Short Communication

Multidrug-resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolated from broiler chickens in Eastern Thailand

Suttitas Tongkamsai[1*] Wachirawit Chatmontee[1] Kulchai Nakbubpa[1]

Abstract

Broiler chickens have been suggested as an important source of antibiotic resistance *Escherichia coli* (*E. coli)* and *Klebsiella pneumoniae* (*K. pneumoniae)*. The coinfection of multiple species of bacteria provides a specific ecological niche for the plasmid-mediated exchange of antibiotic-resistance genes. However, there is limited information about antimicrobial resistance (AMR) phenotypes of *E. coli* and *K. pneumoniae* isolates from broiler chickens. This study aimed to isolate *E. coli* and *K. pneumoniae* from broiler chicken farms in Thailand by observing the antibiotic resistance profiles and detecting the coexistence of these two bacteria. A total of 26 *E. coli* and 29 *K. pneumoniae* isolates were collected from 116 chicken cloacal swabs from four broiler chicken farms. Biochemistry tests and conventional polymerase chain reaction (PCR) were performed to identify the strains. All isolates were tested for antimicrobial susceptibility by disc diffusion against 11 antibiotics. Extended-spectrum β-lactamase (ESBL) production was confirmed using the double disc synergy test. The most detected antibiotic resistance from *E. coli* and *K. pneumoniae* isolates was erythromycin (100%), followed by chloramphenicol (96%) for *E. coli* and amoxycillin (93%) for *K. pneumoniae*. Most isolates of *E. coli* (100%) and *K. pneumoniae* (89.65%) were classified as multi-drug resistance (MDR) bacteria. Ten simultaneous *E. coli* and *K. pneumoniae* isolates were found. The ESBL production was detected from most *E. coli*, whereas it was not detected from *K. pneumoniae*, concurrently. Co-resistance against enrofloxacin was the majority of them. Possibly coexisting resistance genes in plasmids, they transmit between species from the same host. This is the first report on detecting simultaneously isolated multidrug-resistant *E. coli* and *K. pneumoniae* from broiler chickens in Thailand. Surveying the spread of multidrug-resistant bacteria and further studies of genetic communication events are necessary to determine human health risks.

Keywords: *Escherichia coli, Klebsiella pneumoniae*, multidrug-resistant bacteria, simultaneous infection

[1]*Faculty of Veterinary Medicine, Rajamangala University of Technology Tawan-ok, Bangpra, Chonburi, 20110, Thailand*
***Correspondence**: suttitas_to@rmutto.ac.th* (S. Tongkamsai)
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Introduction

Escherichia coli (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are members of the Enterobacteriaceae family, commonly opportunistic pathogens that cause systemic infection in animals and humans (Gelalcha *et al*., 2023). *E. coli* is a normal human and chicken gut flora but may be involved in diseases such as septicemia and urinary tract infections in humans. Avian pathogenic *Escherichia coli* (APEC) is responsible for chickens' respiratory tract and yolk sac infections (Meguenni *et al*., 2019). *K. pneumoniae* can also be found in the intestinal flora, which may potentially infect the urinary system or lungs in humans. *K. pneumoniae* often causes high mortality related to respiratory tract infections in broiler chicks and hens (Daehre *et al*., 2018). Antibiotics are used for disease control and growth promotion in modern livestock production. The continued use of antibiotics in farm animals, including poultry, results in the spread of resistant strains in the environment through fecal contamination (Martinez-Alvarez *et al*., 2022). These resistant strains can be transferred from animals to humans through horizontal gene transfer (Apostolakos *et al*., 2021). Isolates that are not susceptible to at least three or more groups of antibiotics are known as multidrug-resistant (MDR) organisms. Treatment failure due to multi-drug resistance affects human health, animal health, and economic losses (Badr *et al*., 2022). The acquired antimicrobial resistance (AMR) mechanism is associated with the mobility of genetic materials, including plasmid conjugation or mobile elements on plasmids (Falgenhauer *et al*., 2018). Although numerous studies have reported that the exchange of AMR genes among multiple species of pathogens probably occurred within the host (Savin *et al*., 2020), only a few studies have observed AMR between *E. coli* and *K. pneumoniae* isolates within chickens. Due to extended-spectrum beta-lactamases (ESBLs)-producing Enterobacteriaceae has increased dramatically in poultry production (Musa *et al*., 2020), we investigate the resistance event against commonly used antimicrobial agents, including β-lactams, tetracycline, aminoglycosides, and fluoroquinolones. Therefore, the objective of the present study was to determine AMR phenotypes of *E. coli* and *K. pneumoniae* isolates recovered from broiler chickens in Thailand.

