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RESEARCH ARTICLE

Emergence of resistance genes in fecal samples of antibiotic-treated Philippine broilers emphasizes the need to review local farming practices

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ABSTRACT

The poultry industry is a major contributor to the Philippine economy. Given the rising concerns in antibiotic resistance and food security, farmers need to maximize profit and output while ensuring safe and ecologically sustainable farming practices. This study surveyed antibiotic use in 12 commercial poultry farms in the Philippines. All farms reported the use of medically important antibiotics either for prophylaxis (91.67%), metaphylaxis (100%), and growth promotion (50%). Considering the widespread use of medically important antibiotics, we then investigated the potential contribution of local antibiotic treatment protocols to the emergence of antibiotic resistance genes in the fecal samples of broiler chickens under a controlled experimental setup. Significantly, we observed the emergence of antibiotic resistance genes (*ermB*) in fecal samples of antibiotic-treated broilers after 7 days and 21 days in antibiotic-free broilers raised in the same farming environment. These data were corroborated by antibiotic resistance gene profiles of fecal samples from commercial poultry farms. Antibiotic resistance genes (*tetA*, *tetB*, *tetU*, *tetW*, *qnrB*, *qnrS*) were prevalent in the fecal samples of antibiotic-treated broilers treated with tetracycline and quinolone antibiotics. Therefore, our study provides concrete evidence for the strong correlation between the use of medically important antibiotics in poultry farming and the emergence of antibiotic resistance genes. Antimicrobial resistance is a major contributor to failures in infectious disease treatment strategies in humans and animals. Therefore, the cost-benefit ratio of poorly regulated antibiotic treatment protocols in poultry farming could have a long-term detrimental impact on our economy and public health. Our study suggests the need to review our current policies and practices in using medically important antibiotics in the Philippine poultry industry.

Keywords: Antibiotics; antimicrobial resistance; poultry farming.

INTRODUCTION

Poultry industry gross output value has been steadily increasing in the Philippines (Statista, 2021). Among the regions, Central Luzon has the highest total chicken inventory with 26.98 million birds (PSA, 2021). A leading producer in the region is the province of Pampanga, with 164.48 thousand metric tons of chickens a year (Mapa, 2021). Nevertheless, accompanying the rise in poultry production is the challenge of maintaining an environmentally sound and ecologically sustainable means of the industry.

Ever since the empiric discovery of its beneficial effects in animal meat production, antibiotic use in the agricultural sector has long been a concern in mitigating the problem of antibiotic resistance (Verraes *et al*., 2013; Allen & Stanton, 2014). It has been emphasized that the consumption of antibiotics in animals correlates to antibiotic resistance genes isolated in humans (Hu *et al*., 2014; O' Neill, 2015). As a result, developed countries have firmly regulated agricultural application of antibiotics in the hope of curbing the spread of antibiotic resistance (Cogliani *et al*., 2011; European Parliament, 2019; FDA, 2021).

In low and middle-income countries, drivers of the spread of antibiotic resistance are reported to be complex and multi-sectoral, which include: inappropriate socio-ecological behaviors, poverty, overcrowding, lack of surveillance systems, food supply chain safety issues, highly contaminated waste effluents, and loose rules and regulations (Iskandar *et al*., 2020). In the Philippines, an action plan to combat anti-microbial resistance using the "One Health Approach" was launched in 2014 (Department of Health *et al*., 2015) and was followed through by the current government administration (Inter-Agency Committee on Antimicrobial Resistance, 2019). It includes 7 key strategies for both human and animal health

sectors, namely: (1) committing to the Philippine action plan through multi-sectoral engagement and accountability, (2) strengthening surveillance and laboratory capacity, (3) ensuring uninterrupted access to safe and quality-assured antimicrobials, (4) regulating and promoting the rational use of antimicrobials, (5) implementing appropriate measures to reduce infection across all settings, (6) promoting innovation and research on antimicrobial resistance, and (7) improving awareness and understanding of antimicrobial resistance through effective communication and education. Nonetheless, there are still concerns regarding the actual implementation of the aforementioned plan, especially in the agricultural sector.

Regulations related to sale, prescription and distribution of antibiotics for animal use have long been established in the Philippines (Department of Health & Department of Agriculture, 2013). Even so, weaknesses in the implementation of standards for veterinary medicinal products, lack of strict enforcement of animal antibiotic use policies, and poor awareness of farmers on the prudent use of antibiotics, may have contributed to the antibiotic resistance problem in the Philippines (Barroga *et al*., 2020). This is exemplified by the availability of antibiotics through over-the-counter access in local retail stores with limited veterinary oversight. In effect, medically important antibiotics in human medicine are frequently utilized in Philippine poultry farms (Barroga *et al*., 2020). This is in stark contrast to developed countries where medically important antibiotics are strictly regulated for agricultural applications (European Parliament, 2019; FDA, 2021). Fundamentally, there might be a disparity between Philippine's declared animal antibiotic use policies and what is really happening in actual farms.

In the context formerly described, this study aims to demonstrate the emergence of antibiotic resistance genes in the fecal samples of broiler chickens that were raised in an experimental setup, using a local poultry farm antibiotic treatment protocol. In addition, surveys of antibiotic use in 12 commercial poultry farms, as well as collection of 10 fecal samples in 2 volunteer commercial poultry farms, aim to demonstrate the effects of current antibiotic practices with respect to their antibiotic resistance gene contents. However, isolation and identification of resistant bacterial isolates are outside the scope of this study. Demonstrating antibiotic resistance genes from actual bacterial isolates, as well as an expanded sampling size, can be pursued in future studies. Nonetheless, this study may provide additional insights for the effective implementation of the Philippine's comprehensive action plan in combatting the spread of antibiotic resistance.

MATERIALS AND METHODS

Animal Handling and Care

Treatment protocol was adopted from the study of Nicdao (2020). Eighteen commercially-acquired 1-day-old Cobb broiler chicks were separated into 2 treatment groups: antibiotic-treated and antibiotic-free/control group. Each group (n=9) were housed in a cage made of polyvinyl chloride-coated wire mesh with hover-type brooder heat lamps and removable catch trays for animal litter. Both cages were exposed to natural ventilation and were protected from direct sunlight and wet weather conditions. Erythromycin (250mg/L; days 1-3), doxycycline (250mg/L; days 7-10), amoxicillin (250mg/L; days 17-19), and tetracycline (275mg/L; days 22-24) powder were mixed through drinking water using commercially prescribed prophylactic doses for the antibiotic-treated group (Figure 1). Identical schedule of antibiotic-free starter feeds (days 1-21), grower feeds (days 22-28), and finisher feeds (days 28-32) were given *ad libitum* for both treatment groups. Weekly fecal samples were collected and pooled starting at day 0 until termination of the experiment (day 32). Institutional Animal Care and Use Committee approval was acquired from Pampanga State Agricultural University, where all animal experiments were housed and conducted.

