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Molecular Detection and Antibiogram of ESBL-Producing and Carbapenem-Resistant *Escherichia coli* from Rabbit, Swine, and Poultry in Malaysia

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ABSTRACT

The emergence of multidrug-resistance Enterobacteriaceae such as extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* (*E. coli*) and carbapenem-resistant *E. coli* (CREC) has become an urgent veterinary and public health threat. These multidrug-resistant microorganisms are frequently associated with diseases that have high mortality with limited treatment options. This study aims to investigate the prevalence of ESBL producing *E. coli* and CREC from the rabbit, swine, and poultry and to determine the antibiogram profile of these *E. coli* isolates. In this study, 400 fecal swab samples were collected from rabbits, swine, and poultry from several selected animal farms in Malaysia. After incubation and isolation processes, suspected *E. coli* isolates were subjected to a PCR test to confirm the identity of the bacteria. The antibiogram of the *E. coli* isolates was determined via the Kirby Bauer disk diffusion method. A total of 212 (53%) *E. coli* isolates were isolated from rabbits (51 isolates), poultry (110 isolates), and swine (51 isolates). Screening of antimicrobial resistance genes revealed twelve ESBL producing *E. coli* (3%; 12/400). Two ESBL producing *E. coli* were also carrying carbapenemase gene (Bla_{NDM}), indicating ESBL producing and carbapenem-resistant *E. coli* (ESBL-CREC) in poultry fecal swab samples. The bacteria isolates were found to show resistance against nine antibiotics, including ertapenem, ampicillin, and amoxicillin-clavulanate. A total of 3.3% (7/212) of the *E. coli* isolates were found to be multidrug-resistance. This study demonstrated the presence of ESBL-producing *E. coli* and ESBL-producing CREC from poultry fecal swabs in Malaysia.

Keywords: carbapenem-resistant Escherichia coli (CREC); extended-spectrum β-lactamase (ESBL); antibiotic resistance; antibiogram; livestock

INTRODUCTION

The widespread of microorganisms with antimicrobial resistance traits are recognized as serious threats to human and animal health globally (Tian *et al.*, 2018; Chai *et al.*, 2020). These concerns are exemplified by the emergence of Gram-negative bacteria that show multidrug-resistance traits. Similar to the other commensal bacteria, *E. coli* can be commonly found in humans, animals, and the environment, making them an excellent indicator of antimicrobial resistance. Globally, antibiotic resistances were frequently detected among commensal bacteria from food-producing animals (Ramos *et al.*, 2020). The use of critically important antibiotics in food-producing animals has led to the emergence of multidrug-resistant foodborne bacteria, including extended-spectrum β-lactamase (ESBL)-producing *E. coli* (Ramos *et al.*, 2020). ESBL-producing *E. coli* can release ESBL enzymes to hydrolyze all β-lactams, including the third generation of cephalosporins, except cephamycin and carbapenems (Kpoda *et al.*, 2018). In fact, many ESBL-producing *E. coli* are also resistant to the other antimicrobial groups, such as aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole (Abayneh *et al.*, 2018).

Carbapenems having a broad-spectrum antibacterial activity are the most effective β-lactams antibiotics against Gram-positive and Gram-negative bacteria. For years, carbapenems have been considered the most effective antibiotic to treat serious infections caused by multidrug-resistant Gram-negative bacteria (Liu *et al.*, 2018). The mode of action of carbapenem is initiated by penetration of the bacterial cell wall and binding to enzymes known as penicillin-binding protein (PBPs). The resultant lethal effect is the inactivation of an inhibitor of autolytic enzymes within the cell wall, which will lead

to the killing of bacteria (Codjoe & Donkor, 2017). The presence of a carbapenem structure with the β-lactam ring allows the carbapenems to be less vulnerable to most β-lactam resistance determinants. Besides, carbapenems present fewer adverse effects to the patients, making them a safer therapeutic choice than the other last-line antibiotics. However, the rapid increase in the prevalence rate of carbapenemase gene carriage by Enterobacteriaceae has render carbapenems useless against such pathogens (Codjoe & Donkor, 2017).

