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Molecular Detection of Colistin-Resistant *E. coli* in Village Chickens from Kelantan, Malaysia

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Abstract: Pathogenic *Escherichia coli* can cause a variety of intestinal and extra-intestinal infections in humans and animals. The availability and subsequent misuse of antimicrobials, especially in poultry production systems, has contributed immensely to the emergence and spread of multidrug-resistant *E. coli*. This study investigated the genotypic characterization of colistin-resistant *E. coli* and selected antimicrobial-resistance encoding genes along with their phenotypic resistant pattern and the multiple antimicrobial resistant (MAR) index from village chickens in Kelantan. Sixty *E. coli* isolates obtained from a previous study's stock culture were enriched and analyzed using routine microbiological methods: Kirby–Bauer disc diffusion method, minimum inhibitory concentration (MIC), and PCR amplification of *E. coli* species-specific and multidrug-resistance *mcr*-positive *E. coli*. All the isolates were confirmed as *E. coli* and 16.6% (10/60) were positive for *mcr*. Five isolates were positive for *mcr*-1, three for *mcr*-4, and two for *mcr*-9. The *mcr*-positive isolates showed varying degrees of resistance to different antimicrobials. The isolates were resistant to gentamicin (100%), chloramphenicol (100%), and tetracycline (89.4%) and susceptible to ceftaxidime (2.26%) and imipenem (18%). Furthermore, 100%, 94.7%, and 89.4% of isolates from village chickens belonged to phylogroup C, B2, and E, while 21.0% and 42.1% of the isolates belonged to phylogroup A and B1, respectively. Sequence types (STs) of selected *E. coli* isolates were further analyzed using multi-locus sequence typing, and 10 different STs were identified. This study showed the emerging threats of multidrug-resistant *mcr*-positive *E. coli* gene in village chickens that are believed to be raised with minimal or no antibiotics.

Keywords: colistin; *Eschericia coli*; multidrug resistance; village chicken

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1. Introduction

Antibiotic resistance poses an escalating threat to global public health, rendering once effective treatments ineffective and increasing the complexity of managing infectious diseases [1]. Among the antibiotics of last resort, colistin, a polymyxin antibiotic, has been

thrust into the spotlight due to the discovery of plasmid-mediated colistin resistance genes, notably the mobile colistin resistance (*mcr*) gene [2]. The rapid dissemination of *mcr* among diverse bacterial species raises concerns about the potential loss of colistin as a viable therapeutic option, necessitating urgent research efforts to comprehend its prevalence and mechanisms of transmission [3]. *Escherichia coli* (*E. coli*), an ubiquitous Gram-negative bacterium residing in the intestines of humans and animals, has emerged as a sentinel organism for monitoring antibiotic resistance trends. The association between colistin resistance in *E. coli* and the *mcr* gene has been widely documented in clinical settings [4]. However, the dynamics of colistin resistance in *E. coli* strains isolated from agricultural environments, particularly in poultry, demand meticulous scrutiny [5]. Village chickens, commonly found in the agricultural landscapes of areas such as Kelantan, Malaysia, may act as reservoirs for antibiotic-resistant bacteria due to their close interactions with humans and exposure to diverse environmental conditions [6]. Malaysia, like many other countries, faces challenges in combating antibiotic resistance [1,7]. The country's poultry industry plays a crucial role in food production, with local chickens being raised for both meat and egg production. The intensive farming practices, including the use of antibiotics as growth promoters, create an environment conducive to the emergence and dissemination of antibiotic-resistant bacteria [8].

Despite the significance of local chickens in Malaysia's food supply, limited information is available regarding the occurrence and characteristics of multidrug-resistant *E. coli* strains carrying the *mcr*-1 gene in these chicken populations. Understanding the extent and distribution of *mcr*-mediated colistin resistance in local chickens is essential for assessing the potential risks to human health, as these bacteria can enter the food chain and pose a threat to consumers. Therefore, this study aimed to conduct the first PCR detection of the *mcr* gene and phylotyping of multidrug-resistant *E. coli* isolates obtained from local chicken samples in Malaysia. The polymerase chain reaction (PCR) is a highly sensitive and specific molecular biology technique that enables the detection and amplification of target genes, such as *mcr*. Additionally, employing phylotyping techniques will allow for the classification of *E. coli* strains into distinct phylogenetic groups based on genetic markers, providing insights into the genetic diversity and evolutionary relationships among the multidrug-resistant *E. coli* isolates. The findings from this study will bridge the knowledge gap regarding the occurrence and characteristics of multidrug-resistant *E. coli* strains carrying the *mcr* gene in village chicken populations in Malaysia. This knowledge can inform evidence-based strategies for antimicrobial stewardship, surveillance, and control measures to mitigate the spread of antibiotic resistance in both animal and human health settings. Furthermore, the findings of this research have far-reaching implications for public health policies, emphasizing the urgent need for comprehensive One Health approaches that encompass both human and animal health sectors to mitigate the spread of colistin resistance and preserve the efficacy of this vital antibiotic class.

