ESBL resistance genes.

Keywords ESBL, *Escherichia coli*, Broiler chickens, Farm environment, ESBL genes, Kelantan, Malaysia

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Introduction

Antimicrobial resistance (AMR) is an increasing public health threat globally. The emergence and spread of antibiotic-resistant bacteria in food-producing animals are of particular concern. AMR worryingly spreads due to the misuse and excessive use of antimicrobials in humans and food animals [1]. The increasing number of animals bred for food production leads to more use of antimicrobial substances [2]. This overuse of antimicrobials may induce the intestinal microorganisms to acquire resistance and transmit to humans and animals.

Some evidence showed that animal gut microbiota and animal farms are the reservoirs of resistant bacteria and antimicrobial resistance genes (ARGs) because of antibiotic utilization [3–6]. The use of antibiotics for disease prevention and growth promotion in animals has been shown to cause antibiotic-resistant infections in humans [7]. Resistant gut bacteria are shed through feces and urine, causing environmental contaminations [8, 9]. In addition, several reports indicate that animal wastes such as manure play vital role in the spread of antimicrobial-resistant bacteria and ARGs to the environment [10–12].

Multidrug resistance has been increasing all over the world and is considered a public health threat. Several recent investigations reported the emergence of multidrug-resistant bacterial pathogens from different origins that increase the necessity of the proper use of antibiotics [13–19]. Besides, the routine application of antimicrobial susceptibility testing to detect the antibiotic of choice as well as the screening of the emerging MDR strains is crucial.

Extended-spectrum beta-lactamase (ESBL) is one of the most potent enzymes produced by certain resistant bacteria inducing antibiotic resistances that poses the most public health concerns. ESBL is a highly potent bacterial enzyme that confers resistance to β-lactam antibiotics including cephalosporines which are classified as the highest priority as well as critical important antimicrobials for human medicine by WHO [20]. It is mainly produced by *Enterobacterales*, specifically by *E. coli* and *Klebsiella pneumoniae* [21]. According to the CDC 2019 report, ESBL-producing *Enterobacterales* are among the most serious threats to human health [22]. ESBL-producing *E. coli* is one such group of bacteria that is increasingly detected in food-producing animals, including broiler chickens.

Escherichia coli is one of the most common normal inhabitants of human and animal intestines [23]. However, *E. coli* can also be a potentially pathogenic bacterium that colonizes the gastrointestinal tract and may disseminate to extra-intestinal organs of humans and various animals. *Escherichia coli* causes various diseases such as urinary tract infection (UTI), neonatal meningitis, septicemia in humans, and avian colibacillosis in poultry [24, 25]. Avian Pathogenic *E. coli* (APEC) is part of Extraintestinal pathogenic *E. coli* (ExPEC). These bacteria are the main cause of poultry extraintestinal bacterial infection [26]. Virulence factors contribute in the pathogenesis of *E.coli*, can be grouped into adhesion, protection, iron acquisition factors, and toxins production [27]. The iron acquisition systems, colicins (CvaC), increased serum resistance proteins, capsule as well as lipopolysaccharide complexes, and temperature-sensitive hemagglutinin (Tsh) are virulence related to APEC [28, 29]. The P-fimbriae (PAP) helps the bacteria for adhesions to the host cell to initiate infection [30]. Capsule and lipopolysaccharide protect the bacteria from the host immune system [29]. Antibiotic-resistant *E. coli* can cause disease in humans through direct contact or via food chain. *Escherichia coli* can mutate and horizontal transfer antibiotic resistance genes to inter- and intra-bacterial species [31]. Such characteristics of this bacteria facilitate the rapid spread of antibiotic resistance. Poultry is the most common source of ESBL-EC for humans among food-producing animals [32]. ESBL producing *E. coli* is increasingly reported in food-producing animals, including chickens. Furthermore, it is the main cause of infections and death in poultry farms and may transmit to humans and cause life threatening disease [33]. In poultry farms, ESBL-EC can contaminate the farm environment through fecal shedding of these resistant bacteria. It has been shown that ESBL-EC is highly prevalent in chicken meat compared to other meat-producing animals [34].

ESBL-producing strains of bacteria are the most common ESBL-encoding genes of bla_{CTX-M}, bla_{TEM}, and bla_{SHV}. Among the ESBL encoding genes, bla_{CTX-M} has been widely reported and is responsible for the increasing ESBL related resistance in pathogens infecting humans and animals throughout the world [35, 36]. The ESBL genes are encoded by plasmids which can spread easily between commensal and pathogenic bacteria in poultry farms and the environment [37].

According to Clermont O classification, *E. coli* can be classified into eight phylogenetic groups A, B1, B2, C, D, E, F, and clade I [38]. Commensal *E. coli* colonizing the gut lining are usually grouped as phylogroup A or B1. Meanwhile, pathogenic *E. coli* that causes gut infections is usually assigned to groups A, B1, or D. *Escherichia coli* that causes ExPEC-related infections are usually grouped as phylogroup B2 and D. Group E is closely related to group D, while group F is closer to group B2 [39–41]. Multi-locus sequence typing (MLST) is important to determine the phylogenetic relationship and evolution of bacterial lineages.

In Malaysia, ESBL colonization was reported in clinical patients, communities, farm animals, and from food [36, 42–45]. In this study, we investigated the prevalence

and molecular characterization of ESBL-EC in broiler chickens and their respective farms environment. The phylogenetic group distribution and virulence gene of *E. coli* isolates were determined. The presence of bla_{CTX-M}, bla_{TEM} and bla_{SHV} ESBL genes in the *E. coli* isolates were investigated.

Method and materials

Sample collection

A total of four hundred fifty-three samples were collected from six different broilers chicken farms and farm environments in Kota Bharu and nearby areas (Fig. 1). Samples were collected from February to November 2021 using a simple random sampling technique. Two hundred ten cloacal swabs, 95 environmental swabs from feeding and drinking troughs, 27 drinking water, 32 feed, 20 litter, 55 freshly passed fecal samples, and 14 sewage glasses of water were collected. Cloacal, fecal, and environmental swabs samples were collected using a sterile cotton swab with an Amies transport medium (Oxoid, Manchester, UK). Feed and litter were collected in sterile zip lock bags. All samples were collected aseptically using sterile and appropriate containers. Collected samples were labeled individually with identification number and date of collection and were transported to the laboratory using a cold box within 6 h. In the case of delay, samples were stored at 4 °C until processed.

Isolation and identification of *E. Coli*

Collected samples were enriched in Buffered Peptone Water (BPW) (Oxoid, Manchester, UK) by incubating at 37 °C for 24 h as described previously [46]. The isolation and identification of *E. coli* were performed as described previously [36]. The identified presumptive *E. coli* was further tested for nine biochemical characteristics including triple sugar iron agar (TSI) for glucose fermentation,

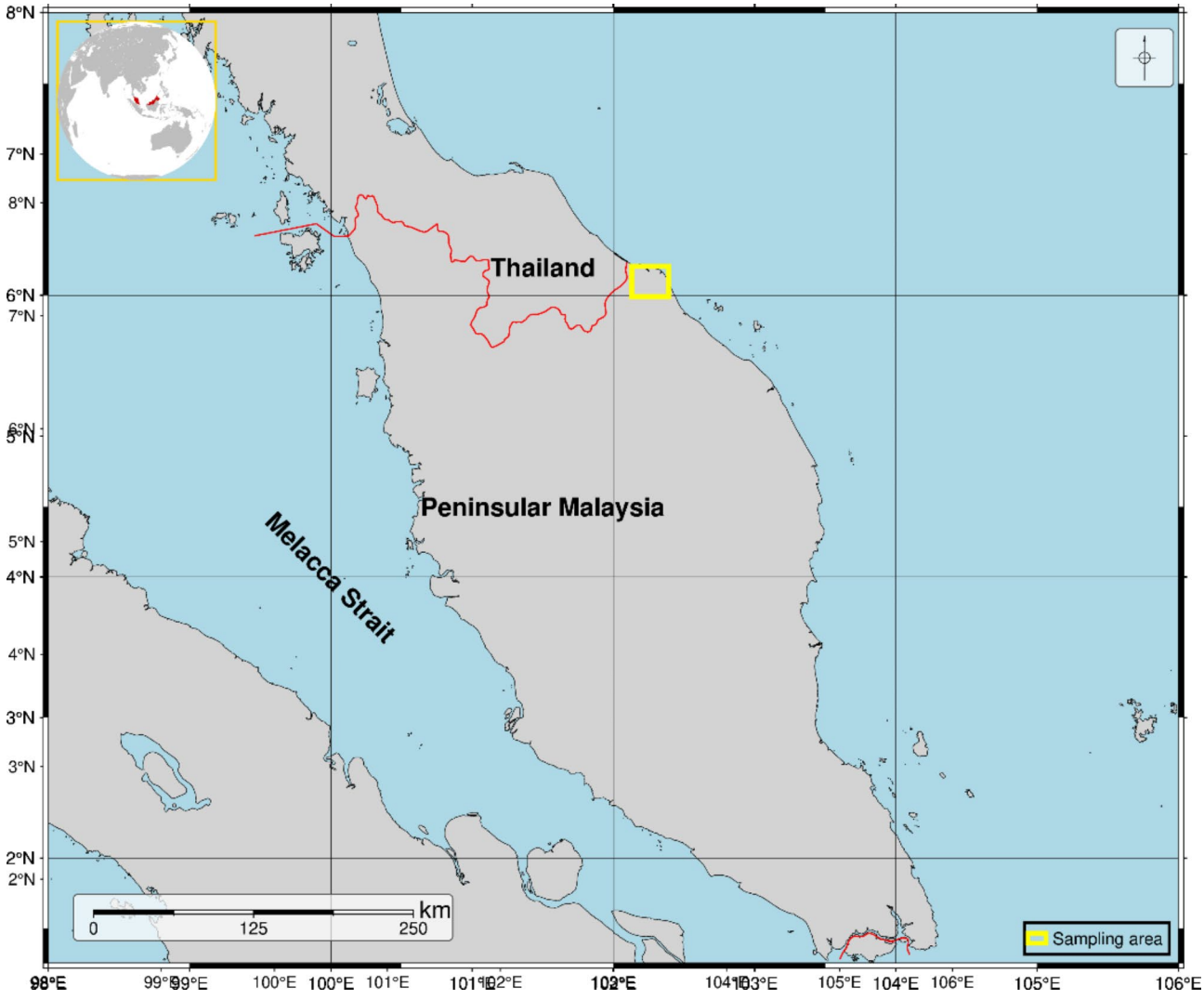

Fig. 1 A map showing location of sampling area for this study and the locality is boxed in yellow, which includes the Kota Bharu city and its surrounding

citrate metabolism, methyl red, Voges-Proskauer, motility, urease production, indole production, gas, and H_2S production as described previously [47]. The media used for the biochemical test were from Oxoid, Manchester, UK. *E. coli* ATCC® 25,922 was used as a positive control strain.