Briefly, resistance to carbapenems can be brought about by various mechanisms, with the most common being the production of carbapenemases, a class of enzymes capable of degrading carbapenems (Sawa *et al.,* 2020). Besides, resistance to carbapenems can also be due to the poor binding of carbapenems to penicillin-binding proteins present in the bacteria, the over-expression of multidrug efflux pumps by the bacteria or lack of porins present in the bacterial cell membrane (Suay-García & Pérez-Gracia, 2019). In fact, carbapenem-resistant Enterobacteriaceae (CRE) bacteria such as carbapenem-resistance *E. coli* (CREC) do not respond to common antibiotics and thus limit the treatment options (Köck *et al.,* 2018). Hence, the emergence of antibiotic resistance in Enterobacteriaceae has significant clinical and socioeconomic consequences (Rodríguez-Baño *et al.,* 2018).

Previous studies reported the presence of ESBL-producing *E. coli* and CREC in food-producing animals (Köck *et al.,* 2018; Hosuru Subramanya *et al.,* 2020). However, little is known regarding the spread of ESBL-producing *E. coli* and CRE among livestock animals in Malaysia. Therefore, this study aims to determine the prevalence rates of ESBL-producing *E. coli* and CREC as well the antibiogram profile of *E. coli* isolated from commercially farmed rabbits, swine, and poultry in Malaysia. Subsequently, the findings from this study shall provide useful insights into the development of strategies to treat and control the spread of multidrug-resistance *E. coli* in livestock.

MATERIALS AND METHODS

Ethical Approval

The study and sampling method were approved by the Universiti Sultan Zainal Abidin Animal and Plant Research Ethnic Committee (UAPREC) under the code: UAPREC/04/018.

Sample Collection and *E. coli* Isolation

A total of 400 fecal swab samples of 100 rabbits, 100 swine, and 200 poultry were collected from various commercial farms and abattoirs in Malaysia. All of the samples were independently collected by swab technique through rectal using sterilized cotton swabs. The fecal samples collected were then placed in the transport media, Brilliant Green Bile broth (HiMedia, India), and incubated at 37 °C for 24 hours. After incubation, the swab samples were streaked onto eosin-methylene blue (EMB) agar plates (Merck KGaA, Germany) and incubated at 37 °C for 24 hours. Bacterial colonies that showed metallic green sheen appearance on EMB were collected and streaked on Muller Hinton Agar (MHA) plates (Oxoid LTD, UK). The suspected bacteria colonies were then subjected to phenotypic identification. The selected bacteria colonies from MHA were tested with Gram staining and several biochemical tests, including Catalase and Triple Sugar Iron (TSI) agar tests. Isolates that were Gram-negative, rod-shaped, and showed biochemical characteristics identical to *E. coli* were further analyzed using genotypic approaches.

Genotypic Identification of *E. coli* and CREC

The genomic DNA of the bacterial isolates was extracted using the simple boiling method described by Rasool *et al.* (2018). The bacterial isolates were identified using primers that detected 16S rRNA of *E. coli* (Mogheiseh *et al.,* 2020). Isolates that showed the presence of DNA bands on 2.0% (w / v) agarose gel (Promega, USA) at the size of 232 bp were considered to be *E. coli*, as shown in Figure 1. *E. coli* isolates were then further subjected to PCR test to screen for the presence of antimicrobial resistance genes using the primers listed in Table 1. *E. coli* isolates that harbored $bla_{CTX\text{-}M1}$ gene were categorized as ESBL-producing *E. coli*. *E. coli* isolates that tested positive for bla_{NDM}, bla_{OXA}, bla_{KPC}, and bla_{VIM} were considered as CREC.

Antibiogram

An antibiotic susceptibility test was performed on the *E. coli* isolates using the Kirby-Bauer disk diffusion method. The susceptibility of the *E. coli* isolates against antimicrobial agents was tested using 11 different antibiotic discs, including doripenem (10 μg), ertapenem (10 μg), imipenem (10 μg), meropenem (10 μg), amoxicillin-clavulanate (10 μg), ampicillin (10 μg), tetracycline (30 μg), doxycycline (30 μg), gentamicin (10 μg), and ciprofloxacin (5 μg). Two duplicates were made for each isolate, and the MHA plates inoculated with *E. coli* were incubated at 37 °C for 24 hours. The diameter of inhibition zones was measured, and the results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) standard guidelines (CLSI, 2018). Isolates that showed resistance against 3 or more categories of antibiotics were classified as multidrug-resistant *E. coli* (Magiorakos *et al.,* 2012).

