Serogroup, pathotype and multiple drug resistance of *Escherichia coli* strains isolated from the cloaca of layer chickens in San Jose, Batangas, Philippines

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Four hundred cloacal swab samples from layer chickens in ten layer farms in San Jose, Batangas, Philippines were obtained for the detection of pathogenic serogroups of *Escherichia coli* and determination of their resistance and susceptibility to different antibiotics and potential production of extended-spectrum β-lactamase (ESBL). A total of 226 isolates were phenotypically identified as *E. coli* through cultural, morphological, physiological and biochemical characterizations with ≥ 99% similarity to the reference *E. coli* in the API®20 E™ database. Only five *E. coli* isolates were serogrouped using four Denka Seiken polyvalent antisera (Polyvalent 1, 3, 4 and 7). Three (FK24, FK26 and FK33) of these isolates belonged to the enterotoxigenic *E. coli* (ETEC) pathotype serogroup consisting of O6, O27, O78, O148, O159 and O168. Another isolate (FK9) was of the enteropathogenic *E. coli* (EPEC) pathotype and could belong to the serogroup O18, O114, O142, O151, O157 and O158. Another isolate (FI22) belonged to the enteroinvasive *E. coli* (EIEC) pathotype serogroup which includes O28ac, O112ac, O124, O136 and O144. All the *E. coli* isolates from five farms (Farms A, E, F, G and K) were found to be multi-drug resistant or non-susceptible to at least 1 antimicrobial agent in at least 3 antimicrobial categories. Out of the 226 isolates, 90.3%, including the serogrouped *E. coli* strains, were multi-drug resistant. About 75.7% exhibited resistance to tetracycline while 74.8%, 72.1% and 65.5% exhibited resistance to trimethoprim/sulfamethoxazole, ampicillin and nalidixic acid, respectively. Susceptibility to imipenem, amikacin, nitrofurantoin, gentamicin, cefotaxime, ceftazidime and cefoxitin was still at least 75%, 100%, 89.4%, 88.5%, 86.7%, 86.3%, 84.5% and 78.8% of the isolates, respectively. Only one isolate, FG27, appeared to be a potential ESBL-producer.

KEYWORDS

Escherichia coli, extended spectrum β-lactamase (ESBL), layer chickens, multi-drug resistant, pathotype, serogroup

INTRODUCTION

In commercial poultry, certain strains of *Escherichia coli*, such as those designated as avian pathogenic *E. coli* (APEC), spread into various internal organs and cause colibacillosis (Kabir 2010). Avian colibacillosis is considered as one of the major bacterial diseases in the poultry industry worldwide causing heavy economic losses.

E. coli from poultry can infect humans both through direct contact with the animal or via ingestion of contaminated poultry and poultry products. Strains of *E. coli* belonging to one of the pathogenic serotypes (pathotypes) enteroaggregative (EAEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), and

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diffusely adherent (DAEC) *E. coli,* may cause diarrhea (van den Bogaard *et al*. 2001).

Aside from being use in the treatment of bacterial infections, antibiotics are utilized as prophylaxis to prevent infection, and as growth promoters to improve feed utilization and production in animals (Barton, 2000). This use and overuse of antibiotics have led to the development of antibiotic resistance (Smith *et al*. 2002) and to the acceleration of the development and prevalence of multi-drug resistant bacteria in livestock and people (Saliu *et al*. 2017).

Extended-spectrum beta-lactamase (ESBL)-producing bacteria, currently considered as one of the main threats for the treatment of infections in humans and animals (Saliu *et al.* 2017), are resistant to a wide range of ß-lactam antibiotics. ESBLs are often plasmid-mediated hydrolytic enzymes that mediate resistance to extended-spectrum cephalosporins, namely, ceftazidime, cefotaxime and ceftriaxone, and to monobactams such as aztreonam (Nteimam 2005). The first plasmid mediated beta-lactamase in Gram negative bacteria was TEM 1 which was designated as such being isolated from the blood culture of a patient in Greece named Temoniera. SHV-1, or sulfhydryl variable type 1, is another beta-lactamase commonly found in *Klebsiella* and *Escherichia coli* (Rawat and Nair 2010). ESBLs are thought to have evolved by mutation of the *tem* and *shv* genes that alter the amino acid configuration around the active site of these beta-lactamases. These are members of the class A beta-lactamases, which are the most common plasmid-mediated beta-lactamases found in Gram negative bacilli of the Enterobacteriaceae family (Nteimam 2005; Bradford 2001). CTX-M enzymes have a potent hydrolytic activity against cefotaxime. Organisms producing CTX-M-type beta-lactamases typically have cefotaxime minimum inhibitory concentrations (MICs) in the resistant range (> 64µg/mL), while ceftazidime MICs are usually in the apparently susceptible range (2 to 8 µg/mL). The OXA-type beta-lactamases are so named because of their oxacillin-hydrolyzing abilities. They predominantly occur in *Pseudomonas aeruginosa* but have been detected in many other Gram-negative bacteria (Paterson and Bonomo 2005).