Materials and Methods

Bacterial isolation and identification: A total of 116 cloacal swab samples were collected from 4 chicken farms during 2022-2023. The four chicken farms are located in the eastern region of Thailand. Each farm collected 29 samples. In Chonburi Province, samples were collected from VC and JL farms, while in Rayong Province, samples were collected from SY and SR farms. In particular, SY, SR, and JL farms (n=87) are antibiotic-free broiler farms, and VC farm (n=29) is an antibiotic-used (tilmicosin) broiler farm. All samples were transported on ice to the laboratory and processed on the same day. Then, samples were streaked onto MacConkey agar and Eosin Methylene Blue (EMB) agar plates and incubated aerobically at 37 ˚C for 18 to 24 h. Round with dark purple and round with dark purple and metallic sheen on surface colonies were subjected to Gram-staining, oxidase, Simmon citrate agar, indole, the methyl red (MR), the Voges-Proskauer (VP), and triple sugar iron (TSI) agar test following the standard protocols (Hiroi *et al*., 2012), after that concluded *E. coli* and *K. pneumoniae* to each samples. All 26 isolates of *E. coli* and 29 isolates of *K. pneumoniae* were selected and confirmative identified using the molecular technique by PCR assay, targeting the 16S rRNA for *E. coli* and the *khe* gene for *K. pneumoniae*, as described elsewhere (Babu *et al*., 2013; Kamaruzzaman *et al*., 2020). The positive control was TISTR 527 (original code: ATCC 11775) for *E. coli* and ATCC 700603 for *K. pneumoniae.* Samples were taken from chickens following the procedures according to the permission of the Institutional Animal Care and Use Committee (RMUTTO-ACUC-2-2023-007).

Antimicrobial susceptibility test and ESBL detection: Antimicrobial susceptibility tests of all *E. coli* and *K. pneumoniae* isolates and both positive controls: *E. coli* TISTR 527 and *K. pneumoniae* ATCC 700603 were performed against 11 antibiotics using the disc diffusion test on Mueller–Hinton agar (MHA) as previously described (CLSI, 2023). The following antibiotic discs were used: Ampicillin (AMP, 10µg), Amoxycillin (AML, 20µg), Chloramphenicol (C, 30µg), Doxycycline (DO, 30µg), Enrofloxacin (ENR, 5µg), Erythromycin (E, 15µg), Gentamicin (CN, 10µg), Imipenem (IPM, 10µg), Sulfamethoxazole-trimethoprim (SXT, 1.25/23.75µg), Streptomycin (S, 10µg), and Tetracycline (TET, 30µg). The plates were incubated at 37 °C for 24 h under aerobic conditions, and then the diameter of the inhibitory zone was measured. ESBL production was screened for all isolates by disc diffusion method using cefotaxime (CTX, 30µg) and ceftazidime (CAZ, 30µg). ESBL production was confirmed by the double disc synergy test using one β-lactamase inhibitor (AMC) disc and two cephalosporin discs (CAZ and CTX), as previously reported (Anago *et al*., 2015).