PCR confirmation of isolated *E. Coli*

The genomic DNA of isolated *E. coli* was extracted by boiling method as described previously by Mahmud et al. [48]. Extracted DNA was amplified with species-specific primer for the *Pho* gene encoding for *E. coli* as described previously [44, 49].

Antimicrobial susceptibility profile

E. coli isolates were tested for their susceptibility to 16 antibiotics belonging to 11 different antibiotic classes (Table 1). Antimicrobial susceptibility test was determined using the Kirby-Bauer disk diffusion method following the recommendation of the Clinical Laboratory Standards Institute (CLSI) [50]. *E. coli* isolate was grown on nutrient agar (NA) (Oxoid, Manchester, UK) at 37 °C for 18 h. Pure colonies from NA were suspended in 2 ml of sterile normal saline (0.85%) with turbidity equivalent to 0.5 MacFarland Standard. The bacterial suspension was then spread evenly on Mueller-Hinton agar (MHA) (Oxoid, Manchester, UK) using a sterile cotton swab. Then antibiotic disks were dispensed on the surface of the agar plate and incubated at 37 °C for 18 h. The susceptibility profile of the *E. coli* isolates was done by measuring the zone of inhibition and interpreted by comparing with CLSI standard breakpoint to determine as sensitive, intermediate, and resistant as per CLSI guidelines [50].

Table 1 List of antibiotics and antibiotic classes used for antibiotic susceptibility test

Antibiotic (µg)	Antibiotic class	Antibiotic (µg)	Antibiotic class
Ampicillin (AMP10)	Penicillin	ceftriaxone (CRO30)	Cephalosporine
Amoxicillin/clavulanic acid (AMC30)	Beta-lactam inhibitor	meropenem (MEM10)	Carbapenem
Gentamicin (GEN10)	Aminoglycoside	cefotaxime (CTX30)	Cephalosporine
Imipenem (IPM10)	Carbapenem	tetracycline (TET30),	Tetracycline
Ciprofloxacin (CIP5)	Fluoroquinolones	ceftazidime (CAZ30)	Cephalosporine
Trimethoprim/sulfamethoxazole (SXT25)	Sulphonamides	cefuroxime (CXM30)	Cephalosporine
Chloramphenicol (CHL30)	Chloramphenicol	Aztreonam (ATM30)	Beta-lactam
Streptomycin (STR10)	Aminoglycoside	nalidixic acid (NAL)	quinolones

All isolates with intermediate resistance were considered as resistance in calculating Multiple antibiotic resistance (MAR) index as well as in defining multi-drug resistance (MDR), extensively drug resistance (XDR), and pan drug resistance (PDR). The MDR is defined as "non-susceptibility" to at least one agent in three or more antibiotic classes and XDR is defined as "non-susceptibility" to at least one antibiotic in all the antimicrobial classes but isolates remain susceptible to only one or two antibiotic classes. PDR is "non-susceptibility" to all antibiotics in all the antibiotic classes based on Magiorakos et al. definition described previously [51]. The MAR index calculation was done as described in Tambekar et al. [52]. All the antibiotic discs used were from Oxoid, Manchester, UK. Standard *E. coli* strain ATCC® 25,922 was used as a control strain.

ESBL production

E. coli isolates with zone of inhibition of cefpodoxime ≤ 17 mm, ceftazidime ≤ 22 mm, aztreonam ≤ 27 mm, ceftriaxone ≤ 25 mm, and cefotaxime ≤ 27 mm considered as a potential ESBL producer according to CLSI, 2021 guideline and in addition, the isolates encoding ESBL genes and/or, virulence genes were selected for ESBL production test using Modified Double Disc Synergy (MDDS). ESBL production of *E. coli* isolates were tested using MDDST using amoxicillin-clavulanate (30 µg) along with four cephalosporins; 3GC-cefotaxime, ceftriaxone, aztreonam, cefpodoxime and 4GC-cefepime as previously described [53]. Briefly, 3–5 freshly grown colonies were suspended into 0.85% sterile normal saline. The turbidity of bacterial suspension was equivalent to 0.5 McFarland standard, the bacterial inoculum was lawn on the surface of Mueller Hinton agar. Amoxicillin-clavulanate (AMC30µg) was placed in the center of the plate with 3GC and 4GC discs placed 15 mm apart around AMC and the 3G and 4G cephalosporin were 20 mm apart from each other. Isolates were determined as ESBL producers when the zone of inhibition increased towards the AMC disc [53]. *Escherichia coli* 25,922 was used as a negative control for ESBL production.

Detection of ESBL encoding genes

All the confirmed *E. coli* isolates were screened for ESBL-encoding genes bla_{CTX}, $bla_{TEM,}$ and $bla_{SHV.}$ The PCR amplification of these ESBL genes were performed following the previous protocol [54]. The PCR amplification protocol used for $bla_{TEM,}$ and bla_{CTX-M} genes was as described previously [36]. The PCR protocol for bla_{SHV} (768 bp) amplification was an initial denaturation step at 95 °C for 15 min, followed by 30 times of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min and the final extension 72 °C for

10 min. Primers used in this study are summarized in Table 2.

E. coli isolates that are positive for the ESBL gene (bla_{CTX-M}, $bla_{TEM,}$ and/or bla_{SHV}) were further confirmed by DNA sequencing and the sequenced DNA was analyzed using BLAST analysis in a website: http://www.ncbi.nlm.nih.gov/. The nucleotide sequence of the ESBL genes is available in GenBank accession no. PQ522631-PQ522632.

Table 2 List of primers and genes used in this study

Gene Name	Sequence	Amplicon size(bp)	Annealing T(°C)	Reference
pho	F: 5′- GTGACAAAAGCCACACCATAAATGCCT-3′	903	56	[49]
	R: 3′-TACACTGTCATTACGTTGCGGATTTGGCGT-5′			
E. coli	F: 5′-TGACGTTACCCGCAGAAGAA-3′	832	55	[44]
	R: 3′-CTCCAATCCGGACTACGACG-5′			
bla_{CTX-M}	F: 5′-ATGTGCAGYACCAGTA-ARGTKATGGC-3′	592	65	[54]
	R: 3′-TGGGTRAARTARGTSAC-CAGAAYSAGCGG-5′			
bla_{TEM}	F: 5′-GCGGAACCCCTATTTG-3′	964	55	
	R: 3′-ACCAATGCTTAATCAGTGAG-5′			
bla_{SHV}	F: 5′-TCGCCTGTGTATTATCTCCC-3′	768	58	
	R: 3′-CGCAGATAAATCACCACAATG-5′			
chu A	F:5′-TGCCATCAACACAGTATATCC-3′	288	59	[38]
	R:3′-TCAGGTCGCGAGTGACGGC-5′			
yja A	F:5′-ATCACATAGGATTCTGCCG-3′	211		
	R:3′-CAGCGGAGTATAGATGCCA-5′			
TspE4.C2	F:5′-AAGGATTCGCTGTTACCGGAC-3′	152		
	R:3′-AACTCCTGATACAGGTGGC-5′			
arpA	F:5′-TGATATCACGCAGTCAGTAGC-3′	400		
	R:3′-CCGGCCATATTCACATAA-5′			
trpAgpC	F:5′-ACAAAAAGTTCTATCGCTTCC-3′	219	62	
	R:3′-CCTGATCCAGATGATGCTC-5′			
ArpAgpE	F:5′-ACTATTCTCTGCAGGAAGTC – 3′	301	59	
	R:3′-CTTCCGATGTTCTGAACGT-5′			
trpBA	F:5′-TCCTGGGACATAATGGTCAG-3′	489		
	R:3′-GTGTCAGAACGGAATTGT-5′			

Phylogenetic typing

Following Clermont O et al. classification, *E. coli* was classified into eight phylogenetic groups A, B1, B2, C, D, E, F, and clade I. Quadruplex PCR was used to assign the isolates into phylogroups A, B1, B2, and D which target *chuA*, *yjaA*, *TspE4.C2*, and *arpA* genes as described previously [38]. The isolates of phylogroup A were separated from phylogroup C by trpAgpC primer which is a C-specific primer. Similarly, phylogroup D isolates were distinguished from E using ArpAgpE primer, and phylogroup F was differentiated from phylogroup D in the quadruplex PCR as F does not contain the *ArpA* gene. The PCR protocol used was based on Clermont et al. [38].

Virulence gene

The ESBL genes-positive *E. coli* isolates were assessed for APEC-associated virulence genes. The APEC virulence factors include genes related to adhesion (*papC* and *tsh*), toxin production (*astA*, *vat* and *cvaC/cvi*), iron uptake (*iucD*, *irp2*), and invasion (*iss*). The *papC* gene encodes for P fimbrial adhesion, *iucD* encodes the aerobactin operon, *irp2* encodes an iron-repressible protein, *tsh* encodes for temperature-sensitive hemagglutinin, *vat* encodes for vacuolating autotransporter toxin, *astA* for enteroaggregative heat-stable toxin, *iss* increases serum survival and cva/cvi encodes for colicin V plasmid operon [55]. Multiplex PCR was performed to test *papC*, *iucD*, *irp2*, *tsh*, *vat*, *astA*, *iss*, and *cva/cvi* virulence genes associated with virulence factors. The PCR protocol used was as described previously [55].

