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EMERGENCE OF MCR *-1, -3, -6, -8* AND *-9*) IN *ESCHERICHIA COLI* ISOLATED FROM LIVE CHICKENS, RAW CHICKEN MEAT AND VEGETABLES FROM KELANTAN, MALAYSIA

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ABSTRACT

The uncontrolled usage of antibiotics, especially colistin as growth promoter in poultry and the manures utilized as fertilizers in vegetable farming serve as the fundamental causes of mobilized colistin resistance (*mcr*) gene emergence in *Escherichia coli (E. coli*). This study was conducted to determine the prevalence, antibiotic susceptibility profile and phylogroups of colistin resistant *E. coli* isolated from poultry farm (217 chicken cloacal swab), 200 raw chicken meat and 100 vegetables samples from markets in Kelantan, Malaysia. The samples were processed using routine microbiological method, polymerase chain reaction (PCR) to detect colistin resistant *E. coli* isolates, disk diffusion antimicrobial susceptibility tests with 13 antibiotics, colistin minimum inhibitory concentration (MIC) test and PCR based phylogroups detection. The overall *E. coli* prevalence was 71.0% (367/517) and 3.9% (20/517) isolates harboured multiple *mcr*-genes (*mcr-1, mcr-3, mcr-6, mcr-8* and *mcr-9*) from chicken origin only, while no *mcr* detected was in vegetables. The findings revealed that 89 – 100% *E. coli* isolates from chickens were resistant towards tetracycline, ampicillin, amoxicillin, and chloramphenicol with multiple antibiotic resistance index score of more than 0.2 yet the vegetable isolates were showing higher sensitivity towards these antibiotics. The ten *mcr*-harbouring isolates exhibited phenotypic colistin resistance at MIC ≥ 4 µg/ml. Meanwhile, phylogroup A (45.1%) and phylogroup B1 (20%) were predominating in chicken whereas phylogroup A (59.4%) was highlighted in vegetable origin isolates. These findings underscore the emerging threat of multidrug resistance and increasing trends of *mcr* in *E. coli*, mainly in food animals in Malaysia.

Keywords: *Escherichia coli*, colistin resistance, antimicrobial resistance, poultry, vegetable, *mcr*, Malaysia

INTRODUCTION

The growing global prevalence of antimicrobial resistance (AMR) largely contributes to annual deaths of human and prominent loss in the food agricultural sectors **(Mansaray *et al.*, 2020).** The antibiotics selection pressure due to the indiscriminate use of antibiotics in humans, livestock production and agriculture are the contributors of bacterial evolution and the rapid spread of AMR **(Kakkar *et al.*, 2018; Aghapour *et al.*, 2019).** The last-option drug, colistin, from polymyxin class is used to treat multi-drug resistance (MDR) infections in Enterobacterales, however the drug was exploited in animal feed additive as growth promoter before being banned in 2016 **(Majewski *et al.*, 2020; Shen *et al.*, 2020; Zhang *et al.*, 2021).** Interestingly, the use colistin as animal growth promoter is very much prevalent among Asian countries like China and India. Similar effect was observed in low-middle income countries (LMICs) of Africa and Europe continents **(Mansaray *et al.*, 2020).**

In Malaysia, the antibiotics used for disease prevention and animal growth promotion are controlled by Ministry of Agriculture through Department of Veterinary **(Thapa *et al.*, 2020).** Besides, according to the Malaysian National Pharmaceutical Control Bureau, β-lactams, tetracycline, sulphonamides, aminoglycoside, macrolides, fluoroquinolones and cephalosporins are the general antibiotics allowed and approved to be prescribed in animal husbandry **(Chuah *et al.*, 2018).** As the animal protein consumption rate escalated abruptly, it contributed to the extensive use of antibiotics in livestock industry **(Dadgostar, 2019).** Overall, in livestock, poultry is the major hotspot of AMR worldwide **(Mansaray *et al.*, 2020)** and *E. coli* is considered the most common pathogen causing enteric diseases via fecal-oral route from contaminated environment **(Montealegre *et al.*, 2018)** and serves as an AMR reservoir by easily transmitting the antibiotic resistance genes (ARGs) and antibiotics residual compounds to and from the environment **(WHO, 2018; Montealegre *et al.*, 2018; Karimi Dehkordi *et al.*, 2020).**

Meanwhile, AMR emergence in fresh vegetables is related to livestock sources where the pathogens easily transmit from animal manures used as fertilizers for vegetable farming **(Holvoet *et al.*, 2013; Mohd Kamaruddin *et al.*, 2021).** The misuse of antibiotics in food-animal industry directly spreads AMR via ARGs and MDR bacterial strains through the human-animal-environment interface **(Furlan & Stehling, 2021).** Evidently, several studies have proven the detection of antibiotic residuals in animal manures from Malaysia, China and Canada **(Quaik *et al.*, 2020).** In Malaysian crop farming, uncomposted chicken manures were used as fertilizers **(Barrow *et al.*, 2009; Shobri *et al.*, 2016; Tan *et al.*, 2021).** Therefore, the chances of *E. coli* contamination in vegetables yielded in Malaysia might have upsurged **(Hisham *et al.*, 2021).** Since the first discovery of plasmid – mediated *mcr-1* gene, eight more homologs of *mcr* were identified; *mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8,* and *mcr-9* **(Oh *et al.*, 2020).** Due to the long-term usage of colistin, clear indications of rapid colistin resistance transmission are in conjunction with the increasing detection of *mcr* genes, exclusively *mcr-1* in *E. coli* **(Gogry *et al.*, 2022).**

Phylogroups of *E. coli* provide further information on the ecological niches, ability to cause diseases as well as history traits of the strain **(Zakariazadeh *et al.*, 2019; Goudarztalejerdi *et al.*, 2020).** The *E. coli* strains can be categorised into eight phylogroups (A, B1, B2, C, D, E, F and Escherichia cryptic clade 1) **(Clermont *et al.*, 2013).** So far in Malaysia, the frequent phylogroups detected in *E. coli* were A followed by B1 that co-harboured *mcr-1* in healthy poultry sources **(Aklilu *et al.*, 2022).** Majority of the *E. coli* from infected chickens belonged to phylogroup B1, followed by D and A **(Roseliza *et al.*, 2017)** while vegetable origin *E. coli* belonged to multiple phylogroups of A, B1, B2 and D without any specifications **(Ortega-Paredes *et al.*, 2018; Janalíková *et al.*, 2018; Massella *et al.*, 2021; Zara & Vital, 2022).** The disparity within these phylogroups distribution in Malaysia and worldwide might be influenced by several environmental factors, health status of the host and the antibiotics used **(Aklilu *et al.*, 2022).** Thus, this study discusses the dissemination level of colistin resistance and *mcr* genes in poultry and vegetable samples by determining the prevalence, antimicrobial resistance patterns and phylogroups of *E. coli* in poultry farms (live chicken), raw chicken meat and vegetables in Kelantan, Malaysia.

