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RESEARCH ARTICLE

Detection of colistin-resistant *Escherichia coli* isolated from broiler chickens in Kelantan, Malaysia

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ABSTRACT

Antimicrobial resistant *Escherichia coli* have become an ever increasing problem in human, and animal health and production. The imprudent use of antibiotics and poor hygienic practices especially in poultry industries have been contributing to the emergence and spread of *E. coli* species resistant to broad spectrum antibiotics including Colistin. This study was conducted to detect colistin – resistance and antibiotic sensitivity patterns in *E. coli* isolated from broiler chickens in Kelantan. A total of 320 cloacal swabs were collected from apparently healthy broiler chickens in different districts of Kelantan and were analysed using routine microbiological methods, Kirby–Bauer method for antimicrobial susceptibility test and PCR amplification of species-specific and colistin - resistance encoding genes. Out of the 320 samples, 91 isolates were confirmed as *E. coli* and 21/91 (23.08%) were positive for colistin - resistant encoding gene, *mcr*-1. Most of the isolates were resistant to tetracycline (95.24%), chloramphenicol (85.71%), and sulphamethoxazole/ trimethoprim (85.71%). However, the isolates were less resistant towards piperacillin/ tazobactam (4.76%) and meropenem (9.52%). The findings from this study reveal the emerging threats of colistin - resistant in local food animal production, particularly in poultry production industry. However, more comprehensive, and large-scale studies focusing on more resistance patterns using determination of minimum inhibitory concentration (MIC), virulence and resistance characteristics and molecular epidemiology of colistin – resistant *E. coli* are recommended for better understanding of the epidemiology and to implement the appropriate control and prevention strategies.

Keywords: *E. coli*; antimicrobial resistance; colistin resistance; poultry.

INTRODUCTION

Escherichia coli is the most frequent and versatile infectious microorganisms due to its genomic plasticity and variability and its ability to survive in diverse ecological niches (da Silva & Mendonça, 2012). Notably, recent reports documented alarming increase of antimicrobial resistant strains of *E. coli. Escherichia coli,* which is a normal flora in humans, animals as well as environment are considered the excellent indicators of antimicrobial resistance (AMR) (Yassin *et al*., 2017). The AMR in *E. coli* developed into the multi-drug resistant (MDR) in the bacterial strains over the past 20 years (Sarker *et al*., 2019). Multiple mechanisms such as failure of drug binding on target, degradation or alteration of the drug, overexpression of the drug, modified permeability of barriers and active exportation of the drugs are known to cause emergence of antibiotic resistance (Moreira *et al*., 2005). Moreover, specific resistance fingerprints (Sayah *et al*., 2005) and genes of mutations, selective pressure and induction, transmission via horizontal and vertical gene transfer are other factors that introduce AMR among *E. coli*, other bacterial strains, and the human food chain (Sarker *et al*., 2019).

Imprudent use of antimicrobial agents caused further ineffectiveness in the bacterial infection treatment due to the AMR development (Yassin *et al*., 2017). Moreover, antibiotics have been extensively used as growth promoters and prophylactic agents in agricultural sectors (Miles *et al*., 2006). Sub-therapeutic doses of antibiotics for bacterial infection treatment were routinely administered for the livestock, mostly in developing countries to handle disease outbreaks in farms ultimately contributing to the development of resistance among pathogens (Wang *et al*., 2013). Those regimes include the fluoroquinolones and cephalosporins that were fed at low dosage, increasing the risk of emergence of resistant pathogen and its transmission of antimicrobial resistant genes (ARGs) from animals to humans (Kumar *et al*., 2019). AMR in *E. coli* have been spreading rapidly to multiple countries, posing threats to animal and human community worldwide (da Silva & Mendonça, 2012).