Results and Discussion

All 116 cloacal swab samples from 4 chicken farms showed 22.41% (26/116) of *E. coli* and 25% (29/116) of *K. pneumoniae*, respectively. In these bacterial isolates, there were 21.55% (25/116) of ESBL-producing *E. coli* and 1.72% (2/116) of ESBL-producing *K. pneumoniae*, respectively. In these bacterial isolates, 100% (26/26) of *E. coli* and 89.65% (26/29) of *K. pneumoniae* were multi-drug resistance (MDR) isolates (Table 1). Farm SY detected 13 isolates of *E. coli* and 16 isolates of *K. pneumoniae*. Farm SR detected four isolates of *E. coli* and one isolate of *K. pneumoniae*. Farm VC did not detect any *E. coli* but found two isolates of *K. pneumoniae*. Farm JL detected nine isolates of *E. coli* and ten isolates of *K. pneumoniae*.

From all isolates of *E. coli* and *K. pneumoniae* that resisted antibiotics, the highest percentage of resistance (100%) was erythromycin, which was found in both *E. coli* and *K. pneumoniae*. When considering ampicillin, amoxycillin, tetracycline, gentamicin, streptomycin, and enrofloxacin, higher percentages of resistance were found in *K. pneumoniae* than in *E. coli* as 82, 93, 65,

27, 34, and 37%, respectively. Still, they found no resistance to *K. pneumoniae* in imipenem. In cases of doxycycline, chloramphenicol, and sulfamethoxazole-trimetoprim had higher percentages of resistance in *E. coli* than *K. pneumoniae*, as 26, 96, and 92%, respectively (Figure 1).

The prevalence of *E. coli* and *K. pneumoniae* was 8.62% (10/116); 90% (9/10) of isolates were recovered from antibiotic-free broiler farms and 10% (1/10) from an antibiotic-used broiler farm. The AMR phenotype characteristics of all simultaneous isolates were presented, including ESBL production and AMR profiles. The proportion of ESBL-producing *E. coli* was 90% (9/10), but none of *K. pneumoniae* isolates was ESBL-production. The highest AMR profile for *E. coli* was C-E-SXT (50%, 5/10) and AMP-E-DO-ENR (20%, 2/10) for *K. pneumoniae* (Table 2).

Table 1 Distribution of *E. coli* and *K. pneumoniae* recovered from broiler chickens in Thailand

Bacterial strains	No. of samples of isolates (%)	No. of ESBL-producing isolates (%)	No. of MDR isolates (%)
E. coli	26/116 (22.41)	25/26 (96.15)	26/26 (100)
K. pneumoniae	29/116 (25)	2/29 (6.89)	26/29 (89.65)