MATERIAL AND METHODS

Ethical approval statement

This study was conducted at the Zoonotic and Public Health Research Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The handling of

animals, study protocols, procedures, and consents were approved by the Institutional Animal Care and Use Committee of University Malaysia Kelantan (Approval code: UMK/FPV/ACUE/PG/2/2019, Approval Date: February 2019).

Sample collection

The sampling of vegetables, chicken meat and chicken cloacal swabs was conducted from January to June 2021. Hundred vegetable and 200 chicken meat samples were collected from selected six wet markets and six supermarkets based on convenience within Kota Bharu, Kelantan. Twenty samples from tomatoes, cucumbers, cabbages, cauliflowers and long beans were collected respectively. Meanwhile, 40 samples of chicken thighs, breasts, drumsticks, livers and skins were collected respectively. Each sample was collected aseptically, kept in a sterile ziplock bag. Four different broiler farms were identified, and 217 chicken cloacal swabs were randomly collected from each farm (intensive management system) based on the chicken population per farm. The farms were selected randomly based on the list of poultry farms provided by the Department of Veterinary Services, Malaysia which were located within Kota Bharu and Bachok districts of Kelantan. The cloacal swabs were collected aseptically with sterile cotton swabs with Amies transport media (Citotest Labware, China) and any chicken showing signs of illness were excluded from the sampling. The samples were transported immediately in (4-8ºC) cold storage boxes to the Zoonotic and Public Health Research Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (UMK), Malaysia.

Isolation and phenotypic identification of *E. coli*

Each vegetable and chicken meat sample in the sterile ziplock bags was soaked and homogenised with 10 ml of 0.85% normal saline. One millilitre from each homogenized sample solution was pipetted into 9 ml Luria Bertani broth (Oxoid, England). Whereas the cloacal swab samples were enriched directly into 10 ml Buffered Peptone Water (Oxoid, England). All 517 samples were incubated in the shaking incubator at 37ºC for enrichment overnight. The enriched samples then cultured on MacConkey (MAC) (Oxoid, England) and Eosin Methylene Blue (EMB) (Oxoid, England) agars to screen and isolate the suspected *E. coli* based on the colony morphology **(Bhowmik & Ahsan, 2019).** The suspected isolates were then subjected to Gram-staining and further confirmed with five biochemical tests: Sulphur Indole Motility (SIM) test, triple sugar iron (TSI) test, Urease test, Citrate test and Methyl red-Voges Proskauer (MR-VP) **(Eshrati *et al.*, 2020; Soomro *et al.*, 2002; Bhowmik & Ahsan, 2019).** Along with, *E. coli* ATCC 25922 strain was used as quality control.

DNA extraction

The DNA extraction of *E. coli* isolates were proceeded using boiling method adapted and optimized from **Mohamed *et al.* (2022).** A loopful of test isolate colonies from Nutrient agar were dissolved into 1 ml of 0.85% normal saline in a 1.5 ml Eppendorf tube. The mixture was centrifuged at 12000 rpm for 5 mins and the supernatant was discarded. The residual was mixed with 500 µl of nuclease free water (NFW) and vortexed vigorously. The suspension was incubated in a 95ºC water bath for 15 mins. After incubation, the suspension was transferred immediately into a 0ºC ice box, incubated for 10 mins. Then, the suspension was centrifuged at 12000 rpm for 5 mins. Four hundred microlitre of the supernatant (DNA) was transferred into another sterile 1.5 ml Eppendorf tube. The DNA quality of each isolate was checked using spectrophotometer (IMPLEN, USA).

PCR for *E. coli* detection

The species-specific gene primer used to detect the *E. coli* was *phoA.* The PCR reaction mix was prepared with 12.5 µl of 2x GreenTaq Master mix (Promega, UK), 8.5 µl of NFW, 1.0 µl of each 10µM primer (Integrated DNA Technologies, USA) dilution and 2 µl of the DNA lysate. The primer sequences of *phoA* gene for forward: 5'-GTG ACA AAA GCC CCG ACA CCA TAA ATG C- 3' and reverse: 5'- TAC ACT GTC ATT ACG TTG CGG ATT TGG CGT-3' **(Kong *et al.*, 1999; Aklilu and Raman, 2020)**. The PCR was performed using a C1000 Touch Thermal cycler (Bio-Rad, USA), with pre-denaturation at 95 ºC for 4 mins and 30 cycles of denaturation at 95 ºC for 30 s, annealing at 68 ºC for 30s and extension at 72 ºC for 60s, and a final extension at 72 ºC for 10 mins.

Multiplex PCR for *mcr*- gene detection in *E. coli*

The multiplex PCR was performed using the respective gene primers as stated in Table 1 and *E. coli* ATCC 25922 strain was used as quality control. The multiplex PCR reaction mix was prepared with 12.5 µl of 2x GreenTaq Master mix (Promega, UK), 4.5 µl and 5.5 µl of NFW (for *mcr*- 1-5 and *mcr*- 6-9 respectively), 0.5 µl of each 10 µM primer (Integrated DNA Technologies, USA) dilution, 3 µl of the DNA lysate. The PCR protocol for *mcr*- 1-5 starts with an initial denaturation at 94 ºC for 15 mins with 25 cycles of amplification at 94 ºC for 30 s, 58 ºC for 90 s, 72 ºC for 60 s and final extension at 72 ºC for 10 mins **(Rebelo *et al.*, 2018).** While the PCR for *mcr*- 6-9 amplified with an initial denaturation at 95 ºC for 3 mins, 30 cycles of denaturation at 95 ºC for 30s, annealing at 55ºC for 30s, elongation at 72 ºC for 60 s, followed by the final elongation at 72ºC for 10 mins **(Borowiak *et al.*, 2020).**

Analysis of PCR products using gel electrophoresis

All of the PCR products were run through 1.5% agarose gel electrophoresis at 100V for 45 mins with 100bp DNA ladder (Vivantis, Malaysia). The gels were prepared with 1.2g of agarose powder (First Base Laboratories, Malaysia) in 80 ml of 1X TBE Buffer (First Base Laboratories, Malaysia) with 1.0µl Midori Green (Nippon Genetics Europe, Germany). The PCR amplification results were analysed using gel electrophoresis (100V for 45 mins) with 100bp DNA ladder (Vivantis, Malaysia) and 1kb DNA ladder (Lucigen, USA) accordingly. The gel images were photographed and analyzed using Gel Doc™ EZ Imager (Bio-Rad, USA).