2. Materials and Methods

2.1. Isolation and Phenotypic Identification of E. coli

This study utilized stock *Escherichia coli* isolates obtained from a previous unpublished study conducted in our laboratory. In that study, samples were collected from the only live-bird market in Kelantan, Malaysia. Cloacal swabs were aseptically collected from live poultry (village chickens) at different vendor stalls within the market to ensure representation of diverse sources. Swabs were immediately placed in sterile transport media and transported on ice to the laboratory for bacterial isolation. Upon arrival, samples were processed following standard microbiological techniques. For this study, we retrieved and revived these stock *E. coli* isolates to investigate their antimicrobial resistance profiles,

presence of colistin resistant genes (*mcr*1 to 9), and phylogenetic distribution using routine microbial techniques. Briefly, 60 stock E. coli isolates were obtained and enriched in buffered peptone water, incubated at 37 °C for 24 h. After enrichment, the bacteria were plated on blood agar and aerobically incubated at 37 °C for an additional 24 h. Presumptive colonies resembling *E. coli*, identified based on characteristics such as small size, round shape, raised elevation, smooth texture, and gray to white color with hemolysis, were selected. These colonies were streaked on freshly prepared MacConkey agar and incubated at 37 °C for 24 h. Colonies fermenting lactose and appearing pink on MacConkey agar were further sub-cultured on eosin methylene blue (EMB) agar. Two to three colonies displaying a green metallic sheen on EMB were chosen and sub-cultured on nutrient agar slants, followed by an incubation period at 37 °C for 18–24 h. To confirm these isolates as *E. coli*, a range of biochemical tests were carried out, including assessments for glucose fermentation, citrate utilization, urease production, indole fermentation, methyl red test, and motility. These tests were performed according to the procedures described in a referenced study [9]. *E. coli* isolates that were phenotypically confirmed were sub-cultured on nutrient agar and preserved in Luria–Bertani (LB) broth containing 50% glycerol at −80 °C for future analysis [9].

2.2. Antimicrobial Susceptibility Test

The antimicrobial susceptibility testing (AST) of *E. coli* isolates was conducted using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar plates (Oxoid, Manchester, UK). A bacterial suspension with a turbidity matching the 0.5 McFarland standard was evenly spread across the surface of Mueller–Hinton agar plates using a sterile cotton swab. Antibiotic discs, including aztreonam (ATM30), cefotaxime (CTX30), amoxicillin-clavulanic acid (AMC30), ceftazidime (CAZ30), ceftriaxone (CRO30), trimethoprim-sulfamethoxazole (SXT25), chloramphenicol (C30), tetracycline (TE30), gentamicin (GEN10), kanamycin (K30), ampicillin (AMP10), ofloxacin (OFX5), imipenem (IPM10), and erythromycin, were placed on the surface of the agar plates. The plates were then incubated at 37 °C for 18 h. The zone of inhibition was measured to the nearest millimeter and interpreted following the guidelines provided by the Clinical and Laboratory Standards Institute [10].

2.3. Determination of Multiple Antibiotic Resistance (MAR) Index

Bacterial strains demonstrating resistance to three or more classes of antimicrobial agents were categorized as multidrug-resistant (MDR), following the methodology outlined in the study [11]. The multiple antibiotic resistance (MAR) index, a metric crucial in assessing bacterial resistance patterns, was calculated using the formula:

$$\text{MAR index} = \frac{\text{Number of antimicrobials to which the isolate showed resistance}}{\text{Number of total antibiotics exposed to the isolate.}}$$

2.4. Minimum Inhibitory Concentration (MIC) Test of Colistin in MCR-EC

The *E. coli* isolates carrying the *mcr* gene (*MCR*-EC) underwent additional testing for colistin minimum inhibitory concentration (MIC) using the colistin broth disk elution method, following the standardized guidelines set by CLSI [10]. The colistin MIC endpoint was determined as the lowest concentration that entirely prevented visible growth of the tested isolate. A MIC of $\geq$4 μg/mL was classified as resistant.