Data Analysis

The prevalence rate of *E. coli*, ESBL producing *E. coli*, and CREC were calculated and presented in percentage (%). Categorical data were analyzed using Chi-square test or Fisher's exact tests via Minitab® 16.1.1 (2010) with 95% confidence interval ($p<0.05$) was set to indicate the significant difference. The antibiotic resistance rates (%) of the *E. coli* isolates were calculated as the proportion of isolates tested that had an inhibition zone below the respective antibiotic breakpoint. The

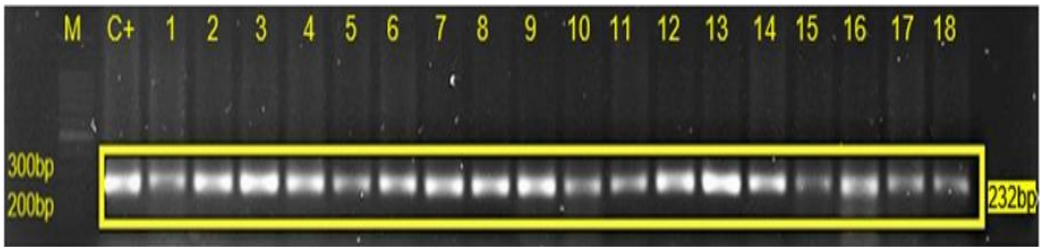

Figure 1. Agarose gel electrophoresis image of the 16 sRNA genes at the size of 232bp from representative isolates of *Escherichia coli* collected from fecal swab samples of chicken, pigs, and rabbits. Samples 1 to 18 were found to contain 16 sRNA genes and considered to be *E. coli*. M= 100 bp DNA marker; C+ = positive control; 1 to 18= bacterial isolates from chicken, pigs, and rabbits.

Table 1. Primer sequences used in this study

No.	Primers	Primer sequence (5′-3′)	Base pair (bp)	References
1	16S rRNA	ATCAACCGAGATTCCCCCAGT TCACTATCGGTCAGTCAGGAG	232	Mogheiseh *et al.*, 2020
2	Bla_{CTX-M1}	AAAAATCACTGCGCCAGTTC AGCTTATTCATCGCCACGTT	415	Naas *et al.*, 2011
3	Bla_{NDM}	GGCCGTATGAGTGATTGC TATTATGCACCCGGTCGC	725	Wang *et al.*, 2012
4	Bla_{OXA}	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	744	Brink *et al.*, 2013
5	Bla_{KPC}	CTGTCTTGTCTCTCATGGCC CCTCGCTGTRCTTGTCATCC	796	Grundmann *et al.*, 2017
6	Bla_{VIM}	AGTGGTGAGTATCCGACAG TCAATCTCCGCGAGAAG	212	Grundmann *et al.*, 2017

relationships between antibiotic exposure and overall antibiotic resistance in *E. coli* isolates were assessed using multiple antimicrobial resistance index (MARI). The MARI was calculated as the proportion of antibiotics tested to which the isolate was phenotypically resistant (Rasool *et al.*, 2018). A MARI index of 2.0 was set as a threshold value to differentiate low and high-risk regions where antibiotics were overused (Rasool *et al.*, 2018). A dendrogram to visualize the relatedness between *E. coli* isolates based on their phenotypic antibiotic resistance was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in BioNumerics 8.0 software (Applied Maths, Texas).

RESULTS

Genotypic Identification of *E. coli* and CREC

The prevalence rate of *E. coli*, ESBL producing *E. coli*, and CREC from rabbit, swine, and poultry were summarized in Table 2. In this study, a total of 212 isolates of *E. coli* (53%) were isolated from rabbit (51/100; 51%), swine (51/100; 51%), and poultry (110/200; 55%). Further screening of bla_{CTX-M1} genes revealed the presence of 12 (12/200; 5.5%) isolates of ESBL producing *E. coli* from poultry (Figure 2). Two CREC (1%; 2/200) were also identified among *E. coli* isolates from poultry fecal swab samples through screening of bla_{NDM} gene (Figure 3). No ESBL producing *E. coli* and *CREC* were detected from rabbits and swine.

