PREVALENCE AND ANTIMICROBIAL RESISTANT PATTERNS OF EXTENDED SPECTRUM BETA-LACTAMASE PRODUCING *Escherichia coli* IN COMMERCIAL SWINE AND BROILER FARMS IN CENTRAL LUZON, THE PHILIPPINES

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DOCTOR OF PHILOSOPHY
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GRADUATE SCHOOL
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A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY SCIENCE

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I would like to dedicate this piece of work to my loving family: my wife Merly and my children Dorothy Kathleen and Nathan Lemuel.

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หัวข้อดุษฎีนิพนธ์	ความชุกและรูปแบบการดื้อต่อยาต้านจุลชีพของเชื้อเอสเชอริเชียโคไล ที่สร้างเอนไซม์ บีตา- แลคทาเมสที่มีฤทธิ์ขยายอำนาจการดื้อยาในฟาร์มสุกรและไก่เนื้อที่เลี้ยงเชิงธุรกิจในตอนกลางของลูซอน ฟิลิปปินส์
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บทคัดย่อ

ปัจจุบันประเทศฟิลิปปินส์ยังไม่มีระบบการเฝ้าระวังเชื้อ อี โคไลชนิดที่สร้างเอ็นไซม์บีตา-แลคทาเมสชนิดฤทธิ์ขยาย หรือ อีเอสบีเอล ในไก่เนื้อและสุกรและมีข้อมูลที่จำกัด ดังนั้นทำการศึกษานี้เพื่อหาความชุก ปัจจัยเสี่ยง และรูปแบบการดื้อสารต้านจุลชีพในด้านลักษณะปรากฏและลักษณะทางพันธุกรรมของเชื้อชนิดนี้

ทำการศึกษาในฟาร์มไก่เนื้อ 78 ฟาร์มและฟาร์มสุกร 54 ฟาร์มในเขตลูซอนกลาง ข้อมูลการใช้สารต้านจุลชีพ รูปแบบฟาร์มและการจัดการฟาร์มได้ถูกรวบรวมและทำการวิเคราะห์ เก็บตัวอย่างอุจจาระจำนวนทั้งหมด 156 ตัวอย่างจากฟาร์มไก่เนื้อ (78 ตัวอย่างแบบรวมด้วยวิธีการป้ายจากโคลเอกา และ 78 ตัวอย่างด้วยวิธีการใช้รองเท้าบูท) และ 162 ตัวอย่างจากฟาร์มสุกร (108 ตัวอย่างแบบรวมจากฟาร์มสุกรขุนและพ่อแม่พันธุ์ และ 54 ตัวอย่างด้วยวิธีการใช้รองเท้าบูท) แบคทีเรียเพาะและคัดกรองด้วยอาหารเลี้ยงเชื้อแมคคองกีและอาหารเลี้ยงเชื้อ อีโอซิน เมทิลลีน บลูที่มีเซฟโฟแท็กซีมความเข้มข้น 1 มิลลิกรัมต่อลิตร การแยกและยืนยันลักษณะแสดงออกของเชื้อแบคทีเรียที่แยกได้ด้วย Vitek 2 Compact and Combined Disc การทดสอบความไวต่อสารต้านจุลชีพของเชื้อด้วย Vitek 2 Compact using minimum inhibitory concentration. ใช้วิธีปฏิกิริยาลูกโซ่พอลิเมอเรสเพื่อหายีนดื้อสารต้านจุลชีพ โดยใช้ไพร์เมอร์ที่จำเพาะสำหรับ $bla_{\text{CTX-M}}$ และกลุ่มที่เกี่ยวข้อง 5 กลุ่ม ได้แก่ bla_{TEM} and bla_{SHV} รวมถึงยีน mcr-1 ทำการวิเคราะห์หาลักษณะปรากฏจากตัวอย่างที่คัดเลือกมา

ผลจากไก่เนื้อพบว่าความชุกในระดับฟาร์มคิดเป็นร้อยละ 66.67 (52/78) ซึ่งเป็นความชุกของตัวอย่างที่เก็บจาก ตัวอย่างแบบรวมด้วยวิธีการป้ายจากโคลเอกาและตัวอย่างที่เก็บด้วยวิธีการใช้รองเท้าบูท คิดเป็นร้อยละ 60.26 (47/78) และ 28.21 (22/78) ตามลำดับ ปัจจัยเสี่ยงที่มีนัยสำคัญทางสถิติประกอบด้วย การใช้อาหารที่มาจากแหล่งการค้า (OR=3.49, p=0.042) การเลี้ยงที่มีวงรอบ 6-8 ครั้งต่อปี (OR=6.62, p=0.003) การไม่ใช้ยาฆ่าเชื้อ (OR=3.91, p=0.033) ในสุกรพบว่าความชุกระดับฟาร์มเป็นร้อยละ 57.41 (31/54) ความชุกในสุกรขุนเป็นร้อยละ 27.78 (15/54) และในแม่พันธุ์เป็นร้อยละ 35.19 (19/54) ความชุกของเชื้อ อี โคไลชนิดที่สร้างเอ็นไซม์บีตาแลคทาเมสชนิดฤทธิ์ขยายจากตัวอย่างที่เก็บด้วยวิธีการใช้รองเท้าบูทคิดเป็นร้อยละ 25.93ร้อยละ (14/54) การขาดการฝึกอบรมระบบการผลิตสุกรเป็นปัจจัยเสี่ยง (OR=4.45, p=0.023)

เชื้ออี โคไลชนิดที่สร้างเอ็นไซม์บีตาแลคทาเมสชนิดฤทธิ์ขยายที่แยกได้จากฟาร์มไก่เนื้อและสุกรแสดงรูปแบบการดื้อต่อเพนนิซิลลิน (ร้อยละ 100) และต่อเซฟาโลสปอรินส์ (ร้อยละ 90-100) การดื้อสารต้านจุลชีพ ต่อ ไตรเมโทพริม/ซัลฟาเมททอคซาโซลพบในระดับสูง (ร้อยละ 72.46 ในไก่เนื้อและ 89.58 ในสุกร) เช่นเดียวกับซิโปรฟลอคซาซิน (ร้อยละ 91.3 ในไก่เนื้อ และ 52 ในสุกร). เชื้อที่แยกได้ส่วนใหญ่มีความไวต่อ เซฟอคซิติน (ร้อยละ 46.4 ในไก่เนื้อและ 25 ในสุกร) และแอมมอคซีซิลิน/กรดคลาวูลานิก (ร้อยละ 43.5 ในไก่เนื้อ และ 39.6 ในสุกร) ลักษณะแสดงออกของรูปแบบการดื้อสารต้านจุลชีพที่พบมากที่สุดของเชื้อที่แยกได้จากไก่เนื้อ คือ เพนนิซิลิน-เซฟพีม-ฟลูโอโลควิโนโลน-กลุ่มที่ขัดขวางกระบวนการสร้างโฟเลทในขณะที่เชื้อที่แยกได้จากสุกรมีลักษณะแสดงออกของรูปแบบการดื้อสารต้านจุลชีพที่พบบ่อย คือ เพนนิซิลิน-เซฟพีม-กลุ่มที่ขัดขวางกระบวนการสร้างโฟเลท

ยีนที่พบเป็นส่วนใหญ่ของกลุ่มเชื้ออีเอสบีเอล คือ $bla_{\text{CTX-M}}$ (ร้อยละ 89.86) ในกลุ่ม $bla_{\text{CTX-M}}$ นั้นพบ $bla_{\text{CTX-M-1}}$ มากที่สุด (ร้อยละ 72.46) ตามด้วย $bla_{\text{CTX-M-2}}$ (ร้อยละ 65.22) และ $bla_{\text{CTX-M-9}}$ (ร้อยละ 52.17) ส่วนยีน bla_{TEM} และ ยีน bla_{SHV} พบร้อยละ57.97 และ 27.54 ของเชื้อที่แยกได้ทั้งหมด ในสุกรยีนที่พบเป็นส่วนใหญ่ของกลุ่มเชื้ออีเอสบีเอล คือ $bla_{\text{CTX-M}}$ และ bla_{TEM} ซึ่งทั้งสองยีนนี้พบร้อยละ 91.67 เท่ากัน และตามด้วยยีน bla_{SHV} พบที่ร้อยละ 60.42 กลุ่มของ $bla_{\text{CTX-Mgroups}}$ ในสุกร พบยีน $bla_{\text{CTX-M-1}}$ มากที่สุด (ร้อยละ 75.0). ยีน $bla_{\text{CTX-M-15}}$ ซึ่งเป็นชนิดย่อยภายใต้กลุ่ม $bla_{\text{CTX-M-1group}}$ และชนิดนี้มีความสำคัญในทางสาธารณสุขโดยเป็นชนิดของยีนที่พบอย่างแพร่หลายในมนุษย์ ซึ่งตรวจพบในตัวอย่างจากสัตว์ไก่เนื้อ (ร้อยละ 72.46) มากกว่าในตัวอย่างที่ได้จากสุกร (ร้อยละ 35.42) จากเชื้อทั้งหมดที่แยกได้ กลุ่มยีนที่มักพบร่วมกัน คือ $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} เชื้อส่วนใหญ่ที่แยกได้จากไก่กระทง (ร้อยละ 33.33) มียีนที่มักพบร่วมกัน คือ $bla_{\text{CTX-M}}$ และ bla_{TEM} ขณะที่เชื้อที่แยกได้จากสุกร

(ร้อยละ 58.33) ส่วนใหญ่พบยีน bla_{CTX-M}, bla_{TEM} และ bla_{SHV} ที่มักพบร่วมกัน ยิ่งไปกว่านั้นเชื้อที่แยกได้จากไก่กระทงมักพบว่ามียีนในกลุ่ม $bla_{CTX-Mgroup}$ 2 ยีน หรือมากกว่า และเก้ารูปแบบที่แตกต่างกัน พบว่ารูปแบบร่วมของ $bla_{CTX-M-1}$ +$bla_{CTX-M-2}$ +$bla_{CTX-M-9}$ มีสูงที่สุด (ร้อยละ 17.39) เชื้อที่แยกได้จากสุกรมีรูปแบบร่วมที่แตกต่างกันในกลุ่มของ bla_{CTX-M} และรูปแบบร่วมที่พบสูงที่สุด คือ $bla_{CTX-M-1}$+ $bla_{CTX-M-8}$ (ร้อยละ25) จากผลการวิเคราะห์ความสัมพันธ์ทางพันธุกรรมของยีน $bla_{CTX-M-1}$ และ $bla_{CTX-M-15}$ แสดงให้เห็นว่าเชื้อที่แยกได้นั้นมีสายสัมพันธ์ร่วมกันกับเชื้อที่แยกได้จากประเทศอื่นๆ นอกจากการพบยีนในกลุ่มของอีเอสีเอลแล้วยังพบยีนดื้อต่อโคลิสตินอีกด้วย เชื้อที่แยกได้ส่วนใหญ่คิดเป็นร้อยละ 84.06 (58 /69) จากไก่เนื้อ และร้อยละ 54.16 (26 /48) จากสุกร พบยีน *mcr-1* ตามลำดับ

อ้างอิงจากผลระดับความชุกเชื้อ อี โคไลชนิดที่สร้างเอ็นไซม์บีตา-แลคทาเมสชนิดฤทธิขยายมีระดับสูงทั้งในฟาร์มไก่เนื้อและฟาร์มสุกรโดยที่เป็นเชื้อที่มีรูปแบบการดื้อสารต้านจุลชีพหลายชนิดร่วมกัน พบยีนดื้อต่อสารต้านจุลชีพ 3 ชนิด (bla_{CTX-M}-bla_{TEM},-bla_{SHV}) และการพบร่วมกันของยีนเหล่านั้นในเชื้อที่แยกได้ตัวเดียวกัน ซึ่งอาจจะเป็นการคงอยู่การการดื้อต่อสารต้านจุลชีพและจากการได้รับความเสี่ยงของการแพร่กระจายเชื้อจากคนงานฟาร์ม สัตว์อื่นๆ เชื้อแบคทีเรียที่ยังคงไวต่อสารต้านจุลชีพอื่นๆ และสิ่งแวดล้อม การศึกษานี้แนะนำการมีระบบเฝ้าระวังและควบคุมที่ได้เริ่มใช้นี้ควรต้องมีต่อไปและความชุก ปัจจัยเสี่ยงและรูปแบบการดื้อต่อสารต้านจุลชีพควรต้องมีการเฝ้าระวังเป็นประจำเพื่อให้เข้าถึงการเปลี่ยนแปลงซึ่งอาจจะใช้เป็นแนวทางสำหรับทิศทางของงานวิจัยในอนาคตและการสร้างข้อกำหนดนโยบายอันที่จะนำไปสู่การตัดสินใจระดับนโยบายจากหลักฐานประจักษ์

Dissertation Title	Prevalence and Antimicrobial Resistant Patterns of Extended Spectrum Beta-Lactamase Producing *Escherichia coli* in Commercial Swine and Broiler Farms in Central Luzon, The Philippines	
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Degree	Doctor of Philosophy (Veterinary Science)	
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ABSTRACT

There is no established surveillance system yet for ESBL-producing *E. coli* for broiler and swine in the Philippines, and data are indeed lacking. Hence, the study was conducted to determine the prevalence, risk factors, phenotypic and genotypic antimicrobial resistance (AMR) patterns of this pathogen.

A total of 78 broiler and 54 swine farms from Central Luzon region were studied. Information on antimicrobial usage, farm characteristics and farm practices were obtained and putative risk factors were analyzed. A total of 156 fecal samples from broiler farms (78 pooled cloacal swabs and 78 boot swabs) and 162 samples from swine farms (108 pooled fecal samples from finishers and breeders and 54 boot swabs) were collected. Bacteria were isolated and screened using MacConkey agar and Eosin Methylene Blue agar plates both supplemented with 1mg/L cefotaxime. Bacterial identification and phenotypic confirmatory tests were done through Vitek 2 Compact and Combined Disc Test. Antimicrobial Susceptibility Test was also done through Vitek 2 Compact using minimum inhibitory concentration. PCR assay was done to detect resistance genes using specific primers for $bla_{\text{CTX-M}}$ and its five major groupings, bla_{TEM} and bla_{SHV}, including mcr-1 gene. Phylogenetic analyses were conducted in the selected samples.

Results showed a broiler farm prevalence of 66.67% (52/78) while pooled cloacal and boot swab samples showed a prevalence of 60.26% (47/78) and 28.21% (22/78), respectively. Significant risk factors observed include commercial source of feeds (OR=3.49, p=0.042), 6-8

growing cycles per year (OR=6.62, p=0.003) and lack of disinfection (OR=3.91, p=0.033). In swine, results showed a prevalence of 57.41% (31/54) among swine farms studied. The prevalence among finishers is 27.78% (15/54) compared to breeders with a prevalence of 35.19% (19/54). ESBL-producing *E. coli* was isolated in boot swabs with 25.93% (14/54) prevalence. Lack of training in pig production was observed to be a risk factor (OR=4.45, p=0.023).

All ESBL-producing *E. coli* isolated from broiler and swine farms showed pattern resistant to penicillin (100%) and cephalosphorins (>90%-100%). High resistance was also recorded in trimethoprim/sulfamethoxazole (72.46% in broilers, 89.58% in swine) as well as ciprofloxacin (91.3% in broilers, 52% in swine). Many isolates are still susceptible to cefoxitin (46.4% in broilers and 25% in swine) and amoxicillin/clavulanic acid (43.5% in broilers and 39.6% in swine). The most common phenotypic pattern in broiler isolates is Penicillin-Cephem-Fluoroquinolone-Folate Pathway Inhibitor while in swine isolates, the most common pattern is Penicillin-Cephem-Folate Pathway Inhibitor.

The most prevalent ESBL encoding gene detected in broilers was $bla_{\text{CTX-M}}$ (89.86%). Among the $bla_{\text{CTX-Mgroups}}$, $bla_{\text{CTX-M-1}}$ has the highest prevalence (72.46%) followed by $bla_{\text{CTX-M-2}}$ (65.22%) and $bla_{\text{CTX-M-9}}$ (52.17%). The genes bla_{TEM} and bla_{SHV} were also identified in 57.97% and 27.54% of isolates, respectively. In swine, the most prevalent ESBL encoding genes detected were $bla_{\text{CTX-M}}$ and bla_{TEM}, which were both observed at 91.67% followed by bla_{SHV} gene at 60.42%. Among the $bla_{\text{CTX-Mgroups}}$ in swine, $bla_{\text{CTX-M-1}}$ was also the most prevalent in this group (75.0%). The $bla_{\text{CTX-M-15}}$, a sub-type under $bla_{\text{CTX-M-1group}}$, and which has a public health importance being the most widespread gene type in humans, is more common in broiler (72.46%) than in swine (35.42%) isolates. The co-existence of three different kinds of resistance genotypes ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) were found common. Majority of broiler isolates (33.33%) have co-existence of $bla_{\text{CTX-M}}$ and bla_{TEM} genes while majority of swine isolates (58.33%) have $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}. Moreover, most of the broiler isolates carry two or more $bla_{\text{CTX-Mgroup}}$ genes and nine different combinations were observed with $bla_{\text{CTX-M-1}}$ +$bla_{\text{CTX-M-2}}$ +$bla_{\text{CTX-M-9}}$ having the highest percentage (17.39%). In swine isolates, only five different $bla_{\text{CTX-Mgroup}}$ combinations were observed with $bla_{\text{CTX-M-1}}$+ $bla_{\text{CTX-M-8}}$ having the highest percentage (25%). The results of phylogenetic analyses of the $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ genes showed that isolates share a common phylogenetic root with strains from other countries. In addition to ESBL genes, colistin resistance gene was also observed. Majority of the isolates, 84.06% (58 out of 69) from broilers and 54.16% (26 out of 48) from swine, were found to be positive for *mcr-1* gene, respectively.

Based on results, high prevalence of ESBL-producing *E. coli* in both broiler and swine farms were observed exhibiting multi-drug resistance patterns. Three different kinds of resistance

genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$) were detected and coexistence of these genes were occurring within the same isolate which could result in retained antimicrobial resistance and may even pose risks of possible transmission to farm workers, other animals, susceptible bacteria and the environment. It is recommended that the surveillance initiated in this study be sustained and that the prevalence, risk factors and AMR patterns be monitored regularly to assess changes that may serve as guide in future research directions, and formulating policy recommendations leading to evidence-based policy decisions.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial Resistance
AMU	Antimicrobial Usage
AST	Antimicrobial Susceptibility Test
CDT	Combined Disc Test
CLSI	Clinical and Laboratory Standards Institute
EMB	Eosin Methylene Blue Agar
ESBL	Extended Spectrum Beta-Lactamase
MAC	MacConkey Agar
PCR	Polymerase Chain Reaction
AM	Ampicillin
AMC	Amoxicillin/Clavulanic Acid
TZP	Piperacillin/Tazobactam
CXM	Cefuroxime
CXMA	Cefuroxime Axetil
FOX	Cefoxitin
CAZ	Ceftazidime
CRO	Ceftriaxone
FEP	Cefepime
ETP	Ertapenem
IPM	Imipenem
MEM	Meropenem
AN	Amikacin
GM	Gentamicin
CIP	Ciprofloxacin
CS	Colistin
SXT	Trimethoprim/Sulfamethoxazole

STATEMENT OF ORIGINALITY

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that this thesis is my own work and that I have not submitted this material in whole or in part for any degree or diploma at any educational institution.

Romeo Sawit Gundran

CHAPTER 1

INTRODUCTION

Antimicrobial resistance (AMR) is now a worldwide problem and one of our most serious health threats. Infections from resistant bacteria are now too common, and some pathogens have even become resistant to multiple classes of antibiotics (CDC, 2013). It has become a rapidly growing public health concern worldwide. The World Health Organization (WHO) and countries around the world are beginning to be threatened by the possibility of reaching a post-antibiotic era, where the most common infectious diseases can kill millions of lives (DOH, 2015). It is estimated that in the United States alone, more than two million people are sickened every year with antibiotic-resistant infections, with at least 23,000 dying as a result (CDC, 2013). The Food and Agriculture Organization of the United Nations (FAO) estimates that around 500,000 human deaths related to antimicrobial resistance occur each year. Since 2003, FAO/WHO/OIE have been meeting jointly and working collaboratively to address the development and spread of antimicrobial resistance as a global public health problem due to human and non-human antimicrobial usage (FAO, 2008).

Antimicrobial resistance is now compromising the treatment of bacterial infections in both human and animals. Commensal bacteria, such as *Escherichia coli* may carry transferable resistance determinants, which can be further transmitted to other pathogenic bacteria. These antimicrobial-resistant bacteria, and their resistance genes, can easily be transmitted to humans through the food chain (Ramos, 2015). The threat of AMR is believed to become more intense by 2050 leading to an estimated 10 million deaths annually and global economic losses approximating 100 trillion US dollars or a reduction in the world's Gross Domestic Product (GDP) by 2-3.5 per year (O'Neill, 2014).

One specific AMR problem with global spread affecting both animals and humans is the extended-spectrum β-lactamase (ESBL)-producing *E. coli* (Chong et al., 2018).

Extended-spectrum β-lactamase is an enzyme that allows bacteria to become resistant to a wide variety of penicillins and cephalosporins. Bacteria that contain this enzyme are known as ESBLs or ESBL-producing bacteria (CDC, 2013). They are resistant to cephalosporins such as cefuroxime, cefotaxime, ceftazidime and ceftriaxone, and, through genetically linked resistance mechanisms, they are often resistant to other antibacterials including quinolones and aminoglycosides. Most ESBL-producing organisms are thus multi-drug resistant and many are only susceptible to carbapenems, along with little-used agents such as fosfomycin (Hunter et al., 2010). The threat level of ESBL-producing *Enterobacteriaceae* is categorized as serious and requires prompt and sustained action to ensure the problem does not grow (CDC, 2013).

The infection caused by Gram-negative bacteria producing extended spectrum beta-lactamases (ESBLs) is increasing worldwide **(**Dierikx, 2013) since their first description in Europe, in the early 1980s (Canton et al., 2008). It has been documented in humans as well as in food-producing birds, including chickens, and for unknown reasons the prevalence has increased significantly during the last decade (Olsen et al., 2014). In Netherlands, it was reported that the prevalence of ESBL-producing *Escherichia coli* in the gastrointestinal tract of healthy food-producing animals, especially broilers, increased from 3% in 2003 to 15% in 2008 and in 2009 ESBL-producing bacteria were detected in 26 of 26 broiler farms (Dierikx et al., 2010). Reports of contamination of retail chicken meat with ESBL-producing Gram-negative bacteria have been documented in several countries (Doi et al., 2010; Overdevest et al., 2010). Hence, the broiler industry has been considered a potential reservoir of ESBL-producing Gram-negative bacteria that may be acquired by humans through handling or consumption of contaminated meat (Dierikx, 2013)**.** With *E. coli* as a major opportunistic pathogen in chickens and with a potential for zoonotic transfer to human beings, ESBL-producing *E. coli* represents a major risk both to broiler production and to human health (Olsen et al., 2014). In Denmark, a study indicated that ESBL-producing *E. coli* may be transferred between pigs and humans with pigs as a reservoir for ESBL genes (Hammerum et al., 2014). For a long time, TEM- and SHV-types were the dominant ESBLs enzymes all over the world but this has changed dramatically since nowadays, CTX-M-enzymes have become the most widespread type of ESBLs (Canton et al., 2012; Canton et al., 2008). It is now present not only in humans,

but also in food, food-producing animals, companion and wild animals, and in the environment (Canton et al., 2012).

In a 2015 study conducted in Portugal, the prevalence of extended-spectrum ß-lactamase-producing *E. coli* isolates recovered in the faecal flora of food-producing animals were 49%, 9.3% and 5.5%, in pigs, cattle and sheep, respectively. The prevalent ß-lactamase detected was the CTX-M-1 enzyme, followed by CTX-M-9, CTX-M-14, SHV-12 and CTX-M-32 and for the first time, CTX-M-enzymes where reported from beef cattle and sheep (Ramos, 2015). Recent studies on the prevalence of ESBL-producing *E. coli* in pig farms in Thailand showed that 79.7% (98/123) of the farms had at least one sample being positive (Tablerk et al., 2015). Similar study in broiler farms has been conducted in Sri Lanka and found a prevalence of 50.6% (42/83) on broiler farms studied (Mahalingam et al., 2015). In Germany, it was reported that ESBL/AmpC-producing *E. coli* was found in all broiler farms studied, in 56.3% of breeding pig farms, in 60% of dairy cattle herds and in 43.8% of fattening pig holdings (Friese et al., 2013).

The Philippines, like other Southeast Asian countries, has already been encountering the many challenges of antimicrobial resistance (AMR) which include increasing social and economic costs and rising patient mortality. Although considered a global threat, it is already an emerging local health concern which calls for an urgent collaboration among different sectors to provide solutions addressing this growing problem. AMR in the Philippines is a national priority (Garin, 2015). AMR Surveillance in humans is being done at the Research Institute for Tropical Medicine (RITM) of the Department of Health and based on the 2014 Data Summary Report, it was shown that out of 5,506 *E. coli* isolates tested, 25% screened positive for ESBL. Other reports were variable but is ranging from 9.4% (n=32) to 51.6% (n=153) (RITM, 2014).

In humans, there were only few studies in the Philippines which reported the molecular characteristics of ESBL determinants. One study reported the CTX-M as the predominant extended-spectrum b-lactamases among *Enterobacteriaceae* (Tian et al., 2010) and another study reported that out of the $bla_{\text{CTX-M}}$-positive isolates, $bla_{\text{CTX-M-15}}$ shows the highest prevalence, followed by $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-14}}$ (Kanamori et al., 2011). Another report states that the prevalence of ESBL-producing *E. coli* significantly increased among in-hospital patients at a tertiary hospital in the Philippines from 2010 to

2014 (So and Mendoza, 2015). With limited reports available, it was suggested that the continuous monitoring and research undertakings on antimicrobial resistance in the Philippines be done.

Currently, there is a lack of information or published studies on the occurrence of ESBL- producing *E. coli* in broilers and swine in the Philippines unlike the regular antimicrobial resistance surveillance program among humans in various hospitals in the country in the past decades (Cruz and Hedreyda, 2017; Cabrera and Rodriguez, 2009; Kanamori et al., 2011). In fact, much work is needed to elucidate the level and status of AMR in food animals. There is therefore a need to determine the prevalence of ESBL-producing *E. coli* in commercial broiler and swine farms, identify the antimicrobial classes to which bacteria have developed resistance and identify the resistance genes including the putative risk factors that may be associated in its occurrence. These information will be useful in formulating evidence-based policies on mitigating antimicrobial resistance, hence this study.

Objectives of the Study

The objectives of this research study are as follows:

1. To determine the prevalence of ESBL-producing *E. coli* in commercial broiler and swine farms in the Central Luzon Region.
2. To determine the risk factors associated with the detection of ESBL-producing *E. coli* in commercial broiler and swine farms.
3. To establish the antimicrobial resistance patterns of ESBL-producing *E. coli.*

CHAPTER 2

REVIEW OF LITERATURE

2.1 The Swine and Broiler Industry in Central Luzon

Central Luzon, or Region 3, is a major industrial and agricultural center just north of Metro-Manila. Central Luzon is also known as the 'central plains' of Luzon. It is the traditional rice granary of the Philippines. It consists of seven provinces: Aurora, Bataan, Bulacan, Nueva Ecija, Pampanga, Tarlac, and Zambales. It occupies a total land area of 18,231 sq km, a vast expanse of which consists of plains. The map below shows its location in the country.

Figure 2.1 Map showing the various regions in the Philippines

A high concentration of commercial hog and broiler producers are found in the region. It has become the Philippines' leading hog producer. Table 2.1 below indicates that it accounts for about 37.1% of pig population (1,678,199 out of 4,518,781 pigs) and the highest in the country as of January 2016 (PSA, 2016).

Table 2.1
Inventory of commercial pigs in the Philippines, by region, from 2012-2016

	2012	2013	2014	2015	2016
PHILIPPINES	**3,881,354**	**4,092,812**	**4,144,831**	**4,217,432**	**4,518,781**
NCR	0	0	0	0	0
CAR	3,091	3,954	3,788	3,654	5,994
ILOCOS REGION	94,182	91,637	89,458	94,328	101,620
CAGAYAN VALLEY	38,790	38,942	35,133	38,243	41,532
CENTRAL LUZON	**1,126,629**	**1,338,622**	**1,388,067**	**1,437,159**	**1,678,199**
CALABARZON	1,202,431	1,186,079	1,188,891	1,197,823	1,261,484
MIMAROPA	80,356	84,668	98,175	107,701	107,755
BICOL REGION	152,187	151,851	154,840	141,504	114,905
WESTERN VISAYAS	134,602	137,908	140,301	141,796	147,177
CENTRAL VISAYAS	240,575	230,541	224,156	237,721	210,380
EASTERN VISAYAS	8,811	9,216	9,517	10,589	9,916
ZAMBOANGA PENINSULA	16,076	14,596	15,881	14,517	9,930
NORTHERN MINDANAO	302,056	298,028	294,518	296,851	347,789
DAVAO REGION	161,387	169,851	162,453	151,001	147,385
SOCCSKSARGEN	312,522	328,897	328,256	328,069	318,369
CARAGA	7,659	8,023	11,397	16,476	16,346
ARMM	0	0	0	0	0

Source: Philippine Statistics Authority (2016)

As of January 2016, the population of commercial pigs is shown in Table 2.2 Bulacan has the largest pig population among the seven provinces comprising 63.8% of the total population in the region, followed by the provinces of Tarlac and Pampanga (PSA, 2016). The profiles for the last five years are as follows:

Table 2.2

Inventory of commercial pigs in Central Luzon, by province, from 2012-2016

	2012	2013	2014	2015	2016
CENTRAL LUZON	1,126,629	1,338,622	1,388,067	1,437,159	1,678,199
Aurora	678	578	529	595	1,052
Bataan	28,669	30,025	31,332	32,043	30,987
Bulacan	861,395	1,014,036	1,014,542	1,010,953	1,071,497
Nueva Ecija	62,500	64,706	66,310	68,408	69,637
Pampanga	41,832	76,108	98,722	96,477	98,138
Tarlac	122,850	143,048	159,868	209,261	386,368
Zambales	8,705	10,121	16,764	19,422	20,520

Source: Philippine Statistics Authority (2016)

Central Luzon is also the top producer of broilers in the Philippines. Table 2.2 below indicates that it accounts for about 29.1% of broiler population (19,143,421 out of 65,713,051 broilers) and also the highest in the country as of January 2016 (PSA, 2016).

As of January 2016, the population of commercial broilers is shown in Table 2.4. Nueva Ecija has the largest broiler population among the seven provinces comprising 31% of the total population in the region, followed by the provinces of Bataan and Pampanga (PSA, 2016). The profiles for the last five years are as follows:

Table 2.3

Inventory of commercial broilers in the Philippines, by region, from 2012-2016

	2012	2013	2014	2015	2016
PHILIPPINES	**57,284,153**	**59,196,045**	**61,582,178**	**66,616,937**	**65,713,051**
NCR	0	0	0	0	0
CAR	3,141	4,898	1,008	28,494	4,300
ILOCOS REGION	3,521,787	5,064,493	4,809,720	5,181,634	5,031,289
CAGAYAN VALLEY	2,802,870	2,574,854	1,193,668	1,730,882	1,718,266
CENTRAL LUZON	**15,273,759**	**16,570,553**	**18,630,455**	**18,734,189**	**19,143,421**
CALABARZON	12,588,202	11,011,336	13,638,790	12,984,104	11,404,949
MIMAROPA	235,570	198,524	156,201	235,788	212,236
BICOL REGION	1,329,911	1,513,044	3,191,300	3,040,308	4,532,982
WESTERN VISAYAS	3,600,453	4,252,158	3,632,810	4,931,079	5,201,191
CENTRAL VISAYAS	2,868,682	2,917,672	2,902,906	3,442,059	3,034,096
EASTERN VISAYAS	1,728,381	1,233,891	262,540	279,717	295,516
ZAMBOANGA PENINSULA	1,376,208	1,503,024	1,065,114	1,205,995	1,269,702
NORTHERN MINDANAO	8,866,291	8,517,220	7,644,910	7,729,669	7,746,405
DAVAO REGION	1,649,022	2,038,968	2,865,234	4,825,211	3,983,322
SOCCSKSARGEN	1,273,723	1,633,084	1,366,566	1,882,349	1,872,161
CARAGA	166,153	161,826	220,956	385,459	263,215
ARMM	0	500	0	0	0

Source: Philippine Statistics Authority (2016)

Table 2.4

Inventory of commercial broilers in Central Luzon, by province, from 2012-2016

	2012	2013	2014	2015	2016
CENTRAL LUZON	15,273,759	16,570,553	18,630,455	18,734,189	19,143,421
Aurora	2,950	11,432	11,460	11,700	14,460
Bataan	3,731,793	3,585,302	3,873,835	4,130,627	3,973,405
Bulacan	**2,536,539**	**2,088,857**	**2,329,314**	**2,404,453**	**3,194,862**
Nueva Ecija	**3,775,450**	**3,694,658**	**3,729,530**	**4,546,550**	**5,868,940**
Pampanga	**3,499,843**	**5,028,027**	**5,516,640**	**3,995,890**	**3,803,848**
Tarlac	**1,278,270**	**1,574,720**	**2,558,640**	**2,765,412**	**2,225,405**
Zambales	448,914	587,557	611,036	879,557	62,501

Source: Philippine Statistics Authority (2016)

2.2 Use of Antimicrobial Agents in Swine and Broilers

Antimicrobials are used in livestock production to maintain health and productivity. These practices contribute to the spread of drug-resistant pathogens in both livestock and humans, posing a significant public health threat and global health crisis of antimicrobial resistance (Van Boeckel et al., 2015; Maron et al., 2013; Acar and Moulin, 2012). New forms of antibiotic resistance can cross international boundaries and spread between continents with ease. In fact, many forms of resistance spread with remarkable speed. World health leaders have described antibiotic-resistant microorganisms as “nightmare bacteria” that “pose a catastrophic threat” to people in every country in the world (CDC, 2013).

