Original Article

Identification of genotype and phenotype of antimicrobial resistance of *Escherichia coli* isolates from pigs in southern Vietnam

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Abstract

Escherichia coli is a primary reservoir of antimicrobial resistance, known chiefly for the container of AMR-encoding genes (ARGs), and poses potential risks to human and animal health. This study investigated AMR phenotypes and ARGs in 90 *E. coli* isolates from different pig groups in 10 farms in southern Vietnam. The minimum inhibitory concentration (MIC) of 19 common antimicrobial agents was determined, and polymerase chain reaction (PCR) was used to investigate seven ARGs (*blaTEM, aadA1, strA, dfrA12, sul3, cmlA and tetA*). Cohen's kappa statistic (κ) was applied to assess the concordance between phenotypic and genotypic profiles. A total of 81.1% of *E. coli* isolates were multi-drug resistant (MDR). The amphenicol class accounted for the highest resistance (100% isolates), followed by the tetracycline class (97.8%), the quinolones and penicillin classes (85.6% each), sulfonamides (67.8%) and aminoglycosides (63.3%). A greater proportion of isolates from weaner pigs showed resistance to multi-antibiotics (43.0%), followed by growers (39.5%) and finishers (36.3%), although the difference was not significant ($P>0.05$). The prevalence of ARGs was greatly variable and was highest for *aadA1* (98.9%), *cmlA* (98.9%), bla_{TEM} (97.8%), *dfrA12* (97.8%), *tetA* (97.8%), *sul3* (97.8%) and *strA* (83.3%). No significant correlation between ARGs and phenotypic resistance was identified. The results indicate a great diversity of genotypic and phenotypic AMR profiles in pig *E. coli* isolates. The lack of correlation might be a reflection of additional genes encoding the observed genotypic profiles or the presence of non-plasmid mediated resistance in many cases.

Keywords: antimicrobial resistance, *Escherichia coli*, genotype, multi-drug resistance, phenotype, pigs

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Introduction

Antimicrobials are widely used in animal food production for the purposes of disease prevention and treatment. In some countries, antimicrobials are added to commercial feed rations to increase growth and productivity (Pagel and Gautier, 2012). Although there are wide-ranging benefits to their strategic use, excessive or inappropriate antimicrobial use (AMU) in animal production encourages the development of antimicrobial resistance (AMR) in organisms (Pagel and Gautier, 2012). In Vietnam, the pig species is quantitatively the target of the greatest AMU, reaching 41.7% of total AMU (3842 tons annually) (Carrique-Mas *et al.*, 2020).

The natural habitat of *Escherichia coli* is the alimentary and genital tract of pigs (Jeffrey *et al.*, 2012). Commensal *E. coli* is commonly used to monitor AMR in surveillance systems because of its ubiquity and its capacity to develop AMR following AMU. *E. coli* has been considered an important bacteria that displays great evidence for a drug-resistant link between animal sources and transmission to humans, especially when the antibiotic resistance to critically important antimicrobials (CIA) is widespread for human medicine, such as polymyxins, quinolones or 3rd - 4th generation cephalosporins (WHO, 2019). AMR-encoding genes can be acquired by mutation or horizontal transfer through plasmid via numerous pathways, such as direct contact, contact with animal secretions or via food and water (Paulo *et al.*, 2013). Numerous studies have described a vast range of AMR types encoded by ARGs in *E. coli* from different geographical regions, e.g. *E. coli* possesses β-lactams resistance $bla_{\text{TEM-1}}$ (Chah *et al.*, 2010), tetracycline resistance *tetA* (Schmidt *et al.*, 2001), sulfonamide resistance *sul3, dfrA12* (Kozak, 2009), aminoglycoside resistance *aadA1, strA* ((Maria *et al.*, 2011; Schmidt *et al.*, 2001), and phenicol resistance *cmlA* (Keyes, 2000).

In this study, we aimed to assess the antimicrobial resistant phenotypes and genotypic prevalence, and their correlation from porcine *E. coli* isolates in southern Vietnam. The results describing levels of AMU and the most common encoding genes should help to improve awareness among producers and veterinary drug sellers on the undesirable consequences of excessive AMU.