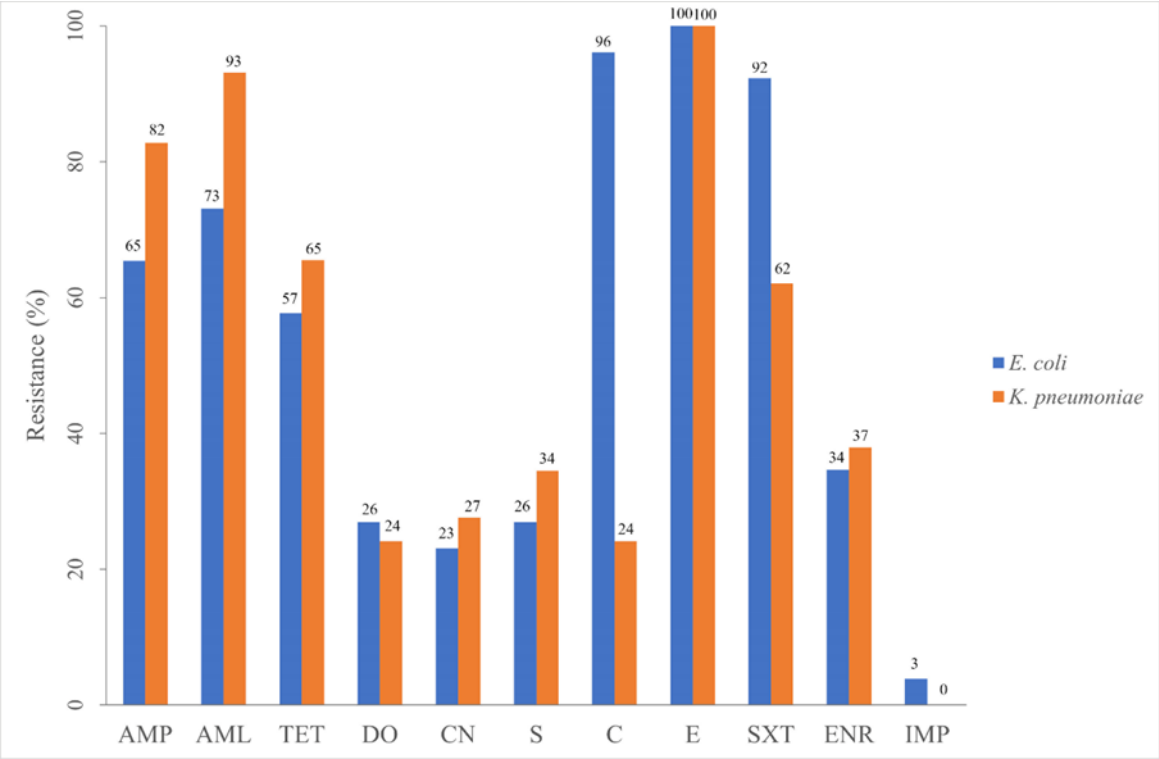

Figure 1 Percentage (%) of resistance against antimicrobial agents total *E. coli* isolates (n=26) and *K. pneumoniae* (n=29). AMP and AML (representative of Penicillins); TET and DO (representative of Tetracycline); CN and S (representative of Amino-glycoside); C (representative of Phenical); E (representative of Monobactam); SXT (representative of Folate pathway inhibitor); ENR (representative of Fluoroquinolone); IMP (representative of Cabapenem).

Table 2 Simultaneous isolates of *E. coli* and *K. pneumoniae* and their characteristics.

Isolate	ESBL-producing *E. coli*	ESBL-producing *K. pneumoniae*	AMR Pattern	
			E. coli	*K. pneumoniae*
SY329	Positive	Negative	AMP-C-CN-E-TET-SXT	AML-E-S
SY331	Positive	Negative	AML-C-E-DO-ENR-SXT	AMP-E-TET-SXT
SR458	Negative	Negative	AMP-C-E-DO-ENR-SXT	AML-CN-E-ENR
VC489	Positive	Negative	AMP-C-CN-E-TET-ENR	AMP-C-CN-E-ENR-SXT
JL503	Positive	Negative	AMP-C-E-TET-ENR-S-SXT	AMP-E-DO-ENR
JL514	Positive	Negative	C-E-SXT	AMP-E-DO-ENR-SXT
JL523	Positive	Negative	C-E-SXT	AMP-E-DO-ENR-S-SXT
JL524	Positive	Negative	C-E-SXT	AMP-E
JL525	Positive	Negative	C-E-SXT	AMP-C-E-DO-SXT
JL529	Positive	Negative	C-E-SXT	AMP-E-DO-ENR