2.5. Genetic Characteristics of E. coli

2.5.1. Genomic DNA Extraction

Genomic DNA extraction followed the boiling method described in the protocol by Chen [12]. In summary, a loopful of bacteria cultured overnight on a nutrient agar plate was transferred into a 1.5 µL microcentrifuge tube containing 100 µL of sterile distilled water. The suspension was allowed to incubate at room temperature for 5 min and then heat-treated at 96 °C for 10 min. After heat treatment, the suspension was centrifuged at 12,000× *g* for 5 min. The resulting supernatant, containing the extracted DNA, was carefully collected using a micropipette and stored at 4 °C until further use.

2.5.2. Molecular Confirmation of *E. coli* Using Specie-Specific Gene

The DNA templates extracted from *E. coli* isolates that tested positive in biochemical assays were used as templates for PCR amplification employing specie-specific primers targeting the phoA gene, following the previously established method [13]. The PCR reaction for the phoA primer followed this protocol: an initial denaturation step at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at an optimized temperature of 56 °C for 30 s, and extension at 72 °C for 60 s. A final extension step was conducted at 72 °C for 10 min. The resulting amplicons were electrophoresed in a 1.5% agarose gel for 45 min at 90 volts. Subsequently, the amplicons were visualized using the Gel Doc™ EZ Imager (Bio-Rad, Hercules, CA, USA) under a UV transilluminator.

2.5.3. Detection of Colistin Resistance Gene (*mcr*) in *E. coli* Isolates

Genomic DNA extracted was subjected to PCR amplification using specific primers designed to identify *mcr*-producing genes in *E. coli*. The PCR protocol for detecting *mcr* encoding genes involved an initial denaturation step at 95 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. A final extension step was carried out at 72 °C for 10 min. Subsequently, agarose gel electrophoresis was performed to visualize the amplicons, and the gel images were analyzed using the Gel Doc™ EZ Imager (Bio-Rad, Hercules, CA, USA).

2.5.4. Phylogenetic Grouping of *E. coli*

The Clermont phylotyping method, which employs quadraplex PCR, was utilized to determine the phylogroups of all *E. coli* isolates [14]. The genes, primer sequences, and their expected band sizes for both quadraplex PCR are listed in Table 1. Each PCR reaction included 12.5 µL of 2× GreenTaq Master mix (Promega, Southampton, Hampshire, UK), 5.5 µL and 7.5 µL of NFW (for quadraplex PCR and allele-specific PCR, respectively), 0.5 µL of diluted forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA), and 3 µL of the DNA lysate. The concentrations of the diluted primers chuA, yjaA, TspE4. C2, ArpAgpE, and trpAgpC were 20 pmol, while arpA was 40 pmol, and trpBA 12 pmol. PCR amplification was performed with the following conditions: initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C (for group E) or 59 °C (for quadruplex) or 62 °C (for group C) for 20 s, and a final extension at 72 °C for 5 min. Subsequently, the PCR products were run on a 1.5% agarose gel electrophoresis at 90 volts for 40 min and analyzed using the Gel Doc™ EZ Imager (Bio-Rad, USA) for visualization.

Table 1. Resistance phenotypes, resistant genes, phylotypes, and sequence types of E. coli isolates from village chickens.

