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Research article

Antibiotic resistance of *Escherichia coli* serotype O8 and O9 isolated from cattle in the Mekong Delta, Vietnam

Nguyen Khanh Thuan[1,*], Nguyen Thanh Lam[1], Nguyen Tran Phuoc Chien[1], Ly Thi Lien Khai[1], Tran Ngoc Bich[1]

[1]Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Can Tho 900000, Vietnam

Abstract

This study was conducted to clarify the antimicrobial susceptibility and antibiotic resistance genes of *Escherichia coli* O8 and O9 isolated from 244 cattle feces collected from December 2021 to March 2022 in the Mekong Delta, Vietnam. *E. coli* was isolated from the feces following the method of TCVN 7686:2007 (ISO16654:2001), and serotypes O8 and O9 were identified by the PCR method. *E. coli* O8 and O9 were identified at a relatively high rate, with 15.98% and 8.20%, respectively. The disc diffusion method was applied to determine the antimicrobial susceptibility of those *E. coli* strains against thirteen antibiotics following the guidelines of the Clinical and Laboratory Standard Institute. Those *E. coli* O8 and O9 strains showed high susceptibility to amikacin (98.31%), doxycycline (96.61%), ofloxacin (94.92%), and levofloxacin (93.22%). However, those isolates exhibited resistance against ampicillin (47.46%), streptomycin (44.07%), and tetracycline (42.37%). Of those 59 *E. coli* strains, 35 strains (59.32%) were multiple resistant against two to twelve antibiotics with 25 resistant patterns. The pattern of Am+Ac+Co+Sm+Te+Cl was the most common type (6.78%). The prevalence of six antibiotic-resistance genes was determined by using PCR. Gene *tetA* was the most prevalent gene (66.10%), while *cat1* was the least one (5.08%). Forty-five *E. coli* O8 and O9 strains (76.27%) harbored from two to four antibiotic resistance genes, and the pattern of *strA+tetA+sulII* was detected at the highest rate (23.73%). Therefore, the prevalence and antibiotic resistance of *E. coli* O8 and O9 should be controlled to protect cattle and human health.

Keywords: Antibiotic resistance, Cattle, *Escherichia coli* O8 and O9, Mekong Delta, Resistance genes.

***Corresponding author:** Nguyen Khanh Thuan, Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Can Tho 900000, Vietnam. Tel: +84919783065. Email: nkthuan@ctu.edu.vn.

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INTRODUCTION

Escherichia coli (*E. coli*), inhabitants of the gastrointestinal tract, are associated with diarrhea and a range of extra-intestinal diseases in humans and animals (West et al., 2007). Several *E. coli* serotypes were considered important pathogens causing diarrhea and septicemic disease in foals, calves, piglets, and lambs, such as O8, O9, O15, O26, O35, O78, O86, O101, O115, and O119 (Dwight, 2004). Those Enterotoxigenic *E. coli* (ETEC) strains are responsible for the significant loss of neonatal animals with high mortality and morbidity rates (Johnson and Nolan, 2009; Rajkhowa et al., 2009). Moreover, a few *E. coli* O8 and O9 strains belong to the Shiga toxin-producing *E. coli* (STEC) group; they can cause severe foodborne diseases in humans (Kobayashi et al., 2001; El-Jakee et al., 2012; Cheney et al., 2015; Navarro et al., 2018). However, the prevalence of *E. coli* O8 and O9 in cattle in Vietnam has not been reported. Therefore, there is a limit to diagnosing and treating cattle and humans effectively.

Moreover, *E. coli* is also considered an indicator of antibiotic resistance levels in Gram-negative bacteria. The misuse of antibiotics can stimulate the amplification of resistant strains in the intestinal tract, whether those strains are direct treatment targets or members of the commensal gut fauna (Cheney et al., 2015). The resistance genes can be transferred between bacterial species via plasmids, transposons, and integrons. Then, humans could be infected with those resistant zoonotic bacteria via the food chain or through contact with infected animals, feces, or contaminated environments (EFSA - ECDC, 2022). Sorum and L'Abee-Lund (2002) reported that the same use of antibiotics in animal husbandry and humans is responsible for the antibiotic-resistant strains occurring in humans. In previous reports, it exhibited that *E. coli* could carry several antibiotic resistance genes and transfer among bacterial species such as fluoroquinolone (*qnr*), tetracycline (*tetA*), ampicillin (*CITM*), sulfonamide (*sulII*), chloramphenicol (*cat1*), and aminoglycosides (*aadA1*) (Momtaz et al., 2013; Bai et al., 2016; Jaja et al., 2020). To aid the reduction of antimicrobial misusage, understanding the prevalence of *E. coli* serotypes and acquiring antimicrobial resistance is of primary concern. The knowledge of antimicrobial resistance patterns is essential for treating colibacillosis and serves as a guide for implementing public health interventions.