Since antimicrobial resistance has now emerged as a serious and complex, worldwide problem, monitoring the prevalence of resistance to antibiotics in human populations and in animals is needed in order to detect the transfer of drug-resistant bacteria or resistance genes from animal origin to humans and vice-versa (WHO 1997). In the Philippines, an antimicrobial resistance surveillance program is conducted yearly for human bacterial isolates (RITM 2016). In 2015, the Bureau of Animal Industry (BAI) spearheaded a training seminar on the development of AMR surveillance and monitoring program for livestock with various Department of Agriculture agencies such as BAI, Bureau of Fisheries and Aquatic Resources (BFAR), National Dairy Authority (NDA), National Meat Inspection Service (NMIS) and the Philippine Carabao Center (PCC). The activity was deemed vital and timely for the Philippine animal industry as there is still no existing AMR Surveillance and Monitoring Program specific for livestock (Morales 2015).

This study was conducted primarily to characterize phenotypically and serologically the *Escherichia coli* isolates from apparently healthy layer chickens in ten layer farms in San Jose, Batangas in order to detect potentially pathogenic serogroups and pathotypes, to determine the prevalence of multi-drug resistant strains among the isolates, and to detect potential extended spectrum β-lactamase (ESBL)-producers.

MATERIALS AND METHODS

Sampling Site and Size

The municipality of San Jose in Batangas, Philippines is known mainly for its livestock, poultry and egg industry. It is the main provider of poultry products inside and outside the province (Baconguis 2007). The municipality, dubbed as the "Egg Basket of the Philippines", generates an estimated five million eggs daily (Bernardo 2014) from 270 commercial poultry farms registered as of June 2011 (A. Basiloy, personal communication).

Furthermore, the average number of layer chickens grown by a farm per age group was 6000 heads. Most of the farms were concentrated in barangays Galamay-Amo, Lumil and Tugtug. During the study, only ten farms, designated as Farms A, B, C, D, E, F, G, I, J and K granted the request for sampling. The layer farms were situated in barangays Galamay-Amo, Lumil, Dagatan, Taysan, Aya, Bagong Pook, Lalayat and Lapu-lapu 2nd. The layer chickens were of the breed DeKalb and Lohmann and were 19 to 30 weeks old at the time of sampling. This range was considered the age when chickens are of high egg productivity. The chickens were chosen based on the absence of signs of respiratory illness and wet droppings and preference of the farm owner on which chickens were to be sampled.

Stratified random sampling, supervised by a resident veterinarian, was followed where the perceived value of prevalence of the microorganism was set at 0.5 (50%) and the measure of reliability at 5%. The following formula was adopted from Thompson (1992):

$$n \geq \frac{NPQ}{(N-1)\,[CV\,(P_{srs})\,P]^2 + PQ}$$

where: n = sample size
P = perceived value of prevalence of the microorganism set at 0.5 (50%)
CV = measure of reliability at 5%
Q = 1-P
N = 60,000*
SRS = stratified random sampling

*based on the average number of layer chickens grown by a farm per age group (6000 heads x 10 farms)

A total of 400 cloacal swab samples, or 40 per farm, were collected.

Cloacal Swab Sample Collection and Pre-enrichment

A sterile cotton swab, moistened with sterile Buffered Peptone Water (BPW) (BD Difco, USA), was inserted into the cloaca of the chicken and rotated gently to obtain a sufficient amount of fecal matter. The cloaca, the common chamber into which the digestive, urinary and reproductive tract open, was chosen so as to obtain fecal sample without sacrificing the chicken. All swabs were returned to their respective BPW tube, kept in a cooler, transported to the laboratory, and incubated at 42°C for 24 hours (Welch 2006).