The first global map (228 countries) of antibiotic consumption in livestock was investigated and a total consumption of 63,151 tons was estimated conservatively. It was projected that antimicrobial consumption will rise by 67% by 2030, and nearly double in Brazil, Russia, India, China, and South Africa. This rise is likely to be driven by the growth in consumer demand for livestock products in middle-income countries and a shift to large-scale farms where antimicrobials are used routinely. This calls for initiatives to preserve antibiotic effectiveness while simultaneously ensuring food security in low- and lower-middle-income countries ((Van Boeckel et al., 2015).

The widespread use of antibiotics particularly at sub-therapeutic dose in food-animal feeds to prevent diseases and to improve production performance in modern animal husbandry in livestock contributes, by means of natural selection, to the emergence of antibiotic resistant bacteria (ARBs) (Cheng at al., 2014) and has significant public health implications: ARBs of animal origin can be transmitted to humans through the environment (Graham et al., 2009) and food products (Price et al., 2005) and to agricultural workers by direct contact (Smith, 2013).

Broiler and swine industry in the Philippines are both significant contributors to the country's agricultural sector. To meet the increasing demand for broilers and livestock, modern intensive farming system with high population density of animals is being widely practiced. This provides an environment conducive for rapid dissemination of infectious agents, thus requiring aggressive approach in disease management. It often involves the administration of antimicrobials to food animals for therapeutic use (treatment of clinical disease), prophylactic use (control of common diseases encountered) and sub-therapeutic use (growth promotant) (Chuanchuen et al., 2014).

Prudent use of antibiotics and the establishment of scientific monitoring systems are the best and fastest way to limit the adverse effects of the abuse of antibiotics and to ensure the safety of animal-derived food and environment (Cheng at al, 2014). Internationally, multiple jurisdictions have responded by restricting antimicrobial use for these purposes, and by requiring a veterinary prescription to use these drugs in food animals (Maron et al., 2013).

In the Philippines two agencies are involved in the regulation of antimicrobial use (AMU): the Department of Agriculture (DA) through the Bureau of Animal Industry (BAI) and the Department of Health through its Food and Drug Administration (DOH-FDA). Both agencies work in cooperation based on their respective regulatory functions. FDA regulates all veterinary drugs for injection and individual administration for animals. The BAI regulates veterinary drugs and products that are used or mixed or incorporated in feeds and drinking water. RA No. 9711 of the Food and Drug Administrative Act of 2009 is the most current legislation for regulating and monitoring of establishments and products including veterinary drugs and other health products. Most veterinary drugs are imported and are usually mixed in feed and water for controlling diseases and infections

in pigs and broilers. Of the products registered at the Department of Agriculture's Bureau of Animal Industry (BAI), in 2011 the most commonly sold antimicrobials were chlortetracycline and tiamulin hydrogen fumarate. No publications on AMU in livestock could be found. Information on national AMR surveillance in livestock and livestock products could not be found (Chuanchuen, et al., 2014).

2.3 Antimicrobial Agents and their Mechanism of Action

There are many different classes of antimicrobial agents such as ß-lactams, tetracyclines, aminoglycosides, macrolides, lincosamides, sulfonamides and quinolones and they may be used at different times in the life cycle of broilers, swine and other livestock (Landers et al., 2012). Table 2.5 below presents examples of antimicrobial agents used in animals and/or humans, and their specific applications, as individual or herd treatment, on the different animal species (Ramos, 2015).

As presented in the table, some antimicrobials are used in both veterinary and human medicine such as tetracyclines, penicillins, macrolides and sulphonamides. Some of these antimicrobials were classified, by the World Organization for Animal Health (OIE), as “Critically Important for Veterinary Practice” (OIE, 2014). Losing any of them because of antimicrobial resistance will have very direct consequences for animals because very few alternatives will be left for macrolides, in treating Mycoplasma infections in pigs and broilers, and fluoroquinolones in the treatment of chronic respiratory disease in broilers (Vaarten, 2012).

Table 2.5

Examples of antimicrobial agents used in animals and/or humans, and their specific applications in different animals.

Class or subclass	**Representative drugs**	**Use**[1]		**Specific veterinary applications**
		V	**H**	
Penicillins	Benzylpenicillin	x	x	Penicillins and cephalosporins are used for individual treatment as intramammary suspension in cow mastitis, or as injection in cattle, pigs and sheep. Amoxicillin is used as oral powder for pigs and chickens with respiratory infections.
	Ampicillin	x	x	
	Amoxicillin	x	x	
Cephalosporins	Cefotaxime		x	Ceftiofur is used for individual treatment, by injection, for respiratory disease in swine and ruminants (cattle, sheep and goats).
	Ceftazidime		x	
	Ceftiofur	x		
	Cefalonium	x		
Glycopeptides	Vancomycin		x	Avoparcin is used as a growth promoter
	Avoparcin	x		
Aminoglycosides	Streptomycin	x	x	Aminoglycosides is used for individual treatment, by injection, of cattle, sheep and pigs, or in intramammary suspension in cattle mastitis. Apramycin is used as premix for herd treatment of bacterial enteritis in pigs
	Dihydrostreptomycin	x	x	
	Apramycin	x		

Table 2.5 (continued)

Class or subclass	Representative drugs	Use[1]		Specific veterinary applications
		V	H	
Tetracyclines	Chlortetracycline	x	x	Chlortetracycline is used as oral powder for treatment of respiratory disease in calves caused by *Pasteurella* spp.. Oxytetracycline is used as a premix for the treatment of furunculosis in salmon farming. Oxytetracycline and tetracycline are used for individual treatment, by injection, in cattle, pigs and sheep.
	Oxytetracycline	x	x	
	Tetracycline	x	x	
Macrolides	Spiramycin	x	x	Tylosin is used as a premix for herd treatment and prevention of pigs dysentery and chickens necrotic enteritis. Spiramycin is used for individual treatment, by injection, of cattle and sows mastitis. Erythromycin is used as powder of oral solution for treatment of Chronic Respiratory Disease in poultry.
	Tylosin	x	x	
	Erythromycin	x		

Table 2.5 (continued)

Class or subclass	Representative drugs	Use[1]		Specific veterinary applications
		V	H	
Phenicol	Chloramphenicol		x	Florfenicol is used in drinking water of pigs respiratory disease associated with *Pasteurella multocida*; or for individual treatment, by injection, in cattle, sheep for respiratory tract infections.
	Florfenicol	x		
Quinolones	Ciprofloxacin		x	Enrofloxacin is used in the treatment of respiratory disease and enteritis, by injection, in cattle and pigs, or as oral solution, used in drinking water in chicken, turkey and rabbit.
	Nalidixic acid		x	
	Enrofloxacin	x		
	Marbofloxacin	x		
Sulfonamides and Trimethoprim	Sulfamethoxazol	x	x	Are used as oral solution, in drinking water, for herd treatment and prevention of respiratory infections in broilers and pigs.
	Trimethoprim	x	x	

[1] Approved for Veterinary (V) or Human (H) Use
Source: http://www.vmd.defra.gov.uk/ProductInformationDatabase

Introduction of Antibiotics and Emergence of Resistance

The introduction of antimicrobials transformed human and animal health systems in the war against infectious diseases, resulting in improved survivability for both humans and domestic animals. However, this immediately subsided because bacterial populations could quickly modify themselves to resist antimicrobials, propagate resistance traits, and even share resistance genes with other existing susceptible bacteria within their environment. Such abilities have seriously compromised the usefulness of antibiotics in

the battle against microbes and warn of a future when antimicrobials may have very limited usefulness to control bacterial infection. The table below shows the timeline of introduction of antibiotics and the emergence of antibiotic resistance.

Table 2.6

Timeline of introduction of antibiotics and emergence of resistance (adapted from AMRLS, 2011)

Year	Introduction of Antibiotics	Emergence of Antimicrobial Resistance
2001 to 2008	Introduction of broader spectrum fluoroquinolones (2001), telithromycin (2002), tigecycline (2006)	Emergence of vancomycin-resistant staphylococcal infections; Spread of extended-spectrum beta-lactamase among Gram negatives; Emergence of more multi-drug resistant organisms
1991 to 2000	Introduction of oral extended spectrum cephalosporins (1998), quinupristin-dalfopristin (1999), linezolid	Emergence of vancomycin-resistant enterococci; emergence of multi-drug resistant Mycobacterium tuberculosis; global emergence of multi- drug resistant *Salmonella enterica* serovar typhimurium DT 104
1981 to 1990	Introduction of cefotaxime (1981), clavulanic acid-amoxicillin (1983), imipenem-cilastatin (1985), norfloxacin (1986), aztreonam (1986)	Spread of methicillin-resistant staphylococcus infections; emergence of AIDS-related bacterial infections
1971 to 1980	Introduction of carbenicillin (1973), cefoxitin (1978), cefaclor (1979)	Increasing trend of nosocomial infections due to opportunistic pathogens; Ampicillin-resistant infections become frequent
1961 to 1970	Introduction of gentamicin (1963), ampicillin (1966), cephalothin (1966), amikacin (1970)	Emergence of gentamicin-resistant Pseudomonas (1968); emergence of methicillin-resistant staphylococcal infections (1968)

Table 2.6 (continued)

Year	Introduction of Antibiotics	Emergence of Antimicrobial Resistance
1951 to 1960	Introduction of erythromycin, vancomycin, tylosin and methicillin	Penicillin-resistant infections become clinically significant
1941 to 1950	Introduction of streptomycin (1944), chloramphenicol (1946) and chlortetracycline (1948)	Penicillin made available to the public; widespread use in animals by 1950.
1930 to 1940	Introduction of sulfonamide	Efficacy of penicillin in humans shown; sulfonamides introduced in food animal use
Before 1930	Discovery of penicillin (1929)	

Similar timeline of antibiotic deployment and emergence of antibiotic resistance is presented in Figure 2.2. Correspondingly, clinically significant antibiotic resistance has evolved against virtually every antibiotic deployed suggesting that new antimicrobial development strategies should be expanded (Clatworthy et al., 2007).

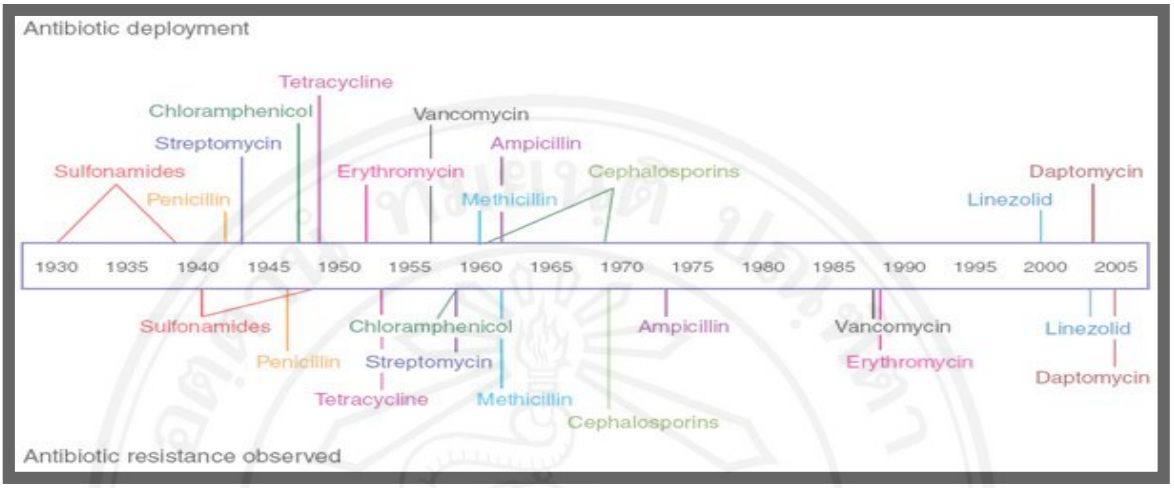


Figure 2.2 Timeline of antibiotic deployment and evolution of antibiotic resistance (adapted from Clatworthy et al., 2007)

The Beta-Lactam Antibiotics

Beta-lactam antibiotics are widely used drugs in human and animal medicine. They are used to treat infections of Gram-positive and Gram-negative bacteria. Beta-lactam antibiotics are named after their structure, the beta-lactam ring, which forms the center and active part of the drug. One can divide this large class of antibiotics in different groups based on their mode of action: penicillins, cephalosporins (1st, 2nd, 3rd, 4th generation), carbapenems, penems, monobactams and beta-lactamase inhibitors like clavulanic acid. This last group has no antibacterial activity on its own but inhibits the activity of beta-lactam degrading enzymes (beta-lactamases) and is often given in combination with other beta-lactam antibiotics. The most famous and oldest beta-lactam antibiotic is penicillin. Penicillin and all later discovered beta-lactam antibiotics, interfere with the last phase of cell-wall synthesis (Caprile, 1988).

There are several penicillin binding proteins (PBPs) which vary with type and species of the bacteria. Differences in affinities of individual beta-lactam agents for certain PBPs determine differences in activity found related to various cephalosporins.

This partly explains the difference in affinity of some compounds for Gram-positive and Gram-negative bacteria. Generally the first discovered beta-lactam antibiotics (penicillins and first generation cephalosporins) are more active against Gram-positives than against Gram-negatives. Later generations of these drugs have a broader activity against Gram-negatives (2nd generation cephalosporins) as well as against both Gram-negative and Gram-positive bacteria (3rd generation cephalosporins, or extended spectrum cephalosporins (ESC) (Caprile, 1988).

Mechanism of Action of Antimicrobials

There are three ways by which antibiotics can be classified: 1) spectrum of activity whether narrow or broad spectrum, 2) effect on bacteria whether bacteriostatic or bactericidal, and 3) mechanism of action.

The different mechanism of actions of antibiotics, owing to the nature of their structure and degree of affinity to certain target sites within bacterial cells, are as follows (Gordoncillo et al., 2011; AMRLS, 2011):

Inhibitors of cell wall synthesis. While the cells of humans and animals do not have cell walls, this structure is critical for the life and survival of bacterial species. A drug that targets cell walls can therefore selectively kill or inhibit bacterial organisms. Examples: penicillins, cephalosporins, bacitracin and vancomycin.

Inhibitors of cell membrane function. Cell membranes are important barriers that segregate and regulate the intra- and extracellular flow of substances. A disruption or damage to this structure could result in leakage of important solutes essential for the cell's survival. Because this structure is found in both eukaryotic and prokaryotic cells, the action of this class of antibiotic are often poorly selective and can often be toxic for systemic use in the mammalian host. Most clinical usage is therefore limited to topical applications. Examples: polymixin B and colistin.

Inhibitors of protein synthesis. Enzymes and cellular structures are primarily made of proteins. Protein synthesis is an essential process necessary for the multiplication and survival of all bacterial cells. Several types of antibacterial agents target bacterial protein synthesis by binding to either the 30S or 50S subunits of the intracellular

ribosomes. This activity then results in the disruption of the normal cellular metabolism of the bacteria, and consequently leads to the death of the organism or the inhibition of its growth and multiplication. Examples: aminoglycosides, tetracyclines, macrolides, lincosamides, tiamulins, virginiamycin, kitasamycin, chloramphenicol.

Inhibitors of nucleic acid synthesis. DNA and RNA are keys to the replication of all living forms, including bacteria. Some antibiotics work by binding to components involved in the process of DNA or RNA synthesis, which causes interference of the normal cellular processes which will ultimately compromise bacterial multiplication and survival. Examples: quinolones, metronidazole, and rifampin.

Inhibitors of other metabolic processes. Other antibiotics act on selected cellular processes essential for the survival of the bacterial pathogens. For example, both sulfonamides and trimethoprim disrupt the folic acid pathway, which is a necessary step for bacteria to produce precursors important for DNA synthesis. Sulfonamides target and bind to dihydropteroate synthase, trimethophrim inhibit dihydrofolate reductase; both of these enzymes are essential for the production of folic acid, a vitamin synthesized by bacteria, but not humans.

2.4 Mechanism of Antimicrobial Resistance

Antimicrobial resistance is resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it (WHO, 2015). Resistant microorganisms are able to withstand attack by antimicrobial drugs so that standard treatments become ineffective and infections persist, increasing the risk of spread to others. The evolution of resistant strains is a natural phenomenon that occurs when microorganisms replicate themselves erroneously or when resistant traits are exchanged between them. The use and misuse of antimicrobial drugs accelerates the emergence of drug-resistant strains. Poor infection control practices, inadequate sanitary conditions and inappropriate food-handling encourage the further spread of antimicrobial resistance (WHO, 2015).

Bacteria survive in the presence of an antibiotic by disrupting one or more of the essential steps required for the effective action of the antimicrobial agent. This may involve preventing antibiotic access into the bacterial cell or perhaps removal or even

degradation of the active component of the antimicrobial agent. No single mechanism of resistance is considered responsible for the observed resistance in a bacterial organism. In fact, several different mechanisms may work together to confer resistance to a single antimicrobial agent. The four major bacterial resistance strategies are as follows (AMLRS, 2011):

By prevention of the antimicrobial from reaching its target by reducing its ability to penetrate into the cell. Some bacteria protect themselves by prohibiting these antimicrobial compounds from entering past their cell walls. For example, a variety of Gram-negative bacteria reduce the uptake of certain antibiotics, such as aminoglycosides and beta lactams, by modifying the cell membrane porin channel frequency, size, and selectivity. Prohibiting entry in this manner will prevent these antimicrobials from reaching their intended targets that, for aminoglycosides and beta lactams, are the ribosomes and the penicillin-binding proteins (PBPs), respectively.

By expulsion of the antimicrobial agents from the cell via general or specific efflux pumps. Some bacteria possess membrane proteins that act as an export or efflux pump for certain antimicrobials, extruding the antibiotic out of the cell as fast as it can enter. This results in low intracellular concentrations that are insufficient to elicit an effect. Some efflux pumps selectively extrude specific antibiotics such as macrolides, lincosamides, streptogramins and tetracyclines.

By inactivation of antimicrobial agents via modification or degradation. A classic example is the hydrolytic deactivation of the beta-lactam ring in penicillins and cephalosporins by the bacterial enzyme called beta lactamase. The inactivated penicilloic acid will then be ineffective in binding to PBPs (penicillin-binding proteins), thereby protecting the process of cell wall synthesis.

By modification of the antimicrobial target within the bacteria. Some resistant bacteria evade antimicrobials by reprogramming or camouflaging critical target sites to avoid recognition. Therefore, in spite of the presence of an intact and active antimicrobial compound, no subsequent binding or inhibition will take place. This strategy has been observed in: *Staphylococci* against methicillin and other beta-lactams (Changes or

acquisition of different PBPs that do not sufficiently bind beta-lactams to inhibit cell wall synthesis) as well as in *Enterococci* against vancomycin (alteration in cell wall precursor

An outline of the mechanisms of antimicrobial resistance against different classes of antimicrobials is presented in Table 2.7 (Forbes et al., 1998; Berger-Bachi, 2002).

Table 2.7

Mechanisms of resistance against different antimicrobial classes

Antimicrobial Class	**Mechanism of Resistance**	**Specific Means To Achieve Resistance**	**Examples**
Beta-lactams Examples: penicillin, ampicillin, mezlocillin, peperacillin, cefazolin,	Enzymatic destruction	Destruction of beta-lactam rings by beta-lactamase enzymes. With the beta-lactam ring destroyed, the antibiotic will no longer have the ability to bind to PBP (Penicillin-binding protein), and interfere with cell wall synthesis.	Resistance of staphylococci to penicillin; Resistance of *Enterobacteriaceae* to penicllins, cephalosporins, and aztreonam
cefotaxime, ceftazidime, aztreonam, imipenem	Altered target	Changes in penicillin binding proteins. Mutational changes in original PBPs or acquisition of different PBPs will lead to inability of the antibiotic to bind to the PBP and inhibit cell wall synthesis	Resistance of staphylococci to methicillin and oxacillin
	Decreased uptake	Porin channel formation is decreased. Since this is where beta-lactams cross the outer membrane to reach the PBP of Gram-negative bacteria, a change in the number or character of these channels can reduce betalactam uptake.	Resistance of Enterobacter aerogenes, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* to imipenem
Glycopeptides Example: vancomycin	Altered target	Alteration in the molecular structure of cell wall precursor components decreases binding of vancomycin so that cell wall synthesis is able to continue.	Resistance of enterococci to vancomycin

Table 2.7 (continued)

Antimicrobial Class	Mechanism of Resistance	Specific Means To Achieve Resistance	Examples
Aminoglyosides Examples: gentamicin, tobramycin, amikacin, netilmicin, streptomycin, kanamycin	Enzymatic modification	Modifying enzymes alter various sites on the aminoglycoside molecule so that the ability of this drug to bind the ribosome and halt protein synthesis is greatly diminished or lost entirely.	Resistance of many Gram- positive and Gram negative bacteria to aminoglycosides
	Decreased uptake	Change in number or character of porin channels (through which aminoglycosides cross the outer membrane to reach the ribosomes of gram-negative bacteria) so that aminoglycoside uptake is diminished.	Resistance of a variety of Gram- negative bacteria to aminoglycosides
	Altered target	Modification of ribosomal proteins or of 16s rRNA. This reduces the ability of aminoglycoside to successfully bind and inhibit protein synthesis	Resistance of Mycobacterium spp to streptomycin
Quinolones Examples: ciprofloxacin, levofloxacin, norfloxacin, lomefloxacin	Decreased uptake	Alterations in the outer membrane diminishes uptake of drug and/or activation of an efflux pump that removes quinolones before intracellular concentration is sufficient for inhibiting DNA metabolism.	Resistance of Gram negative and staphylococci (efflux mechanism only) to various quinolones
	Altered target	Changes in DNA gyrase subunits decrease the ability of quinolones to bind this enzyme and interfere with DNA processes	Gram negative and Gram positive resistance to various

2.5 Molecular Basis of Antimicrobial Resistance

The abilities of bacterial organisms to utilize the various strategies to resist antimicrobial compounds are all genetically encoded and there are molecular basis for such resistance strategies. There are two types: 1) Intrinsic (or natural) and 2) Acquired (or transferable) resistance.

Intrinsic resistance occurs when bacteria lack a structure on which the antibiotics can act on or have enzymes which prevent antibiotic action. For example, Mycoplasma has no cell wall and is naturally resistant to Penicillin. Anaerobic bacteria have natural resistance to aminoglycosides because of lack of oxidative metabolism to drive uptake of aminoglycosides. *Klebsiella spp*. are resistant to ampicillin (a beta lactam) because of production of beta lactamases that destroy ampicillin before the drug can reach the PBP targets (Forbes et al., 1998).

Acquired resistance occurs when a particular organism obtains the ability to resist the activity of an antimicrobial agent to which it was previously susceptible. It involves a genetic change. Changes in bacterial genome occur through *mutation* or *horizontal gene transfer* via transformation, transduction or conjugation. Such change may lead to an alteration in the structural and functional features of the bacteria involved, which may result in changes leading to resistance against a particular antibiotic.

Mutation is a spontaneous change in the DNA sequence within the gene that may lead to a change in the trait which it codes for. Any change in a single base pair may lead to a corresponding change in one or more of the amino acids for which it codes, which can then change the enzyme or cell structure that consequently changes the affinity or effective activity of the targeted antimicrobials.

Horizontal gene transfer, or the process of swapping genetic material between neighboring "contemporary" bacteria, is another means by which resistance can be acquired. Many of the antibiotic resistance genes are carried on plasmids, transposons or integrons that can act as vectors that transfer these genes to other members of the same bacterial species, as well as to bacteria in another genus or species. Horizontal gene transfer may occur via three main mechanisms: transformation, transduction or conjugation.

Transformation involves uptake of short fragments of naked DNA by naturally transformable bacteria. *Transduction* involves transfer of DNA from one bacterium into another via bacteriophages. *Conjugation* involves transfer of DNA via sexual pilus and requires cell to cell contact. DNA fragments that contain resistance genes from resistant donors can then make previously susceptible bacteria express resistance as coded by these newly acquired resistance genes.

2.6 Extended-spectrum beta-lactamases (ESBLs)-producing *Escherichia coli*

Extended-spectrum β-lactamase is an enzyme that allows bacteria to become resistant to a wide variety of penicillins and cephalosporins. Bacteria that contain this enzyme are known as ESBLs or ESBL-producing bacteria. ESBL-producing *Enterobacteriaceae* are resistant to strong antibiotics including extended spectrum cephalosporins. CDC has categorized the threat level of ESBL as serious (CDC, 2013).

A dramatic increase in the number of B-lactamases has been described since the 1980s of the last century. For a long time, TEM- and SHV-types were the dominant ESBLs enzymes all over the world (Bradford, 2001; MShana, 2011). However, this situation changed dramatically in the present century (Canton et al., 2012; Canton et al., 2008). Nowadays, CTX-M-enzymes have become the most widespread type of ESBLs (Canton et al., 2012).

The highest number of variants described in the last years corresponds to the CTX-M family (123 variants until 2011). This explosive dissemination of CTX-Ms around the world has been referred as the "CTX-M pandemic" due to their increasing description worldwide (Canton et al., 2012).

This CTX-M pandemic is a cause for worry since most ESBL-producing isolates are now *E. coli* expressing CTX-M ß-lactamases, that “cross the border” from hospital settings to the community (Canton et al., 2012; Canton et al., 2008). There is now a global epidemic of *E. coli* strains harbouring CTX-M-enzymes that require serious attention, the CTX-M-15 and CTX-M-14 enzymes are by far the most important ones virtually invading all human and animal compartments, as well as the environment, all over the world (Ramos, 2015; Cantón et al., 2008; Hiroi et al., 2012).

2.6.1 Types of ESBL

The first report of plasmid-encoded beta-lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983. Now, the total number of ESBLs that have been characterized exceeds 200 (Paterson and Bonomo, 2005). The different types of ESBLs are as follows:

TEM Type: The TEM-type ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 was first reported in 1965 from an *Escherichia coli* isolate from a patient in Athens, Greece, named Temoneira hence the designation TEM (Sougakoff, et al., 1988; Paterson and Bonomo, 2005). TEM-1 is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid. TEM-2 has the same hydrolytic profile as TEM-1, but differs from TEM-1 by having a more active native promoter and by a difference in isoelectric point (5.6 compared to 5.4). TEM-13 also has a similar hydrolytic profile to TEM-1 and TEM-2. TEM-1, TEM-2, and TEM-13 are not ESBLs. However, in 1987 *Klebsiella pneumoniae* isolates detected in France as early as 1984 were found to harbor a novel plasmid-mediated beta-lactamase coined CTX-1. The enzyme was originally named CTX-1 because of its enhanced activity against cefotaxime but now, it is termed TEM-3. Currently, over 100 TEM-type β-lactamases have already been described of which most of them are ESBLs. Other TEM-type enzymes which are less susceptible to the effects of beta-lactamase inhibitors and which have negligible hydrolytic activity against the extended-spectrum cephalosporins are not considered ESBLs (Paterson and Bonomo, 2005).

SHV Type: SHV refers to Sulfhydryl variable. SHV used to be more frequently found in clinical isolates than any other type of ESBL. The first SHV that hydrolyze extended spectrum β-lactam antibiotics was isolated from *Klebsiella ozaenae* in 1983 in Germany. Unlike TEM-type β-lactamases, there are few derivatives of SHV-1; more than 50 SHV varieties have been described worldwide (MShana, 2011). SHV enzymes have emerged in *Enterobacteriaceae* causing infections in health care in the last decades. They are now observed in isolates in different epidemiological settings both in human, animal and the environment. Not all SHV β-lactamases are ESBLs. Others are non-ESBL and still others remain unclassified variants. SHV-ESBLs are usually encoded

by self-transmissible plasmids that frequently carry resistance genes to other drug classes and have become widespread throughout the world in several *Enterobacteriaceae*, emphasizing their clinical significance (Liakopoulos et al., 2016)

CTX-M Type: CTX-M is a recently described family of ESBLs and has become the most common type of plasmid-mediated ESBL enzymes produced by drug-resistant organisms sharing less than 40% amino acid sequence homology with the TEM- and SHV-type enzymes (He et al., 2016). CTX-M enzymes hydrolyze cefotaxime more than ceftazidime and they also hydrolyze cefepime with high efficiency. CTX-M type has been reported in most parts of the world, and is believed to be the most frequent type of ESBLs in the world. More than 113 CTX-M varieties are currently known. The $bla_{\text{CTX-M-15}}$ allele is considered to be predominant in many countries (MShana, 2011) and the most widely distributed gene encoding extended-spectrum β-lactamases globally (Zhang et al., 2013).

CTX-M enzymes can be subclassified by amino acid sequence similarities. Phylogenic study reveals five major groups of acquired CTX-M enzymes. The members of each group share >94% identity, whereas ≤90% identity is observed between the members belonging to distinct groups. The five groupings are as follows (Bonnet, 2004):

1. CTX-M-1 group includes six plasmid-mediated enzymes (CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15, and FEC-1) and the unpublished enzymes CTX-M-22, CTX-M-23, and CTX-M-28;
2. CTX-M-2 group includes eight plasmid-mediated CTX-M enzymes (CTX-M-2, CTX-M-4, CTX-M-4L, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20, and Toho-1);
3. CTX-M-8 group includes one plasmid-mediated member;
4. CTX-M-9 group includes nine plasmid-mediated enzymes (CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-19, CTX-M-21, CTX-M-27, and Toho-2) and other unpublished enzyme (CTX-M-24); and
5. CTX-M-25 group includes the CTX-M-25 and CTX-M-26 enzymes

OXA-Beta Lactamase Type: The OXA- β-lactamases are so named because of their oxacillin hydrolyzing abilities. These β-lactamases are characterized by their

ability to hydrolyze cloxacillin and oxacillin 50% more than benzyl penicillin. They predominantly occur in *Pseudomonas spp*, but have been detected in many other gram negative bacteria (Paterson and Bonomo, 2005).

Other Types. Other ESBL types include PER1-2, VEB-1-2, GES, SFO and IBC. PER type ESBL share only 25 to 27% homology with known TEM and SHV type ESBLs. This enzyme was first detected in pseudomonas and later in salmonella and acinetobacter. It has higher level resistance to ceftazidime, cefotaxime and aztreonam, which is reversed by clavulanic acid (MShana, 2011; Paterson and Bonomo, 2005).

2.6.2 ESBL Scientific Nomenclature

Beta-lactamase resistance mechanisms are described with a standardized scientific nomenclature. The terminology varies depending on whether the description is of the gene or the enzyme. In description of a gene e.g. *bla*$_{\text{CTX-M-15}}$, the prefix of '*bla'* refers to the gene function (beta-lactamase). The following sub-text e.g. 'CTX-M-15' refers to the sub-family 'CTX-M' and the specific variant '15'. Where the beta-lactamase is referred to as simply 'CTX-M-15' this is a reference to the enzyme rather than the gene. The sub-family naming comes from various original scientific descriptions. Within beta-lactamases it is a common practice to designate the sub-family based on the hydrolysis substrate of the enzyme and the geographical location where the gene was first identified. For instance CTX-M is a 'cefotaximase' from 'Munich'. Newly identified variants of the gene are submitted to a centralized repository for numerical classification e.g CTX-M-13, CTX-M-14 or CTX-M-15 (Rogers, 2014).

2.7 ESBL-producing *E. coli* in Animals and Food Products

Previous studies have reported the wide presence of extended-spectrum beta-lactamases (ESBLs) in bacteria recovered from a diversity of animals and food products in different countries. These include detection of CTX-M-1 and TEM-52 beta-lactamases in *Escherichia coli* strains from healthy pets (Costa et al., 2004) and wild animals (Costa et al, 2006) in Portugal; from food animals in Denmark (Olesen et al., 2004); from meat and meat products in Norway (Sunde and Norstrom, 2006); from diseased chickens and swine in China (Yang et al., 2004), among many others.

ESBL bacteria are frequently present in the gastro-intestinal tract of animals and have been isolated from swine, cattle, turkey, cats, dogs, broilers, wild animals and horses. The gastrointestinal tract of animals is seen as an important reservoir for bacteria that produce beta-lactamases, and a potential source for human pathogens to take up these resistance genes. ESBL are located on plasmids which enable them to spread very rapidly (Dierikx, 2013).