Multi-locus sequence typing (MLST) of ESBL producing *E. Coli*

The *E. coli* isolates positive for the ESBL gene and/or carry virulence genes were selected for MLST analysis. The detailed MLST analysis method was done as described previously [36]. The PCR conditions used were as follows: initial denaturation at 95 °C for 2 min; 30 cycles of initial denaturation at 95 °C for 1 min, annealing at 57 °C (*adk*) or 64 °C (*fumC*, and *purA*) or 68 °C (*recA*), 72 °C (*gyrB*), 69 °C(*icd*) and 71 °C (*mdh*) for 1 min and elongation 72 °C for 2 min; followed by a final extension step at 72 °C for 5 min. The amplified PCR products were sent to Apical Scientific SDN. BHD, Malaysia, for a sequence analysis. The alleles and Sequence types (ST) were assigned based on the *E. coli* database at the MLST website, http://enterobase.warwick.ac.uk/.

Statistical analysis

The presented data set is categorical data type since it has a well-known non-numerical set of values. Hence tools

Table 3 Isolation and PCR confirmed *E. Coli* strains in broiler chickens from Kelantan Malaysia

Sample type	Isolated E.coli	PCR-confirmed E. coli	Percentage%
Cloacal Swab	205	203	99.0
Drinking water	24	23	95.8
Environmental	85	83	97.6
Fecal	55	55	100.0
Food	31	31	100.0
Litter	20	20	100.0
Sewage	11	10	90.9
Grand Total	431	425	98.6

applicable to this type of data were utilized. Correlations between the phenotypic antibiotic resistance, and antibiotic resistance genes as well as correlation among the antibiotics were determined using Python version 3.11. Python-Pandas, One-hot-encoding approach use applied to generate the correlation matrix between the phenotypic antibiotic resistance, antibiotic resistance genes as well as the correlation among the antibiotics as explained previously [56]. The correlation ratio was calculated based on the Pearson correlation coefficient (r) using the same matrix table generated using the one-hot-encoding of the Pandas library. The resulting matrix was plotted using the Python seaborn library heat map visualization tool [57].

The Venn diagram showing the overlap of the ESBL genes such as bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes was plotted using R software version 4.3.3, the package of ggVennDiagram [58].

Results

Phenotypic characteristics of the recovered isolates

Out of the 453 samples tested, 431 (95%) were positive for *E. coli* using routine microbiological methods. Of the isolated *E. coli*, 98.6% (425/431) were confirmed as *E. coli* by PCR (Table 3). Majority of the confirmed *E. coli* were assigned to phylogenetic group A accounting for 52.2% (222/425) followed by B1 (11%, 48/425).

Of the *E. coli* confirmed by PCR, 334 of them were evaluated for phenotypic ESBL production. Out of the 334 *E. coli* isolates, 14.7% (49/334) were phenotypically ESBL producers. Among these, 57.1% (28/49) of the ESBL-EC were detected from cloacal samples. Meanwhile, 42.9% of the ESBL-EC were identified from various farm environment samples. These include environmental swabs (14.3%, 7/49), litter samples (6.1%, 3/49), drinking water (6.1%, 3/49), freshly passed fecal samples (10.2%, 5/49) and food (6.1%, 3/49) were found positive for ESBL-EC.

Antimicrobial susceptibility profile

The 334 *E. coli* isolates were subjected to antimicrobial susceptibility profiles of sixteen antibiotics belonging to eleven different classes. *E. coli* isolates were highly resistant to tetracycline, streptomycin, chloramphenicol, ampicillin, ciprofloxacin, and trimethoprim/sulfamethoxazole (Fig. 2). Meanwhile, the isolates showed least resistance towards meropenem and imipenem in both ESBL-EC and non-ESBL-EC. ESBL-EC were shown to be highly resistant towards ciprofloxacin (100%), nalidixic acid (88%), cefotaxime (57%), cefuroxime (47%), ceftriaxone (43%), aztreonam (31%), amoxicillin/

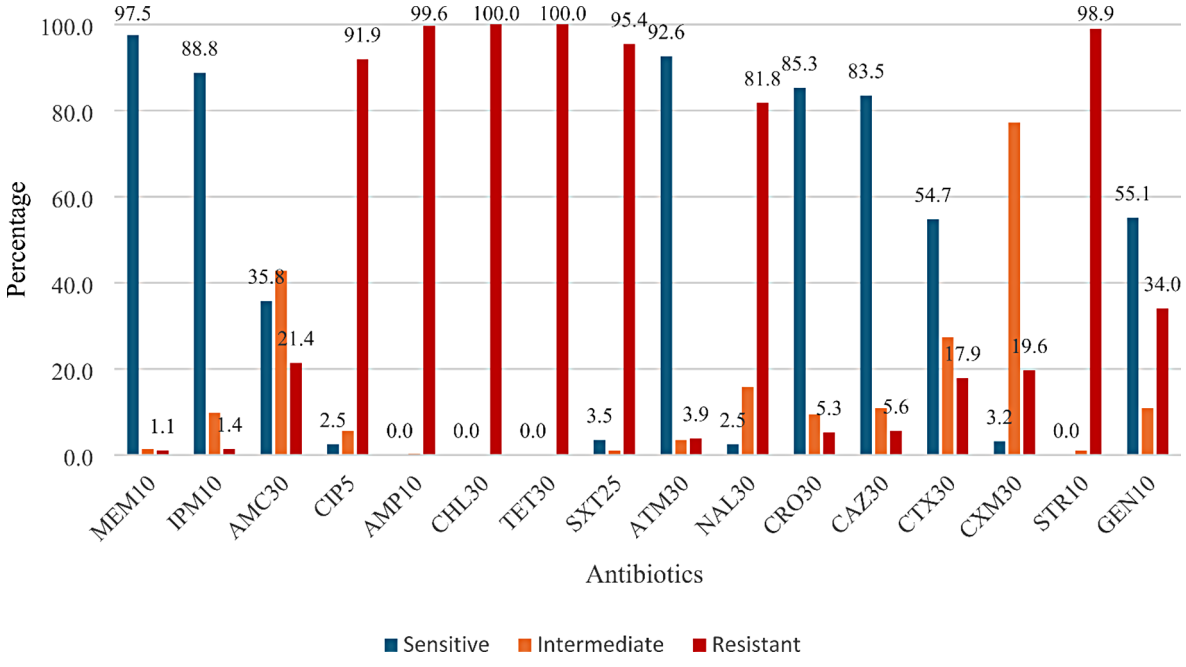

Fig. 2 Antimicrobial resistance profiles of non-ESBL-EC isolates from broiler chicken and farm environment in Kelantan, Malaysia ($n = 285$)

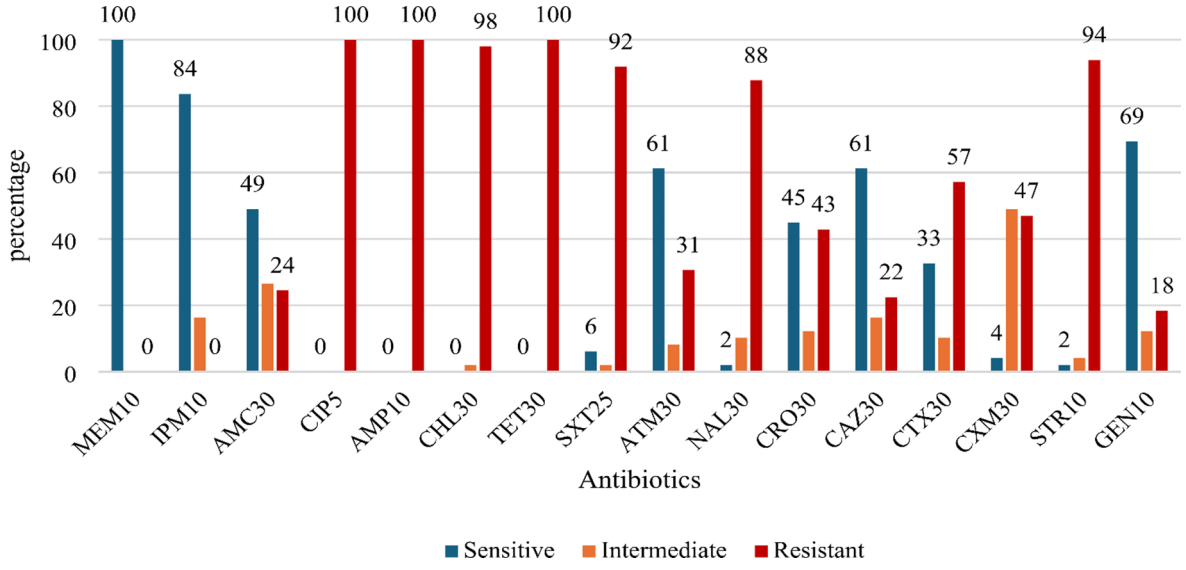

Fig. 3 Antimicrobial resistance profiles of ESBL-EC isolates from broiler chicken and farm environment in Kelantan, Malaysia ($n=49$)

Table 4 ESBL encoding genes in ESBL-EC from broiler chicken and farm environment in Kelantan, Malaysia

ESBL genes	Cloacal	EVT	Food	Litter	Drinking water	Fecal	Grand total	Percentage (%)
bla_{TEM}	11	4	2	2	-	1	20	40.82
bla_{CTX-M}, bla_{TEM}	8	-	1	-	-	1	10	20.41
bla_{SHV}, bla_{TEM}	-	-	-	1	3	-	4	8.16
bla_{CTX-M}, bla_{SHV}	1	-	-	-	-	-	1	2.04
bla_{CTX-M}, bla_{SHV}, bla_{TEM}	1	3	-	-	-	-	4	8.16
bla_{CTX-M}	1	-	-	-	-	-	1	2.04
Negative	6	1	1	-	-	1	9	18.37
Grand Total	**28**	**8**	**4**	**3**	**3**	**3**	**49**	**100.00**

clavulanic acid (24%), and ceftazidime (22%) compared to non-ESBL producing *E. coli* (Fig. 3). AST profile of the antibiotic ciprofloxacin, tetracycline, ampicillin, chloramphenicol streptomycin, and trimethoprim/sulfamethoxazole showed similarly high resistance among all sample types. In addition, isolates were highly susceptible to meropenem in all sample types. However, amoxicillin-clavulanic acid showed higher resistance and intermediate (non-susceptible) rate in sewage (71%), cloacal swabs (66%), Environmental swab (62%), litter (55%), food (53%), and fecal (51%) compared to drinking water (33%). The detail antibiotic susceptibility profile of each sample type is described in supplementary material (Supplementary Fig. 1).