Materials and Methods

Study design: A total of 90 fecal samples were collected from 10 different farms in southern Vietnam from July to September 2019. There was a wide range of pig farms from the aspect of management methods, herd size (small, medium and large-scale), commercial or backyard farms. From each farm, samples were conducted stratified by age, including piglets (3-10 weeks of age), growers (10-15 weeks of age) and finishers (15-22 weeks of age). From each age-group, 3 pooled fecal samples were collected; each pooled fecal sample (25g) consisted of fecal material collected from 3 different pigs of the same age-group.

Fecal samples were collected directly from the rectum, labeled on separate falcon tubes (50 ml) and stored in the ice box (2 - 8^{o}C) before submitting to the microbiology laboratory of the Veterinary Hospital, Nong Lam University for isolation.

Sample inoculation and isolation of E. coli: The fecal sampling dilutions at 10^{-3} - 10^{-6} concentration in 0.9% saline (NaCl) were inoculated on to tryptic soy agar (TSA, HiMedia, India) containing 5% sheep blood and Eosin-Methylene Blue agar (EMB, HiMedia, India). The specific pure colonies of *E. coli* were confirmed using standard biochemical tests IMViC (HiMedia, India) including Indole, Methyl Red, Voges-Proskauer and Citrate.

Identified E. coli isolates were used for AMR phenotypes and ARGs determination.

Plasmid DNA extraction and PCR protocol: The procedure for plasmid extraction and purification was carried out in accordance with the guidelines of alkaline lysis method from Birnboim *et al.*, 1979 and the plasmid DNA kit (Thermo, USA). 600 μl Luria-Bertani (LB) broth was prepared before adding 100 μl cell lysis buffer and 350 ml neutralization solution. The tube was then centrifuged for 20 seconds at 13,000 rpm. The clear supernatant was transferred to a freshly labeled 1.5 ml tube.

The extracted DNA was then screened for seven ARGs using referenced specific primers (Table 1), in 25 μl PCR reaction (12 μl go taq master mix (GoTaq® Green Master Mix: Cat#M7122; Promega, USA), 0.5 μl forward primer, 0.5 μl reverse primer, 3 μl template DNA and 9 μl PCR water). The amplified conditions for PCR reaction were 94°C for 10 minutes; 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 90 seconds, replicated for 30 cycles; and then finally extended at 72°C for 10 minutes. Three microliters of PCR products were mixed with gelled DNA stain then analyzed by electrophoresis in a 1% (weight/volume) agarose gel in 1X Tris-Boric-EDTA (TBE). A 1 Kb Plus DNA ladder (Invitrogen) was used as the molecular weight marker to indicate the specific sizes of the PCR products.

Determination of phenotypic resistance: The identified isolates of *E. coli* were inoculated on to nutrient broth, refrigerated (2 - 8^{o}C) and transferred to the testing laboratory (KU Veterinary Medicine KPS, Thailand). AMR phenotypes to antibiotics were identified by the method of determining the minimum inhibitory concentration, according to the guidelines of the commercial kit (Vitek-2-system, Global CLSI2014). *E. coli* ATCC 25922 was used as the quality control strain. The VITEK® 2 Gram Negative Susceptibility Card in this project was AST GN65.

A total of 19 commonly used antimicrobials belonging to 11 classes were investigated. According to CIA standard (WHO, 2019), these antimicrobials were categorized into three groups: critically important, highly important and important. Antimicrobials within the critically important category were cephalosporins 3rd and 4th generation (cefovecin, cefpodoxime, ceftiofur); quinolones (enrofloxacin, marbofloxacin); polymyxins (polymyxin B); aminoglycosides (amikacin, gentamicin, tobramycin); carbapenems (imipenem); and penicillins (ampicillin, amoxicillin, amoxicillin/ clavulanic acid, piperacillin). Highly important antimicrobials for human medicine

were amphenicols (chloramphenicol); 1st generation cephalosporins (cephalexin); tetracyclines (tetracycline); sulfonamides (trimethoprim/ sulfamethoxazole). Nitrofuran derivatives (nitrofurantoin) are termed as an important antimicrobial used in humans.

Statistical analyses: Binomial 95% confidence intervals (95% CI) were calculated around prevalence estimates. Comparisons of the prevalence of resistance between age groups were performed using Chi-square (χ^2). Cohen's kappa statistic was used to investigate the relationship between phenotypic AMR and ARGs in *E. coli* isolates (McHugh, 2012).