Isolation of *E. coli* Strains from Cloacal Swabs

E. coli isolation was done by streaking a loopful of the pre-enriched sample in BPW onto plates of MacConkey Agar (MAC) (BD Difco, USA) and Levine Eosin Methylene Blue Agar (Levine EMBA) (BD Difco, USA). Plates were incubated at 37°C for 24 hours. Pink to red colonies on MAC and blue-black colonies with a green, metallic sheen on Levine EMBA, typical of *E. coli* colonies, were selected for further re-streaking on Levine EMBA plates and Tryptic Soy Agar (TSA) (BD

Difco, USA). Purified colonies were selected for Gram-staining, catalase and oxidase tests (Herrera 2001; Health Protection Agency 2007).

Phenotypic Identification of *E. coli* Isolates

The putative *E. coli* isolates were phenotypically identified using cultural, morphological and physiological/biochemical tests, the latter mainly through the use of API®20 E™ strips (Biomerieux, France) (API®20 E™ Manual 2010).

O-Serogrouping and Possible Pathotype Determination of *E. coli* Strains

Two loopfuls of a 24-hour old pure culture was suspended in three milliliters of sterile 0.85% saline solution and heated at 121°C for 15 minutes. The heated suspension was centrifuged at 900 x *g* for 20 minutes. The supernatant was discarded while the pellet was re-suspended in 0.5 mL sterile 0.85% saline solution and used as antigenic suspension (Denka Seiken *Escherichia coli* Antisera Manual 2009).

A test for autoagglutination was first performed to determine if further serotyping could be done with the isolates.

a. Autoagglutination test. A clean glass slide was partitioned into five parts. An aliquot of 30 µL physiological saline was placed in one of the sections and 10 µL of the antigenic suspension was added and mixed into the saline. The glass slide was tilted back and forth for one minute after which agglutination was observed (Denka Seiken *Escherichia coli* Antisera Manual 2009). If autoagglutination occurred, the culture was considered "rough" indicating that the strain did not make an O antigen. This strain will either not agglutinate or give weak cross-agglutination in a variety of antisera. Consequently, the strain cannot be tested with its respective antiserum (Ellermeier and Slauch 2006). In such case, it is recommended that the isolate be subcultured on a nonselective agar, incubated, and tested again for autoagglutination. If no autoagglutination occurs this time, the isolate can be further serotyped. If autoagglutination occurs, it is deemed unlikely to be the bacterium of interest and serotyping should not be carried out (Hendriksen and Larsen 2004).

b. Serogrouping of the isolates. The possible O serogroup and pathotype of the isolates were determined using four polyvalent antisera : *E. coli* Polyvalent 1 antiserum (Denka Seiken, Japan) for the detection of the serogroups O1, O26, O86a, O111, O119, O127a and O128 of the EPEC pathotype ; *E. coli* Polyvalent 3 antiserum (Denka Seiken, Japan) for detecting serogroups O18, O114, O142, O151, O157 and O158 of another EPEC pathotype; *E. coli* Polyvalent 4 antiserum (Denka Seiken, Japan) for serogroups O6, O27, O78, O148, O159 and O168 of the ETEC pathotype; and *E. coli* Polyvalent 7 antiserum (Denka Seiken, Japan) for the following serogroups of the EIEC pathotype: O28ac, O112ac, O124, O136 and O144. The four, out of nine commercially available, polyvalent antisera used encompassed 24 O serogroups as these contained many EPEC and ETEC pathotypes frequently associated with avian colibacillosis in domestic poultry (Kariuki *et al*. 2002; Kabir 2010). The O serogroups in the Polyvalent 7 antiserum were traditionally considered as EIEC strains (Rosario *et al*. 2004).

A clean glass slide was partitioned into four parts. A drop of each polyvalent antiserum was placed into each section and 30 µL of physiological saline was added and mixed into each of the polyvalent antiserum. The glass slide was tilted back and forth for one minute after which agglutination was observed. This served as the negative control (Denka Seiken *Escherichia coli* Antisera Manual 2009).

For the serogroup determination, a glass slide was partitioned into four parts as described above. A drop of each polyvalent antiserum was then placed onto each of the sections in the glass slide. An aliquot of 10 µL antigenic suspension was added and mixed into each of the drops. The glass slide was tilted back and forth for one minute after which agglutination was observed. Only strong agglutination (i.e., formation of precipitate) observed within one minute was regarded as positive. Delayed or weak agglutination was regarded as negative. Each isolate was classified as EPEC, ETEC or EIEC according to the reactions with these polyvalent antisera (Denka Seiken *Escherichia coli* Antisera Manual 2009).