Hence, the prevalence of *E. coli* serotypes O8 and O9 in cattle should be a concern. These serotypes affect cattle health and are associated with human diseases. This study determined the prevalence and antibiotic resistance of those serotypes in cattle to enhance antibiotic-using management in animal husbandry and human public health in the Mekong Delta.

MATERIALS AND METHODS

Isolation of *Escherichia coli* in cattle feces

A total of 244 feces samples were collected randomly from healthy cattle raised in small-scale farms in the Mekong Delta, Vietnam, from December 2021 to March 2022. The cattle were of all ages, breeders, and genders, including beef cattle (n = 168) and dairy cattle (n = 76). Those fresh feces (100 g/sample) were taken instantly after the cattle had excreted using sterilized gloves. Then,

those feces were put in sterilized plastic bags separately and kept in a cool box to transport to the laboratory within one day.

At the laboratory, 25 g of feces was incubated in 250 mL of buffered peptone water (BPW, Merck, Germany) at 37°C for 24 h. Then, one loopful of enriched broth was cultured on MacConkey agar (MC, Merck, Germany) and further incubated at 37°C for 24 h to isolate *E. coli* in feces. After that, suspicious *E. coli* isolates on MC were picked up and subcultured on nutrient agar (NA, Merck, Germany) to examine the biochemical tests. The procedure followed the guidelines of TCVN 7686:2007 (ISO16654:2001) standard (Vietnam) and Barrow and Feltham (2003).

For further experiments, those identified E. coli isolates were subcultured on trypticase soy agar (TSA, Merck, Germany).

Identification of *E. coli* serotypes O8 and O9.

The PCR assay was used to identify serotypes O8 and O9 of *E. coli* isolates. DNA of *E. coli* isolates on TSA were extracted by the heat shocking method following the guidelines of Soumet et al. (1994).

The ingredients of one PCR mixture contained Master Mix 2X (Promega, USA) (12.5 µL); forward and reverse primers (IDT, USA) at 10 µM (0.5 µL/primer); distilled water (9.5 µL), and DNA template (2.0 µL). The primer sequences and thermocycling PCR reactions followed the previous reports. For detecting *E. coli* serotype O8, the primers were 5'-CCAGAGGCATAATCAGAAATAACAG-3' (forward) and 5'-GCAGAGTTAGTCAACAAAAGGTCAG-3' (reverse) (Li et al., 2010). For detecting *E. coli* serotype O9, the primers were 5'-TGGGTGTTAAAAGACATCAA-3' (forward) and 5'-CCCAGAAATCCATGCTC-3' (reverse) (Yadegari et al., 2019).

In this study, *E. coli* serotype O8 and O9 strains, previously isolated from cattle in some provinces of the Mekong Delta, were used as the positive controls. Those strains were kept in the Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Vietnam. The PCR products were electrophoresed in 1.5% agarose gels at 50V for 60 minutes. Then, gels were dyed in ethidium bromide, and figures were captured under UV to detect the results.

Antimicrobial susceptibility testing

The *E. coli* strains were examined for their antimicrobial susceptibility following the method of the Kirby-Bauer disk diffusion test (Bauer et al., 1966). The results of the antibiotic resistance zone were determined following the standard of Clinical Laboratory Standards Institute procedure M02-M07 (CLSI, 2022). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as controls.

Based on the results of antibiotics used in cattle in the Mekong Delta previously by Nguyen et al. (2022) and recommendations in the guidelines of CLSI (2022), a total of thirteen antibiotics were examined in this study. The antibiotic discs were supplied by Nam Khoa Biotek Ltd. (Vietnam) including ampicillin (Am, 10 µg), amoxicillin/clavulanic acid (Ac, 20/10 µg), cefuroxime (Cu, 30 µg), ceftazidime (Cz, 30 µg), colistin (Co, 10 µg), gentamycin (Ge, 10 µg), amikacin (Ak, 30 µg), streptomycin (Sm, 10 µg), tetracycline (Te, 30 µg), doxycycline (Dx, 30 µg), chloramphenicol (Cl, 30 µg), levofloxacin (Lv, 5 µg), ofloxacin (Of, 5 µg).

