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ANTIMICROBIAL SENSITIVITY PROFILE OF FECAL *Escherichia coli* ISOLATED FROM BROILERS FED WITH DIFFERENT LEVELS OF CHLORTETRACYCLINE

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ABSTRACT

The objectives of the study were to determine and compare the susceptibility of *E. coli* isolated from cloacal fecal samples of broilers fed with 0 ppm, 75 ppm (subtherapeutic dose) and 400 ppm (therapeutic dose) of chlortetracycline (CTC). *E. coli* were isolated, screened, cultured from the 48 fecal samples collected at day 10 and 33, and subjected to sensitivity test against nine antibiotics by disc diffusion on Mueller-Hinton agar. Agar plates which developed zones of inhibition (ZOI) were counted and the diameter of the zone was measured. Data were analyzed using descriptive statistics. Results indicated that at day 10, *E. coli* samples from broilers fed with 75 ppm already developed resistance to trimethoprim sulfamethoxazole (TMPS) and tetracycline. However, susceptibility was still noted with the other seven antibiotics (14-20 mm). At day 33, *E. coli* showed resistance against ampicillin, chloramphenicol and TMPS at both 75 ppm and 400 ppm. Moreover, reduced percentage of plates with ZOI was noted among aminoglycosides at 75 ppm indicating resistance. When fed with 400 ppm, susceptibility of *E. coli* against tetracycline and aminoglycosides tended to increase (9-20 mm). This indicates that the use of subtherapeutic levels of CTC in feeds may cause *E. coli* multi-drug resistance in broilers.

Key words: broilers, chlortetracycline, disc diffusion, *Escherichia coli*, multi-drug resistance

INTRODUCTION

Antibiotics have been widely used both in animals and humans. Antimicrobials have been commonly used in animal production to prevent and treat diseases or to promote growth and has led to the development of antimicrobial resistance. Resistance against certain antibiotics can be transmitted in humans by handling and eating of food animals exposed to resistant microbes which pose a public health concern. This would lead to potential failure of treating chronic diseases, lack of treatment options, and increase in the disease severity or seriously may lead to deaths from infections (Jimenez-Belenguer *et al*., 2016).

Antimicrobial resistance in different bacterial species has been reported in several countries. A study conducted by Yassin *et al*. (2017) on *E. coli* isolates found in livestock and

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poultry species showed that the isolates (94%) were mostly resistant to at least one drug while 83% being multi-resistant to at least three different classes of antibiotics. These *E. coli* isolates were mostly resistant to tetracycline, nalidixic acid, sulfamethoxazole, trimethoprim/sulfamethoxazole and ampicillin, and showed increasing resistance to amikacin, aztreonam, ceftazidime, cefotaxime, chloramphenicol, and ciprofloxacin. However, these isolates were least resistant to amoxicillin plus clavulanic acid and ertapenem. Multidrug resistance was most common in isolates found from ducks, followed by chickens, pigs and cows. These findings conclude that antibiotic resistance in *E. coli* can serve as carriers of resistant genes in livestock and poultry and can be transferred to other microbes which pose a public health concern. To prevent the spread of bacteria especially *E. coli* with multidrug-resistant genes, it is important to improve antibiotic use in animal production.

While the Philippines has established a regulatory body to monitor drugs and medicines, the implementation of the monitoring system both in human and animal sectors is insufficient. Moreover, there are no standard guidelines in treating animals and some antibiotics are not registered in the Philippines. Veterinarians will have no choice but to use the same antibiotics that are also being used in the treatment of some diseases found in humans. According to the report on antibiotics which are sold or distributed in animal production, the largest class of antibiotic used in food animals are tetracyclines at 41% of the total volume (FDA, 2011). This study was conducted to determine and compare the susceptibility of *E. coli* collected from cloacal fecal samples of broilers fed with 0, 75 and 400 ppm of chlortetracycline (CTC).

MATERIALS AND METHODS

One hundred forty-four (144) straight-run day-old Cobb chicks (Class A- NLT 42 grams) were purchased from a reliable hatchery. Day-old chicks were randomly distributed in 24 cages and were fed with booster feeds (basal diet) for 10 days. Birds were assigned to 3 treatments following completely randomized design (CRD). Treatments were replicated 8 times with 6 birds per replicate. Chlortetracycline was withdrawn during the finisher stage for treatment 2 and 3. The treatments given were 1) Basal diet without CTC 2) Basal diet with 75 ppm CTC (subtherapeutic dose) given from day 0 to day 33 and 3) Basal diet with 75 ppm CTC given from day 0 to day 24, then added with 325 ppm CTC (therapeutic dose) from day 25 up to day 33.