Colistin is the ultimate last-resort antimicrobial agent used to treat multidrug-resistant Gram- negative bacterial infection such as carbapenem-resistant Enterobacteriaceae (Arcilla *et al*., 2016). The antibiotic was mainly administered as antibiotic growth promoters through food and drinking water, where the whole herd of animals generally receive the antibiotics regardless of their health condition (Andrade *et al*., 2020). The gene encoding colistin - resistant, the mobilized colistin - resistance (*mcr*) genes are plasmid-borne

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(Carroll *et al*., 2019). Since the first discovery of plasmid mediated *mcr-1* in Enterobacteriaceae, from food animal origin (Gharaibeh & Shatnawi, 2019) reported in 2015 from China, many studies reported the prevalence of *mcr* genes in South Asia, Africa, Europe and South America (Yu *et al*., 2016). The drastic trend could potentially develop into colistin - resistant gene reservoir affecting other Enterobacteriaceae, especially in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. This may lead to the ineffectiveness of currently available therapeutic antibiotics and development of pan-drug resistance, among the MDR bacteria (Mat Zin *et al*., 2017).

Meanwhile, in Malaysia, the recent discovery of the horizontally transferable *mcr-1* gene in *E. coli* from swine samples reported by Liu *et al*. (2016) have significantly impacted the perception of the current situation of colistin – resistance in the animal meat industry. Apart from animals, human clinical specimens were the contributing origin of the *mcr-1* gene found in the *E. coli* isolates retrieved from 2013, together with pigs, chickens and environmental specimens (Yu *et al*., 2016). Besides, distinct report of the identified *TnAs2-mcr-3* element of *mcr-3* gene in *E. coli* from pig isolates (Yin *et al*., 2017) also explains the widespread transmission of colistin - resistant genes in Malaysia. Recently, Aklilu and Raman (2020) reported a 52.1% detection rate of *mcr-1* gene in *E. coli* isolated from chicken meat obtained from Kelantan thereby indicating that the potential sources of origin of colistin resistant *E. coli* were untraceable and unavailable. Therefore, this study is conducted to detect the colistin - resistance in *E. coli* isolated from poultry chickens in Kelantan as well as to determine the antibiotic sensitivity patterns of the isolates.

MATERIALS AND METHODS

Ethical approval statement

The current study was conducted at the Zoonotic Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The study protocols, procedures, and consents were approved by the Institutional Animal Care and Use Committee of Universiti Malaysia Kelantan (UMK/FPV/ACUE/PG/2/2019).

Sample collection

A total of 320 cloacal swab samples were collected from different poultry farms in five districts of Kelantan, Tumpat (n = 40), Pasir Mas (n = 40), Jeli (n = 40), Bachok (n = 100) and Machang (n = 100). The samples were collected from the selected broiler farms listed by the Department of Veterinary Services, Malaysia. The cloacal swabs were collected with sterile cotton swab with Amies transport media (Oxoid, UK) and immediately transported to the laboratory, in (4-8°C) cold storage container. Samples were stored in chiller (4-8°C) and processed within 18 hours upon collection.

Bacterial isolation and identification

Each cloacal swabs were incubated in Alkaline Peptone water (APW) at 37°C, overnight for the primary enrichment of the bacteria. The enriched bacterial growth was cultured on MacConkey agar (Oxoid, UK) twice and subsequently on Eosin Methylene Blue (Oxoid, UK) to screen and isolate presumptive *E. coli* based on their colony morphology. Colonies displaying green metallic sheen on Eosin Methylene Blue agar were presumptively identified as *E. coli* and were subjected to biochemical tests. The biochemical tests used include the SIM (Sulphur, Indole, Motility) and (TSI) Triple Sugar Iron tests. The suspected *E. coli* isolates were then sub-cultured in Luria Bertani broth and stored as glycerol stock by adding 1:1 ratio of 50% glycerol kept at –80°C until further use.