Isolate Identification	Resistance Phenotype	MIC Colistin	Resistance Genes Detected	Phylogenetic Group	MLST Sequence Type	MLST Clonal Complex
C5	Ery-tet-Ofl-Amp-Kan-Amk-Chl-Ctz-Amc-AmS-Cfx	≥4	*mcr*1, sul1, tetA, genta (aac3), chloram (catA1)	A, C, E, B2, B1	345	-
C6	Ery-tet-Ofl-Amp-Kan-Chl-Ctz-Amc-AmS-Cfx	≥4	*mcr*1, sul1, tetA, genta (aac3),	A, C, E, B2	770	-
C7	Ery-tet-Ofl-Amp-Kan-Amk-Chl-AmS-Cfx	≥4	*mcr*4, sul1, tetA, genta (aac3),	A, C, E, B1	10	-
C9	Ery-tet-Ofl-Amp-Kan-Amk-Chl-Amc-AmS-Cfx	≥4	*mcr*1, sul1, tetA, genta (aac3), chloram (catA1)	A, C, E, B2, B1	540	-
C10	Ery-tet-Ofl-Amp-Kan-Amk-Chl-Ctz-Amc-AmS-Cfx	≥4	*mcr*4, sul1, tetA, genta (aac3), chloram (catA1)	A, C, E, B1	155	ST155cplx
C11	Ery-tet-Ofl-Amp-Kan-Chl-AmS-Cfx	≥4	*mcr*9, sul1, tetA, genta (aac3),	A, C, E, B2	31	-
C12	Ery-tet-Ofl-Amp-Kan-Chl-Ctz-Amc-AmS-Cfx	≤4	*mcr*1, sul1, tetA, genta (aac3),	A, C, E, B1	1638	ST10cplx
C15	Ery-tet-Ofl-Amp-Kan-Amk-Chl-Amc-AmS	≥4	*mcr*4, sul1, tetA, genta (aac3),	A, C, E, B1	906	ST11cplx
C18	Ery-Tet-Ofl-Amp-Kan-Chl-Ctz-Amc-Ams	≥4	*mcr*9, sul1, tetA, genta (aac3), chloram (catA1)	A, C, E, B1	206	-
C19	Tet-Cft-Amp-Kan-Chl-Amc-Ams	≥4	*mcr*1, sul1, tetA, genta (aac3), chloram (catA1)	A, C, E, B1	162	-

Key: Ery-erythromycin, tet-tetracycline, Ofl-ofloxacin, Amp-ampicillin, Kan-kanamycin, Amk-amikacin, Chl-chloramphenicol, Ctz-ceftriaxone, Amc-amoxicillin-clavulanic acid, AmS-ampicillin sulbactam, Cfx-cefotaxime.

2.5.5. Multi-Locus Sequence Typing (MLST)

The MLST analysis of specific isolates (C5, C6, C7, C9, C10, C11, C12, C15, C18, C19), selected based on their resistance profiles, was conducted following established procedures [15]. The PCR amplification and sequencing of the seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA, and recA) were carried out using recommended protocols for *E. coli*. Allelic numbers and sequence types (STs) were assigned using the MLST database https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search (accessed on 26 March 2025. The primer sequences for all seven housekeeping genes can be found at https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html (accessed on 26 March 2025). The PCR reactions were prepared in a 50 µL amplification mixture containing 5 µL of template DNA, 1 µL of each primer (25 pmol/µL), 25 µL GoTaq® Master Mix (Promega, Madison, WI, USA), and 18 µL molecular-grade water. Amplification conditions included an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 56 °C (adk) or 64 °C (fumC and purA) or 68 °C (recA, gyrB, icd and mdh) for 1 min, extension at 72 °C for 2 min and with a final extension at 72 °C for 5 min. The amplified PCR products were sent to Apical 1st base

sequencing service (Apical Scientific SDN. BHD., Seri Kembangan, Selangor, Malaysia) for sequencing.

3. Results

3.1. Molecular Occurrence of E. coli and Colistin Resistant mcr-E. coli (MCREC)

In our research, PCR analysis confirmed the existence of the phoA gene (903 bp) in all 60 *E. coli* isolates identified through phenotypic means. Using the multiplex PCR detection method, we identified a total of 10 out of 60 *E. coli* isolates carrying *mcr* genes, indicating an occurrence rate of 16.6%. Notably, the *mcr* gene variants discovered in this study included *mcr*-1, *mcr*-4, and *mcr*-9 (see Table 1).

3.2. Phylogenetic Grouping of E. coli and MCREC

The results from the multiplex PCR phylogenetic grouping showed that the majority of the examined isolates were pathogenic *E. coli*, with 94.7% falling into phylogenetic group B2 and 89.4% in group E. Commensal *E. coli*, identified as group C (100%), A (21.0%), and B1 (42.1%), were also observed. In the specific case of the four *MCR*-EC isolates, all of them (100%) belonged to phylogroup C, with a smaller proportion falling into groups B2 and E (Table 1).

