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All communications should be addressed to:

The Editor-in-Chief
Philippine Journal of Veterinary Medicine
College of Veterinary Medicine
University of the Philippines Los Baños
Laguna, Philippines 4031
Telefax Nos. +63-49-536-2727, +63-49-536-2730
Email: pjvm1964@gmail.com, pjvm.uplb@up.edu.ph

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ORIGINAL ARTICLE

PHENOTYPIC ANTIMICROBIAL RESISTANCE PATTERNS IN *Escherichia coli* ISOLATED FROM SLAUGHTERED HEALTHY PIGS AND CATTLE IN NUEVA VIZCAYA, PHILIPPINES

Chrysler D. Bakakew, DVM , Jayson V. Tabuac, DVM and Haidee E. Torio, DVM, MVE

Department of Clinical Sciences, College of Veterinary Medicine, Nueva Vizcaya State University, Bayombong, Nueva Vizcaya, 3700, Philippines

ABSTRACT

This cross-sectional study investigated antimicrobial resistance (AMR) prevalence and characterized and compared phenotypic resistance in intestinal *Escherichia coli* from healthy pigs and cattle at slaughter in Solano, Nueva Vizcaya. A single *Escherichia coli* isolates from pig and cattle fecal samples were tested by the disk diffusion method to a panel of eight antimicrobials important to human therapy. A total of 83 *E. coli* were isolated in 111 fecal samples from pigs and cattle. Of these, 75 (90.36%) were found to be resistant to at least one antimicrobial agent with rates in pigs at 95% and in cattle at 88.6%. Over-all, high resistance rates to amoxicillin (78.3%), tetracycline (63.8%), and trimethoprim sulfamethoxazole (50.6%) were observed. Isolates from pigs showed higher percentage resistance compared to cattle for tetracyclines (86.4% vs 38.5%) ($p<0.00001$), trimethoprim-sulfamethoxazole (77.3% vs 20.5%) ($p<0.00001$), amoxicillin (81.8% vs 74.3% ($p>0.05$), and chloramphenicol (43.2% vs. 5.1%) ($p<0.0001$). Multidrug resistance was significantly higher in pig isolates at 88.1% compared to 39.4% in cattle ($p<0.00001$). Thirty-one resistance patterns were observed in all isolates. The most common resistance pattern in cattle isolates is to a single antimicrobial, amoxicillin at 24.24% while in pigs, chloramphenicol-amoxicillin-trimethoprim sulfamethoxazole-tetracycline is common at 14.28%. Resistance to ciprofloxacin was not observed in both species. Results revealed high percentage resistance in *Escherichia coli* from both pigs and cattle. Both species could be major sources of *Escherichia coli* resistant to multiple antimicrobials used in human therapy.

Keywords: *antimicrobial resistance, cattle, Escherichia coli, pigs, Philippines*

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INTRODUCTION

Antimicrobial resistance (AMR) is an increasingly serious public health threat worldwide (WHO, 2017). Resistance in common bacteria to antimicrobials of clinical significance to human therapy, especially to critically important ones like β-lactams, fluoroquinolones, and aminoglycosides, has reached alarming levels in many parts of the world (WHO, 2017). With this increase in frequency, untreatable human infections arise (Cameron and McAllister, 2016) contributing significantly to a number of morbidities and mortalities each year (WHO, 2014; CDC, 2019).

The widespread use of antimicrobials in food animal production for therapeutic and non-therapeutic purposes has been implicated as a major driver in the emergence of antimicrobial-resistant bacteria that may spread to humans and the environment (FAO, 2016, Robinson *et al.,* 2016). In the Philippines, existing regulations on the proper use of antimicrobials in animals are lacking but large quantities have been consistently used for growth promotion, disease prevention, and treatment (DOH and DA, 2015). These uses of antimicrobials are now standard practices particularly for pigs, poultry, and aquaculture industries in the country (Cassou *et al.*, 2018). Among the most commonly used antimicrobials in food animals, particularly pigs, are tetracycline, penicillin, and amoxicillin (DOH and DA, 2015). In Southeast Asia, all

***FOR CORRESPONDENCE:**
(e-mail: hetorio@nvsu.edu.ph)

classes of antimicrobials important for human medicine are used in animals (Nguyen *et al.*, 2016). Antimicrobials administered to the herd for the purposes of therapy, prophylaxis, or growth promotion may lead to increased antimicrobial selection pressure and thus, to a high level of resistant bacteria (Padilla and Amatorio, 2017).