Birds were fed with booster feed (10 days), starter (14 days) and finisher (11 days) diets. Broilers were housed in conventional open-sided housing. Feeds were given *ad libitum* with clean drinking water available at all times. Farm immunization program against New Castle Disease at days 7 and 21 and Infectious Bursal Disease at day 10 was followed. A total of 48 fecal samples were collected using sterile cotton buds and immediately placed in sterile nutrient broth at days 10 and 33. Samples were grown in Eosin Methylene Blue (EMB) agar. The colonies with green metallic sheen were subjected to different biochemical tests such Methyl-Red and Voges-Proskauer (MR-VP) test for confirmatory test. Nutrient agar slants were used to maintain the stock culture for each of the *E. coli* isolate. The isolated *E. coli* were inoculated on the slant by streaking and were incubated at 37°C for 24 hours. Finally, glycerol was overlaid and the culture was kept at room temperature. Isolates were subjected to antimicrobial susceptibility testing against Cephalothin (30 µg), Ampicillin (10 µg), Penicillin G (10 µg), TMPS (25 µg), Chloramphenicol (30 µg), Tetracycline

(30 µg), Neomycin (30 µg), Gentamicin (10 µg) and Kanamycin (30 µg) by disc diffusion on Mueller-Hinton agar (Oxoid) as recommended by NCCLS (2001). The zones of inhibition were measured using calipers to the nearest millimeter. *E. coli* ATCC 25922 was used as a reference strain for quality control of the antimicrobial susceptibility testing. Agar plates which developed zones of inhibition (ZOI) were counted and the diameter of the zones was measured. The data were analyzed using descriptive statistics.

RESULTS AND DISCUSSION

Results indicated that at day 10, *E. coli* samples from broilers fed with 75 ppm already developed resistance to trimethoprim sulfamethoxazole (TMPS) and tetracycline (Table 1). However, susceptibility was still noted with the other seven antibiotics (14-20 mm) (Table 2). At day 33, *E. coli* showed resistance against ampicillin, chloramphenicol and TMPS at both 75 and 400 ppm. Moreover, reduced percentage of plates with ZOI was noted among aminoglycosides at 75 ppm indicating resistance. When fed with 400 ppm, susceptibility of *E. coli* against tetracycline and aminoglycosides tended to increase (9-20 mm) (Table 1 and 2).

E. coli strains which are normal residents of the lower intestines are genetically flexible and can adapt to environmental stress. With the continuous antibiotic use in its environment, commensal *E. coli* acquire resistant genes and develop mutant genes to maintain a balanced population and to survive in the distal intestinal tract (Szmolka and Nagy, 2013). Non-pathogenic *E. coli* isolated from animals, food products and people show different classes of resistant genes and may contribute to increasing number of resistant bacteria by either accepting or sharing transmissible antimicrobial resistance mechanisms. The genetic transfer of resistant bacteria by mobile genetic elements such as transposons, plasmids, integrons and the alteration of marlocus regulation contribute to the increase of bacteria with multiresistance pattern. Resistant genes inside integrons such as class 1 and 2 were mostly found in seventeen multiple-antibiotic resistant nonpathogenic *E.coli* strains from human, animal and food origin. The amino acid changes in MarR and marO locus regulation were found in 15 and 14 of these isolates, respectively (Saenz *et al*., 2004). In a study conducted by Diarra *et al*. (2007) which characterized *E. coli* isolates resistant to tetracyclines, it showed that resistance against tetracycline is regulated by genes encoding for efflux pumps found on large plasmids that also carry both antimicrobial and heavy metal resistant genes. It was also found that at least one of the three tetracycline resistance genes (tetA, tetB and tetC) was present in all of these isolates. Efflux pumps and resistance transfer mechanisms carried by plasmids or other genetic mobile elements are the most common tools used by *E. coli*. Co-existence and co-transfer of these resistant genes in bacterial population especially in the environment poses a public health concern. Multidrug resistant (MDR) in normal *E. coli* found in the environment and animals will serve as a reservoir for intra- and inter-specific gene exchange which leads to possible transmission of MDR bacteria from food to humans (Szmolka and Nagy, 2013). Exchange can be done by conjugation, transduction and transformation in bacterial strains of the same species or different species or genera (Tenover, 2003). The persistence of a common multiresistance pattern between several important antimicrobial classes and corresponding genes such as in β-lactams (blaTEM), sulfonamides (class 1 integrons), aminoglycosides (aadA1-like and strA/B), and tetracyclines [tet(A) and tet(B)] is found in animal production (Szmolka and Nagy, 2013). In addition,