Determination of Antibiotic Susceptibility and Resistance Profiles of *E. coli* Isolates

The modified Kirby-Bauer Method was employed in determining antibiotic susceptibility and resistance profiles of *E. coli* isolates. Briefly, three to five colonies of an 18-24 hour pure culture were suspended in sterile 0.85% saline solution resulting in a bacterial suspension with turbidity comparable to 0.5 McFarland which corresponded to approximately 1.5 x 10^8 CFU/mL). A sterile cotton swab was dipped into the suspension and the excess liquid was removed by pressing it against the side of the tube. Starting from the top of the Mueller-Hinton Agar (MHA) (BD Difco, USA), the surface was inoculated with the swab. The entire plate was covered with the inoculum by streaking back and forth from edge to edge (BSAC 2011). The choice of antibiotics was based on a list recommended against *E. coli* by the British Society for Antimicrobial Chemotherapy (2011), and on the list of antibiotics used in poultry feeds and treatment (Shane 2005; PVET 2014).

The antibiotic discs (HiMedia, India) and the respective concentrations used were amikacin (AK 30µg), amoxicillin/clavulanic acid (AMC 30µg) (Bioanalyse, Turkey), ampicillin (AM 10µg), cefotaxime (CTX 30µg) (Bioanalyse, Turkey), cefoxitin (CX 30µg), ceftazidime (CAZ 30µg) (Bioanalyse, Turkey), cephalothin (CEP 30µg), ciprofloxacin (CIP 5µg), gentamicin (GEN 10µg), imipenem (IPM 10µg), nalidixic acid (NA 30µg), nitrofurantoin (NIT 300µg), spectinomycin (SPC 100µg), trimethoprim/sulfamethoxazole (SXT 23.75µg/ 1.25µg) and tetracycline (TE 30µg). Duplicate plates were prepared for each isolate. The test was monitored by the use of *E. coli* ATCC 8739 as susceptible strain. Interpretation was based on the comparison of the diameter of the zone of inhibition observed with published criteria of the Clinical and Laboratory Standards Institute (2011) for zone diameters. Briefly, inhibition zones less than or equal to the following values were interpreted as resistant: amikacin–14mm, amoxicillin/clavulanic acid-13mm, ampicillin-13mm, cefotaxime-22 mm, cefoxitin-14mm, ceftazidime-17mm, cephalothin-14 mm, ciprofloxacin-15mm, gentamicin-12 mm, imipenem- 19mm, nalidixic acid-13 mm, nitrofurantoin-14 mm, spectinomycin -15 mm, trimethoprim/sulfamethoxazole – 10mm and tetracycline -11mm.

Detection of Potential Extended-Spectrum β-lactamase (ESBL) Production by *E. coli* Isolates

The modified double-disc diffusion test was employed in the detection of potential extended-spectrum β-lactamase (ESBL) production by *E. coli* isolates. Essentially it followed the procedure described above in the determination of susceptibility and resistance profiles of *E. coli* isolates. Three antibiotic discs (Bioanalyse, Turkey), separately containing cefotaxime (CTX 30µg), ceftazidime (CAZ 30µg), and amoxicillin/clavulanic acid (AMC 20µg /10µg), were placed 25-30 mm apart on the plate in a straight line, with the amoxicillin/clavulanic acid disc in the middle. Enhancement of the zone of inhibition around the ceftazidime disc facing the amoxicillin-clavulanic acid disc (i.e.,

zone of inhibition greater than 5 mm compared to the zone without amoxicillin/clavulanic acid) was interpreted as a positive test indicating that the isolate was likely to be an ESBL producer. For TEM and SHV variants, the synergy of amoxicillin-clavulanic acid with ceftazidime is reportedly more obvious while for CTX-M types, the synergy of amoxicillin-clavulanic acid with cefotaxime is the one more obvious (BSAC 2011; Livermore and Brown 2001; Bradford 2001; Nteimam 2005). Triplicate plates were prepared for each isolate. Test performance was monitored by the use of *E. coli* ATCC 8739 as a non ESBL-producer strain. The unavailability of an ESBL-producer strain at the time makes it a limitation of the present study.