Poultry Farm Data and Fecal Samples Collection

Representatives from 12 commercial broiler poultry farms in the Philippines were interviewed between the years 2018-2019. Antibiotic practices, as well as projected annual industry output, were elicited using a prepared questionnaire. Interviews were conducted in coordination with the local municipal agriculture office. Among the interviewed farms, only five agreed to participate in fecal sample collection. Fecal samples from ten broilers were individually collected using sterile cloacal plastic bags fastened in the caudal region to directly catch fecal material with minimal environmental contamination. Between the five poultry farms, only two farms that reported the use of the most frequently utilized medically important antibiotics (quinolones and tetracyclines) were included in the study. Commercial poultry farm 1 administered prophylactic doses of Doxycycline everyday *per orem* mixed with daily feeds (250mg/kg of feeds), except during the recommended 7-day antibiotic withdrawal period before culling. Commercial poultry farm 2 utilized Doxycyline (250mg/L) and Enrofloxacin (50mg/L) once a week while using Levofloxacin (50mg/L) only once for growth promotion. Aforementioned 3

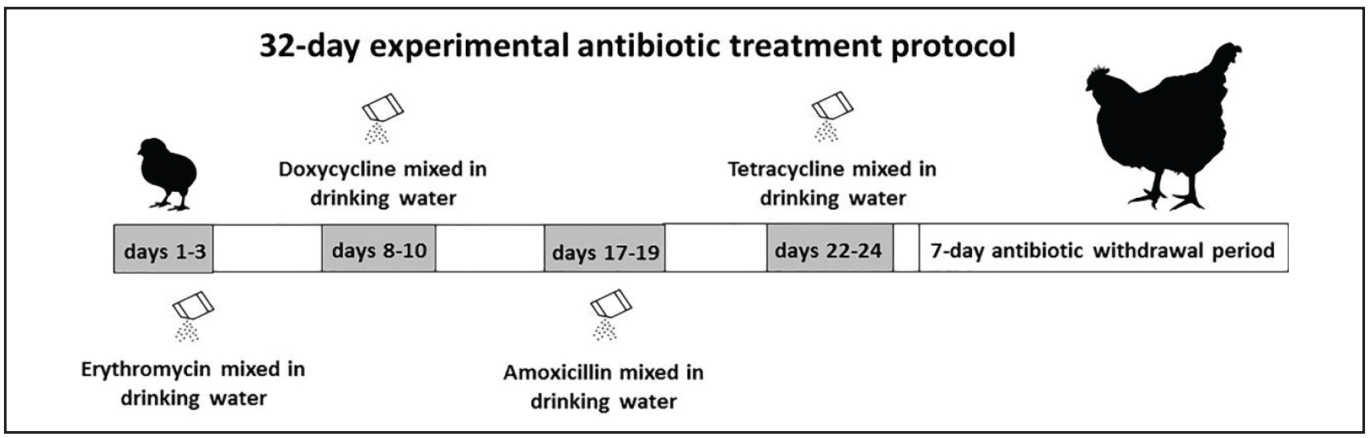

Figure 1. In the experimental setup, Erythromycin (250mg/L), Doxycycline (250mg/L), Amoxicillin (250mg/L) and Tetracycline (275mg/L) were individually administered *per orem* by mixing them with drinking water while following commercially prescribed prophylactic doses.

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Screening of Antibiotic Resistance Genes

Fecal samples were frozen (-18°C) until ready for DNA extraction. Fecal DNA were isolated using Quick-DNA Fecal/Soil Microbe Miniprep Kit (Cat# D6010) as instructed in the product manual. Isolated DNA were subjected to initial denaturation (95°C x 1-minute) and 30 cycles of uniplex PCR (denaturation: 95°C x 15-seconds, annealing: 52°C x 15-seconds, extension: 72°C x 10-seconds) using MyTaq Red DNA Polymerase (Cat# BIO-21108). Plasmid-encoded antibiotic resistance genes (*ereB, ermB, tetA, tetB, tetU, tetW, tla-1, ctx-M-1, qnrB, qnrS*) were screened using their appropriate primer pairs and positive controls (Table 1). All positive controls were synthesized (Kinovett Scientific Solutions Co.) based on primer sequences available in literature. Resulting amplicons were then run at 75-volts for 40-minutes in 1% agarose in 2.5x TBE buffer gel-electrophoresis with pre-cast addition of 1x GelRed stain.

Table 1. Antibiotic resistance genes, primer pairs and their respective positive controls

Genes	Primer pairs (5' to 3')	References	Positive Control Sequences (5' to 3')
ereB	forward (TCTGCATTATGCCAACGGTA) reverse (TCTGCTCACTTTGTGGGTTT)	Szczepanowski *et al*., 2009	ATGAGACAATTCTGCATTATGCCAACGGTATGATATACTTGGACTATAAC ATTCAAGCTATGTCGGGCTTTATTTCAGGAGGCGGAATGCAGGGCGATA TGGGTGCAAAAGACAAATACATGGCAGATTCTGTGCTGTGGCATTTAAA AAACCCACAAAGTGAGCAGAAAGTGATAGT
ermB	forward (CTATCTGATTGTTGAAGAAGGATT) reverse (GTTTACTTTTGGTTTAGGATGAAA)	Akanbi *et al.*, 2017	CGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTG GATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCA GCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAA ACAGTGTCTTAA
tetA	forward (CCTGATTATGCCGGTGCT) reverse (TGGCGTAGTCGACAGCAG)	Szczepanowski *et al*., 2009	TCGGCATCGGCCTGATTATGCCGGTGCTGCCGGGCCTCCTGCGCGATCT GGTTCACTCGAACGACGTCACCGCCCACTATGGCATTCTGCTGGCGCTGT ATGCGTTGATGCAATTTGCCTGCGCACCTGTGCTGGGCGCGCTGTCGGA TCGTTTCGGGCGGCGGCCGGTCTTGCTCGTCTCGCTGGCCGGCGCTGCT GTCGACTACGCCATCATGGCGAC
tetB	forward (TACGTGAATTTATTGCTTCG) reverse (ATACAGCATCCAAAGCGCA)	Srinivasan *et al.*, 2009	CCAACGTTATTACGTGAATTTATTGCTTCGGAAGATATCGCTAACCACTTT GGCGTATTGCTTGCACTTTATGCGTTAATGCAGGTTATCTTTGCTCCTTGG CTTGGAAAAATGTCTGACCGATTTGGTCGGCGCCCAGTGCTGTTGTTGTC ATTAATAGGCGCATCGCTGGATTACTTATTGCTGGCTTTTTCAAGTGCGC TTTGGATGCTGTATTTAGGCCGTT
tetU	forward (GCAGCTAAGACGTGGCAAA) reverse (TGCTTCAGCAAATTCCGATA)	Puhler *et al.*, 2007	ATGCAGCTAAGACGTGGCAAAGCAACGGATTGGCATGCGATGGTTCAG GAAAGCTTAGATAGTTTTGCAAGCCCGCATTTTTTGCCGATTGATATAAA ACCTATTGATAAAATAGTTATTGAAGGTTTGATAGCTGAGCCTTCTAATT GGTCGATAATTGCTAGACATACAAAATATAAATATCGGAATTTGCTGAA GCAAGAAAGTCAA
tetW	forward (GTCGAAAAAGGGACAACGAG) reverse (CTAAAACAGCCAAAGAGCGG)	Szczepanowski *et al*., 2009	ACCGGGGAGCGTCGAAAAAGGGACAACGAGGACGGACACCATGTTTTT GGAGCGGCAGCGTGGGATTACCATTCAAGCGGCAGTCACTTCCTTCCAG TGGCACAGATGTAAAGTTAACATTGTGGATACGCCCGGCCACATGGATT TTTTGGCGGAGGTGTACCGCTCTTTGGCTGTTTTAGATGGGGCCAT
tla-1	forward (GACGCTACCGTTCAGCTCTT) reverse (GTGGCAGCAGTAATGCCTTT)	Puhler *et al.*, 2007	GACAACTCCCGACGCTACCGTTCAGCTCTTAAAGAAGTTCTACAAAAATG AAATACTCTCAAAAAATAGTTACGACTATTTGCTTAATACTATGATTGAA ACTACTACCGGACCGAAACGACTCAAAGGACTTTTGCCCGATGGAACTG TTGTTGCTCATAAAACCGGAAGCTCCGATACTAACGATAAAGGCATTACT GCTGCCACAAATGATATC
ctx-M-1	forward (ACCAACGATATCGCGGTGA) reverse (ACATCGCGACGGCTTTCT)	Calero-Caceres *et al.*, 2014	CTATGGCACCACCAACGATATCGCGGTGATCTGGCCAAAAGATCGTGCG CCGCTGATTCTGGTCACTTACTTCACCCAGCCTCAACCTAAGGCAGAAAG CCGTCGCGATGTATTAGCGTCG
qnrB	forward(GATCGTGAAAGCCAGAAAGG) reverse (ACGATGCCTGGTAGTTGTCC)	Qin *et al*., 2017	TCAGTTCTATGATCGTGAAAGCCAGAAAGGGTGCAATTTTAGTCGTGCG ATGCTGAAAGATGCCATTTTTAAAAGCTGTGATTTATCCATGGCGGATTT TCGCAATTCCAGTGCGCTGGGCATTGAAATTCGCCACTGCCGCGCACAA GGCGCAGATTTCCGCGGCGCAAGCTTTATGAAATATGATCACCACGCGCA CCTGGTTTTGTAGCGCATATATCACGAATACCAATCTAAGCTACGCCAAT TTTTCGAAAGTCGTGTTGGAAAAGTGTGAGCTGTGGGAAAACCGTTGGA TAGGTGCCCAGGTACTGGGCGCGACGTTCAGTGGTTCAGATCTCTCCGG CGGCGAGTTTTCGACTTTCGACTGGCGAGCAGCAAACTTCACACATTGC GATCTGACCAATTCGGAGTTGGGTGACTTAGATATTCGGGGCGTTGATT TACAAGGCGTTAAGTTGGACAACTACCAGGCATCGTTGCTCATGGA
qnrS	forward (ACGACATTCGTCAACTGCAA) reverse (TAAATTGGCACCCTGTAGGC)	Qin *et al*., 2017	CTTGCGTGATACGACATTCGTCAACTGCAAGTTCATTGAACAGGGTGATA TCGAAGGCTGCCACTTTGATGTCGCAGATCTTCGTGATGCAAGTTTCCAA CAATGCCAACTTGCGATGGCAAACTTCAGTAATGCCAATTGCTACGGTAT AGAGTTCCGTGCGTGTGATTTAAAAGGTGCCAACTTTTCCCGAACAAACT TTGCCCATCAAGTGAGTAATCGTATGTACTTTTGCTCAGCATTTATTTCTG GATGTAATCTTTCCTATGCCAATATGGAGAGGGTTTGTTTAGAAAAATGT GAGTTGTTTGAAAATCGCTGGATAGGAACGAACCTAGCGGGTGCATCAC TGAAAGAGTCAGACTTAAGTCGAGGTGTTTTTTCCGAAGATGTCTGGGG GCAATTTAGCCTACAGGGTGCCAATTTATGCCACGCCG