2.8 ESBL-producing *E. coli* in Vegetables

Recently, a study was conducted in Italy to investigate the occurrence of extended-spectrum β-lactamase (ESBL), AmpC, and carbapenemase-producing Gram-negative bacteria from 160 samples of fresh vegetables (*n*=80) and ready-to-eat (RTE) prepacked salads (*n* = 80). Results showed that resistance to β-lactam antibiotics was found in 44 (24 from fresh vegetables and 20 from RTE salads) of a total of 312 Gram-negative strains (14.1%). The prevalence of ESBL-producing strains from fresh vegetables was 83.3% (20/24) and 16.7% (4/24) for AmpC. Among the 20 bacterial isolates from RTE salads, 80% (16/20) were identified as ESBL-producing strains and the remaining 20% (4/20) as MBL-producing strains. The study suggests the possible public health risks associated with the consumption of these fresh vegetable products (Iseppi, et al., 2018).

2.9 Detection of ESBL

A variety of methods have been used to screen and confirm the presence of Extended Spectrum β–Lactamase. The Clinical and Laboratory Standards Institute (CLSI) recommended the disk diffusion methods for screening ESBL- producing *Escherichia coli* (Patel et al., 2014). Cefpodoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone can be used, and the use of more than one of these discs increases the sensitivity of detection (Murray et al., 1999). With any zone of diameter that may indicate suspicion of ESBL production, phenotypic confirmation should be done. Cefpodoxime 10μg has been found to be more sensitive than other cephalosporins for screening ESBL production, CLSI recommends, the isolate with zone diameter ≤17mm should be confirmed for ESBL production (Patel et al., 2014).

Disk Approximation Method (Double Disc Synergy). This is a simple and reliable method for detection of ESBL production. The disc that contains oxyimino β lactam (30μg) is placed 30mm apart (center-center) from amoxicillin/clavulanate disk (20/10μg) clear extension of the edge of the inhibition zone towards amoxicillin/clavulanate disk is interpreted as positive ESBL production. The sensitivity of the test can be increased by reducing the distance to 20mm (Murray et al., 1999, Patel et al., 2014). Three dimensional tests can also be used to confirm ESBL production (Menon et al., 2006). In this method the standard inoculum of test organisms is inoculated on Muller Hinton agar plate, a slit is cut on agar plate in which a broth suspension of test organism is placed. Antibiotic disc is placed 3-4mm from the slit (Menon et al., 2006). Distortion of circular inhibition zone is interpreted as positive ESBL production. This method is very sensitive in detecting ESBL production, but is more labor intensive than other methods (MShana, 2011).

Combined Disc Test (Inhibitor-Potentiated Disc Test). Cephalosporins discs (cefotaxime 30μg, ceftazidime 30μg, Cefpodoxime 30μg) with and without 10μg clavulanic acid are placed on Muller Hinton agar inoculated with test organisms (Carter et al., 2000, Patel, et al., 2014). An increase in the inhibition zone diameter of ≥ 5mm in cephalosporins disc combined with clavulanic acid, compared to cephalosporins alone, indicates ESBL production (Patel et al., 2014). MIC reduction test can also be used; an 8 fold reduction in the MIC of cephalosporin in the presence of clavulanic acid, using E Test or broth micro/macro dilution indicates ESBL production. There is commercially available E tests for ESBL detection; one side contains a gradient of cephalosporin (MIC 0.5-32μg/ml) and other side the same gradient with a constant concentration of 4μg/ml clavulanic acid (MShana, 2011).

BD Phoenix Automated Microbiology system. The phoenix ESBL test uses the growth response to cefpodoxime, ceftazidime and cefotaxime to detect ESBL production. VITEK ESBL Cards: Wells containing cards are inoculated, the reduction in growth of cephalosporins well contains clavulanic acid; when compared to with level of growth in well with cephalosporin alone indicates presence of ESBL production (Leverstein-van Hall et al., 2002).

Molecular Detection Methods. These include DNA probes, PCR, oligotyping, PCR-RFLPs and nucleotide sequencing. Molecular methods can detect different variants

of ESBL but they can be labor intensive and expensive to be adopted as routine methods (Karisiki, et al., 2006; MShana, 2011).

MALDI-TOF MS. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is emerging as a rapid, inexpensive, and accurate method for bacterial identification. It is a promising tool for the identification of bacteria. One study reported that MALDI-TOF MS had high detection performance for the ST131-whole, ST131-O25b, and ST405 clonal groups and should be considered as an alternative method to monitor the epidemiology of the ESBL-producing *E. coli* ST131 and ST405 clonal groups (Matsumura, et al., 2014).

Today, matrix-assisted laser desorption ionization–time of flight MALDI-TOF MS is adapted for use in microbiology laboratories, where it serves as a paradigm-shifting, rapid, and robust method for accurate microbial identification. Multiple instrument platforms, marketed by well-established manufacturers, are beginning to displace automated phenotypic identification instruments and in some cases genetic sequence-based identification practices. There is future for MALDI-TOF MS in the clinical microbiology laboratory to accelerate diagnosis and microbial identification (Clark, et al., 2013).

2.10 Prevalence of ESBL-producing *E. coli* in Pigs and Broilers

Since 2003, beta-lactam resistance has increased in broilers although its occurrence has been reported earlier. In Portugal, the first report of ESBLs from chickens and swine highlighted the antibiotic-resistant bacteria and/or resistance genes that might be acquired by humans through the food chain. ESBL-producing *Enterobacteriaceae* were identified in 60% of chicken carcasses, 10% of feces of healthy chicken and 5.7% of feces of healthy swine samples, mostly corresponding to *E. coli*. TEM-52, SHV-2 and CTX-M-1 were detected from chicken and SHV-12 from swine samples. High clonal diversity was observed and most *bla*ESBL genes were transferable (67%) (Machado et al., 2008).

The table below shows the summary of studies on the prevalence of ESBL-producing *E. coli* reported by various authors in different countries:

Table 2.8

Prevalence of ESBL-producing E. coli reported in other countries

Country	Animal	Prevalence	Reference
Netherlands	Broilers	15%	Dierikx et al., 2010
Sri Lanka	Broilers	50.6%	Mahalingam et al., 2015
Portugal	Broilers	60%	Machado et al., 2008
China	Broilers	88.8%	Li et al., 2016
Iran	Broilers	53%	Khoshbakht, et al., 2016
Romania	Broilers	69%	Maciuca, et al., 2015
Malaysia	Broilers	48.8%	Aliyu, et al., 2016
Thailand	Pigs	79.7%	Tablerk et al., 2015
Vietnam	Pigs	89%	Dang et al., 2018
Portugal	Pigs	49%	Friese et al., 2013
Germany	Pigs (Breeders)	56.3%	Friese et al., 2013
Germany	Pigs (Finishers)	43.8%	Friese et al., 2013
Thailand	Pigs	44.4%	Changkaew et al., 2015
China	Pigs	43.2%	Xu et al., 2015
Taiwan	Pigs	19.7%	Lee and Yeh 2017
South Korea	Pigs	4.98%	Shin et al., 2017

2.11 Risk Factors for ESBL-producing *E. coli* in Pigs and Broilers

The use of prophylactic antimicrobials has been shown to be a risk factor in the occurrence of ESBL-producing *E. coli.* in some studies (Dohmen et al., 2017; Cameron-Veas et al., 2015; Lugsomya et al., 2017).

In Netherlands, thirty six (36) Dutch conventional pig farms were studied during 2011-2013 to determine associations between the presence of ESBL-E. *coli*-positive pigs and farm management practices. They reported that the risk is strongly determined by the cephalosporin use at the farm (OR = 46.4, p = 0.006). Other farm management factors (e.g. presence of a hygiene lock, pest control delivered by a professional), related with improved biosecurity, were also plausibly related to lower probabilities for ESBL-E. *coli*-positive farms. Conclusively, ESBL-*E. coli* prevalence decreased in pigs during 2011 and 2013 in the Netherlands by having improved biosecurity (Dohmen et al., 2017).

In Thailand, a study examined the antimicrobial resistance (AMR) profiles in commensal *E. coli* derived from healthy fattening pigs that used prophylactic and therapeutic antimicrobials and pigs without usage of antimicrobials. Although there was a high level of multidrug resistance in all three categories of farm, the study revealed that the isolates with an extended-spectrum beta-lactamase phenotype (ESBLP) and with resistance to aminoglycosides, chloramphenicol, fluoroquinolones, nitrofurantoin, tiamulin, and trimethoprim/sulfamethoxazole were significantly more common among the farms that used prophylactic antimicrobials ($p < 0.05$) than the other farms. The routine use of prophylactic antimicrobials increased the resistance rates to other important antimicrobials (Lugsomya, et al., 2017)

In a longitudinal study of 27 Norwegian broiler farms including 182 broiler flocks, it was reported that the risk for occurrence of cephalosporin-resistant E. *coli* was associated with the status of the previous flock in the broiler house (OR=12.7), number of parent flocks supplying the broiler flock with day-old chickens (OR=6.3), routines for disinfection of floor between production cycles (OR=0.1), and transport personnel entering the room where the broilers are raised (OR=9.3). The study recommended the implementation of a high level of biosecurity with a minimal number of people entering the broiler house during production cycles, as well as rigorous cleaning and disinfection

routines between production cycles in order to decrease the occurrence of cephalosporin-resistant *E. coli* in broiler flocks (Mo et al., 2016). Cleaning and disinfection proves vital since contamination of broiler houses with ESBL-producing *E. coli* is an important risk factor (Hiroi, et al., 2012)

2.12 Antimicrobial Resistance Patterns

Phenotypic AMR Patterns. Extended-spectrum β-lactamase allows ESBL-producing *E. coli* to become resistant to a wide variety of penicillins and cephalosporins and through genetically linked resistance mechanisms, they are often resistant to other antibacterials including quinolones and aminoglycosides. Because of this, most ESBL-producing organisms become multidrug resistant and many are only susceptible to carbapenems (Hunter et al., 2010). Thus, the Centers for Disease Control and Prevention (CDC) has categorized the threat level of ESBL-producing *Enterobacteriaceae* as serious and which requires prompt and sustained action to ensure that the problem does not grow (CDC, 2013).

ESBLs are often encoded by genes located on large plasmids, and these also carry genes for resistance to other antimicrobial agents which explains multi-drug resistance in the isolates (Rawat and Nair, 2010). Fluroquinolone resistance is plasmid-mediated, mainly by Qnr proteins (Strahilevitz *et. al.*, 2009) while trimethoprim-sulfamethoxazole resistance *E. coli* often correlates with the presence of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes in integrons (White *et. al.*, 2001).

Genotypic AMR Patterns. For a long time, TEM- and SHV-types were the dominant ESBLs enzymes all over the world but this has changed dramatically. Nowadays, CTX-M-enzymes have become the most widespread type of ESBLs (Ewers *et. al.*, 2012; Canton et al., 2012; Canton et al., 2008). It is now present not only in humans, but also in food, food-producing animals, companion and wild animals, and in the environment (Canton et al., 2012). Recently, a study in Italy confirmed the presence of $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, in fresh vegetables and ready-to-eat salads in the market where consumption of these fresh products could pose public health risks (Iseppi, et al., 2018).

The table below presents the ESBL genes in *E. coli* isolated from fecal samples of broilers in various countries worldwide. Bacteria become resistant to beta-lactam antimicrobials by producing certain enzymes that inactivate these antimicrobials. The genes encoding these enzymes are located on mobile genetic elements, named plasmids. These plasmids and the associated resistance genes, can easily be exchanged between bacteria, causing spread between bacteria, not only between bacteria of the same species but also between different bacterial species **(**Dierikx, 2013).

Table 2.9

ESBL genes in E. coli isolated from fecal samples of broilers in various countries.

Continent	**Country**	**Year Isolated**	**ESBL Enzymes**	**References**
Asia	China	2000-2010	CTX-M-3, -14, -15, -24, -27, -55, -64, -65, -102, -104 TEM -52, SHV-12	Li et al., 2010; Ho et al., 2012; Zheng et al., 2012
	Japan	1999-2002, 2006, 2007	CTX-M-2, -14, -15, -18, SHV-2, -12	Hiroi et.al., 2011 Hiroi et.al., 2012
Europe	Spain	2000-2001, 2003	CTX-M-1, -9, -14, -32 TEM-52, SHV-2	Blanc et al., 2006 Brinas et al., 2003 Cortes et al., 2010
	Portugal	2004, 2005	CTX-M-14, -32, TEM-52	Costa et al., 2009 Machado et al., 2008
	France	2005	CTX-M-1	Girlich et al., 2007
	Italy	2007	CTX-M-1, -32 SHV-12	Bortolaia et al., 2010
	Belgium	2007	CTX-M-1, -2, -14, -15, TEM-52, -106	Smet et al., 2008
	Czech Republic	2008	CTX-M-14	Kolar et al., 2010
	Denmark	2010	SHV-2	DANMAP, 2010 DANMAP, 2011
	Sweden	2010, 2011	CTX-M-1	SVARM 2010 SVARM 2011

Table 2.9 (continued)

Continent	Country	Year Isolated	ESBL Enzymes	References
	UK	2010	CTX-M-1, -3, -15, TEM-52	Randall et al., 2011
	Switzerland	2009	CTX-M-1 TEM-52, SHV-12	Geser, 2012
Africa	Tunisia	2011	CTX-M-1	Ben et al., 2012

A study in Netherlands indicated that the mechanism by which bacteria develop resistance in broilers is similar to what is found in bacteria causing urinary tract infections in people. In addition, 94% of broiler meat purchased at different stores in the Netherlands contained similar ESBL-producing bacteria. One in five of ESBL-producing *E. coli* isolates from human infection is genetically related to isolates from broilers. The transfer via broiler meat to humans is considered a likely transmission route. Approximately 10% of people carry ESBL-producing bacteria in their intestines; for broiler farmers this percentage is 33% (Dierikx, 2013). In the same study, beta-lactamase genes were identified through PCR using the primers and product sizes presented in Table 2.10.

Table 2.10

Primers and product sizes for PCR used in identifying beta-lactamase genes (adapted from Dierikx, 2013).

Targets	Primer	Nucleotide sequence 5' - 3'	Size (bps)
TEM	TEM-F	GCG GAA CCC CTA TTT G	964
	TEM-R	ACC AAT GCT TAA TCA GTG AG	
CTX-M	CTX-M-F	ATG TGC AGY ACC AGT AAR GTK ATG GC	59
	CTX-M-R	TGG GTR AAR TAR GTS ACC AGA AYS AGC GG	
CTX-M-1	CTX-M-1-F	GGT TAA AAA ATC ACT GCG TC	863
	CTX-M-1-R	TTG GTG ACG ATT TTA GCC GC	
CTX-M-2	CTX-M-2a-F	GAT GAG ACC TTC CGT CTG GA	397
	CTX-M-2a-R	CAG AAA CCG TGG GTT ACG AT	
SHV	SHV-F	TTA TCT CCC TGT TAG CCA CC	795
	SHV-R	GAT TTG CTG ATT TCG CTC GG	

There are major gaps in surveillance and sharing of data on resistant bacteria that are transmitted through the food chain. Surveillance in food-producing animals, as for surveillance in humans, is hampered by lack of harmonized global standards and platforms for data sharing. A multi-sectoral approach is needed to contain AMR in food-producing animals and the food chain. The tripartite collaboration between WHO, FAO and OIE, in the spirit of the 'One Health' approach, provides a coordinating platform for work in this area (WHO, 2014).

2.13 Action Plan to Combat Antimicrobial Resistance

Resistance to antibiotics has recently increased dramatically worldwide. The pipeline of new classes of antibiotics is dry for at least the next few years. Therefore antibiotic resistance represents one of the most problematic public health issues of our time. Treatment failures already happen in increasing numbers for common community-acquired infections, such as urinary tract infections or intra-abdominal infections. This is due in particular to *Enterobacteriaceae* harboring extended-spectrum beta-lactamases (ESBL). *Enterobacteriaceae* harboring carbapenemases are also highly prevalent in many countries. In the future, difficult surgical procedures, transplants, and other immunosuppressive therapies may become very risky. Resistance is mainly due to an

excessive usage of antibiotics, in both humans and animals, and to cross-transmission of resistant bacteria. Action is urgently needed and thus, the World Alliance Against Antibiotic Resistance (WAAAR) was created in 2011 (Carlet et al., 2014).

Prevalence of MRSA infections decreased in the last few years in many European countries, and this can be considered as a very positive and promising result. Vancomycin-resistant enterococci (VRE) are also very frequent, with large differences between countries. However, the prevalence of *Escherichia coli* and *Klebsiella pneumoniae* harboring extended-spectrum beta lactamases (ESBL) is increasing regularly worldwide reaching 50 to 70% for *Escherichia coli* in some European or Asian countries (Lowe, et al., 2012).

The World Health Organization (WHO) has long recognized AMR as a growing global health threat, and the World Health Assembly, through several resolutions over two decades, has called upon Member States and the international community to take measures to curtail the emergence and spread of AMR (WHO, 2012). During the 2011 World Health Assembly, the WHO endorsed the 6-point policy package as part of its Global Action on AMR. It contains the following key strategies adhering to the One Health Approach:

1. Committing to a comprehensive, financed national plan with accountability and civic society engagement
2. Strengthening of surveillance and laboratory capacity
3. Ensuring the uninterrupted access to essential medicines of assured quality
4. Regulation and promotion of rational use of medicines, including in animal husbandry, and ensuring proper patient care
5. Enhancing infection prevention and control
6. Fostering innovations and research and development for new tools

In the Philippines, the government has successfully formulated an action plan named as “Philippine Action Plan to Combat AMR: One Health Approach”. This was officially launched as Philippine AMR Summit last November 2015 that brought together all key partners across many sectors to showcase their contribution and plans to mitigate and control AMR. Outputs from this research will surely enrich information to this action

plan where policies will be developed with the purpose of promoting responsible use of antimicrobial products. This serves as the country road map towards containing, controlling and preventing AMR which provides an intervention strategy in order to facilitate the mechanisms of combating the growing problem of AMR as one nation through political commitment and leadership, institutionalizing integrated surveillance systems, regulating access to quality antimicrobials, rational use of antimicrobials, establishing measures to prevent and control further spread of AMR, and strengthening research and development initiatives. This comprehensive plan emphasizes the **"One Health Approach"** as it recognizes that the causation of AMR is inter-related and inter-sectoral thereby requiring collaborative multidisciplinary work at local, national, and global levels to attain optimal health for humans, animals and the environment (DOH, 2015).

With a vision of “A nation protected against the threats of antimicrobial resistance”, the action plan has seven key strategies:

Key Strategy 1	Commit to a comprehensive, financed national plan with accountability and civic society engagement
Key Strategy 2	Strengthen surveillance and laboratory capacity
Key Strategy 3	Ensure uninterrupted access to essential medicines of assured quality
Key Strategy 4	Regulate and promote rational use of medicines, including in animal husbandry and ensure proper patient care
Key Strategy 5	Enhance infection prevention and control across all settings
Key Strategy 6	Foster innovations, research, and development
Key Strategy 7	Development of a Risk Communication Plan to combat AMR

CHAPTER 3

MATERIALS AND METHODS

3.1 The Study and Its Reference Population

A cross-sectional study was conducted from March to October 2017 to determine the prevalence of ESBL-producing *E. coli* in commercial broiler and swine farms in the Central Luzon Region, to determine the risk factors associated with its detection and to establish the phenotypic and genotypic antimicrobial resistance patterns. All broiler and swine population in commercial farms in Central Luzon region were considered as the reference population in this study.

3.2 Sampling Strategy and Sample Size Determination

Table 3.1 presents the estimated number of commercial broiler and swine farms in the highest producing provinces of Central Luzon (Figure 3.1) and served as the study population. The number of broilers and pigs in these four provinces comprise almost 78.80% (out of 19,143,421 birds) and 96.8% (out of 1,678,199 heads) of the total broiler and pig population in the entire region, respectively, based on the inventory of commercial pigs in Central Luzon, by province, in January 2016 (PSA, 2016).

From the four provinces, a sampling frame of all commercial broiler and swine farms were constructed. The information on the number of existing farms was obtained from the Provincial Veterinary Offices of each province. From the sampling frame, representative samples of commercial farms were chosen based on at least two eligibility criteria such as willingness to participate in the study and accessibility of the farm. Each selected farm was contacted for the collection of samples and interview using a prepared survey questionnaire (see Appendix).

The sample size was calculated using the software WinEpiscope using the following assumptions: 80% prevalence for swine farms (based on the previous study of

Tablerk et al., 2015 in Thailand), 50% prevalence for broiler farms (based on the previous study of Mahalingam et al., 2015 in Sri Lanka), 10% accepted error and 95% level of confidence. The reported prevalence for both swine and broiler farms from other countries were used as assumptions in sample size calculations because of the absence of any report on previous prevalence in the Philippines during the time of the study. Using the information on the number of existing swine and broiler farms obtained from the Provincial Veterinary Office of each province (Table 3.1), and the aforementioned assumptions, the calculated sample sizes were78 for broiler farms and 54 for swine farms. Utilizing the probability proportional to size sampling, the number of farms that were included and randomly selected in each province is presented in Table 3.2

Table 3.1

Number of commercial broiler and swine farms in selected provinces of Central Luzon.

Provinces	No. Broiler Farms	No. Swine Farms
Bulacan	44	196
Nueva Ecija	197	113
Pampanga	101	37
Tarlac	49	60
TOTAL	**391**	**406**

Source: Provincial Veterinary Offices, 2016

Table 3.2

Number of farms in selected provinces of Central Luzon that were included in the study based on the sample size calculation.

Provinces	No. Broiler Farms	No. Swine Farms
Bulacan	9	26
Nueva Ecija	39	15
Pampanga	20	5
Tarlac	10	8
TOTAL	**78**	**54**

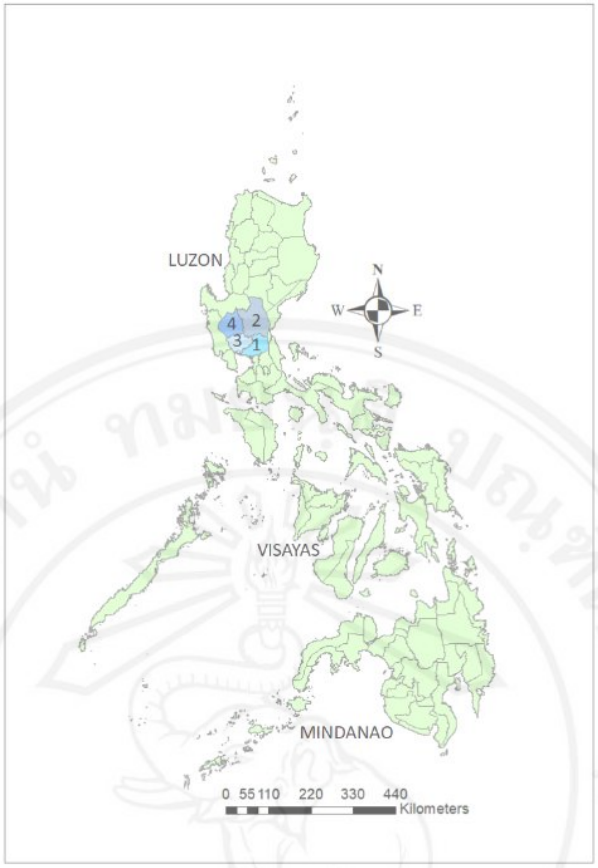


Figure 3.1 Map of the Philippines showing the location of the study provinces (1= Bulacan, 2 = Nueva Ecija, 3 = Pampanga, 4 = Tarlac)

3.3 Collection of Samples

Broilers. For each selected broiler farm, fresh fecal samples were collected using sterile cotton swabs directly from cloaca of ten (10) randomly selected birds following the method described in a previous study (Mahalingam et al., 2015) with some modifications. The cloacal swab samples in each farm were pooled in two Falcon tubes each containing 25 ml Luria-Bertani (LB) broth (Merck, Darmstadt, Germany). Five cloacal swabs were pooled in each tube following the recommendation that five swabs per vial should be used as a maximum recommended number (Spackman, 2013). At the same time, a paired boot swab samples were obtained. This was done by wearing a sterile shoe cover pre-moistened with sterile normal saline and by walking along the whole length of the inside area of the broiler house. Boot swabs were carefully removed so as not to dislodge the adherent material and kept inverted to retain the attached material. The samples were properly labeled for identification, placed in sterile plastic bags, then

transferred later to 500ml beaker containing 250ml of LB broth for enrichment following the procedure described in previous study (Laube et al., 2014).

Samples were kept in cool boxes with ice packs to keep contents cool and protected against external contamination during transport. The samples were then transported to the laboratory and microbiological analysis were performed within 12 hours after sample collection. At the laboratory, all samples were incubated aerobically at 37^{o}C for 24 hours.

Swine. For each chosen swine farm, separate samples of ten samples each from both groups of breeders and finishers were collected randomly. Around 2.5 grams of fresh feces were collected directly from the rectum of each animal and were pooled using sterile plastic bags following the procedure described in previous studies (Laube et al., 2014; Tablerk et al., 2015). Moreover, one pair of boot swab samples was also collected from each swine farm. This was done by wearing a sterile shoe cover pre-moistened with sterile normal saline and by walking along the whole length of the inside passageway area of the pig house measuring approximately 1.2m width and 30-40m length depending on the size of the pig house. The boot swab samples were properly labeled and placed in sterile plastic bags. Samples were then kept in cool boxes with ice packs and transported to the laboratory for microbiological analysis within 12 hours after sample collection.

At the laboratory, twenty five (25) grams of pooled feces each from 10 breeder pigs and 10 finisher pigs were prepared and placed in a 250ml Erlenmeyer flask and then enriched by adding 225 ml of Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) and mixed properly. Similarly, boot swab samples were transferred to a 500ml beaker containing 250ml of LB broth for enrichment. Both enriched fecal and boot swab samples were incubated aerobically at 37^{o}C for 24 hours.

Overall, a total of 318 samples were collected. There were 156 samples collected from broiler farms (78 pooled cloacal swabs and 78 boot swabs) and 162 samples collected from swine farms (54 pooled fecal samples from finishers, 54 pooled fecal samples from breeders and 54 boot swabs).

3.4 Farm Interview

Immediately after collection of samples from each farm, personal interviews using a prepared and pre-tested structured questionnaire (see Appendix) were conducted from key persons (either farm owner or farm manager) in all selected broiler and swine farms regarding their farm production and farm practices and to determine the possible risk factors associated with the occurrence of antimicrobial resistance to ESBL-producing *E. coli*. Questions that were asked included farm information such as population inventory, housing, breeds, other farm characteristics and risk factor information which included a range of possible risk factors such as length of farming, sources of stocks, feed source, vaccination history, farm management practices, biosecurity status, antimicrobial usage, disease management, knowledge of antimicrobial resistance. The information given by the key persons were validated by asking the farm veterinarian. All information obtained during the interview were recorded carefully.

3.5 Bacterial Isolation and Confirmation

The procedure for the isolation of *E. coli* is presented in Figure 3.2 following the method described in a previous study (Padilla and Amatorio, 2017). Briefly, from fecal and boot swab samples enriched with LB broth and incubated aerobically at 37^{o}C for 24 hours, a loopful (10µl) of each sample was streaked onto MacConkey agar plate (Oxoid, United Kingdom) supplemented with 1mg/L cefotaxime (AppliChem GmbH, D-64291Darmstadt, Germany) and incubated aerobically at 37˚C for 24 hours. Subsequently, two bright pink colonies, suggestive of lactose-fermenting bacteria and morphologically indicative of ESBL *E. coli*, were picked and streaked in a selective and differential medium, Eosin Methylene Blue agar plate (HiMedia, Mumbai, India) also supplemented with 1mg/L cefotaxime. The plates were then incubated at 37˚C for 24 hours. Thereafter, a single isolated colony with characteristic metallic green sheen from EMB agar was picked and inoculated in Nutrient agar, incubated aerobically at 37˚C for 24 hours, to obtain a pure culture.

Pure cultures were preserved using 20% glycerol in Mueller-Hinton Broth and stored at -80^{0}C freezer for later confirmatory testing using Combined Disc Test, Vitek 2 Compact and PCR assay for molecular characterization. For all preserved isolates, the

recovery was done using Nutrient agar. The automated bacterial ESBL-*E. coli* identification and confirmatory testing using Vitek 2 Compact equipment was done at the National Meat Inspection Service (NMIS) Microbiology Laboratory. Molecular characterization was done at the Molecular Biology Laboratory of the College of Veterinary Science and Medicine at Central Luzon State University.

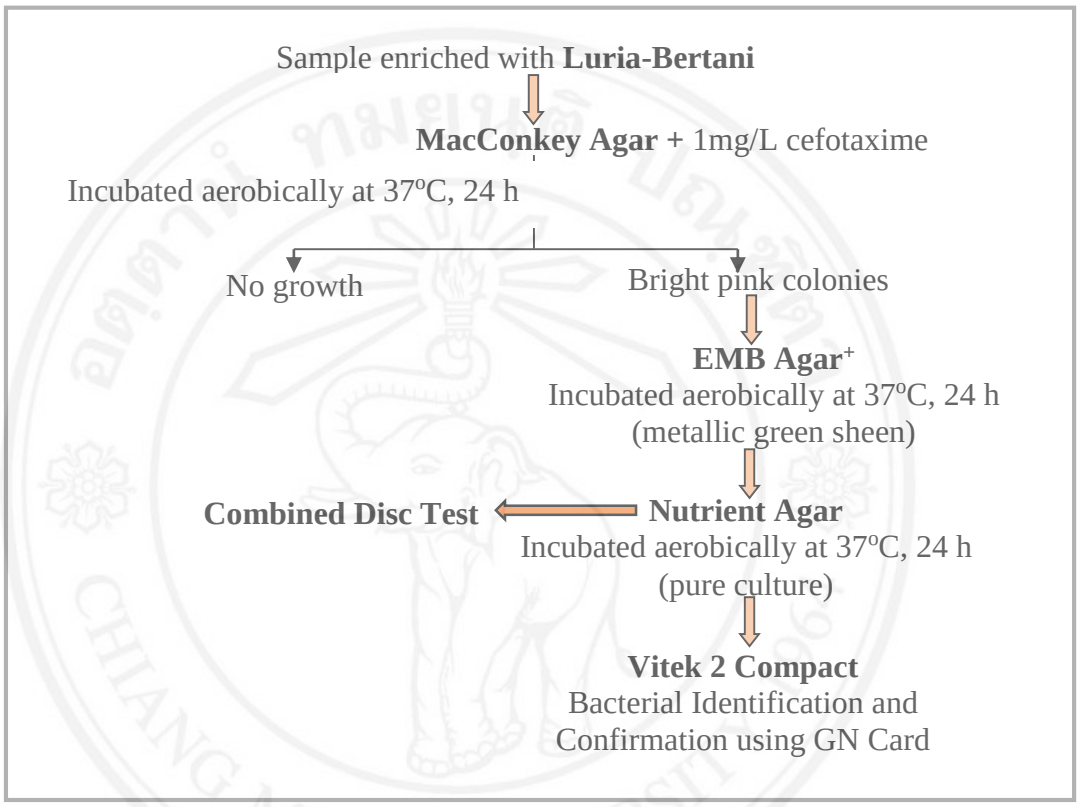


Figure 3.2. Schematic diagram for the isolation, identification and confirmatory testing of ESBL-producing *E. coli*.

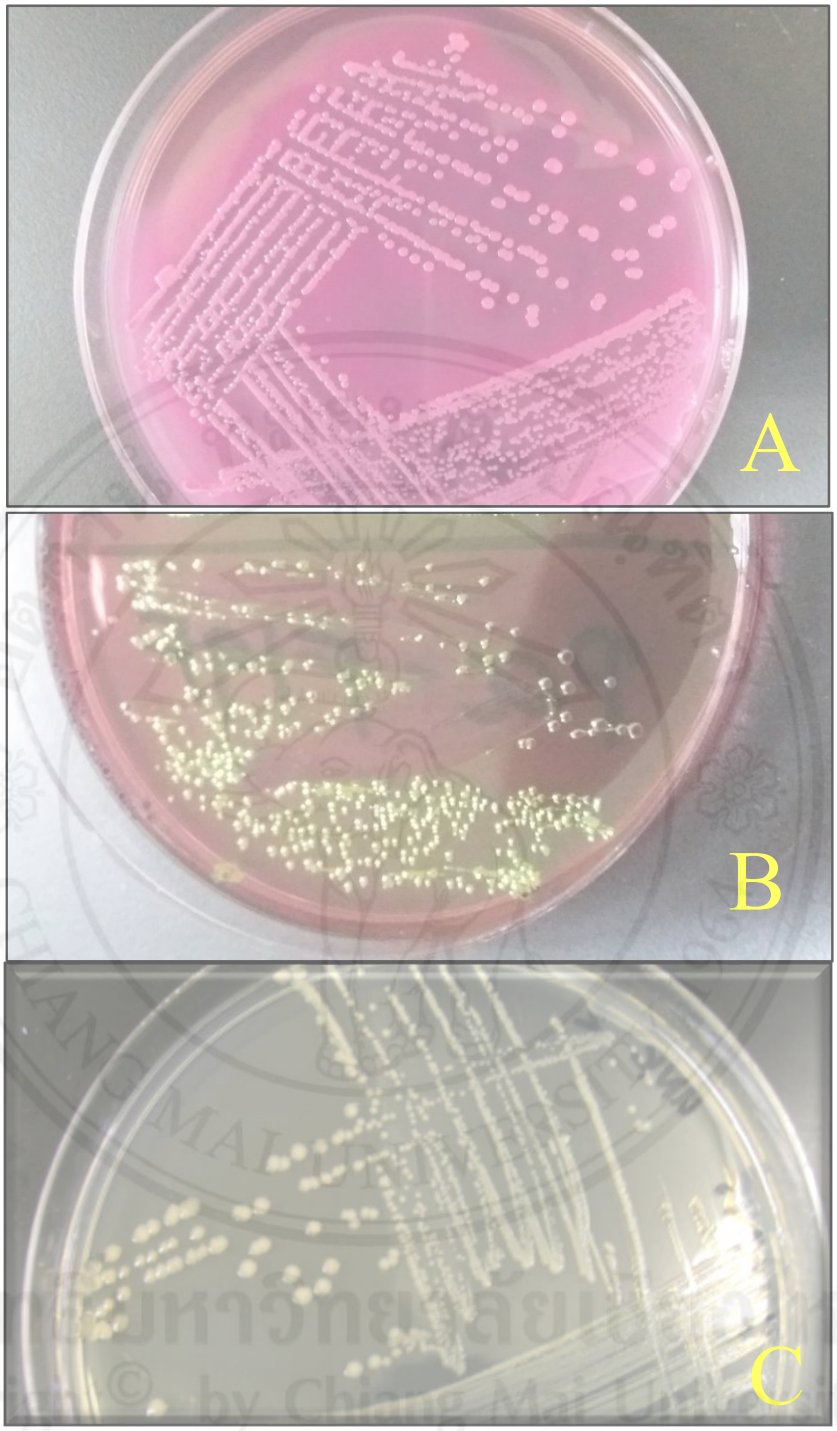


Figure 3.3. Typical colonies of ESBL-producing *E. coli* isolates in (A) MacConkey agar plate (bright pink colonies), (B) EMB agar plate (metallic green sheen) and (C) Nutrient agar plate (pure culture).