Detection of antibiotic resistance genes by PCR method

The PCR method was also used to detect the prevalence of antibiotic-resistance genes of *E. coli* O8 and O9 isolates. The procedure of the PCR assay was similar to the one described for identifying serotypes of *E. coli* isolates.

In this experiment, a total of six resistance genes against different groups of antibiotics were examined, including aminoglycoside (*strA*), beta-lactam (*blaTEM*), chloramphenicol (*cat1*), quinolone (*qnrA*), sulfonamide (*sulII*), tetracycline (*tetA*). Those primer sequences and PCR conditions were set up following the research of Boerlin et al. (2005), Cattoir et al. (2007), Jouini et al. (2007), Gow et al. (2008), Van et al. (2008), and Abdelgader et al. (2018). The positive controls were *E. coli* strains harboring antibiotic resistance genes, isolated from animals in the Mekong Delta previously. Those strains were kept in the Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Vietnam.

Data analysis

Data were inserted and expressed as percentages using Microsoft Excel software (Microsoft, USA). Then, the statistical analysis was performed using the Statistical Package (the Social Sciences statistical package), version 7.1 (IBM, USA), with a statistical significance level of 95%.

RESULTS

Prevalence of *E. coli* serotypes O8 and O9 in cattle

E. coli was detected in all 244 feces samples (100%). Of 244 positive *E. coli* samples, the prevalence of *E. coli* serotypes O8 and O9 were 15.98% and 8.20%, respectively (Table 1). The rest of the *E. coli* strains could not be determined serotypes in this study.

Table 1 The prevalence of *E. coli* O8 and O9 in cattle in the Mekong Delta

Cattle	No. of examined samples	*E. coli* O8		*E. coli* O9	
		No. of positive samples	Percentage (%)	No. of positive samples	Percentage (%)
Beef cattle	168	31	18.45	13	7.74
Dairy cattle	76	8	10.53	7	9.21
Total	244	39	15.98	20	8.20

There was no significant difference in the prevalence of E. coli O8 and O9 in beef and dairy cattle (P>0.05)

E. coli O8 and O9 were present at 18.45% and 7.74%, respectively, in beef cattle, while those serotypes were 10.53% and 9.21% in dairy cattle. However, there was no significant difference in the prevalence of E. coli O8 and O9 in both beef and dairy cattle ($P>0.05$).

Antimicrobial susceptibility

The results exhibited that *E. coli* O8 and O9 were relatively susceptible to all examined antibiotics (Table 2), especially to amikacin (98.31%), doxycycline (96.61%), ofloxacin (94.92%), and levofloxacin (93.22%). However, those strains showed resistance to ampicillin (47.46%), streptomycin (44.07%), and tetracycline (42.37%). There was no significant difference in the antibiotic resistance rates between *E. coli* O8 and O9 ($P>0.05$).

Table 2 Antibiotic susceptibility of the *E. coli* O8 and O9 isolated from cattle (n = 59)

Antibiotics	Susceptibility		Resistance	
	No. of isolates	Percentages (%)	No. of isolates	Percentages (%)
Ampicillin	31	52.54	28	47.46
Streptomycin	33	55.93	26	44.07
Tetracycline	34	57.63	25	42.37
Amoxicillin/clavulanic acid	40	67.80	19	32.20
Colistin	41	69.49	18	30.51
Chloramphenicol	42	71.19	17	28.81
Gentamicin	51	86.44	8	13.56
Cefuroxime	53	89.83	6	10.17
Ceftazidime	53	89.83	6	10.17
Levofloxacin	55	93.22	4	6.78
Ofloxacin	56	94.92	3	5.08
Doxycycline	57	96.61	2	3.39
Amikacin	58	98.31	1	1.69

There was no significant difference in the antibiotic resistance between E. coli O8 and O9 (P>0.05)

Of the 59 *E. coli* O8 and O9 isolates examined, thirty-five isolates(59.32%) were multi-resistant from two to twelve antibiotics examined, andtwenty-five antibiotic resistance patterns were obtained. Among them, theantibiotic resistance pattern of Am+Ac+Co+Sm+Te+Cl was the most commontype (6.78%), and ampicillin (Am) was present in most antibiotic resistance patterns (Table 3). Moreover, except for amikacin in this study, one *E. coli* O8 isolate showed resistance to twelve antibiotics.