Confirmation of *E. coli* using species-specific gene

The suspected *E. coli* isolates were processed for DNA extraction using NucleoSpin® Tissue kit (Macherey-Nagel, Germany) and polymerase chain reaction was performed with *E. coli* species-specific, *phoA* gene with the following primers and amplification protocol. Forward sequence: 5'-GTG ACA AAA GCC CGG ACA CCA TAA ATG CCT-3', reverse sequence: 5'-TAC ACT GTC ATT ACG TTG CGG ATT TGG CGT-3' (Thong *et al*., 2011), denatured at 95°C for 4 mins, amplified for 30 cycles of 95°C for 30 s, 56°C for 30s and 72°C for 1 min with final extension of 72°C for 10 mins. The PCR products were analysed using gel electrophoresis, prepared 1.5% agarose gel with 1.2 g of agarose powder (Agarose Vivantis, Malaysia) dissolved in 80 ml TBE Buffer, added with 1.2 ul Midori Green (Nippon Genetics Europe, Germany). The results of the PCR amplification were photographed and analysed using Gel Doc™ EZ Imager (Bio-Rad, USA).

Detection of colistin - resistance encoding gene*, mcr-1*

For the detection of colistin - resistant encoding gene, *mcr-1*, PCR was performed using *mcr-1* gene primers, forward sequence: 5'-AGTCCGTTTGTTCTTGTGGC-3'; reverse sequence: 5'-AGATCCTTGGTCTCGGCTTG-3' (Rebelo *et al*., 2018). The DNA samples were initially denatured at 94°C for 15 mins with 25 cycles of amplification at 94°C for 30 s, 58°C for 90 s and 72°C for 1 min and at 72°C for 10 mins for final extension. Similarly, the PCR products were run through agarose gel electrophoresis and analysed using Gel Doc™ EZ Imager (Bio-Rad, USA).

Antibiotic susceptibility testing (AST)

Kirby–Bauer antibiotic susceptibility testing (AST) or disk diffusion method was performed on all *mcr-1* positive isolates with 12 antibiotics of different classes. The isolates were pre-cultured on Luria Bertani agar and incubated 18–24 hours at 37°C before performing AST. A 0.5 McFarland turbidity standard was used to match the dilution of bacterial isolates in 3ml of 0.85% normal saline and was inoculated on Muller Hinton Agar (MHA) and incubated at 37°C for 18 hours. The antibiotics tested include tetracycline (30 ug), amoxicillin (10 ug), meropenem (10 ug), cefotaxime (30 ug), imipenem (10 ug), ceftazidime (30 ug), ofloxacin (5 ug), aztreonam (30 ug), sulphamethoxazole /trimethoprim (25 ug), chloramphenicol (30 ug), amoxicillin/ clavulanic acid (30 ug) and tazobactam/ piperacillin (110 ug). The zone of inhibition of each antibiotic for each isolate were measured and the antimicrobial resistance was evaluated based on Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). *Escherichia coli* ATCC 25922 strain was used as negative control.

Determination of multiple antibiotic resistance (MAR) index

The susceptibility of each *mcr-1* carrying *E. coli* isolates were determined using MAR index formula (Sandhu *et al*., 2016; Akande *et al*., 2019).

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.

The results were interpreted according to the MAR index standard, where the isolates with the MAR index of ≥ 0.2 were considered as the isolate with high risk of antibiotic contamination (Sandhu *et al*., 2016; Akande *et al*., 2019).

RESULTS

Isolation and identification of *E. coli*

From the routine microbiological and biochemical identification method, 121 (37.81%) isolates were detected as presumptive *E. coli*, from the 320 cloacal swab samples retrieved from different poultry farms in Kelantan districts. The suspected *E. coli* isolates showed positive production of indole, absence of blackening of medium and motile condition in SIM test. Whereas the isolates exhibited acidic or yellow agar slant and butt with presence of gas production in TSI test indicating as lactose fermenters.