Table 1 Features of PCR primers used for detection of ARGs in this study

Antimicrobial class	ARGs	Primers	Sizes (bp)	References
Aminoglycosides	*aadA1*	F: CATTTGTACGGCTCCGCAGT R: AGAATGTCATTGCGCTGCCA	259	Maria *et al*., 2011
Beta-lactams	*bla*$_{TEM}$	F: TACGATACGGGAGGGCTTAC R: TTCCTGTTTTTGCTCACCCA	716	Belaaouaj, 1994
Chloramphenicol	*cmlA*	F: CCGCCACGGTGTTGTTGTTATC R: CACCTTGCCTGCCCATCATTAG	698	Keyes, 2000
Sulfonamides	*dfrA12*	F: CGGGTTATTGGCAATGGTCC R: CTTGAATGGTTTCGGTTGAG	400	Virve *et al*., 2008
Aminoglycosides	*strA*	F: ATGGTGGACCCTAAAACTCT R: CGTCTAGGATCGAGACAAAG	893	Kozak, 2009
Sulfonamides	*sul3*	F: CAACGGAAGTGGGCGTTGTGGA R: GCTGCACCAATTCGCTGAACG	244	Kozak, 2009
Tetracyclines	*tetA*	F: GTAATTCTGAGCACTGTCGC R: CTGCCTGGACAACATTGCTT	937	Schmidt *et al*., 2001

Results

Phenotypic antimicrobial resistance (AMR): Figure 1 shows the AMR prevalence of *Escherichia coli* isolates collected from pig farms in southern Vietnam. The highest resistance (±95% CI) corresponded to chloramphenicol (90/90, 100 ± 0%), tetracycline (97.8 ± 3.1%), ampicillin (85.6 ± 7.3%) and amoxicillin (85.6 ± 7.3%), followed by trimethoprim-sulphamethoxazole (67.8 ± 9.7%) and piperacillin (54/90, 60 ± 10.1%%). All *E. coli* isolates were sensitive to nitrofurantoin and amikacin. Fewer than 10% of the isolates were resistant to cefovecin (8.9 ± 5.9%), imipenem (4.4 ± 4.3%), cefpodoxime (4.4 ± 4.3%), ceftiofur (3.3 ± 3.7%), cefalexin (3.3 ± 3.7%) and polymyxin B (2.2 ± 3.0%). In particular, imipenem resistance, which had not been reported in animal *E. coli* in Vietnam previously, accounted for 4.4% + 4.3% (4/90) in this study.

Of all antimicrobial classes, amphenicols (chloramphenicol) accounted for the highest resistance levels (100%), followed by tetracyclines (97. 8%), quinolones and penicillin class (85.6%), sulfonamides (67.8%) and aminoglycosides (63.3%). The multi-drug resistance, i.e. resistance to at least three antimicrobial classes, was present in 81.1% of isolates (Figure 2). The average prevalence of MDR among weaners, growers and finishers was 43.0%, 39.5% and 36.3%. *E. coli* isolates from finishers were more likely to be susceptible than those from weaners and growers. In particular, none of the *E. coli* isolates of finishers were resistant to amoxicillin/clavulanate, amikacin and cephalosporins, while *E. coli* isolates of weaners and growers were prevalently resistant to those drugs ranging from 6.7 to 20.0%.

Prevalence of ARGs: Table 2 presents the findings regarding the presence of each ARG in the 90 *E. coli* pool isolates. The highest ARGs identification (97.6%) was found in grower pigs, followed by weaners (95.3%) and the lowest in finisher pigs (94.8%). There were at least 80% of *E. coli* isolates in pigs found positive for all seven tested ARGs. The lowest incidence was *strA* genes (83.3%) while the highest were the *aadA1, cmlA, tetA* (100%). Specifically, *bla*$_{TEM}$ genes which confer decreased susceptibility to the 3rd generation cephalosporins accounted for a considerable proportion (97.8%) from samples originated from all farms.

Co-existence of 2 to 7 ARG in various combinations was identified in 89/90 of all tested *E. coli* isolates. There were 21 different kinds of coexistence of resistance genes (Table 3). The most frequently co-existing ARGs were *sul3* and *cmlA* of sulfonamide resistant genes (97.8%).