Table 1 Primer sequences used for the detection of *mcr* - 1 – 9 genes in *E. coli* using multiplex PCR

Primer	Sequence(5'-3')	Genes	Expected amplicon size (bp)	Reference
mcr-1F	AGTCCGTTTGTTCTTGTGGC	*mcr*-1	320	**(Rebelo *et al.*, 2018)**
mcr-1R	AGATCCTTGGTCTCGGCTTG			
mcr-2F	CAAGTGTGTTGGTCGCAGTT	*mcr*-2	715	
mcr-2R	TCTAGCCCGACAAGCATACC			
mcr-3F	AAATAAAAATTGTTCCGCTTATG	*mcr*-3	929	
mcr-3R	AATGGAGATCCCCGTTTTT			
mcr-4F	TCACTTTCATCACTGCGTTG	*mcr*-4	1116	
mcr-4R	TTGGTCCATGACTACCAATG			
mcr-5F	ATGCGGTTGTCTGCATTTATC	*mcr*-5	1644	
mcr-5R	TCATTGTGGTTGTCCTTTTCTG			
mcr-6F	AGCTATGTCAATCCCGTGAT	*mcr*-6	252	
mcr-6R	ATTGGCTAGGTTGTCAATC			
mcr-7F	GCCCTTCTTTTCGTTGTT	*mcr*-7	551	
mcr-7R	GGTTGGTCTCTTTCTCGT			
mcr-8F	TCAACAATTCTACAAAGCGTG	*mcr*-8	856	
mcr-8R	AATGCTGCGCGAATGAAG			
mcr-9F	TTCCCTTTGTTCTGGTTG	*mcr*-9	1011	**(Borowiak *et al.*, 2020)**
mcr-9R	GCAGGTAATAAGTCGGTC			

Antibiotic Susceptibility Test (AST)

All confirmed *E. coli* isolates were subjected to Kirby–Bauer disk diffusion AST with 13 antibiotics from different classes as summarized in Table 2. The isolates were pre-cultured on Nutrient agar (Oxoid, England) and incubated for 18 – 24 hours at 37ºC before performing AST. Few bacterial colonies of test isolate from Nutrient agar were diluted into 3 ml of 0.85% sterile normal saline to create the inoculum suspension, adjusted to the density of 0.5 McFarland standard. A sterile cotton swab was used to lawn the inoculum four times at different rotation angles onto Muller Hinton agar (Oxoid, England) to ensure even distribution of the inoculum on the entire agar plate **(Hudzicki, 2009)**. The antibiotic disks were placed on the inoculated MHA plate and incubated at 37ºC for 18 hours while *E. coli* ATCC 25922 strain was used as quality control. The zone of inhibition diameter of each antibiotic disk on the agar was measured and interpreted as sensitive, intermediate and resistant toward the antibiotics based on the Clinical and Laboratory Standards Institute (CLSI) guidelines **(CLSI, 2021).**

Table 2 Antibiotics used in AST and their corresponding possible mechanisms of AMR development in *E. coli.*

Mechanism	Classes of antibiotics	Antibiotics used, Abbreviation (Dosage)
Inhibition of bacterial cell wall synthesis	Penicillin	Amoxicillin, AMX10 (10 µg)
		Ampicillin, AMP10 (10 µg)
	Carbapenems	Meropenem, MEM10 (10 µg)
		Imipenem, IPM10 (10 µg)
	Monobactam	Aztreonam, ATM30 (30 µg)
	Cephems (Parental)	Cefotaxime, CTX30, (30 µg)
	Cephalosporins III	Ceftazidime, CAZ30 (30 µg)
	β – lactams combination agents	Amoxicillin/ Clavulanic acid, AMC30 (30 µg)
Disruption of DNA synthesis and during DNA replication	Quinolones	Ciprofloxacin, CIP5 (5 µg)
	Fluoroquinolones	Nalidixic acid, NA30 (30 µg)
	Folate pathway antagonists	Sulphamethoxazole/ trimethoprim, SXT25 (25 µg)
Inhibition of protein synthesis	Tetracyclines	Tetracycline, TE30 (30 µg)
	Phenicol	Chloramphenicol, C30 (30 µg)

Multiple Antibiotic Resistance (MAR) Index

The antibiotic susceptibility of all *E. coli* isolates was further determined using MAR index formula, MAR= a/b, where a represents the number of antibiotics the test isolate is resistant to, and b stands for the total number of antibiotics tested in this study. MAR is an inexpensive and valid tool used for bacterial source tracking. The isolates with MAR index of more than or equal to 0.2 are considered isolates with higher risk of antibiotic contamination where multiple antibiotics are often used in that environment **(Sandhu *et al.*, 2016 ; Akande *et al.*, 2019)**.

Minimum inhibitory concentration (MIC) test of colistin in MCR-EC

The *mcr*-harbouring *E. coli* (MCR-EC) isolates were further tested for colistin minimum inhibitory concentration (MIC) using colistin broth disk elution method as standardized by CLSI guidelines **(CLSI, 2021).** The end point of the colistin MIC was based on the lowest concentration that completely inhibited the visible growth of tested isolate, where MIC of ≤ 2 µg/ml and ≥ 4 µg/ml were considered intermediate and resistant respectively.

Statistical analysis

The prevalence of *E. coli* among vegetables, chicken meat and cloacal swabs samples and prevalence of MCR-EC detected in chicken meat and chicken cloacal swabs were analysed statistically using IBM SPSS® (version 29) software and Microsoft Excel. The Chi-square test and Fisher's exact test were performed based on the sample's distribution. The differences were considered statistically significant at p-value < 0.05 and p < 0.001 accordingly, with 95% confidence level.

Multiplex PCR for to detect phylogroups of *E. coli*

Clermont phylotyping method using quadraplex PCR was used to determine the phylogroups of all *E. coli* isolates **(Clermont *et al.*, 2013)** while *E. coli* ATCC 25922 strain was used as quality control. The genes, primer sequences and their expected amplicon sizes of quadraplex PCR and allele-specific PCR are tabulated in Table 3. Each PCR reaction consists of 12.5 µl of 2x GreenTaq Master mix (Promega, UK), 5.5µl and 7.5µl of NFW (for quadraplex PCR and allele-specific PCR respectively), 0.5 µl of diluted forward and reverse primers (Integrated DNA Technologies, USA) and 3 µl of the DNA lysate. The concentration of diluted primers *chuA, yjaA, TspE4.C2, ArpAgpE* and *trpAgpC* were 20 pmol, *arpA* was 40 pmol and *trpBA* was 12 pmol. The PCR amplification was performed with the following conditions: initial denaturation at 94°C for 4 mins, 30 cycles of denaturation at 94°C for 30s and annealing at 57°C (group E) or 59°C (quadruplex) or 62°C (group C) for 20s and a final extension at 72°C for 5 mins. The PCR products were analysed using gel electrophoresis too.