Table 1. Antimicrobial resistance rate of *E. coli* isolates with *mcr-1* gene, n = 21 with 12 antibiotics and their corresponding possible mechanisms of AMR development in *E. coli*

Classes of antibiotics	Antibiotics used (Dosage)	Resistance rate (%), n = 21
Mechanism: Inhibition of bacterial cell wall synthesis		
Penicillin	Amoxicillin (10 ug)	80.95
Carbapenems	Meropenem (10 ug)	9.52
	Imipenem (10 ug)	33.33
Monobactam	Aztreonam (30 ug)	42.86
Cephems (Parental) Cephalosporins III	Cefotaxime (30 ug)	28.57
	Ceftazidime (30 ug)	23.81
β–lactams combination agents	Tazobactam / piperacillin (110 ug)	4.76
	Amoxicillin/ Clavulanic acid (30 ug)	52.38
Mechanism: Disruption of DNA synthesis and during DNA replication		
Fluoroquinolones	Ofloxacin (5 ug)	76.19
Folate pathway antagonists	Sulphamethoxazole/ trimethoprim (25 ug)	85.71
Mechanism: Inhibition of protein synthesis		
Tetracyclines	Tetracycline (30 ug)	95.24
Phenicol	Chloramphenicol (30 ug)	85.71

Molecular identification with Polymerase Chain reaction amplification

The PCR analysis revealed that 91 isolates from the presumptive 121 *E. coli* isolates were positive for *phoA* gene at 903bp, which indicates the prevalence rate of 28.44% (91/320). Meanwhile, 21 isolates from the confirmed *E. coli* isolates were tested positive for *mcr-1* gene, giving an overall prevalence of 23.08% (21/91) *E. coli* isolates carrying *mcr-1* gene.

Antibiotic susceptibility testing

The results showed that the *mcr-1* positive *E. coli* isolates exhibited higher resistance level towards three antibiotics from three different classes, with tetracycline (95.24%) being the most resistant toward these isolates. Meanwhile, 85.71% of the isolates showed resistance to chloramphenicol from phenicol class and sulphamethoxazole/ trimethoprim. Whereas the isolates were less susceptible towards the combination of tazobactam/piperacillin (4.76%) and meropenem (9.52%). However, all isolates showed resistance to at least three antibiotics except for one isolate that showed resistance to aztreonam alone (Table 1).

MAR index

Based on the MAR index analysis of 21 *mcr-1* carrying *E. coli* isolates, 20 isolates (95.2%) were showing MAR > 0.2, which indicates the multidrug-resistant nature of the isolates which have acquired this resistance due to exposure to higher concentrations of the specific antibiotics. The results of MAR index of these isolates were tabulated in Table 2.

Table 2. MAR indices of *mcr-1* carrying *E. coli* isolates, n = 21

MAR Index	Number of isolates (%)
0.0	00
0.1	1 (4.8)
0.2	00
0.3	3 (14.3)
0.4	1 (4.8)
0.5	5 (23.8)
0.6	2 (9.5)
0.7	3 (14.3)
0.8	5 (23.8)
0.9	1 (4.8)
1.0	00

DISCUSSIONS

According to the National Surveillance of Antimicrobial Resistance (NSAR) reports, Malaysia was among the initial countries that reported the emergence of *mcr-1* gene in Enterobacteriaceae, especially from animal and environmental samples (Hsu *et al*., 2017). Our findings revealed a prevalence rate of 28.44% (91/320) of *E. coli* isolates from poultry farms from five districts of Kelantan. The species-specific gene, *phoA*, which is the housekeeping gene of *E. coli* was used to confirm the *E. coli* strains. The *phoA* target gene has been proven as the universal marker of *E. coli,* and this has been confirmed by several studies where the gene was not detected in any non- *E. coli* DNA templates (Kong *et al*., 1999), and explained the usefulness and stability of *phoA* gene for the identification of *E. coli* from other enteric bacteria (Rathi *et al*., 2009; Thong *et al*., 2011). Previous studies (Hu *et al*., 2011; Alnahass *et al*., 2016) used this gene to detect pathogenic *E. coli* in samples collected from healthy and diseased chickens. In contrast to previous reports from the same study area, lower *E. coli* prevalence rate was recorded. Ibrahim *et al.* (2021) and Aklilu and Raman (2020) reported *E. coli* prevalence rates of 50% and 46% from poultry cloacal samples and raw chicken meat samples respectively. Meanwhile, 23.08% prevalence of *mcr*-carrying *E. coli* was recorded in our study yet recent report from the same area stated a 52.1% prevalence of *mcr-1* gene in *E. coli* isolates from raw chicken meat collected from Kota Bharu, Kelantan (Aklilu & Raman, 2020). These findings indicate that the detection of *mcr-1* gene in *E. coli* from poultry origin still exist moderately in Malaysia.