RESULTS AND DISCUSSION

Phenotypic Identification of the Suspected *E. coli* Isolates

Only 226 out of 394 putative *E. coli* isolates were phenotypically identified, using cultural, morphological, and biochemical/physiological characterizations, to be *E. coli*. The *E. coli* isolates were all pink in color when grown on MAC and blue-black with a green metallic sheen when grown on Levine EMBA, both at 37°C for 24 hours. These were all Gram-negative, short and straight rods, catalase-positive and oxidase-negative. The isolates also showed positive reactions to the enzymes ONPG (orthonitrophenyl-β-D-galactopyranoside), LDC (lysine decarboxylase), and ODC (ornithine decarboxylase); to IND (indole production); and the fermentation of the sugars GLU (glucose), MAN (mannitol), SOR (sorbitol), RHA (rhamnose), MEL (melibiose) and ARA (arabinose) using the API®20 ETM strip. Their similarity to the reference *E. coli* used in the API®20 E™ database was ≥ 99%.

O-Serogroup and Possible Pathotypes of the Isolates

Of the 226 isolates subjected to serogrouping, only five exhibited agglutination within one minute using the four polyvalent antisera used (Table 1). The other 221 *E. coli* isolates should be tested with the remaining five Denka Seiken Polyvalent Antisera (Polyvalent 2, 5, 6, 8 and 9 antisera) to determine their pathotype and possible O serogrouping. Polyvalent 2 antiserum contains six O serogroups that belong to the EPEC pathotype; Polyvalent 5 and 6 antisera contain nine O serogroups that belong to the ETEC pathotype; and Polyvalent 8 antiserum contains four O serogroups that belong to the EIEC pathotype. Polyvalent 9, the newly added antiserum in the Denka Seiken set, contains seven O serogroups that belong to the EHEC pathotype (Denka Seiken *Escherichia coli* Antisera Manual 2009).

Table 1: Determination of the possible O serogroup and pathotype of the typeable *E. coli* isolates obtained from the cloaca of apparently healthy layer chickens from ten layer farms in San Jose, Batangas

FARM CODE	NUMBER OF *E. coli* ISOLATES TESTED	TYPEABLE *E. coli* ISOLATES*		POSSIBLE O-SEROTYPES	PATHOTYPES DETECTED**
		Number	Isolate Code		
FA	14	0		-	-
FB	28	0		-	-
FC	26	0		-	-
FD	21	0		-	-
FE	22	0		-	-
FF	24	0		-	-
FG	16	0		-	-
FI	28	1	FI22	O28ac, O112ac,O124, O136, O144	EIEC
FJ	26	0		-	-
FK	21	4	FK9	O18, O114, O142, O151, O157, O158	EPEC
			FK24 FK26 FK33	O6, O27, O78, O148, O159, O168	ETEC
Total	**226**	**5**			

*Exhibited agglutination in any of the four polyvalent antisera
**Polyvalent 1 antiserum: EPEC; Polyvalent 3 antiserum: EPEC; Polyvalent 4 antiserum: ETEC; Polyvalent 7 antiserum: EIEC

E. coli isolates FK24, FK26 and FK33 exhibited agglutination using Polyvalent 4 antiserum. These could, therefore, belong to any of the ETEC pathotype serogroups O6, O27, O78, O148, O159 and O168. Another isolate (FK9) gave a positive agglutination reaction with Polyvalent 3 antiserum indicating that it could belong to any of the EPEC pathotype serogroups O18, O114, O142, O151, O157 and O158. Isolate FI22, on the other hand, which agglutinated Polyvalent 7 antiserum, could belong to serogroups O28ac, O112ac, O124, O136 and O144, all of the EIEC pathotype.

The three isolates from Farm K, FK24, FK26 and FK33, serotyped possibly to the ETEC group may pose a health threat to chickens and to humans. ETEC is known to be the common cause of "traveler's diarrhea". It induces watery diarrhea that can be mild in nature, or, in some instances, can be a severe, cholera-like illness that can be life- threatening (Brooks *et al.* 2010).

Another possible threat to human health is that caused by an isolate from Farm I that belongs to the enteroinvasive *E. coli* (EIEC) pathotype. This group causes invasive inflammatory colitis and bloody stools with mucus accompanied by fever and severe cramps (Brooks *et al.* 2010).

Table 2: Susceptibility and resistance of the five serogrouped *E. coli* isolates obtained fromthe cloaca of layer chickens from ten layer farms in San Jose, Batangas, Philippines.