All the AST-tested *E. coli* isolates (334) were MDR. Of these 15% (50/334) of them were XDR and one *E. coli* isolate was classified as PDR. In addition, 100% of the ESBL-EC were MDR of which 28.6% (14/49) of them were classified as XDR. However, none of the ESBL-EC were classified as PDR.

All (100%) of the *E. coli* isolates were with MAR index value>0.2. The majority of the isolates (64.4%) had MAR index values in the range 0.3–0.6, while the remaining isolates (35.7%) had MAR index values in the range 0.7-1. Among the ESBL-EC isolates, 46.9% had MAR index values ranging from 0.4 to 0.6, while the majority, 53.1%, had MAR index values ranging from 0.7 to 0.9. None of the isolates showed a MAR index value ≤ 2. The phenotypic multi-drug resistance type, resistance antibiotics MAR index values and the antibiotic resistance genes in the ESBL-EC isolates is summarized in supplementary material (Supplementary Table 1).

Extended spectrum beta lactamase (ESBL) encoding genes

All the isolated *E. coli* were screened for bla_{CTX-M}, bla_{TEM} and bla_{SHV} ESBL encoding genes. 79.5% (338/425) of the isolated *E. coli* were positive for the bla_{TEM} gene. In addition, 9.9% (42/425) of the isolates were positive for the bla_{SHV} gene, and 7.3% (10/425) were positive for bla_{CTX-M} genes. Out of the ESBL-EC, 81.6% were positive for at least one ESBL-encoding gene. In this study, bla_{TEM} (77.6%, 38/49), bla_{CTX-M} (32.7%; 16/49) and bla_{SHV} (18.4%; 9/49) were found harboring ESBL genes in the ESBL-EC isolates (Table 4). The bla_{TEM} was the predominant ESBL gene detected. Moreover, 20.41% (10/49) of the ESBL-EC isolates were positive for both bla_{CTXM} and

bla_{TEM}, 8.16% (4/49) carried both bla_{SHV} and bla_{TEM}, and another 8.16% (4/49) carried all the ESBL bla_{CTX-M}, bla_{TEM} and bla_{SHV} genes (Fig. 4). However, 18.4% (9/49) of the ESBL-EC were negative for the ESBL genes. The co-occurrence of both *mcr* and ESBL encoding genes was observed in eight isolates.

The correlation coefficient of the phenotypic (the resistant antibiotics) and the genotypic (positive resistance genes) resistance of the ESBL-EC showed that CRO as well as ATM have a strong correlation with an ESBL gene bla_{CTX-M} positive. In addition, CTX30 and CAZ30, CRO30 and CAZ30, ATM30 and CAZ30, NAL30 and SXT25, ATM30 and CTX30, CRO30 and CTX30, CRO30 and ATM30 resistant antibiotics showed strong correlation pattern (Fig. 5).

Multilocus sequence typing (MLST)

The MLST result of the isolated *E. coli* in this study is highly diverse. The most dominantly identified sequence types were ST10 ($n=3$) and ST206 ($n=3$). Two isolates with ST10 were detected from cloacal and one was from fecal samples. Isolates assigned to ST48, ST10, ST648, ST469, and ST165 were positive for more than three virulence genes. ST10 Cplx comprises ST10, ST1638 and ST48 sequence types (Table 5).

Phylogenetic group and the virulence genes distribution

The PCR identification of phylogenetic typing of the ESBL-EC showed that phylogroup A was the most frequent group followed by B1 in all cloacal and farm environment samples, accounting for 44.9% ($n=22$) and 12.2% ($n=6$) of the ESBL-EC (Table 6). Moreover, B2 (6.1%; $n=3$), D (8.2%; $n=4$), and F (4.1%; $n=2$) phylogroups of ESBL-EC isolates were detected. The virulence genes distribution in the *E. coli* isolates were *astA* (33.3%; 22/66), *iss* (15.2%; 10/66), *irp2* (15.2%, 10/66), *Papc* (10.6%; 7/66), *iucD* (10.6%; 7/66) and *tsh* (1.5%; 1/66).

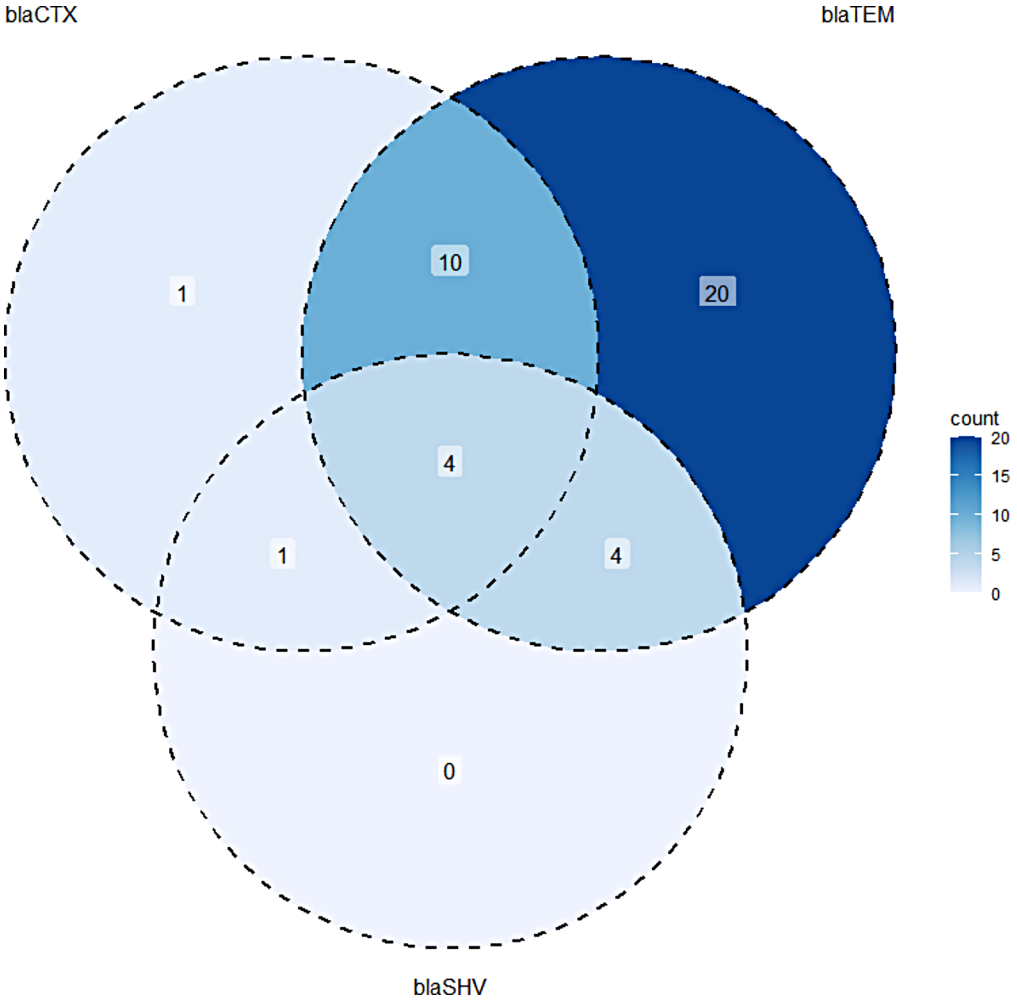

Fig. 4 A Venn diagram showing overlap ESBL genes such as bla_{CTX-M}, bla_{TEM}, and bla_{SHV} in ESBL-EC. Each circle is labeled with the respective gene name, the numbers in the non- overlapping region show the count of isolates carrying respective single ESBL gene. Numbers in the overlapping region indicate the number of isolates carrying the respective genes commonly