The antimicrobial resistance (AMR) patterns of our *mcr-1* positive isolates revealed that 20 out of 21 isolates were resistant to at least three classes of antibiotics, indicating the existence of MDR strains (Adelowo *et al*., 2014; Dominguez *et al*., 2018). The findings in this study also showed that most of the isolates (95.24%) were

resistant to tetracycline (Table 1). The increased rate of resistance towards tetracycline is similar to the latest study from Tai'an, China showing 100% of *mcr-1* carrying *E. coli* isolates were resistant to tetracycline (Song *et al*., 2020). Tetracycline resistance in *E. coli* were developed or regulated by the mechanism of conjugation of larger plasmids that carries other antibiotics resistant genes or other pathogenic factors. The plasmids are capable of selecting any factors or genes, hence successful transformations will be executed easily leading to the development of resistance of bacteria (Miles *et al*., 2006). Likewise, corresponding patterns of tetracycline resistance were observed in MDR *E. coli* without *mcr-1* gene. A study conducted on the prevalence of *E. coli* and *Salmonella spp.* in poultry farms in Malaysia reported that 90 % of the *E. coli* isolates were resistant to tetracycline (Ibrahim *et al*., 2021). An overall review of *E. coli* epidemiology in Southeast Asian countries announced in 2016, showed more than 70% of the isolates were resistant to tetracycline (Nhung *et al*., 2016) along with the hiked prevalence of 95.25% on tetracycline resistant isolates carrying encoding genes; *tet A* and *tet B* were detected in Bangladesh poultry samples (Al Azad *et al*., 2019).

Followed by the highest resistance rate towards tetracycline, similar patterns of resistance towards both chloramphenicol and sulphamethoxazole/ trimethoprim were observed as 85.71% of the *mcr-1* carrying *E. coli* were resistant to these antibiotics. This finding is in agreement with the previous study documented in East Coast states of Peninsular Malaysia which reported the prevalence range of 83 - 85% (Ibrahim *et al*., 2021). Whereas study by Geidam *et al*. (2012) conducted in Selangor, Malaysia, reported a prevalence rate of 77 - 79% of *E. coli* isolates from the poultry samples with similar resistance patterns. However, studies from other countries reported different patterns of resistance in *E. coli* isolates from poultry. Those studies reported consistent rate of higher resistance towards sulphamethoxazole/ trimethoprim than to chloramphenicol (Moreira *et al*., 2005; Nhung *et al*., 2016). Along with the significant antibiotic pressures developed from inappropriate usage of antibiotics in animals, irrational dose regimens are forcing bacterial strains to become MDR within shorter period (Muktan *et al*., 2020).

On the other hand, the isolates from this study showed lower resistance rates of 4.76% and 9.52% towards tazobactam/piperacillin and meropenem respectively. This finding is consistent with the NSAR, Malaysia report where carbapenem resistance remained stable within 4 years (2010 to 2014) at around 0.5% to 0.2% among *E. coli* strains (Hsu *et al*., 2017). Likewise, lower resistance towards tazobactam/ piperacillin (0%) and meropenem (2.6%) were reported in a study conducted in Nepal investigating the antibiotic resistance of *mcr-1* gene positive *E. coli* isolates from poultry (Muktan *et al*., 2020). These antibiotics are the last generation of broad - spectrum beta-lactamase inhibitor class, generally reserved to treat serious infection as an alternative for suspected resistant antibiotics (Malchione *et al*., 2019). The resistance of bacterial strains to extended spectrum beta- lactamases (ESBLs) may have developed by adapting the mechanism of producing carbapenemases (Atterby *et al*., 2019) as well as the biofilm production that provides suitable domains for the antibiotic – resistant genes to facilitate its dissemination extensively (Surgers *et al*., 2018). These ranges of drastic rise of carbapenem resistance are worrisome as it has started to coexist with colistin - resistance (Muktan *et al*., 2020) which may lead to the emergence of pan-resistant or superbug resistant strains (Song *et al.,* 2020).