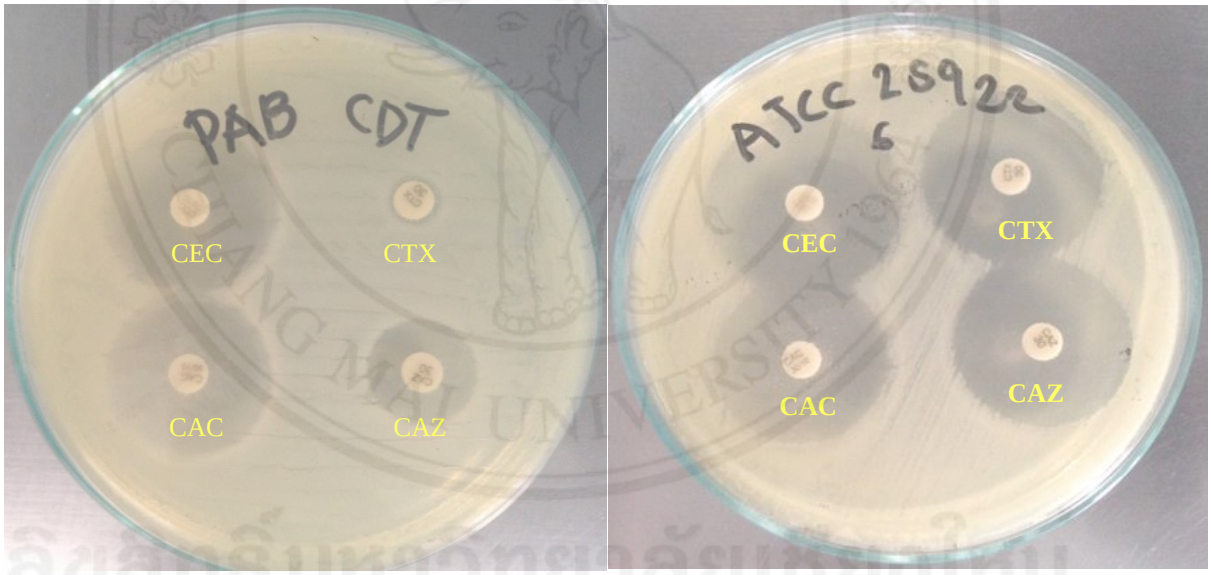
3.6 Bacterial Identification and Confirmatory Testing using Vitek 2 Compact

Bacterial identification and confirmatory testing were performed through Vitek 2 Compact (bioMérieux, Craponne, France), an automated microbiology system utilizing growth-based technology, using GN card (Figure 3.4). Fresh pure culture of the organism was prepared in Nutrient agar plate. After overnight incubation, a sterile swab was used to transfer sufficient number of colonies of a pure culture of *E. coli* in a glass tube containing 3ml of sterile 0.45% saline. The turbidity was adjusted to 0.50-0.63 McFarland using DensiChek™. The test tube containing the microorganism suspension was placed into the GN cassette and the identification card is placed in the neighbouring slot while inserting the transfer tube into the corresponding suspension tube. For quality control, *E. coli* ATCC 25922 was used. The filled cassette was placed manually into a vacuum chamber station. The card is sealed and incubated automatically at 35.5 ± 1.0^{0}C. The GN card is based on established biochemical methods (utilizing 47 biochemical tests) and newly developed substrates similar to Analytic Profile Index (API®) kit phenotypic identification system. The final identification results became available in approximately 10 hours or less (Pincus, 2010).

Figure 3.4. Loading of GN card and AST card in Vitek 2 Compact machine for bacterial identification and ESBL detection and antimicrobial profiling.

3.7 Phenotypic Confirmatory Testing using Combined Disc Test (CDT)

Aside from Vitek 2 identification of ESBL-producing *E. coli*, phenotypic confirmatory testing was done using CDT to confirm ESBL production. Both ceftazidime (30 µg) and cefotaxime (30 µg) alone and in combination with 10µg clavulanic acid were tested through disc diffusion. The zone of Inhibition (ZOI) diameters were recorded and interpreted accordingly. A ≥ 5mm increase in the zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirmed the presence of an ESBL. For quality control, *E. coli* ATCC 25922 was used in both Vitek 2 Compact and Combined Disc Test for the screening and confirmatory testing of ESBL-producing *E. coli* (Figure 3.5). Table 3.3 outlines the screening and confirmatory tests that were employed for ESBL-producing *E. coli* as recommended by the Clinical and Laboratory Standards Institute (Patel et al., 2014).



ESBL-Positive Sample	**Negative Control**
Cefotaxime (CTX)=8.09; Cefotax/Clav (CEC) = 27.64 Ceftazidime(CAZ)=16.93; Ceftaz/Clav (CAC) =25.71 *Interpretation: A ≥ 5-mm increase in zone diameter is considered ESBL+*	Cefotaxime (CTX)=30.96; Cefotax/Clav (CEC)=32.74 Ceftazidime(CAZ)=28.13; Ceftaz/Clav (CAC)=28.64 *Interpretation: A ≤ 2-mm increase in zone diameter is considered ESBL-*

Figure 3.5. Phenotypic confirmatory testing of ESBL-producing *E. coli* through Combined Disc Test.

Table 3.3

Screening and confirmatory tests for ESBL-producing E. coli (as recommended by Clinical and Laboratory Standards Institute).

Test	Initial Screen Test	Phenotypic Confirmatory Test
Test method	Disk diffusion	Disk diffusion
Medium	Mueller Hinton Agar	Mueller Hinton Agar
Antimicrobial concentration	Ceftazidime30 μg or Cefotaxime30 μg	Ceftazidime30 μg Ceftazidime-clavulanate 30/10 μg and Cefotaxime30 μg Cefotaxime-clavulanate 30/10 μg (Confirmatory testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulanate)
Inoculum	Standard disk diffusion procedure	Standard disk diffusion Procedure
Incubation conditions	35 ± 2 °C; ambient air	35 ± 2 °C; ambient air
Incubation length	16–18 hours	16–18 hours
Results	Ceftazidime zone≤22mm Cefotaxime zone≤27mm Zones above may indicate ESBL production.	A ≥ 5-mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanate vs the zone diameter of the agent when tested alone=ESBL (eg, ceftazidime zone=16; ceftazidime-clavulanate zone=21).
Quality Control recommendations	*E. coli* ATCC 25922: Ceftazidime (25-32mm) Cefotaxime (29-35mm)	*E. coli* ATCC 25922: ≤ 2-mm increase in zone diameter for antimicrobial agent tested in combination with clavulanate vs the zone diameter when tested alone.

3.8 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility test was also performed through Vitek 2 Compact (bioMérieux, Craponne, France), using AST-N261card. The susceptibility of ESBL-producing *E. coli* to eight classes of antibiotics (Penicillins, Beta Lactam-Beta Lactamase Inhibitor Combination, Cephems, Carbapenems, Aminoglycosides, Lipopeptides, Flouroquinolones, and Folate Pathway Inhibitors) or to a total of seventeen (17) different antimicrobial agents was tested (Table 3.4). In the same table, the interpretation of the results was based on the minimum inhibitory concentration interpretive standards published in the Clinical and Laboratory Standards Institute. For quality control, *E. coli* ATCC 25922 was also used in the susceptibility testing.

Table 3.4

Minimal Inhibitory Concentration (MIC) Interpretive Standards for Enterobacteriaceae

Antibiotics	Concentration mg/ml	Qlty Control *E. coli* ATCC 25922	MIC Interpretive Criteria (mg/ml)		
			Susceptible	Intermediate	Resistant
Penicillins					
Ampicillin (AM)	4, 8, 32	≤2 – 8	≤8	16	≥32
Beta Lactam-Beta Lactamase Inhibitor Combination					
Amoxicillin/Clav Acid (AMC)	4/2, 16/8, 32/16	≤2/1 – 8/4	≤8/4	16/8	≥32/16
Piperacillin/Tazobactam (TZP)	2/4, 8/4, 24/4, 32/4, 32/8, 48/8	≤4/4	≤16/4	32/4–64/4	≥128/4
Cephems					
Cefuroxime (CXM)	2, 8, 32	2 – 8	≤8	16	≥32
Cefuroxime Axetil (CXMA)	2, 8, 32	2 – 8	≤8	16	≥32
Cefoxitin (FOX)	8, 16, 32	≤4 – 8	≤8	16	≥32
Ceftazidime (CAZ)	1, 2, 8, 32	≤1	≤4	8	≥16
Ceftriaxone (CRO)	1, 2, 8, 32	≤1	≤1	2	≥4
Cefepime (FEP)	2, 8, 16, 32	≤1	≤2	4–8	≥16
Carbapenems					
Ertapenem (ETP)	0.5, 1, 6	≤0.5	≤0.5	1	≥2
Imipenem (IPM)	1, 2, 6, 12	≤0.25	≤1	2	≥4
Meropenem (MEM)	0.5, 2, 6, 12	≤0.25	≤1	2	≥4

Table 3.4 (continued)

Antibiotics	Concentration mg/ml	Qlty Control *E. coli* ATCC 25922	MIC Interpretive Criteria (mg/ml)		
			Susceptible	Intermediate	Resistant
Aminoglycosides					
Amikacin (AN)	8, 16, 64	≤2 – 4	≤16	32	≥64
Gentamicin (GM)	4, 16, 32	≤1	≤4	8	≥16
Flouroquinolones					
Ciprofloxacin (CIP)	0.5, 2, 4	≤0.25	≤1	2	≥4
Lipopeptides					
Colistin (CS)	4, 16, 32	≤0.5 – 1	≤2	-	≥4
Folate Pathway Inhibitors					
Trimethoprim/Sulfamethoxazole (SXT)	1/19, 4/76, 16/304	≤20 (1/19)	≤2/38	-	≥4/76

3.9 DNA Extraction

Bacterial isolates from plates were cultured each in 5-mL broth media in culture tubes for 24 h at 37°C with agitation at 100 rpm. The next day, cell suspensions were transferred in 1.5-mL microcentrifuge tubes and centrifuged at 12,000 rpm for 5 min at 4°C, and the pellets obtained were resuspended in 100µL of 100 mM Tris-Cl pH 8.0 and mixed by tapping and then the pellet was collected using the microcentrifuge at 14,000 rpm for 10 min. The supernatant was decanted and the pellets were resuspended in 50 µl of 100 mM Tris-Cl to be subjected to boiling at 100°C in a heatblock for 10 min. It was then cooled in crushed ice, centrifuged at 1000 rpm for 5 minutes, and stored at -20°C until PCR amplification. To determine DNA concentration, total extracted DNA was quantified using UV/VIS spectrophotometer, at 260/280 wavelength (Implen, Nanophotometer) . Aliquots of 1 µl of template DNA were used for PCR.

3.10 PCR Assay and Gene Detection

PCR amplifications were carried out using the optimized conditions from published studies (Table 3.5). Nine (9) primers were used to detect ESBL-producing *E. coli* resistance genes. One additional primer was included to detect colistin resistance gene. All isolates were screened for target genes. The PCR assay was performed in BioRad T100 thermal cycler (BioRad, Herts, United Kingdom) individually for each primer set according to the following amplification conditions: initial denaturation at 95^0C for 3 min, 35 cycles of denaturation at 94^0C for 1 min, and optimized annealing temperature for each primer set (Table 3.5). Elongation was set at 72^0C for 1 min with final elongation at 72^0C for 7 minutes. One microliter of *E. coli* DNA lysate was used as template for the PCR reaction mixture containing 0.5U DNA taq polymerase, 1x PCR buffer, 2 Mm $MgCl_2$, 1mM dNTP, 1 uM each of primer pair. A mixture of 3µl of PCR products and 2µl of loading buffer was loaded in 1.5% agarose gel and separated through electrophoresis using 0.5x TBE buffer to determine the molecular size of the amplified products per target gene. *E. coli* strains of ATCC 25922 and ATCC 35218 (β-lactamase-producing strain) (Microbiologics, Minnesota, USA) were used as negative and positive controls in the PCR Assay, respectively.

Table 3.5

Primers used to detect ESBL-producing E. coli resistance genes in broiler and swine farm isolates.

Target gene	Primer	Sequence (5 → 3)	Anneal Temp (^{0}C)	Size (bp)	Reference
bla$_{\text{CTX-M}}$	CTX-M-F	ATGTGCAGYACCAGTAARGTKATGGC	55	592	(Moubareck et al., 2005)
	CTX-M-R	TGGGTRAARTARGTSACCAGAAYSAGCGG			
bla$_{\text{CTX-M-1group}}$	CTX-M-1-F	GGTTAAAAAATCACTGCGTC	50	873	(Moubareck et al., 2005)
	CTX-M-1-R	TTACAAACCGTYGGTGACGA			
bla$_{\text{CTX-M-15}}$	CTX-M-15-F	CACACGTGGAATTTAGGGACT	50	995	(Muzaheed et al., 2008)
	CTX-M-15-R	GCCGTCTAAGGCGATAAACA			
bla$_{\text{CTX-M-2group}}$	CTX-M-2-F	ATGATGACTCAGAGCATTCGCCGC	56	876	(Celenza et al., 2006)
	CTX-M-2-R	TCAGAAACCGTGGGTTACGATTTT			
bla$_{\text{CTX-M-8group}}$	CTX-M-8-F	TGATGAGACATCGCGTTAAG	52	666	(Jouini et al., 2007)
	CTX-M-8-R	TAACCGTCGGTGACGATTTT			
bla$_{\text{CTX-M-9group}}$	CTX-M-9-F	GTGACAAAGAGAGTGCAACGG	55	856	(Sabaté et al., 2000)
	CTX-M-9-R	ATGATTCTCGCCGCTGAAGCC			
bla$_{\text{CTX-M-25group}}$	CTX-M-25-F	GCACGATGACATTCGGG	52	327	(Woodford et al., 2005)
	CTX-M-25-R	AACCCACGATGTGGGTAGC			
bla$_{\text{TEM}}$	TEM-F	TTGGGTGCACGAGTGGGTTA	55	506	(Melano 2003)
	TEM-R	TAATTGTTGCCGGGAAGCTA			
bla$_{\text{SHV}}$	SHV-F	TCGGGCCGCGTAGGCATGAT	52	628	(Melano 2003)
	SHV-R	AGCAGGGCGACAATCCCGCG			
mcr-1*	CLR5-F CLR5-R	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTA GGG	58	305	Vila et al., 2015

* Target gene for colistin resistance

3.11 DNA Sequencing and Analysis

Purified PCR products from few representative isolates were sent to ASIAGEL Corporation (Quezon City, Philippines) for DNA sequencing analysis to confirm the target genes. Matches were analysed using Basic Local Alignment Search Tool (BLAST) and the phylogenetic tree analysis was done using Molecular Evolution Genetic Analysis (MEGA) Version 7.

3.12 Data Management

All information obtained in this study were recorded and numerically coded and were entered into appropriate files using MS Excel. A coding manual was devised for the recoding of data particularly the risk factor information. Data were prepared for 2x2 table analysis. The continuous independent variables such as broiler population, age of birds during collection of samples, length of broiler farming and others were dichotomized using median as cutpoint. Table 3.6 and Table 3.7 present the definitions of variables used in the risk factor analysis for broiler and swine, respectively.

Table 3.6

Description of independent variables for the risk factor analysis in broilers.

Variable	Definition	Code
Population	Broiler population during the time of visit at the farm	0 = <38000 1 = ≥38000
Age	Age of birds during the collection of samples	0 = <24 days 1 = ≥24 days
Housing	Type of housing	0 = Tunnel vent 1 = Conventional
Farming	Length of broiler farming in years	0 = ≥ 6 years 1 = < 6 years
Feeds	Source of feeds	0 = Company source 1 = Commercial source
Source	Source of broiler chicks	0 = Company source 1 = Commercial source
Training	Attended a training program on broiler production	0 = No 1 = Yes
Harvest	Age of broilers at harvest	0 = 30-35days 1 = 36 days and above
Cycles	Number of growing cycles per year	0 = 3-5 cycles 1= 6-8 cycles
Animals	Presence of other animals in the farm	0 = No 1 = Yes

Table 3.6 (continued)

Variable	Definition	Code
Disinfection	Disinfection upon entry of visitors and vehicles at the farm	0 = No 1 = Yes
Antibiotics	Source of antibiotics being used in the farm	0 = Company source 1 = Commercial source
Diarrhea[1]	Occurrence of diarrhea among broilers in the farm during the days surrounding the visit	0 = No 1 = Yes
Migrate	Presence of migrating birds in the farm	0 = No 1 = Yes
AMR	Awareness about antimicrobial resistance	0 = No 1 = Yes
Overuse	Awareness on adverse effect of antibiotic overuse	0 = No 1 = Yes

[1] Diarrhea is defined as a change in the consistency of droppings from the normal firm and brown to watery, mucoid and sometimes colored (whitish, yellowish, greenish) feces. All responses regarding the occurrence of diarrhea was validated through the farm veterinarian.

Table 3.7

Description of independent variables for the risk factor analysis in swine.

Variable	Definition	Code
Sow Level	Pig population in the farm in terms of sow level	0 = ≤150 sows 1 = >150 sows
Housing	Type of housing	0 = Tunnel vent 1 = Conventional
Farming	Length of swine farming in years	0 = ≤19 years 1= >19 years
Training	Attended a training program on pig production	0 = No 1 = Yes
Stocks	Purchase of stocks in the farm	0 = No 1 = Yes

Table 3.7 (continued)

Variable	Definition	Code
Feeds	Type of feeds used in the farm	0 = Commercial 1 = Own Mix
Vaccine	Vaccination of swine in the farm	0 = No 1 = Yes
E. coli	Vaccination of animals against *E. coli* (ETEC) vaccine	0 = No 1 = Yes
Vet	Employment of veterinarian in the farm	0 = No 1 = Yes
Grow	Number of growing months for pigs in the farm	0 = 4-5 months 1 = 6-7 months
Animals	Presence of other animals in the farm	0 = No 1 = Yes
Disinfection	Disinfection of vehicles and visitors upon farm entry	0 = No 1 = Yes
Promotants	Use of growth promotants in feeds in the farm	0 = No 1 = Yes
Antibiotics	Source of antibiotics supply in the farm	0 = Direct purchase 1 = From farm vet
Diarrhea	Frequency of diarrhea observed in the farm during the days surrounding the visit	0 = Sometimes 1 = Often
Duration	Usual duration of antibiotic treatment	0 = 1-4 days 1 = 5-7 days
Rotation	Practice of rotational use of antibiotics	0 = No 1 = Yes
AMR	Awareness about antimicrobial resistance	0 = No 1 = Yes
Adverse	Awareness on adverse effect of antibiotic overuse	0 = No 1 = Yes
Usage	Usage of the same antibiotics for every batch of pigs	0 = No 1 = Yes

3.13 Statistical Analysis

The data were analyzed using Stata version 13 (StataCorp LP, College Station, Texas 77845 USA) and Statistix version 10 (Analytical Software, Tallahassee Florida, USA). Descriptive statistics, including frequency, mean, standard deviation, minimum and maximum were done to describe the general information on the farms. Farm prevalence was calculated as the number of farms with at least one sample being positive for ESBL-producing *E. coli*, either from pooled fecal sample or boot swab sample, over the total number of farms studied. Odds ratios were also calculated. The 95% confidence intervals were determined using exact binomial confidence limits for the proportion with a significance level (alpha) of 0.05, to test for the difference in proportions.

Risk factor analysis was done in two stages. Initially, univariate analysis was done for each independent variable to calculate crude odds ratios with 95% confidence intervals to determine any association between the risk factors and the occurrence of ESBL-producing *E. coli* in the farms. In the next stage, independent variables that were significantly associated at $p<0.15$ from the univariate analysis were included in the subsequent statistical modelling. Stepwise logistic regression analysis was done using both forward and backward selection. In case of collinearity of variables, the one with higher biological plausibility was retained for multivariate analysis. Model best fit was assessed by testing the relationship between each of the independent variables and the outcome and by selecting the statistically significant variable ($P<0.05$). Odds ratios were obtained to quantify the relative importance of the different risk factors in the model.

CHAPTER 4

RESULTS

4.1 Description of Farm Production and Husbandry Practices

The number of farms included in the study is presented in Table 4.1. Farm questionnaires were used to document the production and husbandry practices, health status, and antimicrobial usage in all broiler and swine farms visited.

Table 4.1

Number of farms in selected provinces of Central Luzon that were included in the study.

Provinces	No. Swine Farms	No. Broiler Farms
Bulacan	26	9
Nueva Ecija	15	39
Pampanga	5	20
Tarlac	8	10
TOTAL	**54**	**78**

4.1.1 Farm Production and Husbandry Practices in Broiler Farms

There were 78 broiler farms visited and these farms have a mean broiler population of 68,872 birds ranging from 10,000 to 342,000 birds in each farm. The broiler farmers have been in the broiler farming business for several years, with a mean of 8.9 years, ranging from 1 to 30 years. Out of 78 farms, 36 farms (54%) operate using a tunnel vent housing while the rest are still conventional. All farms vaccinate against Newcastle Disease (ND), Infectious Bursal Disease (IBD) and Infectious Bronchitis (IB). All farms have their own water source through a deep well. The source of feeds, chicks and antibiotics are from various broiler contract growing companies (Danway, New Hope,

Bounty, Magnolia, Foster) in 50 of the farms (64%) while the other 28 farms purchase their feeds, chicks and antibiotics from commercial source.

Some farms (44.8%) are more aggressive in growing broilers with 6-8 growing cycles per year while others have only 3-5 cycles in a year's time. Despite the utmost importance of disinfection, there are farms (37%) that do not strictly observe disinfection prior to entry of people or vehicles in the farm although majority follow this important biosecurity procedure conscientiously. Larger farms in particular utilize an automatic sprayer to disinfect the entire vehicle before entry. There are still a large percentage of farms that are not aware of antimicrobial resistance (38.5%) and the adverse effects of overuse or inappropriate use of antibiotics (39.7%). The other information on the broiler production and husbandry practices are found in Table 4.2.

Table 4.2

Summary of farming practices observed in the broiler farms (n=78)

Farming Practice	Number of Farms	Proportion of Farms
Broiler Population		
<38000	30	38.46%
≥38000	48	61.54%
Type of Housing		
Conventional	36	46.15%
Tunnel vent	42	53.85%
Length of Farming		
≥ 6 years	38	48.72%
< 6 years	40	51.28%
Source of Feeds		
Commercial source	28	35.90%
Company source	50	64.10%
Source of Broiler Chicks		
Commercial source	28	35.90%
Company source	50	64.10%
Training on Broiler Production		
No	61	78.21%

Table 4.2 (continued)

Farming Practice	Number of Farms	Proportion of Farms
Yes	17	21.79%
Age at Harvest		
30-35days	59	75.64%
36 days and above	19	24.36%
Growing Cycles per Year		
6-8 cycles	35	44.87%
3-5 cycles	43	55.13%
Presence of Other Animals in the Farm		
No	11	14.10%
Yes	67	85.90%
Disinfection at Farm Entry		
No	29	37.18%
Yes	49	62.82%
Source of Antibiotics		
Commercial source	28	35.90%
Company source	50	64.10%

4.1.2 Farm Production and Husbandry Practices in Swine Farms

There were 54 swine farms visited and these farms have a mean sow population of 298 sows ranging from 10 to 1000 sow levels. Majority of the swine farmers have been in the pig farming business for many years with a mean of 18 years, ranging from 2 to 40 years. There are two farms (3.7%) that operate using tunnel vent housing, the rest are all conventional. All farms vaccinate against Hog Cholera but they vary in their vaccination programs for other diseases. In decreasing order, the percentage of farms that vaccinate for other diseases are as follows: Mycoplasma (65.22%), PRRS (56.52%), PCV2 (45.65%), Pseudorabies (41.30%), *E. coli* (39.13%), Actinobacillus pleuropneumonia (28.25%), Parvovirus (23.91%), Swine Influenza (23.91%), Leptospirosis (15.22%), Hemophilus (15.22%), Atrophic Rhinitis (4.35%), and Swine Erysipelas (2.17%).

All farms have their own water source and majority of them (55.6%) mix their own feeds. Fifteen farms (27.8%) confirmed that they mix antibiotic growth promotant in their feeds such as chlortetracycline and amoxicillin. A few farms uses the non-antibiotic growth promotant Triquinol. Almost all farms directly purchase their own antibiotics from various sources while three farms acquire drugs from their farm veterinarians. One third (33.33%) of the farmers are not aware of antimicrobial resistance and 27.8% are not informed about the adverse effects of overuse or inappropriate use of antibiotics. The other information on swine production and husbandry practices are found in Table 4.3.

Table 4.3

Summary of farming practices observed in swine farms (n=54)

Farming Practice	Number of Farms	Proportion of Farms
Sow Level		
≤150	25	46.30%
>150	29	53.70%
Housing		
Conventional	52	96.30%
Tunnel Vent	2	3.70%
Length of Pig Farming		
≤19 years	33	61.11%
>19 years	21	38.89%
Purchasing of stocks		
No	20	37.04%
Yes	34	62.96%
Type of Feeds		
Own Mix	30	55.56%
Commercial	24	44.44%
Presence of Farm Veterinarian		
No	18	33.33%
Yes	36	66.67%

Table 4.3 (continued)

Farming Practice	Number of Farms	Proportion of Farms
Number of Growing Months		
4-5 months	24	44.44%
6-7 months	30	55.56%
Presence of Other Animals in the Farm		
Yes	29	53.70%
No	25	46.30%
Disinfection		
No	12	22.22%
Yes	42	77.78%
Use of Growth Promotant in Feeds		
Yes	15	27.78%
No	39	72.22%
Supply of Antibiotics		
Direct Purchase	49	90.74%
From Farm Vet	5	9.26%
Duration of Use of Antibiotic During Treatment		
1-4 days	23	42.59%
5-7 days	31	57.41%
Rotational use of Antibiotics		
No	15	27.78%
Yes	39	72.22%
Use of Same Antibiotics		
Yes	29	53.70%
No	25	46.30%
***E. coli* (Enterotoxigenic) Vaccination**		
No	36	66.67%
Yes	18	33.33%

4.2 Antimicrobial Usage in the Farms

4.2.1 Antimicrobial Usage in Broiler Farms

The antimicrobial usage in broiler farms based on the farm survey conducted is presented in Table 4.4. The most commonly used antimicrobials in broiler farms is trimethoprim-sulfonamide (TMPS), 84.62% (CI: 74.67-91.79%) followed by tilmicosin, 80.77% (CI: 70.27-88.82%) and pefloxacin, 70.51% (CI: 59.11-80.30%). The three most commonly used antimicrobials belong to three different groups of antibiotics. TMPS is a potentiated sulfonamide and a folic acid synthesis inhibitor while tilmicosin and pefloxacin belong to the macrolide and fluoroquinolone groups, respectively.

Table 4.4

Antimicrobial usage in broiler farms (n=78) based on farm survey

Antibiotic	No. Farms that Uses Antibiotic	Proportion	95% Conf Interval	
			Lower Limit	Upper Limit
Amoxicillin	24	30.77%	20.81%	42.24%
Cefpodoxime	12	15.38%	8.21%	25.33%
Ceftiofur	11	14.10%	7.26%	23.83%
Colistin	2	2.56%	0.31%	8.96%
Doxycycline	16	20.51%	12.20%	31.16%
Enrofloxacin	12	15.38%	8.21%	25.33%
Gentamicin	22	28.21%	18.59%	39.53%
Levofloxacin	13	16.67%	9.18%	26.81%
Norfloxacin	23	29.49%	19.70%	40.89%
Ofloxacin	12	15.38%	8.21%	25.33%
Pefloxacin	55	70.51%	59.11%	80.30%
Tilmicosin	63	80.77%	70.27%	88.82%
Trimethoprim-Sulfonamide	66	84.62%	74.67%	91.79%
Tylosin	35	44.87%	33.59%	56.56%

However, quinolone drugs are the most widely used since all farms (100%) used at least one type of quinolones in their production units. In addition to pefloxacin,

other farms also have increasing use of other quinolone drugs including ofloxacin (15.38%), enrofloxacin (15.38%), levofloxacin (16.67%) and norfloxacin (29.49%).

Combined usage of macrolides is also very high with a total of 68 farms (80.77%) using at least one tilmicosin or tylosin. It was observed that most of these farms have both macrolides available for prevention or treatment of broiler diseases.

4.2.2 Antimicrobial Usage in Swine Farms

The antimicrobial usage in swine farms based on farm survey is shown in Table 4.5. The most commonly used antimicrobials in swine farms is amoxicillin, 54.55% (CI: 38.85-69.61%) followed by tiamulin, 34.09% (CI: 20.49- 49.92%) and both chlortetracycline and florfenicol, 27.27% (CI: 14.96-42.79%). The four most commonly used antimicrobials belong to four different groups of antibiotics. Amoxicillin belongs to penicillin group while tiamulin, chlortetracycline and florfenicol are macrolide, tetracycline and chloramphenicol (thiamphenicol derivative), respectively.

However, penicillin drugs are the most widely used since 34 farms (77.27%) used at least one type of penicillin in their production units. In addition to amoxicillin, other farms also have increasing use of penicillin-streptomycin (18.18%) and penicillin (15.91%). Usage of penicillin is still promoted as a first choice of treatment of unknown infections.

Combined usage of macrolides is also very high with a total of 27 farms (61.36%) using at least one tilmicosin, tylosin or tiamulin. Moreover, a total of 24 farms (54.55%) used at least one type of tetracyclines (doxycycline, chlortetracycline, oxytetracycline). Monitoring of macrolide and tetracycline usage should be performed as increasing resistance to these antibiotics are observed in the recent years.

The information on the usage of antimicrobials is very important since the use of prophylactic antimicrobials has been shown to be a risk factor in the occurrence of ESBL-producing *E. coli* in pigs (Dohmen, et al., 2017; Cameron-Veas et al., 2015; Lugsomya, et al., 2017).

Table 4.5

Antimicrobial usage in swine farms (n=44) based on farm survey

Antibiotic	No. of Farms that Uses Antibiotic	Proportion	95% Confidence Interval	
			Lower Limit	Upper Limit
Amoxicillin	24	54.55%	38.85%	69.61%
Apramycin	1	2.27%	0.06%	12.02%
Bacitracin	1	2.27%	0.06%	12.02%
Ceftiofur	2	4.55%	0.56%	15.47%
Cephalexin	2	4.55%	0.56%	15.47%
Ciprofloxacin	1	2.27%	0.06%	12.02%
Chlortetracycline	12	27.27%	14.96%	42.79%
Colistin	8	18.18%	8.19%	32.71%
Doxycycline	6	13.64%	5.17%	27.35%
Enrofloxacin	9	20.45%	9.80%	35.30%
Florfenicol	12	27.27%	14.96%	42.79%
Gentamicin	6	13.64%	5.17%	27.35%
Lincomycin	4	9.09%	2.53%	21.67%
Linco-Spectin	11	25.00%	13.19%	40.34%
Neomycin	2	4.55%	0.56%	15.47%
Norfloxocin	3	6.82%	1.43%	18.66%
Oxytetracycline	9	20.45%	9.80%	35.30%
Penicillin	7	15.91%	6.64%	30.07%
Pen-Strep	8	18.18%	8.19%	32.71%
Sulfamethazine	2	4.55%	0.56%	15.47%
Tiamulin	15	34.09%	20.49%	49.92%
Tilmicosin	6	13.64%	5.17%	27.35%
TMPS	6	13.64%	5.17%	27.35%
Tylosin	9	20.45%	9.80%	35.30%

4.3 Detection of ESBL-producing *E. coli*

A total of 117 presumptive ESBL-producing *E. coli* isolates (69 broiler, 48 swine) identified in the bacterial culture and isolation yielded the following results in the confirmatory testing (Table 4.6).