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Commensal *Escherichia coli* is a natural flora of the gut and though usually non-pathogenic, the concern about these bacteria is that it could serve as a reservoir of resistance genes that can be transferred to other enteric pathogens (Szmolka and Nagy, 2013) and eventually may spread to humans directly or indirectly through the food supply (Oguttu *et al*., 2008). During slaughter and distribution processes, *E. coli* may contaminate carcasses contributing to the spread of AMR. As *E. coli* is ubiquitous in both livestock and humans, it is considered as an ideal organism to study the level and spread of AMR due to its wide range of resistance phenotypes (Muloi *et al.,* 2019).

A few studies on AMR available in the Philippines have reported isolation of resistant commensals and pathogens in food animals focusing primarily on pigs and poultry (Ng and Rivera, 2014; Sison *et al.,*2015; Padilla and Amatorio, 2017; Torio and Padilla, 2018). In cattle however, there is lack of data locally as to prevalence of AMR in bacteria particularly in commensal *E. coli*. Because of the limited information available on AMR in food animals, this study was conducted to investigate and compare the level of AMR in *E. coli* in cattle and pigs at slaughter and to characterize the AMR patterns in both species. Data from this study may contribute in assessing AMR in bacteria from food animals that can be used as scientific basis for reviewing guidelines or policies to combat AMR.

MATERIALS AND METHODS

Study design and sample collection

A cross-sectional survey was employed in this study. Sample collection was carried out in the lone slaughterhouse of Solano, Nueva Vizcaya. The slaughterhouse was chosen due to its high volume of throughput being one of the largest slaughterhouses providing meat to the markets of Nueva Vizcaya. A total of 111 fecal samples, 68 from pigs and 43 from cattle, were collected from the colon at the evisceration area after post-mortem inspection. Systematic sampling was performed. Every sample collection which was conducted every Monday of the week where significantly the larger volume of slaughter is available as this coincides with the market day in the area.

The samples were placed individually in sterile zipper-locked sample bags, properly labeled, and were transported in insulated polystyrene container with ice packs to the Nueva Vizcaya State University-College of Veterinary Medicine Microbiology Laboratory for processing within four hours of collection.

E. coli isolation

Ten grams of fecal samples was transferred into sterile container and mixed with 90 ml buffered peptone water. The resultant homogenate was used for bacterial culture.

Isolation and identification of *E. coli* followed a standard procedure (Quinn *et al.,* 2009). A loopful of the homogenate was inoculated in MacConkey (MAC) agar and incubated aerobically at 37^{o}C for 24 hours. A single isolated lactose-fermenting colony from MAC was selected and streaked in eosin methylene blue (EMB) agar and incubated overnight at 37^{o}C. Gram-staining was also performed on a selected colony. One greenish metallic sheen colony on EMB was subcultured in nutrient agar. Biochemical tests to confirm *E. coli* employed triple sugar iron, urease test, indole, methyl red, Vogues-Proskauer and Simmon's citrate tests.

Antimicrobial susceptibility testing (AST)

One *E. coli* isolate from each sample was subjected to AST using the Kirby-Bauer disk diffusion method and according to Clinical and Laboratory Standards Institute (CLSI, 2014). The antimicrobials selected for the disk diffusion assay include eight antimicrobial agents belonging to seven antimicrobial classes namely: (1) Penicillin (amoxicillin, 10 µg); (2) 3rd generation cephalosporins (ceftriaxone, 30 µg and cefotaxime, 30 µg); (3) fluoroquinolones (ciprofloxacin, 5 µg); 4) phenicols (chloramphenicol, 30 µg); (5) aminoglycosides (gentamicin, 10 µg); (6) tetracyclines (tetracycline, 30 µg); and (7) folate pathway inhibitors (trimethoprim-sulfamethoxazole, 23.75/1.25 µg). These agents are in the CLSI recommended list of antimicrobials for susceptibility testing of *E. coli* and other Enterobacteriaceae. Antimicrobials belonged to either the critically important antimicrobials (CIA) or the highly important antimicrobials list of the World Health Organization (WHO, 2017). All test antimicrobials, however, are classified as veterinary critically important antimicrobials (VCIA) (OIE, 2007).