This study showed a high (22.41%) prevalence of *E. coli*, where 21.55% (25/116) produced ESBL. Indeed, all isolates (100%) were multidrug-resistant. This is lower than that of other similar studies carried out in Thailand. Tansawai *et al.* (2019) reported a 25.9% prevalence of ESBL-*E. coli* from backyard poultry fecal samples. Rodroo *et al.* (2020) also reported the occurrence of 96.2% MDR *E. coli* in a northern province in Thailand. However, another study in China showed that *E. coli* strains had a high MDR prevalence (87.88%) (Li *et al.*, 2022). Also, vertical transmission of ESBL-*E. coli* down the poultry production pyramid was previously reported from different geographical locations (Bastidas-Caldes *et al.*, 2023b). The results show that 1.72% (2/116) of *K. pneumoniae* isolates from broiler chickens were ESBL producers, and 89.65% of our isolates were multidrug-resistant. Similar results (20%) were obtained from broiler chickens in Indonesia (Safika *et al.*, 2022); according to a similar study, All isolates of chicken origin in this country were identified as MDR (Hayati *et al.*, 2019). Antimicrobial susceptibility tests were performed against nine different chemical classes. This study revealed that the highest *E. coli* and *K. pneumoniae* resistance rate was recorded for erythromycin (100%). This finding follows Rahman *et al.* (2020), who reported that 89.5% of *E. coli* isolated from chicken meat in Bangladesh exhibited resistance to erythromycin. In addition, Safika *et al.* (2022) demonstrated high resistance rates (100%) of *K. pneumoniae* to erythromycin in Indonesia. Erythromycin is one of the most widely used antibiotics in food animals, which might result in antimicrobial resistance among the isolates. *E. coli* tested in this study was highly resistant to chloramphenicol (96%) and 93% to Sulfamethoxazole-trimethoprim, consistent with Martinez-Alvarez *et al.* (2022), who reported the highest resistance rate of ESBL- *E. coli* from broiler farm environment to chloramphenicol (100%). Similarly, Liaqat *et al.* (2022) showed that *E. coli* strains in Pakistan were entirely resistant (100%) to sulfamethoxazole-trimethoprim. Moreover, high resistance rates of *K. pneumoniae* to the β-lactam group (93% for amoxycillin and 82% for ampicillin) were shown in this study. Resistance to Amoxycillin and Ampicillin was lower than previous results in Poland and Indonesia. Kowalczyk *et al.* (2022) and Hayati *et al.* (2019) found high resistance (100%) to Amoxycillin and Ampicillin. The aminoglycoside group noted low resistance of *E. coli* (23% for gentamicin and 26% for streptomycin) and *K. pneumoniae* (27% for gentamicin and 34% for streptomycin). Regardless, these findings disagreed with a previous report in Nigeria, which showed higher resistance (100%) to Gentamicin for *E. coli* and 46.66% for *K. pneumoniae*. Our survey focused on the coexistence of *E. coli* and *K. pneumoniae* isolates from the same chicken because transferable plasmids harboring resistance genes are possible among multiple bacteria species. Interestingly, none of *K. pneumoniae* was ESBL-production, whereas 90% of ESBL- *E. coli* was observed. However, some studies suggest otherwise; the transfer of mobile drug-resistance genes might contribute to the proliferation of ESBLs (Bastidas-Caldes *et al.*, 2023a), and they can cross between animals and humans through chicken meat (Projahn *et al.*, 2019). In the present study, bacterial species were resistant to multiple antibiotics. Three simultaneous isolates demonstrated overlapping AMR patterns: AMP-C-CN-E-ENR for VC489, AMP-E-ENR for JL503, and C-E-SXT for JL525. This also aligns with data from a previous study investigating how quinolone resistance could be transferred by conjugating *E. coli* and *K. pneumoniae* isolates from the same specimen (Quan *et al.*, 2023). We found both strains from VC489 and JL503 were resistant to ampicillin. In a similar study conducted in Nigeria, the highest prevalence of *E. coli* and *K. pneumoniae* were resistant to Ampicillin (Jesumirhewe *et al.*, 2023). Additionally, most isolates exhibited sulfamethoxazole-trimethoprim resistance; a similar pattern was previously reported in clinical samples from Poland (Majewski *et al.*, 2021). The spread of multidrug-resistant bacteria is a major problem in human and veterinary medicine. In conclusion, our current study characterized the high prevalence of *E. coli* and *K. pneumoniae* associated with ESBL and MDR strains among broiler chickens in Thailand. Most *E. coli* isolates were ESBL-producing bacteria, and All *E. coli* were classified as MDR. Few *K. pneumoniae* were ESBL-production, but most were MDR-*K. pneumoniae*. All *E. coli* and *K. pneumoniae* were resistant to Monobactam (Erythromycin). Resistance to Fluoroquinolone (Enrofloxacin) was mostly identified in simultaneous *E. coli* and *K. pneumoniae* isolates. The results show that broiler chickens might be an important source of antibiotic-resistant pathogenic bacteria. Moreover, further conjugation studies are required to determine the exchange of the genetic elements, especially antibiotic resistance gene transmission during coinfection.

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