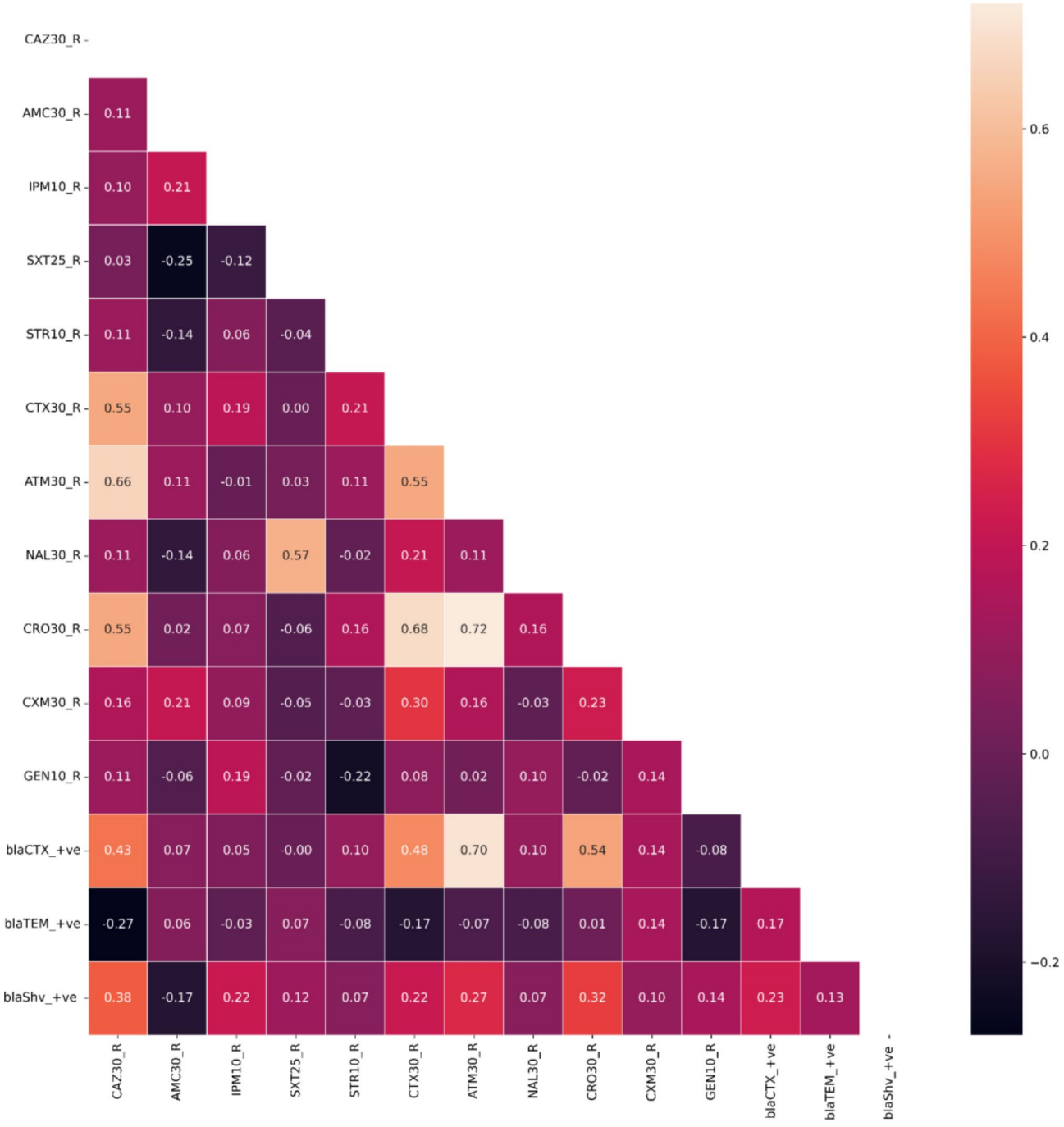

Fig. 5 Correlation heatmap of resistant antibiotic with resistant genes

Discussion

Escherichia coli is a common inhabitant of warm-blooded animal guts and has the potential to cause serious infections in humans and animals. The ability of *E. coli* to mutate, and acquire mobile genetic elements carrying resistance genes through horizontal gene transmission is a major contributing factor in the emergence and spread of antibiotic resistance [31].

In our study, among the presumptive *E. coli* isolates 14.7% (49/334) were phenotypically ESBL-EC. This is in line with a previous study from South Korea, where ESBL-EC was 14% [59]. But, this prevalence is lower than those reported in previous studies from Malaysia (48.8%), Indonesia (28.75%), India (25%) and Zambia (20.1%) [44, 60, 61]; however the current prevalence is higher than what has been reported from Tanzania (4.7%) and Mexico (5%) [62, 63]. These differences in the prevalence of

Table 5 Phenotypic and genotypic characteristics of selected isolated *E.coli* positive for ESBL genes

Sample ID	Source	ESBL- EC	ESBL gene	ST	ST Complex	Phylogenetic group	Virulence genes	MAR I
1b	Cloacal	Positive	bla_{CTX-M},bla_{TEM}, bla_{SHV}	ST117	-	F	*ast*	0.8
B6H2	Cloacal	Positive	bla_{CTX-M}, bla_{TEM}	ST48	ST10 Cplx	B2	*ast, papC, iuc D*	0.9
C34H4	Cloacal	Positive	bla_{CTX-M}, bla_{SHV}	ST5229	ST101 Cplx	B1		0.8
C39H4	Cloacal	Negative	bla_{CTX-M}, bla_{TEM}	ST1771		A	*ast*	0.5
E24	EVT swab	Positive	bla_{CTX-M},bla_{TEM}, bla_{SHV}	ST1638	ST10 Cplx	A		0.9
E43	EVT swab	Negative	bla_{CTX-M},	ST398	ST398 Cplx	F	*ast, iss*	0.6
F3	Fecal	Negative	bla_{CTX-M},bla_{TEM}, bla_{SHV}	ST10	ST10 Cplx	A	*ast*	0.8
Food9	Feed	Positive	bla_{CTX-M},bla_{TEM}, bla_{SHV}	ST3896	-	Unknown	*papc, ast*	0.8
KB25	Cloacal	Positive	bla_{CTX-M}, bla_{TEM}	ST10	ST10 Cplx	C	*ast, irp2,papC, iucD*	0.8
KB29	Cloacal	Positive	bla_{CTX-M}, bla_{TEM}	ST648	ST648 Cplx	D	*ast, iss, papC, iucD, tsh*	0.8
KB4	Cloacal	Positive	bla_{CTX-M},	ST162	ST469 Cplx	B1	*ast, iss, iuc D*	0.7
KBF1	Fecal	Positive	bla_{CTX-M}, bla_{TEM}	ST165	ST165 Cplx	A	*ast, iss, irp2*	0.8
L25	Litter	Positive	bla_{CTX-M},bla_{TEM}, bla_{SHV}	ST106	ST69 Cplx	D	*iss, irp2*	0.9
Drw3	Drinking water	Positive	bla_{CTX-M}, bla_{TEM}	ST206	ST206 Cplx	A	*ast*	0.8
Drw7	Drinking water	Negative	bla_{TEM}, bla_{SHV}	ST1285	-	B1	-	0.8
E26	EVT swab	Negative	bla_{TEM}, bla_{SHV}	ST206	ST206 Cplx	A	*iss, irp2*	0.7
Food1(2)	Feed	Negative	bla_{SHV}	ST155	ST155 Cplx	B1		0.5
KB10	Cloacal	Negative	bla_{TEM}, bla_{SHV}	ST10	ST10 Cplx	A		0.8
ZC4H1	Cloacal	Negative	bla_{TEM}, bla_{SHV}	ST206	ST206 Cplx	C	*ast*	0.5

Table 6 Phylogenetic typing of ESBL-EC from broiler chickens and farm environments in Kelantan, Malaysia $n=49$

Row labels	A	B1	B2	C	Clad I or II	D	E	F	Unknown	Grand total
Cloacal	11	4	3	3	1	1	1	2	2	28
Drinking Water	1	1	-	-	-	1	-	-	-	3
EVT	4	-	-	1	-	1	-	-	1	7
Fecal	3	1	-	-	1	-	-	-	-	3
Food	2	-	-	-	1	-	-	-	-	5
Litter	1	-	-	-	-	1	1	-	-	3
Grand Total	**22**	**6**	**3**	**4**	**3**	**4**	**2**	**2**	**3**	**49**

ESBL-EC might be due to the difference in geographic location, time study difference, and different methods used. In this study, we determined the phenotypic ESBL-EC by MDDS, while most of the above studies used double disk synergy (DDS). The majority of the ESBL-EC were recovered from cloacal swab samples, 53.1% followed by environmental swab samples (14.3%). In addition to broiler chickens, feces, litter, water, and food were positive for ESBL-EC. Similarly, feces and litter were reported as a source of ESBL-EC in Germany [64]. It was also reported that healthy dairy cattle, farm environment, and milk were reservoirs of ESBL-EC [65]. This highlights that healthy livestock animals could be the reservoirs and sources of ESBL-EC to humans. A study from Singapore showed that ESBL-EC prevalence was high in chicken meat compared to pork and beef [66].

In the current study, we observed that the resistance patterns of *E. coli* isolates against tetracycline, ampicillin, meropenem, streptomycin, trimethoprim-sulfamethoxazole, and chloramphenicol antibiotics are similar in all sample types. The ESBL-EC strains were 100% resistant to ciprofloxacin, ampicillin, and tetracycline as well as 98% resistant to chloramphenicol. This 100% correlation between ESBL-EC and resistance towards these antibiotics might be due to the cross-resistance of different antibiotic classes in the plasmids and could result in multi-drug resistance [67, 68]. Higher resistance to streptomycin, ampicillin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole is repeatedly reported in several studies from poultry origin *E.coli* [36, 69, 70]. This might be caused by the common use of these antibiotics in food animal production. Isolates also showed resistance to trimethoprim-sulfamethoxazole (92%), and nalidixic acid (88%). Furthermore, 100% of the ESBL-EC were multi-drug resistant and 14% of them were XDR based on the classification of Magiorakos et al. [51]. In agreement with our findings, 96.2% of ESBL-EC were reported to be MDR from a study on broiler chickens from Thailand [71]. This could be due to the excessive use of antibiotics in animal production which may result in limited options for the treatment of multi-drug-resistant infections. ESBL-EC showed higher resistance to ciprofloxacin, cefotaxime, cefuroxime, ceftazidime, amoxicillin/clavulanic acid, aztreonam, ceftriaxone and nalidixic

acid compared to none ESBL-EC isolates. In the current study, the MAR index value of all the *E. coli* isolates was greater than 0.2, indicating that the *E. coli* strains including the ESBL-EC might have originated from highly antibiotic resistant bacteria contaminated environments or overuse of antibiotics.

The isolates from the current study were found to contain the most common ESBL genes, bla_{CTX-M}, bla_{TEM}, and bla_{SHV}. Majority of the isolated *E. coli* (79.5%) were positive for the bla_{TEM} gene followed by bla_{SHV} (9.9%) and then bla_{CTX-M} (7.3%). Meanwhile, 77.6% (38/49) of the ESBL-EC isolates harbored the bla_{TEM} gene, 32.7% (16/49) contained the bla_{CTX-M} gene, and 18.4% (9/49) contained the bla_{SHV} genes. The prevalence bla_{CTX-M} is higher in ESBL-EC compared to the prevalence of bla_{CTX-M} in the non-ESBL-EC isolates. This could be due to the fact that bla_{CTX-M} is related to cefotaxime resistance, cefotaxime resistance is higher in ESBL-EC than non ESBL-EC. Moreover, 20.41% (10/49) of the ESBL-EC isolates were positive for both bla_{CTX-M} and bla_{TEM}, 8.16% (4/49) carried both bla_{SHV} and bla_{TEM}, and another 2.04% (1/49) had bla_{CTX-M}, and bla_{SHV} genes. In addition, another 8.16% (4/49) carry all the three ESBL genes, bla_{CTX-M}, bla_{TEM} and bla_{SHV} genes. A study from Spain reported 20.7% ESBL producing *E. coli* harboring bla_{SHV-12} gene from broiler chicken [72]. In a similar study, isolates were typed ST68 with phylogroup-E and ST117 with phylogroup-B2 from chicken manure. The results also showed that 18.4% of the ESBL-EC did not harbor any of the tested ESBL genes, this discrepancy of the phenotypic ESBL production and genotypic result might be due to other mechanisms of resistance or mutations that increase ESBL activity including other ESBL types such as AmpC β-lactamase or mutation in the outer membrane porin [73]. AmpC β-lactamase is a chromosomal mediated resistance that enhance the ESBL production but does not encode these specific ESBL genes [74].