Correlation of phenotypic AMR and ARGs: The correlation between phenotypic resistance and associated ARGs for *E. coli* isolates is presented in Table 4. Cohen's kappa was the highest for *bla*$_{TEM}$ vs ampicillin and *bla*$_{TEM}$ vs amoxicillin (κ=0.098), followed by *dfrA12* vs tri/sulfa (κ=0.047), and *sul3* vs tri/sulfa (κ=0.024). In general, no significant correlation was seen between the surveyed ARGs versus antibiotic phenotype classifications.

Discussion

Our studies have shown the phenotypic and genotypic features of *E. coli* from southern Vietnamese pigs. Overall, finisher pigs (15 to 22 weeks) had the lowest resistance rates, while weaners (3 to 10 weeks) had the highest. A possible explanation for this is that recent legislation has banned antimicrobial growth promoters (AGP) in commercial feeds intended for older pigs (Law No. 13/202020/ND-CP on veterinary medicine, Vietnam).

Among *E. coli* isolates, we observed resistance levels >85% against chloramphenicol, tetracycline,

ampicillin, amoxicillin, levels from 25 to 70% against trimethoprim/sulfamethoxazole, piperacillin, enrofloxacin, marbofloxacin, gentamicin, and tobramycin. Since choramphenicol is not approved for use in Vietnamese farming, the resistance levels of chloramphenicol suggest cross-resistance with florfenicol or fluorinated derivatives (Ministry of Agriculture, 2009; Schwarz *et al*., 2004). The resistance results were similar with the *E. coli* study in the Mekong Delta area (Huynh and Ly, 2018) where ampicillin and trimethoprim/sulfamethoxazole belonged to the most resistant drugs, and amikacin and cephalosporins remained resistant at lower levels. Even in many European countries and Australia, where AGP is restricted, tetracycline and penicillin were reported to be commonly seen in animals (Garcia *et al*., 2014; Smith *et al*., 2016). Additionally, 60% and 26.7% isolates, respectively, displayed resistance against piperacillin and tobramycin, antibiotics prescribed for critical importance in human usage.

We found that some *E. coli* isolates displayed MDR involving 3-9 antibiotics, which was similar to MDR detected among pig isolates in previous studies (Nhung *et al*., 2014; Huynh and Ly, 2018). The variety in the level of multi-drug resistance indicates the diversity of antibiotic usage in Vietnamese pig farms. The suggestion for such variation can be better understood by some resistance not conferring fitness costs and remaining even in the absence of AMU (Anita *et al*., 2015).

The greatest number of phenotypic and ARGs resistant isolates was seen in weaner pigs. Diseases are most common in pigs 1-2 weeks of age after weaning and develop most strongly in 3-5 weeks after weaning due to small farming household practices (Jeffrey *et al*., 2013) and thus, early weaning pigs are at a greater risk of acquired disease and more likely to be treated with antimicrobials. Our results showed that the majority of *E. coli* isolates carried $bla_{\text{TEM-1}}$, *tetA*, *sul3*, *dfrA12*, *aadA1*, *strA* and *cmlA*. All the seven tested ARGs associated with frequently-used antimicrobials were in line with previous studies (Sengeløv *et al*, 2003; José *et al*., 2014; Ahmad and Khalil, 2019) and explained the enormously common resistance across pig farms.