For disk diffusion assay, turbidity of *E. coli* inoculum was visually compared and adjusted to 0.5 McFarland standard. *E. coli* inoculated Mueller-Hinton agar (MHA; Hi-Media) plates were dispensed with the antimicrobial disks zone of inhibition diameter was measured. Results were categorized according to the exhibiting resistance to at least three antimicrobial classes tested were regarded as multidrug resistant (MDR) (Magiorakos *et al*., 2012). *E. coli* ATCC® 25922™ was used as reference strain for quality control of AST.

Statistical Analysis

Data were analyzed descriptively. The percentage resistance in *E. coli* isolates was calculated using the OpenEpi software version 3.03a. Antimicrobial resistance rates were compared using Chi-squared test. Differences at $P<0.05$ were considered significant.

RESULTS

E. coli was isolated and identified in 83 out of 111 fecal samples. Significantly higher isolation rate was observed in cattle at 39 of the 43 (90.7%) compared to pigs with 64.7% (44/68) rate (P=0.00107). The identification was based on cultural characteristics and the results of biochemical tests.

Seventy-five out of 83 (90.4%) *E. coli* isolates from cattle and pigs were found to be resistant to least one antimicrobial tested in this study. Over-all percentage resistance was high in isolates from both species at 95.4% (42/44) from pigs and 88.6% (33/39) from cattle. Though higher percentage was observed in pigs, this was not significantly different with that of cattle ($P>0.05$) Table 1 shows distribution of *E. coli* from both species exhibiting resistance to each antimicrobial tested.

Table 1. Distribution of *E. coli* isolated from pigs and cattle at slaughter in Solano, Nueva Vizcaya exhibiting resistance to the antimicrobials tested.

Antimicrobial agents	Antimicrobial Class	Number and proportion of resistant *E.coli*		
		Over-all (%) (n=83)	Pigs (%) (n=44)	Cattle (%) (n=39)
Amoxicillin	Penicillin	65 (78.3)	36 (81.8)	29 (74.3)
Cefotaxime (3G)	Cephalosporin	38 (45.8)	21 (47.7)	17(43.6)
Ceftriaxone (3G)	Cephalosporin	30 (36.1)	15 (34.1)	15 (38.5)
Ciprofloxacin	Fluoroquinolone	0	0	0
Gentamicin	Aminoglycosides	11(13.2)	11 (25.0)	0
Chloramphenicol	Amphenicol	21 (25.3)	19 (43.2)	2 (5.10)
Tetracycline	Tetracycline	53 (63.8)	38 (86.4)	15 (38.5)
Trimethoprim-sulfamethoxazole	SDHFRIC	42 (50.6)	34 (77.3)	8 (20.5)

Overall, high percentage resistance was observed to amoxicillin, tetracycline, and trimethoprim sulfamethoxazole at 78.3%, 63.5%, and 50.6%, respectively. As shown in Figure 1, isolates from pigs showed higher rates of resistance compared to that of cattle for tetracyclines (pig: 86.4% vs cattle: 38.5%) ($p<0.0001$), amoxicillin (pigs: 81.8% vs cattle:74.3%) ($p>0.05$), trimethoprim-sulfamethoxazole (pig:77.3% vs cattle: 20.5%) ($p<0.00001$), and chloramphenicol (pig: 43.2% vs cattle: 5.1%) ($p<0.001$). On the other hand, resistance was not observed for ciprofloxacin in isolates from both species and to gentamicin in cattle isolates. Resistance to 3rd generation cephalosporins (cefotaxime, and ceftriaxone) was observed at a moderate rate for both species.

Two thirds of the total number of isolates (66.7%) displayed resistance to multiple antimicrobials, hence are called multidrug resistant (MDR). MDR isolates are those that showed resistance to at least three antimicrobial classes. MDR is significantly higher in pigs where 37 out of 42 (88.1%) isolates have multiple resistance compared to that of cattle at 39.4% (13 of 33) ($p<0.00001$). Percentage of *E. coli* isolates according to number of antimicrobial classes where resistance was observed is shown in Figure 2. Resistance to only one class was not observed for isolates in pigs, in cattle however, it is the most common among the isolates at 33.3%. Multiple resistance commonly involved three to five classes. Resistance to six and seven classes simultaneously was not observed.