Table 3 Primer sequences used for the detection of phylogroups of *E. coli* isolates using quadraplex PCR and allele-specific PCR.

Primer	Sequence (5'-3')	Gene	Expected Amplicon size (bp)
*chuA*F	ATGGTACCGGACGAACCAAC	*chuA*	288
*chuA*R	TGCCGCCAGTACCAAAGACA		
*yjaA*F	CAAACGTGAAGTGTCAGGAG	*yjaA*	211
*yjaA*R	AATGCGTTCCTCAACCTGTG		
*TspE4.C2*F	CACTATTCGTAAGGTCATCC	*TspE4.C2*	152
*TspE4.C2*R	AGTTTATCGCTGCGGGTCGC		
*ArpA*F	AACGCTATTCGCCAGCTTGC	*arpA*	400
*ArpA*R	TCTCCCCATACCGTACGCTA		
*trpAgpC*F	AGTTTTATGCCCAGTGCGAG	*trpA* (Group C)	219
*trpAgpC*R	TCTGCGCCGGTCACGCCC		
*ArpAgpE*F	GATTCCATCTTGTCAAAATATGCC	*arpA* (Group E)	301
*ArpAgpE*R	GAAAAGAAAAAGAATTCCCAAGAG		
*trpBA*F	CGGCGATAAAGACATCTTCAC	*trpA* (internal control)	489
*trpBA*R	GCAACGCGGCCTGGCGGAAG		

RESULTS

Isolation and phenotypic identification of *E. coli*

Based on the routine microbiological isolation and identification method, using MAC and EMB agar, the suspected *E. coli* isolates displayed red or dark pink, smooth, circular colonies surrounded by red precipitation on MAC agar and green metallic sheen, smooth, circular colonies on EMB agar and Gram-negative with pink rod-shaped bacterium in Gram staining. All presumptive isolates that were subjected to five biochemical tests were confirmed as *E. coli* isolates with positive production of indole by displaying bright pink layer at the meniscus level and diffused zone of growth flaring into the medium indicating positive motility in the SIM test. The isolates also exhibited acidic slant and butt with positive gas production in TSI agar, positive for Methyl red and negative for Voges Proskauer in MRVP test as well as no change in citrate test **(Eshrati et al., 2020).** Overall, 44.0% (44/100) *E. coli* isolates were detected in vegetable, 68.5% (137/200) in raw chicken meat and 98.6% (214/217) in chicken cloacal swab samples. Thus, the overall *E. coli* isolates detected phenotypically in this study was 76.4% (395/517), as summarized in Table 4.

Table 4 Phenotypic and molecular identification of *E. coli* and detection of *mcr* genes

Types of samples		Vegetable	Chicken meat	Chicken cloacal swab	Chicken origin only	Overall samples
Number of samples collected		100	200	217	417	517
Total number of *E. coli* isolates detected by phenotypic identification (%)		44 (44.0)	137 (68.5)	214 (98.6)	351 (84.2)	395 (76.4)
Total number of *E. coli* isolates detected by genotypic identification (%)		32 (32.0)	128 (64.0)	207 (95.4)	335 (80.3)	367 (71.0)[a)]
Total number of *mcr* genes detected in *E. coli* (%)		-	5 (2.5)	15 (6.9)	20 (4.8)[b)]	20 (3.9)
mcr genes detected in *E. coli* (%)	*mcr*-1	-	4 (2.0)	7 (3.2)	11 (2.6)	11 (2.2)
	mcr-3	-	1 (0.5)	-	1 (0.2)	1 (0.2)
	mcr-6	-	-	4 (1.9)	4 (1.0)	4 (0.8)
	mcr-8	-	-	3 (1.4)	3 (0.7)	3 (0.6)
	mcr-9	-	-	1 (0.5)	1 (0.2)	1 (0.2)

* Chi square test value, *p*-value: a) 144.44, 0.00 (statistically significant *p*-value < 0.001); b) 4.44, 0.04 (statistically significant *p*-value < 0.005)

Molecular identification of *E. coli* and *mcr* genes using PCR

Out of 395 phenotypically identified isolates, 367 isolates were confirmed as *E. coli* with the presence of *phoA* gene using PCR showing an overall prevalence of 71.0% (367/517) of the total samples (Fig 1). Whereas the prevalence of *E. coli* isolates was 32.0% (32/100), 64.0% (128/200) and 95.4% (207/217) in vegetables, raw chicken meat and chicken cloacal swab samples respectively as summarized in Table 4.

Based on multiplex PCR detection method, the overall *E. coli* isolates carrying *mcr*-genes were 20 out of 517, with a prevalence of 3.9%. Yet, the *mcr*-carrying *E. coli* (MCR-EC) isolates were only detected among chicken origin samples, in raw chicken meat and chicken cloacal swab, recording the separate prevalence of 2.5% (5/200) and 6.9% (15/217) respectively. None of the *E. coli* isolates from vegetables were MCR-EC. Interestingly, the *mcr*-gene variants detected in this study were *mcr*-1, *mcr*-3, *mcr*-6, *mcr*-8 and *mcr*-9 as summarised in Table 4 and the PCR results were shown in Fig 2 (a), (b) and (c).

Antibiotic Susceptibility Test (AST)

The AST results revealed that most of the vegetable isolates were sensitive towards meropenem (96.9%), followed by aztreonam (93.8%), and ceftazidime (90.6%). The highest resistance with 28.1% isolates was shown towards ampicillin and amoxicillin, followed by 25.0% towards tetracycline as simplified in Fig 3. Similar to the vegetable samples, 89.8% and 89.1% *E. coli* from chicken meat were sensitive towards meropenem and aztreonam respectively. However, 96.1% were showing highest resistance towards tetracycline, followed by 94.5% towards both ampicillin and amoxicillin and 89.8% to tetracycline. Meanwhile, majority 98.6%, 90.3% and 88.9% *E. coli* from chicken cloacal swab were sensitive towards meropenem, aztreonam and imipenem. Yet, all (100%) isolates of chicken cloacal swabs were showing resistance towards tetracycline, ampicillin and amoxicillin and 99.5% towards chloramphenicol.

Overall, the *E. coli* isolates from chicken sources showed higher AMR towards tetracycline, ampicillin, amoxicillin and chloramphenicol. However, AMR towards tetracycline, ampicillin and amoxicillin in vegetable origin *E. coli* were low as the isolates were still sensitive. Besides, 100% MCR-EC isolates were resistant towards tetracycline, ampicillin and amoxicillin. On the other hand, 100% MCR-EC isolates were sensitive towards meropenem, followed by 90% towards aztreonam as well. The distribution of AMR of MCR-EC was summarized in Table 5.