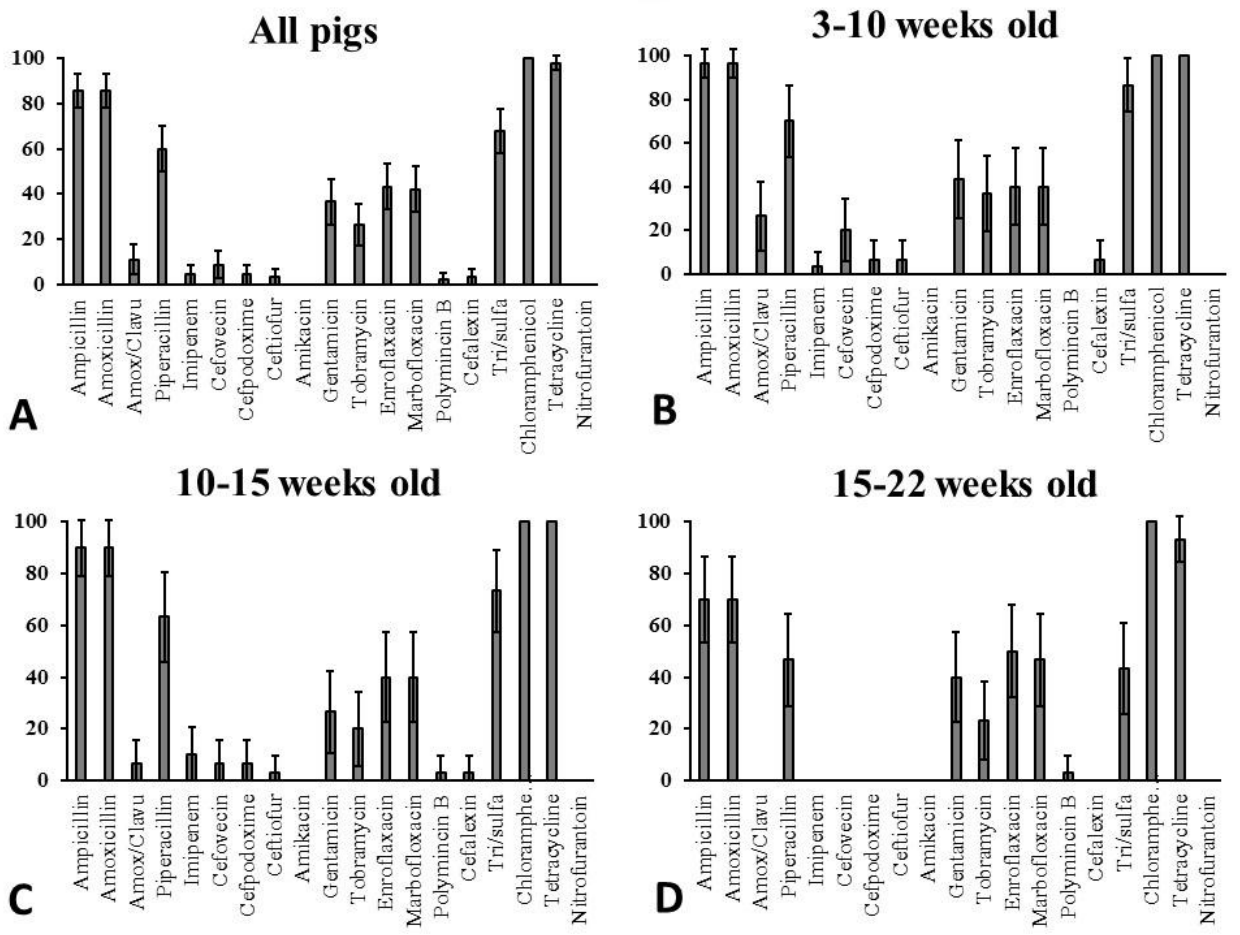

Figure 1 Prevalence (%) of AMR among *Escherichia coli* isolates collected from 3 different age groups in pig farms of Southern, Vietnam. (A) AMR rate of all age pigs (n=90); (B) AMR rate of weaner pigs (n=30); (C) AMR rate of grower pigs (n=30); (D) AMR rate of finishers (n=30). The error bars indicate 95% confidence intervals. Phenotypic resistance count indicates the number of drugs for which isolates expressed resistance out of 19 that were evaluated.