Antibiotic Susceptibility Test

The antibiotic resistance rate of isolated *E. coli* (n=212) against 11 different antibiotics was summarized in Table 3 and Table 4. Based on the antibiogram, the tested *E. coli* isolates showed resistance against nine different antibiotics, with a high resistance rate towards ertapenem (48.1%; 102/212). None of the *E. coli* was resistant against doripenem and imipenem. Further analysis revealed that 3.3% (7/212) of the *E. coli* isolates were categorized as multidrug resistance as they showed resistance to antibiotics from three different categories of antimicrobials. Additionally, 34% (72/212) of *E. coli* displayed resistance behavior against agents from two different antibiotic groups, while 70 (33%) isolates were only resistant to antibiotics from one category of antimicrobial. The MARI assessment (Table 5) revealed that 16 (7.5%; 16/212) isolates of *E. coli* had MARI values of 0.2 and above, where the isolates showed resistance against three and above antibiotics. The dendrogram generated using UPGMA, BioNumerics version 8.0 (Applied Maths, Texas) was shown in Figure 4.

DISCUSSION

The ongoing increase of antimicrobial resistance (AMR) among Gram-negative bacteria, especially Enterobacteriaceae in livestock animals, and their potential transmissions to humans represent a major

Table 2. Prevalence rate of *Escherichia coli*, extended-spectrum β-lactamase (ESBL) producing *E. coli*, and carbapenem-resistant *E. coli* (CREC) according to animal species

Animal species	Number of *Escherichia coli* positive isolates (%)	Significance	Number of ESBL producing *Escherichia coli* (%)	Number of CREC (%)
Rabbit	51 (51)	p>0.05	0 (0)	0 (0)
Swine	51 (51)	p>0.05	0 (0)	0 (0)
Poultry	110 (55)	p>0.05	11 (5.5)	2 (1)

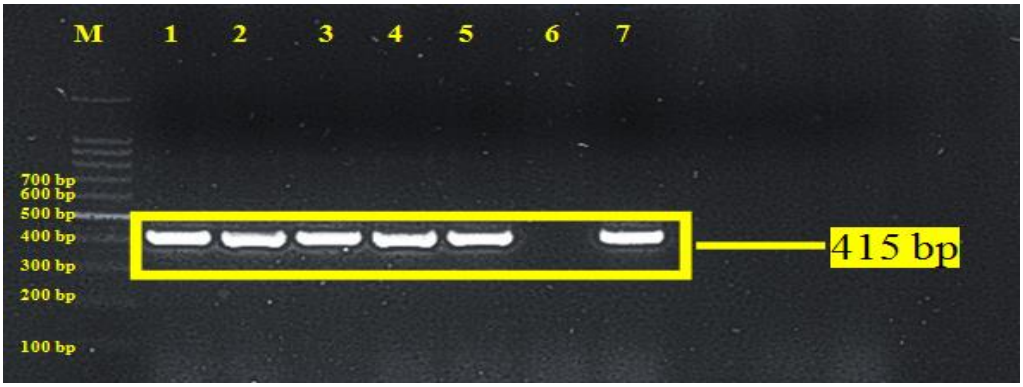

Figure 2. Agarose gel electrophoresis image of bla_{CTX-M1} gene at the size of 415bp from representative isolates of *Escherichia coli* collected from rectal swab samples of chicken. *E. coli* isolates 1, 2, 3, 4, 5, and 7 were found to be bla_{CTX-M1} genes positive and considered to be ESBL-producing *E. coli*. M = 100 bp DNA marker; 1 to 5 and 6 = *E. coli* isolates from chicken.

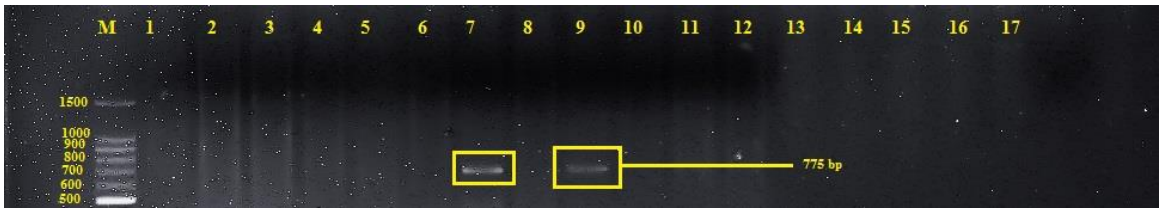

Figure 3. Agarose gel electrophoresis image of bla_{NDM} genes at the size of 775bp from eleven extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* isolated in chicken. Bacterial isolates labeled 7 and 9 produced DNA band for bla_{NDM} genes at size approximately 775 bp. M= 100 bp DNA marker; 1 to 11 = ESBL-producing *E. coli* from chicken.

concern to public health. This is because the treatments of infections caused by AMR bacteria are much more challenging, with limited antibiotic options and limited evidence of their efficacy (Rodríguez-Baño *et al.*, 2018). Commensal enteric bacteria, such as *E. coli* which reside for prolonged periods inside the intestinal tract, may act as reservoirs for AMR dispersion in the food chain (Lugsomya *et al.*, 2018).