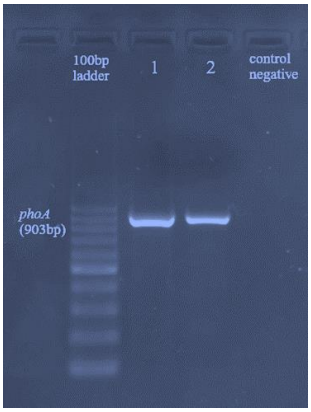

Figure 1 Image showing positive bands of *phoA* gene detected in *E. coli* at 903bp in lanes 1 and 2, on 1.5% agarose gel after electrophoresis at 100V for 45 mins.

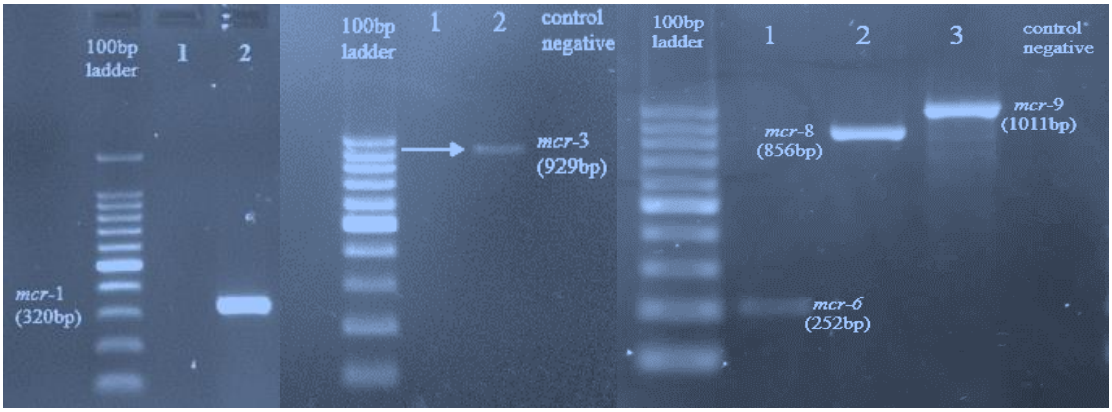

Figure 2 PCR amplification products showing detection of *mcr* genes (a) *mcr-1* gene at 320bp, (b) *mcr-3* gene at 929bp and (c) *mcr-6*, *8*, and *9* at 252bp, 856bp and 1011bp respectively detected in *E. coli* isolates on 1.5% agarose gel after electrophoresis at 100V for 45 mins.

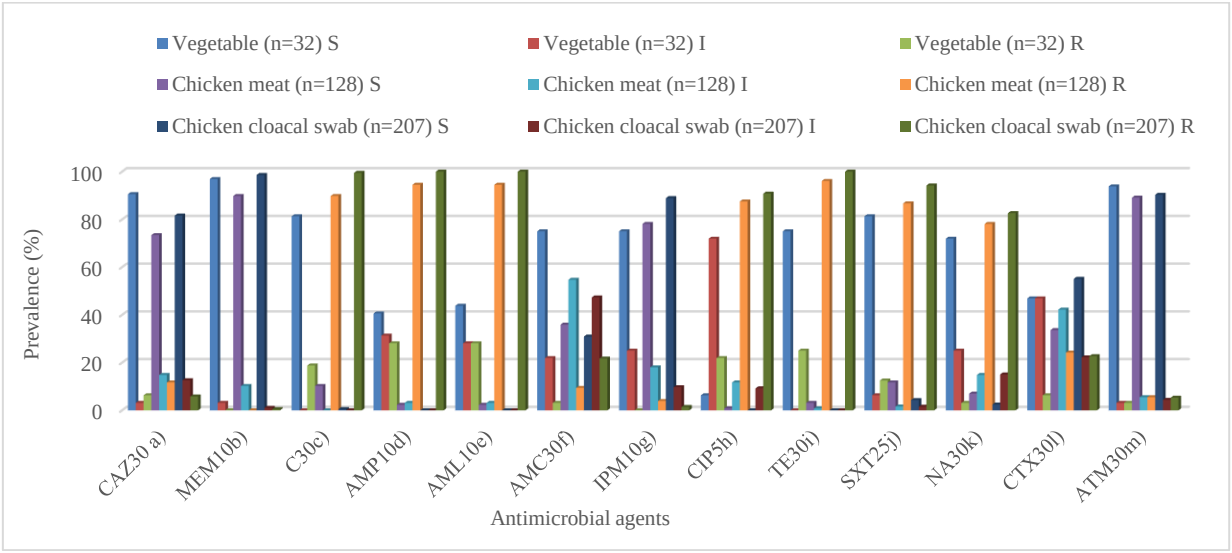

Figure 3 Distributions of antibiotic susceptibility profile of *E. coli* from vegetables, chicken meat, chicken cloacal swab samples

Table 5 Distributions of antibiotic susceptibility profile, MAR index and MIC test of MCR-EC isolates (n = 20).

Isolate Id	Source	*mcr* genes detected	AMR Profile	Colistin MIC	MAR Index	Phylogroup
SK3	Chicken meat	*mcr-1*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	<4	0.5	A
TG27B	Chicken meat	*mcr-3*	C30, AMP10, TE30, AML10, SXT25	>1	0.4	D
BT7B	Chicken meat	*mcr-1*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	4	0.5	B1
TG16B	Chicken meat	*mcr-1*	CAZ30, C30, CIP5, AMP10, TE30, AML10, CTX30, SXT25, ATM30, NA30	<4	0.8	A
DS13B	Chicken meat	*mcr-1*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	4	0.5	A
KB4A	Chicken cloacal	*mcr-9*	CIP5, AMP10, TE30, AML10, CTX30, SXT25, ATM30, NA30	> 2	0.6	B1
B8H4	Chicken cloacal	*mcr-6*	C30, CIP5, AMP10, AMC30, TE30, AML10, CTX30, SXT25, NA30	> 1	0.7	B1
B7H4	Chicken cloacal	*mcr-1*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	4	0.5	D
B9H2	Chicken cloacal	*mcr-1*	C30, CIP5, AMP10, TE30, AML10, SXT25	<4	0.5	E
12b	Chicken cloacal	*mcr-1*	C30, CIP5, AMP10, AMC30, TE30, AML10, CTX30, SXT25, NA30	>1	0.7	Clade I/11
3bA	Chicken cloacal	*mcr-1*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	> 1	0.5	A
ZC4B	Chicken cloacal	*mcr-8*	C30, AMP10, TE30, AML10	> 1	0.3	C
ZC5B	Chicken cloacal	*mcr-8*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	> 1	0.5	C
C39H4	Chicken cloacal	*mcr-8*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	> 2	0.5	A
YC1	Chicken cloacal	*mcr-6*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	4	0.5	F
YC23	Chicken cloacal	*mcr-1*	C30, CIP5, AMP10, TE30, AML10, SXT25	>1	0.5	A
ZC22	Chicken cloacal	*mcr-1*	C30, AMP10, TE30, AML10	4	0.3	B1
B2H1	Chicken cloacal	*mcr-6*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	4	0.5	B2
ZC13B	Chicken cloacal	*mcr-1*	C30, CIP5, AMP10, TE30, AML10, SXT25, NA30	>1	0.5	C
7a-B	Chicken cloacal	*mcr-6*	C30, CIP5, IPM10, AMP10, TE30, AML10, SXT25, NA30	4	0.6	F