In our study, bla_{TEM} was the predominant ESBL gene followed by bla_{CTX-M} and bla_{SHV}. Similarly, a study from China reported bla_{TEM} gene (82.3%) as the predominant ESBL type, followed by bla_{CTX-M} (43.5%) and bla_{SHV} (19.4%) [75]. In agreement with our findings, recent studies from Ecuador and Nigeria show that bla_{TEM} was the most prevalent ESBL gene for *E.coli* [67, 76]. A study conducted from hospital patients in Malaysia reported that 87.5% of ESBL-EC were harboring bla_{TEM} genes [77]. Moreover, we found high prevalence of bla_{TEM} genes in 100% ampicillin resistance ESBL-EC, in agreement with our finding, literatures reported in 98–100% ampicillin-resistant *E. coli* with high bla_{TEM} prevalence from chickens and humans [76, 78]. However, most of the broiler chicken origin studies around the globe including our previous study reported that bla_{CTX-M} was the dominant ESBL gene [36, 60, 61]. It has been reported that bla_{CTX-M} is an increasingly reported type of ESBL gene in food-producing animals. In general, the spread of these ESBL genes among the chickens and farm environment could be due to the fact these genes being plasmid mediated so that they can spread easily.

Four ESBL-EC isolates in the current study were positive for all bla_{CTX-M}, bla_{TEM}, and bla_{SHV} ESBL genes. These isolates were assigned to sequence types and phylogroup, ST117-F, ST1638-A, ST106-D, and ST3896-unknown, respectively. In addition, these isolates were sourced from cloacal swabs, environmental swabs, and litter. This indicates that these ESBL genes could spread easily from the chickens to the environment and vice versa. The ESBL-EC isolate ST117 was positive for *astA*, and ST3896 was found positive for *papC* and *astA* virulence genes. This indicates that these virulent ESBL-EC strains have an elevated risk of causing serious infection to the birds and possibility of zoonotic transmission to humans through the food chain.

In our study, Phylogenetic group A (44.9%) was the most frequent group followed by B1 (12.2%) among the ESBL-EC isolates. On top of that, we found 6.1% ($n=3$) of B2, 8.2% ($n=4$) of D, and 4.1% ($n=2$) of F phylogroups. In agreement with these findings, another similar studies from Malaysia reported phylogroup A was the dominant phylogroup accounting for 45.1% [79–81]. It is widely reported that phylogenetic groups B2, F, and D are potential pathogens that can cause ExPEC infections in humans. Meanwhile, phylogenetic groups A and B1 are associated with commensal bacteria [82]. Even though phylogroups A and B1 are commensals these isolates were positive for virulence genes and ESBL genes. This could be due to the character of *E. coli* serving as a transporter of resistance genes to other or the same species of bacteria. This indicates that commensal *E.coli* isolates can be the reservoir of ESBL determinants that can spread to other pathogenic bacteria and disseminate antibiotic resistance.

The sequence typing of the *E.coli* isolates in this study showed high diversity. The most common STs we found were ST206 ($n=3$) and ST10 ($n=3$). Among the identified STs in this study, ST10, ST48, and ST117 were the most frequently reported STs in *E.coli* isolates from poultry [83]. Of these ST48 and ST10 were found in both poultry and human ExPEC isolates [84]. Moreover, ST10 and ST117 *E. coli* isolates harbored bla_{CTX-M}, bla_{TEM}, and bla_{SHV}, meanwhile, ST48 was positive for bla_{CTX-M}, and bla_{TEM}. ST117 has been reported in Avian pathogenic *E. coli* (APEC) associated in study from Italy [85]. Moreover, ST117 was reported as an emerging ExPEC lineage [86].

In the present study, an ESBL-EC with ST648 was found harboring both bla_{CTX-M}, *and* bla_{TEM}, ESBL genes

and it was positive for *astA*, *iss*, *papC*, *iucD*, and *tsh* APEC related virulence genes with phylogenetic group D. This lineage was reported previously in highly virulent multidrug resistant pathogenic ESBL-EC with multiple resistant genes, and it is an emerging high-risk virulent strain with similar features to the ST131 [87]. Similarly, this lineage was reported to carry antibiotic resistance genes and has been reported from human in China [14]. The most dominant APEC related virulence genes detected were in the *E. coli* isolates were *ast* A (33.3%; 22/66), followed by *iss* (15.2%; 10/66), and *irp2* (15.2%, 10/66).

In this study, we initiated a detailed investigation into the prevalence and molecular characterization of ESBL-EC in broiler chickens and their environment. Our main objective was to determine the existence of ESBL-EC and the molecular characteristics of these strains. We postulated that both the broiler chickens and their environment might serve as significant sources of ESBL-EC, thereby facilitating the spread of antibiotic resistance. To test our hypothesis, we conducted a comprehensive field study involving the collection of samples from broiler chickens and their surrounding farm environment. From these samples *E. coli* was isolated, and were tested for antimicrobial resistance profile, phenotypic ESBL production, ESBL gene, phylogenetic group, virulence genes, and sequence typing to identify and characterize the ESBL-EC strains.

Conclusion and recommendations

This study showed both the broiler chickens and their contaminated farm environments could be significant sources of ESBL-producing *E. coli* in Kelantan, Malaysia. Our results show that feces, litter, drinking water, as well as chicken's food were reservoirs for ESBL-EC and ESBL genes. The bla_{TEM} was the most common ESBL gene detected. All the ESBL-EC were multi-drug resistant and more than half of them were XDR. Moreover, the *E. coli* isolates were highly resistant to tetracycline, ampicillin, streptomycin, and chloramphenicol. Multi-drug resistant ESBL-EC have been found positive for virulence genes that can cause disease in birds, and humans. ESBL-EC from broiler chickens and their contaminated farm environment may pose a potential risk of transmission to humans through food chain or direct /indirect contact. Based on our findings, we recommend the implementation of a strict regulation of antibiotic use in broiler chicken production focusing on both the chickens and their contaminated environments.

Supplementary Information

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Supplementary Material 1

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Author contributions
ML conducted the sample collection and laboratory analysis, wrote the main manuscript text, and prepared figures and tables. EA supervised the laboratory and provided funding for the laboratory consumables. EA, MM and NFK, supervise and edit the manuscript. SSD and HL assist in laboratory analysis and sample collection. AAK= assists in sample collection. All authors have reviewed the manuscript. ML=Mulu Lemlem, EA= Erkihun Aklilu, MM=Maizan Mohamed, NFK=Nor Fadhilah Kamaruzzaman, SSD=Susmita Seenu Devan, HL= Habiba Lawal, AAK= Abubakar Abdulkarim Kanamma.

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Data availability
Data is provided within the manuscript as well as attached in the supplementary section. The nucleotide sequence of the ESBL genes is available in GenBank accession no. PQ522631-PQ522632.

Declarations

Ethics approval and consent to participate
The Institutional Animal Care and Use Committee of Universiti Malaysia Kelantan approved this study (UMK/FPV/ACUE/PG/2/2019). The animal subjects (chicken from commercial poultry farms) were only used to collect cloacal swabs, and no invasive or harmful procedures were used to handle the birds. Informed consent was obtained from the farm owners for the use of their animals in the study prior to study commencement. All methods conducted in this manuscript are based on standard guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Chantziaras I, Boyen F, Callens B, Dewulf J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J Antimicrob Chemother. 2014;69(3):827–34.
2. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R. Global trends in antimicrobial use in food animals. Proc Natl Acad Sci U S A. 2015;112(18):5649–54.
3. Zhu Z, Cao M, Wang W, Zhang L, Ma T, Liu G, Zhang Y, Shang Z, Chen X, Shi Y, et al. Exploring the prevalence and distribution patterns of Antibiotic Resistance genes in bovine gut microbiota using a Metagenomic Approach. Microb Drug Resist. 2021;27(7):980–90.
4. Bai H, He LY, Wu DL, Gao FZ, Zhang M, Zou HY, Yao MS, Ying GG. Spread of airborne antibiotic resistance from animal farms to the environment: dispersal pattern and exposure risk. Environ Int. 2022;158:106927.
5. Wang Y, Lyu N, Liu F, Liu WJ, Bi Y, Zhang Z, Ma S, Cao J, Song X, Wang A, et al. More diversified antibiotic resistance genes in chickens and workers of the live poultry markets. Environ Int. 2021;153:106534.
6. Larsson DGJ, Flach CF. Antibiotic resistance in the environment. Nat Rev Microbiol. 2022;20(5):257–69.