Table 3 Antibiotic resistance patterns of *E. coli* O8 and O9 isolates (n = 59)

No. of antibiotics	Antibiotic resistance pattern	No. of patterns	No. of isolates	Percentage (%)
2	Ac+Cu	4	1	1.69
	Am+Te		1	1.69
	Co+Ge		1	1.69
	Te+Dx		1	1.69
3	Ac+Cu+Co	7	1	1.69
	Am+Ac+Sm		2	3.39
	Am+Sm+Te		1	1.69
	Am+Cu+Co		2	3.39
	Am+Ac+Sm		1	1.69
	Am+Sm+Cl		1	1.69
	Co+Ak+Lv		1	1.69
4	Ac+Sm+Te+Cl	4	2	3.39
	Am+Ac+Sm+Te		1	1.69
	Am+Co+Ge+Sm		1	1.69
	Ge+Te+Lv+Of		1	1.69
5	Am+Ac+Sm+Te+Cl	6	3	5.08
	Am+Cz+Co+Sm+Cl		1	1.69
	Am+Ac+Co+Sm+Te		1	1.69
	Am+Cz+Sm+Te+Cl		2	3.39
	Am+Ge+Cl+Lv+Of		1	1.69
	Am+Ac+Ge+Sm+Te		1	1.69
6	Am+Cz+Cu+Co+Sm+Te	2	1	1.69
	Am+Ac+Co+Sm+Te+Cl		4	6.78
7	Am+Ac+Co+Ge+Sm+Te+Cl	1	2	3.39
12	Am+Ac+Cz+Cu+Co+Ge+Sm+Te+Dx+Cl+Lv+Of	1	1	1.69
Total		25	35	59.32

Ac: amoxicillin/clavulanic acid; Am: ampicillin; Cz: ceftazidime; Cu: cefuroxime; Co: colistin; Ge: gentamicin; Ak: amikacin; Te: tetracycline; Cl: chloramphenicol; Lv: levofloxacin; Dx: doxycycline; Sm: streptomycin; Of: ofloxacin

Prevalence of antibiotic resistance genes

All 6 antibiotic resistance genes were detected in 59 *E. coli* O8 and O9 isolates. Of the 6 examined genes, gene *tetA* was prevalent at the highest rate (66.10%), followed by *sulII* (38.98%) and *strA* (37.29%) (Table 4). There was no significant difference in the antibiotic resistance genes between *E. coli* O8 and O9 ($P>0.05$) in this study. The high prevalence of *tetA* and *strA* genes reflected the high resistance of *E. coli* O8 and O9 to tetracycline and streptomycin in the antimicrobial susceptibility test.

Table 4 Prevalence of antibiotic resistance genes in *E. coli* O8 and O9 (n = 59)

Genes	No. of positive isolates	Percentage (%)
tetA	39	66.10
sulII	23	38.98
strA	22	37.29
blaTEM	16	27.12
qnrA	8	13.56
cat1	3	5.08

**The prevalence of antibiotic-resistance genes between E. coli O8 and O9 (P>0.05)*

Of 59 *E. coli* O8 and O9 isolates, 14 isolates did not harbor or harbor one antibiotic resistance gene. In contrast, forty-five isolates (76.27%) were obtained from two to four antibiotic resistance genes, and 10 combined patterns of antibiotic resistance genes were created (Table 5). The most prevalent pattern was *strA+tetA+sulII* (23.73%), followed by *tetA+sulII* (16.95%) and *strA+tetA* (15.25%). Those combined patterns also indicated the ability of *E. coli* O8 and O9 isolates to be resistant to multiple antibiotics in this study.