ANTIBIOTIC (DOSE)	Serogrouped *Escherichia coli* Isolates				
	FK9	FK24	FK26	FK33	FI22
Amikacin (AK 30µg)	S	S	S	S	S
Amoxicillin/clavulanic acid (AMC 30 µg)	I	I	S	**R**	S
Ampicillin (AM 10µg)	**R**	**R**	**R**	**R**	S
Cefotaxime (CTX 30µg)	S	S	S	S	S
Cefoxitin (CX 30µg)	S	S	S	**R**	S
Ceftazidime (CAZ 30µg)	S	S	S	S	S
Cephalothin (CEP 30µg)	S	S	S	**R**	S
Ciprofloxacin (CIP 5µg)	S	S	S	I	S
Gentamicin (GEN 10µg)	S	S	S	S	S
Imipenem (IPM 10µg)	S	S	S	S	S
Nalidixic Acid (NA 30µg)	S	**R**	S	**R**	S
Nitrofurantoin (NIT 300µg)	S	S	S	S	S
Spectinomycin (SPC 100µg)	S	**R**	I	I	I
Trimethoprim/ sulfamethoxazole (SXT 23.75µg/1.25µg)	**R**	**R**	**R**	**R**	**R**
Tetracycline (TE 30µg)	**R**	**R**	**R**	**R**	**R**

*Based on the zone size interpretative chart for antibiotics published by the Clinical and Laboratory Standards Institute (CLSI), January 2011.
S: susceptible or sensitive; R: resistant; I: immediate

The detection of these potential *E. coli* pathogenic serotypes in apparently healthy layer chickens is significant as it suggests that the intestinal tract of chickens may be an important natural reservoir for these pathotypes. Although, they may not currently be pathogenic to chickens, they can pose a threat to humans when transmitted.

Antibiotic Susceptibility and Resistance Profiles of the *E. coli* Isolates

Majority of the *E. coli* isolates, including four of the five serogrouped isolates (FK9, FK24, FK26, FK33), were found to be multi-drug resistant (Table 2), defined herein as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al*. 2012). The isolates showed considerable degree of resistance to tetracycline (75.7%), trimethoprim/sulfamethoxazole (74.8%), ampicillin (72.1%) and nalidixic acid (65.5%) (Table 3). Moderate degrees of resistance to ciprofloxacin, cephalothin, spectinomycin and amoxicillin/clavulanic acid at 31.0%, 22.6%, 22.1% and 21.2%, respectively were also observed. Degrees of susceptibility to imipenem, amikacin, nitrofurantoin, gentamicin, cefotaxime, ceftazidime and cefoxitin were relatively high at 100%, 89.4%, 88.5%, 86.7%, 86.3%, 84.5% and 78.8%, respectively.

The major factor affecting the development of antimicrobial resistance in bacteria is antibiotic use (van den Bogaard *et al*. 2001). The degree of resistance to an antibiotic is related to the extent of antibiotic use. It has been established that the longer an antimicrobial drug has been used, the likelihood of the emergence of microbial resistance is higher, as has been observed with older drugs including sulfonamides, tetracyclines and quinolones (such as nalidixic acid) (WHO 1998).

Antibiotics are seldom administered to laying hens producing eggs for human consumption due to possible antibiotic residues in eggs. However, during rearing, antibiotics are commonly used (van den Bogaard *et al*. 2001). It is said that resistance to tetracyclines in pathogenic, zoonotic and indicator bacteria, such as *E. coli*, is common probably as a consequence of selection pressure by the massive use of these drugs (San Martin *et al*. 2005). In the Philippines, tetracyclines and trimethoprim/sulfamethoxazole are used in animal feeds for prophylaxis and as growth promoters (PVET 2014; Jiao *et al.* 2007) Antibiotic susceptibility testing of the *E. coli* isolates from all the farms in the present study revealed resistance to tetracycline and trimethoprim/sulfamethoxazole at 75.66% and 74.78%, respectively. Nalidixic acid also showed a resistance rate of 65.49%. Quinolones and fluoroquinolones are mostly used also in poultry (Jiao *et al.* 2007).

Another major concern discovered was resistance to cephalothin, a first generation cephalosporin. In human

Table 3: The general antibiotic susceptibility profile* of the 226 *E. coli* isolates obtained from the cloaca of layer chickens from ten layer farms in San Jose, Batangas, regardless of farm.