Data Analysis

Predicted rates of screened antibiotic resistance genes in participating commercial poultry farms were reported as confidence intervals. Confidence intervals for a population proportion at 95% confidence level were calculated using the formula:

$$\rho \pm z^* \sqrt{\frac{\rho(1-\rho)}{n}},$$

where ρ is the sample proportion, n is the sample size, and z * is the appropriate value (1.96) for the standard normal distribution at 95% confidence level (Rumsey, 2021).

RESULTS

Local poultry farms utilize medically important antibiotics

Representatives from 12 commercial poultry farms were interviewed to elicit their respective antibiotic practices. All interviewed farms (100%) reported the use antibiotics for broiler production. Eleven out of the 12 farms (91.67%) use antibiotics for prophylaxis while all farms (100%) use antibiotics for metaphylaxis. Six out of the 12 farms (50%) use antibiotics for growth promotion. All 12 farms (100%) utilize antibiotics that are classified under the 2017 WHO list of medically important antimicrobials (Table 2). Among the antibiotic classes, nitrofurantoins (25%) were the most frequently reported for prophylaxis (Figure 2A); aminoglycosides (23.08%) were the most frequently reported for metaphylaxis (Figure 2B); quinolones (33.33%) were the most frequently reported for growth promotion (Figure 2C). In consideration of their reported poultry farm productions, quinolones (39.94%) are the most utilized class of antibiotics, followed by tetracyclines (29.33%) and aminoglycosides (18.78%) (Figure 2D). With widespread use of medically important antibiotics, it is important to investigate its direct effect to the emergence of antibiotic resistance genes in fecal samples for broiler chickens.

Antibiotic resistance gene (ermB) appeared as early as day 7 in antibiotic-treated broilers within the experimental setup

Broilers were grown in an experimental setup to investigate weekly changes in their antibiotic resistance gene contents. Pooled fecal samples from the antibiotic-treated and antibiotic-free experimental groups were initially free of any of the screened antibiotic resistance genes. After the first week (day 7), *ermB* appeared in the antibiotic-treated group and was consistently present until termination of the experiment. In the antibiotic-free group, no antibiotic resistance genes were found until the third week (day 21), when *ermB* also persisted until the termination of the experiment (Figure 3A). No other screened antibiotic resistance genes were found in both treatment groups (Figure 3B). With the emergence of antibiotic resistance genes in the experimental setup, investigating antibiotic resistance gene contents of broilers in actual commercial poultry farms becomes even more significant.

Tetracycline resistance genes (*tetA*, *tetB*, *tetU*, *tetW*) were demonstrated in fecal samples acquired from commercial poultry farms

Fecal samples from two participating commercial poultry farms were acquired to investigate the effect of their respective antibiotic treatments to broiler antibiotic resistance gene contents. In the first commercial poultry farm where only doxycycline was used, 8 out of the 10 fecal samples (80%) contained *tetA* (Figure 4A), 10 out of 10 (100%) contained *tetB* (Figure 4B), 9 out of 10 (90%) contained *tetU* (Figure 4C), 8 out of 10 (80%) contained *tetW* (Figure 4D). In the second commercial poultry farm where doxycycline, enrofloxacin and levofloxacin were used, 3 out of the 10 fecal samples (30%) contained *tetA* (Figure 4A), 3 out of 10 (30%) contained *tetB* (Figure 4B), 6 out of 10 (60%) contained *tetU* (Figure 4C), 7 out of 10 (70%) contained *tetW* (Figure 4D). It appears that tetracycline resistance genes are more prevalent in the first commercial poultry farm when compared to the second.

Quinolone resistance genes (*qnrB*, *qnrS*) were demonstrated only in fecal samples acquired from the commercial poultry farm that used tetracycline and quinolone antibiotics

Interestingly, in the second commercial poultry farm, which included quinolones in their antibiotic regimen, 2 out of the 10 (20%) fecal samples contained *qnrB* (Figure 5A) while 4 out of the 10 (40%) contained *qnrS* (Figure 5B). These genes were not detected in the farm which did not use quinolones in their farming practice. No *ermB* antibiotic resistance gene was found in fecal samples from both commercial poultry farms (Figure 5C).