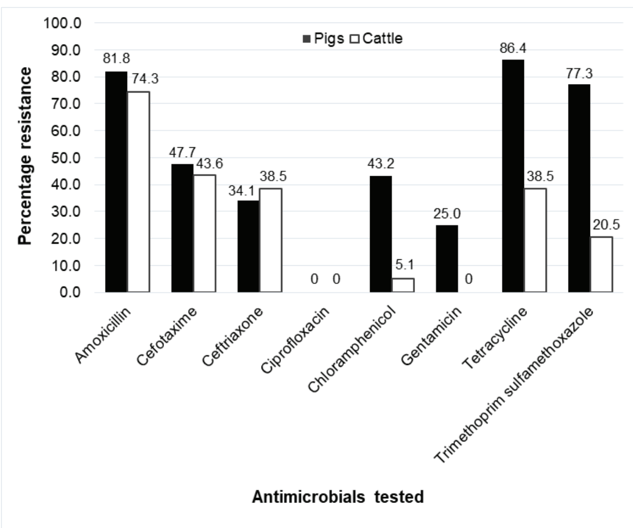

Figure 1. Percentage resistance in *E. coli* isolates from pigs and cattle to individual antimicrobials tested.

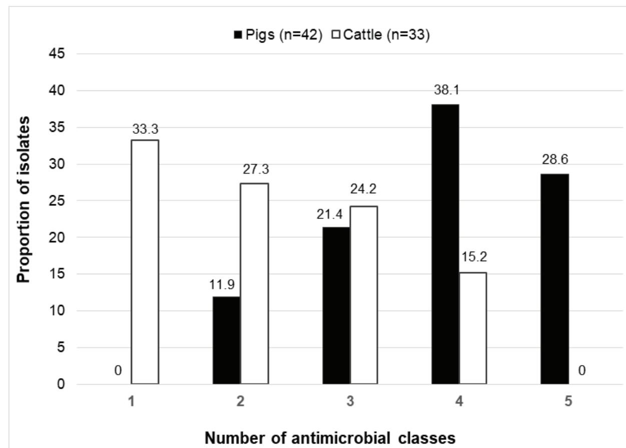

Figure 2. Percentage of *E. coli* isolates from pigs and cattle according to number of antimicrobial classes to which resistance was observed .

Patterns of resistance to one or more antimicrobial agents are presented in Table 2. A total of 31 resistance patterns were observed in all isolates. Six of these patterns are common in isolates from both species. Twenty-two different resistance phenotypes were demonstrated in pigs compared to 15 in cattle. The multiple resistance patterns were most frequent at 67.7% (21/31).

The most common resistance phenotype in *E. coli* from cattle was to a single antimicrobial (AX) found in eight (24.2%) isolates while for pigs, C-AX-SXT-TE is the most frequent pattern at 14.3%, showing resistance to four drug classes namely amphenicols, penicillin, SDHFRIC, and tetracyclines.

DISCUSSION

AMR in bacteria from food animals has emerged as a major threat in both animal and human health (Cameron and McAllister, 2016). In the Philippines, however, information on AMR in bacteria originating from food animals is extremely limited considering that antimicrobials are widely used in animal production. This study investigated the level of AMR and characterized and compared the phenotypic resistance in *E. coli* isolates sourced from healthy pigs and cattle at slaughter. *E. coli* from cattle as well as other food production animals that include pigs serve as indicator of AMR prevalence (Cameron and McAllister, 2016; Caruso, 2018) and antibiotic use (Yassin *et al.,* 2017).

Table 2. Distribution of antimicrobial resistance patterns found in *E. coli* isolates from cattle and pigs according to the number of antimicrobial classes.

No. of Antimicrobial Classes	Resistance Patterns	Pigs (n=42)	Cattle (n=33)
1	C		1
	CTX		1
	AX		8
	CTX-CRO		1
2	C-TE	1	
	CTX- TE	1	1
	AX-TE	1	4
	C-CTX	1	
	CTX-CRO-SXT	1	
	AX-CTX-CRO		4
3	C-CRO-TE	1	
	C-AX-TE	1	
	AX-CTX-SXT	1	
	C-SXT-TE	1	
	AX-SXT-TE	4	1
	AX-CTX-TE		1
	AX-CRO-TE		2
	AX-CTX-CRO-SXT	1	2
	AX-CTX-CRO-TE		2
4	C-AX-CN-TE	1	
	AX-CTX-SXT-TE	1	
	AX-CN-SXT-TE	1	
	AX-CRO-SXT-TE	1	1
	C-AX-SXT-TE	6	
	C-AX-CTX-SXT		1
	C-AX-CTX-CRO-TE	1	
	AX-CTX-CRO-SXT-TE	5	3
5	AX-CTX-CN-SXT-TE	1	
	C-AX-CN-SXT-TE	3	
	C-AX-CTX-SXT-TE	3	
	AX-CTX-CRO-CN-SXT-TE	5	
Total isolates with MDR phenotype (with resistance to ≥3 classes)		37(88.1%)	13(39.4%)