Table 5 The combined patterns of antibiotic-resistance genes in *E. coli* O8 and O9 (n = 59)

No. of genes	Gene patterns	No. of patterns	No. of isolates	Percentage (%)
2	*strA+tetA*	6	9	15.25
	tetA+sulII		10	16.95
	tetA+qnrA		2	3.39
	strA+sulII		3	5.08
	strA+qnrA		1	1.69
	tetA+blaTEM		2	3.39
3	*strA+tetA+sulII*	3	14	23.73
	tetA+sulII+qnrA		1	1.69
	sulII+qnrA+cat1		1	1.69
4	*strA+tetA+sulII+qnrA*	1	2	3.39
Total		10	45	76.27

DISCUSSION

E. coli is a typical intestinal fauna in most animals and maintains intestinal physiology (El-Jakee et al., 2012); therefore, *E. coli* was quite detected in 100% of feces samples in this study. *E. coli* includes several serotypes that are pathogenic or non-pathogenic strains. In this study, *E. coli* O8 and O9 isolates were present at a high proportion of 15.98% and 8.20%, respectively. Those serotypes represented Enterotoxigenic *E. coli* and Shiga-toxin *E. coli* groups that could cause severe diseases in cattle and humans. Liu et al. (2010) reported *E. coli* strains belonging to serogroups O8, O9, O15, O26, O35, O78, O86, O101, O115, and O119 were commonly associated with septicemia or

diarrhea in calves and became a significant threat to the cattle farming. *E. coli* O8 was one of the predominant serovars (18.46%) isolated from diarrhoeic calves and lambs in India (Manzoor et al., 2015), and *E. coli* O9 was detected frequently in cattle in Mexico (Navarro et al., 2018). In humans, Rodas et al. (2011) isolated *E. coli* O8 from diarrhea children in hospitals in Bolivia at a relatively high rate (14.0%). It indicated that cattle might be a natural reservoir of pathogenic *E. coli* in the Mekong Delta, Vietnam.

Moreover, *E. coli* O8 and O9 isolates were detected in both dairy and beef cattle in this study. In young cattle, those serotypes are agents causing diarrhea, bacteremic blood in neonatal calves (Ghanbarpour and Nazem, 2010), or fatal enteritis in calves (Feuerstein et al., 2022). In addition, those serotypes could also cause mastitis in dairy cattle (Spînu et al., 2012). Further, cattle and sheep were determined as natural reservoirs of Shiga-toxin *E. coli*, including some *E. coli* O8 serotypes causing foodborne diseases in humans in Japan and China (Kobayashi et al., 2001; Liu et al., 2020). It showed that the prevalence of *E. coli* O8 and O9 in cattle could become a risk to animal health and a source of disease transmission to humans. Therefore, further research should be focused on controlling the contamination and infection of *E. coli* O8 and O9 between cattle and humans.

Although *E. coli* O8 and O9 were relatively sensitive to most examined antibiotics in this study, those isolates exhibited resistance to ampicillin, streptomycin, and tetracycline, which have been used in animal production in the Mekong Delta for a long time. Antibiotics were not used frequently in raising cattle. Hence, there were numerous reasons for this resistance. Long-term antibiotic use could improve bacteria's antibiotic-resistant ability (da Costa et al., 2007). Antimicrobial substances or compounds may be licensed for use in humans and animals. The uncontrolled use of an antibiotic could trigger the development of antimicrobial resistance in bacteria (WHO, 2016). A report in Bolivia indicated that a high level of multi-resistance of ETEC, including *E. coli* O8, might emerge to ampicillin, tetracycline, and trimethoprim-sulfamethoxazole, which had been widely used in this country (Rodas et al., 2011). In Germany, Feuerstein et al. (2022) reported that *E. coli* isolated from calves showed high resistance to tetracycline (61.9%), spectinomycin (62.2%), and ampicillin (69.7%). A previous report indicated that EHEC isolated from cattle in the Mekong Delta were also highly resistant to ampicillin (64.10%) and colistin (53.85%) (Nguyen et al., 2022). Thus, the use of antibiotics in animals, including cattle, must be managed strictly to prevent the increasing and spreading of antibiotic-resistant strains in environmental farming and the human community.