In summary, our data demonstrated that the presence of resistance genes were reflective of the corresponding antibiotic treatment protocols of participating commercial poultry farms (Figure 6A and 6B). Multiple tetracycline resistance genes were present in all fecal samples from the first commercial poultry farm while both tetracycline and quinolone resistance genes appeared only in the second commercial poultry farm (Figure 6C). Calculating for their

Table 2. Reported antibiotics utilized in interviewed local poultry farms

Antibiotics	ATC Classification	2017 WHO Classification of Medically Important Antimicrobials
Amoxicillin	(J01C) beta-lactam penicillins	(critically important) aminopenicillins
Bacitracin	(J01X) other antibacterials	(important) cyclic polypeptides
Colistin	(J01X) other antibacterials	(critically important) polymyxins
Co-trimoxazole	(J01E) sulfonamides and trimethroprim	(highly important) sulfonamides, dihydrofolate reductase inhibitors and combinations
Doxycycline	(J01A) tetracyclines	(highly important) tetracyclines
Enrofloxacin	n/a	(critically important) quinolones
Fosfomycin	(J01X) other antibacterials	(critically important) phosphonic acid derivatives
Gentamycin	(J01G) aminoglycosides	(critically important) aminoglycosides
Levofloxacin	(J01M) quinolones	(critically important) quinolones
Nifuroxazide	(J01X) other antibacterials	(important) nitrofurantoins
Ofloxacin	(J01M) quinolones	(critically important) quinolones
Penicillin	(J01C) beta-lactam penicillins	(critically important) natural penicillins
Tylosin	n/a	(critically important) macrolides

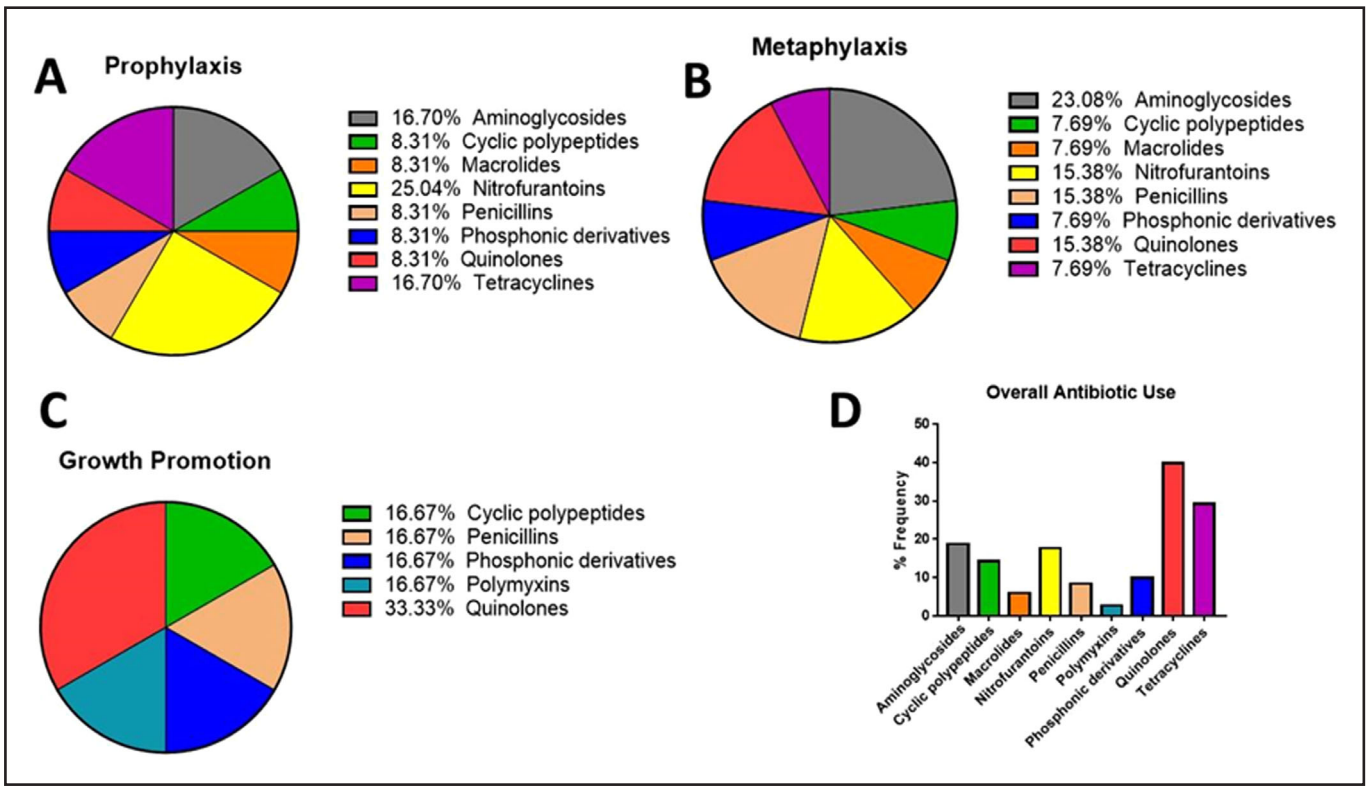

Figure 2. (A-C) All interviewed commercial poultry farms reported the use of medically important antibiotics for different applications. (D) In consideration of their reported poultry farm outputs (N=5,643,000 broilers), quinolones are the most frequently applied antibiotics to broiler chickens, followed by tetracyclines and aminoglycosides.

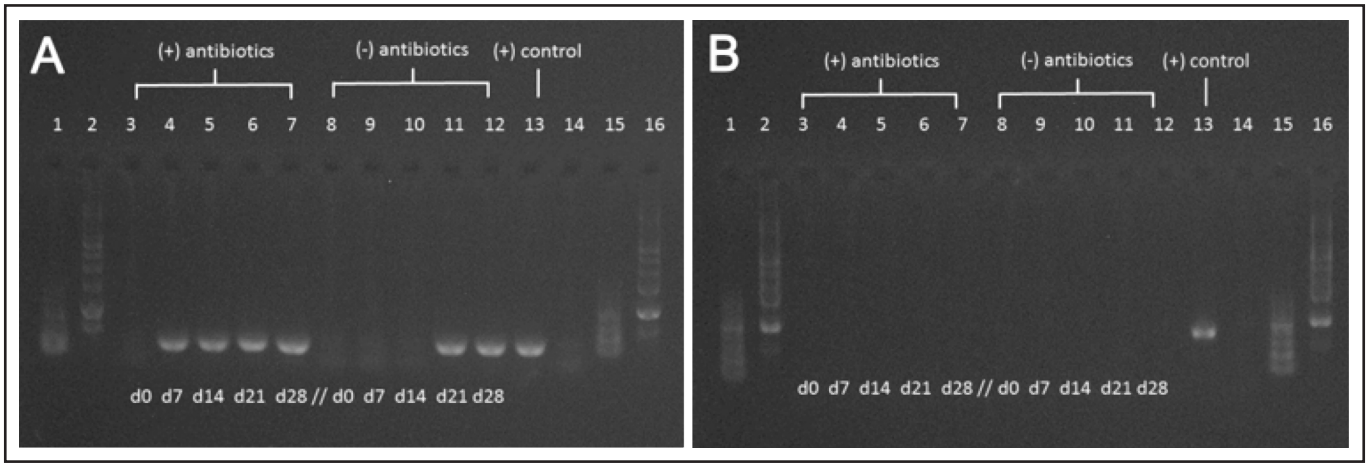

Figure 3. (A) Antibiotic resistance gene *ermB* appeared in fecal samples of antibiotic-treated broilers as early as the 7th day (d7) and persisted throughout the experiment (n=9). In contrast, antibiotic resistance gene *ermB* appeared later in fecal samples of antibiotic-free broilers at d21 (n=9) (lanes 1 and 15=100bp ladder; 2 and 16=1kbp ladder; 13=*ermB* positive control; 14=blank). (B) No antibiotic resistance genes appeared when screened for *qnrB* as no quinolone antibiotic was utilized in the experimental setup (lane 13=*qnrB* positive control).

predicted antibiotic resistance rates, confidence intervals for tetracycline resistance are all higher for the first commercial poultry farm when compared to that of the second (Table 3).