Abbreviations: AX- amoxicillin, CIP – ciprofloxacin, TE – tetracycline, CN – gentamicin, C – chloramphenicol, SXT – trimethoprim sulfamethoxazole, CRO- ceftriaxone, CTX - cefotaxime

In the present study, the over-all percentage resistance in *E. coli* isolates was high at 90.4% with resistance rate in pigs at 95.4% and in cattle at 88.4%. This is in consonance with the previous report in *E. coli* in pigs in the country at 95% which was attributed to the wide use of antimicrobials in pigs and easy access of farmers to antimicrobials even in the absence of veterinary prescription (Padilla and Amatorio, 2017). In cattle, the high over-all prevalence was unexpected and can be attributed to high resistance observed to one of the antimicrobials tested, amoxicillin. This antimicrobial is a commonly used drug in the penicillin class for treatment of respiratory infections in cattle. To the knowledge of the authors, this is the first local report on the prevalence and characterization of phenotypic resistance in *E. coli* in healthy cattle.

Tetracycline, amoxicillin, and trimethoprim-sulfamethoxazole showed the highest percentage resistance among the antimicrobials tested in both species. The rates observed were higher in pigs compared to that of cattle which may indicate the more extensive use of these antimicrobials in pig production. In pigs, tetracyclines and amoxicillin are the leading choices of antibiotic medication in the country (DOH and DA, 2015) as well as trimethoprim sulfamethoxazole (Jiao *et al.,* 2007). Previous studies in Southeast Asia also reported high resistance rates in *E. coli* to these older classes of antimicrobials (Mainda *et al*., 2015; Nhung *et al*., 2016 and Trongit *et al*., 2016).

Tetracyclines is a broad-spectrum antimicrobial which has a long history of use in animals since its discovery in the 1940's. Chlortetracycline is commonly administeredorally as these are incorporated in feeds or in drinking water for growth promotion and prophylaxis. According to Aasmae *et al.* (2019), there is a higher probability for commensal *E. coli* to become a reservoir of resistance when antibiotics used are given orally. In addition, oxytetracycline is available as a long-acting antibiotic with wide spectrum of activity. These tetracyclines derivatives, because of their preparation, are often the preferred antimicrobial particularly in backyard swine farms locally where availability of services of veterinarian and veterinary technicians is limited. These may probably account for high percentage resistance to this antimicrobial in isolates from pigs. Several studies locally and in other countries have also reported similar findings of resistance in *E. coli* to this drug (Jiao et al, 2007; Padilla and Amatorio, 2017; and Yassin *et al,* 2017). In the USA, China, and Kenya, resistance to tetracylines was explained by its common use as first line antibiotic treatment (Tadesse *et al*., 2012), promoter of feed efficiency (Yassin *et al*, 2017) as well as its ready availability at a low cost (Kikuvi *et al.,* 2006). In cattle, tetracyclines have been routinely used for treatment of mastitis, however, the oral administration of this drug is not as common as in pigs, which may account for the lower prevalence of resistance to this antimicrobial. Previous studies had confirmed the correlation between route of administration and level of antimicrobial resistance in *E. coli* isolated from livestock (Chantziaras *et al*., 2014; Gibbons *et al.,* 2014).

On the other hand, resistance to trimethoprim-sulfamethoxazole was expected and can be accounted for by their common and lengthy use in animals for infections caused by *E. coli*, *Salmonella,* or *Mycoplasma* (PVET, 2019) or to co-resistance.

Resistance to amoxicillin is high in both species which is not uncommon. Other studies also demonstrated high percentage resistance against ampicillin, an analogue of amoxicillin, in *E. coli* from pigs locally where increasing rates were reported from a low 23% in 2007 (Jiao *et al.)* to more than double this rate at 62% in 2017 (Padilla and Amatorio). This may indicate the rate at which resistance may increase over a short period of time making it more worrisome particularly, for this kind of antimicrobial considered as critically important in human therapy. Being a first line antimicrobial, effectiveness of this drug should be preserved and so it is important that prudent use in animals is observed.

Another noteworthy finding in this study is the resistance found to third generation cephalosporins - ceftriaxone and cefotaxime, which are at a moderate rate in both species. This is unexpected as these agents are for human use only. As *E. coli* are transmissible to humans, resistance to these drugs in isolates from food animals may compromise their critical importance in human medicine.