In the present study, the overall prevalence rate of *E. coli* from the three species of animals was 53% (212/400). The prevalence rate of *E. coli* found in this study is higher than the prevalence rate of 6.3% in swine, 15.7% in chicken, and 40% in rabbit samples reported elsewhere (Abd El Tawab *et al.*, 2015; Eldin & Reda, 2016; Zhang *et al.*, 2019). Further genotypic screening of bla_{CTX-M1} revealed the presence of ESBL-producing *E. coli* (5.5%; 11/200) from poultry fecal swab samples. This finding is lower than the 20.1% to 52.14% occurrence rates of ESBL-producing *E. coli* reported in chicken by previous studies elsewhere (Chishimba *et al.*, 2016; Rahman *et al.*, 2018; Baran *et al.*, 2020). In 2016, a study conducted by Chishimba *et al.* (2016) reported 20.1% of the *E. coli* isolates from poultry swab samples collected in a poultry abattoir located in Lusaka, Zambia, were confirmed to be ESBL-producing *E. coli.* Another study by Rahman *et al.* (2019) reported that 47.6% of *E. coli* isolates recovered from chicken meats in live bird markets of District Peshawar, Pakistan, were found to be ESBL producers. Meanwhile, Baran *et al.* (2020) stated the prevalence of 52.1% ESBL-producing *E. coli* in broiler meats obtained from supermarkets in Erzurum, Turkey. In addition, two of the ESBL-producing *E. coli* isolates were harboring bla_{NDM} genes, indicating the presence of ESBL-producing CREC. This result is different from the study by Hosuru Subramanya *et al.* (2020) that reported the absence of carbapenemase-producing *E. coli* from rectal swab samples collected among healthy chickens in Kaski District of Western Nepal. The presence of ESBL-producing CRE is worrisome since isolates that harbor both ESBL and carbapenemase genes often confer a

Table 3. Antibiogram of *Escherichia coli* against 11 selected antibiotics (n=212)

No	Antibiotics	Potency (μg)	Number of isolates (%)		
			Resistant	Intermediate	Susceptible
1	Ertapenem	10	102 (48.1)	0 (0)	110 (51.9)
2	Ampicillin	10	51 (24.1)	0 (0)	161 (75.9)
3	Amoxicillin-clavulanate	10	27 (12.7)	20 (9.4)	165 (77.8)
4	Tetracycline	30	22 (10.4)	4 (1.9)	186 (87.7)
5	Ciprofloxacin	5	12 (5.7)	13 (6.1)	187 (88.2)
6	Amikacin	30	6 (2.8)	2 (0.9)	204 (96.2)
7	Doxycycline	30	3 (1.4)	4 (1.9)	205 (96.7)
8	Meropenem	10	6 (2.8)	7 (3.3)	199 (93.7)
9	Gentamicin	10	4 (1.9)	2 (0.9)	206 (97.2)
10	Doripenem	10	0 (0)	3 (1.4)	209 (98.6)
11	Imipenem	10	0 (0)	2 (0.9)	210 (99.1)

Table 4. Antibiogram of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* against 11 selected antibiotics (n=12)

No	Antibiotics	Potency (μg)	Number of isolates (%)		
			Resistant	Intermediate	Susceptible
1	Ciprofloxacin	5	3 (25)	2 (16.7)	7 (58.3)
2	Amoxicillin-clavulanate	10	1 (8.3)	1 (8.3)	10 (100)
3	Ertapenem	10	0 (0)	0 (0)	12 (100)
4	Ampicillin	10	0 (0)	0 (0)	12 (100)
5	Tetracycline	30	0 (0)	0 (0)	12 (100)
6	Amikacin	30	0 (0)	0 (0)	12 (100)
7	Doxycycline	30	0 (0)	0 (0)	12 (100)
8	Meropenem	10	0 (0)	0 (0)	12 (100)
9	Gentamicin	10	0 (0)	0 (0)	12 (100)
10	Doripenem	10	0 (0)	0 (0)	12 (100)
11	Imipenem	10	0 (0)	0 (0)	12 (100)