DISCUSSION

Poultry industry is an important component of the Philippine economy. Assisting our farmers to maximize their profits and outputs while ensuring ecologically sustainable methods for food sustainability should be a top priority. Given the difference of actual antibiotic practices in the Philippines when compared to international standards and recommendations, more researches should be done to determine if current local antibiotic practices are indeed beneficial for our farmers or may actually be detrimental in the long run.

Looking at the reported poultry farm antibiotic applications, local farmers use antibiotics to prevent undiagnosed diseases (prophylaxis), as well as to control

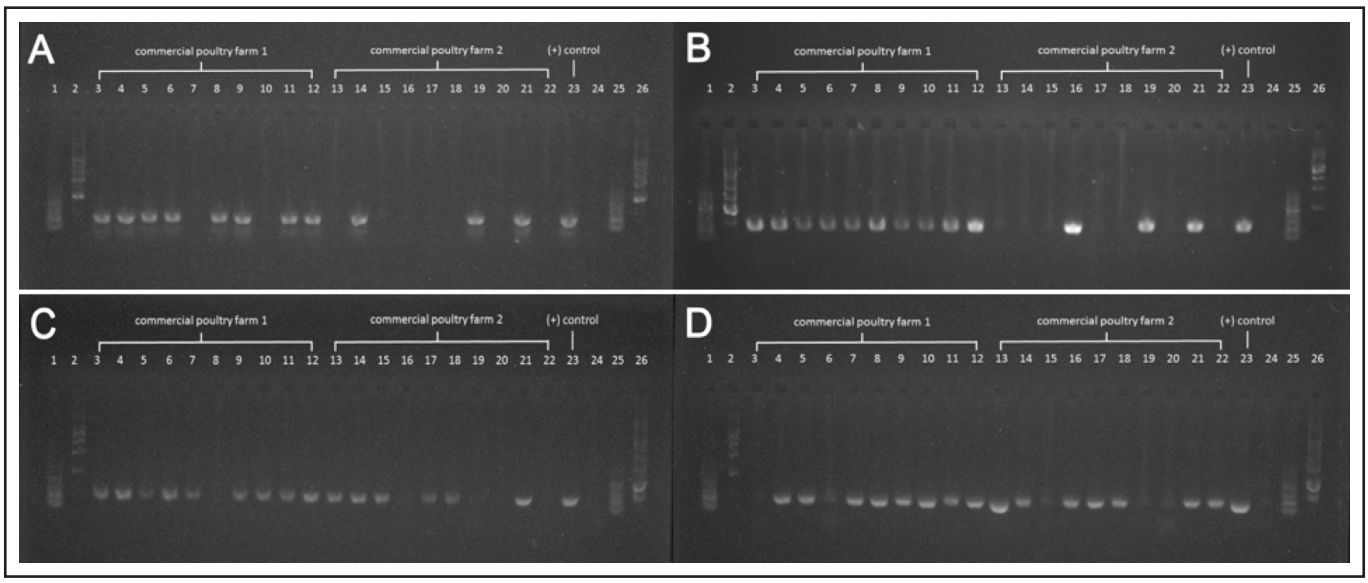

Figure 4. (A) Antibiotic resistance gene *tetA* appeared in 80% and 30% of fecal samples from commercial poultry farms 1 and 2, respectively (lanes 1 and 25=100bp ladder; 2 and 26=1kbp ladder; 3 to 12=samples from commercial poultry farm 1; 13 to 22=samples from commercial poultry farm 2; 23=*tetA* positive control; 24=blank). (B) Antibiotic resistance gene *tetB* appeared in 100% and 30% of fecal samples from commercial poultry farms 1 and 2, respectively (lane 23=*tetB* positive control). (C) Antibiotic resistance gene *tetU* appeared in 90% and 60% of fecal samples from commercial poultry farms 1 and 2, respectively (lane 23=*tetU* positive control). (D) Antibiotic resistance gene *tetW* appeared in 80% and 70% of fecal samples from commercial poultry farms 1 and 2, respectively (lane 23=*tetW* positive control).

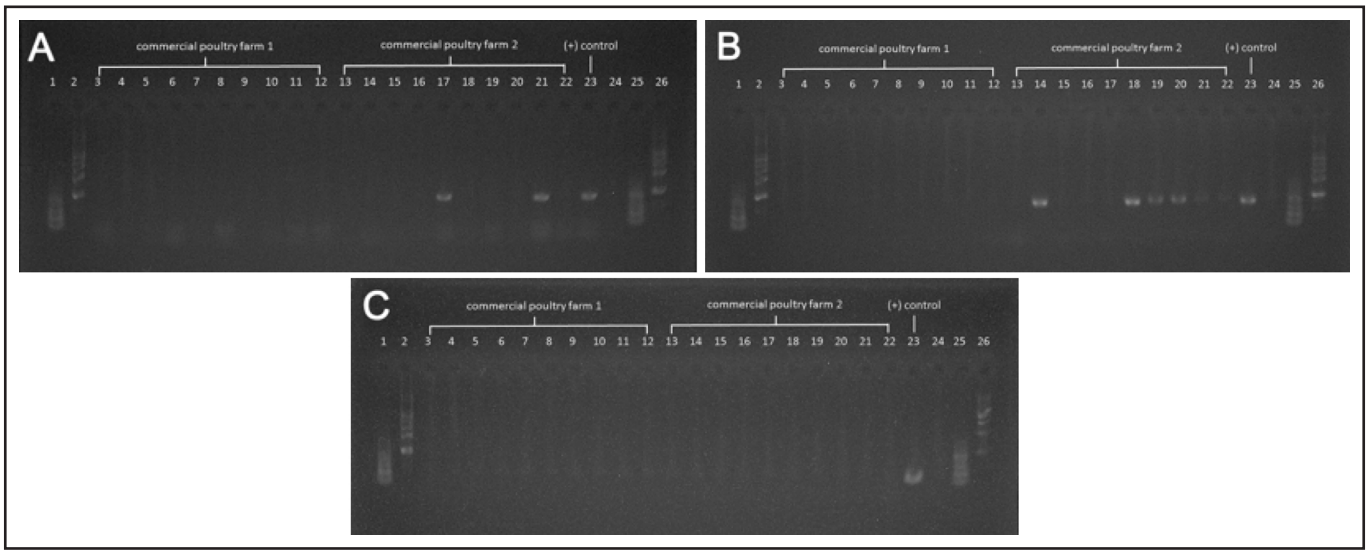

Figure 5. (A) Antibiotic resistance gene *qnrB* appeared only in 20% of fecal samples from commercial poultry farm 2 (lanes 1 and 25=100bp ladder; 2 and 26=1kbp ladder; 3 to 12=samples from commercial poultry farm 1; 13 to 22=samples from commercial poultry farm 2; 23=*qnrB* positive control; 24=blank). (B) Antibiotic resistance gene *qnrS* appeared only in 40% of fecal samples from commercial poultry farm 2 (lane 23=*qnrS* positive control). (C) Antibiotic resistance gene *ermB* did not appear in any of the fecal samples collected from participating commercial poultry farms (lane 23=*ermB* positive control).

the spread of diagnosed diseases by treating animals as a group (metaphylaxis). In the recent study of Barroga *et al*. (2020), respiratory diseases, followed by enteric diseases, are the most common indications for animal antibiotic treatment in Philippine farms. The third reported application in this study, growth promotion, is the most concerning as they usually entail the use of sub-therapeutic doses to improve animal meat output and profit (O' Neill, 2015). The latter practice may promote further the spread of antibiotic resistance genes as microbes are exposed to antibiotic selective pressures without necessarily eliminating undesired pathogens. With the emergence of antibiotic resistance genes using local antibiotic treatment protocols, it is possible that local farmers may encounter more antibiotic resistant infections that negatively affects their output and net profit.