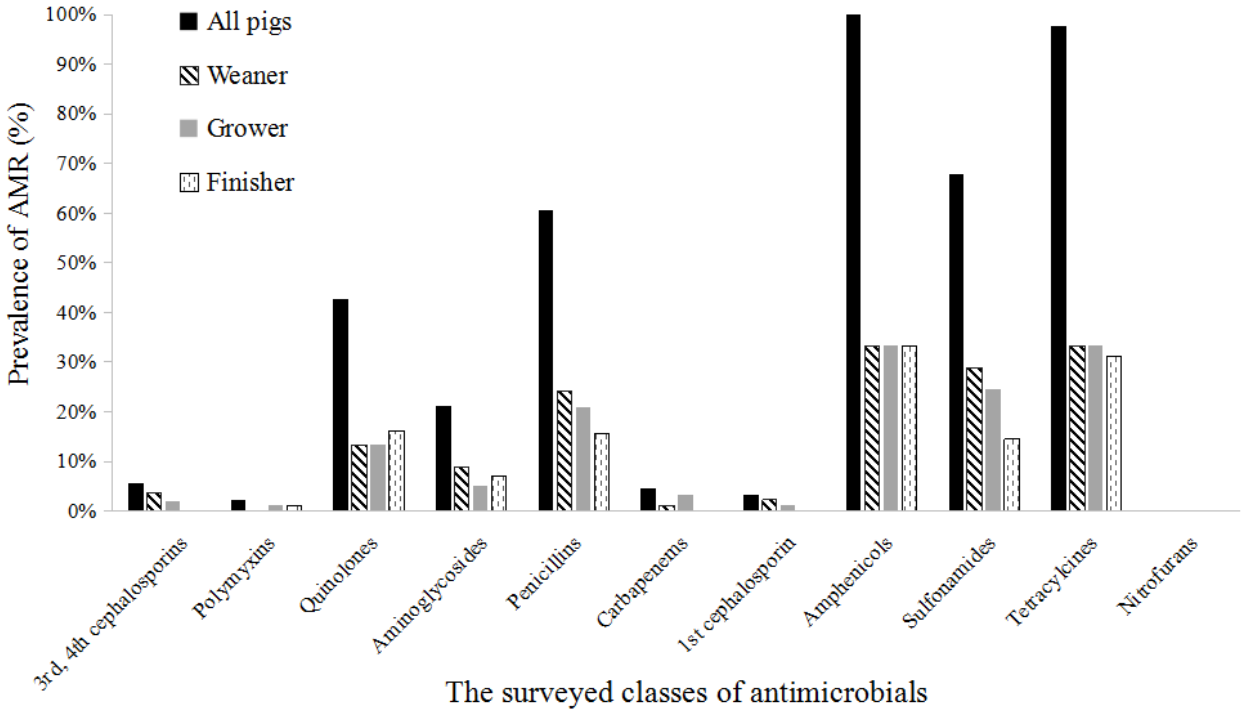

Figure 2 Prevalence of antibiotic resistance (AMR) of *Escherichia coli* isolates from weaners, growers and finishers based on 11 antibiotic classes.

Table 2 The percentage and number (in brackets) of ARGs detection from *E. coli* isolates of different age-group of pigs in Southern, Vietnam.

ARGs	Age groups of pigs (each group with n =30)			All pigs (n=90)
	Weaner	Grower	Finisher	
aadA1	100 (30)	100 (30)	96.7 (29)	98.9 (89)
bla_{TEM}	96.7 (29)	100 (30)	96.7 (29)	97.8 (88)
cmlA	100 (30)	100 (30)	96.7 (29)	98.9 (89)
dfrA12	96.7 (29)	100 (30)	96.7 (29)	97.8 (88)
strA	76.7 (23)	86.7 (26)	83.3 (25)	82.2 (74)
sul3	96.7 (29)	100 (30)	96.7 (29)	97.8 (88)
tetA	100 (30)	96.7 (29)	96.7 (29)	97.8 (88)

Table 3 Frequency of antibiotic resistant genes in *E. coli* isolates (n=90).

ARGs coexistence	Frequency	
	n	%
bla$_{TEM}$	88	97.8
bla$_{TEM}$ *aadA1*	86	95.6
bla$_{TEM}$ *aadA1 strA*	72	80.0
bla$_{TEM}$ *aadA1 strA dfrA12*	70	77.8
bla$_{TEM}$ *aadA1 strA dfrA12 sul3*	69	76.7
bla$_{TEM}$ *aadA1 strA dfrA12 sul3 cmlA*	69	76.7
bla$_{TEM}$ *aadA1 strA dfrA12 sul3 cmlA tetA*	68	75.6
aadA1	89	98.9
aadA1 strA	74	82.2
aadA1 strA dfrA12	72	80.0
aadA1 strA dfrA12 sul3	71	78.9
aadA1 strA dfrA12 sul3 cmlA	71	78.9
aadA1 strA dfrA12 sul3 cmlA tetA	70	77.8
strA	75	83.3
strA dfrA12	72	80.0
strA dfrA12 sul3	71	78.9
strA dfrA12 sul3 cmlA	71	78.9
strA dfrA12 sul3 cmlA tetA	70	77.8
dfrA12	88	97.8
dfrA12 sul3	85	94.4
dfrA12 sul3 cmlA	85	94.4
dfrA12 sul3 cmlA tetA	84	93.3
sul3	88	97.8
sul3 cmlA	88	97.8
sul3 cmlA tetA	87	96.7
cmlA	89	98.9
cmlA tetA	87	96.7
tetA	88	97.8