The MAR index of these *E. coli* isolates in Table 2. showed that 95.2% are highly categorised as originated from antimicrobial-contaminated environment. Similar findings were reported in the study by Ibrahim *et al*. (2021) where 96% of the *E. coli* isolates obtained from the poultry farms in the states of East – Coast of Peninsular Malaysia were having MAR > 0.2. These indicates that the higher selection pressure due to over-usage of antibiotics in poultry farms. The transfer of the resistant strains of *E. coli* to human, other animals and environment can become highly threatening if the negative implications of the uncontrolled growth-promoters usage in food-animal production is not taken into considerations (Abdalla *et al*., 2021).

Further studies are encouraged to investigate on the coherent relationship of AMR and colistin – resistance (*mcr* genes) in *E. coli.* The possible mechanisms causing the development of AMR in these classes of antibiotics (Table 1) are inhibition of bacterial cell wall synthesis, inhibition of protein synthesis and disruption of DNA synthesis of *E. coli* (Tenover, 2006). However, the resistance of these antibiotics in *mcr*-carrying *E. coli* may have enhanced due to the mobilized plasmid *mcr-1* gene that encodes for phosphoethanolamine transferase (sulfatase) (Baron *et al*., 2016; Olaitan *et al*., 2016). This plasmid characteristic enables the transfer of resistant gene compound even to other Enterobacteriaceae and *Pseudomonas aeruginosa*. The clonal transmission adds on the possibilities of highly sensitive and colistin - resistant bacteria to subsequently acquire AMR – encoding plasmids such as ESBL plasmids (Olaitan *et al*., 2016). This mechanism might get elevated in intestinal *E. coli* with the frequent exposure towards antibiotic stress (Sekyere & Asante, 2018). The co-localization of other antibiotic resistant genes like *tetA*, *tetB* of tetracyclines and *sul1, sul3* of sulphonamides on *mcr-1* carrying plasmid may enhance the selective pressure of being resistant towards any antibiotics other than colistin (Maamar *et al*., 2018). Another possibility may be mediated by non – antibiotic resistance genes (NARGs) such the *mcr-1, blaNDM-1* and *blaCTX-M-15.* These genes express resistance by single nucleotide polymorphism (SNP) mutations, which usually occurs in bacterial isolates without the ARGs (Sekyere & Asante, 2018). Yet, few reports remained with the *mcr-1* being the sole resistant gene on plasmids that is responsible to choose other AMR mainly due to selective pressure (Poirel *et al*., 2018).

CONCLUSION

The poultry industry in Malaysia has been thriving and is the major source of animal protein in the country. Commercialized domestic producers and integrators has been developing and improving the industry by enhancing food nutrition and adopting quality facilities of the livestock production. Yet, they need to consider the pertinent issues related to antimicrobial resistance especially with the presence of MDR *E. coli* which extremely exhibits the resistant phenotypes and antibiotic resistant genes in recent times. Based on our findings, the *mcr-1* carrying *E. coli* may be resolved soon or might end up adapting to newer mechanisms to disseminate into new surroundings and other pathogens. Serious implementations on establishing major integrated surveillance programme with the collaboration of panels of human health, animal health and food safety are highly recommended to limit the possible ways of spreading AMR and to facilitate discovery of new serotypes or clones of the colistin - resistant *E. coli*. Besides, it is important to generate a critical database of AMR and latest potential status of *mcr* genes in Malaysia that will be useful for future exploration of alternatives in food animal production and health.

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Conflict of Interest

All the authors declares that they have no conflict of interests.

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