ANTIBIOTIC (DOSE)	*E. coli* ISOLATES					
	Sensitive		Intermediate		Resistant	
	Number	%	Number	%	Number	%
Amikacin (AK 30µg)	202	89.38	15	6.64	9	3.98
Amoxicillin/clavulanic acid (AMC 30 µg)	94	41.59	84	37.17	48	21.24
Ampicillin (AM 10µg)	55	24.34	8	3.54	163	72.12
Cefotaxime (CTX 30µg)	195	86.28	29	12.83	2	0.88
Cefoxitin (CX 30µg)	178	78.76	23	10.18	25	11.06
Ceftazidime (CAZ 30µg)	191	84.51	18	7.96	17	7.52
Cephalothin (CEP 30µg)	111	49.12	64	28.32	51	22.57
Ciprofloxacin (CIP 5µg)	90	39.82	66	29.20	70	30.97
Gentamicin (GEN 10µg)	196	86.73	11	4.87	19	8.41
Imipenem (IPM 10µg)	226	100.00	0	0.00	0	0.00
Nalidixic Acid (NA 30µg)	66	29.20	12	5.31	148	65.49
Nitrofurantoin (NIT 300µg)	200	88.50	18	7.96	8	3.54
Spectinomycin (SPC 100µg)	127	56.19	49	21.68	50	22.12
Trimethoprim/ sulfamethoxazole (SXT 23.75µg/1.25µg)	53	23.45	4	1.77	169	74.78
Tetracycline (TE 30µg)	26	11.50	29	12.83	171	75.66

*Based on the zone size interpretative chart for antibiotics published by the Clinical and Laboratory Standards Institute (CLSI), January 2011.

medicine, cephalosporins, especially of the third generation, are important as they are used to treat seriously-ill patients with life-threatening diseases, many of which are due to organisms that reside in the gastrointestinal tract. They are the antibiotics of choice in the treatment of serious *Salmonella* and *Shigella* infections particularly in children where fluoroquinolones are avoided due to their potential for toxicity (U.S. FDA 2012). In animals, cephalosporins are not available for herd-wide or flock-wide use via medicated feed or medicated drinking water. Only two cephalosporins, ceftiofur and cephapirin, are currently approved for use in food-producing animal species (U.S. FDA 2012; U.S. FDA 2017).

The resistance of the isolates to cephalothin may be due to their acquisition of a gene that encodes for the β-lactamase enzyme although such was not proven in this study. Gram-negative bacterial resistance to cephalosporins occurs mainly through inactivation of the cephalosporin by β-lactamases. These enzymes can be both innate and acquired. Among bacteria of human health concern, the two most important classes of β-lactamase enzymes are the AmpC cephalosporinases and ESBL. The *cmy-2,* a type of AmpC gene, is found on the chromosome of most members of Family Enterobacteriaceae and is also currently found on promiscuous plasmids in *Salmonella, E. coli* and other members of this family. These enzymes provide resistance to first, second and third generation cephalosporins (U.S. FDA 2012).

On the other hand, the greater susceptibility of *E. coli* to imipenem and amikacin observed in this study might be due to the recommended controlled usage of these antibiotics as they belong to the clinically-important antimicrobials. Carbapenems may be the sole therapy or are one of a few remaining alternatives to treat serious human diseases such as those caused by organisms that may be transmitted via non-human sources or diseases caused by organisms that may acquire resistance genes from non-human sources. The use of carbapenems (e.g. imipenem) is also being regulated to limit their exposure to multi-drug resistant Gram-negative bacteria (Collignon *et al.* 2009).

Prevalence of Multi-Drug Resistant *E. coli* Strains

Multi-drug resistant *E. coli* strains were confirmed to be present in asymptomatic layer chickens from the ten farms sampled (Table 4). Of the 226 *E. coli* isolated, 204 were found multi-drug resistant (90.3%) while only three isolates were susceptible to all 15 antibiotics tested. It is alarming to note that all the isolates from Farms A, E, F, G and K were resistant to at least one drug in at least three antimicrobial categories, or were multi-drug resistant while the isolates from one farm, Farm J, had the lowest percentage of such strains at 53.9. The latter remains a major concern as more than one half are considered MDR strains. Most of the *E. coli* isolates were resistant to antibiotics in four antimicrobial categories: tetracyclines (e.g. tetracycline), folate pathway inhibitors (e.g. trimethoprim/ sulfamethoxazole), penicillins (ampicillin) and quinolones (nalidixic acid) (Table 2).