Table 5. Multiple antimicrobial resistance index (MARI) assessment of *Escherichia coli* isolates (n=212)

Number of antibiotics	Number of isolates	Percentages (%)	MARI
0	60	28.3	**0**
1	67	31.6	**0.09**
2	69	32.5	**0.18**
3	13	6.1	**0.27**
4	3	1.4	**0.36**

higher resistance level to both carbapenem and cephalosporin (Tian *et al.,* 2018). Nevertheless, it is important to note that the presence of these multidrug-resistant bacteria in food-producing animals as food contaminated with ESBL-producing and carbapenem resistance *E. coli* is a potential risk factor for their widespread dissemination in humans (Nahar *et al.,* 2018).

Antibiotic-susceptibility testing revealed that isolated *E. coli* showed a relatively low resistance level against tested antibiotics, except for ertapenem (48.1% resistance rate). This finding is different from the study carried by Yassin *et al.* (2017) that reported a low ertapenem resistance rate (0.2%) by *E. coli* isolated from livestock in China. Since ertapenem belongs to the carbapenem group, the appearance of such a number of ertapenem-resistance *E. coli* (102/212) isolates suggested the emergence of carbapenem resistance *E. coli*. Bacteria that are resistant against carbapenem often produce carbapenemases, an enzyme that can inactivate carbapenems together with the other beta-lactam antibiotics and therefore called carbapenemases (Meletis, 2016). Some of the most effective and geographically widespread carbapenemases include KPC, VIM, NDM, OXA-48 types, and IMP (Meletis, 2016). Surprisingly, further genotypic screening of carbapenem resistance genes only detected the presence of bla_{NDM} from two isolates. Other carbapenem resistance genes, such as bla_{OXA}, bla_{VIM}, or bla_{KPC} genes, were not detected. It is possible that these ertapenem-resistant isolates may harbor the other resistance genes that were not tested in this study or recruited the other mechanisms to overcome the effect of carbapenem antibiotics. Thus, further investigation needs to be carried out to determine the possible mechanisms of resistance.

In this study, none of the *E. coli* isolates were resistant towards imipenem and doripenem, suggesting imipenem can be safely used to treat *E. coli* infections in swine, rabbit, and chicken. In addition, only a small portion of the isolates showed resistance against amikacin, doxycycline, meropenem, and gentamicin. It is noteworthy that the development of antimicrobial resistance in bacteria is frequently associated with the