Multiple Antibiotic Resistance (MAR) Index

The MAR index showed obvious outcome of the chicken origin isolates being the possible high-risk source of antibiotic-contamination. Based on Table 6, 63.3% (81/128) and 67.1% (139/207) *E. coli* from chicken meat and chicken cloacal swab respectively were showing MAR index of 0.5. The results indicated an overall insight of the chicken origin samples representing a highly antibiotic-contaminated source. Similarly, 60% (12/20) MCR-EC isolates, which were the chicken origins, scored MAR 0.5 as well. In contrast, a majority 59.4% (19/32) *E. coli* isolates from vegetables did not score the MAR index, indicated that the samples originated from lower antibiotic-exposed environment.

Table 6 MAR indices of *E. coli* from vegetables, chicken meat, chicken cloacal swab samples and MCR-EC

MAR index	Number of isolates (%)			
	Vegetable (n=32)	Chicken meat (n=128)	Chicken cloacal swab (n=207)	MCR-EC (n=20)
0	**19 (59.4)**	1 (0.8)	-	-
0.1	4 (12.5)	3 (2.3)	-	-
0.2	2 (6.3)	3 (2.3)	-	-
0.3	1 (3.1)	-	3 (1.4)	2 (10)
0.4	3 (9.4)	10 (7.8)	9 (4.3)	1 (5)
0.5	2 (6.3)	**81 (63.3)**	**139 (67.1)**	**12 (60)**
0.6	-	16 (12.5)	24 (11.6)	2 (10)
0.7	-	4 (3.1)	19 (9.2)	2 (10)
0.8	1 (3.1)	10 (7.8)	13 (6.3)	1 (5)
0.9	-	-	-	-
1.0	-	-	-	-

Colistin MIC test for MCR-EC

Based on the colistin broth disk elution test Table 5, 50% (10/20) MCR-EC isolates showed phenotypic resistance towards colistin with MIC ≥ 4 µg/ml. Categorically, 80% (4/5) and 40% (6/15) MCR-EC from chicken meat and chicken cloacal swab respectively were colistin resistant phenotypically. The rest of the MCR-EC were showing intermediate results with colistin MIC < 2 µg/ml.

Multiplex PCR for detection of phylogroups of *E. coli* and MCR-EC

Based on the PCR for phylogroups detection, most of the *E. coli* isolates were from phylogroup A, followed by B1 (Fig 4). Out of 32 vegetable *E. coli* isolates, 59.4% were from phylogroup A and the rest of them were from groups D, B1, B2, C and E as summarized in Table 7. Whereas, from 335 chicken origin *E. coli* isolates, 45.1% and 20.0% belonged to phylogroup A and B1 respectively, followed by phylogroups D, F, clade I/II, E, C and B2 in descending order. Meanwhile, 2.1% isolates were of unknown phylogroup.

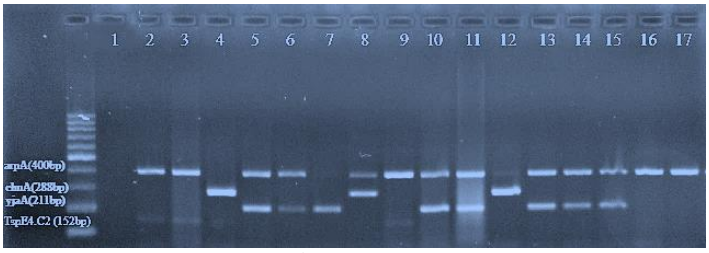

Figure 4 Image showing bands of phylogroup genes detected in *E. coli* isolates using Clermont quadraplex PCR. Lane 5 (+ - + -) and 16 (+ - - -) represent phylogroup A; lane 2 and 3 (+ - - +) represents B1; lane 13 (+ - + -) represents C; lane 8 (+ + - -) represents D; lane 4 (+ + + -) represents F on 1.5% agarose gel after electrophoresis at 100V for 45 mins.

Table 7 The percentage of detected phylogroups of *E. coli* from vegetable and chicken – origin isolates.

Phylogroups	Number of isolates detected per phylogroup (%)			
	Sample types			
	Vegetables (n = 32)	Chicken Meat (n = 128)	Cloacal Swabs (n = 207)	Chicken origin (n = 335)
A	19 (59.4)	63 (49.2)	88 (42.5)	151 (45.1)
B1	3 (9.4)	35 (27.3)	32 (15.5)	67 (20.0)
B2	3 (9.4)	2 (1.7)	8 (3.9)	10 (3.0)
C	2 (6.3)	2 (1.7)	9 (4.3)	11 (3.3)
D	4 (12.5)	8 (6.25)	19 (9.2)	27 (8.1)
E	2 (6.3)	3 (2.3)	14 (6.8)	17 (5.1)
F	-	11 (8.6)	12 (5.8)	23 (6.9)
Clade I/II	-	4 (3.1)	18 (8.7)	22 (6.6)
Unknown	-	-	7 (3.4)	7 (2.1)

The phylogroups detected in MCR-EC

The overall distribution of phylogroups detected in 20 MCR-EC isolates revealed that 30% belonged to phylogroup A, followed by B1 (20%), C (15%), D and F (10%) and B2, E and clade I/II (5%) as tabulated in Table 5.