3.3. Antibiotic Susceptibility Testing

The findings demonstrated that *E. coli* isolates with the *mcr* gene exhibited remarkably high resistance levels against six antibiotics belonging to four main classes. Among these isolates, 100% resistance was observed to ceftiofur, imipenem, ampicillin/sulbactam, chloramphenicol, gentamicin, and kanamycin. Additionally, among the tetracyclines and macrolides class, a sizable number of the isolates showed resistance to both erythromycin (89.4%) and tetracycline (89.4%). Ceftazidime was particularly ineffective, as only 5.26% of the isolates were susceptible to it (Table 2).

Table 2. Antimicrobial resistance rate of *E. coli* isolates with *mcr* gene (*n* = 10).

Class of Antibiotics	Mechanism of Action	Antibiotics (Dosage)	Resistance Rate (%)
	Inhibition of Cell Wall Synthesis		
Penicillin		Amoxicillin (10 µg)	0%
Carbapenems		Imipenem (10 µg)	18%
Cephems (Parental)		Cefotaxime (30 µg)	63.15%
Cephalosporins III		Ceftazadime (30 µg)	5.26%
		Ceftriazone (30 µg)	63.15%
		Ceftiofur (30 µg)	0%
β–lactams combination agents		Amoxicillin/Clavulanic acid (30 µg)	63.15%
		Ampicillin/Sulbactam (20 µg)	100%
	Disruption of DNA synthesis and DNA replication		
Fluoroquinolones		Ofloxacin (5 µg)	63.15%
	Inhibition of protein synthesis		
Tetracyclines		Tetracycline (30 µg)	89.4%

Table 2. *Cont.*

Class of Antibiotics	Mechanism of Action	Antibiotics (Dosage)	Resistance Rate (%)
Phenicols		Chloramphenicol (30 µg)	100%
Macrolides		Erythromycin (30 µg)	89.4%
Aminoglycoside		Kanamycin (30 µg)	100%
Aminoglycoside		Gentamicin (10 µg)	100%
Aminoglycoside		Amikacin (10 µg)	26.31%

Key: µg: microgram, %: percentage.

3.4. Multiple Antimicrobial Resistance Index

All the isolates (100%) had an MAR index value surpassing 0.2. This outcome indicates that these isolates are multidrug-resistant, possibly due to prolonged exposure to certain antibiotics or selective pressures.

3.5. Minimum Inhibitory Concentration (MIC)

Result showed 16.6% (10/60) of the *E. coli* isolates exhibited phenotypic resistance to colistin, with a MIC of ≥4 µg/mL (Table 1).

3.6. Identification of STs for the Colistin Resistant E. coli

MLST analysis of 10 *MCR-E. coli* isolates of village chickens revealed 10 different sequence type (ST), namely, ST345, ST155, ST31, ST770, ST10, ST540, ST1638, ST906, ST162, and ST206. Phylogenetic analysis of the 10 different sequence types showed that the origin of the isolates was from different countries according to the Phyloviz and grape tree analysis (Figures 1 and 2).