Table 1. Percentage (frequencies in parenthesis) of plates inoculated with *E. coli* that developed zone of inhibition in the nine antibiotics tested.

Feeding Period	Antibiotics CTC Levels	Beta-Lactams				TMPS	Chlor	Tetra	Aminoglycosides			
		Ceph	Amp	PenG	Overall				Neo	Genta	Kana	Overall
0-24 days	0 ppm	12.50 (1/8)	12.50 (1/8)	0 (0/8)	8.33	25 (2/8)	37.50 (3/8)	12.5 (1/8)	62.50 (5/8)	75 (6/8)	37.5 (3/8)	58.33
	75ppm	33.33 (5/15)	20 (3/15)	6.67 (1/15)	20.00	13.33 (2/15)	46.67 (7/15)	0 (0/15)	53.33 (8/15)	66.67 (10/15)	53.33 (8/15)	51.11
	0 ppm	28.57 (2/7)	14.29 (1/7)	0 (0/7)	14.29	0						
25-33 days	(0/7)	57.14 (4.7)	0 (0/7)	71.43 (5/7)	85.71 (6/7)	71.43 (5/7)	76.19					
	75 ppm	50 (2/4)	0 (0/4)	0 (0/4)	16.67	0 (0/4)	0 (0/4)	0 (0/4)	50 (2/4)	75 (3/4)	50 (2/4)	58.33
	400 ppm	50 (3/6)	0 (0/6)	16.67 (1/6)	22.22	0 (0/6)	16.67 (1/6)	33.33 (0/6)	83.33 (5/6)	100 (6/6)	83.33 (5/6)	76.19

Ceph- Cephalothin (30 µg), Amp- Ampicillin (10 µg), Pen- Penicillin G (10 µg), TMPS – trimethoprim-sulfamethoxazole (25 µg), Chlor- Chloramphenicol (30 µg), Tetra- Tetracycline (30 µg), Neo - Neomycin (30 µg), Genta - Gentamicin (10 µg) and Kana - Kanamycin (30 µg)

Table 2. Mean diameter (mm) of zone of inhibition of *E. coli* in agar plates on nine antibiotics tested.

Feeding Period	Antibiotics CTC Levels	Beta-Lactams				TMPS	Chlor	Tetra	Aminoglycosides			
		Ceph	Amp	PenG	Overall				Neo	Genta	Kana	Overall
0-25 days	0 ppm	14±0.00	18±0.00	0	16±2.83	17±4.95	24±1.15	22±0.00	14±1.14	20.0±0.82	19±1.15	18±2.73
	75 ppm	15±1.67	17±2.31	10±0.00	16±2.03	27±2.12	26±2.34	0	15±0.97	18.1±2.69	18±2.07	17±2.58
	0 ppm	17±2.12	20±0.00	0	18±2.52	0	26±3.11	0	14±0.45	20.0±0.82	19±1.10	18±2.57
26-33 days	75 ppm	18±3.54	0	0	18±3.54	0	0	0	15±0.71	18.0±2.89	17±2.12	17± 2.56
	400 ppm	14±1.15	0	0	14±1.15	0	24±2.12	9±2.12	14±1.30	17.6±3.36	18±1.79	16±2.75

Ceph- Cephalothin (30 µg), Amp- Ampicillin (10 µg), Pen- Penicillin G (10 µg), TMPS – trimethoprim-sulfamethoxazole (25 µg), Chlor- Chloramphenicol (30 µg), Tetra- Tetracycline (30 µg), Neo - Neomycin (30 µg), Genta - Gentamicin (10 µg) and Kana - Kanamycin (30 µg)