In general, reported rampant use of medically important antibiotics reaffirms the discrepancy between current WHO guidelines and the actual situation in the Philippines. In their policy brief, WHO recommends (1) an overall reduction

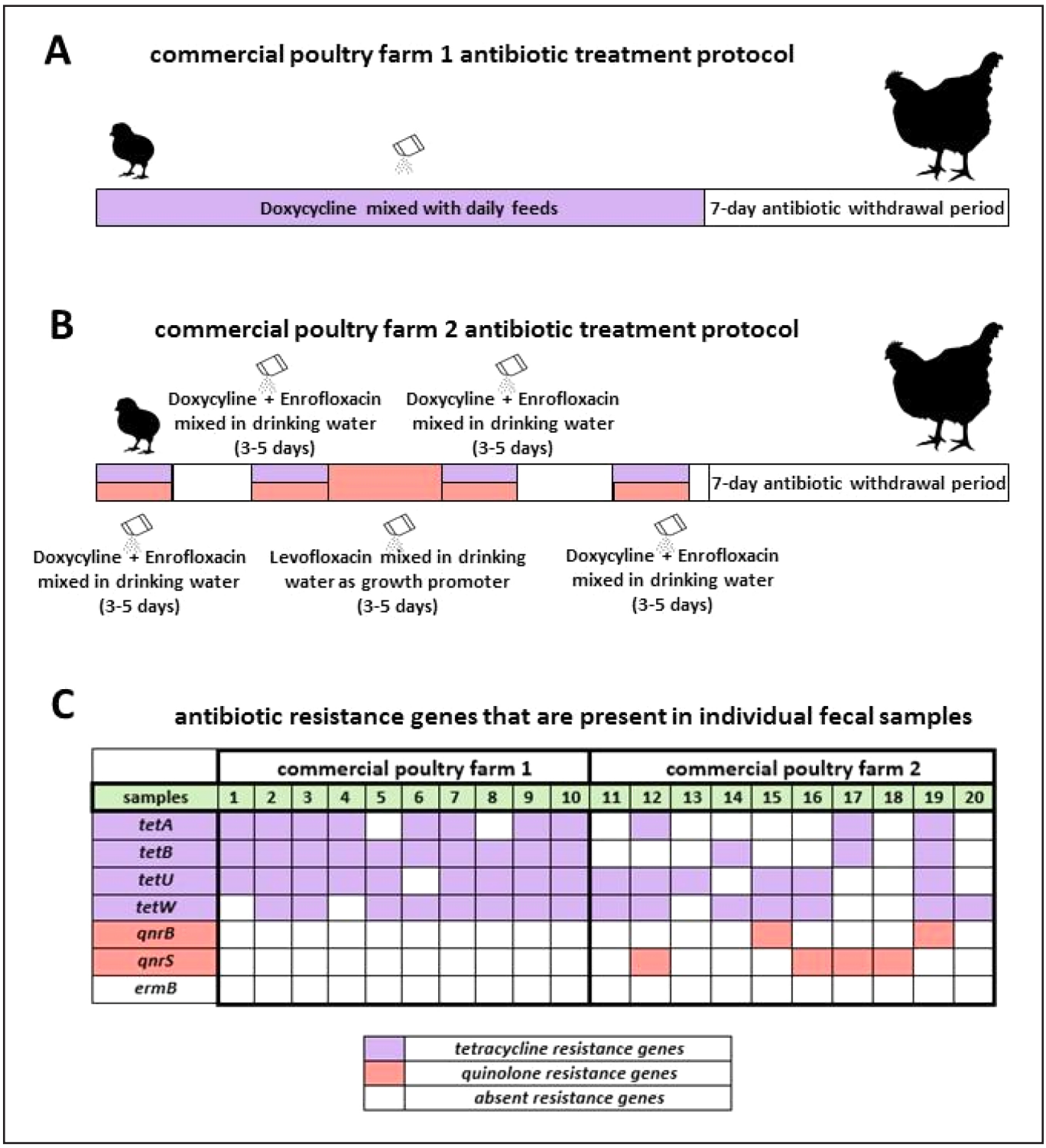

Figure 6. (A) In the first commercial poultry farm, Doxycycline (250mg/kg of feeds) was reported to be administered everyday *per orem* as it was mixed in the daily feeds except during the recommended antibiotic withdrawal period. (B) In the second commercial poultry farm, Doxycycline (250mg/L), Enrofloxacin (50mg/L) and Levofloxacin (50mg/L) were reported to be administered *per orem* by mixing with drinking water while following commercially prescribed prophylactic doses. (C) All collected fecal samples contain at least 1 antibiotic resistance gene. Fecal samples from broilers that were treated everyday with Doxycycline exhibited multiple resistance genes that correspond to the appropriate class of antibiotic (tetracyclines). In contrast, resistance genes for both tetracyclines and quinolones were observed in the fecal samples of the second commercial poultry farm as both classes of antibiotics were utilized. No resistance genes appeared when screened for *ermB* as no macrolide antibiotic was reported to be used in the 2 commercial poultry farms.

Table 3. Predicted antibiotic resistance rates using confidence intervals for population proportion at 95% confidence level

Antibiotic Resistance Gene	Commercial Poultry Farm 1 Fecal SampleResistance Rate	Confidence Interval	Commercial Poultry Farm 2 Fecal SampleResistance Rate	Confidence Interval
tetA	80%	55.21% to 100%	30%	1.60% to 58.40%
tetB	100%	–	30%	1.60% to 58.40%
tetU	90%	89.81% to 100%	60%	29.64% to 90.36%
tetW	80%	55.21% to 100%	70%	41.60% to 98.40%
qnrB	–	–	20%	0% to 44.79%
qnrS	–	–	40%	9.64% to 70.36%

in use of all classes of medically important antimicrobials in food-producing animals, (2) complete restriction of use of all classes of medically important antimicrobials in food-producing animals for growth promotion, (3) complete restriction of use of all classes of medically important antimicrobials in food-producing animals for prevention of infectious disease that have not yet been clinically diagnosed, and (4) the cessation of use of critically important antimicrobials in human medicine for the control or treatment of a clinically diagnosed infectious disease in food-producing animals (2017). With the reported use of critically important antibiotics (i.e. aminoglycosides, macrolides, phosphonic acid derivatives, polymixins, penicillins, and quinolones) for prophylactic, metaphylactic and growth promotion purposes, interviewed poultry farms are much different from international poultry farms in terms of antibiotic practices. In the perspective of global trade, this may become a major concern as countries become more conscious regarding the use of antibiotics in agriculture (Maron *et al*., 2013). Therefore, more consideration should be given if declared Philippine policies on animal antibiotic use are indeed effectively implemented and are in line with WHO recommendations.