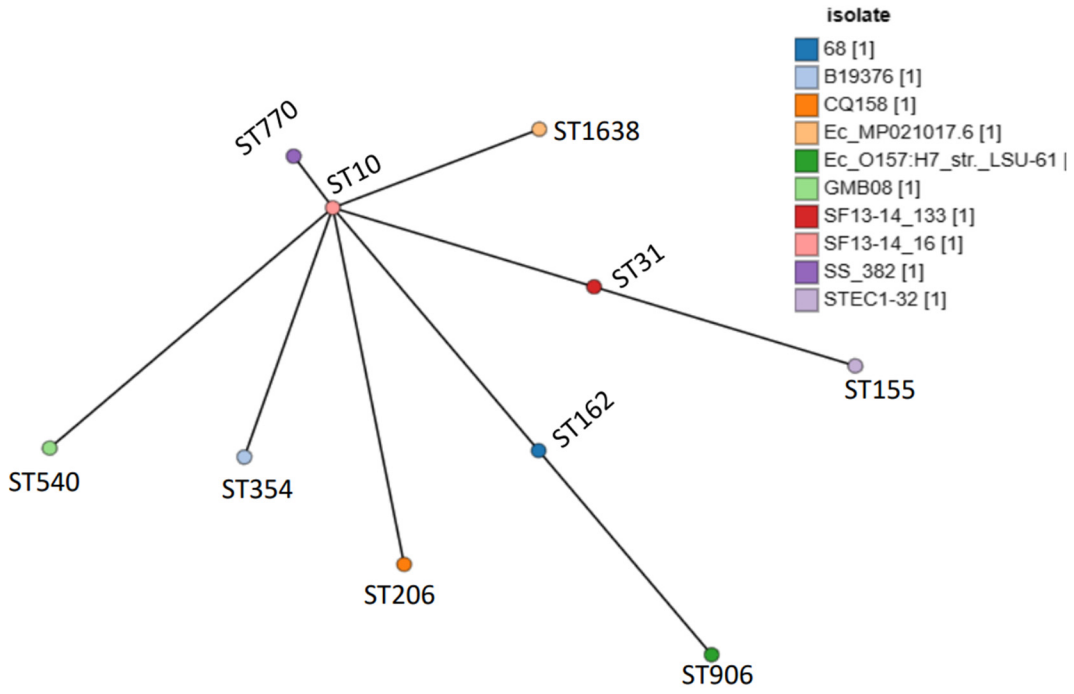


Figure 1. Grape tree phylogeny showing different sequence types (ST).

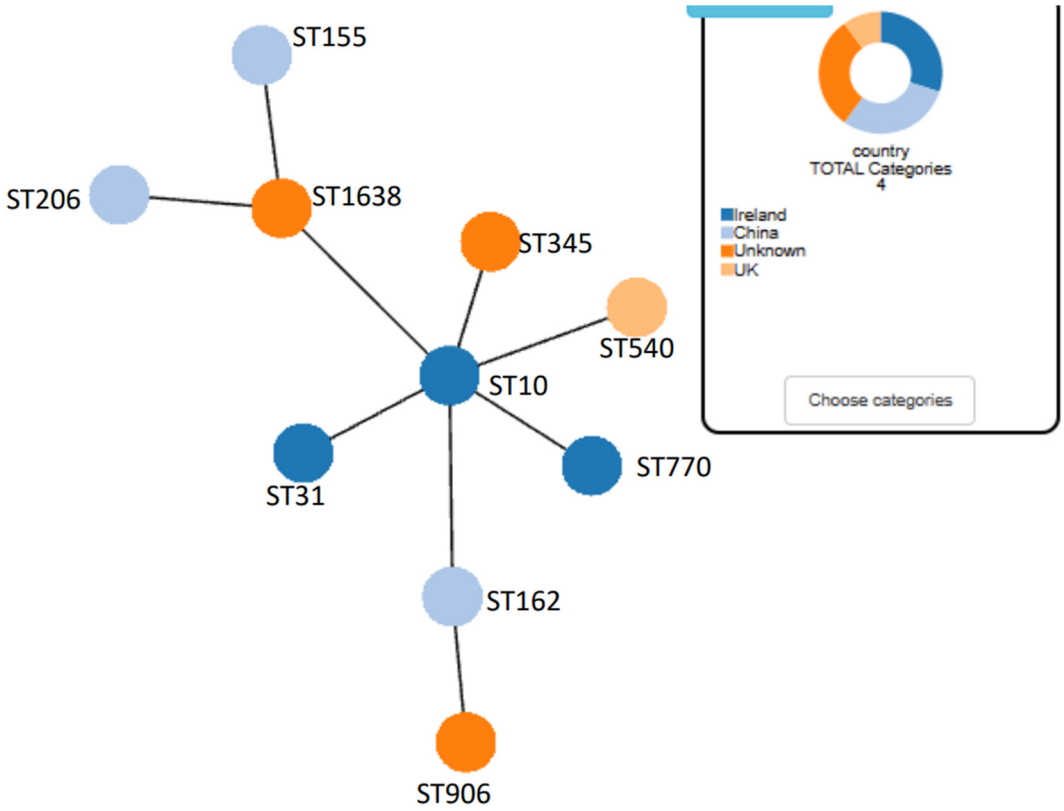


Figure 2. Phyloviz tree phylogenetic analysis showing the country origin of the sequence types (ST).