Previous studies locally did not find resistance in *E. coli* from pigs to these antimicrobials (Jiao *et al.,* 2007; Padilla and Amatorio 2017). Resistance to these drugs may be attributed to cross-resistance to ceftiofur, a third generation cephalosporins, approved for veterinary use in both cattle and pigs primarily for the treatment of respiratory diseases.

Chloramphenicol resistance was also

observed at different rates with significantly higher level in pig isolates compared to that of cattle. A similar finding was reported in other studies in Southeast Asia (Nhung *et al.,* 2016, Tronjit *et al.,* 2016) even though this drug has been banned in food-producing animals in the region including the Philippines (DA and DOH, 1990). Aside from the use of other phenicol derivatives like florfenicol, use of other antimicrobials like tetracyclines at low concentrations may also probably explain resistance observed as has been reported by Mirzaagha *et al* (2011).

Resistance to fluoroquinolone ciprofloxacin was not observed in both species. Previous findings in *E. coli* isolates locally and in other countries reported low levels of resistance to this antimicrobial (Wasyl *et al*., 2013, Padilla and Amatorio 2017). Results of this study could probably be explained by non-usage of the drug in sampled animals. However, enrofloxacin which is a ciprofloxacin analogue, is routinely used in pigs for swine respiratory diseases. Non-usage of such, especially for pigs, is highly unlikely. It is therefore appropriate that resistance to these antimicrobials should be continuously monitored in future studies to ascertain the level of resistance in bacteria.

MDR has become a significant problem because of the alarming levels found in isolates sourced from food animals (Rzewuska *et al.,* 2015, Torio and Padilla, 2018; Hang *et al*., 2019). In this study, two-thirds of the isolates are MDR with a significantly higher rates observed in pig isolates compared to that from cattle. This high proportion can be attributed to the diversity of antimicrobials used in farms (Strom *et al*., 2017) or to co-selection (Harada *et al*., 2006). Difference in rates between species can probably be explained by variation and frequency in usage of antimicrobials in these two populations. Similar findings of higher MDR rates in isolates from pigs compared to cattle were also reported in other studies (Wasyl *et al.,* 2013; Yassin *et al.,* 2017., Aasmae *et al.,* 2019.) High MDR rates in *E. coli* is an important finding as it may pose a serious threat since these bacteria are able to transfer resistance determinants to other enteric pathogens (Wasyl *et al.,* 2012) like Salmonella, which, when transferred to humans, may cause severe infections. The dissemination of MDR bacteria or their genes in mobile genetic elements have narrowed the available options for therapy. Thus, MDR can lead to life-threatening infections (Pormohammad *et al.,* 2019).

Several resistance patterns were found in this study, with swine having more diverse patterns compared to cattle. Resistance patterns common in swine include four antimicrobial classes with C-AX-SXT-TE as the most frequent phenotype while with cattle isolates, the most frequent pattern, involves only one antimicrobial (AX). This finding may somehow suggest the differences in the antimicrobial usage between these species with wider range and variety of antimicrobials use in pigs compared to cattle. As explained by Wasyl *et al.* (2013), the source of *E. coli* isolates will considerably influence the complexity and diversity of resistance patterns in animals.

In conclusion, the current study found a high prevalence of AMR in *E. coli* from pigs and cattle with significantly higher MDR rates in pigs compared to cattle. Diverse resistance patterns were also observed in both species. This study confirmed the previous findings of high level AMR in *E. coli* in pigs and provided the first description of AMR profile in commensal *E. coli* from healthy cattle which were slaughtered locally. It also confirmed reports that indeed, food animals, not only poultry and pigs, but also cattle, are important reservoirs of *E. coli* with various resistance phenotypes including multi-drug resistant types that can potentially be transferred to humans, to other animals, and to the environment. Findings highlight the need for strong nationwide AMR surveillance programs that will effectively and continuously monitor the levels and trends in AMR in bacteria from all major food animals. Furthermore, there is also a need to implement surveillance of antimicrobial usage in food animals and correlate it with AMR prevalence.

STATEMENT OF COMPETING INTEREST

The authors have no competing interest to declare.

AUTHOR'S CONTRIBUTION

Dr. Chrysler Bakakew and Dr. Jayson Tabuac conception, collection of samples, laboratory analysis, manuscript drafting. Dr. Haidee E. Torio conception, design, laboratory analysis, analysis of data, manuscript editing for final approval.

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