Vitek 2 Compact. A total of 101 isolates were identified as ESBL-producing *E. coli* using Vitek 2 Compact, following the CLSI standards. Compared to phenotypic confirmatory testing below, there were some isolates which yielded negative ESBL test results. However, this should be interpreted with caution since a negative ESBL test result does not rule out the presence of an ESBL masked by an AmpC beta-lactamase (Biomerieux, 2015). Possibly, it may have just been masked by the expression of AmpC-type enzymes (Bradford et al., 1997). This means that Vitek may have possibly classified some isolates as ESBL negatives even if it is a true positive because of the co-existence of AmpC beta lactamase in the isolate.

Combined Disc Test (Phenotypic). A total of 113 isolates were identified as ESBL-producing *E. coli* in the phenotypic confirmatory testing using CDT. Compared with the results of bacterial isolation and PCR assay, four presumptive isolates were classified as negatives. CDT is the recommended phenotypic confirmatory testing by CLSI, however, previous report pointed out that none of the available tests for phenotypic detection of ESBL are 100% sensitive or specific and the need for improved detection is well recognized (Paterson and Yu, 1999). Similar to Vitek, one of the possible reasons for a false-negative result in CDT is the presence of a high-level expression of AmpC β-lactamases in the isolate which may mask the presence of ESBLs (Bradbord, 2001; Bradford et al., 1997). The co-existence of ESBL and AmpC beta lactamases in one single isolate has been well documented in many previous studies (Kolar et al., 2010; Reich et al., 2013; Friese, et al., 2013; Laube, et al., 2014; Huijbers, et al., 2014), thus, it is highly possible that some of our isolates may have co-existence of ESBL and AmpC beta lactamases. In one study on the coexpression of β-lactamases, it was reported that ESBL and AmpC co-expression was detected in 9.77% of the isolates (Kolhapure et al., 2015). Thus, in future studies it is recommended that AmpC detection be included as well.

Despite some limitations, double disc approximation test, combined disc test and broth dilution MIC method are still the easiest and cost effective methods for use by many clinical laboratories (Bradbord, 2001). One study have documented the sensitivity of double discs with ceftazidime plus clavulanic acid as being 86% and with cefotaxime plus clavulanic acid as being 65% and recommended the use of both ceftazidime and cefotaxime combinations to increase the sensitivity up to 93%. (M'Zali et al., 2000). Based on the result of the phenotypic confirmatory test in this study, it is recommended that CDT be used as the standard diagnostic protocol for the detection of ESBL-producing *E. coli* in Regional Animal Disease Diagnostic Laboratories (RADDL) in the Philippines.

If the combined results of both phenotypic and genotypic confirmatory tests would be taken as the denominator, or if the result of the ESBL-encoding gene detection by PCR will be used as the gold standard as described in a previous published report (Garrec et al., 2011), then the diagnostic sensitivity of Vitek in this study would then be 86.32% (101/117) and the CDT would be 96.58% (113/117). The diagnostic sensitivity of CDT obtained in this study is quite similar with the result obtained in a previous comparative study of nine phenotypic methods for detection of ESBL production by *Enterobacteriaceae* where a sensitivity of 97% for CDT was reported (Garrec, et al., 2011). In that same study, the sensitivity of Vitek 2 in detecting *E. coli* was reported to be 80% and may reach up to 92% depending on the card used.

PCR Assay (Genotypic). A total of 117 isolates were identified as ESBL-producing *E. coli* in the PCR assay and gene detection using specific primers shown in Table 3.5. Using PCR, at least one ESBL-encoding gene has been detected in all isolates and a coexistence of ESBL-encoding genes ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) within the same isolate was commonly observed in many samples (Tables 4.24, 4.25 and 4.26).

Based on the above combination of test results to detect ESBL-producing *E. coli*, a total of 117 samples were considered to be ESBL-producing *E. coli* positive. Thus, a positive sample for this study is defined as a presumptive isolate which was confirmed ESBL-producing *E. coli* positive through phenotypic and genotypic confirmatory tests.

Table 4.6

Comparison of the number of samples identified and confirmed to be ESBL-producing E. coli.

Samples	Bacterial Isolation	Vitek 2 Compact	Combined Disc Test	PCR Assay[1]
Broiler				
Pooled Fecal Samples	47	37	45	47
Boot Swabs	22	19	21	22
Swine				
Pooled Fecal Samples	34	31	33	34
Boot Swabs	14	14	14	14
TOTAL	**117**	**101**	**113**	**117**

[1] PCR yielded positive results for ESBL-encoding genes using the primers shown in Table 3.5

4.4 Prevalence of ESBL-producing *E. coli* in the Farms

4.4.1 Prevalence of ESBL-Producing *E. coli* in Broiler Farms

The number of farms with at least one sample being positive for ESBL-producing *E. coli*, either from pooled cloacal sample or boot swab sample is 52 while the total number of positive samples is 69 (Table 4.7). Results of the study showed a prevalence of 66.67% (52 out of 78) among broiler farms studied (95% CI: 55.08-76.94). The prevalence in pooled cloacal samples is 60.26% (95% CI: 48.54-71.17) while the prevalence in boot swabs is much lower, 28.21% (95% CI: 18.59-39.53). There is a significant difference in the prevalence between pooled cloacal and boot swab samples (p=0.0001).

Table 4.7

Prevalence of ESBL-producing E. coli in broiler farms (n=78) in selected provinces in Central Luzon.

Farm/Samples	No. of Positives	Prevalence %	95% Conf Interval	
			Lower	Upper
Farm	52	66.67	55.08	76.94
Pooled Cloacal Samples	47	60.26	48.54	71.17
Boot Swabs	22	28.21	18.59	39.53

Further analysis of the farm prevalence in four provinces showed that the highest occurrence was recorded in Pampanga (80%), followed by Nueva Ecija (66.67%), Tarlac (60%) and Bulacan (44.44%). Table 4.8 below also shows the Odds Ratio (OR). With Bulacan having the lowest prevalence, it was taken as a reference against which the prevalence of other provinces were compared. An OR of 5 indicate that Pampanga is five times more likely to have ESBL-producing *E. coli* in the farms suggesting the presence of risk factors in the area such as those observed and described in this study. On the other hand, Nueva Ecija is 2.5 times more likely to have ESBL-producing *E. coli* in the farms compared to Bulacan province.

Table 4.8. Prevalence and Odds Ratios for ESBL-producing *E. coli* in broiler farms in four provinces in Central Luzon.

	ESBL-EC		Total	Prevalence	Odds Ratio	95% Conf Interval	
	Positive	Negative				Lower	Upper
Bulacan	4	5	9	44.44	1.00	0.262	3.824
Nueva Ecija	26	13	39	66.67	2.50	1.221	5.118
Pampanga	16	4	20	80.00	5.00	1.620	15.435
Tarlac	6	4	10	60.00	1.87	0.515	6.829

4.4.2 Prevalence of ESBL-Producing *E. coli* in Swine Farms

The number of farms with at least one sample being positive for ESBL-producing *E. coli*, either from pooled fecal sample or boot swab sample is 31 while the total number of positive samples is 48 (Table 4.9). The swine farm prevalence was observed at 57.41% (31 out of 54) (95% CI: 43.21-70.77). Seven farms have positive isolates both from fecal and boot swab samples. The rest of the farms were either positive for fecal or boot swab samples. The prevalence of ESBL-producing *E. coli* isolated from pooled fecal samples from breeders (19/48) and finishers (15/48) and from boot swabs (14/48) were observed at 35.19% (95% CI: 22.68-49.38), 27.78% (95% CI: 16.46-41.64) and 25.93% (95% CI: 14.96-39.65), respectively (Table 4.9). There is no significant difference in the prevalence between pooled fecal samples from both breeders and finishers (p=0.407). Likewise, there is no significant difference in the prevalence between fecal and boot swab samples (p=0.466).

Table 4.9

Prevalence of ESBL-producing E. coli in swine farms (n=54) in selected provinces in Central Luzon.

Farm/Samples	No. of Positives	Prevalence %	95% Conf Interval	
			Lower	Upper
Farm	31	57.41	43.21	70.77
Pooled Fecal Samples from Breeders	19	35.19	22.68	49.38
Pooled Fecal Samples from Finishers	15	27.78	16.46	41.64
Boot Swabs	14	25.93	14.96	39.65

Further analysis of the farm prevalence in four provinces showed that the highest was recorded in Tarlac (100%), followed by Nueva Ecija (73.33%), Pampanga (60%) and Bulacan (34.62%). Table 4.10 below also shows the OR in three provinces (the OR in Tarlac cannot be estimated because of a zero value in one of the cells). With Bulacan having the lowest prevalence, it was taken as a reference against which the prevalence of other provinces were compared. In the table, Nueva Ecija is five times more likely to have ESBL-producing *E. coli* in the farms compared to Bulacan province.

Table 4.10

Prevalence and Odds Ratio for ESBL-producing E. coli in swine farms in four provinces in Central Luzon.

	ESBL-EC		Total	Prevalence	Odds Ratio	95% Conf Interval	
	Positive	Negative				Lower	Upper
Bulacan	9	17	26	34.62	1.000	0.427	2.340
Nueva Ecija	11	4	15	73.33	5.194	1.605	16.814
Pampanga	3	2	5	60.00	2.833	0.464	17.291
Tarlac	8	0	8	100.00	-	-	-

4.5 Risk Factors for Antimicrobial Resistance in the Farms

4.5.1 Risk Factors in Broilers

There were 16 variables considered in the analysis to identify risk factors associated with the occurrence of ESBL-producing *E. coli* in all broiler farms. Separate analysis was done for fecal samples and boot swab samples. Table 4.11 shows the univariate analysis for risk factors in the farm. All variables were not significantly associated with the occurrence of ESBL-EC. However, this observed lack of association could have been due to small number of negative farms. As discussed earlier, there are 52 farms that were positive for ESBL-producing *E. coli* and the 2x2 table analysis did not give any meaningful result. The same result was observed for the risk factor analysis in fecal samples.

Table 4.11

Results of univariate analysis for possible risk factors that may be associated with the occurrence of ESBL-producing E. coli in broiler farms (n=78).

Variables	Positive	Total	Prevalence	Odds Ratio	95% Conf Int		P value
Broiler Population							
<38000	22	30	73.33%	1.65	0.608	4.477	0.324
≥38000	30	48	62.50%				
Age of Birds during Collection							
<24 days	24	37	64.86%	0.86	0.334	2.200	0.748
≥24 days	28	41	68.29%				
Type of Housing							
Conventional	24	36	66.67%	1.00	0.389	2.571	1.00
Tunnel vent	28	42	66.67%				
Length of Farming							
≥ 6 years	26	38	68.42%	1.17	0.454	2.997	0.747
< 6 years	26	40	65.00%				
Source of Feeds							
Commercial source	21	28	75.00%	1.84	0.657	5.143	0.243

Table 4.11 (continued)

Variables	Positive	Total	Prevalence	Odds Ratio	95% Conf Int		P value
Company source	31	50	62.00%				
Source of Broiler Chicks							
Commercial source	21	28	75.00%	1.84	0.657	5.143	0.243
Company source	31	50	62.00%				
Training Program on Broiler Production							
No	40	61	65.57%	0.79	0.246	2.556	0.698
Yes	12	17	70.59%				
Age at Harvest							
30-35days	39	59	66.10%	0.90	0.297	2.724	0.852
36 days and above	13	19	68.42%				
Growing Cycles per Year							
6-8 cycles	26	35	74.29%	1.89	0.713	5.002	0.198
3-5 cycles	26	43	60.47%				
Other Animals in the Farm							
No	8	11	72.73%	1.39	0.337	5.764	0.645
Yes	44	67	65.67%				
Disinfection at Farm Entry							
No	22	29	75.86%	1.99	0.713	5.556	0.185
Yes	30	49	61.22%				
Source of Antibiotics							
Commercial source	21	28	75.00%	1.84	0.657	5.143	0.243
Company source	31	50	62.00%				
Diarrhea Observed							
Yes	35	56	62.50%	0.49	0.158	1.524	0.213
No	17	22	77.27%				
Presence of Migrating Birds in the Farm							
Yes	33	52	63.46%	0.64	0.227	1.800	0.396
No	19	26	73.08%				

Table 4.11 (continued)

Variables	Positive	Total	Prevalence	Odds Ratio	95% Conf Int		P value
Awareness about AMR							
No	22	30	73.33%	1.65	0.608	4.477	0.323
Yes	30	48	62.50%				
Awareness on Adverse Effect of Antibiotic Overuse							
No	23	31	74.19%	1.78	0.660	4.810	0.252
Yes	29	47	61.70%				

Table 4.12 shows the result of univariate analysis for possible risk factors that may be associated with the occurrence of ESBL-producing *E. coli* in boot swab samples collected from broiler farms. There were eight independent variables that were found significantly associated with the occurrence of ESBL-producing *E. coli* at $p<0.15$. These include source of feeds (OR=3.95, 95%CI 1.40- 11.12, p=0.007), source of broiler chicks (OR=3.95, 95%CI 1.40-11.12, p=0.007), number of growing cycles per year (OR=3.86, 95%CI 1.35-11.03, p=0.009), lack of disinfection before entry at the farm (OR=3.61, 95%CI 1.29-10.10, p=0.012), source of antibiotics (OR=3.95, 95%CI 1.40-11.12, p=0.007), lack of awareness about antimicrobial resistance (OR=2.53, 95%CI 0.94-6.86, p=0.067), lack of training on broiler production (OR=3.66, 95%CI 0.82-16.27, p=0.088) and lack of awareness on the possible adverse effect of overuse or inappropriate use of antibiotics (OR=2.34, 95%CI 0.87- 6.31, p=0.094).

Table 4.12

Results of univariate analysis for possible risk factors that may be associated with the occurrence of ESBL-producing E. coli in boot swab samples collected from broiler farms (n=78).

Variables	Positive	Total	Prevalence	Odds Ratio	95% Conf Int		P value
Broiler Population							
<38000	10	30	33.33%	**1.50**	0.551	4.084	**0.426**
≥38000	12	48	25.00%				
Age of Birds during Collection							
<24 days	11	37	29.73%	**1.15**	0.430	3.096	**0.776**
≥24 days	11	41	26.83%				
Type of Housing							
Conventional	12	36	33.33%	**1.60**	0.593	4.315	**0.351**
Tunnel vent	10	42	23.81%				
Length of Farming							
≥ 6 years	12	40	30.00%	**1.20**	0.450	3.230	**0.712**
< 6 years	10	38	26.32%				
Source of Feeds							
Commercial source	13	28	46.43%	**3.95**	1.402	11.119	**0.0074**
Company source	9	50	18.00%				
Source of Broiler Chicks							
Commercial source	13	28	46.43%	**3.95**	1.402	11.119	**0.0074**
Company source	9	50	18.00%				
Training Program on Broiler Production							
No	20	61	32.79%	**3.66**	0.820	16.270	**0.0885**
Yes	2	17	11.76%				
Age at Harvest							
30-35days	18	59	30.51%	**1.65**	0.479	5.657	**0.425**
36 days and above	4	19	21.05%				

Table 4.12 (continued)

Variables	Positive	Total	Prevalence	Odds Ratio	95% Conf Int		P value
Growing Cycles per Year							
6-8 cycles	15	35	42.86%	**3.86**	1.35	11.03	**0.0095**
3-5 cycles	7	43	16.28%				
Other Animals in the Farm							
No	2	11	18.18%	**0.52**	0.103	2.636	**0.425**
Yes	20	67	29.85%				
Disinfection at Farm Entry							
No	13	29	44.83%	**3.61**	1.291	10.103	**0.0121**
Yes	9	49	18.37%				
Source of Antibiotics							
Commercial source	13	28	46.43%	**3.95**	1.402	11.119	**0.0074**
Company source	9	50	18.00%				
Diarrhea Observed							
Yes	6	22	27.27%	**0.94**	0.311	2.825	**0.908**
No	16	56	28.57%				
Presence of Migrating Birds in the Farm							
Yes	8	26	30.77%	**1.21**	0.430	3.390	**0.722**
No	14	52	26.92%				
Awareness about AMR							
No	12	30	40.00%	**2.53**	0.940	6.860	**0.0672**
Yes	10	48	20.83%				
Awareness on Adverse Effect of Antibiotic Overuse							
No	12	31	38.71%	**2.34**	0.870	6.310	**0.0941**
Yes	10	47	21.28%				

However, statistical modelling using the stepwise logistic regression analysis revealed that only three important risk factors were significantly associated with the occurrence of ESBL-producing *E. coli* in boot swabs collected from broiler farms (Table 4.13). These include: 1) commercial source of feeds (OR=3.49, p=0.042) compared to

feeds provided by companies; 2) 6-8 growing cycles per year (OR=6.62, p=0.003) compared to 3-5 cycles per year only; and 3) lack of disinfection at farm entry (OR=3.91, p=0.033) compared to farms that strictly implement the said biosecurity procedure.

Table 4.13

Best fit logistic regression model for the occurrence of ESBL-producing E. coli in boot swab samples collected from broiler farms.

Variables[a]	Coefficient	Std Error	Odds Ratio	95% Conf Interv		P value
Constant	-1.4491	0.657				
Source of Feeds	1.2525	0.617	3.49	1.04	11.74	0.0426
Growing Cycles	1.8894	0.650	6.62	1.85	23.71	0.0037
Disinfection	1.3646	0.641	3.91	1.11	13.77	0.0334

[a] See Table 3.6 for the description of variables

[b] Exponential value of the coefficient equals the odds ratio.

Deviance on 74 DF = 72.93

4.5.2 Risk Factors in Swine

Table 4.14 shows the result of univariate analysis for possible risk factors that may be associated with the occurrence of ESBL-producing *E. coli* in swine farms. Four putative risk factors were found significantly associated at p<0.15. These include lack of training in pig production (OR=4.45, 95%CI 1.23-16.14, p=0.018), non-sourcing of new and replacement stocks from outside sources (OR=3.38, 95%CI 1.0-11.38, p=0.044), employment of a farm veterinarian (OR=2.6, 95%CI 0.76-8.81, p=0.119) and lack of awareness on the adverse effects of overuse or inappropriate use of antibiotics (OR=4.21, 95%CI 1.02-17.28, p=0.037).

Table 4.14

Results of univariate analysis for possible risk factors that may be associated with the occurrence of ESBL-producing E. coli in swine farms (n=54).

Variables	Positive	Total	Prev	OR	95% CI		P Value
Sow Level							
≤150 sows	16	25	64.00%	1.66	0.555	4.956	**0.363**
>150 sows	15	29	51.72%				
Housing							
Conventional	30	52	57.69%	1.36	0.081	23.014	**0.829**
Tunnel vent	1	2	50.00%				
Length of Pig Farming							
≤19 years	20	33	60.61%	1.40	0.463	4.223	**0.551**
>19 years	11	21	52.38%				
Training in Pig Production							
No	15	19	78.95%	4.45	1.228	16.144	**0.018**
Yes	16	35	45.71%				
Purchase of stocks							
No	15	20	75.00%	3.38	1.001	11.383	**0.044**
Yes	16	34	47.06%				
Type of Feeds							
Own Mix	17	30	56.67%	0.93	0.315	2.768	**0.902**
Commercial	14	24	58.33%				
Vaccination History							
Yes	21	38	55.26%	0.74	0.224	2.454	**0.623**
No	10	16	62.50%				
***E. coli* Vaccination**							
No	21	36	58.33%	1.12	0.358	3.508	**0.845**
Yes	10	18	55.56%				
Farm Veterinarian							
No	13	18	72.22%	2.60	0.767	8.815	**0.119**
Yes	18	36	50.00%				

Table 4.14 (continued)

Variables	Positive	Total	Prev	OR	95% CI		P Value
Number of Growing Months							
4-5 months	14	24	58.33%	1.07	0.361	3.172	**0.902**
6-7 months	17	30	56.67%				
Other Animals in the Farm							
Yes	18	29	62.07%	1.51	0.510	4.472	**0.455**
No	13	25	52.00%				
Disinfection							
No	8	12	66.67%	1.65	0.430	6.343	**0.462**
Yes	23	42	54.76%				
Growth Promotant in Feeds							
Yes	8	15	53.33%	0.80	0.240	2.635	**0.707**
No	23	39	58.97%				
Supply of Antibiotics							
Direct Purchase	28	49	57.14%	0.89	0.136	5.805	**0.902**
From Farm Vet	3	5	60.00%				
Frequency of Diarrhea							
Sometimes	28	46	60.87%	2.59	0.551	12.203	**0.217**
Often	3	8	37.50%				
Antibiotic Treatment Duration							
1-4 days	14	23	60.87%	1.28	0.428	3.834	**0.657**
5-7 days	17	31	54.84%				
Rotational Use of Antibiotics							
No	10	15	66.67%	1.71	0.494	5.951	**0.393**
Yes	21	39	53.85%				
AMR Awareness							
No	11	18	61.11%	1.26	0.397	3.984	**0.697**
Yes	20	36	55.56%				
Aware of Adverse Effects							
No	12	15	80.00%	4.21	1.025	17.288	**0.037**
Yes	19	39	48.72%				

Table 4.14 (continued)

Variables	Positive	Total	Prev	OR	95% CI		P Value
Use of Same Antibiotics							
Yes	18	29	62.07%	1.51	0.510	4.472	**0.455**
No	13	25	52.00%				

However, subsequent statistical modelling using the stepwise logistic regression analysis of the four putative risk factors showed that only one variable, training in pig production (OR=4.45, p=0.023), was found significantly associated with the occurrence of ESBL-producing *E. coli* in swine farms (Table 4.15).

Table 4.15

Best fit logistic regression model for the occurrence of ESBL-producing E. coli in swine farms.

Variables[a]	Coefficient	Std Error	Odds Ratio	95% Conf Interv		P value
Constant	-0.1718	0.339				
Training in Pig Production	1.4936	0.656	4.45	1.23	16.14	0.0230

[a] See Table 3.7 for the description of variable

[b] Exponential value of the coefficient equals the odds ratio.

Deviance on 52 DF = 67.82

Table 4.16 presents the result of univariate analysis for possible risk factors that may be associated with the occurrence of ESBL-producing *E. coli* in boot swab samples collected from swine farms. Only one variable, lack of training in pig production (OR=3.52, 95%CI 0.99-12.46, p=0.045), was identified.

Table 4.16

Results of univariate analysis for possible risk factors that may be associated with the occurrence of ESBL-producing E. coli in boot swab samples collected from swine farms (n=54).

Variables	Positive	Total	Prev	OR	95% CI		P Value
Sow Level							
≤150	8	25	32.0%	1.80	0.53	6.13	**0.344**
>150	6	29	20.69%				
Housing							
Conventional	13	52	25.00%	0.33	0.019	5.716	**0.454**
Tunnel vent	1	2	50.00%				
Length of Pig Farming							
≤19 years	9	33	27.27%	1.20	0.339	4.243	**0.777**
>19 years	5	21	23.81%				
Training in Pig Production							
No	8	19	42.11%	3.52	0.991	12.464	**0.045**
Yes	6	35	17.14%				
Purchase of stocks							
Yes	7	34	20.59%	0.48	0.139	1.662	**0.243**
No	7	20	35.00%				
Type of Feeds							
Own Mix	8	30	26.67%	1.09	0.319	3.725	**0.889**
Commercial	6	24	25.00%				
Vaccination History							
Yes	8	38	21.05%	0.44	0.123	1.594	**0.207**
No	6	16	37.50%				
Farm Veterinarian							
Yes	8	36	22.22%	0.57	0.162	2.006	**0.379**
No	6	18	33.33%				
Number of Growing Months							
4-5 months	6	24	25.00%	0.92	0.268	3.130	**0.889**
6-7 months	8	30	26.67%				

Table 4.16 (continued)

Variables	Positive	Total	Prev	OR	95% CI		P Value
Other Animals in the Farm							
Yes	7	29	24.14%	0.82	0.241	2.768	**0.746**
No	7	25	28.00%				
Disinfection							
No	3	12	25.00%	0.94	0.214	4.112	**0.934**
Yes	11	42	26.19%				
Growth Promotant in Feeds							
Yes	3	15	20.00%	0.64	0.150	2.698	**0.537**
No	11	39	28.21%				
Supply of Antibiotics*							
Direct Purchase	14	49	28.57%	-	-	-	**0.165**
From Farm Vet	0	5	0.00%				
Frequency of Diarrhea							
Sometimes	11	46	23.91%	0.52	0.107	2.553	**0.413**
Often	3	8	37.50%				
Antibiotic Treatment Duration							
1-4 days	7	23	30.43%	1.50	0.441	5.100	**0.514**
5-7 days	7	31	22.58%				
Rotational Use of Antibiotics							
Yes	9	39	23.08%	0.60	0.162	2.216	**0.441**
No	5	15	33.33%				
AMR Awareness							
No	6	18	33.33%	1.75	0.498	6.145	**0.379**
Yes	8	36	22.22%				
Aware of Adverse Effects							
No	6	15	40.00%	2.58	0.709	9.411	**0.143**
Yes	8	39	20.51%				

Table 4.16 (continued)

Variables	Positive	Total	Prev	OR	95% CI		P Value
Use of Same Antibiotics							
Yes	9	29	31.03%	1.80	0.512	6.325	**0.356**
No	5	25	20.00%				

*OR cannot be estimated because of a zero value in one cell.

4.6 Phenotypic Antimicrobial Resistance

4.6.1 Results of Antimicrobial Susceptibility Testing (AST)

The AST-N261, was used to determine the susceptibility of ESBL-producing *E. coli* to seventeen (17) antimicrobial agents. The interpretation of the results was based on the minimum inhibitory concentration interpretive standards as presented previously in Table 3.4. The results of the AST for broiler isolates are presented in Table 4.17 below. A graphical presentation of the AST results is also presented in Figure 4.1.

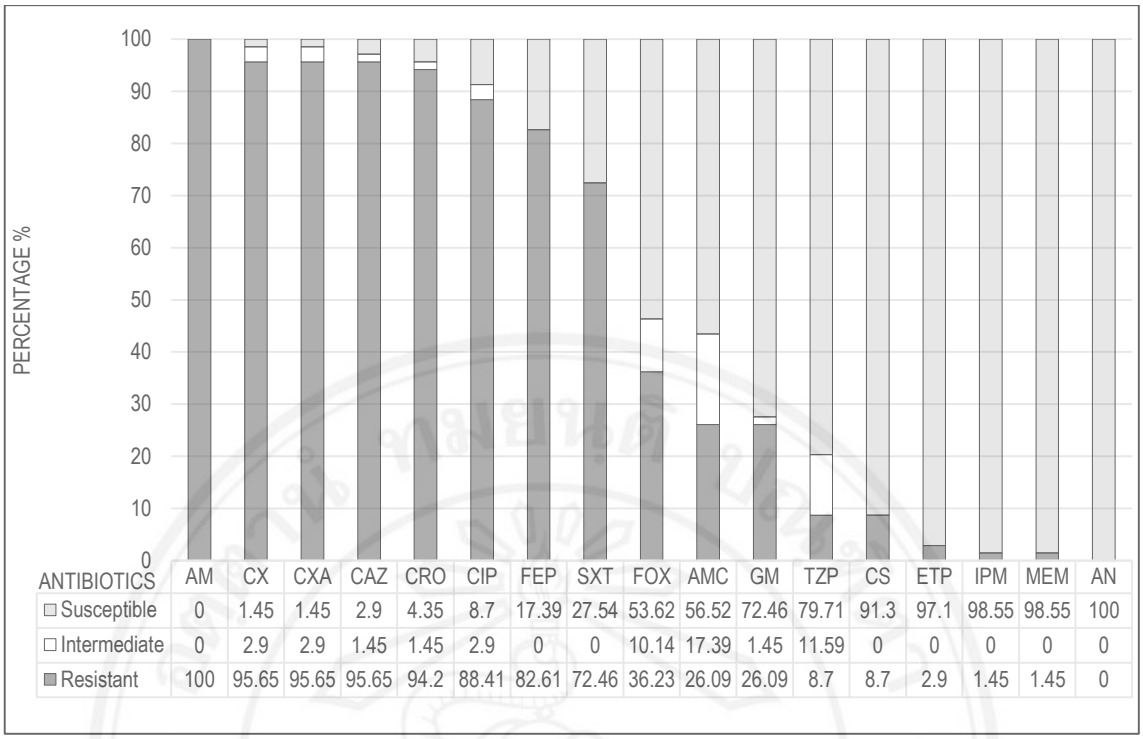
Table 4.17

Antibiotic susceptibility test results (%) of ESBL-producing E. coli in samples collected from broiler farms (n=69).

Antibiotics	MIC Interpretive Criteria		
	Susceptible %	Intermediate %	Resistant %
Penicillins			
Ampicillin (AM)	0.00	0.00	100.00
Beta Lactam-Beta Lactamase Inhibitor Combination			
Amoxicillin/Clavulanic Acid (AMC)	56.52	17.39	26.09
Piperacillin/Tazobactam (TZP)	79.71	11.59	8.70
Cephems			
Cefuroxime (CXM)	1.45	2.90	95.65
Cefuroxime Axetil (CXMA)	1.45	2.90	95.65
Cefoxitin (FOX)	53.62	10.14	36.23
Ceftazidime (CAZ)	2.90	1.45	95.65

Table 4.17 (continued)

Antibiotics	MIC Interpretive Criteria		
	Susceptible %	Intermediate %	Resistant %
Ceftriaxone (CRO)	4.35	1.45	94.20
Cefepime (FEP)	17.39	0.00	82.61
Carbapenems			
Ertapenem (ETP)	97.10	0.00	2.90
Imipenem (IPM)	98.55	0.00	1.45
Meropenem (MEM)	98.55	0.00	1.45
Aminoglycosides			
Amikacin (AN)	100.00	0.00	0.00
Gentamicin (GM)	72.46	1.45	26.09
Flouroquinolones			
Ciprofloxacin (CIP)	8.70	2.90	88.41
Lipopeptides			
Colistin (CS)	91.30	0.00	8.70
Folate Pathway Inhibitors			
Trimethoprim/Sulfamethoxazole (SXT)	27.54	0.00	72.46



ANTIBIOTICS	AM	CX	CXA	CAZ	CRO	CIP	FEP	SXT	FOX	AMC	GM	TZP	CS	ETP	IPM	MEM	AN
Susceptible	0	1.45	1.45	2.9	4.35	8.7	17.39	27.54	53.62	56.52	72.46	79.71	91.3	97.1	98.55	98.55	100
Intermediate	0	2.9	2.9	1.45	1.45	2.9	0	0	10.14	17.39	1.45	11.59	0	0	0	0	0
Resistant	100	95.65	95.65	95.65	94.2	88.41	82.61	72.46	36.23	26.09	26.09	8.7	8.7	2.9	1.45	1.45	0

Figure 4.1. Antimicrobial resistance profile of ESBL-producing *E. coli* isolates from broiler farms. Ampicillin (AM), amoxicillin/clavulanic acid (AMC), piperacillin/tazobactam (TZP), cefuroxime (CX), cefuroxime axetil (CXA), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), ertapenem (ETP) imipenem (IPM), meropenem (MEM), amikacin (AN), gentamicin (GM), ciprofloxacin (CIP), colistin (CS), trimethoprim/sulfamethoxazole (SXT).

4.6.1.1 Antimicrobial Susceptibility Test Results in Broiler Isolates

The ESBL-producing *E. coli* isolates from broilers showed phenotypic resistance to ampicillin (100%) and cephems (average 87.25%). Additionally, the isolates also showed very high resistance to fluoroquinolones (91.31%) and trimethoprim/sulfamethoxazole (72.46%). On the contrary, all isolates remain susceptible to amikacin (100%).

Based on the results of antimicrobial susceptibility testing using Vitek 2 method, the isolates are still fairly susceptible to colistin (91.30%), piperacillin/tazobactam (79.71%) and gentamicin (72.46%). Susceptibility to amoxicillin/clavulanic acid (56.52%) is low considering that its usage is reported at 30.77% (24/78) in the broiler farms included in the study. On the other hand, despite an increased usage of gentamicin (28.21%) in the broiler farms, the said antibiotic is still fairly effective.

4.6.1.2 Antimicrobial Susceptibility Test Results in Swine Isolates

The results of the AST for swine isolates are presented in Table 4.18 and in Figure 4.2. The ESBL-producing *E. coli* isolates from swine showed phenotypic resistance to ampicillin (100%) and to almost all cephems tested (100%) except to cefoxitin. Additionally, the isolates also showed very high resistance to trimethoprim/ sulfamethoxazole (89.58%). Conversely, the isolates were very susceptible to amikacin (100%) and carbapenems (100%). In comparison to isolates from broilers, all ESBL *E. coli* of swine showed susceptibility to carbapenems which indicates that transmission of resistance to this group of antibiotics is of least concern in swine at the time of the study.

Amoxicillin/clavulanic acid and aminoglycosides (gentamycin, streptomycin, neomycin, apramycin) are commonly used in swine for treatment of various diseases based on our farm survey with usage of 43.64% and 30.91%, respectively. Interestingly, only two farms declared the usage of colistin, which could possibly explain the susceptibility of isolates to colistin (95.83%). On the contrary, increasing resistance is observed in amoxicillin/clavulanic acid (39.58%) which is possibly due to its increasing use in swine farms.