Table 4 The occurrence of phenotypic resistant (AMR) and associated antimicrobial resistant genes (ARGs) for *E. coli* isolates in this

AMR genes vs. antibiotics	Gene (+)		Gene (-)		Kappa
	Phenotype (+)	Phenotype (-)	Phenotype (+)	Phenotype (-)	
bla$_{TEM}$ vs ampicillin	76	12	1	1	0.0986
bla$_{TEM}$ vs amoxicillin	76	12	1	1	0.0986
bla$_{TEM}$ vs amoxicillin/clavulanic acid	10	78	0	2	0.0057
bla$_{TEM}$ vs piperacillin	53	35	1	1	0.0110
bla$_{TEM}$ vs imipenem	4	84	0	2	0.0021
bla$_{TEM}$ vs cefovecin	8	80	0	2	0.0044
bla$_{TEM}$ vs cefpodoxime	4	84	0	2	0.0021
bla$_{TEM}$ vs ceftiofur	3	85	0	2	0.0016
bla$_{TEM}$ vs cephalexin	4	84	0	2	0.0021
aadA1 vs amikacin	0	89	0	1	-
aadA1 vs gentamicin	32	57	1	0	-
aadA1 vs tobramycin	23	66	1	0	-
strA vs amikacin	0	74	0	16	-
strA vs gentamicin	24	50	9	7	-0.1188
strA vs tobramycin	16	58	8	8	-0.1276
dfrA12 vs tri/sulfa	59	27	2	2	0.0467
sul3 vs tri/sulfa	60	28	1	1	0.0239
cmlA vs chloramphenicol	89	0	1	0	-
tetA vs tetracycline	89	0	1	0	-

There were significant isolates with decreased susceptibility lacking a relevant genetic resistant determinant from our database. As all known resistance genes were screened for, the *bla*$_{TEM}$, which is primarily associated with broad spectrum ß-lactam resistance (Fatima *et al.*, 2017), it has been found to have slight correlation with phenotypic resistance of ampicillin and amoxicillin in this study (κ=0.0986; p=0.01). In the absence of valid genotype-phenotype correlation, these findings are hypothesized to be

influenced by selection, publication bias and the limited utility of existing measures. Previous studies have found phenotype-genotype discrepancies are affected by the prevalence of pseudogenes in the *E. coli* populations, thus eliminating their ability to be expressed (Hartl DL and Clark AG 1997, Margaret *et al*., 2011). Johnson, 2010 indicated multidrug resistance genes may be linked to other selectively advantageous genes, in case selection for one resistance trait would lead to the propagation of all of the linked resistance traits that maintained the ARGs in a population. There are numerous ARGs and variations in expression that can result in resistance that were not investigated in this study, for example, *gyrA* gene encodes a protein forming a subunit of a DNA gyrase that causes resistance to the antibiotic ciprofloxacin (Moran *et al*., 2017) or the *mcr* gene on plasmids that can be readily transferred between bacteria and confer resistance to polymyxins (Mohammad and Shoroq, 2019). Other limitations were due to the plasmid DNA extraction approach making up part of the total DNA present in complex samples (Kav *et al*., 2013). A larger population of *E. coli* to give insight into the ARGs on plasmid and genomic DNA, and further investigation of the expression levels of large numbers of genes or to genotype multiple regions of a genome would be the next step in AMR reference laboratories for routine surveillance activities.

In conclusion, overall the study investigated the prevalence of AMR and ARGs of *E. coli* isolates from pigs in southern Vietnam. The isolates were resistant to a wide range of antimicrobial agents with considerable MIC values. Resistance levels over 85% were observed against chloramphenicol, tetracycline, ampicillin, amoxicillin. The most common ARG in *E. coli* isolates were *aadA1*, *cmlA*, *bla*$_{\text{TEM}}$, *dfrA12*, *tetA*, *sul3* and *strA*. We did, however, not find any correlation between ARGs and the AMR phenotypes among *E. coli* isolates. A large number of isolates and more genetic information should be carried on as improved confidence in the estimation of the levels of AMR in the Vietnamese pig population.

Competing interests: The authors declare that they have no competing interests.

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