On the other hand, those *E. coli* O8 and O9 isolates showed multi-drug resistance. It indicated a potential risk in treating diseases for cattle and humans. The accumulation of antibiotic-resistance ability in *E. coli* strains has been reported. In a previous report, *E. coli* originating from calves could show multi-drug resistance significantly (84.2%) to most veterinary antibiotics used in Germany (Feuerstein et al., 2022). Wani et al. (2013) reported that *E. coli* originating from calves and lambs in India showed multiple resistance against ampicillin, co-amoxiclav, co-trimoxazole, and cefalosporins. The multidrug resistance of those isolates could be the consequence of the indiscriminate use of antibiotics in clinical practice there. Sharma et al. (2017) also reported that *E. coli*, including serotypes O8 and O9, showed multiple resistance to

amoxicillin, ceftriaxone, cephalexin, cloxacillin, enrofloxacin, and gentamicin as well as the multi-drug resistance was observed in 84.6% in total of *E. coli* isolates. Hence, the prevalence of *E. coli* O8 and O9 isolates in this study that showed multiple antibiotic resistance in cattle was a concerning issue. In the intestinal fauna, those strains could transfer their antibiotic-resistance patterns to other bacteria, causing failure in treatment. Among the examined antibiotics, ampicillin (Am) was determined as the most resistant antibiotic found in most resistance patterns, followed by streptomycin (Sm) and tetracycline (Te). It could be due to the long-term use of ampicillin in animal husbandry that *E. coli* strains have launched a natural resistance to those antibiotics.

A total of six antibiotic genes were examined, and the tetA gene was detected at the highest frequency (66.10%). It revealed that *E. coli* O8 and O9 could have harbored this gene for a long time and expressed resistance. Although those *E. coli* isolates were highly resistant to ampicillin and streptomycin, the prevalence of related genes (*blaTEM*, *strA*) was not significant. The expression of resistance might rest on environmental conditions or genetic characteristics of this gene. Even though those antibiotics were not used or were non-resistant, the prevalence of multiple antibiotic-resistant genes in bacterial populations could still exist (Bengtsson-Palme, 2018). Moreover, the prevalence of those antibiotic resistance genes could also be related to gene-coding plasmids or other genetic materials (Maynard et al., 2003; Yamamoto et al., 2014). Anes et al. (2020) clarified the molecular characteristics of bovine multi-drug resistant *E. coli* and indicated different plasmid replicon types for the horizontal transfer of antimicrobial resistance.

In this study, those *E. coli* O8 and O9 isolates did not show various combined antibiotic-resistance gene patterns. This could be due to the limited number of resistant genes observed; however, the results demonstrated that those isolates could be a tank of combined antibiotic resistance genes that obstruct treatment effectiveness for cattle diseases. Those isolates could also become hazards to human health if humans were infected with those strains from cattle via direct or indirect contact. Among most combined patterns, the regular prevalence of *strA* and *tetA* genes was compatible with the antimicrobial susceptibility test results. It indicated that those *E. coli* O8 and O9 isolates harbored antibiotic resistance genes and expressed their resistance. Those antibiotic resistance genes combined and produced different patterns distributed in treatment obstacles of colibacillosis in cattle and humans in the Mekong Delta, Vietnam. Penders et al. (2013) indicated that the presence of multi-drug resistant strains as part of the intestinal fauna of cattle could cause difficulty in treating extra-intestinal infections caused by *E. coli* strains that received antibiotic-resistance genes through horizontal transfer in the intestinal environment. Thus, the management of antibiotic use is essential to prevent the formation of antibiotic-resistant *E. coli* strains.

CONCLUSIONS

There was a high prevalence of *E. coli* O8 and O9, which were potential pathogens that caused diseases in cattle and humans in the Mekong Delta. Those serotypes were highly prevalent in both beef and dairy cattle. Although *E. coli* O8 and O9 isolates still exhibited sensitivity to all examined antibiotics,

they showed relative resistance to ampicillin, streptomycin, and tetracycline with diverse antibiotic resistance patterns obtained. Moreover, those serotypes harbored multi-antibiotic resistance genes, which expressed resistance to many antibiotics used in treating animal and human diseases. Therefore, the prevalence and antibiotic resistance of *E. coli* O8 and O9 should be controlled to protect cattle and human health in the Mekong Delta, Vietnam.

AUTHOR CONTRIBUTIONS

This work was conducted with the contribution of all authors. **Nguyen Khanh Thuan, Nguyen Thanh Lam, and Nguyen Tran Phuoc Chien** designed the experimental procedures. **Nguyen Khanh Thuan**, **Nguyen Thanh Lam,** and **Nguyen Tran Phuoc Chien** performed the experiments. **Nguyen Khanh Thuan**, **Nguyen Thanh Lam**, **Nguyen Tran Phuoc Chien, Ly Thi Lien Khai,** and **Tran Ngoc Bich** interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

We have no conflict of interest.

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