Increasing resistance to fluoroquinolones (52.08%) from isolates in swine is observed which is still lower compared to that of broiler isolates (91.31%). Usage of flouroquinolones is lower in swine farms (23.64%) compared to broiler farms wherein 100% usage in at least one antibiotic under this group (pefloxacin, norfloxacin, ofloxacin) is reported.

Table 4.18

Antibiotic susceptibility test results (%) of ESBL-producing E. coli in samples collected from swine farms (n=48).

Antibiotics	MIC Interpretive Criteria		
	Susceptible %	Intermediate %	Resistant %
Penicillins			
Ampicillin (AM)	0.00	0.00	100.00
Beta Lactam-Beta Lactamase Inhibitor Combination			
Amoxicillin/Clavulanic Acid (AMC)	60.42	27.08	12.50
Piperacillin/Tazobactam (TZP)	93.75	4.17	2.08
Cephems			
Cefuroxime (CXM)	0.00	0.00	100.00
Cefuroxime Axetil (CXMA)	0.00	0.00	100.00
Cefoxitin (FOX)	75.00	10.42	14.58
Ceftazidime (CAZ)	0.00	2.08	97.92
Ceftriaxone (CRO)	0.00	2.08	97.92
Cefepime (FEP)	6.25	0.00	93.75
Carbapenems			
Ertapenem (ETP)	100.00	0.00	0.00
Imipenem (IPM)	100.00	0.00	0.00
Meropenem (MEM)	100.00	0.00	0.00
Aminoglycosides			
Amikacin (AN)	100.00	0.00	0.00
Gentamicin (GM)	52.08	8.33	39.58
Flouroquinolones			
Ciprofloxacin (CIP)	47.92	8.33	43.75
Lipopeptides			
Colistin (CS)	95.83	0.00	4.17

Similar to isolates from broilers, 4.7% of swine isolates (2 out of 48) showed phenotypic resistance to colistin. However, molecular testing of the colistin resistance gene showed that 54.17% (95%CI: 39.17, 68.63) of the isolates (26 out of 48) were found positive for *mcr-1* gene, the gene responsible for colistin resistance (Liu *et. al.*, 2016).

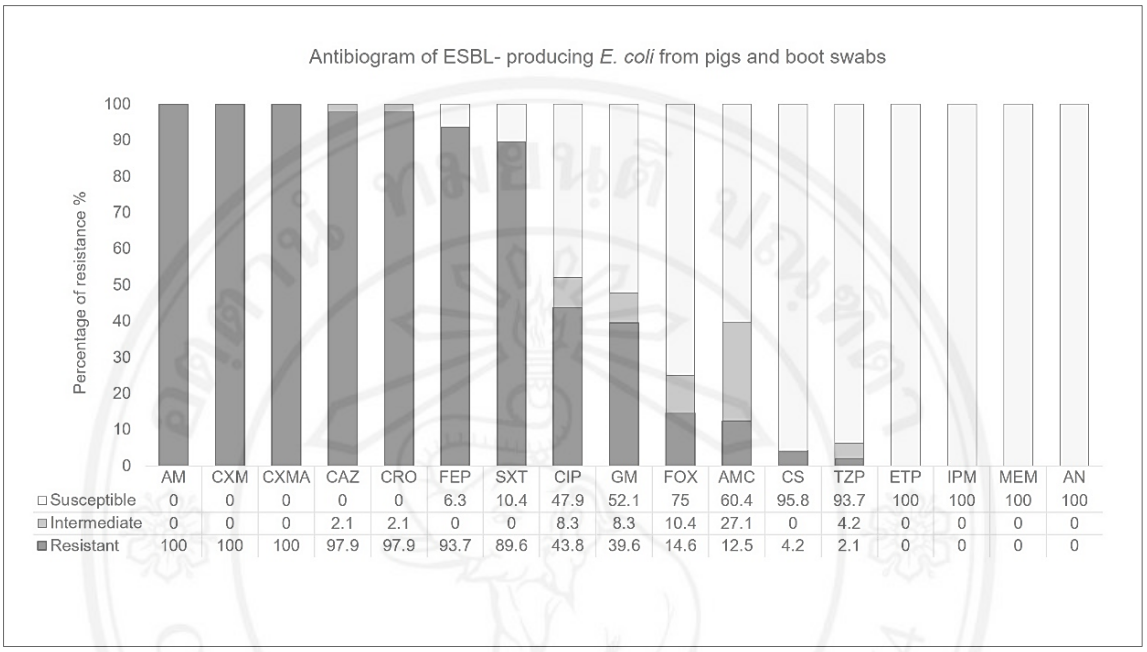


Figure 4.2. Antimicrobial resistance profile of ESBL-producing *E. coli* isolates from swine farms. Ampicillin (AM), amoxicillin/clavulanic acid (AMC), piperacillin/tazobactam (TZP), cefuroxime (CXM), cefuroxime axetil (CXMA), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), ertapenem (ETP) imipenem (IPM), meropenem (MEM), amikacin (AN), gentamicin (GM), ciprofloxacin (CIP), colistin (CS), trimethoprim/sulfamethoxazole (SXT)

4.6.2 Prevalence of Antimicrobial Resistance

4.6.2.1 Prevalence of Antimicrobial Resistance in Broilers

Table 4.19 shows the decreasing order of prevalence of antibiotic resistance of isolates from broilers. All isolates are resistant to ampicillin and susceptible to amikacin. Results also indicate that cephems (represented by cefuroxime, cefuroxime axetil, ceftazidime, ceftriaxone, cefepime), ciprofloxacin, trimethoprim/ sulfamethoxazole are antibiotics that are no longer effective in treating broiler diseases caused by ESBL-producing *E. coli*.

Routine usage of these antibiotics has increased the risk of developing antimicrobial resistance. Usage of at least one flouroquinolones (pefloxacin, norfloxacin, ofloxacin, levofloxacin) is reported in all 78 (100%) broiler farms followed by usage of trimethoprim/sulfamethoxazole in 66 (84.62%) broiler farms. Likewise, usage of cephalosphorin (ceftiofur and cefpodox) is reported in 23 (29.49%) broiler farms.

Table 4.19

Prevalence of antimicrobial resistance (phenotypic) among ESBL- producing E. coli isolates collected from broiler farms (n=69) and swine farms (n=48).

Broiler		**Swine**	
Antibiotics	**Prevalence**	**Antibiotics**	**Prevalence**
Ampicillin	100.00	Ampicillin	100.00
Cefuroxime	98.55	Cefuroxime	100.00
Cefuroxime Axetil	98.55	Cefuroxime Axetil	100.00
Ceftazidime	97.10	Ceftazidime	100.00
Ceftriaxone	95.65	Ceftriaxone	100.00
Ciprofloxacin	91.30	Cefepime	93.75
Cefepime	82.61	Trimethoprim/Sulfamethoxazole	89.58
Trimethoprim/Sulfamethoxazole	72.46	Ciprofloxacin	52.08
Cefoxitin	46.38	Gentamicin	47.92
Amoxicillin/Clav Acid	43.48	Amoxicillin/Clav Acid	39.58
Gentamicin	27.54	Cefoxitin	25.00
Piperacillin/Tazobactam	20.29	Piperacillin/Tazobactam	6.25
Colistin	8.70	Colistin	4.17
Ertapenem	2.90	Ertapenem	0.00
Imipenem	1.45	Imipenem	0.00
Meropenem	1.45	Meropenem	0.00
Amikacin	0.00	Amikacin	0.00

4.6.2.2 Prevalence of Antimicrobial Resistance in Swine

Table 4.19 also presents in decreasing order the prevalence of antibiotic resistance in swine isolates. All isolates are resistant to ampicillin and susceptible to amikacin. Also, cephems (cefuroxime, cefuroxime axetil, ceftazidime, ceftriaxone, cefepime), trimethoprim/sulfamethoxazole are antibiotics that are no longer effective in treating swine diseases being caused by ESBL-producing *E. coli*. Though, none of these cephems are registered veterinary product in the Philippines, ceftiofur and cefalexin are readily available. Among cephems tested, prevalence of cefoxitin resistance is lower in swine (25%) compared to that of broilers (46.38%).

The use of flouroquinolones, aminoglycosides and amoxicillin/ clavulanic should be monitored and regulated since increasing usage and resistance were observed in these antibiotics. Reported usage of flouroquinolones, aminoglycosides and amoxicillin/clavulanic acid were 23.64%, 30.91% and 43.64%, respectively.

On the other hand, the usage of gentamicin (10.91%) and colistin (14.54%) in swine farms are lower compared to other antibiotics which could still be effective in the treatment of gram-negative infections. However, since phenotypic resistance to gentamicin and colistin is observed, the usage of this antibiotic should also be monitored and regulated. Contrary to broiler isolates, carbapenem resistance was not observed in swine isolates.

All isolates are susceptible to amikacin, however, this antibiotic is not also commonly used in swine and none of the sampled swine farms reported its usage which could explain the observed susceptibility.

4.6.3 Multi-drug Resistance Patterns

4.6.3.1 Multi-drug Resistance Patterns in Broilers

Table 4.20 shows the antimicrobial-drug resistance patterns of ESBL-producing *E. coli* isolates from broilers. A total of 17 multi-drug resistance patterns were observed in isolates from broiler samples. The combinations of PCN-BLIC-CEPH-FRQL-FPI (16/69) is the most common among isolates followed by PCN-CEPH-FRQL-FPI (13/69), PCN-CEPH-FRQL-(11/69) and PCN-BLIC-CEPH-AMGL-FRQL-FPI (9/69). It was observed that the common denominator of these patterns is Penicillin-

Cephems-Fluoroquinolones with a total of 49 isolates followed by PCN-CEPH-Folic acid inhibitor with 38 isolates.

All except one isolate followed a typical resistance pattern for ESBL-producing *E. coli* which includes acquired resistance to penicillins (100%) and cephems (100%). Additionally, it was observed also that 91.30% of the isolates have fluoroquinolone resistance and followed by folic acid inhibitor (72.46%). In addition, two isolates have a pattern of PCN-CEPH-CARB which implies greater risks as infection caused by these bacterial isolates will limit treatment options.

Table 4.20

Antimicrobial-drug resistance patterns of ESBL-producing E. coli isolates from broilers (n=69).

No. of Isolates	Multi-drug Resistance Patterns	No. of Antibiotics
1	PCN-CEPH-FPI	3/8
1	PCN-CEPH-LPP	3/8
11	PCN-CEPH-FRQL	3/8
13	PCN-CEPH-FRQL-FPI	4/8
1	PCN-AMGL-FRQL-LPP	4/8
2	PCN-CEPH-AMGL-FRQL	4/8
3	PCN-BLIC-CEPH-FPI	4/8
3	PCN-BLIC-CEPH-FRQL	4/8
1	PCN-BLIC-CEPH-AMGL-FPI	5/8
1	PCN-CEPH-CARB-FRQL-FPI	5/8
1	PCN-CEPH-FRQL-LPP-FPI	5/8
2	PCN-CEPH-AMGL-FRQL-FPI	5/8
16	PCN-BLIC-CEPH-FRQL-FPI	5/8
1	PCN-BLIC-CEPH-CARB-FRQL-FPI	6/8
2	PCN-CEPH-AMGL-FRQL-LPP-FPI	6/8
9	PCN-BLIC-CEPH-AMGL-FRQL-FPI	6/8
1	PCN-BLIC-CEPH-CARB-AMGL-FRQL-LPP-FPI	8/8

Note: PCN, penicillin; BLIC, beta-lactam inhibitor combinations; CEPH, cephems; CARB, carbapenems; AMGL, aminoglycosides; FRQL, fluoroquinolones; LPP, lipopeptides; FPI, folate pathway inhibitor.

4.6.3.2 Multi-drug Resistance Patterns in Swine

Table 4.21 shows the antimicrobial-drug resistance patterns of ESBL-producing *E. coli* isolates from swine. A total of 14 multi-drug resistance patterns were observed in isolates from swine samples. The patterns were less diverse compared to broiler isolates. The combinations of PCN-CEPH-FRQL-FPI (9/48) is the most common among isolates followed by PCN-CEPH-AMGL-FPI (8/48) and PCN-BLIC-CEPH-AMGL-FRQL-FPI (5/48). It was observed that the common denominator of these patterns is Penicillin-Cephems-Folic Acid Inhibitor with a total of 22 isolates followed by PCN-CEPH-FRQL with 14 isolates.

Table 4.21

Antimicrobial-drug resistance patterns of ESBL-producing E. coli isolates from swine (n=48)

No. of Isolates	Multi-drug Resistance Patterns	No. of Antibiotics
2	PCN-CEPH	2/8
1	PCN-CEPH-AMGL	3/8
1	PCN-CEPH-FRQL	3/8
4	PCN-CEPH-FPI	3/8
3	PCN-BLIC-CEPH-FPI	4/8
8	PCN-CEPH-AMGL-FPI	4/8
9	PCN-CEPH-FRQL-FPI	4/8
1	PCN-BLIC-CEPH-AMGL-FRQL	5/8
1	PCN-BLIC-CEPH-LPP-FPI	5/8
4	PCN-BLIC-CEPH-AMGL-FPI	5/8
4	PCN-BLIC-CEPH-FRQL-FPI	5/8
4	PCN-CEPH-AMGL-FRQL-FPI	5/8
1	PCN-BLIC-CEPH-FRQL-LPP-FPI	6/8
5	PCN-BLIC-CEPH-AMGL-FRQL-FPI	6/8

Note: PCN, penicillin; BLIC, beta-lactam inhibitor combinations; CEPH, cephems; CARB, carbapenems; AMGL, aminoglycosides; FRQL, fluoroquinolones; LPP, lipopeptides; FPI, folate pathway inhibitor.

All isolates followed a typical resistance pattern for ESBL which includes acquired resistance to penicillins (100%) and cephems (100%). In addition, frequency of resistance to folic acid inhibitor (89.58%) is very high followed by fluoroquinolones (52.08%) and aminoglycosides (47.92%). No pattern involving carbapenems was observed in the swine isolates. Therefore, the most common antibiotic resistance in swine isolates is PCN-CEPH-FPI since they present in most combinations. Interestingly, only 8 (14.55%) of the sampled swine farms use trimethoprim/sulfamethoxazole.

Increasing resistance to aminoglycosides and fluoroquionolones is probably due to the routine use of antibiotics belonging to these groups. Aminoglycosides such as gentamicin, neomycin, apramycin and streptomycin (in combination with penicillin) comprises 30.91% of antimicrobial usage in the sampled swine farms. On the other hand, combined usage of enrofloxacin, norfloxacin, ciprofloxacin comprises 23.64% of antimicrobial usage of swine farms included this study.

4.7 Genotypic Antimicrobial Resistance

4.7.1 Prevalence of Resistance Genes

4.7.1.1 Prevalence of Resistance Genes in Broilers

The most prevalent ESBL encoding gene observed in this study is *bla*$_{\text{CTX-M}}$, at 89.86%. Of the five *bla*$_{\text{CTX-Mgroups}}$ studied, four groups were detected with *bla*$_{\text{CTX-M-1group}}$ having the highest prevalence (72.46%) followed by *bla*$_{\text{CTX-M-2group}}$ (65.22%) and then by *bla*$_{\text{CTX-M-9group}}$ (52.17%) and lastly by *bla*$_{\text{CTX-M-8group}}$ (21.74%). In addition to *bla*$_{\text{CTX-M}}$ genes, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ genes were also identified in 57.97% and 27.54% of broiler isolates, respectively (Table 4.22).

Table 4.22

Prevalence of ESBL-producing E. coli resistance genes detected in broiler farms (n=69).

Genotype	No. of Positives	Prevalence %	95% Conf Interval	
			Lower	Upper
bla$_{\text{CTX-M}}$	62	89.86	80.21	95.82
bla$_{\text{CTX-M-1group}}$	50	72.46	60.38	82.54
bla$_{\text{CTX-M-15}}$	50	72.46	60.38	82.54
bla$_{\text{CTX-M-2group}}$	45	65.22	52.79	76.29
bla$_{\text{CTX-M-8group}}$	15	21.74	12.71	33.31
bla$_{\text{CTX-M-9group}}$	36	52.17	39.80	64.35
bla$_{\text{CTX-M-25group}}$	0	-	-	-
bla$_{\text{TEM}}$	40	57.97	45.48	69.76
bla$_{\text{SHV}}$	19	27.54	17.46	39.62
mcr-1*	58	84.06	73.26	91.76

*Colistin resistance gene

4.7.1.2 Prevalence of Resistance Genes in Swine

Table 4.23 shows the prevalence of ESBL-producing *E. coli* resistance genes detected in swine. The most prevalent ESBL encoding genes observed in swine are *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$, both observed at 91.67%. The most prevalent *bla*$_{\text{CTX-Mgroup}}$ in swine isolates was *bla*$_{\text{CTX-M-1group}}$, observed at 75.0%, followed by *bla*$_{\text{CTX-M-8group}}$ at 45.83%, then by *bla*$_{\text{CTX-M-9group}}$, 18.75% and lastly, *bla*$_{\text{CTX-M-2group}}$, 6.25%. In addition to *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ genes, *bla*$_{\text{SHV}}$ gene was also identified in 60.42% of swine isolates.

Table 4.23

Prevalence of ESBL-producing E. coli resistance genes detected in swine farms (n=48).

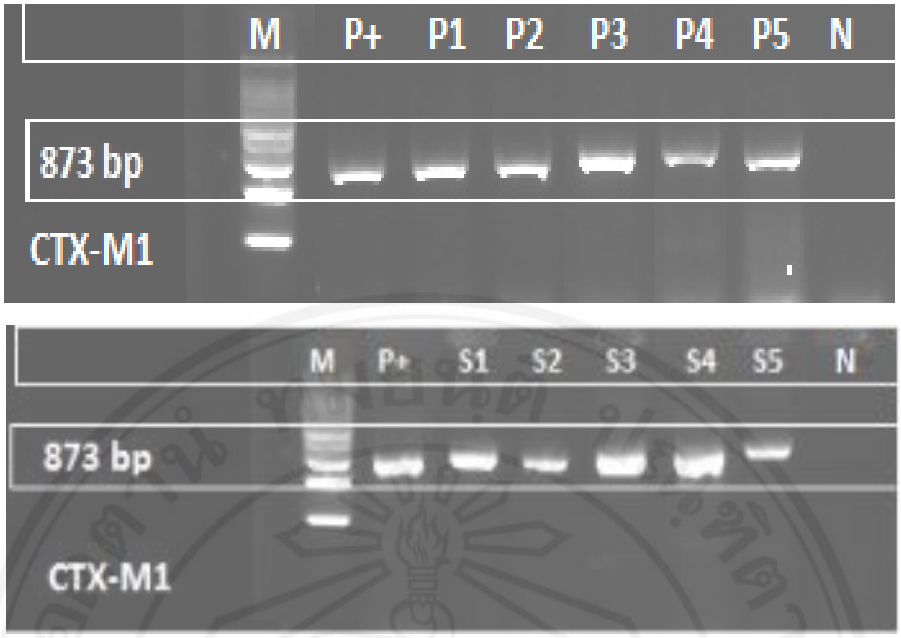
Genotype	No. of Positives	Prevalence %	95% Confidence Interval	
			Lower	Upper
bla$_{\text{CTX-M}}$	44	91.67	80.02	97.68
bla$_{\text{CTX-M-1group}}$	36	75.00	60.40	86.36
bla$_{\text{CTX-M-15}}$	17	35.42	22.16	50.54

Table 4.23 (continued)

Genotype	No. of Positives	Prevalence %	95% Confidence Interval	
			Lower	Upper
bla$_{\text{CTX-M-2group}}$	3	6.25	1.31	17.20
bla$_{\text{CTX-M-8group}}$	22	45.83	31.37	60.83
bla$_{\text{CTX-M-9group}}$	9	18.75	8.95	32.63
bla$_{\text{CTX-M-25group}}$	0	-	-	-
bla$_{\text{TEM}}$	44	91.67	80.02	97.68
bla$_{\text{SHV}}$	29	60.42	45.27	74.23
mcr-1*	26	54.17	39.17	68.63

*Colistin resistance gene

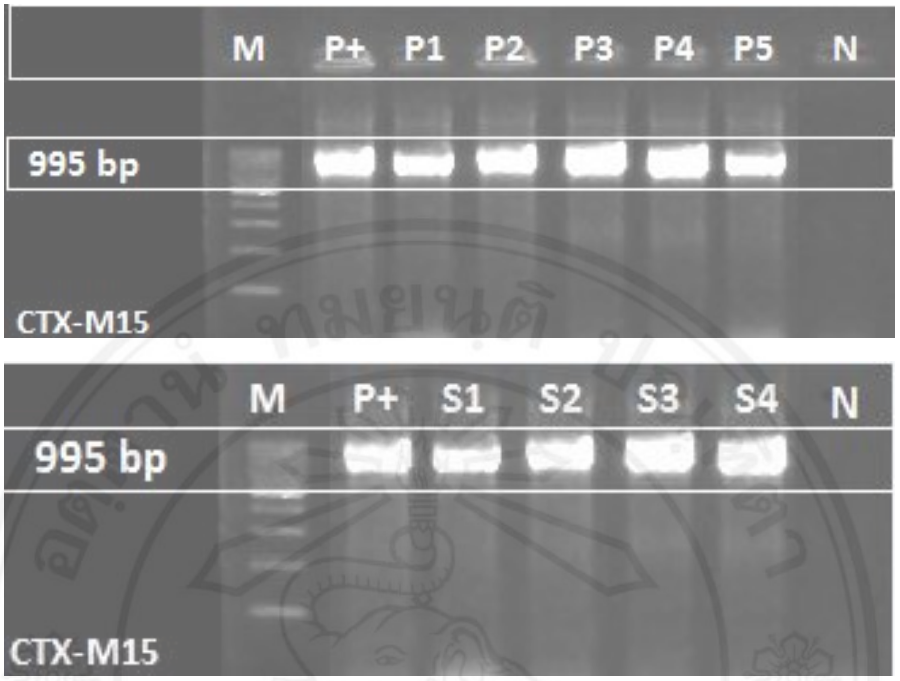
CTX-M-type ESBLs have become the most common type of plasmid-mediated ESBL enzymes produced by drug-resistant organisms (He et al., 2016). Sharing less than 40% amino acid sequence homology with the TEM- and SHV-type enzymes (He et al., 2016), the CTX-M enzymes are subclassified into five groups (Bonnet, 2004). All these five groups of CTX-M enzymes were included in this study. CTX-M-1 group has six plasmid-mediated enzymes including CTX-M-15 which has a public health importance and has been reported as the most widely distributed gene encoding extended-spectrum β-lactamase globally (Zhang et al., 2013). CTX-M-2 group has eight plasmid-mediated enzymes. CTX-M-8 group includes one plasmid-mediated member. CTX-M-9 group includes nine plasmid-mediated enzymes including the CTX-M-14 which have been linked directly or indirectly with animals in different countries. The last group is the CTX-M-25 group which was not detected in this study.



Legend:
M = Marker (1 kb Ladder)
P+ = Positive Control (*E. coli* ATCC® 35218, ESBL-producing)
P = Broiler Samples
S = Swine Samples
N = Negative Control (*E. coli* ATCC® 25922)

Figure 4.3. Amplification of *bla*$_{CTX-M-1}$ gene from broiler and swine ESBL-producing *E. coli* isolates.

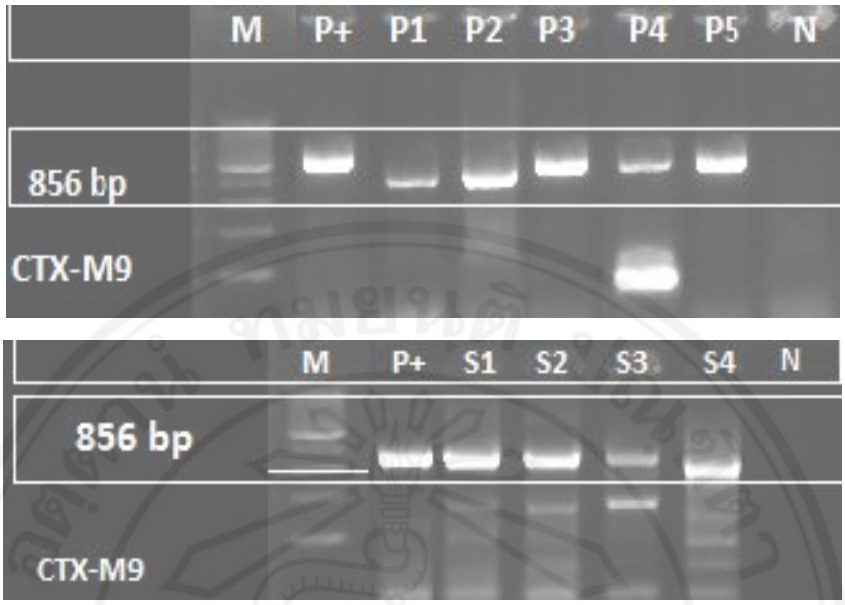
Figure 4.3 shows the amplified *bla*$_{CTX-M-1}$ gene with prevalence of 72.46% in broiler isolates and 75% in swine isolates. This is the major type of isolates both from swine and broilers in this study. The *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-15}$ were the most prevalent *bla*$_{CTX-M}$ variants in humans. The *bla*$_{CTX-M-1}$ has recently been identified also in food-producing animals worldwide thus suggesting a potential risk of diffusion through zoonotic pathogens. These observations strongly suggest the wide diffusion of this gene variant among bacteria circulating in humans and animals (Carattoli, 2008).



Legend:
M = Marker (1 kb Ladder)
P+ = Positive Control (*E. coli* ATCC® 35218, ESBL-producing)
P = Broiler Samples
S = Swine Samples
N = Negative Control (*E. coli* ATCC® 25922)

Figure 4.4. Amplification of *bla*$_{\text{CTX-M-15}}$ gene from broiler and swine ESBL-producing *E. coli* isolates.

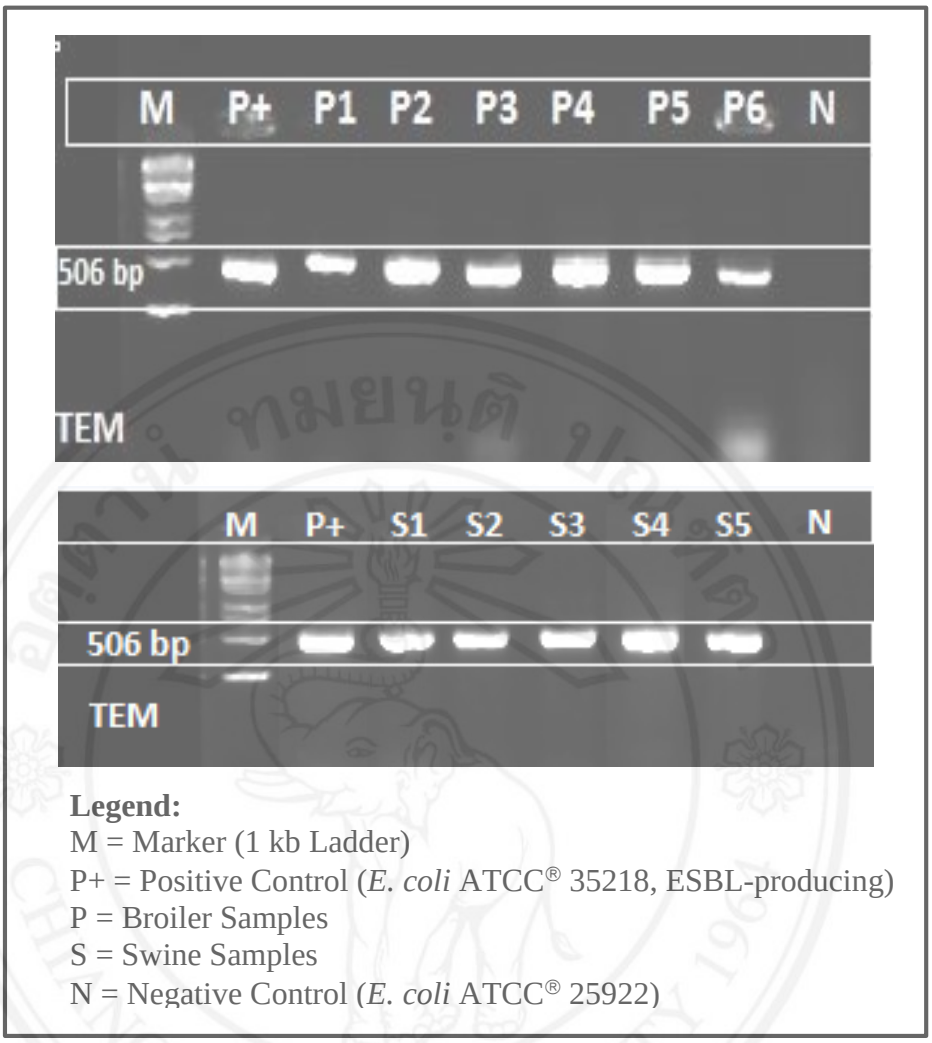
Figure 4.4 shows the amplification of *bla*$_{\text{CTX-M-15}}$ gene. The prevalence of *bla*$_{\text{CTX-M-15}}$ gene is 72.46% of all broiler isolates which is higher compared to swine isolates with only 35.42% prevalence. The said gene has the highest public health importance since it is the most widespread gene type of ESBL-producing *E. coli* in humans (Canton *et. al.*, 2008). The CTX-M15 enzyme is also first reported in the UK in 2003, initially co-existed with CTX-M-9, SHV-variants (mainly SHV-12), and to a lesser extent with TEM derivatives both in the hospital and in the community (Coque *et. al.* 2008).



Legend:
M = Marker (1 kb Ladder)
P+ = Positive Control (*E. coli* ATCC® 35218, ESBL-producing)
P = Broiler Samples
S = Swine Samples
N = Negative Control (*E. coli* ATCC® 25922)

Figure 4.5. Amplification of *bla*$_{\text{CTX-M-9}}$ gene from broiler and swine ESBL-producing *E. coli* isolates.

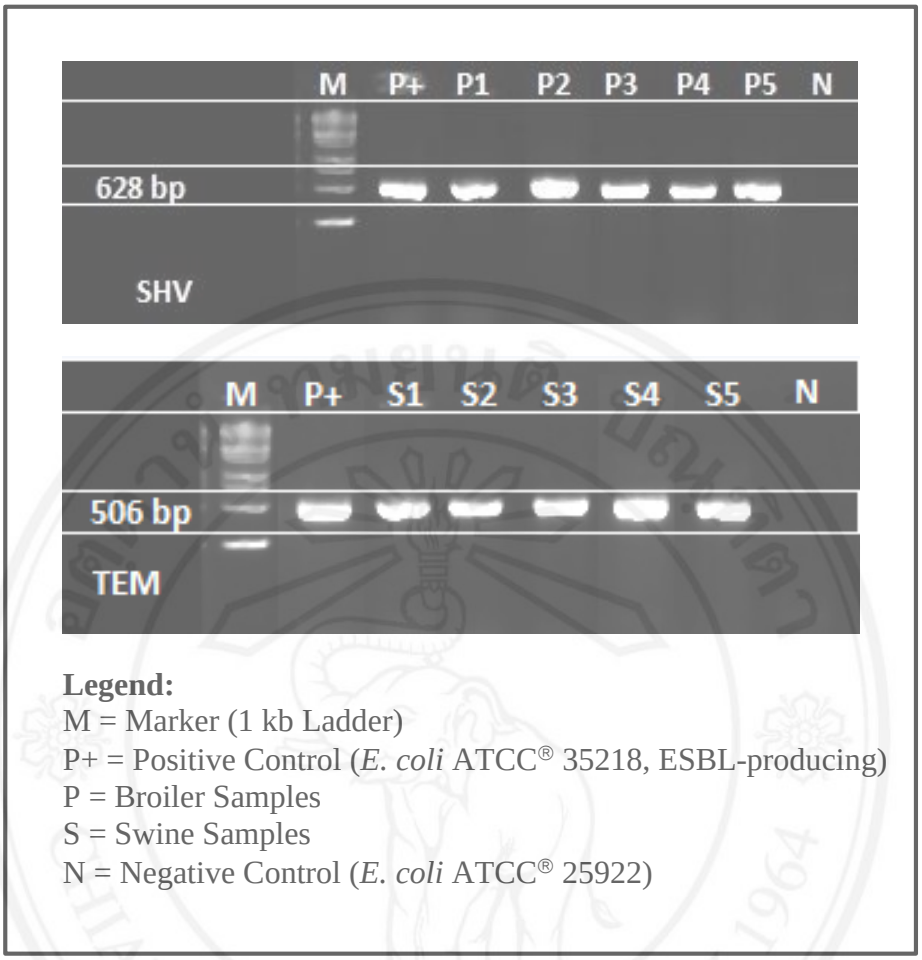
Figure 4.5 shows the amplification of *bla*$_{\text{CTX-M-9}}$ gene both in broiler and swine isolates. This gene was observed in 52.17% and in 18.75% of broiler and swine isolates, respectively. The *bla*$_{\text{CTX-M-9}}$ gene is widely reported in earlier studies in human infections in Europe particularly in Spain and UK. A study in 2003 also reported occurrence of these genes in broiler isolates in France. The CTX-M-9-like enzymes (CTX-M-9 and CTX-M-14) have been linked directly or indirectly with animals in different countries. CTX-M-9 producers have been detected among healthy and sick animals in Spain since 1997 (Coque *et. al.*, 2008).