7. Martin MJ, Thottathil SE, Newman TB. Antibiotics overuse in animal agriculture: a call to action for Health Care Providers. Am J Public Health. 2015;105(12):2409–10.
8. Mackie RI, Koike S, Krapac I, Chee-Sanford J, Maxwell S, Aminov RI. Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. Anim Biotechnol. 2006;17(2):157–76.
9. Zhou J, Yun X, Wang J, Li Q, Wang Y. A review on the ecotoxicological effect of sulphonamides on aquatic organisms. Toxicol Rep. 2022;9:534–40.
10. Van Epps A, Blaney L. Antibiotic residues in Animal Waste: occurrence and degradation in Conventional Agricultural Waste Management practices. Curr Pollution Rep. 2016;2(3):135–55.
11. Xie WY, Shen Q, Zhao FJ. Antibiotics and antibiotic resistance from animal manures to soil: a review. Eur J Soil Sci. 2017;69(1):181–95.
12. Menz J, Olsson O, Kummerer K. Antibiotic residues in livestock manure: does the EU risk assessment sufficiently protect against microbial toxicity and selection of resistant bacteria in the environment? J Hazard Mater. 2019;379:120807.
13. Algammal A, Hetta HF, Mabrok M, Behzadi P. Editorial: emerging multidrug-resistant bacterial pathogens superbugs: a rising public health threat. Front Microbiol. 2023;14:1135614.
14. Shafiq M, Zeng M, Permana B, Bilal H, Huang J, Yao F, Algammal AM, Li X, Yuan Y, Jiao X. Coexistence of *bla (NDM-5)* and *tet(X4)* in international high-risk *Escherichia coli* clone ST648 of human origin in China. *Front Microbiol* 2022, 13:1031688.
15. Elbehiry A, Marzouk E, Aldubaib M, Moussa I, Abalkhail A, Ibrahem M, Hamada M, Sindi W, Alzaben F, Almuzaini AM, et al. *Pseudomonas* species prevalence, protein analysis, and antibiotic resistance: an evolving public health challenge. AMB Express. 2022;12(1):53.
16. Algammal AM, Eidaroos NH, Alfifi KJ, Alatawy M, Al-Harbi AI, Alanazi YF, Ghobashy MOI, Khafagy AR, Esawy AM, El-Sadda SS, et al. oprL gene sequencing, resistance patterns, virulence genes, Quorum Sensing and Antibiotic Resistance genes of XDR *Pseudomonas aeruginosa* isolated from broiler chickens. Infect Drug Resist. 2023;16:853–67.
17. Algammal AM, Ibrahim RA, Alfifi KJ, Ghabban H, Alghamdi S, Kabrah A, Khafagy AR, Abou-Elela GM, Abu-Elala NM, Donadu MG et al. A first report of molecular typing, virulence traits, and phenotypic and genotypic resistance patterns of newly emerging XDR and MDR *Aeromonas veronii* in Mugil Seheli. Pathogens 2022, 11(11).
18. Algammal AM, Abo Hashem ME, Alfifi KJ, Al-Otaibi AS, Alatawy M, ElTarabili RM, Abd El-Ghany WA, Hetta HF, Hamouda AM, Elewa AA, et al. Sequence analysis, Antibiogram Profile, Virulence and Antibiotic Resistance genes of XDR and MDR Gallibacterium anatis isolated from layer chickens in Egypt. Infect Drug Resist. 2022;15:4321–34.
19. Algammal AM, Alfifi KJ, Mabrok M, Alatawy M, Abdel-Moneam DA, Alghamdi S, Azab MM, Ibrahim RA, Hetta HF, El-Tarabili RM. Newly Emerging MDR B. cereus in Mugil seheli as the First Report Commonly Harbor *nhe, hbl, cytK*, and *pc-plc* Virulence Genes and *bla1*, *bla2*, *tetA*, and *ermA* Resistance Genes. *Infect Drug Resist* 2022, 15:2167–2185.
20. WHO. Critically Important Antimicrobials for Human Medicine. In., vol. ISBN 9789241515528, 6th ed edn. Genève, Zwitserland: WHO; 2019.
21. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev. 2005;18(4):657–86.
22. CDC: Antibiotic Resistance Threats in the United States. 2019. U.S. Department of Health and Human Services. Atlanta. 2019.
23. Tenaillon O, Skurnik D, Picard B, Denamur E. The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol. 2010;8(3):207–17.
24. Riley L. Distinguishing pathovars from nonpathovars: *Escherichia coli*. Microbiol. Spectr. 8. https://doi.org/10.1128/microbiolspec. In.: AME-0014-2020; 2020.
25. de Mesquita Souza Saraiva M, Lim K, do Monte DFM, Givisiez PEN, Alves LBR, de Freitas Neto OC, Kariuki S, Junior AB, de Oliveira CJB, Gebreyes WA. Antimicrobial resistance in the globalized food chain: a One Health perspective applied to the poultry industry. Braz J Microbiol. 2022;53(1):465–86.
26. Guabiraba R, Schouler C. Avian colibacillosis: still many black holes. FEMS Microbiol Lett. 2015;362(15):fnv118.
27. Dziva F, Stevens MP. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol. 2008;37(4):355–66.
28. Ewers C, Antao EM, Diehl I, Philipp HC, Wieler LH. Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. Appl Environ Microbiol. 2009;75(1):184–92.
29. Sarowska J, Futoma-Koloch B, Jama-Kmiecik A, Frej-Madrzak M, Ksiazczyk M, Bugla-Ploskonska G, Choroszy-Krol I. Virulence factors, prevalence and potential transmission of extraintestinal pathogenic *Escherichia coli* isolated from different sources: recent reports. Gut Pathog. 2019;11(1):10.
30. Dho-Moulin M, Fairbrother JM. Avian pathogenic *Escherichia coli* (APEC). Vet Res. 1999;30(2–3):299–316.
31. Chroma M, Kolar M. Genetic methods for detection of antibiotic resistance: focus on extended-spectrum beta-lactamases. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2010;154(4):289–96.
32. Murphy EMA, Ricci D, Auce A, Beechinor Z, Bergendahl JG, Breathnach H, Bures R, Duarte Da Silva J, Hederova JP. EMA and EFSA Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA). EFSA J. 2017;15(1):e04666.
33. Laube H, Friese A, von Salviati C, Guerra B, Rosler U. Transmission of ESBL/AmpC-producing *Escherichia coli* from broiler chicken farms to surrounding areas. Vet Microbiol. 2014;172(3–4):519–27.
34. Kaesbohrer A, Bakran-Lebl K, Irrgang A, Fischer J, Kampf P, Schiffmann A, Werckenthin C, Busch M, Kreienbrock L, Hille K. Diversity in prevalence and characteristics of ESBL/pAmpC producing *E. Coli* in food in Germany. Vet Microbiol. 2019;233:52–60.
35. Castanheira M, Simner PJ, Bradford PA. Extended-spectrum beta-lactamases: an update on their characteristics, epidemiology and detection. JAC Antimicrob Resist. 2021;3(3):dlab092.
36. Lemlem M, Aklilu E, Mohammed M, Kamaruzzaman F, Zakaria Z, Harun A, Devan SS. Molecular detection and antimicrobial resistance profiles of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* in broiler chicken farms in Malaysia. PLoS ONE. 2023;18(5):e0285743.
37. Islam MS, Rahman A, Hassan J, Rahman MT. Extended-spectrum beta-lactamase in *Escherichia coli* isolated from humans, animals, and environments in Bangladesh: a One Health perspective systematic review and meta-analysis. One Health. 2023;16:100526.
38. Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep. 2013;5(1):58–65.
39. Kohler CD, Dobrindt U. What defines extraintestinal pathogenic *Escherichia coli*? Int J Med Microbiol. 2011;301(8):642–7.
40. Tivendale KA, Logue CM, Kariyawasam S, Jordan D, Hussein A, Li G, Wannemuehler Y, Nolan LK. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. Infect Immun. 2010;78(8):3412–9.
41. Dale AP, Woodford N. Extra-intestinal pathogenic *Escherichia coli* (ExPEC): Disease, carriage and clones. J Infect. 2015;71(6):615–26.
42. Fazlul MKKFY, Najnin A, Rashid MA, Nazmul MHM. Detection of *CTX-M*- type ESBLs from *Escherichia coli* Clinical Isolates from a Tertiary Hospital, Malaysia. Baghdad Sci J. 2019;16(3Suppl):0682.
43. Dwiyanto J, Hor JW, Reidpath D, Su TT, Lee SWH, Ayub Q, Mustapha FB, Lee SM, Foo SC, Chong CW, et al. Pan-genome and resistome analysis of extended-spectrum ss-lactamase-producing *Escherichia coli*: a multi-setting epidemiological surveillance study from Malaysia. PLoS ONE. 2022;17(3):e0265142.
44. Aliyu AB, Saleha AA, Jalila A, Zunita Z. Risk factors and spatial distribution of extended spectrum beta-lactamase-producing- *Escherichia coli* at retail poultry meat markets in Malaysia: a cross-sectional study. BMC Public Health. 2016;16(1):699.
45. Habiba L, Erkihun A, Nor Fadhilah K, Zarizal S, Gaddafi Mohammed S, Mulu L. Livestock and environment as potential sources and reservoirs for multi-drug resistant *Escherichia coli* in Malaysia: a systematic review. Veterinary Integr Sci. 2023;22(2). https://doi.org/10.12982/VIS.2024.028.
46. Gazal LES, Medeiros LP, Dibo M, Nishio EK, Koga VL, Goncalves BC, Grassotti TT, de Camargo TCL, Pinheiro JJ, Vespero EC, et al. Detection of ESBL/AmpC-Producing and fosfomycin-resistant *Escherichia coli* from different sources in Poultry Production in Southern Brazil. Front Microbiol. 2020;11:604544.
47. Megersa R, Mathewos M, Fesseha H. Isolation and identification of *Escherichia coli* from dairy cow raw milk in Bishoftu Town, Central Ethiopia. Arch Vet Anim Sci 2019, 1(1).
48. Mahmud S, Nazir K, Rahman MT. Prevalence and molecular detection of fluoroquinolone-resistant genes (*qnrA* and *qnrS*) in *Escherichia coli* isolated from healthy broiler chickens. Vet World. 2018;11(12):1720–4.
49. Yu K, Xin. Thong, Kwai Lin: Multiplex PCR for simultaneous detection of virulence genes in *Escherichia coli*. Malays J Sci. 2009;28(1):1–14.