Appearance of *ermB* at day 7 in the fecal samples of the antibiotic-treated group demonstrates that the use of a local poultry farm antibiotic treatment protocol may lead to the emergence of antibiotic resistance genes. The gene *ermB* is an acquired antibiotic resistance gene that confers resistance against macrolides and other classes of antibiotics with similar mechanism of action, such as lincosamides, streptogramin B and oxazolidinones. It encodes for rRNA methylases that acts on adenine residues and prevents binding of the antibiotics to the 50S ribosomal subunit of their target bacteria (Van Hoek *et al*., 2011). It has been reported that antibiotic resistance genes such as *ermB*, can be directly acquired from natural reservoirs in the environment with their highly mobile genetic platforms such as plasmids and transposons through horizontal gene transfer (Cantón, 2009). Furthermore, *ermB* expression is also induced by the presence of erythromycin (Wang *et al*., 2021). With the day 1 application of erythromycin in the antibiotic-treated group, antibiotic selective pressure may have promoted its acquisition and prevalence from day 7 onwards. Interestingly, the unexpected presence of *ermB* in the antibiotic-free group from day 21 onwards may also be attributed to the possibility of horizontal gene transfer through abiotic surfaces, as reported in the study of Warnes *et al.* (2012). Inadvertent contamination in a common physical environment may have occurred during feeding, animal handling and cage cleaning within the experimental setup. Alternatively, it can also be argued that the use of antibiotics in the experimental setup simply hastened the eventual acquisition of *ermB* that is already present in its natural reservoir. As such, *ermB* also eventually appeared in the antibiotic-free group. Applying either concepts in actual poultry farms, it is very probable that antibiotic resistance genes may easily spread among antibiotic-treated broilers, as well as in their immediate environment.

In commercial poultry farm fecal samples, the role of antibiotic treatments in the spread of antibiotic resistance genes is again reiterated. It is noteworthy that multiple tetracycline resistance genes appeared more frequently in samples treated with daily doses of Doxycycline (commercial poultry farm 1) while only those treated with Enrofloxacin and Levofloxacin (commercial poultry farm 2) demonstrated the presence of quinolone resistance genes (Figure 6C). Additionally, higher predicted rates (Table 3) of tetracycline resistance *per se* in the first commercial poultry farm may also be attributed to the daily administration of Doxycycline as sequential use of the same antibiotic promote further the development of antibiotic resistance through persistent antibiotic selective pressure (D'Agata *et al*., 2008).

For tetracyclines, prevalence of their resistance genes has long been considered a contributory factor to the decline of their clinical applications (Speer *et al*., 1992). Genes *tetA, tetB* and *tetU* are established to code for energy-dependent efflux proteins that are prevalent in gram-negative bacteria, while *tetW* encodes for ribosomal protection proteins that directly hinder the 30s ribosomal subunit inhibition of tetracyclines (Roberts, 2005; CARD, 2021). In contrast, quinolone resistance genes were initially considered less of a concern given the synthetic nature of quinolones and the supposed absence of corresponding natural resistance genes (Hernández *et al*., 2011). However, mutations in quinolone target-enzymes DNA gyrase and topoisomerase IV, as well as mutations in regulatory genes of efflux pumps, were initially discovered as the main mechanisms of quinolone resistance. Recently, plasmid-encoded quinolone resistance genes have also emerged. Included in the latter are *qnrB* and *qnrS*, which both encode for proteins that protect DNA gyrase (Hooper & Jacoby, 2015; CARD, 2021). All the aforementioned tetracycline and quinolone resistance genes can also be directly acquired from natural reservoirs in the environment through highly mobile genetic platforms (Cantón, 2009). Altogether, the demonstration of tetracycline and quinolone antibiotic resistance genes in the fecal samples of commercially-available antibiotic-treated broilers support the current thrust of WHO in terms of regulating agricultural antibiotic applications.

Most developed countries have previously conducted their own studies regarding risk and cost-benefit analyses of agricultural antibiotic use, whether using an economical perspective (Graham *et al*., 2007; Teillant & Laxminarayan, 2015; Bergevoet *et al*., 2019) or considering its influence on human clinical antibiotic-resistant infections (Cogliani *et al*., 2011; Forslund *et al*., 2013; Hu *et al*., 2014). As a result, WHO and most developed countries have somewhat reached a consensus with their existing policies and recommendations

regarding regulating agricultural antibiotic use (European Parliament, 2019; FDA, 2021; WHO, 2017). In the Philippines, it appears that we have yet to conduct our own substantial studies that investigate similar aspects of local agricultural antibiotic use. Hence, it is encouraging that the need for more studies was also highlighted in the Philippines' current key strategies in combatting antimicrobial resistance (Inter-Agency Committee on Antimicrobial Resistance, 2019). Still, the heterogeneity of data and limitations of data collection hinder the effective comparison of antibiotic practices whether in humans or animal applications, as well as the effective demonstration of a possible correlation between antibiotic resistant rates in both. Demonstrating that rampant poultry antibiotic use has a direct effect on reported human clinical antibiotic resistant rates remains to be a challenge in the Philippine setting.

A possible solution is to improve antibiotic surveillance data through the systemic inclusion of antibiotic consumption in terms of the Defined Daily Dose (DDD) of the WHO Collaborating Center for Drug Statistics Methodology. DDD is the assumed average maintenance dose per day for a drug used for its main indication in human adults and thus, is different from the actual prescribed dose. It can be utilized to standardize the comparison of antibiotic usage between different health care environments in conjunction with Anatomical Therapeutic Chemical (ATC) classification of drugs (Hutchinson *et al*., 2004). With DDD values, we can directly compare antibiotic consumption within and between institutions across time and effectively correlate their reported clinical antibiotic resistance rates. For animal antibiotic use, applying the same standardization concept of DDD is more challenging as animal antibiotic practices are extensively more varied as opposed to the relative uniformity of human medical practice. Consequently, some developed countries have recently published their own veterinary values (DDD_{vet}) in order to have more effective surveillance of their animal antibiotic practices (Fujimoto *et al*., 2021). Should we have our own set of DDD_{vet} values and a systemic monitoring of animal antibiotic consumption, we can also directly compare within and between institutions across time and also effectively correlate to reported clinical antibiotic resistance rates.

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Conflict of Interest

The authors declare that they have no conflict of interests.

REFERENCES

Allen, H.K. & Stanton, T.B. (2014). Altered egos: antibiotic effects on food animal microbiomes. *Annual Review of Microbiology* **68**: 297-315. https://doi.org/10.1146/annurev-micro-091213-113052

Barroga, T.R.M., Morales, R.G., Benigno, C.C., Castro, S.J.M., Caniban, M.M., Cabullo, M.F.B., Agunos, A., de Balogh, K. & Dorado-Garcia, A. (2020). Antimicrobials used in backyard and commercial poultry and swine farms in the Philippines: A qualitative pilot study. *Frontiers in Veterinary Science* **7**: 329. https://doi.org/10.3389/fvets.2020.00329

Bergevoet, R., van Asseldonk, M., Bondt, N., van Horne, P., Hoste, R., de Lauwere, C. & Puister-Jansen, L. (2019). Economics of antibiotic usage on Dutch farms: The impact of antibiotic reduction on economic results of pig and broiler farms in the Netherlands. *Wageningen Economic Research*. https://library.wur.nl/WebQuery/wurpubs/fulltext/475403

Cantón, R. (2009). Antibiotic resistance genes from the environment: A perspective through newly identified antibiotic resistance mechanisms in the clinical setting. *Clinical Microbiology and Infection* **15**: 20-25. https://doi.org/10.1111/j.1469-0691.2008.02679.x

CARD. (2021). Comprehensive Antibiotic Resistance Database. https://card.mcmaster.ca/. Accessed 13 December 2021.