Legend:
M = Marker (1 kb Ladder)
P+ = Positive Control (*E. coli* ATCC® 35218, ESBL-producing)
P = Broiler Samples
S = Swine Samples
N = Negative Control (*E. coli* ATCC® 25922)

Figure 4.6. Amplification of bla_{TEM} gene from broiler and swine ESBL-producing *E. coli* isolates.

The bla_{CTX-M} gene with bla_{TEM} gene is the most common combination in both broilers and swine either with or without SHV in this study. Compared to bla_{SHV} gene, bla_{TEM} gene is more common in swine than broilers with prevalence of 91.67% and 57.97%, respectively. TEM-1 has the capability to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid (Paterson and Bonomo, 2005).



Legend:
M = Marker (1 kb Ladder)
P+ = Positive Control (*E. coli* ATCC® 35218, ESBL-producing)
P = Broiler Samples
S = Swine Samples
N = Negative Control (*E. coli* ATCC® 25922)

Figure 4.7. Amplification of *bla*$_{SHV}$ gene from broiler and swine ESBL-producing *E. coli* isolates.

The *bla*$_{SHV}$ gene is more common in swine (60.42%) compared to broiler isolates (27.54%) in this study. Within 15 years of the discovery of SHV-2 enzyme, organisms harboring this enzyme were found in every inhabited continent implying that selection pressure from third-generation cephalosporins in the first decade of their use was responsible (Paterson and Bonomo, 2005).

4.7.2 Genotypic Resistance Patterns in Broilers and Swine

The most common genotypic resistance pattern in broiler isolates as presented in Table 4.24 was *bla*$_{CTX-M}$ + *bla*$_{TEM}$ with a total of 23 isolates (33.33%) and this agrees with other studies (Oteo *et. al.*, 2010; Khoshbakht et al., 2016). On the other hand, the most common pattern in swine is *bla*$_{CTX-M}$ + *bla*$_{TEM}$ + *bla*$_{SHV}$ with a total of 28 isolates (58.33%) followed by *bla*$_{CTX-M}$ + *bla*$_{TEM}$ with a total of 15 isolates (31.25%).

Clearly, the *bla*$_{CTX-M}$ gene with *bla*$_{TEM}$ gene with or without SHV is the most common combination observed and this corroborates with the previous report detecting these three genotypes in fecal samples (Selma et al., 2017). The co-existence of different β-lactamases genes within the same isolates has been reported also by several investigators (He et al., 2013; Li et al., 2016).

Table 4.24

Distribution of ESBL genotype among ESBL-positive E.coli isolates.

Patterns of ESBL genotype	Broiler		Swine	
	No. of isolates	Percentage	No. of isolates	Percentage
bla$_{CTX-M}$ + *bla*$_{TEM}$ + *bla*$_{SHV}$	15	21.74	28	58.33
bla$_{CTX-M}$ + *bla*$_{TEM}$	23	33.33	15	31.25
bla$_{CTX-M}$ + *bla*$_{SHV}$	4	5.80	1	2.08
bla$_{CTX-M}$ only	26	37.68	3	6.25
bla$_{TEM}$ only	1	1.45	1	2.08
Total	**69**	**100**	**48**	**100**

In livestock and animal products, broiler and broiler products show the highest prevalence of ESBL-producers with CTX-M-1, TEM-52 and SHV-12 being the most common ESBL-types in broilers (Saliu et al., 2017).

Most of the isolates (46/69 or 66.67%) from broilers (Table 4.25) carry two or more *bla*$_{CTX-M}$ groups. On the other hand, only 21 out of 48 isolates (43.75%) from swine (Table 4.26) have two or more *bla*$_{CTX-M}$ groups and many carries only *bla*$_{CTX-M1}$. Multiple CTX-M types in a single isolate could imply that infections caused by these isolates may be more difficult to treat since ESBL expression is more likely to occur

phenotypically. In addition to this, higher isolation rate of ESBL in broilers could be due to strong genotypic resistance pattern compared to that of swine isolates.

Table 4.25

Distribution of $bla_{CTX\text{-}Mgroups}$ in ESBL-producing E. coli isolates from broilers

Patterns of $bla_{\text{CTX-M groups}}$	No. of isolates	Percentage
$bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-9}}$	12	17.39
$bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-2}}$	10	14.49
$bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-8}}$ + $bla_{\text{CTX-M-9}}$	8	11.59
$bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-9}}$	5	7.25
$bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-9}}$	4	5.80
$bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-8}}$ + $bla_{\text{CTX-M-9}}$	3	4.35
$bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-8}}$	2	2.90
$bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-8}}$	1	1.45
$bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-8}}$ + $bla_{\text{CTX-M-9}}$	1	1.45
$bla_{\text{CTX-M-1}}$	9	13.04
$bla_{\text{CTX-M-2}}$	9	13.04
$bla_{\text{CTX-M-9}}$	3	4.35
$bla_{\text{CTX-M-8}}$	1	1.45
Others (non-$bla_{\text{CTX-Mgroups}}$)	1	1.45
Total	**69**	**100**

In Table 4.25, a total of nine different patterns of $bla_{\text{CTX-Mgroups}}$ were observed in broilers with $bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-9}}$ combination with the highest percentage (17.39%). In contrast, only five different patterns of $bla_{\text{CTX-Mgroups}}$ were observed in swine isolates (Table 4.26) with $bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-8}}$ combination having the highest percentage (25%). The co-existence of two or more CTX-M-type β-lactamases in the same strain is more common in broilers than in swine. Co-existence of different types of CTX-M are no longer an unusual event since they have many homologous regions, other recombinant enzymes may emerge in the near future (He et. al.,2013).

Table 4.26

Distribution of $bla_{CTX\text{-}Mgroups}$ in ESBL- producing E. coli isolates from swine.

Patterns of $bla_{CTX\text{-}M\ groups}$	No. of isolates	Percentage
$bla_{CTX\text{-}M\text{-}1}$	20	41.67
$bla_{CTX\text{-}M\text{-}1}$ + $bla_{CTX\text{-}M\text{-}8}$	12	25.00
$bla_{CTX\text{-}M\text{-}1}$ + $bla_{CTX\text{-}M\text{-}8}$ + $bla_{CTX\text{-}M\text{-}9}$	3	6.25
$bla_{CTX\text{-}M\text{-}8}$ + $bla_{CTX\text{-}M\text{-}9}$	3	6.25
$bla_{CTX\text{-}M\text{-}2}$ + $bla_{CTX\text{-}M\text{-}8}$ + $bla_{CTX\text{-}M\text{-}9}$	2	4.17
$bla_{CTX\text{-}M\text{-}1}$ + $bla_{CTX\text{-}M\text{-}2}$	1	2.08
$bla_{CTX\text{-}M\text{-}8}$	2	4.17
$bla_{CTX\text{-}M\text{-}9}$	1	2.08
Others (non-$bla_{CTX\text{-}Mgroups}$)	4	8.33
Total	**48**	**100**

4.7.3 DNA Sequencing from Representative Samples

Representative samples that yielded positive PCR results for three resistance genes were chosen for DNA sequencing and the results are presented below:

The nucleotide comparison of 15 ESBL-producing *E. coli* $bla_{CTX\text{-}M\text{-}1group}$ genetic sequences that include 10 sequenced samples in the study is shown in Figure 4.8 below. Cluster I includes two groups, one from the Philippines and one sample from the Philippines grouped with the 5 strains reported from Austria, Iran, Germany, Poland and Portugal.

In Cluster 2, one sample from the Philippines had a monophyletic relationship showing distant relationship with the other sequences, hence it clustered separately. This was found out to be matched with the *Escherichia coli* strain Ah01 insertion sequence ISEcp1 ISEcp1 transposase (tnpA) gene, partial cds; class A $bla_{CTX\text{-}M\text{-}123}$ gene, $bla_{CTX\text{-}M\text{-}123}$ allele. It has undergone mutation with an insertion sequence (also known as an IS, an insertion sequence element, or an IS element) which is a short DNA sequence that acts as a simple transposable element. Insertion sequences have two major characteristics:

they are small relative to other transposable elements (generally around 700 to 2500 bp in length) and only code for proteins implicated in the transposition activity, which also carry accessory genes such as antibiotic resistance genes). These proteins are usually the transposase which catalyzes the enzymatic reaction allowing the IS to move, and also one regulatory protein which either stimulates or inhibits the transposition activity.

Figure 4.8. Phylogenetic relationships among 10 strains of ESBL-producing *E. coli* $bla_{\text{CTX-M-1group}}$ gene obtained from four (4) broiler and six (6) swine samples and selected five published strains from different countries using Maximum Likelihood method, available in MEGA 7.0.26 (Kumar et al., 2016). Bootstrap (1000 replicates) values are placed at the major nodes on the tree. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

The phylogenetic tree analysis of the ESBL-producing *E. coli* $bla_{\text{CTX-M-15}}$ gene sequence (Figure 4.9) revealed that six Philippine strains of *E. coli* were confirmed to share common phylogenetic root with strains from India and also with Germany, Russia, United Kingdom and Brazil. On the other hand, one Philippine strain (Pampanga) shared common phylogenetic root with the strain from China but also associated with the rest of the isolated strains. Moreover, a Philippine strain from Tarlac has a different phylogenetic root from the rest of the isolated strains and strains from other countries.

Existing ESBL-producing *E. coli* strains of the Philippines, regardless of the animal origin (broiler or swine) except one isolated strain, are closely related to the strains in other countries. The one isolated strain is most closely related to *Klebsiella pneumoniae* strain KPB-967/16 insertion sequence ISEcp1, partial sequence; 48-bp intergenic spacer, complete sequence; and *bla*$_{\text{CTX-M-15}}$ gene.

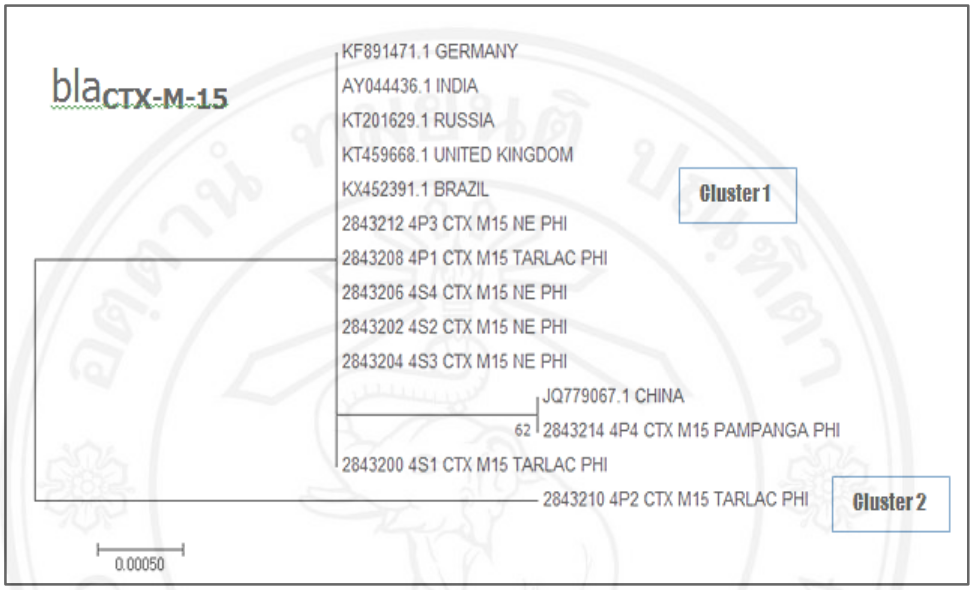


Figure 4.9. Phylogenetic relationships among 8 field isolates obtained from 4 broiler and 4 swine samples and 6 selected *E. coli* isolates in other countries based on the ESBL-producing *E. coli bla*$_{\text{CTX-M-15}}$ gene nucleotide sequences obtained from GenBank using Maximum Likelihood Analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

The nucleotide comparison of 8 ESBL-producing *E. coli bla*$_{\text{CTX-M-9group}}$ gene sequences that include two phylogenetic clusters (Figure 4.10). Cluster 1 with six sequences encompasses the indigenous strains isolated in the Philippines while Cluster II is comprised of two selected sequences of *bla*$_{\text{CTX-M-9group}}$ from previous studies, from NCBI database. Upon phylogenetic analysis, results revealed that the 6 isolated representative strains formed a separate monophyletic cluster with the existing selected strains from different countries.

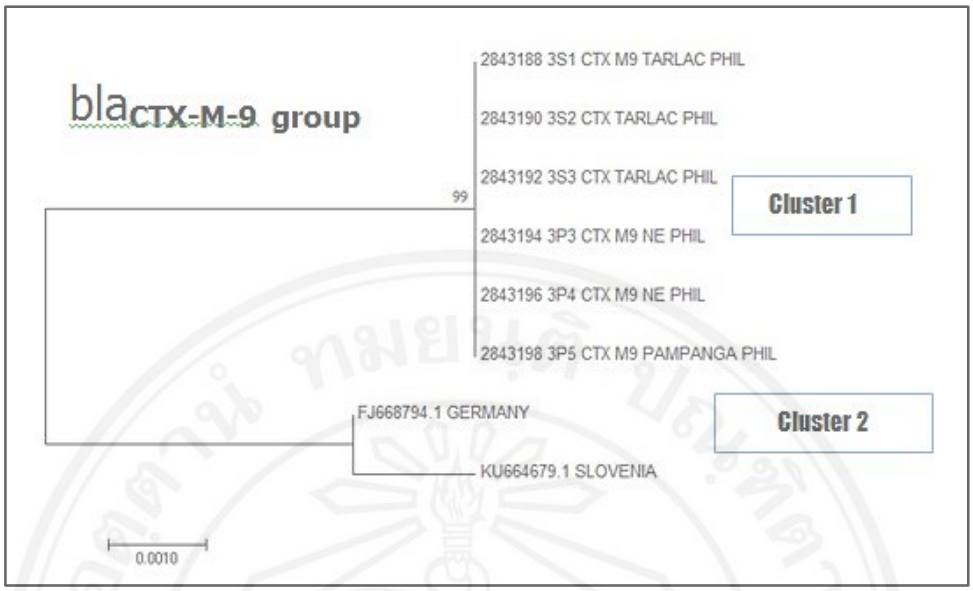


Figure 4.10. Phylogenetic tree indicating the relationships between ESBL-producing *E. coli* $bla_{\text{CTX-M-9group}}$ representative broiler and swine samples and selected CTX M9 strains from GenBank using Maximum Likelihood Analysis, available in MEGA 7.0.26 Tamura-Nei model (Tamura and Nei, 1993). Bootstrap (1000 replicates) values are placed at the major nodes on the tree. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Kumar et al., 2016).

CHAPTER 5

DISCUSSION

5.1 Prevalence of ESBL-producing *E. coli*

ESBL-producing *E. coli* isolated from livestock and poultry animals is of public health importance since infections with these bacteria are resistant to treatment with penicillin and cephalosporins which increases the risk of mortality and delay in appropriate treatment (Chong et al., 2018).

Broilers. This is the first report of ESBL-producing *E. coli* in broiler farms in the Philippines with a very high prevalence. A farm prevalence of 66.67% (52/78) is alarming and requires risk assessments and appropriate risk management to minimize the occurrence and spread of this resistant pathogen. Seventeen farms have positive isolates both from cloacal and boot swab samples. The rest of the farms were either positive for cloacal swabs or boot swabs.

In Netherlands, it was reported that the prevalence of ESBL-producing *Escherichia coli* in the gastrointestinal tract of healthy food-producing animals, especially broilers, increased from 3% in 2003 to 15% in 2008 and in 2009 ESBL-producing bacteria were detected in 26 of 26 broiler farms (Dierikx et al., 2010). A study conducted in Sri Lanka reported a prevalence of 50.6% (42/83) among broiler farms (Mahalingam et al., 2015). In Germany, it was reported that ESBL/AmpC-producing *E. coli* was found in all broiler farms studied (Friese et al., 2013). A high prevalence was also reported in China with 88.8% (Li et al., 2016) as well as in Romania, with 69% (Maciuca, et al., 2015). In Iran, the prevalence among broilers was reported at 53% (Khoshbakht, et al., 2016).

With *E. coli* as a major opportunistic pathogen in chickens and with a potential for zoonotic transfer to human beings, ESBL-producing *E. coli* represents a major risk both to broiler production and to human health (Olsen et al., 2014).

The detection of ESBL-producing *E. coli* in boot swabs in this study (28.21%) suggests the possible spread of the pathogen in the environment which could be a factor for a successful transmission in farm workers and in the community as previously reported (Bui et al., 2018; Huijbers et al., 2014). In this study, more ESBL-producing *E. coli* were isolated from cloacal swabs compared to boot swabs. This can be expected especially when the farms have good management practices and the floorings are kept dry (Olsen et al., 2014).

Swine. Similar with broilers, this is the first report of ESBL-producing *E. coli* in swine farms in the Philippines. The prevalence obtained in this study (57.41%) is lower compared to previous published studies in Southeast Asia. Recent studies on the prevalence of ESBL-producing *E. coli* in pig farms in Thailand showed that 79.7% (98/123) of the farms had at least one sample being positive (Tablerk et al., 2015). The prevalence observed in Vietnam is a bit higher (89%) (Dang et al., 2018). In a 2015 study conducted in Portugal, the prevalence of extended-spectrum ß-lactamase-producing *E. coli* isolates recovered in the faecal flora of pigs is 49%. In Germany, it was reported that ESBL/AmpC-producing *E. coli* was found in 56.3% of breeding pig farms, and in 43.8% of fattening pig holdings (Friese et al., 2013).

Moreover, the prevalence and characteristics of ESBL-producing *E. coli* in pigs have been documented by other studies in Thailand (44.4%) (Changkaew et al., 2015), and China (43.2%) (Xu et al., 2015). The calculated prevalence in this study is comparable to the results of Changkaew (2015) and Xu et al. (2015). Lower levels were reported in Taiwan (19.7%) (Lee and Yeh 2017) and South Korea (4.98%) (Shin et al., 2017).

Similar to broilers, the detection of ESBL-*E. coli* in boot swabs (25.93%) in this study suggests the possible spread of the pathogen in the environment which could be a factor for a successful transmission in farm workers and in the community as previously reported (Zhang et al., 2016).

Hence, appropriate interventions should be implemented to decrease the prevalence observed in this study considering the public health implication of ESBL-producing *E. coli*. In Netherlands for example, it has been shown that the prevalence among swine

farms decreased from 27% in 2011 to 13% in 2013 when antimicrobial usage was restricted (Dohmen et al., 2017).

5.2 Risk Factors for the Occurrence of ESBL-producing *E. coli*

The use of prophylactic antimicrobials has been shown to be a risk factor in the occurrence of ESBL-producing *E. coli.* in some studies (Dohmen et al., 2017; Cameron-Veas et al., 2015; Lugsomya et al., 2017).

In this study, there were three important risk factors found significantly associated with the occurrence of ESBL-producing *E. coli* in broiler farms: commercial source of feeds, 6-8 growing cycles per year and lack of disinfection at farm entry. Both commercial source of feeds and the more aggressive practice of growing broilers in 6-8 cycles per year may indicate the possible use of prophylactic antimicrobials or addition of Antibiotic Growth Promotant (AGP) in feeds which has been a well-documented risk factor for the occurrence of antimicrobial resistance. Thus, this practice should be strictly monitored and a policy to phase out the use of AGP in feeds should be put in place.

On the other hand, the lack of necessary disinfection upon entry in the farm may result to compromised biosecurity. This underscores the importance of disinfection as a very important biosecurity procedure. In a longitudinal study of Norwegian broiler farms, it was reported that disinfection of floor between production cycles served as a protective factor in the occurrence of cephalosporin-resistant *E. coli*. The said study highlights the implementation of a high level of biosecurity with a minimal number of people entering the broiler house during production cycles, as well as rigorous cleaning and disinfection routines between production cycles in order to decrease the occurrence of cephalosporin-resistant *E. coli* in broilers (Mo et al., 2016). Cleaning and disinfection proves vital since contamination of broiler houses with ESBL-producing *E. coli* is an important risk factor (Hiroi, et al., 2012). In a study of 36 pig farms in Netherlands from 2011-2013, the prevalence of ESBL-producing *E. coli* decreased because of improved biosecurity (Dohmen et al., 2017).

In swine farms, the lack of training in pig production as a risk factor for the occurrence of ESBL-producing *E. coli*, highlights the importance of educating the

farmers particularly on the application of Good Animal Husbandry Practices (GAHP), the practice of the prudent use of antibiotics including the observance of the proper withdrawal periods and the use of antibiotics only when prescribed by a licensed veterinarian.

5.3 Phenotypic Antimicrobial Resistance Patterns

Broilers. All isolates are resistant to ampicillin. This resistance is conferred by the gene AmpC which is also a public health concern (Ewers *et. al.*, 2012). Although AMU survey among broiler farms in this study showed that none of the farms uses ampicillin, however, 30.77% (24 out of 78) of the farms use amoxicillin instead, which is quite the same in its basic composition.

In addition, the cephems tested in the study are not used in farm animals and no broiler product having these cephems is registered in the Philippines. However, ceftiofur, a 4th generation cephalosporin, is available in the market in at least seven registered veterinary products. Other cephalosphorins i.e. cephalexin and cepfodox, are registered and available for veterinary use. A total of 23 (29.49%) broiler farms reported the use of either cefpodox or ceftiofur which is higher compared to only 4 (7.27%) swine farms that reported the use of either ceftiofur or cefalexin. Usage of cephalosporins (especially 3rd and 4th generation) lead to emergence of ESBL-producing *E. coli* (EFSA, 2010), hence this could explain more ESBL *E. coli* being isolated in broiler samples.

The use of cephalosphorins and amoxicillin/clavulanic acid should be monitored and regulated since increasing usage and resistance is observed in these antibiotics. On the contrary, the usage of gentamicin and colistin in broilers can still be effective. However, since phenotypic resistance to these two were observed, their usage should also be monitored and regulated. Disturbingly, carbapenem resistance was observed despite the fact that these antibiotics are not used in farm animals.

All isolates are susceptible to amikacin. However, this antibiotic is not routinely used in broiler chickens which could explain the observed susceptibility. Only one product is registered in the Philippines with amikacin as its primary active ingredient and used for intramuscular and intrauterine infusion. Similarly, no veterinary product

containing piperacillin/tazobactam and carbapenems as active ingredient is registered in the Philippines (PVET, 2015).

Most of the ESBL *E. coli* isolates worldwide are still susceptible to carbapenems. However, two of our isolates showed resistance to ertapenem (2.9%) while one of these two isolates is resistant to all carbapenems tested. Increasing concern has been emphasized by the World Health Organization (WHO) on the emergence of carbapenem-resistant ESBL-producing *E. coli* (Lutgring and Limbago, 2016; WHO, 2017).

Some isolates showed some susceptibility to some types of cephems. The reasons for this apparent susceptibility to some cephalosporins is the result of various degrees of hydrolysis of cephalosporins by different β-lactamases and enhanced penetration through the bacterial outer membrane of some cephalosporins compared to others (Paterson and Bonomo, 2005).

Another drug resistance being monitored most recently is the emergence of colistin-resistant *E. coli* wherein colistin is considered to be the last drug of choice for treatment of multi-drug resistant *E. coli* (Liu et. al., 2016; Sibhghatulla et. al., 2016). In this study, 8.7% of broiler isolates (6 out of 69) were found to be phenotypically resistant to colistin. However, the result of PCR shows that 84.06% (95%CI: 73.26, 91.76) of the isolates (58 out of 69) were found positive for mcr-1 gene, the gene responsible for colistin resistance (see Table 4.22). *Mcr* (Mobile COL-R)-1 gene was originally described in 2015 in China in commensal *E. coli* from animals. It encodes a phosphor ethanol amine transferase, which confers transferable plasmid-mediated COL-R. Subsequently *mcr-1* gene was reported in *Enterobacteriaceae* isolated from animals and meat for human consumption in Germany, Vietnam, Japan, Denmark, Canada and France (Vila et al., 2016). Evidence suggests that the spread of *mcr-1* is from animals to human beings (Liu et al., 2016; Webb et al. 2016). In China, it was reported that the outbreak of *mcr-1*-containing *E. coli* of chicken origin started in 2009. The proportion of *mcr-1*-positive *E. coli* increased from 5.2% in 2009, to 5.9% in 2010, 11.9% in 2011, 20.9% in 2012, 25.4% in 2013, and 30.0% in 2014 (Shen et al., 2016)

The most common antibiotic resistance pattern in broiler isolates is PCN-CEPH-FRQL-FPI since they present in most combinations. Possible explanation of high

resistance to FPI and FRQL is the antimicrobial usage of sampled broiler farms. A total of 66 (84.62) broiler farms use trimethoprim/sulfamethoxazole while 55 (70.51%) use pefloxacin and other fluoroquinolones such as norfloxacin (29.49%), levofloxacin (16.67%) and ofloxacin (15.38%). Co-selection with other resistance mechanisms, especially to fluoroquinolones, aminoglycosides and sulfonamides, seems to have contributed to the problem caused by ESBL-producing *E. coli* infections (Coque *et. al.*, 2008).

Extended-spectrum β-lactamase allows ESBL-producing *E. coli* to become resistant to a wide variety of penicillins and cephalosporins and through genetically linked resistance mechanisms, they are often resistant to other antibacterials including quinolones and aminoglycosides. Most ESBL-producing organisms are thus multidrug resistant and many are only susceptible to carbapenems (Hunter et al., 2010). The threat level of ESBL-producing *Enterobacteriaceae* is categorized as serious and requires prompt and sustained action to ensure the problem does not grow (CDC, 2013).

ESBLs are often encoded by genes located on large plasmids, and these also carry genes for resistance to other antimicrobial agents which explains multi-drug resistance in the isolates (Rawat and Nair, 2010). Fluroquinolone resistance is plasmid-mediated, mainly by Qnr proteins (Strahilevitz *et. al.*, 2009) while trimethoprim-sulfamethoxazole resistance *E. coli* often correlates with the presence of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes in integrons (White *et. al.*, 2001).

Swine. The isolates fit the characteristics of ESBL described by Pitout and Laupland (2008). They are resistant to cefuroxime, cefuroxime, ceftazidime, ceftriaxone, and cefepime. They are still sensitive to cefoxitin, imipenem, and meropenem. Their resistance is related to their ability to produce extended spectrum β-lactamases that can hydrolyze the β-lactam ring. There is absolute resistance against ampicillin. This conforms with the result of Michael et al. (2014). The resistance level against amoxicillin/clavulanic acid is close to the report of Gundogan and Avci (2013).

The resistance against ciprofloxacin is comparable to the previous report (Geser et al., 2011). Fluoroquinolone resistant ESBL-producing *E. coli* is a concern in human medicine since this group of antibiotics are commonly used to treat urinary tract

infections. Hence, monitoring and characterization of this plasmid-mediated resistance is of public health importance (Kock et al., 2016).

The resistance against gentamicin is comparable to the previous report (Geser et al., 2011; Li et al., 2015). In contrast, Ugwu et al. (2015) reported 100% resistance while Gundogan and Avci (2013) and Michael et al. (2017) reported a lower resistance compared to this study. Apparently, gentamicin is the most common antibiotic under the aminoglycoside family used within the study area.

High resistance to trimethorprim/sulfamethoxazole was reported previously (Michael et al., 2017). The high rate of resistance to some of the non-cephalosporin drugs (trimethoprim/sulfamethoxazole, ampicillin, ciprofloxacin, and gentamicin) indicate a role for swine commensal *E. coli* strains as reservoirs of antimicrobial resistance (Chah et al., 2018).

All isolates followed a typical resistance pattern for ESBL-producing *E. coli* which includes acquired resistance to penicillins (100%) and cephems (100%). In addition, frequency of resistance to folic acid inhibitor (89.58%) is very high followed by fluoroquinolones (52.08%) and aminoglycosides (47.92%).

5.4 Genotypic Antimicrobial Resistance Patterns

Broilers. The $bla_{\text{CTX-M}}$ being the most prevalent gene in this study is similar to published studies in broilers (Bui et al., 2018; Li et al, 2016; Shin et al., 2017). In humans, however, the recent report revealed that TEM-type is more prevalent in clinical isolates from Filipinos (Cruz & Hedreyda, 2017) which is contrary to earlier reports wherein $bla_{\text{CTX-M}}$ is the most prevalent type in hospitalized patients (Tian et al., 2010). Previous studies suggest that ESBL genotypes can vary between regions and geographical location. Therefore, it is warranted to conduct wider scope and regular surveillance study to determine the prevalence and distribution of these enzymes among broiler farms in the Philippines.

The study identified $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ genes as the most prevalent $bla_{\text{CTX-M}}$ variants in this study which is similar to other reports on broilers (Li et al., 2016) and humans (Maciuca et al., 2015). In fact, $bla_{\text{CTX-M-15}}$, has been reported as the most widely

distributed gene encoding extended-spectrum β-lactamases globally (Zhang et al., 2013). Some studies also established the relationships of broiler isolates from human isolates suggesting a potential zoonotic transmission (Huijbers et al., 2014). This could be a result of contamination of fecal materials in broiler meat during slaughter, processing, selling and cooking of broiler products (Aliyu et al., 2016; Boonyasiri et al., 2014). Moreover, the high prevalence of *bla*$_{\text{CTX-M-15}}$ gene in this study has public health importance since it is the most widespread gene type of ESBL-producing *E. coli* in humans (Cantón et al., 2012)

The *bla*$_{\text{CTX-M-2}}$ is the second most common CTX-M type β-lactamase in this study. It was previously isolated in chicken meat and in healthy chickens (Aliyu et al., 2016; Huijbers et al., 2016). The presence of *bla*$_{\text{CTX-M-2}}$ in food animals has been observed in Japan since 1999 (Carattoli, 2008). Spread of plasmids of *bla*$_{\text{CTX-M-2}}$ between Belgium and France has been reported (Coque *et. al.*, 2008). It was isolated in chicken meat in Ghana (Rasmussen *et. al.*, 2015), in healthy chickens in Denmark (Bortolaia *et. al.*, 2011) and in broiler chickens in Japan (Hiroi *et. al.*, 2012)

The *bla*$_{\text{CTX-M-9}}$ gene is observed in 52.17% of isolates in this study. The *bla*$_{\text{CTX-M-9}}$ gene is widely reported in earlier studies in human infections in Europe, particularly in Spain and UK. A study in 2003 also reported the occurrence of these genes in broiler isolates in France. The CTX-M9-like enzymes (CTX-M-9 and CTX-M-14) have been linked directly or indirectly with animals in different countries (Coque, Baquero, & Canton, 2008). The *bla*$_{\text{CTX-M-8}}$ gene was observed in 21.74% of isolates in this study. Though detected in lower proportion in broilers, this is an emerging genotype in human (Eller et al., 2013). Moreover, no *bla*$_{\text{CTX-M-25}}$ gene was detected in this study. However, it is still recommended that this be included in any future surveillance. Either, the gene is not really present during the time of study or an improvement can be done in the optimization procedure for the PCR condition employed.

The PCR amplification of *bla*$_{\text{CTX-M}}$ specific products alone and without sequencing, in an isolate that produces an ESBL, usually provides sufficient evidence that a *bla*$_{\text{CTX-M}}$ gene is responsible for this phenotype (Sibhghatulla, 2016).

The coexistence of different β-lactamase genes within the same isolates has been reported by several investigators (He et al., 2013; Li et al., 2016). The most common ESBL genotype among our isolates was *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ (33.33%) which agrees with other studies (Khoshbakht et al., 2016). The *bla*$_{\text{CTX-M}}$ with *bla*$_{\text{TEM}}$ combination may occur with or without SHV and this corroborates with the previous report detecting these three genotypes in poultry fecal samples (Selma et al., 2017). To my knowledge, this is the first report of high co-resistance pattern among broiler isolates in the Philippines. The presence of multiple ESBL resistance genes could result in retained resistance to β-lactamases despite the reduced expression of one or two genes.

For a long time, TEM- and SHV-types were the dominant ESBLs enzymes all over the world but this has changed dramatically since nowadays, CTX-M-enzymes have become the most widespread type of ESBLs (Ewers *et. al.*, 2012; Canton et al., 2012; Canton et al., 2008). It is now present not only in humans, but also in food, food-producing animals, companion and wild animals, and in the environment (Canton et al., 2012).

The name CTX reflects the potent hydrolytic activity of these β-lactamases against cefotaxime and they are not very closely related to TEM or SHV β-lactamases (Sibhghatulla, 2016). SHV refers to sulfhydryl variable. This designation was made because it was thought that the inhibition of SHV activity by p-chloromercuribenzoate was substrate-related, and was variable according to the substrate used for the assay. On the other hand, over 100 TEM-type β-lactamases have been described, of which the majority are ESBLs (Paterson and Bonomo, 2005).