50. CLSI. *Performance Standard for Antimicrobial Suseceptibility Testing.* 31st ed. In: *CLSI supplement M100Clinical and Laboratory Standards Institute.* 2021.
51. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268–81.
52. Tambekar D, Dhanorkar D, Gulhane S, Khandelwal V, Dudhane M. Antibacterial susceptibility of some urinary tract pathogens to commonly used antibiotics. Afr J Biotechnol 2006, 5(17).
53. Kaur J, Chopra S, Sheevani, Mahajan G. Modified double disc synergy test to detect ESBL production in urinary isolates of *Escherichia coli* and *Klebsiella pneumoniae*. J Clin Diagn Res. 2013;7(2):229–33.
54. Dierikx C, van Essen-Zandbergen A, Veldman K, Smith H, Mevius D. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet Microbiol. 2010;145(3–4):273–8.
55. Ewers C, Janssen T, Kiessling S, Philipp HC, Wieler LH. Rapid detection of virulence-associated genes in avian pathogenic Escherichia coli by multiplex polymerase chain reaction. Avian Dis. 2005;49(2):269–73.
56. Reback J, McKinney W, Van Den Bossche J, Augspurger T, Cloud P, Klein A, Hawkins S, Roeschke M, Tratner J, She C. pandas-dev/pandas: Pandas 1.0. 5. *Zenodo* 2020.
57. Waskom ML. Seaborn: statistical data visualization. J Open Source Softw. 2021;6(60):3021.
58. Gao C, Yu G, Dusa A. ggVennDiagram: a 'ggplot2'implement of Venn diagram. *R package* 2019.
59. Oh SS, Song J, Kim J, Shin J. Increasing prevalence of multidrug-resistant *mcr-1*-positive *Escherichia coli isolates* from fresh vegetables and healthy food animals in South Korea. Int J Infect Dis. 2020;92:53–5.
60. Wibisono FJ, Sumiarto B, Untari T, Effendi MH, Permatasari DA, Witaningrum AM. *CTX* gene of Extended Spectrum Beta-Lactamase (ESBL) Producing *Escherichia coli* on broilers in Blitar, Indonesia. Syst Reviews Pharm 2020, 11(7).
61. Chowdhury M, Bardhan R, Pal S, Banerjee A, Batabyal K, Joardar SN, Mandal GP, Bandyopadhyay S, Dutta TK, Sar TK, et al. Comparative occurrence of ESBL/AmpC beta-lactamase-producing *Escherichia coli* and *Salmonella* in contract farm and backyard broilers. Lett Appl Microbiol. 2022;74(1):53–62.
62. Mandujano A, Cortes-Espinosa DV, Vasquez-Villanueva J, Guel P, Rivera G, Juarez-Rendon K, Cruz-Pulido WL, Aguilera-Arreola G, Guerrero A, Bocanegra-Garcia V, et al. Extended-spectrum beta-lactamase-producing *Escherichia coli* isolated from Food-Producing animals in Tamaulipas, Mexico. Antibiot (Basel). 2023;12(6):1010.
63. Mgaya FX, Matee MI, Muhairwa AP, Hoza AS. Occurrence of Multidrug Resistant *Escherichia coli* in raw meat and Cloaca swabs in Poultry processed in Slaughter slabs in Dar Es Salaam, Tanzania. Antibiot (Basel). 2021;10(4):343.
64. Laube H, Friese A, von Salviati C, Guerra B, Kasbohrer A, Kreienbrock L, Roesler U. Longitudinal monitoring of extended-spectrum-beta-lactamase/AmpC-producing *Escherichia coli* at German broiler chicken fattening farms. Appl Environ Microbiol. 2013;79(16):4815–20.
65. Kamaruzzaman EA, Abdul Aziz S, Bitrus AA, Zakaria Z, Hassan L. Occurrence and characteristics of extended-spectrum β-Lactamase-producing *Escherichia coli* from Dairy Cattle, milk, and Farm environments in Peninsular Malaysia. Pathogens. 2020;9(12):1007.
66. Guo S, Aung KT, Leekitcharoenphon P, Tay MYF, Seow KLG, Zhong Y, Ng LC, Aarestrup FM, Schlundt J. Prevalence and genomic analysis of ESBL-producing *Escherichia coli* in retail raw meats in Singapore. J Antimicrob Chemother. 2021;76(3):601–5.
67. Bastidas-Caldes C, Cisneros-Vasquez E, Zambrano A, Mosquera-Maza A, Calero-Caceres W, Rey J, Yamamoto Y, Yamamoto M, Calvopina M, de Waard JH. Co-harboring of Beta-lactamases and *mcr-1* genes in *Escherichia coli* and *Klebsiella pneumoniae* from Healthy Carriers and Backyard animals in Rural communities in Ecuador. Antibiot (Basel) 2023, 12(5).
68. Dominguez JE, Redondo LM, Figueroa Espinosa RA, Cejas D, Gutkind GO, Chacana PA, Di Conza JA, Fernandez Miyakawa ME. Simultaneous carriage of *mcr-1* and other Antimicrobial Resistance determinants in *Escherichia coli* from Poultry. Front Microbiol. 2018;9:1679.
69. Elmi SA, Simons D, Elton L, Haider N, Abdel Hamid MM, Shuaib YA, Khan MA, Othman I, Kock R, Osman AY. Identification of risk factors Associated with resistant *Escherichia coli* isolates from Poultry farms in the East Coast of Peninsular Malaysia: A Cross Sectional Study. Antibiot (Basel) 2021, 10(2).
70. Rizal S, Nurhapsari I, Fauziah I, Masrukhin M, Jatmiko YD. Prevalence of multidrug-resistant and extended-spectrum β-lactamase producing *Escherichia coli* from local and broiler chickens at Cibinong market, West Java, Indonesia. 2021.
71. Rodroo J, Intanon M, Kreausukon K, Kongkaew A, Bender J, Awaiwanont N. Occurrence of extended-spectrum beta-lactamase producing *E. Coli* in broiler farm workers and the farm environment in Chiang Mai-Lamphun, Thailand. Veterinary Integr Sci 2021, 19(1).
72. Martinez-Alvarez S, Sanz S, Olarte C, Hidalgo-Sanz R, Carvalho I, Fernandez-Fernandez R, Campana-Burguet A, Latorre-Fernandez J, Zarazaga M, Torres C. Antimicrobial Resistance in *Escherichia coli* from the Broiler Farm Environment, with Detection of *SHV-12*- Producing Isolates. Antibiot (Basel). 2022;11(4):444.
73. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev. 2009;22(1):161–82.
74. Bradford PA. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev. 2001;14(4):933–51. table of contents.
75. Wang Y, Zhou J, Li X, Ma L, Cao X, Hu W, Zhao L, Jing W, Lan X, Li Y, et al. Genetic diversity, antimicrobial resistance and extended-spectrum beta-lactamase type of *Escherichia coli* isolates from chicken, dog, pig and yak in Gansu and Qinghai Provinces, China. J Glob Antimicrob Resist. 2020;22:726–32.
76. Aworh MK, Kwaga JKP, Hendriksen RS, Okolocha EC, Thakur S. Genetic relatedness of multidrug resistant *Escherichia coli* isolated from humans, chickens and poultry environments. Antimicrob Resist Infect Control. 2021;10(1):58.
77. Lim KT, Yasin R, Yeo CC, Puthucheary S, Thong KL. Characterization of multidrug resistant ESBL-producing Escherichia coli isolates from hospitals in Malaysia. J Biomed Biotechnol. 2009;2009:165637.
78. Hemeg HA. Molecular characterization of antibiotic resistant *Escherichia coli* isolates recovered from food samples and outpatient clinics, KSA. Saudi J Biol Sci. 2018;25(5):928–31.
79. Devan SS, Aklilu E, Zakaria Z, Hamdan RH, Lemlem M, Kamaruzzaman NF, Kamaruzaman INA, Reduan MFH. Emergence of *Mcr – 1, -3, -6, -8* and *– 9*) in *Escherichia Coli* Isolated from Live Chickens, Raw Chicken Meat and Vegetables from Kelantan, Malaysia. *Journal of microbiology, biotechnology and food sciences* 2023, 12(6):e9829.
80. Lemlem M, Aklilu E, Mohamed M, Kamaruzzaman NF, Zakaria Z, Harun A, Devan SS, Kamaruzaman INA, Reduan MFH, Saravanan M. Phenotypic and genotypic characterization of colistin-resistant *Escherichia Coli* with *mcr-4, mcr-5, mcr-6*, and *mcr-9* genes from broiler chicken and farm environment. *BMC Microbiol* 2023, 23(1):392.
81. Aklilu E, Harun A, Singh KKB. Molecular characterization of *bla(NDM), bla(OXA-48), mcr-1* and *bla(TEM-52)* positive and concurrently carbapenem and colistin resistant and extended spectrum beta-lactamase producing *Escherichia coli* in chicken in Malaysia. *BMC Vet Res* 2022, 18(1):190.
82. Denamur E, Clermont O, Bonacorsi S, Gordon D. The population genetics of pathogenic *Escherichia coli*. Nat Rev Microbiol. 2021;19(1):37–54.
83. Ronco T, Stegger M, Olsen RH, Sekse C, Nordstoga AB, Pohjanvirta T, Lilje B, Lyhs U, Andersen PS, Pedersen K. Spread of avian pathogenic *Escherichia coli* ST117 O78:H4 in nordic broiler production. BMC Genomics. 2017;18(1):13.
84. Borges CA, Tarlton NJ, Riley LW. Escherichia coli from commercial broiler and backyard chickens share sequence types, Antimicrobial Resistance profiles, and Resistance genes with human extraintestinal pathogenic *Escherichia coli*. Foodborne Pathog Dis. 2019;16(12):813–22.
85. Tofani S, Albini E, Blasi F, Cucco L, Lovito C, Maresca C, Pesciaroli M, Orsini S, Scoccia E, Pezzotti G et al. Assessing the load, virulence and antibiotic-resistant traits of ESBL/Ampc *E. Coli* from broilers raised on conventional, Antibiotic-Free, and Organic farms. Antibiot (Basel) 2022, 11(11).
86. Xia F, Jiang M, Wen Z, Wang Z, Wang M, Xu Y, Zhuge X, Dai J. Complete genomic analysis of ST117 lineage extraintestinal pathogenic *Escherichia coli* (ExPEC) to reveal multiple genetic determinants to drive its global transmission: ST117 *E. coli* as an emerging multidrug-resistant foodborne ExPEC with zoonotic potential. *Transbound Emerg Dis* 2022, n/a(n/a).
87. Schaufler K, Semmler T, Wieler LH, Trott DJ, Pitout J, Peirano G, Bonnedahl J, Dolejska M, Literak I, Fuchs S et al. Genomic and functional analysis of emerging virulent and multidrug-resistant *Escherichia coli* Lineage sequence type 648. Antimicrob Agents Chemother 2019, 63(6).

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