Cogliani, C., Goossens, H. & Greko, C. (2011). Restricting antimicrobial use in food animals: Lessons from Europe. *Microbe* **6**: 274-279. https://doi.org/10.1128/microbe.6.274.1

D'Agata, E.M.C., Dupont-Rouzeyrol, M., Magal, P., Olivier, D. & Ruan, S. (2008). The impact of different antibiotic regimens on the emergence of antimicrobial-resistant bacteria. *PLoS ONE* **3**: e4036. https://doi.org/10.1371/journal.pone.0004036

Department of Health & Department of Agriculture. (2013). Rules on the regulation of veterinary drugs and products, veterinary biological products, and veterinary drug establishments. Philippines. https://www.fda.gov.ph/wp-content/uploads/2021/04/JOINT-DOH-and-DA-Administrative-Order-No.-2013-0026.pdf

Department of Health, Department of Agriculture, Department of Science and Technology, Department of Trade and Industry & Department of Interior and Local Government. (2015). The Philippine Action Plan to Combat Antimicrobial Resistance: A One Health Approach. *Philippine Action Plan to Combat AMR: One Health Approach*: 1-40.

European Parliament. (2019). Veterinary medicines: fighting antibiotic resistance. https://www.europarl.europa.eu/news/en/headlines/society/20181018STO16580/veterinary-medicines-fighting-antibiotic-resistance. Accessed 24 August 24 2021.

FDA (United States Food and Drug Administration)(2021). Antibiotic Resistance. https://www.fda.gov/animal-veterinary/safety-health/antimicrobial-resistance# Antimicrobial Resistance. Accessed 24 August 24 2021.

Forslund, K., Sunagawa, S., Kultima, J.R., Mende, D.R., Arumugam, M., Typas, A. & Bork, P. (2013). Country-specific antibiotic use practices impact the human gut resistome. *Genome Research* **23**: 1163-1169. https://doi.org/10.1101/gr.155465.113.23

Fujimoto, K., Kawasaki, M., Abe, R., Yokoyama, T., Haga, T. & Sugiura, K. (2021). Establishing defined daily doses (DDDs) for antimicrobial agents used in pigs, cattle and poultry in Japan and comparing them with European DDD values. *PLoS ONE* **16**: e0245105. https://doi.org/10.1371/journal.pone.0245105

Graham, J.P., Boland, J.J. & Sibergeld, E. (2007). Growth promoting antibiotics in food animal production: An economic analysis. *Public Health Report* **122**: 79-87. https://doi.org/10.1177/003335490712200111

Hernández, A., Sánchez, M.B. & Martínez, J.L. (2011). Quinolone resistance: much more than predicted. *Frontiers in Microbiology* **2**: 22. https://doi.org/10.3389/fmicb.2011.00022

Hooper, D. & Jacoby, G.A. (2015). Mechanisms of drug resistance: quinolone resistance. *Annals of the New York Academy of Sciences* **1354**: 12-31. https://doi.org/10.1111/nyas.12830

Hu, Y., Yang, X., Lu, N. & Zhu, B. (2014). The abundance of antibiotic resistance genes in human guts has correlation to the consumption of antibiotics in animal. *Gut Microbes* **5**: 245-249. https://doi.org/10.4161/gmic.27916

Hutchinson, J.M., Patrick, D.M., Marra, F., Ng, H., Bowie, W.R., Heule, L., Muscat, M. & Monnet, D.L. (2004). Measurement of antibiotic consumption: A practical guide to the use of the anatomical therapeutic chemical classification and defined daily dose system methodology in Canada. *Canadian Journal of Infectious Diseases and Medical Microbiology* **15**: 29-35. https://doi.org/10.1155/2004/389092

Inter-Agency Committee on Antimicrobial Resistance. (2019). The Philippine Action Plan to Combat Antimicrobial Resistance 2019-2023, 102. https://www.who.int/publications/m/item/philippines-the-philippine-action-plan-to-combat-antimicrobial-resistance-one-health-approach

Iskandar, K., Molinier, L., Hallit, S., Sartelli, M., Catena, F., Coccolini, F., Hardcastle, T.C., Rocques, C. & Salameh, P. (2020). Drivers of antibiotic resistance transmission in low-and middle-income countries from a "one health" perspective – a review. *Antibiotics* **9**: 372. https://doi.org/10.3390/antibiotics9070372

Mapa, D.S. (2021). *Chicken Situation Report: January-December 2020*. Philippine Statistics Authority. https://psa.gov.ph/sites/default/files/3_SR Chicken Annual Situation Report_signed.pdf

Maron, D.F., Smith, T.J.S. & Nachman, K.E. (2013). Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. *Globalization and Health* **9**: 48. https://doi.org/10.1186/1744-8603-9-48

Nicdao, M.A.C. (2020). A cocktail of *Lactobacillus* species controls *Salmonella* infection and maintains animal productivity in poultry farming. *Philippine Science Letters* **13**: 62-71. https://scienggj.org/2020-special-issue-6/

O' Neill, J. (2015). Antimicrobials in Agriculture and the Environment: Reducing Unnecessary Use and Waste. Review on Antimicrobial Resistance. https://amr-review.org/sites/default/files/Antimicrobials in agriculture and the environment – Reducing unnecessary use and waste.pdf

PSA. (2021). Philippine Statistics Authority: Republic of the Philippines. https://psa.gov.ph/livestock-poultry-iprs/chicken. Accessed 24 August 2021.

Roberts, M.C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters* **245**: 195-203. https://doi.org/10.1016/j.femsle.2005.02.034

Rumsey, D.J. (2021). How to determine the confidence interval for a population proportion. In: Statistics for Dummies, Rumsey, D. (editor) 2nd edition. New Jersey: John Wiley & Son Inc. https://www.dummies.com/article/academics-the-arts/math/statistics/how-to-determine-the-confidence-interval-for-a-population-proportion-169356. Accessed 12 December 2021.

Speer, B.S., Shoemaker, N.B. & Salyers, A.A. (1992). Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clinical Microbiology Reviews* **5**: 387-399. https://doi.org/10.1128/CMR.5.4.387

Statista. (2021). Philippines: gross output value of poultry industry. Statista Research Department. https://www.statista.com/statistics/1171582/philippines-gross-output-value-poultry-sector/. Accessed 24 August 2021.

Teillant, A. & Laxminarayan, R. (2015). Economics of antibiotic use in U.S. swine and poultry production. *Choices* **30**: 1-11.

Van Hoek, A.H.A.M., Mevius, D., Guerra, B., Mullany, P., Roberts, A.P. & Aarts, H.J.M. (2011). Acquired antibiotic resistance genes: An overview. *Frontiers in Microbiology* **2**: 203. https://doi.org/10.3389/fmicb.2011.00203

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., De Schaetzen M-A., Van Huffel, X., Imberechts, H., Dierick, K. *et al.* (2013). Antimicrobial resistance in the food chain: A review. *International Journal of Environmental Research and Public Health* **10**: 2643-2669. https://doi.org/10.3390/ijerph10072643

Wang, S., Jiang, K., Du, X., Lu, Y., Liao, L., He, Z. & He, W. (2021). Translational attenuation mechanism of *ErmB* induction by erythromycin is dependent on two leader peptides. *Frontiers in Microbiology* **12**: 690744. https://doi.org/10.3389/fmicb.2021.690744

Warnes, S.L., Highmore, C.J. & Keevil, C.W. (2012). Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: Implications for public health. *MBio* **3**: e00489-12. https://doi.org/10.1128/mBio.00489-12

WHO (World Health Organization)(2017). WHO guidelines on use of medically important antimicrobials in food-producing animals. https://apps.who.int/iris/bitstream/handle/10665/258970/9789241550130-eng.pdf?sequence=1%0Ahttp://apps.who.int/iris/bitstream/handle/10665/258970/9789241550130-eng.pdf?sequence=1. Accessed 24 August 24 2021.