Swine. The high prevalence of *bla*$_{\text{CTX-M}}$ encoding gene in this study is similar to published studies in pigs (Cameron-Veas et al., 2015; Changchaew et al., 2015; Xu et al., 2015; Lee and Yeh 2017; Dang et al., 2018). The *bla*$_{\text{CTX-M-1}}$ being the most prevalent *bla*$_{\text{CTX-M}}$ group in this study is similar to other reports on swine (García-cobos et al., 2015). Moreover, the high prevalence of *bla*$_{\text{CTX-M-15}}$ (35.42%) gene in this study has public health importance since it is the most widespread gene type of ESBL-producing *E. coli* in humans (Cantón et al., 2012). This gene was reported in swine feces in China (Hu et. al., 2013; Xu et. al., 2015), Ireland (Wang et. al., 2016), Korea (Tamang et. al., 2013) and UK (Randall et. al., 2014). In a 2015 study conducted in Portugal in the faecal

flora of food-producing animals, the most prevalent ß-lactamase detected was the CTX-M-1 enzyme, followed by CTX-M-9 and for the first time, CTX-M-enzymes where reported from beef cattle and sheep (Ramos, 2015).

The *bla*$_{\text{CTX-M-8}}$ is the second most prevalent CTX-M type among isolates observed in this study. This is an emerging genotype in human (Eller et al., 2013) but no report can be found in pigs to date. The *bla*$_{\text{CTX-M-9}}$ gene was also previously isolated in a healthy pig (Changkaew et al., 2015; Lugsomya et al., 2017). In contrast to the findings in this study, *bla*$_{\text{CTX-M-9}}$ group was the most prevalent genotype in isolates from pigs in previous reports (Xu et al., 2015; Liu et al., 2018). The *bla*$_{\text{CTX-M-9}}$ group, particularly *bla*$_{\text{CTX-M-14}}$ subtype was the most prevalent in healthy swine in Korea with 75.20% prevalence (Tamang et. al., 2013). The *bla*$_{\text{CTX-M-2}}$ being the least prevalent CTX-M type in isolates of porcine origin agrees with the studies of Biasino et al. (2018).

The prevalence of *bla*$_{\text{TEM}}$ genotype in this study is similar to the report of Valentina (2015) and higher than reported by Xu et al. (2015). The *bla*$_{\text{TEM}}$ gene was isolated by Wang et. al. (2016) in farrowing houses and piglet while Xu et. al. (2015) reported its presence in diarrheic piglets in China. The prevalence of *bla*$_{\text{SHV}}$ genotype in the isolates is high in contrast to other reports where *bla*$_{\text{SHV}}$ was not detected. (Changkaew et al., 2015; Xu et al., 2015). This indicates that *bla*$_{\text{SHV}}$ genotype have lower prevalence compared to *bla*$_{\text{CTX-M}}$ groups. This supports other studies that *bla*$_{\text{CTX-M}}$ groups is more widely distributed and prevalent among ESBL genotypes.

Some studies also established the relationships of swine isolates from human isolates suggesting a potential zoonotic transmission (Dohmen et al., 2015; Zhang et al., 2016; Dang et al., 2018). This could be a result of contamination of fecal materials in pork during slaughter, processing, selling and cooking of pork products (Biasino et al., 2018; Schill et al., 2017).

Most of the isolates from pigs and boot swabs carry two or more *bla*$_{\text{CTX-M-}}$ groups. In this study, co-existence of two or more CTX-M type β-lactamases in the same strain is common and has been previously reported (Changkaew et al., 2015; Lee and Yeh 2017; Lugsomya 2017). This coexistence of different types of CTX-M- can be a normal scenario since they have many homologous regions which may result in the emergence of

recombinant enzymes (He et al., 2013). The multiple CTX-M- types in single isolate could imply that infections caused by these isolates may be more difficult to treat since ESBL expression is more likely to occur phenotypically.

The coexistence of different β-lactamase genes within the same isolates has been reported by several investigators (He et al., 2013). While the majority of swine isolates (58.33%) in this study have *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$, still the most common ESBL genotypes are *bla*$_{\text{CTX-M-}}$ and *bla*$_{\text{TEM}}$ and this agrees with other studies (Changkaew et al., 2015; Xu et al., 2015). The *bla*$_{\text{CTX-M-}}$ and *bla*$_{\text{TEM}}$ combination may occur with or without SHV and this corroborates with the previous report detecting three genotypes in fecal samples (Selma et al., 2017). Again, to my knowledge, this is the first report of high co-resistance pattern among isolates from pigs in the Philippines. The presence of multiple ESBL resistance genes could result in retained antimicrobial resistance despite the reduced expression of one or two genes.

Based on results, this study presents a baseline information on the prevalence of *ESBL-producing E. coli* in both broiler and swine farms in Central Luzon, the Philippines. In addition, the occurrence of this resistant bacteria in healthy broilers and pigs and their houses pose a great risk of transmission to the workers, environment and other animals with access to the animals and their manure. Regulations and policies on antimicrobial usage should be strictly implemented and monitored while further surveillance studies should be conducted on other high broiler and pig-producing regions in the Philippines. Isolation and phylogenetic evaluation of this bacteria in farm workers, chicken meat and pork and other possible fomites will improve the understanding on the transmission, prevention and control of the spread of these resistant bacteria.

The presented data in this study confirmed that ESBL-producing *E. coli* is highly prevalent in broiler and swine farms in the study area. The isolates are multi-drug resistant with diverse combinations and belong to the three main ESBL genotypes, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, and *bla*$_{\text{SHV}}$. The varied genotypic resistance profile of the isolates implies the diversity of ESBL-producing *E. coli* present in broilers and pigs in the study area which warrants further typing to detect possible presence of mutated and new subtype circulating in the country.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The study was able to establish the prevalence of ESBL-producing *E. coli* in 78 broiler and 54 swine farms in the Central Luzon region, with a total of 318 samples analyzed (156 from broilers, 162 from swine). Also, it generated information on the antimicrobial usage at the farm level and determined the risk factors that are putatively associated with the detection of ESBL-producing *E. coli*. Further, both phenotypic and genotypic AMR patterns were clearly observed after both microbiological and molecular analyses of all 117 positive ESBL-producing *E. coli* isolates.

***On Prevalence*.** The farm prevalence in broilers was determined at 66.67% (52 out of 78 farms) while the prevalence for fecal samples and boot swab samples are observed at 60.26% (47/78) and 28.21% (22/78), respectively. The swine farm prevalence was recorded at 57.41% (31 out of 54 farms) while that of finishers, breeders and boot swabs are 27.78% (15/54), 35.19% (19/54) and 25.93% (14/54), respectively. The prevalence in broiler farm is highest in Pampanga (80%, OR=5) while the prevalence in swine farm is highest in Tarlac (100%) and Nueva Ecija (73.33%).

The high prevalence recorded in both broiler and swine farms is alarming and requires risk assessments and appropriate risk management to minimize the occurrence and spread of ESBL-producing *E. coli*. The detection of the resistant pathogen in boot swabs suggests the possible spread of the pathogen in the environment which could be a factor for a possible transmission to farm workers and in the community. Hence, appropriate interventions such as restriction in antimicrobial usage and promotion of good animal husbandry practices should be implemented to decrease the prevalence considering the public health implication of ESBL-producing *E. coli*.

***On Risk Factors*.** Both univariate and logistic regression analyses indicate that there were three important risk factors found significantly associated with the occurrence of ESBL-producing *E. coli* in broiler farms. These include: 1) commercial source of feeds (OR=3.49, p=0.042) compared to feeds provided by companies; 2) 6-8 growing cycles per year (OR=6.62, p=0.003) compared to 3-5 cycles per year only; and 3) lack of disinfection at farm entry (OR=3.91, p=0.033) compared to farms that strictly implement the said biosecurity procedure. In swine, lack of training in pig production was observed to be a risk factor (OR=4.45, p=0.023).

Both commercial source of feeds and a more aggressive practice of growing broilers in 6-8 cycles per year may indicate the possible use of prophylactic antimicrobials or addition of Antibiotic Growth Promotant (AGP) in feeds which has been a well-documented risk factor for the occurrence of ESBL-producing *E. coli*. This practice should be strictly monitored and a policy to phase out the use of AGP in feeds should be put in place. The lack of disinfection before entry at the farm emphasizes the importance of strictly following disinfection as a very important biosecurity procedure. The lack of training in pig production emphasizes the importance of educating the farmers particularly on the application of Good Animal Husbandry Practices (GAHP), the practice of the prudent use of antibiotics including the observance of the proper withdrawal periods of antibiotics and the use of antimicrobials only when prescribed by a licensed veterinarian.

***On Phenotypic AMR Pattern*.** All positive isolates from broilers and swine showed typical AST pattern expected from ESBL-producing *E. coli*, i.e. resistant to penicillin (100%), cephalosphorins (>90%-100%). High resistance was also recorded in trimethoprim/sulfamethoxazole (72.46% in broilers, 89.58% in swine) as well as ciprofloxacin (91.3% in broilers, 52% in swine). Many isolates are still susceptible to cefoxitin (46.4% in broilers and 25% in swine) and amoxicillin/clavulanic acid (43.5% in broilers and 39.6% in swine). The most common phenotypic pattern in broiler isolates is Penicillin-Cephem-Fluoroquinolone-Folate Pathway Inhibitor while in swine isolates, the most common pattern is Penicillin-Cephem-Folate Pathway Inhibitor.

All isolates in the study followed a typical resistance pattern for ESBL-producing *E. coli* which includes acquired resistance to penicillins and cephalosphorins. In addition, frequency of resistance to folic acid inhibitor is very high followed by fluoroquinolones and aminoglycosides. This is because extended-spectrum β-lactamase allows ESBL-producing *E. coli* to become resistant to a wide variety of penicillins and cephalosporins and through genetically linked resistance mechanisms, they are often resistant to other antibacterials including quinolones and aminoglycosides. Hence, most isolates in this study were observed to be multidrug resistant.

***On Genotypic AMR Pattern*.** In broilers, the most prevalent ESBL encoding gene detected was $bla_{\text{CTX-M}}$ (89.86%). Among the $bla_{\text{CTX-Mgroups}}$, $bla_{\text{CTX-M-1}}$ has the highest prevalence (72.46%) followed by $bla_{\text{CTX-M-2}}$ (65.22%) and $bla_{\text{CTX-M-9}}$ (52.17%). The genes bla_{TEM} and bla_{SHV} were also identified in 57.97% and 27.54% of isolates, respectively. In swine, the most prevalent ESBL encoding genes detected were $bla_{\text{CTX-M}}$ and bla_{TEM}, which were both observed at 91.67% followed by bla_{SHV} gene at 60.42%. Among the $bla_{\text{CTX-Mgroups}}$ in swine, $bla_{\text{CTX-M-1}}$ was also the most prevalent $bla_{\text{CTX-M}}$ gene, 75.0%. The $bla_{\text{CTX-M-15}}$, a sub-type under $bla_{\text{CTX-M-1group}}$, and which has a public health importance being the most widespread gene type in humans, is more common in broiler (72.46%) than in swine (35.42%) isolates.

Majority of broiler isolates (33.33%) have co-existence of $bla_{\text{CTX-M}}$ and bla_{TEM} genes while majority of swine isolates (58.33%) have co-existence of $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV}. Moreover, most of the broiler isolates carry two or more $bla_{\text{CTX-Mgroups}}$ with a total of nine patterns while swine isolates have only five different $bla_{\text{CTX-M}}$ patterns. The co-existence of three different kinds of resistance genotypes ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) and different $bla_{\text{CTXMgroups}}$ within the same isolate could result in retained antimicrobial resistance and may even pose risks of possible transmission to farm workers, other animals, susceptible bacteria and the environment.

Based on the results of the study, it is recommended that the AMR surveillance initiated in this study be sustained and that the AMR prevalence be monitored regularly to assess changes that may serve as guide in future research directions and policy decisions. Documentation of antimicrobial usage at the farm level and correlating this with the occurrence of antimicrobial resistance should be continued. Further study should

be made on the putative risk factors so that the information can be used for evidence-based policy prescriptions.

It is further recommended that AMR awareness campaigns especially among broiler and swine farmers be enhanced to inform them about the prudent use of antibiotics. Some of the farmers that have been interviewed in this study were not even aware of the antimicrobial resistance. It is envisioned that a more aggressive awareness campaigns will help minimize the development of antimicrobial resistance through a more rational and targeted use particularly in the utilization of antibiotics that are critically important to human health such as colistin.

It is likewise recommended that the result of animal studies particularly on the detection of resistance genes be correlated with human studies. Since *bla*$_{\text{CTX-M-15}}$ (P=72.46%), a resistance gene of public health importance, is found highly prevalent in broiler farms, detection among broiler farm workers can establish potential transmission of this resistance gene to humans.

Strong collaboration with relevant sectors, stakeholders and development partners is encouraged in order to sustain the surveillance efforts towards the implementation of the national AMR surveillance plan. This important undertaking should produce reports, evidence-based outputs and vital information that can be used as basis for future research directions, policy recommendations and important high-level decisions.

On the area of research, it is recommended that efforts be done to reduce the use of antibiotics in animal agriculture by exploring novel technologies by way of developing antibiotic alternatives for use in animals to combat the global increase in antibiotic resistance. Some examples of alternatives to antibiotics that may be explored include antimicrobials engineered against multi-resistant pathogens, immune enhancers, naturally occurring antibacterial lytic enzymes, organic acids, phytochemicals, prebiotics, probiotics, therapeutic antibodies as well as vaccines.

The following points are also recommended based on the consultation and discussions with the Philippine Interagency Committee on Antimicrobial Resistance (ICAMR).

1. To draft guidelines on the usage of veterinary antimicrobials to include treatment guide in common bacterial diseases of livestock and poultry with reference to the WHO-FAO-OIE Joint Guidelines on critically important antimicrobials with particular emphasis on the antibiotics critical to human health.

2. To intensify awareness of various stakeholders on the prudent use of antibiotics, recognizing their importance in animal health and thus maximizing their therapeutic effect while cautiously minimizing the development of antimicrobial resistance through a more rational and targeted use particularly in the use of antibiotics that are critically important to human health such as colistin.

3. To enhance capacities on AMR surveillance through a network of laboratories (regional/national government laboratories, universities, private laboratories) all over the Philippines in order to sustain the surveillance and monitoring activities. Through this capacity building effort, the reporting of AMR prevalence at a larger scale is possible.

4. To continue the documentation of antimicrobial usage at the farm level and correlate this with the occurrence of antimicrobial resistance while promoting good animal husbandry practices, helping farmers improve their husbandry and biosecurity programs and developing viable alternatives.

5. To foster strong collaboration with relevant sectors, stakeholders and development partners in order to sustain the surveillance efforts and implement the national AMR surveillance plan covering healthy animals, diseased animals, aquatic animals including animal settings. These undertakings should produce reports, evidence-based outputs and vital information that will be the basis for future research directions, policy recommendations and important high-level decisions.

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APPENDICES

APPENDIX 1

QUESTIONNAIRE FOR BROILER FARM

Farm No._____

1. IDENTIFYING INFORMATION

1.1 Name of the Farm _____

1.2 Complete Address of the Farm

_____

_____

(indicate barangay, municipality/city, province)

1.3 Name of Owner

_____

1.4 Contact Details Mobile: _____

1.5 No. of years Farm is Established _____

1.6 GPS Coordinates Latitude_____ Longitude_____

2. FARM INFORMATION

2.1 Fill up the following **broiler population inventory**

Bldg. No.	Number of Birds	Total
Total		

Age at Visit = _____days

2.2 Date of Visit/Inventory: _____

2.3 Type of housing

[ ] Tunnel Vent [ ] Conventional (Open-sided)

2.4 Breed of broilers raised in the farm? _____

3. RISK FACTOR INFORMATION

Length of Broiler Farming

3.1 How long have you been raising broilers in your broiler farm? _____years

3.2 Do you have any training in broiler production?

[ ] Yes [ ] No

Sources of Stocks & Growing Cycles

3.3. Where do you get your supply of broiler chicks in the farm? _____

[ ] Danway [ ] New Hope [ ] Others,_____

3.5 What is the age of broilers before harvest?

[ ] 30-35 [ ] 36-38 [ ] 39 and Up

3.6 How many growing cycles do you have in your farm each year?

[ ] 3-5 cycles [ ] 4-6 cycles [ ] 6-8 cycles

Feed and Water Source

3.6 What type of feed do you use?_____

[ ] Own mix [ ] Commercial Feeds [ ] Company Feeds

3.7 Where do you get your feeds?

[ ] Danway [ ] New Hope [ ] Others,_____

3.8 What is your water source?

[ ] Own Water pump [ ] Municipal/City water supply [ ] Other Source

Vaccination History

3.9 Do you vaccinate your birds against *E. coli* and other diseases?

[ ] Yes [ ] No

3.10 Please list down your vaccination programs (use the table below)

Vaccinated Against:	Name of Vaccine Given	Age at Vaccination	Date of Last Vaccination
NCD			
IBD			
IB			

[ ] Tunnel Vent [ ] Conventional [ ] Commercial

Farm Veterinarian

3.11 Is there any veterinarian who frequently visits your farm?

[ ] Yes [ ] No

3.12 If yes, frequency of visit of veterinarian in the farm

[ ] Once a week [ ] Every 2 weeks [ ] Once a month [ ] Others _____

Farm Management Practices

3.13 Are there other animals raised in the farm or have access to your farm?

[ ] No [ ] Broiler [ ] Ruminants [ ] Dogs/Cats [ ] Others _____

3.14 Before entering your farm, do visitors or vehicles undergo any disinfection?

[ ] Yes [ ] No

3.15 Do you observe any overcrowding of birds in your farm?

[ ] Yes [ ] No

3.16 Where do you dispose your manure?

[ ] Farm [ ] Within locality [ ] Buyer, _____

Antimicrobial Usage in the Farm

3.17 For what purpose do you use antibiotics in the farm?

[ ] Treatment [ ] Prevention [ ] Growth Promotion

3.18 If growth promotion, in what route of administration do you usually administer the antibiotics?

[ ] Feeds [ ] Drinking water [ ] Both

3.19 Does the feed that you are currently using in the farm contain antibiotic growth promotant?

[ ] Yes [ ] No

3.20 If yes, what is the name of the antibiotic growth promotant? _____

3.21 At what age do you usually administer the antibiotic growth promotant?

[ ] 1st week [ ] 2nd week [ ] 3rd week

3.22 How long do you administer the antibiotic growth promotant?

[ ] 1 week [ ] 2 weeks [ ] 3 weeks

3.23 Who prescribes the use of antibiotic growth promotant?

[ ] Veterinarian [ ] None, just a usual practice

3.24 Where do you get your supply of antibiotics?

[ ] From Farm Vet [ ] Purchased directly Made in _____(country)

3.25 Are you aware that antibiotics have withdrawal periods?

[ ] Yes [ ] No

3.26 Do you strictly follow the withdrawal periods for antibiotics?

[ ] Yes [ ] No

3.27 Does the feed for the last week of production contain antibiotic growth promotant?

[ ] Yes [ ] No

If yes, what is the name of the antibiotic growth promotant? _____

Broiler Disease Management

3.28 What are the diseases of broilers you have encountered in the farm in the past 6 months?

Disease	Antibiotics Used in TREATMENT	Route of Administration	Duration of Treatment

Disease	Antibiotics Used in PREVENTION	Route of Administration	Duration of

Drugs: Enrofloxacin, Gentamicin, Colistin, STP, Tylosin, Lincomycin, Neomycin, Pefloxacin, Tilmicosin, etc

3.29 Do you observe diarrhea in your farm?

[ ] No [ ] Sometimes [ ] Often [ ] Very Often

3.30 When diarrhea occurs, do you always use antibiotics to treat birds in your farm?

[ ] Yes [ ] No

3.31 Who prescribes the use of antibiotics in the farm?

[ ] Farm Vet [ ] Government Vet

3.32 Do you always follow the prescription of the veterinarian?

[ ] Yes [ ] No

3.33 Do you always complete the prescribed treatment regimen of 7 days

[ ] Yes, 7 days [ ] Up to 3 days [ ] Up to 5 days

3.34 Do you send samples for antibiotic susceptibility testing at RADDL or elsewhere?

[ ] Yes [ ] No [ ] Company

3.35 Which antibiotics did you use most frequently during the past year?

Antibiotic	**Reason for Use**	**Route of Administration**	**Duration of Use**

3.36 How do you manage the birds if being treated?

[ ] Isolated [ ] Stays in the group

3.37 For how long do you use the antibiotics when treating birds?

[ ] 1-2 days [ ] 3-4 days [ ] 5 days only

3.38 Do you rotate the use of antibiotics when treating a disease?

[ ] Yes [ ] No

3.39 Do you record the treatment when using antibiotics?

[ ] Yes [ ] No

Presence of Migrating Birds in the Area

3.40 Do you observe any migrating birds in the area?

[ ] Yes [ ] No

Knowledge of Antimicrobial Resistance

3.41 Are you aware of antimicrobial resistance?

[ ] Yes [ ] No

3.42 If yes, where did you learn about antimicrobial resistance?

[ ] Farm Veterinarian [ ] Broiler Raisers Association

3.43 Are you aware that overuse or inappropriate use of antibiotics may have adverse effects?

[ ] Yes [ ] No

3.44 Do you use the same antibiotics for every batch of broilers you grow in a year?

[ ] Yes [ ] No

3.45 Do you change your antibiotics after each batch of broilers?

[ ] Yes [ ] No

3.46 How often do you change the antibiotics that you use in the farm?

[ ] Every 6 months [ ] Every year [ ] Every 2 years

4. SOURCE OF INFORMATION

Name of Person who provided the Information: _____

Contact Number _____

Date of Interview: _____

_____

Printed Name & Signature of Investigator

APPENDIX 2

QUESTIONNAIRE FOR SWINE FARM

No._____

1. IDENTIFYING INFORMATION

1.1 Name of the Farm

_____

1.2 Complete Address of the Farm

_____
_____

(indicate barangay, municipality/city, province)

1.3 Name of Owner _____

1.4 Contact Details Mobile: _____

Email: _____

1.5 No. of years Farm is Established _____

1.6 GPS Coordinates Latitude_____ Longitude_____

2. FARM INFORMATION

2.1 Fill up the following **population inventory**

Number of Buildings: _____

Number of Pigs per age group:

	Sows	**Boars**	**Gilts**	**Piglets/ Sucklings (0-2 wks)**	**Weanlings (2wks-1 mo)**	**Starters (1-2mos)**	**Growers/ Fatteners (2-3.5mos)**	**Finishers (3.5-4mos)**
Total								

TOTAL PIG POPULATION: _____

2.2 Date of Inventory as of _____

2.3 Type of housing

[ ] Cages [ ] Pens [ ] Others (please specify)_____

2.4 Breed of pigs raised in the farm? _____

3. RISK FACTOR INFORMATION

Length of Pig Farming

3.1 How long have you been raising pigs in your swine farm? _____years

3.2 Do you have any training in pig production?

[ ] Yes [ ] No

Sources of Stocks

3.3. Do you purchase animals/breeders? [ ] Yes [ ] No

If No, proceed to 3.6

If yes…

3.4 Where is your source? _____

(Please indicate name of farm source including complete address)

3.5 When was the last time you purchased new stocks? _____

[ ] Last week [ ] Last month [ ] Others, please specify _____

Feed Source

3.6 What type of feed do you use?_____

[ ] Own mix [ ] Commercial Feeds [ ] Others, pls specify_____

3.7 Where do you get your feeds? _____

Vaccination History

3.8 Do you vaccinate your animals against *E. coli* and other diseases?

[ ] Yes [ ] No

3.9 Please list down your vaccination programs (use the table below)

Vaccinated Against:	Name of Vaccine Given	Age at Vaccination	Date of Last Vaccination

Farm Veterinarian

3.10 Do you have a Veterinarian in your farm?

[ ] Yes [ ] No

3.11 If yes, what is the frequency of visit of veterinarian in the farm?

[ ] Once a week [ ] Once a month [ ] Others, pls specify____

Farm Management Practices

3.12 How many months do you grow your pigs before slaughter?

[ ] 4-5 months [ ] 6-7 months [ ] 7-8 months

3.13 Are there other animals raised in the farm or have access to your farm?

[ ] Broiler [ ] Ruminants [ ] Dogs/Cats [ ] Others _____

3.14 Before entering your farm, do visitors or vehicles undergo any disinfection?

[ ] Yes [ ] No

Antimicrobial Usage in the Farm

3.15 For what purpose do you use antibiotics in the farm?

[ ] Treatment [ ] Prevention [ ] Growth Promotion

3.16 If growth promotion, in what route of administration do you usually administer the antibiotics?

[ ] Feeds [ ] Drinking water [ ] Both

3.17 Does the feed that you are currently using in the farm contain antibiotic growth promotant?

[ ] Yes [ ] No

3.18 If yes, what is the name of the antibiotic growth promotant? _____

3.19 At what age do you usually administer the antibiotic growth promotant?

[ ] 1st month [ ] 2nd -3rd month [ ] 4th -5th month

3.20 How long do you administer the antibiotic growth promotant?

[ ] 1 month [ ] 2 months [ ] 3 or more months

3.21 Who prescribes the use of antibiotic growth promotant?

[ ] Veterinarian [ ] None, just a usual practice

3.22 Where do you get your supply of antibiotics?

[ ] From Farm Vet [ ] Purchased directly

3.23 Are you aware that antibiotics have withdrawal periods?

[ ] Yes [ ] No

3.24 Do you strictly follow the withdrawal periods for antibiotics?

[ ] Yes [ ] No

3.25 Does the feed for the last month of production contain antibiotic growth promotant?

[ ] Yes [ ] No

If yes, what is the name of the antibiotic growth promotant? _____

Swine Disease Management

3.26 What are the diseases of pigs you have encountered in the farm in the past year?

Disease	Yes/No	Antibiotics Used	Route of Administration
Respiratory Symptoms (Coughing, sneezing, deformed nose, etc)	[ ] Yes [ ] No		
Gastrointestinal Symptoms (Diarrhea: watery, with blood/mucus, etc)	[ ] Yes [ ] No		
Reproductive Symptoms (Abortion, stillbirths, mummification, mastitis, etc)	[ ] Yes [ ] No		
Skin/Bodily Symptoms (Abscesses, skin discoloration, skin ulceration, poor growth, etc)	[ ] Yes [ ] No		
Locomotion/Nervous Symptoms (Lameness, arthritis, paralysis, etc)	[ ] Yes [ ] No		
Others			

Drugs: Enrofloxacin, Gentamicin, Colistin, STP, Tylosin, Lincomycin, Neomycin, Pefloxacin, Tilmicosin, etc

3.27 How often do you observe diarrhea in your farm?

[ ] Sometimes [ ] Often [ ] Very Often

Note: The frequency of occurrence is categorized as sometimes if diarrhea occurs sporadically and often/very often if it occurs frequently or endemically.

3.28 When diarrhea occurs, do you always use antibiotics to treat animals in your farm?

[ ] Yes [ ] No

3.29 Who prescribes the use of antibiotics in the farm?

[ ] Farm Vet [ ] Government Vet

3.30 Do you always follow the prescription of the veterinarian?

[ ] Yes [ ] No

3.31 Do you always complete the prescribed treatment regimen of 7 days

[ ] Yes [ ] No

3.32 Do you send samples for antibiotic susceptibility testing at RADDL or elsewhere?

[ ] Yes [ ] No

3.33 Which antibiotics did you use most frequently during the past year?

Antibiotic	**Reason for Use**	**Route of Administration**	**Duration of Use**

3.34 How do you manage the animals if being treated?

[ ] Isolated [ ] Stays in the group

3.35 For how long do you use the antibiotics when treating animals?

[ ] 1-2 days [ ] 3-4 days [ ] 5-7 days

3.36 Do you rotate the use of antibiotics when treating a disease?

[ ] Yes [ ] No

If yes, what is the reason: _____

3.37 Do you record the treatment when using antibiotics?

[ ] Yes [ ] No

Knowledge of Antimicrobial Resistance

3.38 Are you aware of antimicrobial resistance?

[ ] Yes [ ] No

3.39 If yes, where did you learn about antimicrobial resistance?

[ ] Farm Veterinarian [ ] Swine Association

3.40 Are you aware that overuse or inappropriate use of antibiotics may have adverse effects?

[ ] Yes [ ] No

3.41 Do you use the same antibiotics for every batch of pigs you grow in a year?

[ ] Yes [ ] No

3.42 How often do you change the antibiotics that you use in the farm?

[ ] Every 6 months [ ] Every year [ ] Every 2 years

4. SOURCE OF INFORMATION

Name of Person who provided the Information: _____

Contact Number _____

Date of Interview: _____

_____

Printed Name & Signature of Investigator

LIST OF PUBLICATIONS

Gundran, R.S., Cardenio, P.A., Villanueva, M.A., Sison, F.B., Benigno, C.C., Kreausukon, K., Pichpol, D., Punyapornwithaya, V. (2019). Prevalence and distribution of *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ genes in extended- spectrum β- lactamase-producing *E. coli* isolates from broiler farms in the Philippines. *BMC Veterinary Research*.

Gundran, R.S., Cardenio, P.A., Salvador, R.T., Sison, F.B., Benigno, C.C., Kreausukon, K., Pichpol, D., Punyapornwithaya, V. (2019). Prevalence, antibiogram and resistance profile of extended-spectrum β-lactamase-producing *Escherichia coli* isolates from pig farms in Luzon, Philippines. *Microbial Drug Resistance*.

CURRICULUM VITAE

Name Mr. Romeo Sawit Gundran

Date of Birth August 9, 1963

Education

- **Doctor of Philosophy in Veterinary Science** *(candidate),* Faculty of Veterinary Medicine, Chiang Mai University, Thailand, 2019
- **Doctor of Public Health (39 units),** College of Public Health, University of the Philippines Manila, 2007
- **Fellow,** Philippine College of Veterinary Epidemiologists, 2019
- **Fellow,** Philippine College of Veterinary Public Health, 2017
- **Diplomate**, Philippine College of Ruminant Practitioners, 2006
- **Qualified Certificate,** Deutsche Stiftung fur Internationale Entwicklung, Munich, Leipzig and Berlin, Federal Republic of Germany, 2002
- **Master of Veterinary Science,** University of Queensland, St. Lucia, Brisbane, Queensland, Australia, 1998
- **Postgraduate Diploma in Veterinary Studies,** University of Queensland, St. Lucia, Brisbane, Queensland, Australia, 1995
- **Doctor of Veterinary Science and Medicine,** Central Luzon State University, 1985
- **Bachelor of Science in Animal Husbandry,** Central Luzon State University, 1983

Some Positions Held

- **College Dean,** College of Veterinary Science and Medicine, Central Luzon State University, Science City of Muñoz, Nueva Ecija, May 2007 to December 2015.
- **Professor III**, College of Veterinary Science and Medicine, Central Luzon State University (Accredited Professor by the Development Council of State Universities and Colleges, March, 2007).

- **Vice President**, Philippine College of Veterinary Epidemiologists, May 2015 to present
- **Member**, Commission on Higher Education Technical Committee for Veterinary Medicine (CHED-TCVM), Technical Panel for Agricultural Education (TPAE), January 2010 to December 2018.

Awards and Recognitions

- **2nd Best Paper Research Category.** Development of LAMP Assay and Quick Test Kit for Gastrointestinal Infections of Swine. Department of Science and Technology (DOST) 2016 National Agriculture, Aquatic, and Resources Research and Development (NSAARRD) Competition, PCAARRD Headquarters, Los Baños. Laguna, July, 2016
- **Best Paper on Developmental Research.** Development of LAMP Assay and Quick Test Kit for Gastrointestinal Infections of Swine. CLAARRDEC 26th Regional Symposium on Research and Development Highlights. Philippine Center for Postharvest Development and Mechanization (PHILMEC), Science City of Munoz. October, 2015.
- **Best Paper Award (Livestock Research).** Development of LAMP Assay and Quick Test Kit for Gastrointestinal Infections of Swine. 27th Agency In-House Review of Completed and On-Going R and D Projects, July, 2015.
- **2013 PRC Outstanding Veterinarian of the Year Award**, Professional Regulation Commission (PRC), June 2013
- **2011 PVMA Outstanding Veterinarian in Education**, Philippine Veterinary Medical Association, February 2011
- **2008 VPAP Most Outstanding Veterinary Practitioner in the Academe**, Veterinary Practitioners Assoc of the Philippines, June 2008
- **2005 CLSU Gintong Butil Award for Professional Achievement**, Central Luzon State University (CLSU) Alumni Association, April, 2005.

- **Plaque of Recognition (Board Topnotcher, 1986**) – Central Luzon State University.
- **CLSU Best Student Organization Adviser of the Year**, 1986-1991
- **Career Executive Service Eligible (80.65%).** Career Executive Service Board. December 6, 2015.
- **Licensed Agriculturist,** Professional Regulation Commission, July 2016
- **15th Place (80.63%),** Veterinary Licensure Examination, Professional Regulation Commission, August 1986.

Membership in Professional and Other Organizations

- **Member**, EPICORE (Worldwide Network of Health Experts), Philippine College of Veterinary Epidemiologists, Philippine Veterinary Medical Association (PVMA), Philippine Society of Animal Science (PSAS), Veterinary Practitioners Association of the Philippines (VPAP), Philippine College of Ruminant Practitioners (PCRP), Philippine College of Veterinary Public Health (PCVPH), Philippine Association of Veterinary Medicine Educators and Schools (PAVMES).
- **National President,** The Gideons International in the Philippines, Feb 2018 to Present.
- **Ministry Area Leader**, CLSU-Lakas Angkan Ministry, 1999 to Present