4. Discussion

Colistin-resistant *Escherichia coli* (CREC) has become a significant global public health threat, with documented cases in various environmental niches, including poultry habitats and live bird markets, posing a potential transmission risk to humans [16]. While poultry production systems have been frequently cited as sources of CREC [17], the presence of CREC in village chickens remains largely unexplored, particularly in the study area. This study investigated the occurrence of CREC in village chickens and explored the genetic characteristics of CREC strains carrying *mcr* genes, shedding light on a previously unexamined facet of antibiotic resistance in poultry farming. However, the presence of CREC in village chickens in Kelantan, Malaysia, has not been explored to date, according to our knowledge. This absence of research might be attributed to the subpar hygienic conditions in village chicken farms, where flocks often reside in spaces contaminated with manure. In this study, 16.6% (10/60) of the village chicken isolates were found to be positive for colistin-resistant *E. coli* (CREC). Surprisingly, half of these CREC samples carried the *mcr*-9 gene, while the *mcr*-4 and *mcr*-1 genes were each detected in 25% of the *E. coli* isolates positive for colistin resistance. This discovery indicates a widespread presence of potential colistin-resistant strains, marked by the identification of *mcr*-1, -4, and -9 genes for the first time in village chickens in Malaysia. The dissemination of these genes was notably evident in poultry and live-animal trade environments due to the presence of poultry feces [18]. Plasmids, which are capable of transferring *mcr* genes with their high transfer capacity, play a significant role in spreading these genes from the environment to a wide range of hosts globally [1]. Despite the ban on colistin use in food animal production in Malaysia, the use of colistin in food-producing animals has been subject to increasing restrictions over the years. Malaysia banned the use of the antibiotic colistin in animal feed starting 1 January 2019. This prohibition applies to both therapeutic purposes and

growth promotion in animal production. The increased prevalence of multiple variants of *mcr* genes in *E. coli* could be attributed to lingering colistin residues in the environment, specifically in the form of plasmid-mediated *mcr* or antibiotic resistance genes (ARGs), as reported by [2].

The ten *E. coli* isolates (CREC) carrying *mcr* genes displayed comparable antibiotic resistance patterns and MAR index profiles. This outcome indicated that these strains were multidrug-resistant (MDR) due to the presence of plasmid-mediated *mcr* genes, aligning with previous findings in Malaysia [19,20]. The co-existence of *mcr* genes facilitated the transfer or localization of various resistance gene compounds among bacteria or different Enterobacterales species, promoting rapid dissemination of antimicrobial resistance, particularly under antibiotic stress conditions [21,22].

A strong correlation was observed between phenotypic and genotypic colistin resistance; all CREC isolates exhibited colistin minimum inhibitory concentrations with MIC $\geq$ 4 μg/mL, confirming clear-cut colistin resistance [10]. Similar findings were reported in China and Bangladesh, where 100% of CREC strains exhibited colistin MIC $\geq$ 4 μg/mL [23]. In contrast, a study revealed that only 50% of phenotypically positive CREC carried the *mcr* gene [3,24]. These disparities emphasize that relying solely on the presence of *mcr* genes does not confirm phenotypic colistin resistance. This study revealed a higher prevalence of colistin-resistant *E. coli* (CREC) carrying *mcr* genes compared to reports from Brazil (19.5%), and Japan (9.7%) [25,26] while similar occurrence rates were noted in the Netherlands (24.8%) [27] and Nepal (26.6%) [28]. However, a higher rate was found in Vietnam (43.3%) [29].

Livestock, particularly poultry, have been identified as potential sources for the transmission of mobile colistin-resistant *E. coli* to humans. This transmission can occur through direct contact or consumption of contaminated meat products, leading to colonization of the intestinal tract and, eventually, severe infections. Recent reports in the study area (Kelantan) indicated the presence of *mcr*-encoding genes in commercial poultry intended for consumption, highlighting the persistence of *mcr*-producing genes in *E. coli* from poultry sources in Kelantan, Malaysia, despite efforts to control the issue. Although no previous research has reported the presence of CREC in village chickens in Malaysia, *mcr*-producing *E. coli* genes have been detected in cloacal samples collected from commercial poultry, their products, and the surrounding environment in the study area [19,20,24]. This discovery is concerning, given that colistin is not used in poultry production systems, especially in village chickens in the study area [8]. This situation may be partially attributed to the imprudent use of antimicrobials in commercial poultry production, as bacteria can develop resistance in areas where there is high or irrational use of antimicrobial agents through horizontal gene transfer.