DISCUSSION

In this study, the *E. coli* prevalence of chicken sample sources were higher when compared to the vegetable samples, with the raw chicken meat and chicken cloacal swabs recording 64.0% and 95.4% respectively. Parallel trends were reported in Bangladesh (65.7%), Brazil (58.7%) and Korea (50.5%) (**Rahman *et al.*, 2020; Crecencio *et al.*, 2020; Seo and Lee, 2018)** and previously in Kelantan with 46% *E. coli* prevalence for raw chicken meat **(Aklilu & Raman, 2020).** These studies clearly indicated the rise in *E. coli* contamination in chicken meat produce sold in local markets and retail shops all around the globe. The possible contamination might have taken place during the unhygienic practices of handling the chickens at farm, during slaughtering and processing the meat from broiler farms to retail shops **(Aklilu & Raman, 2020; Rahman *et al.*, 2020).** This finding also suggests that *E. coli* transmission from chicken to humans presumably happens through food-chain **(Leverstein-van Hall et al., 2011).** Besides, almost all the cloacal swab samples (95.4%) hosted *E. coli*. However, latest studies conducted in Kelantan documented discrepant prevalence of *E. coli* of 28.4% **(Devan *et al.*, 2022)** and 40.8% **(Aklilu *et al.*, 2022)**. In previous years, the prevalence was ranging from 88-89% **(Mahmud *et al.*, 2018; Das *et al.*, 2020**), 72-87% **(Liu *et al.*, 2021)** and 72.0% **(Elmi *et al.*, 2021)** in Bangladesh, China and Malaysia respectively. The increased emergence of *E. coli* in chickens may be the result of poorly maintained hygiene conditions of chicken farms where large flocks of chickens withheld in excreta and manure contaminated spaces, most of the time. This condition exposes the cloaca of chickens to the contaminated floor-bed within shorter proximity which increases possible transmission **(Chelaghma *et al.*, 2022; Suleman *et al.*, 2022).**

Meanwhile, the *mcr*-genes detected in chicken – origin samples were considered highly prevalent (3.9%) and revealed the vast dissemination of potential colistin resistant reservoirs in the form of *mcr*-3, -6, -8 and -9 detected for the first time in Malaysia. So far, *mcr-1* is commonest *mcr*-gene detected in *E. coli* of animal-origin in Malaysia while *mcr-3* from a single study from pig isolate (**Yin *et al.*, 2017)**, which in fact were detected in raw chicken meat and chicken cloacal swabs from Kelantan as well **(Aklilu and Raman, 2020; Aklilu *et al.*, 2022; Devan *et al.*, 2022).** These trends of *mcr-1* presence in poultry showed that this variant still exists in Malaysia. Whereas the detection of the other *mcr* genes in poultry source from *E. coli* were parallel with a study in Thailand from pig source **(Khanawapee *et al.*, 2021).** The *mcr-3* was the next commonly distributed variant in multiple sources including human **(Khanawapee *et al.*, 2021; Hameed *et al.*, 2022),** while *mcr-6* and *mcr-8* were frequently detected in *Moraxella spp*. and *Klebsiella spp*. respectively in swine **(Hussein *et al.*, 2021; Xu *et al.*, 2022)** and *mcr-9* in human and horse isolates **(Khanawapee *et al.*, 2021; Coppola *et al.*, 2022).** These potential plasmid-mediated genes transmission was very manifesting in poultry or live-animal trade due to the feces surrounded farm environment **(Wang *et al.*, 2021).** Notably, the plasmids are capable of transferring *mcr* genes with their high

transfer capacity feature which accounts for the wide dissemination from environment to larger variety of hosts across the globe **(Al-Mir *et al.*, 2021).** Despite the ban of colistin in food animal production in Malaysia, the increased distribution of multiple variants of *mcr*-genes in *E. coli* could be the results of remained colistin residues in the environment in the form of plasmid-mediated *mcr* or ARGs **(Valiakos *et al.*, 2021).** Although the control of antibiotic usage is legislated in Malaysia, many studies are still acknowledging the probable acts of discriminated use of the antibiotics going on in the chicken farms **(Geidam *et al.*, 2012).**

Based on the AST distribution of the *E. coli*, the overall prevalence of AMR towards tetracycline (TE30), ampicillin (AMP10), amoxicillin (AML10) and chloramphenicol (C30) were high and consistent among the chicken origin samples. The observed pattern of AMR was in agreement with earlier studies from Northern (Kedah) and East Coast (Kelantan, Terengganu and Pahang) Peninsular Malaysia states where 80% - 90% *E. coli* from broiler farm chickens were resistant towards those antibiotics **(Ibrahim *et al.*, 2021; Mohamed *et al.*, 2022; Elmi *et al.*, 2021).** However, among the studies, less than 27% *E. coli* were showing resistance towards amoxicillin and chloramphenicol simultaneously **(Elmi *et al.*, 2021),** which might be the indirect consequences of violating the permitted antibiotics residues standards. Indeed, based on the National Pharmaceutical Regulatory Agency under Department of Veterinary Science of Malaysia, chloramphenicol was classified under prohibited drugs in food animals due to their severe toxic effects to animals and humans (Hassali et al., 2018; Chuah et al., 2018) meanwhile amoxicillin was permitted in poultry farms, yet the maximum residual limit of the antibiotic was supposed to be monitored. Yet, this finding clearly shows that events of drug regime violation by certain farms might be a major contributor of this AMR variation within different farms from the same region **(REGOVP, 2020)**. To add with, the MAR index showed that almost all chicken - origin isolates originated from frequently exposed environment to multiple drugs **(Sandhu *et al.*, 2016).** Correspondingly, **Ibrahim *et al.* (2021)** reported that 96% of the *E. coli* isolates also scored MAR more than 0.2 in Malaysia. Essentially, an environment with increased exposure to multidrug creates a natural selection pressure in bacteria which enhances the spreading of MDR to different ecological areas and multiple hosts **(Kagane *et al.*, 2021).**

The collection of 20 *mcr*-gene carrying *E. coli* isolates (MCR-EC) were exhibiting similar antibiogram and MAR index profile of chicken origin samples. This finding revealed that those were MDR carrying plasmid-mediated *mcr* genes and apparently similar to the MCR-EC detected in previous study from Malaysia **(Devan *et al.*, 2022).** The co-presence of *mcr* enabled the plasmid to transfer or localize other resistant gene compounds from one bacterium to another, or among different species of Enterobacterales **(Olaitan *et al.*, 2016).** This mechanism promotes even faster dissemination of AMR with frequent exposure towards antibiotic stress **(Sekyere & Asante, 2018).** Despite carrying the *mcr* gene, only 50% MCR-EC isolates exhibited colistin MIC $\geq$ 4 µg/ml indicating a clear cut colistin resistance **(CLSI, 2021).** Similar finding was reported by **Aklilu *et al.* (2022),** but in China and Bangladesh, 100% MCR-EC exhibited colistin MIC $\geq$ 4 µg/ml **(Amin *et al.*, 2020).** These discrepancies showed that relying on the presence of *mcr* genes alone do not confirm phenotypical colistin resistance.