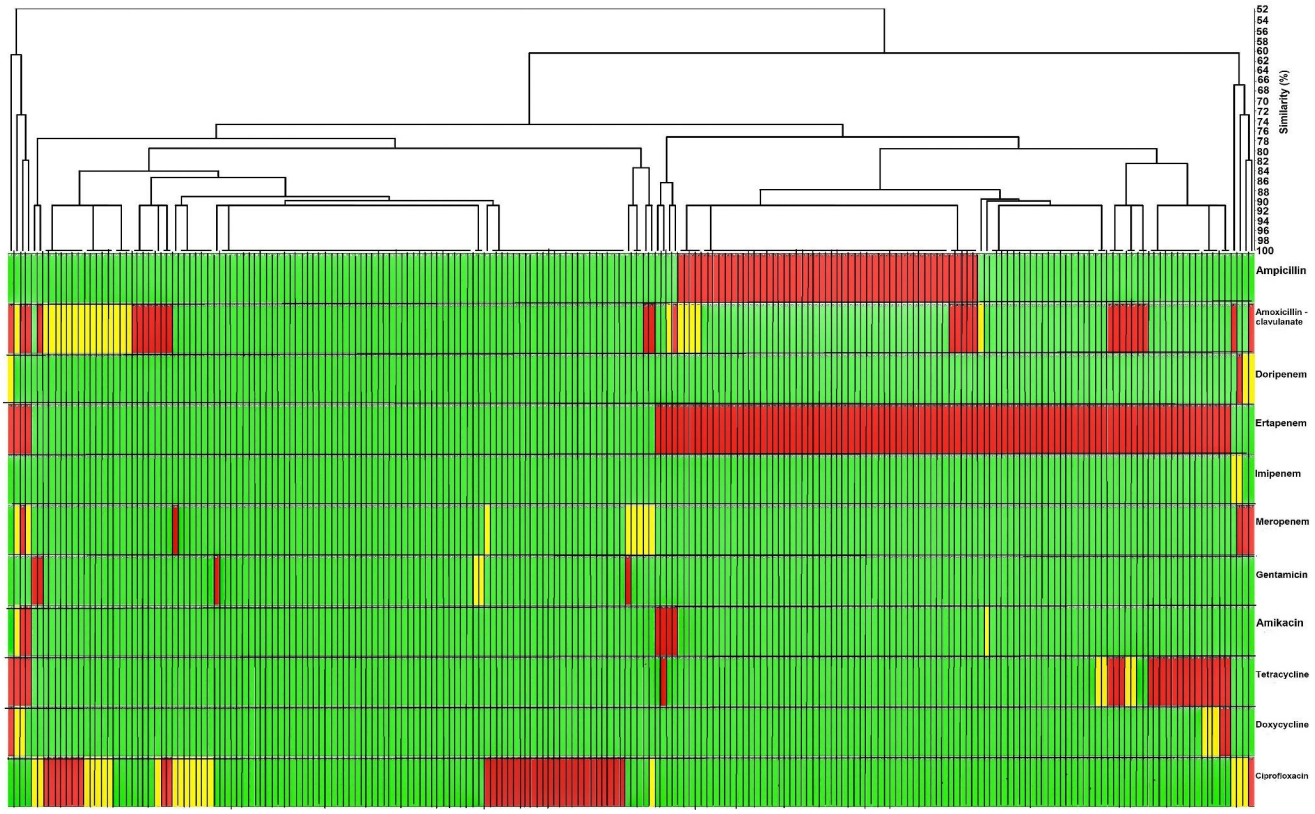

Figure 4. Dendrogram illustrating the relatedness of *Escherichia coli* isolates based on phenotypic antimicrobial resistance pattern (red= resistance; yellow= intermediate resistance, and green= susceptible).

repeated therapeutic or indiscriminate uses of antibiotics (Ariffin *et al.,* 2019; Ariffin *et al.,* 2020). Most of the antibiotics tested in this study were considered to be important critical antimicrobials (amikacin, meropenem, gentamicin, doripenem, and imipenem) and highly important antimicrobials (doxycycline) by World Health Organization (Scott *et al.,* 2019). Therefore, the usage of these critically important antibiotics in livestock animals should be regulated to prevent further emergence of multidrug–resistance bacteria (Scott *et al.,* 2019).

According to Magiorakos *et al.* (2012), multidrug resistance is defined as showing resistance to at least one agent in three or more antimicrobial categories. The present study showed that 3.3% (7/212) of the isolates could be categorized as multidrug resistance. This finding is lower than the 50.9% prevalence rate of multidrug resistance *E. coli* reported in diarrhea rabbits from farms in Ningyang, Xintai, and Dongping regions of China (Zhao *et al.,* 2018). Dendrogram generated using UPGMA also showed that the *E. coli* isolates from different species of animals appeared to show different antibiotic resistance patterns, suggesting these isolates may have different genetic backgrounds or exposure to different antibiotics. Further MARI assessment revealed that 16 (7.5%; 22/212) isolates of *E. coli* had MARI value of 0.2 and above, with resistance against three and above antibiotics. This result indicated that a small portion of the isolates was exposed to an environment with high antibiotic usage. Even though the number of *E. coli* isolates that showed resistance to multiple antibiotics is low, the veterinary authorities and farmers should be alerted to rationalize any usage of these antimicrobials in small and commercially producing livestock farms.

CONCLUSION

In this study, the overall prevalence of *E. coli* in fecal swab samples collected was 53% (212/400). The presence of ESBL-producing *E. coli* and two ESBL-producing CREC were detected from poultry fecal swabs. The prevalence of resistance rate against antibiotics was relatively low, except for ertapenem (48.1%; 102/212). The *E. coli* isolates did not display antibiotic resistance against imipenem and doripenem. In addition, 3.3% (7/212) of the *E. coli* isolates were considered to be multidrug-resistance based on their phenotypic antibiotic resistance profiles.

CONFLICT OF INTEREST

None of the authors have any potential conflict of interest to declare.

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