The analysis of our *E. coli* isolates carrying *mcr* genes revealed that all of these isolates displayed resistance to a minimum of three classes of antibiotics, indicating the presence of multidrug-resistant (MDR) strains, a phenomenon noted in previous studies [30,31]. Our research demonstrated that most of the *mcr*-positive colistin-resistant *E. coli* (CREC) isolates exhibited resistance to antibiotics such as cefotaxime, ceftriaxone, amoxicillin/clavulanic acid, ofloxacin, tetracycline, erythromycin, gentamicin, chloramphenicol, kanamycin, and ampicillin/sulbactam, with varying rates of resistance. This resistance pattern indicates the widespread use of antibiotics in both village chicken and commercial poultry farming, as well as other agricultural practices in Kelantan. Furthermore, many of these antibiotics are commonly prescribed in human medicine, especially chloramphenicol, tetracycline, and gentamicin, indicating a concerning overlap between antibiotic use in humans and poultry. Our findings align with those of previous studies conducted in Kelantan, which

reported varying levels of resistance among *mcr*-producing CREC isolates within the poultry production system [20].

In this study, the phylotyping analysis revealed that the most frequently detected phylogroup in our samples was phylogroup C, followed by B2 and phylogroup E. Generally, *E. coli* assigned to phylogroups B2 and E are known for their high pathogenic potential and are often found in healthy poultry rather than in birds affected by colibacillosis, as reported in studies conducted in India and Japan [32,33]. The prevalence of these specific phylogroups in village chicken isolates could be attributed to inadequate cleaning practices in the poultry pens, making them susceptible to fecal contamination [34]. Recent genomic data on *E. coli* group identification indicated that phylogroup C is closely related to phylogroups A and B1, which are considered commensals. In contrast, phylogroup E along with phylogroups B2 are classified as pathogenic [14,35]. Most of the *E. coli* isolates from village chickens in our study were found to be commensals with opportunistic pathogenic traits. It has been suggested that phylogroup B1 is linked to environmental *E. coli* and is evenly distributed among avian pathogenic *E. coli* (APEC) and non-APEC isolates, indicating the adaptability of pathogenic traits in poultry [36]. Our findings point toward potential environmental contamination of the cloacae. Phylogroup B2 was more prevalent than phylogroup E in our samples. Both strains are considered sister groups due to their shared bacterial colonization characteristics [37]. The predominance of phylogroup B2 is associated with human ExPEC (extraintestinal pathogenic *E. coli*) as they pose a higher risk of transmitting foodborne diseases to humans. In fact, this strain has been detected in poultry carcasses and meat samples from various countries, including Vietnam, Australia, and Denmark [38]. In this research, various *E. coli* strains were identified, including ST345, ST155, ST770, ST10, ST540, ST31, ST1638, ST906, ST206, and ST162. Notably, *E. coli* ST345, ST155, ST31, and ST206 are the strains detected carrying *mcr* genes. All the four sequence types carrying *mcr* genes come from two continents namely Europe and Asia. According to Phyloviz analysis, the detailed source of the ST is unknown, with the exception of ST345 whose detailed source is sea water. Also, most of the ST has been associated with numerous antimicrobial resistance mechanisms such as ESBL, carbapenemases, and colistin resistance genes. The presence of multiple STs from different geographical locations suggests that drug-resistant bacteria are not confined to specific regions but are spreading globally. This pattern of spread may be facilitated by international trade, travel, and animal migration. In addition, the findings reinforce the One Health perspective, emphasizing how antimicrobial resistance spreads across human, animal, and environmental interfaces. Resistant bacteria may be transmitted through food supply chains, contaminated water, or direct human–animal contact. In the same vein, detection of diverse STs could indicate that resistant strains can evolve independently in different settings, leading to genetic diversification. This could complicate tracking and controlling AMR, as new variants may emerge with enhanced resistance or virulence. The implication of these findings could suggest that there is need for international collaboration in genomic surveillance, which is crucial for tracking the emergence and spread of resistant strains in different countries [18].

5. Conclusions

This study marks the initial investigation into the presence and features of the colistin-resistant gene *mcr* in *E. coli* isolates from village chickens in Kelantan, Malaysia, utilizing stock cultures for the first time. The identified *mcr*-positive *E. coli* isolates exhibited multidrug-resistant characteristics. Despite some limitations, the gathered data offer valuable insights into the *mcr* gene related to colistin resistance, shedding light on the issue of transferable colistin resistance and its public health implications in Malaysia. It is imperative to closely monitor and rigorously regulate antimicrobial usage in Kelantan's

chicken production system. The resistance observed in *E. coli* to colistin may stem from the inappropriate use of colistin sulfate in the local poultry industry.

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