MDR *E. coli* strains present in conventional broiler chicken production is independent of specific antibiotic pressure. In the study of Diarra *et al*. (2007) commensal *E. coli* were found resistant to ceftiofur, spectinomycin and gentamicin in chickens fed with salinomycin supplemented diet. Gentamicin resistant *E. coli* were found in chickens fed with diets with bacitracin. Coliforms from birds fed diets with salinomycin were resistant to many antibiotics such as streptomycin, ampicillin, carbenicillin and cephalothin. In a study conducted by Smith *et al*. (2007), *E. coli* isolates were resistant to sulfonamides (50-100%), streptomycin (53-100%) and tetracycline (36-97%) in chicken given with oxytetracycline, sarafloxacin and enrofloxacin. This indicates that transmissible genetic elements can contribute significantly to the spread of resistance and is independent of specific antibiotic selection pressure.

On a study conducted by Diarra *et al*. (2007), it was found that as the bird grows the resistance levels decrease which was also observed in this study from day 0 to 33 at 0 ppm level (14-26 mm) on most of antibiotics tested. Their study suggested that day-old chicks colonized initially with resistant bacterial populations were replaced by normal susceptible bacteria as the bird ages.

There are several factors that contribute to the persistence of resistance. These include direct selection for resistance at high or sub-minimum inhibitory concentration (MIC), co-selection between resistance markers, simple and multistep mutations, compensatory mutation and plasmid resistance (Andersson and Hughes, 2011). Resistance can develop both at high and low concentrations as shown in this study at day 33, wherein *E. coli* showed resistance against ampicillin, chloramphenicol and TMPS at both 75 and 400 ppm. Moreover, reduced percentage of plates with ZOI was noted among aminoglycosides at 75 ppm indicating resistance. On a study conducted by Zhang *et al.* (2015), pathogenic *E. coli* isolates associated with higher prevalence of resistance than commensal *E. coli* isolates across most antibiotics examined was due to excessive use of antibiotics in the treatment of pathogenic strains. While it shows that bacteria with above MIC against antibiotics can select for resistant strains, resistance can also be selected under sub-MIC conditions as shown by mutant *Escherichia coli* and *Salmonella enterica* which exposed under several hundred folds below MIC or at ultra-low antibiotic concentrations found in natural environments developed resistant to tetracycline, fluoroquinolones and aminoglycosides (Andersson and Hughes, 2011). It shows that selection pressure of antibiotics on microflora contributes to the development of resistance as shown in the study where broilers were fed with 75 ppm of CTC in the diet wherein most of antibiotics tested tended to increase resistance (Table 2).

Resistance may be reversed by using the dilution effect wherein antibiotics used were reduced which can be observed from week to months. Antibiotic-resistant bacteria can be displaced by susceptible ones if it cannot reproduce and spread in the host population (Andersson and Hughes, 2011). The same observation was shown in this study wherein susceptibility of *E. coli* against tetracycline and aminoglycosides tended to increase (9-20 mm) when fed diets with 400 ppm in which resistant *E. coli* may not able survive and spread in the population.

Commensal *E. coli* can act as reservoirs for multidrug resistant genes that can be transferred from food to humans which imposes a public health concern. Multiresistant drug patterns in *E. coli* strains are associated with different resistant genes found in the bacterial mobile genetic elements such as integrons, plasmids, and transposons. At day 10, *E. coli* samples from broilers fed with 75 ppm already developed resistance to trimethoprim

sulfamethoxazole (TMPS) and tetracycline. Susceptibility was still noted with the other seven antibiotics (14-20 mm). At day 33, *E. coli* showed resistance against ampicillin, chloramphenicol and TMPS at both 75 ppm and 400 ppm. Reduced percentage of plates with ZOI was noted among aminoglycosides at 75 ppm indicating resistance. This indicates that multiresistance pattern is independent on specific antibiotic selection pressure and that transmissible genetic elements contributed to the spread of resistance in poultry. However, MDR bacteria can be outcompeted by susceptible ones if it cannot reproduce and spread in the host population as observed in this study wherein susceptibility of *E. coli* against tetracycline and aminoglycosides tended to increase (9-20 mm) when diets were fed with 400 ppm. This study indicates that the use of subtherapeutic levels of CTC in feeds may cause *E. coli* multi-drug resistance in broilers.

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