On the contrary, the prevalence of *E. coli* was 32.0% in vegetable samples which corresponds to the studies in Cambodia, Thailand **(Chanseyha *et al.*, 2018),** Iran **(Mashak, 2018)** and India **(Saksena *et al.*, 2020)**, with the respective prevalence of 39.17 %, 31.25 % and 17.5 %. Meanwhile, no MCR-EC was detected in vegetables from this study as parallel to previous study on bean sprouts in Kelantan **(Aklilu and Raman, 2020).** Majority *E. coli* isolates from vegetables were susceptible towards chloramphenicol (81.3%) and tetracycline (75.0%), yet according to **Bahri *et al.* (2019),** the *E. coli* from ulam (fresh herbal vegetable) in Terengganu, reported 52.2% AMR prevalence in ampicillin and tetracycline while 17.4% in chloramphenicol. This trend clearly defines a noticeable decline in the AMR of tetracycline and chloramphenicol among vegetables with the MAR index also represented a low-risk source of MDR contamination. Similar to **Bahri *et al.*, (2019),** the AMR in vegetables from Malaysian markets were less affected than the poultry. The presence of AMR in vegetables revealed that the antibiotics residual compounds may have disseminated in fresh vegetables through poultry manure fertilizers but within a smaller amount. After all, the application of raw animal manures, mainly from poultry farms were disclosed as the main source of *E. coli* outbreaks among humans due to the consumption of raw or unwashed vegetables **(Berger *et al.*, 2010; Sapkota *et al.*, 2019)**. However, there are few more other possibilities of the AMR getting spread in vegetables such as from contaminated crop irrigation water and cross-contamination from poultry farms located within the same region.

Furthermore, the most detected phylogroup was phylogroup A and followed by B1 in both chicken and vegetable sources. Usually, *E. coli* assigned from these phylogroups were found to have low pathogenic potential characteristics and isolated mostly from healthy poultry than the colibacillosis infected as reported in India and Japan **(Hussain *et al.*, 2017; Murase & Ozaki, 2022).** These predominating phylogroups in the chicken isolates might be the result of unhygienic handling of chickens which were prone to fecal contamination during the transportation from farms to consumers **(Rouger *et al.*, 2017).** The updated genomic data of *E. coli* group identification claimed that phylogroup C is highly related to phylogroups A and B1 which are considered commensals, while phylogroups E and F were referred as pathogenic as phylogroups B2 and D **(Clermont *et al.*, 2013; Bhowmik *et al.*, 2022; Bhave *et al.*, 2019).** Hence, the 68.4% chicken – origin *E. coli* isolates in our study were proved to be commensals yet opportunistically pathogenic. Several more studies suggested that phylogroup B1 also associated with environmental origin *E. coli*, and fairly distributed in APEC and non-APEC isolates, which defines the adaptability of pathogenic traits of *E. coli* in poultry **(Mittal *et al.*, 2022).** Thus, our findings indicate possible environmental contamination of the cloacal and probable contamination during sampling. Meanwhile, in chicken meat samples, phylogroup F was much prevalent than phylogroup D **(Ferraresso *et al.*, 2022)** and phylogroup F and B2 strains were considered sister groups as suggested by **Raimondi *et al.* (2019)** due to their shared bacterial colonization. The predomination of phylogroup F was considered related to human ExPEC because they possess higher risk of transmitting foodborne diseases to humans. In fact, this strain has been detected in poultry carcasses and meats from Vietnam, Australia and Denmark **(Sary *et al.*, 2019).**

Phylogroup A alone was the most prevalent (59.4%) in vegetable *E. coli* isolates and it was parallel with ESBL- gene carrying *E. coli* from vegetables in Mexico **(Corzo-Ariyama *et al.*, 2019)** and Ecuador **(Ortega-Paredes *et al.*, 2018)** respectively. However, phylogroup B1 was the most detected in *E. coli* from vegetables as reported in Italy **(Massella *et al.*, 2021)** and Philippines **(Zara & Vital, 2022).** Meanwhile, *E. coli* from fresh vegetable from Czech Republic markets (2015 to 2016) showed a distinctively highest detection of the pathogenic phylogroup B2 (73.3%) followed by both B1 and D (13.3%), while no trace of phylogroup A **(Janalíková *et al.*, 2018).** These non-uniform phylogroup patterns in vegetable could be happening due to the contamination of untreated manure carrying multiple phylogroup strains of *E. coli* **(Bhowmik *et al.*, 2022),** and fecal material contamination from water irrigations used for crop farming **(Zara & Vital, 2022).** Other predisposing factors such as geographical locations and sources of the vegetable may alter the phylogroup distribution of *E. coli* among vegetables. Thus, further investigation on the source of *E. coli* contamination in vegetables need to be implemented to find out the association of vegetable production using poultry manure as fertilizer.

CONCLUSION

Based on our findings, the implication of colistin ban did not hinder the emergence of plasmid-mediated *mcr* genes in *E. coli*, due to increased emergence of the multi-variants, especially *mcr-1*. Obviously, this study revealed that *mcr-3*, *mcr-6*, *mcr-8* and *mcr-9* genes were detected in Malaysia for the first time in poultry source. Meanwhile, the AMR of tetracycline, amoxycillin, ampicillin and chloramphenicol in chickens were apparently getting increased when compared to the colistin resistance in *E. coli* as those antibiotics are still being used as therapeutic agents of several infectious diseases in poultry and other food animal industry. Moreover, based on the MAR index, majority of the *E. coli* from poultry sources were potential reservoirs of MDR *E. coli* strains and if possible measures are not implemented, these strains might colonize wild birds, other animals and vulnerable hosts including humans through several possible routes of transmission. This is because of the previous long-term extensive usage of colistin and other antibiotics that might have contributed significantly to natural selection pressure of antibiotics that promotes the dissemination of AMR from poultry to human-animal-environment chain. Collectively, the *E. coli* isolates from poultry were considered commensal strains that can be opportunistic pathogens as they predominantly belonged to phylogroups A, B1, and D. Meanwhile, the molecular characteristics of vegetables-origin *E. coli* isolates suggested that the AMR transmission may have occurred through poultry manure fertilizers or through other possible reservoir such as contaminated crop irrigation water and cross-contamination from nearby poultry farms. However, more studies need to be conducted to further investigate the usage of poultry manures as fertilizers for vegetable crop farming in Malaysia. Therefore, healthcare providers, physicians and microbiologists should play vital roles in foregrounding the surveillance of the *mcr*-gene variants and other MDR strains prevalence and transmission as a first step to reduce the emergence of *mcr*-carrying colistin resistance and AMR strains from food animal production as well as in clinical medicine. These investigations may contribute as templates to monitor the global clonal epidemiology as well as the major sources and routes of transmission of the E. coli detected in Malaysia. The imperative studies will help to create awareness to the poultry breeders regarding the threatening consequences of the improper usage of antibiotics in the poultry husbandry. At the same time, the database will be an effective source of antibiotics usage guidelines in Malaysia and other Asian countries in future. Moreover, more studies on monitoring the profile and traces of potential colistin-resistant E. coli reservoirs are recommended.

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