Detection of Potential Extended-Spectrum β-lactamase (ESBL) Production by the *E. coli* Isolates

Among the *E. coli* isolates tested, only isolate FG27 was phenotypically-determined as a possible ESBL-producer. The average diameter of the zone of inhibition around the ceftazidime disc facing the amoxicillin/clavulanic acid disc was

Table 4: Percentage of multi-drug resistant *E. coli* isolates obtained from the cloaca of apparently healthy layer chickens from ten layer farms in San Jose, Batangas.

FARM CODE	NUMBER OF *E. coli* ISOLATES TESTED	*E.coli* ISOLATES SUSCEPTIBLE TO ALL ANTIMICROBIAL AGENTS USED		MULTI-DRUG RESISTANT *E. coli* ISOLATES	
		Number	%	Number	%
FA	14	0	0	14	100.00
FB	28	0	0	27	96.43
FC	26	0	0	23	88.46
FD	21	0	0	17	80.95
FE	22	0	0	22	100.00
FF	24	0	0	24	100.00
FG	16	0	0	16	100.00
FI	28	0	0	26	92.86
FJ	26	3	11.54	14	53.85
FK	21	0	0	21	100.00
Total	**226**	**3**	**1.33**	**204**	**90.27**

Table 5: The diameter of the zones of inhibition obtained for *E. coli* FG27 using the modified double-disc diffusion test.

Antibiotic	*E. coli* FG27			
	Diameter of Zone of Inhibition (mm)			
	With synergy with AMC 30µg (mm)	Average (mm)	Without synergy with AMC 30µg (mm)	Average (mm)
Cefotaxime (CTX 30µg)	21, 23, 22	22	21, 20,18	20
Ceftazidime (CAZ 30 µg)	18,19,19	19	15, 13,13	14
Amoxicillin/clavulanic acid (AMC 30µg)	25, 22, 23	23	Not applicable	Not applicable

5 mm greater than the diameter of the zone of inhibition without amoxicillin/clavulanic acid (Table 5). This indicated that isolate FG27 could possibly be one of the producers of the TEM and SHV variants of β-lactamase as these variants are reported to show a more obvious synergistic effect between amoxicillin/clavulanic acid and ceftazidime (Livermore and Brown 2001).

Positive controls are needed to ascertain the performance of ESBL confirmatory test in Enterobacteriaceae. These controls could be one of the *E. coli* strains with the following ESBLs that are available from the National Collection of Type Cultures, London, UK: CTX-M-15 (cefotaximase) NCTC 13353, TEM=3 (broad-spectrum) NCTC 13351 and TEM-10 (ceftazidimase) NCTC 13352. The unavailability of one of these positive controls at the time of the study is a limitation of the study. Since the identified potential production of ESBL of *E. coli* FG27 was only presumptive, it is recommended, for confirmation, that PCR amplification of the oligonucleotide primers that are specific for a β-lactamase gene (i.e., bla_{TEM}, bla_{SHV} and bla_{CTX-M}, bla_{CMY}, bla_{OXA-1} group and bla_{OXA-2} group) be performed for the isolate FG27 as well as for the positive control. These primers are usually chosen to anneal to regions where various point mutations are not known to occur (Bradford, 2001; Yuan *et al.,* 2009).

Extended spectrum β-lactamase (ESBL) producers are particularly of increasing concern as their activities can lead to resistance to third generation cephalosporins such as ceftazidime (Zhanel *et al.* 2005). These extended-spectrum cephalosporins are the treatment of choice for invasive Gram-negative infections, including salmonellosis in children. Because *Salmonella* transmission is primarily foodborne, there is also concern that resistant enteric bacteria from livestock can be transferred through the food supply to consumers (Wittum *et al.* 2010).

Current therapy against strains of Enterobacteriaceae that express ESBLs is limited to such broad-spectrum agents as imipenem. However, there have already been reports of therapeutic failures of this drug with strains that produce multiple β-lactamases (Bradford 2001). In the present study, all the *E. coli* isolates were susceptible to imipenem.

A continuing surveillance of pathogenic serotypes, multi-drug resistance and ESBL-production in bacteria in food-producing animals such as chickens is very important as it will aid in the understanding of the distribution of these isolates and in the establishment of the proper prevention protocols. Data collected can also serve as guides in the use of antibiotics in food animal production and infection chemotherapy (Li *et al.* 2010).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Both authors did the conceptualization and planning of the research as well as data analysis and manuscript writing. The senior author did the sampling and experimentation.

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